Discovery and Mechanistic Study of Novel PBD-Targeted Inhibitors of PLK1 for Prostate Cancer Treatment

Danda Pani Chapagai

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DISCOVERY AND MECHANISTIC STUDY OF NOVEL
PBD-TARGETED INHIBITORS OF PLK1 FOR PROSTATE CANCER TREATMENT

by

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DEDICATION

I would like to dedicate this work to my family. First and foremost, special gratitude to parents, Murari Chapagai and Vima Chapagai who continue to support, love, encourage throughout my life and whose good example taught me to work hard to achieve anything that aspired to. I would also like to dedicate my work to my late grandmother, Khantari Chapagai, who taught me meaning kindness and good.

I would also dedicate this work to my wife, Ganga Poudel Chapagai, who has been a constant support and encouragement during the challenging time in graduate school and life. I am truly thankful for having you in my life. My brother Mukti Chapagai, my sisters Laxmi Chapagai Rijal and Kalpana Chapagai Tiwari, and my sisters in law Deepa Kandel Chapagai, always supported me and they are very special.
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ABSTRACT

Polo-like kinase 1 (PLK1) is a central player in regulating entry into and progression through mitosis. Inhibition of sub-cellular localization and kinase activity of PLK1 through the Polo-box domain (PBD) is emerging as a viable alternative to ATP binding site directed drugs for which the development of resistant mutants and inhibition of closely related members of the PLK family (tumor suppressor roles) are primary concerns. I describe related novel non-peptidic PBD binding inhibitors, termed abbapolins, identified through successful application of the REPLACE strategy and demonstrate their potent antiproliferative activity in prostate tumors and other cell lines. Furthermore, the abbapolins show PLK1-specific binding and inhibitory activity as measured by a cellular thermal denaturation assay and their ability to block phosphorylation of TCTP, a key marker of PLK1 mediated kinase activity. I also made a novel observation that abbapolins upon binding to PLK1 induced its intracellular loss in a mechanism at least partially dependent on the proteasome. The therapeutic potential of these compounds was further indicated through their antiproliferative activity on a cell line (C67V PLK1 mutation) which is dramatically resistant to ATP competitive PLK1 inhibitors. I report novel findings during mitosis inferred from our collective data, namely that catalytic site binding by BI2536 or volasertib unexpectedly decreased the stability of PLK1 as determined by thermal stability assay, suggesting an induction of a conformational change in intracellular PLK1.
Intriguingly, these differential effects on PLK1 thermal stability have opposing impacts on the fate of intracellular PLK1. Binding by catalytic inhibitors cause accumulation of PLK1, whereas PBD binding by abbapolins ultimately lead to its loss in cells. Collectively, the results shed further insight into the unique mechanism of action for abbapolins potentially due to their engagement of a cryptic hydrophobic pocket of the PBD. Furthermore in vivo pharmacokinetic studies showed that optimized abbapolins have promising oral bioavailability and inhibited the growth of prostate tumors in a mouse xenograft experiment. Abbapolins are thus a compelling alternative to catalytic-based inhibitors as the basis for the development of novel therapeutics targeting PLK1.
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LIST OF ABBREVIATIONS

Abbaolin .................................................................................................. 2-(4-alkylbenzamido) benzoic acid

AC50 ................................................. Concentration of compound eliciting 50% of maximal activation of enzyme

AR .................................................................................................................. Androgen Receptor

ATP .................................................................................................................. Adenosine Triphosphate

BubR1 ............................................................. Budding Uninhibited by Benzimidazole-Related 1

CD .................................................................................................................. Catalytic Domain

Cdc25C ................................................................. Cell Division Cycle 25C

Cdk .................................................................................................................. Cyclin Dependent Kinase

Colcemid .......................................................................................................... Colcemid

CPRC .............................................................................................................. Castration-resistant prostate cancer

Cysteine ............................................................................................................. Cysteine

DC50 ................................................................................................................. 50% Degradation Concentration

EC50 ................................................................................................................. 50% of maximal Effective Concentration

Fetal Bovine Serum ............................................................................................... Fetal Bovine Serum

Full length ........................................................................................................... Full length

Fragment Ligated Inhibitory Peptides ................................................................ Fragment Ligated Inhibitory Peptides

Fluorescence Polarization .................................................................................. Fluorescence Polarization
G1, G2 phase .......................................................... Gap 1, Gap 2 phase
IC_{50} ........................................................................ 50% of maximal Inhibitory Concentration
K_0 .............................................................................. Dissociation Constant
Map205 .................................................................. Microtubule-Associated Protein 205
mP ................................................................................ Milipolarization
MTT .......................................................... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
µM ............................................................................... Micromole
nM ............................................................................... Nanomole
PBD .......................................................... Polo Box Domain
PBD .......................................................... Polo Box Domain
PCa .......................................................... Prostate cancer
PLK1 .......................................................... Polo Like Kinase
Pon .......................................................... Adaptor Partner of Drosophila Numb
PTEN .......................................................... Phosphatase and tensin homolog
REPLACE ...... REPLacement with Partial Ligand Alternatives by Computational Enrichment
SAR .......................................................... Structure Activity Relationship
T_{1/2} .......................................................... Half-Life
Tagg .......................................................... Aggregation Temperature
TCTP .......................................................... Translationally Controlled Tumor Protein
Val .......................................................... Valine
V_{ss} .......................................................... Volume of distribution
CHAPTER 1

INTRODUCTION

1.1 GENERAL OVERVIEW

Cancer arises from accumulation of genetic and epigenetic alternations [1]. Cancer cells are characterized by a common set of properties including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, resistance to cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion [2]. In addition, genomic instability and tumor promoting inflammation are two enabling characteristics that facilitate acquisition of the hallmark. Genomic instability generates random mutations and chromosome rearrangements and that drive tumor progression. Inflammation resulting from the innate immune response is designed to fight infections and to heal wounds, but it can inadvertently facilitate to acquire hallmark capabilities. Inflammation can contribute to multiple hallmark capabilities including growth factors that sustain proliferative signaling, survival factors that limit apoptosis, extracellular modifying enzymes that promote angiogenesis and metastasis [3]. It is proven that cancer is a disease initiated and driven by genetic alternation. But now it is clear that epigenetics also plays a very critical role in oncogenesis. Epigenetics is described as the study of chromatin-based activities that regulate DNA-templated processes such as transcription,
DNA repair, and replication without altering DNA sequence. DNA and histone modifying enzymes can alter chromatin (DNA and histone macromolecule complex) structure by modifying the non-covalent interaction between and within nucleosomes which is a fundamental unit of chromatin. The epigenetic pathway can have very critical role in regulating the DNA-templated processes such as transcription, DNA repair and replication. The abnormal expression pattern or chromatin alternation in chromatin regulators can induce and maintain several cancers [4] Interestingly, the hallmarks of cancer such as self-sufficiency, limitless replicative potential, resistance to cell death and tissue invasion are greatly affected by changes in the epigenome [4]. In recent years, epitranscriptomics have been studied for its role in cancer. It is a biochemical modification in RNA without changing the RNA sequence and as result, regulates gene expression and biologicals process required for cancer cells growth and survival [5].

Polo Like Kinase 1 (PLK1)

Polo Like Kinase 1 (PLK1) is only expressed in dividing cells and plays a critical role in several stages of mitosis [6]. It is highly expressed in tumors of various origins while its expression is largely absent in surrounding normal tissues [7]. PLK1 inhibition can selectively kill cancer cells that are addicted to PLK1 overexpression [6, 8-9]. For example, prostate cancer cells harboring mutations on the tumor suppressor Pten are very sensitive to PLK1 inhibition (Figure 1.2) [10]. PLK1 expression is low throughout G0, G1, and S phase and starts to increase in G2 and rises further in M phase. The PLK1 PBD found in the C-terminus (Figure 1.1) recognizes a consensus STP motif present on substrates, MAGPMQ-S-pT-P-LNGAKK, which is essential for PLK1’s intracellular localization and mitotic
functions [25]. In mitotic cells, PLK1 localizes to several places via the PBD such as centrosome, kinetochore, spindle midzone, centromere and post-mitotic bridge. PLK1 activity is essential in several stages of mitosis such as centrosome maturation, bipolar spindle assembly, M phase entry, sister chromatid cohesion, formation of kinetochore-microtubule attachment and cytokinesis and mitotic exit [11]. PLK1 PBD docks PLK1 substrate via the STP prior to phosphorylation by catalytic domain. PLK1 substrate phosphorylation is essential for its mitotic functions. For example, PLK1 is involved in chromosome alignment onto the metaphase plate during prometaphase via BubR1 (Budding Uninhibited by Benzimidazole-Related 1). Cyclin dependent kinase 1 (Cdk1) phosphorylates at T620 on STP motif of BubR1 localized on unattached kinetochores. Then, PLK1 PBD docks the BubR1 via the STP motif and then PLK1 catalytic domain phosphorylates at S676 of BubR1, an important modification for stability of the kinetochore-microtubule interaction, timely mitotic progression, and alignment of chromosomes onto the metaphase plate [12]. PLK1’s other substrate includes TCTP (Translationally Controlled Tumor Protein), Cdc25C, and Cdh1 [13-15].

PLK1 overexpression in murine fibroblast NIH3T3 cells results in oncogenic transformation. The transformed cells show a high proliferation index, grow in soft agar and form tumors in nude mice [18]. However, PLK1 is rarely mutated in tumors (about 1% of 74402 tumoral samples) [19]. That might be since PLK1 is a gene responsible for proliferation and therefore cells cannot tolerate loss of PLK1 function. Because of the essential role of PLK1 in cell proliferation, it is highly expressed in proliferative tissues such as thymus, testis, developing embryos, spleen, etc. In addition, tumors have higher
PLK1 expression compared to paired adjacent tissues which may be reflection of the higher proliferation rate. Whether the higher expression of PLK1 in tumors compared to the paired adjacent tissues is the cause or consequence of oncogenic transformation is an open question at present.

Prostate cancer (PCa) is one of the most common cancers in males. The American Cancer Society estimates about 268,490 new cases of prostate cancer will be diagnosed and about 34,500 will die of this disease in 2022. Current therapeutic approaches are not enough to manage cancer, so efforts are focusing on novel targets and strategies to manage it. The connection between PLK1 and prostate cancer will be discussed in greater detail later in the section titled, "Androgen receptor signaling in prostate cancer”.

PBD structure and its functions

PLK1 consists of a highly conserved N-terminal catalytic kinase domain, and less conserved, functionally essential, C-terminal Polo Box Domain (PBD) and an Inter domain linker (IDL) (Figure 1.1). PBD comprises two polo-box motifs (PB1 and PB2) containing identical β6α folds [11] forming a phosphopeptide-binding heterodimeric module. A similar structure is adopted by related human polo-like kinases PLK2, PLK3 and PLK4 (Figure 1.1). A secondary linker region (L2) separates PB1 and PB2 of PLK1 PBD and can sense presence of phosphopeptide [21-23]. The human PLK1 PBD recognizes a consensus motif present on its substrates, (contained in the optimized poloboxtide peptide MAGPMQ-S-pT-P-LNGAKK), which is essential for PLK1’s intracellular localization and mitotic functions [25]. PLK1 PBD comprises a Ser/pThr binding cavity and a hydrophobic cavity. Ser/pThr residues in the phosphopeptide-substrate binds to the Ser/pThr binding
cavity with the phosphate group contacting the side chains of His538 and Lys540. Mutation of these key residues (His538A and K540M) disrupts phosphopeptide-substrate engagement and PLK1 functions [24]. Residues Tyr417, Tyr421, Tyr481, and Tyr485 form the sides, seven hydrophobic amino acid residues line the pocket and Val415, Leu478, and Phel482 cover the bottom of the PBD binding groove. The four Tyr residues are conserved across the eukaryotic members of the polo-like kinase family and this section of the PBD groove was named “Tyr pocket” [21, 26]. This pocket can adopt an open conformation to accommodate PBD substrate or a closed conformation without such interaction and thus is considered a cryptic binding site. The combination of the adaptable Tyr pocket with the very strong Ser/pThr binding cavity suggests that the co-ordination of these pockets can be important in binding a subset of PBD-interacting proteins [26].

Polo-box interacting protein 1 (PBIP1) is essential for recruiting PLK1 to the interphase and mitotic kinetochores. PLK1 phosphorylates PBIP1 at Thr78 and binds to the resulting S77-pT78 motif via the PBD. Quite interestingly, pThr78 containing peptides interact with PBD of PLK1 but not with those of PLK2 and PLK3 suggesting the specificity of the interaction. Once PLK1 is localized to mitotic kinetochores, PBIP1 is degraded later in mitosis in a PLK1 pThr78 dependent manner thus enabling PLK1 to interact with other partners important for proper kinetochore functions. The pThr78 PBIP1 dependent PLK1 localization to kinetochores is required for chromosome congression and spindle assembly checkpoint [27]. Phe71 of PBIP1 interacts with Tyr417 and Tyr421 of the PBD located in the hydrophobic-Tyr pocket as determined by isothermal titration calorimetry (ITC) and crystal structures of PLK1 PBD complexes with 71-FDPPLHSpTA-79 (3P37) or 72-
DPPLHSpTA-79 (3P36). In the 72-DPPLHSpTA-79-PBD crystal structure, the hydrophobic pocket adapts a closed conformation and does not interact with DPPLHSpTA while in the 71-FDPPLHSpTA-79-PBD crystal structure, Phe71 inserts into the hydrophobic cavity [26].

Further studies carried out by Sharma et al., 2019 using PLK1-depleted cells overexpressing either GFP-PLK1\textsubscript{AAD} (mutation in tyrosine pocket Tyr421 to Ala, Leu478 to Ala and Tyr481 to Asp) or GFP PLK1\textsubscript{AM} (mutation in Ser/pThr binding cavity, His538 to Ala, and Lys540 to Met). GFP-PLK1\textsubscript{AAD} and GFP PLK1\textsubscript{AM} mutants show impaired PLK1 localization to kinetochores whereas PLK1 localization to centrosomes remains unaffected by the mutations. These results suggest that mutations selectively inhibit PBD interaction with substrates required for PLK1 localization to kinetochore such as BubR1 [12, 27-29]. PBIP1, CLASP2 and CLIP-170. HeLa cells overexpressing GFP-PLK1\textsubscript{WT} were transfected with either PBIP1-V5 (V5 was C-terminally tagged to protein), PBIP1-F71A-V5 (Phe71 to Ala), PBIP1-T78A-V5 (Thr 78 to Ala) and lysate was prepared for a pull-down assay. Both mutants PBIP1-T78A-V5 (relative intensity 0.1) and PBIP1-F71A-V5 (relative intensity 0.7) weakly bind to GFP-PLK1\textsubscript{WT} compared to wildtype PBIP1-V5 (relative intensity 2.5). This result suggests that both Phe71 and Thr78 are essential for the PBIP1-PLK1 interaction during mitosis. Co-immunoprecipitation was carried out with PLK1 PBD substrate NEDD1 (Neural precursor cell expressed developmentally downregulated gene 1) with the cell lysate prepared from cells overexpressing GFP PLK1\textsubscript{AM} or GFP-PLK1\textsubscript{AAD} or GFP PLK1\textsubscript{WT}. The GFP PLK1\textsubscript{AM} fails to bind the NEDD1 while the GFP-PLK1\textsubscript{AAD} retains binding to NEDD1. In summary, the cryptic hydrophobic pocket can act in concert with the Ser/pThr binding cavity to specifically recognize a subset of PLK1 substrates [26].
The overexpression study by Sharma et al., 2019 shed some light on the subtle distinctions between the contribution of the PLK1 kinase domain versus the Ser/pThr binding cavity and the Tyr pocket of the PBD. Several studies show small molecules targeting the kinase domain results in mitotic arrest with monopolar spindles whereas small molecules targeting the PBD cause bipolar spindles with misaligned chromosomes [30-31,39]. Depletion of endogenous PLK1 causes monopolar spindle arrest with misaligned chromosomes. Induced expression of the GFP-PLK1 WT restores both bipolar spindle formation and chromosome congressional defects. While the induced expression of GFP-PLK1 AAD or GFP PLK1 AM restores the bipolar spindle formation but does not effectively counteract the chromosome congressional defects. These results suggest that the both the Ser/pThr binding cavity and Tyr pocket of the PBD are essential for the chromosome congression but not for the bipolar spindle formation, whereas the kinase domain is essential for both chromosome congression and bipolar spindle formation [26]

**PLK1 Catalytic Inhibitors**

BI2536

BI2536 was initially identified by screening of a diverse library of organic compounds for their ability to inhibit PLK1 catalytic activity and this was followed by their optimization for potency and selectivity [56]. It is selective for the PLK family against a panel of 63 tyrosine and serine/threonine kinases; however, it is nonselective between PLK family members (PLK1 IC$_{50}$: 0.83 nM, PLK2 IC$_{50}$: 3.5 nM, and PLK3 IC$_{50}$: 9 nM) as determined by In vitro kinase assay [56]. Lack of selectivity of ATP competitive PLK inhibitors is problematic as functions of PLK2, PLK3 and PLK4 are not completely
understood and potentially have opposing functions to those of PLK1. BI2536 causes a mitotic arrest and induces apoptosis in human cancer cell lines irrespective of tissue origins and oncogenic status with EC\textsubscript{50} values ranging from 2 to 25 nM. BI2536 showed a strong inhibition of growth or regression of multiple human carcinomas in mouse xenograft studies [56]. In a phase II study of BI2536 with relapsed small cell lung cancer (SCLC) patients, it was found to be ineffective. Moreover, BI2536 showed side effects such as neutropenia, fatigue, nausea, vomiting and constipation. The study was terminated for a lack of efficacy [32].

Volasertib (BI6727)

BI6727 is an ATP-competitive inhibitor from the dihydropteridinone class of compounds, and it is an optimized analogue of BI2536 with better pharmacological and pharmacodynamic properties. As residues in the ATP binding pocket of PLKs are highly conserved, it potently inhibits PLK1 (IC\textsubscript{50}: 0.87 nM) and other closely related kinases PLK2 (IC\textsubscript{50}: 5 nM) and PLK3 (IC\textsubscript{50}: 56 nM) [33]. BI6727 in a combination with low dose of cytarabine was granted a breakthrough therapy designation by US Food and Drug Administration (FDA) in 2013 for the treatment of Acute Myeloid Leukemia (AML) patients who were not eligible for intensive remission induction therapy [33]). BI6727 induces a characteristic prometaphase arrest and subsequent apoptosis in panel of human cancers in \textit{In vitro} and \textit{In vivo}. In a xenograft study, tumor tissues showed sustained exposure to BI6727 with a high volume of distribution, a long half-life in mice (V\textsubscript{ss}: 7.6 L/kg and T\textsubscript{1/2}: 46 h) and good oral bioavailability. It inhibited the growth of several human cancers in xenograft studies. However, in Phase I clinical trial of BI6727 in
combination with decitabine in patients with acute myeloid leukemia, the objective response rate of 23% was reported. The adverse side effects were neutropenia, pneumonia, and reduced appetite. The study was discontinued [34].

*PLK1 PBD dimerization regulates PLK1 kinase activity*

Phospho-peptide binding to PLK1 PBD partially actives PLK1 possibly via relieving an autoinhibited closed confirmation [35]. It is proposed the interactions between the kinase domain and the conformationally rigid L2 upon phospho-peptide binding, relieves autoinhibition [35]. Sharma et al., 2019 showed that Tyr-hydrophobic pocket in co-ordination with adjacent Ser/pThr binding domain involves in recognition of PLK1 PBD substrates and mitotic chromosome segregation. The study by Zhu et al., 2016, based on biochemical and *in vitro* assays, showed that the Tyr pocket in co-ordination with phospho-substrate binding groove involved in PLK1 PBD dimerization is responsible for fine tuning of PLK1 catalytic activity. Cdk1 phosphorylates Drosophila protein Pon (adaptor Partner of Drosophila Numb) at Thr63 thus creating a PLK1 PBD docking site. The phospho-Pon (pPon) binding induces human PLK1 dimerization, and this relieves an PLK1 autoinhibition caused by the closed conformation. Binding between the pPon and PBD is mediated by polar and hydrophobic interactions. In the crystal structure of PLK1 PBD-pPon complex, the phosphate group of pT63 of pPon forms a salt bridge with Lys540 PBD and a hydrogen bond with His538 within the PBD. Similarly, Ser62 of pPon forms a hydrogen bond with the main chain of Trp414 PBD. In addition to the polar interactions, hydrophobic interactions further strengthen the PBD-pPon interaction. The bulky aromatic ring of the Phe60 pPon inserts into the hydrophobic cavity formed by side chain
of some conserved hydrophobic residues Val415, Tyr417, Phe482 and Tyr485. In the crystal structure, two identical pPon-PLK1 PBD further pack with each other forming a dimer of dimers. The dimerization interface is mainly mediated by extensive hydrophobic interactions. The side chain of Leu505 (Leu505 from PBD of another PLK1 protein) from L2 of PBD inserts into the hydrophobic pocket formed by Val415, Tyr417, Tyr421, Leu478, Tyr481, Phe482 and Tyr485 of the other PBD monomer which partially overlaps with the Phe60 pPon bonding pocket. Interestingly, the bulky aromatic ring of the Phe60 pPon interacts with the PBD and contributes to the integrity of hydrophobic cavity in accommodating Leu505 of PBD. The interaction between PBD bound Phe60-pPon and Leu505 from L2 of PBD is proposed to stabilize dimeric PLK1 and to enhance the kinase activity. The dimerization induced by the pPon peptide is lost by a single point mutation at position 505 (L505E). Intriguingly, changing a Phe to Ala of pPon (pPonF60A) inhibits the PLK1 kinase activity, which is opposite to the effect mediated by pPon. So, it is understandable that the consequence of phospho-peptide binding directly correlates with the primary sequence of the phosphopeptide. The L2 loop can sense presence of a specific phosphopeptide, promotes the PBD dimerization and thus relieves the autoinhibited KD (Kinase Domain)-PBD interaction [23].

With regards to recruitment to kinetochores, a recent study [39] indicated that phospho CENP-U (the PLK1 receptor in the kinetochore core) promotes PLK1 docking through PLK1 dimerization. Another recent study [50] showed that Bora transiently promotes dimerization of cytoplasmic PLK1 during entire G2 phase of cell cycle and the dimerization is lost following the phosphorylation of PLK1 at T210 by Aurora A. Following
the phosphorylation of PLK1 in the cytoplasm, the monomeric PLK1 localizes to nucleus. Priming phosphorylation by Cdk1 on S252 of Bora strongly enhances Bora’s interaction with the PBD [50]. Previous study showed that the motif centered around on S252 is a major Cdk1-dependent PLK1-PBD binding site on Bora. Bora S252A showed marked reduction in binding to the PLK1 PBD. PBD mutant (GST-PBD-AA) incapable of binding with phosphopeptide substrate does not interact with WT Bora. This strongly suggests that the phospho Bora (pS252) interacts with Ser/pThr binding groove in PBD [37-38].

According to Zhu et al., 2016, dimeric or oligomeric PLK1 is active, while based on the Raab et al., 2022 study, monomeric PLK1 is active and the dimeric form PLK1 is held in an inactive state.

**Allosteric mechanism of PLK1 regulation**

The crystal structure complex of the polo-like kinase KD from zebrafish (Danio rerio) and PBD with a Map205 (PBD-binding motif of Drosophila melanogaster microtubule-associated protein 205) shows that the KD is inhibited by the PBD and the IDL by either reduction of flexibility of the hinge region or sequestration of the activation loop. PBD interaction with the KD reduces flexibility of the ATP cleft as the L1 of the PBD docks into the concave surface of the KD formed by the β1 strand of the N lobe, the hinge region and helix αD of the C lobe [35]. PBD binding to a phosphorylated substrate can disrupt the intramolecular interaction between the PBD and KD and relieve the autoinhibition of the PBD. However, PBD binding to Map205 stabilizes the autoinhibited state of Polo (Drosophila) and sequesters it from its substrates.
Using a chemical biology approach, Raab et al., 2018 showed that PLK1 is regulated by an allosteric mechanism. Using an Alpha Screen assay, they discovered small molecules that enhance or inhibit the interaction between PLK1 PBD and a phosphorylated peptide (PoloBoxtide, Biotin-MAGPMQSp[T]PLNGAK). The small molecule, FM0024 enhances the interaction between full length PLK1 (FL PLK1) and PoloBoxtide ($AC_{50}$, concentration of compound eliciting 50% of maximal activation = 10 µM) while other small molecules such as SCR01010 and AW00551 diminish the interaction between PoloBoxtide and FL PLK1 ($IC_{50}$ = 20 µM and 1 µM respectively). However, none of these compounds directly inhibit the interaction between PBD and PoloBoxtide suggesting that these compounds allosterically modulate (positively or negatively) of the activity of PLK1 but not by direct competition with PoloBoxtide binding with PBD. In *in-vitro* kinase assay, FM0024, AW00551 and SCR01010 partially inhibit kinase activity of FL PLK1 and isolated KD resistant prostate cancer. In an *in-vitro* thermal stability assay by differential scanning fluorimetry (DSF), BI6726 strongly stabilizes the catalytic domain ($\Delta T_m = 20.8 \pm 0.02 ^\circ C$) but overall destabilizes the FL-PLK1. The thermal denaturation curve for FL-PLK1 in presence of BI6727 showed a distinct biphasic denaturation pattern. The early section of the curve showed that the binding of BI67237 destabilized PLK1 ($\Delta T_m = -11.1 ^\circ C$) as evidenced by the left shift at lower temperatures, whereas the later section of curve reveals a stabilization at higher temperatures. The complete denaturation of PLK1 in the presence of BI6727 is shifted to the right by 6.6°C. One explanation for the biphasic curve is that BI6727 binding to the KD relieves the PLK1 closed conformation, facilitating the destabilization of the PBD of which is detected by the binding of the fluorophore in DSF.
PLK1. In agreement with this result, BI6727 enhanced the interaction between the PBD of PLK1 and PoloBoxtide in the AlphaScreen assay, which also suggests that BI6727 binding to the KD is promoting an open conformation of PLK1. FM0024 mildly stabilized the KD of PLK1 (ΔTm =1.8 ± 0.25 °C) but did not significantly stabilize the FL PLK1 in the thermal stability assay. This suggests that FM0024 stabilizes the KD and as a result relieves the autoinhibition of the KD by the PBD. The denaturation curve obtained in the presence of FM0024 also shows a distinct denaturation pattern where the first part of curve indicates stabilization, and the second part of curve destabilization similar to BI6727. On other hand, AW00551 stabilizes the FL-PLK1 (ΔTm 4.98 ± 0.7 °C) and also the isolated KD (ΔTm 2.51 ± 0.09 °C). This observation is inconsistent with the finding that AW00551 decreases the interaction between the PBD of PLK1 and the PoloBoxtide in AlphaScreen assay. In agreement with the findings from biochemical assays, FM0024 augments the interaction between the PLK1-Bora complex while the AW00551 inhibits the interaction in a co-immunoprecipitation assay carried with cell lysate from synchronized HeLa cells. Moreover, cells treated with FM0024, which enhanced the interaction between PBD of PLK1 and PoloBoxtide, reveals enhanced phosphorylation of PLK1 specific substrates such as TCTP, Myt1, Cdc25c in HeLa cells. On other hand, AW00551 reduces the phosphorylation of Myt1. SCR01010 shows pronounced mis localization of PLK1 from centrosomes and kinetochores (42%) compared to FM0024 and Poloxin (31% and 32 % respectively). Both FM0024 and AW00551 partially inhibit kinase activity and cause PLK1 conformational change in *in vitro* assays, but they are exceptionally good inhibitor/enhancer of PLK1 signaling in cells [49].
In summary, the study by (Raab et al., 2018) described how small molecules with different modes of action can produce varying outcomes in cells. PLK1 mediated signaling in cells can be inhibited by compounds that are potent inhibitors of enzymatic activity or by allosteric modulators that are weak inhibitors of enzymatic activity. BI2537 strongly inhibits catalytic activity of PLK1 however in contrast, AW00551 and SCR01010 showed different mechanisms of action. They stabilize the KD-PBD interaction and inhibit binding of PBD with phosphopeptides. These results suggest that compounds binding outside of the ATP binding site (allosteric site) are alternative for catalytic based inhibitors of protein kinases to increase selectivity and thus avoid side effects and improve the outcome in patients.

*Androgen receptor signaling in prostate cancer*

The androgen receptor (AR) is essential for the growth of PCa *in vitro* and *in vivo*. Effects of androgens such as testosterone and dihydrotestosterone (DHT) are mediated via the AR, a ligand dependent type 1 nuclear receptor. It comprises a N-terminal transactivation domain (NTD), a DNA-binding domain (DBD), a flexible hinge region and a C-terminal ligand binding domain (LBD). The NTD and LBD are required for transcriptional activity and binding to androgens and other agonists, respectively. Androgen binding to the LBD leads to a series of events including conformational change, nuclear localization of AR and increasing the binding of AR dimers via their DBD with the Androgen response elements (ARESs) located on the promoter and enhancer sequences of target genes. Another layer of regulation of AR activity has been reported as LBD comprises Activation function-2 (AF-2) where co-activators and co-repressors can be recruited. AR has
important roles in advanced PCa. The proposed mechanism of continued transcriptional activity of AR even following castration includes: most importantly expression of constitutively active splice variants lacking the LBD, increased expression levels of AR, altered expression of co-regulator of AR, gain-of-function mutation in the LBD, ligand-independent activation of AR by its NTD in absence of ligand by kinases, cytokines and residual androgens from adrenal gland and tumors. For example, AR variants lacking the LBD were reported in PCa cells such as 22Rv1 and tissues from castration-resistant prostate cancer (CRPC) patients [8, 36]. Constitutively active LBD splice variant can interact with full-length AR (FL-AR). The LBD splice variant (V567es) can localize to nucleus and bind to AREs in absence of androgen potentially via interacting with FL-AR. The constitutively active splice variant can contribute the development of castration-resistant prostate cancer [40]. AR target genes in CRPC are unique from those that are induced by androgens in androgen sensitive PCa. It may be due that AR can differentially associate with transcription factors and co-regulators [41].

**PLK1 in Prostate cancer**

PLK1 is elevated in prostate cancer and is linked to higher tumor grade. Elevated PLK1 is critical to adapt to mitotic stress caused by deletion of a tumor suppressor gene called *Pten* (*Figure 1.2*). This suggests PLK1 is a promising drug target for prostate cancer patients harboring *Pten* deletions [10, 52-53]. *Pten* is mutated in a wide variety of solid tumors such as prostate, glioblastomas, breast, and endometrial. *Pten* phosphatase activity inhibits the phosphoinositide 3-kinase (PI3K)/AKT signaling cascade.
Loss of *Pten* leads to the de-repression of PI3K/AKT pathway and stimulation of cell growth and proliferation [10].

*Pten* encompasses five functional domains including a N-terminal phosphatase domain, a middle C2 domain and a C-terminus PDZ (postsynaptic density protein) - interaction motif [42]. Nuclear *Pten* ensures centromere stability which is independent of the phosphatase activity. *Pten* localizes at centromeres and associates with CENP-C via its C-terminal domain. CENP-C is an integral component of inner kinetochore and is necessary for the kinetochore action and accurate segregation during mitosis. Interaction of *Pten* with CENP-C can be necessary for the localization of *Pten* at centromere and that can target the *Pten* to inner centromere/kinetochore complex. Given the CENP-C binds the back of DNA, the CENP-C associated *Pten* can play a key role in maintaining chromosome structure through the stabilization of centromere [43, 55]. In addition to that, *Pten* in co-ordination with E2F-1 regulates Rad51 at a transcriptional level to alleviate DNA damage and maintains chromosome integrity [55]. Nuclear function of *Pten*, but not its phosphate activity, is required to reverse the mitotic stress suffered by *Pten* depleted PCa cells [10].

X. S. Liu et al., 2011 demonstrated that PLK1 conferred the tumor formation competence of *Pten*-depleted prostate cancer cells. They compared the dependence of PLK1 in *Pten*-depleted DU145 and *Pten* WT DU145 cells on the formation mouse xenograft tumors. *Pten*-depleted DU145 cells overexpress PLK1 compared to *Pten* WT DU145. *Pten*-depleted DU145 cells without BI2536 treatment form aggressive xenograft tumors compared to *Pten*-depleted DU145 cells treated with BI2536 (25 mg/kg, for 3
weeks). Growth of tumors derived from the Pten-depleted DU145 cells are completely inhibited, however, tumors derived from DU145 cells with Pten-WT are unaffected [10]. Untransformed prostate epithelial cells RWPE-1 express very low level of PLK1 protein whereas androgen sensitive C2-4, androgen insensitive LNCaP and androgen insensitive and metastatic PC3 cells express elevated levels of PLK1 with an order of RWPE-1 < C2-4 < LNCaP < PC3 [6]. RWPE-1 cells show resistance to BI2536 treatment (20 nM, 24 hours). However, C2-4, LNCaP and PC3 show cleaved-PARP in co-relation with PLK1 protein expression levels (order of cleaved-PARP: C2-4 < LNCaP < PC3). These results suggest that PLK1 levels are correlated with tumorigenesis and survival of prostate cancer cells. A study was carried out to see whether protein and mRNA levels of AR and its splice variants are affected by inhibition of PLK1 in C4-2 (androgen sensitive, express AR) and 22Rv1 (androgen insensitive cells, expresses both AR and splice variant). Genetic and pharmacologic inhibition of PLK1 reduces AR levels in C4-2 cells and AR and its splice variant levels in 22Rv1 cells. On the other hand, AR inhibition by AR antagonist enzalutamide for 12-hours, decreases PLK1 protein levels in both AR sensitive C2-4 cells and AR insensitive LNCaP synchronized cells. Enzalutamide does not significantly affect the cell cycle suggesting that the reduction in PLK1 level is not because of cell cycle alteration [6]. CPRC cells can produce androgen via the de novo steroidogenesis. Two enzymes CYP17A1 (Cytochrome P450 17A1/steroid 17-a-monooxygenase) and CYP11A1 (Cytochrome P450 11A cholesterol side-chain cleavage enzyme) have important roles in androgen synthesis, and they are under control of SREBP pathway. CYP11A1 converts the substrate cholesterol to precursor pregnenolone then finally CYP17A1 and other enzymes
convert testosterone to dihydrotestosterone in a series of reactions. As expected, BI2536 (200 nM, 4 hours) treatment reduces mRNA level of CYP17A1, CYP11A1 and SREBP in C2-4, 22Rv1 and LNCaP cells. Following the BI2536 treatment, CYP11A1, CYP17A1 and SREBP transcripts are significantly reduced in C2-4 and 22Rv1 cells compared to LNCaP cells [6].

In an elegant experiment, LNCaP cells were inoculated into nude mice which were then castrated when the tumors reached 200–300 mm$^3$. From the day of the castration, mice were treated with BI2536 (25 mg/kg, intravenously) or saline twice per week for up to 8 weeks. A week after castration, tumor volume decreased. Interestingly, after that point, tumors continued to grow in BI2536-untreated mice while tumor volume dramatically reduced in BI2536-treated mice. As expected, tumors in the castrated mice showed resistance to androgen as AR signaling seemed somehow re-activated and continued to be important for prostate tumor growth. This result agrees with clinical data. Patients’ survival is only improved by a 2 to 5 month by treatment of FDA approved drugs abiraterone and enzalutamide (AR antagonists). So, PLK1 level is co-related with PCa growth. Androgen insensitive PCa cells are more sensitive to PLK1 inhibition than androgen sensitive PCa suggesting that PLK1 dependency on survival of PCa cells increases as they transform from androgen sensitive to insensitive [6].

1.2 OUR APPROACH OF TARGETING POLO BOX DOMAIN (PBD)

PLK1 is highly expressed in tumors of various origins while its expression is largely absent in surrounding normal tissues [7]. PLK1 inhibition can selectively kill cancer cells that are addicted to PLK1 overexpression [9-10, 44]. PLK1 consists of a highly conserved N-terminal catalytic kinase domain and less conserved, functionally essential, C-terminal
PBD [11]. PLK1 kinase catalytic inhibitors have advanced to clinical trials but they inhibit the other kinases including PLK1 family members PLK2 and PLK3 and PLK1 selective inhibitors with reduced off target effects have not been identified yet. Although similar in sequence, PLK3 has an opposing function to that of PLK1 and acts directly or indirectly as a tumor suppressor [45]. Moreover, the roles of other PLK family members PLK2 and PLK4 are not clearly understood. The PBD domain binds a STP motif, which is essential for PLK1’s intracellular localization and mitotic functions. ATP binding domains are found on approximately 500 protein kinases and many other ATP hydrolases and are highly related in terms of sequence and 3D structure. The PBD is only found on PLK1-5, not on other kinases, and which makes the PDB a unique druggable domain [11, 45]. HeLa cells overexpressing PLK1 PBD caused mitotic arrest and chromosome congressional defects, suggesting inhibition of PBD blocks PLK1 activity [46]. Therefore, targeting the PBD holds a great promise for better clinical outcomes with fewer side effects in cancer therapy and overcome drug resistance because of mutations in the ATP binding site [6, 46].

A previous study from the laboratories of Dr. McInnes and Dr. Wyatt [47] and newer preliminary work showed that the PBD inhibitors (peptides and FLIPs) are very selective for PLK1 PBD over PLK3 PBD and exhibited anti-proliferative activity against cancer cells. Starting from the PBD-Interacting Protein (PBIP) (PLHSpTAI) and Cdc25C (LLCspTPNGL), natural substrate peptides of PLK1, we identified a peptide library that showed selectivity for PLK1 PBD over PLK3 PBD. Individual amino acids of the substrate peptide were replaced with more drug like small molecules to generate Fragment Ligated Inhibitory Peptides (FLIPs).
FLIPs were then further modified by REPLACE to optimize potency and drug-likeness and also to generate non-peptidic compounds named abbpapolins (2-(4-alkylbenzamido) benzoic acids.

This work seeks to advance the understanding of binding determinants of PLK1 PBD inhibitors and produce a lead molecule to exploit for cancer therapy. In addition, this work investigates molecular mechanism of PBD inhibitors (FLIPs and abbpapolins). Moreover, this work compares the binding mode of catalytic inhibitors and PBD inhibitors. Chapter Two will discuss the structure activity relationship of some FLIPs with better cell permeability. In addition, it will show TCTP phosphorylation, a known PLK1 specific substrate, is blocked by FLIPs. This work has been published [51] and Chapter Two will show my contribution to that paper.

Chapter Three will present the structure activity relationship of abbpapolins and investigation of the unique mechanism action. Chapter Four will further discuss the mechanism of action of select abbpapolins. In addition, it will discuss the different modes of interaction of catalytic based inhibitors and PBD inhibitor abbpapolins with PLK1 in-vitro and in cells. Overall conclusions and future directions of the study will be discussed in Chapter Five.
1.3 FIGURES

Figure 1.1 Polo Like Kinases (PLKs) in human cells. Schematic representation of the human PLK (1-4) family members. Position of Kinase domains (KD) are shown in red, and position of Polo box-1 (PB1) and PB2 are shown in blue. PLK1 amino acid sequence involved in nuclear localization (nuclear localization signal, NLS) is depicted in green, and the destruction box (D-box) that is ubiquitinated for PLK1 degradation at the end of mitosis is shown in yellow. Amino acids that are important for ATP binding and enzymatic activity (T-loop) within the KD and phospho-selectivity within the Polo Box domains (PBDs) are also shown. Figure is drawn from [11].

Figure 1.2 PLK1 inhibition selectively kills prostate cancer cells addicted to PLK1. Schematic representation showing PLK1 inhibition genetically or pharmacologically can selectively kill PLK1-overexpressing prostate cancer (PCa) cells, which makes it an attractive target for therapeutic intervention [10]. Size of PLK1-word in PLK1 addicted PCa cells is shown in bigger compared to in normal cells to reflect the expression level of the PLK1 in those cells. Centrosomes are shown in green circles.
1.4 CHAPTER 1 REFERENCES


Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *Journal of Clinical Investigation, 120*(8).


CHAPTER 2

PEPTIDOMIMETIC POLO-BOX-TARGETED INHIBITORS THAT ENGAGE PLK1 IN TUMOR CELLS AND ARE SELECTIVE AGAINST THE PLK3 TUMOR SUPPRESSOR

ABSTRACT

The polo-box domain (PBD) of PLK1 determines mitotic substrate recognition and subcellular localization. A structure-activity analysis of the PBD phosphopeptide binding motif has identified potent peptides that delineate the determinants required for mimicry by nonpeptidic inhibitors and provide insights into the structural basis for the selectivity of inhibitors for the PLK1 PBD. Fragment-ligated inhibitory peptides (FLIPs) obtained through REPLACE have been optimized to enhance in vitro binding and a systematic analysis of selectivity for PLK1 vs PLK3 has been carried out for peptidomimetics. Furthermore, these more drug-like non-ATP-competitive inhibitors had on-target engagement in a cellular context, as evidenced by inhibition of the phosphorylation of TCTP, a target of PLK1. These results further validate targeting the PBD binding site in the move towards PLK1 inhibitors that are selective to PLK1 and block catalytic activity [1].

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2.1 INTRODUCTION

The polo-like kinases play key roles in mitosis [1] and inhibition of PLK1 activity leads to anti-proliferative effects [1 2]. The four-known human PLKs (with kinase domains, PLK5 does not) have distinct functions despite their sequence similarity especially in the kinase domain. PLK1 is frequently overexpressed in cancer and is a negative prognostic indicator for patient outcomes [3] especially in prostate [4] and colorectal cancers [5]. Furthermore, PLK1 has been demonstrated to be a potential therapeutic target for tumors with inactivated p53 [6] with evidence showing that PLK1 is oncogenic when p53 is mutated [7]. The therapeutic rationale for PLK inhibition has been validated in vitro and in vivo [8] and numerous inhibitors of the ATP binding site of PLKs have been identified, with some entering clinical trials after showing significant anti-tumor activity in preclinical models. Results from two compounds suggest acceptable toxicity profiles warranting further investigation in phase II trials [9]. BI-6727 (volasertib) was granted FDA breakthrough therapy designation for Acute Myeloid Leukemia, however, did not show good efficacy in subsequent trials. Moreover, there are numerous drawbacks to targeting the ATP cleft, including prominently the inhibition of the three other known members of the mammalian PLKs [10]. Due to its tumor suppressor roles, PLK3 inhibition may lead to diminution of the anti-tumor effect mediated by blocking PLK1 [11] suggesting that inhibiting PLK3 can be deleterious. ATP competitive inhibitors will not necessarily block critical non-catalytic functions of PLK1 necessitating alternative approaches.

The sub-cellular targeting binding site in the polo-box domain (PBD) which interacts with phosphosubstrates such as Cdc25C (a phosphatase activating Cdk1 allowing
mitotic entry) and PBIP (plays a central role in the assembly of kinetochore proteins and facilitates chromosome segregation), is amenable to small molecule inhibitor development [12] and high-throughput screening approaches have been used to generate small molecule inhibitors of the PBD-peptide interaction. For the most part however, these are either weakly binding or non-drug-like in nature [13] although one compound, Poloxin, has been improved through the addition of a hydrophobic tag (still relatively weak in terms of anti-proliferative activity) [14]. Some inhibitors possess a contrasting phenotype to PLK1 knockdown and catalytic inhibition [10, 15] suggesting that their mechanism is not exclusively through on target activity. Derivatized peptides that occupy a novel site in the PBD binding groove [16] have been reported however are extensive modified, complex molecules and overall are non-drug-like. In addition, the concentrations required for cellular activity indicate inefficient cell uptake.

Peptide can selectively bind to PLK1 and hence provides a guide to develop metabolically stable and cell permeable compounds. REPLACE, a validated strategy for the iterative discovery of non-peptidic protein-protein interaction inhibitors, has been utilized to discover fragment alternatives for the N-terminal hydrophobic motif in a Cdc25C PBD substrate peptide [17]. In this present study structural determinants for Fragment Ligated Inhibitory Peptides (FLIPs) binding to the PBDs of PLK1 have been defined. In addition, a detailed evaluation of affinity of ligands for the PBD of PLK3 has been completed and used to generate a selectivity index for PLK1, a novel analysis of selectivity of PBD ligands. Cellular studies with FLIPs demonstrate progress towards obtaining cell permeable compounds that are structurally much less complex than
previously described peptidomimetics since they do not require pegylation or masking of the phosphothreonine. These FLIPs engage PLK1 at a cellular level and have antiproliferative activities consistent with PLK1 inhibition. Such compounds make excellent starting points for development as non-ATP competitive PLK1 inhibitors since they preserve selectivity and potency towards the PLK1 PBD while imparting characteristics for drug-likeness. This chapter reports my contribution to the ChemMedChem publication, which included in vitro and cellular characterization of the activity of two compounds,

2.2 METHODS

FLIP Synthesis

FLIPs were synthesized and purified using standard Fmoc chemistry by GenScript (Piscataway, NJ) and unless stated otherwise. HPLC and MS (Mass Spectro) were used to confirm the purity and structure of each FLIPs. FLIPs generated are based on endogenously binding ligands for the PBD and therefore there is not issue with them being false positives from aggregation or spurious fluorescence signals. This is further unlikely due to the sequence degeneracy of the peptides studied and also the availability of crystal structures for the parent motifs studied.

Fluorescent Polarization Binding Assay

FLIPs to be tested were dissolved in DMSO (10 mM) and diluted to working concentrations in assay buffer (a maximum of 600 μM, which equates to a maximum 6% DMSO tolerance determined for the assay). Assays were optimized following standard guidelines (http://www.ncbi.nlm.nih.gov/books/NBK92000/). The PLK1 PBD (367–603)
and PLK3 PBD (335–646) proteins were obtained from BPS Bioscience Inc. (San Diego, CA); 147 ng/well PLK1 and 345 ng/well PLK3 were used per reaction. The fluorescein-tracer phospho-peptides (MAGPMQS[pT]PLNGAKK for PLK1, and GPLATS[pT]PKNG for PLK3) were used at a final concentration of 10 nM. Incubation was carried out at room temperature for 45 min on a shaker. Fluorescence was measured using either a DTX 880 plate reader and Multimode Analysis software (Beckman Coulter, now Molecular Devices, Brea, CA) or a SpectraMax i3 (Molecular Devices, Brea, CA). The polarization values in millipolarization (mP) units were measured at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. Each data point was performed in triplicate for every experiment, and experiments were performed at least three times. An IC$_{50}$ value for each compound was calculated from non-linear regression analysis of the plots of mP values relative to PBD-tracer mP values alone versus FLIP concentrations.

**Cell Culture**

HeLa cervical cancer cells and PC-3 prostate cancer cells were obtained from ATCC (Manassas, VA) and were not authenticated by the authors for this study. HeLa and PC-3 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO2 at 37 °C.

**Cell Viability Assay**

Cells were maintained in 10% FBS and cell proliferation assays were conducted in 10% Nu Serum. Exponentially growing cells were plated in 96-well plates for 24 hours prior to treatment with FLIPs for 72 hours. Following treatment, cell viability was
measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. Cells were incubated for 4 hours with MTT and absorbance at 595 nm was measured on a DTX 880 plate reader. IC_{50} values were generated from normalized absorbance data using GraphPad Prism.

**Western Blotting**

Cells were synchronized in prometaphase by treatment with 100 ng/ml nocodazole for 18 hours. Detached cells were collected, centrifuged and rinsed with PBS. Attached cells were also rinsed with PBS. Media containing test compounds was added to the cells and incubated for 23 hours. Cells were harvested, centrifuged, rinsed with PBS supplemented with protease and phosphatase inhibitors (# A32959 and A32961, Pierce, Rockford, IL, USA) and centrifuged again. Cells were suspended in RIPA buffer supplemented with protease and phosphatase inhibitors and then sonicated on ice. The lysate was shaken for 20 min at 4 °C then centrifuged at 12,000 rpm for 20 min. Supernatant was aspirated and the cell pellet transferred into fresh chilled tubes. Protein quantity was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Proteins (20 μg) were separated by 4–15% SDS-PAGE. Immunoblotting analysis was done using following antibodies: anti-PLK1 (#05–844, EMD Millipore, Temecula, CA, USA), anti-phospho-TCTP-Ser46 (#5251, Cell Signaling Technology, Danvers, MA, USA), anti-TCTP (#8441, Cell Signaling Technology), anti-β-tubulin (#NB600–936, Novus Biologicals, Centennial, CO, USA), mouse HRP-conjugated secondary antibody (#NA931, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and mouse HRP-conjugated secondary antibody (#NA934, GE Healthcare).
2.3 RESULTS

A previous study from the laboratories of Dr. McInnes and Dr. Wyatt [17] have reported initial SAR studies on peptides from the Cdc25C (LLCSpTPNGL) and PBIP (PLHSpTAI) phosphopeptide substrate motifs, two key PBD interacting proteins involved in mitotic regulation [12]. In order to further establish structure-activity relationships for the PBD binding sequence, a FLIP library was designed to probe the contributions of the N- and C-terminal residues of the recognition sequences from Cdc25C and PBIP in a systematic fashion and these compounds were tested in a fluorescence polarization (FP) assay to quantify competitive binding of phosphopeptides to the PBD domain of PLK1 [17]. Further to this, a similar assay format was developed for the PLK3 PBD to determine the selectivity of PBD inhibitors which has not previously been investigated in depth. Yun et al. looked at the binding of limited number of peptides to PLK2 by ITC but not PLK3[12a]. In the first instance, the binding of the fluorescein labeled tracer peptides used for both assays was evaluated to see if there was a difference in affinity for their respective PBD. A titration curve for each tracer/PLK PBD complex was generated to determine $K_D$ values for each and therefore compare the relative binding affinities. The PLK1 PBD fluorescent tracer was found to bind to the PLK1 PBD with a $K_D$ of 4.6 nM, while the corresponding value for the PLK3 binding tracer to the PLK3 PBD was determined to be 27.2 nM. These results demonstrate that the tracer peptide for the PLK1 PBD bound with a 5-fold higher affinity than the PLK3 tracer to its PBD thereby suggesting that this should be accounted for when comparing the selectivity of FLIPs and other drug-like PBD inhibitors. A selectivity index was calculated to provide novel insights into the true
selectivity of both compounds previously described and those generated in this study (Table 2.1).

The affinities of the FLIPs for PLK1 and PLK3 were subsequently measured by plotting the loss of polarization against increasing concentration of competitor FLIPs building on previous work through use of an optimized assay and selectivity determination [17].

Using REPLACE, low molecular weight fragments are computationally docked into the volume of a binding site known to interact with key peptidic determinants in order to identify more drug-like alternatives and iteratively convert a peptidic compound into a non-peptidic inhibitor [17–18]. Through use of the peptide SAR data, REPLACE was applied to identify fragment alternatives to the N-terminal tripeptide of Cdc25C. This resulted in the identification of PBD inhibitor peptide-small molecule hybrids that, when transfected into cells, recapitulate a PLK1 deficient phenotype [17]. These FLIPs were based on substituted benzamide capped peptides and demonstrated that modification of the 4 n-alkyl substituent contributed to binding with a hydrophobic slot observed in crystal structures of the PBD of PLK1[16a, 16b, 19].

Optimization of N-cap and truncation on residues C-terminal to the phosphothreonine

Previous studies reveal that the 4-position substituent increased potency in isosteric series[16c], further homologation of the n-alkyl series was the obvious next step in the SAR (Table 2.1). A FLIP series generated by lengthening the 4-n-alkyl substituent were then tested in the FP assay (19, n-butyl, IC50 = 2.5 μM; 20, n-hexyl, IC50 = 1.0 μM; 21, n-octyl, IC50 = 0.36 μM) and overall resulted in significantly improved affinity for the PLK1
PBD with increasing length of the alkyl substituent. Notably, the n-octyl derivative (21) was found to essentially recapitulate the activity of the native Cdc25C peptide (IC$_{50}$: 0.17 μM). Although 21 measurably bound to the PBD of PLK3 (IC$_{50}$ = 148 μM), it possesses a 2000-fold selectivity index and thereby retains high specificity for PLK1.

Based on the results for 21 generated as a PNGL capped FLIP, further C-terminal modifications were explored in the context of the octyl-benzamide fragment alternatives (Table 2.1). Since the peptide SAR results with 4 (0.064 μM) showed that the PNGL sequence could be replaced with two residues without significant potency loss, a 4-octylbenzamide capped S[pT]AI compound was constructed. As expected, this molecule (23, IC$_{50}$ = 0.41 μM) possessed very similar activity to 21 while retaining its selectivity (SI = 2000). Additionally, the consequences of further truncation were determined by deleting the C-terminal Ile residue. The resulting FLIP (22, IC$_{50}$ = 15.2 μM) containing a single alanine C-terminal to the phospho-Thr bound to the PLK1 PBD with a ~40-fold weaker affinity revealing a substantial potency loss, which was greater than expected based on the peptide SAR. The binding of 22 to PLK3 was measurable (IC$_{50}$ = 201 μM) and therefore had dramatically reduced selectivity compared to 21 (SI = 66). When the C-terminal AI of 23 was replaced with proline and leucine, the resulting FLIP (24) bound to both PLK PBDs with 3–4 fold weaker affinity and therefore retained selectivity for PLK1. The C-terminal amide version of 23 was synthesized to determine the impact of removing this charge. Compound 25 was found to have similar competitive binding activity. Furthermore, the n-nonylbenzamide FLIP was synthesized also with a C-terminal amide
(26) to further the n-alkyl chain SAR and the resulting assay determination showed equipotency to the octyl derivative (25).

**Cellular Activity of FLIPs**

Previously, phosphopeptides and FLIPs from this laboratory were demonstrated to have significant anti-proliferative activity and phenotypes consistent a PLK1 deficient phenotype [17]. PBD peptidomimetics have been shown to possess weak cellular activity and PLK1 phenotypes however require structural complexity through pegylation, histidine derivatization and/or masking of the phosphothreonine for this effect[16b–e].

With the increased activity in binding to the PBD and as the next generation FLIPs described here have greater logP values through addition of the 4-octylbenzamide capping group and decreased overall size (after deletion of two C-terminal residues), it was hypothesized that these next generation FLIPs may possess cellular activity without the use of a delivery agent. Accordingly, cell viability was measured using an MTT assay to determine the anti-proliferative activity for 23 and 24. As expected, despite still having peptidic composition and a negatively charged phosphothreonine, these FLIPs demonstrated cellular activity. FLIP 23 had anti-proliferative IC$_{50}$ values in Pten deficient prostate cancer (PC3) cells of 55.7 μM, in HeLa cells of 128.1 μM, and Kras mutant (A-549) lung cancers cells of 79.5 μM. The anti-proliferative activity of 24 in the same cells was approximately 2-fold lower yet clearly measurable and consistent with its decreased binding to PLK1 PBD. Interestingly removal of the charge on the C-terminus resulted in significantly increased cellular activity with up to a 3-fold increase being observed with the octyl (25) and nonyl (26) versions of 23 (Table 2.1). A non-phosphorylated version of
(Figure 2.3) was determined to have no significant anti-proliferative activity in line with known SAR dictating critical charge-charge interactions of the phosphate. To examine whether the FLIPs interact with and inhibit PLK1 in the cellular context, two different approaches were taken to determine engagement of the target and the anti-target PLK3. First, the stability of PLK1 and PLK3 in cellular extracts was determined by a thermal shift assay [20]. Briefly, the thermal shift assay measures the effect of ligands on the thermal stability of target proteins such as PLK1. A positive control dose response with BI-2536 showed an approximately 1.4-fold increase in stabilization of PLK1 against thermal denaturation across a dose range of 0.03 μM to 3.0 μM. Further data (Figure 2.1) shows that C-terminally amidated FLIP 25 stabilizes PLK1 with an EC₅₀ value of 0.17 μM. PLK3 stabilization was also measured, and an effect was seen, albeit to a lower level with the observed EC₅₀ of 0.58 μM. Interestingly FLIP 26 had increased engagement of PLK1 and decreased affinity for PLK3 as shown by the cellular thermal denaturation assay results (Figure 2.1). The non-phospho version of 26 as a negative control compound did not engage PLK1 to a significant degree (Figure 2.1). Next, the phosphorylation of a known direct target of PLK1, TCTP (translationally controlled tumor protein), was measured by Western blotting [21]. PC3 cells were synchronized by nocodazole (Noc) block then treated with 25, and p-TCTP was measured with a phosphospecific antibody. The results clearly demonstrate that with increasing dose of 25, decreasing levels of pTCTP are detected, while the total TCTP levels remain unchanged (Figure 2.2). The catalytic inhibitor BI2536 served as a positive control for catalytic PLK1 inhibition.
Peptides and particularly phosphopeptides are generally not active in cells due to their instability and lack of permeability. Previous PBD peptidomimetics have required extensive modification including pegylation, histidine alkylation and/or phosphothreonine masking to generate compounds with cellular activity [16d, 16e]. The two FLIPS that were optimized in terms of potency and decreased MW (23 and 24) displayed a measurable anti-proliferative effect without such modification or the use of or a drug delivery agent. The cells utilized were chosen due to reported synthetic lethal interactions with PLK1 inhibition (PC-3 Pten deficient, A549 K-RAS mutant) [24]. The hypothesis that neutralizing the negative charge on the C-terminus might improve cell permeability was proven by the increased potency of 25 and 26 on cells with a respectable level of activity for a phosphate containing molecule being observed. Confirmation of on target anti-proliferative activity was obtained through the a cellular thermal denaturation assay and revealed potent engagement of PLK1 at the cellular level. Interestingly the EC\textsubscript{50} for engagement was greater than the competitive binding observed in the FP binding assay suggesting that interaction with the isolated PBD was not reflective of the intact full length PLK1 protein. It also indicates that the FLIPs do not get into cells efficiently since the difference between the cellular IC\textsubscript{50} and the PLK1 engagement is 2 orders of magnitude. Furthermore, in the intact PLK context, the FLIPs appear to be somewhat less selective. Compound 25 had only 3-fold selectivity for PLK1 vs 3 in the a cellular thermal denaturation assay although 26 had considerably enhanced specificity. It is possible that its extra carbon in the alkyl chain prevents potent interaction with the hydrophobic groove of the cryptic pocket in the PLK3 context. As a negative control to further
determine the validity of these results, a non-phosphorylated version of 26 was found to have no appreciable anti-proliferative activity and also did not lead to significant stabilization of PLK1 in the a cellular thermal denaturation assay. The increased cellular potency also allowed further investigation of the FLIPs to examine PLK1 phosphorylation markers that are reflective of inhibition of the PBD. TCTP (translationally controlled tumor protein) is phosphorylated on serine 46 by PLK1 in vitro and this site has been validated as a direct readout of PLK1 [21a]. PLK1 phosphorylation of TCTP results in its nuclear localization and mitotic regulation through inducing an increase in microtubule dynamics. Western blotting analysis showed that treatment of PC3 cells with FLIP 25 resulted in a dose-dependent decrease in the amount of pTCTP thus confirming cellular inhibition of PLK1 activity. This observation is novel since it has not previously been shown that blocking the PBD results in inhibition of PLK1 mediated phosphorylation of TCTP.

2.4 DISCUSSION

In summary, this study further validates the use of the REPLACE method to develop more drug like small molecule PBD-inhibitors that have a high binding affinity for PLK1 while retaining selectivity against other PLKs including PLK3, a known tumor suppressor. It should be noted that this study is the first to experimentally measure and report PLK3 binding for peptidomimetics and explicitly compare PLK1 versus PLK3 selectivity both in vitro and at the cellular level. SAR in FLIP contexts demonstrate that both the N and C-termini contribute to the high selectivity of the PBIP peptide and that truncated compounds lose both potency and selectivity for PLK1. Cellular studies with the optimized FLIPs show significant anti-proliferative activity and potent on target
engagement thus providing impetus for medicinal chemistry efforts aimed at incorporation of further drug-like properties through REPLACE mediated optimization. Future studies will focus on further application of the REPLACE strategy to the C-terminus and to generating phosphate prodrugs of the FLIPs to improve potency and anti-tumor efficacy of the more drug-like PBD inhibitors.
2.5 TABLES

Table 2.1 PLK1 PBD *in vitro* binding and cellular activity for optimized FLIPs

<table>
<thead>
<tr>
<th>Compound</th>
<th>FLIP</th>
<th>Sequence</th>
<th>PLK1 PBD IC50 [µM]</th>
<th>PLK3 PBD IC50 [µM]</th>
<th>Selectivity Index</th>
<th>MTT IC50 [µM] HeLa</th>
<th>MTT IC50 [µM] PC-3</th>
<th>MTT IC50 [µM] A549</th>
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<td>19</td>
<td>4-butyl</td>
<td>S[pT]PNG</td>
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<td>&gt;2830</td>
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<td>148.8±38.2</td>
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<td>0.3±0.02</td>
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<td>27.0±6.4</td>
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2.6 FIGURES

Figure 2.1 Graph representing densitometry of the western blots of PLK1 and PLK3
Figure 2.2 Western blotting analysis of PC-3 cells following treatment with FLIPs. Cells were synchronized with nocodazole for 18 h prior to treatment with 25 or BI-2536 for 23 h. Cell lysates were harvested, separated by SDS-PAGE, then immunoblotted for TCTP, p-TCTP, and β-tubulin.

Figure 2.3 Anti-proliferative activity of FLIPs.
2.7 CHAPTER 2 REFERENCES


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CHAPTER 3

NON-PEPTIDIC, POLO BOX DOMAIN-TARGETED INHIBITORS OF PLK1 BLOCK KINASE ACTIVITY, INDUCE ITS DEGRADATION AND TARGET RESISTANT CELLS

ABSTRACT

PLK1, polo-like kinase 1, is a central player regulating mitosis. Inhibition of the sub-cellular localization and kinase activity of PLK1 through the PBD, polo-box domain, is a viable alternative to ATP-competitive inhibitors for which the development of resistance and inhibition of related PLK family members are concerns. We describe novel non-peptidic PBD binding inhibitors, termed abbapolins, identified through successful application of the REPLACE strategy and demonstrate their potent anti-proliferative activity in prostate tumors and other cell lines. Furthermore, abbapolins show PLK1-specific binding and inhibitory activity as measured by a cellular thermal shift assay and an ability to block phosphorylation of TCTP, a validated target of PLK1-mediated kinase activity. Additional evidence for engagement of PLK1 was obtained through the unique observation that abbapolins induce PLK1 degradation. Moreover, abbapolins target cells dramatically resistant to ATP-competitive PLK1 inhibitors [2].

3.1 INTRODUCTION

PLKs, polo like kinases, are a conserved family of Serine/Threonine kinases that regulate several key components of cell cycle and proliferation from yeast to humans.\textsuperscript{1,2} Five mammalian members of the PLK family (PLK1-5) each have a distinct expression pattern, physiological functions, and localization [1,3,4]. PLKs 1-3 have similar structures, namely a highly conserved N-terminal kinase domain and a C-terminal PBD, polo box domain comprised of two polo box motifs, whereas PLK4 possesses only one polo box [5]. PLKs 1-4 regulate cell cycle progression and response to stress but each member has distinct functions and cellular substrates [2,4,6]. PLK1 is the most characterized member and has several critical roles including regulating entry into mitosis, centrosome maturation, bipolar spindle formation, sister chromatin splitting, activation of APC/C, anaphase-promoting complex/cyclosome and cytokinesis [1,7]. Correspondingly, PLK1 localizes to key mitotic structures including centrosomes, kinetochores, the central spindle and midbody [8]. The PBD, which binds an STP, phosphoserine/phosphothreonine motif, is critical for its sub-cellular localization and for substrate recognition prior to phosphorylating target substrates [9–12].

PLK1 is highly expressed in many tumor types while its expression is minimal in surrounding normal tissues [13]. PLK1 inhibition genetically or pharmacologically can selectively kill PLK1-overexpressing cancer cells, which makes it an attractive target for therapeutic intervention [14–16]. In preclinical studies, cancer cell lines deficient in p53, RAS, and PTEN that also overexpress PLK1 were sensitive to PLK1 inhibition [15,17]. A large fraction of cancer patients have such genetic abnormalities, indicating that many
may benefit from PLK1 inhibition therapy. Moreover, PLK1 overexpression is an unfavorable prognosis, thus providing further validation as a therapeutic target [18].

Intense effort has been undertaken to develop PLK1 inhibitors, mostly ATP-competitive, and some have advanced to clinical trials. Lack of specificity for PLK1 versus PLK2 or PLK3 may present an explanation for the lack of clinical progress. For example, the most selective PLK1 kinase inhibitor, BI6727 (volasertib), also inhibits PLK2 and PLK3 activity (IC$_{50}$ values of 0.87, 5, and 56 nmol/L, respectively) [19]. This is problematic because PLK3 acts as a tumor suppressor via roles in the cell cycle and apoptosis in response to cellular stress [19]. Therefore, even partial inhibition of PLK3 could interfere with anti-tumor efficacy. In clinical trials, PLK1 catalytic inhibitors showed minimal anti-tumor activity and caused hematological effects that might be due to their lack of selectivity [20-21]. Second, catalytic inhibitors might not block substrate recruitment and subcellular localization functions of the PBD of PLK1. Third, a single point mutation (C67V) in the PLK1 kinase domain confers substantial cellular resistance against catalytic inhibitors [22]. While this has not yet been observed clinically, point mutants in other kinases are well known to cause therapeutic drug resistance.

While ATP binding sites are ubiquitous in (more than 500) known human kinases, the phosphorecognition motifs in the PBD are unique to the PLK family, making it a target for developing more selective inhibitors [5,23]. HeLa cells overexpressing the PBD of PLK1 arrested in mitosis and possessed chromosome congression defects [8]. Phosphopeptides bind selectively to the PLK1 PBD over other PLKs despite conservation of the PBD binding groove; thus, PBD targeting provides a promising strategy for blocking PLK1 functions.
Small molecule inhibitors of the PLK1 PBD have been identified through HTS (High Throughput Screening), high throughput screening [24–26]. Several of these turned out to be non-specific protein alkylators that have little or no potential to be PLK1 PBD specific inhibitors [27]. Other PBD inhibitors have been reported, although they generally lack evidence for direct engagement of PLK1 in cells and none have progressed beyond initial discovery [24,25,28].

REplacement with Partial Ligand Alternatives through Computational Enrichment, REPLACE, is a validated strategy for the iterative conversion of peptide inhibitors to generate pharmaceutically acceptable lead molecules and involves docking low molecular weight fragments into the vacated portion of a receptor after deleting critical residues of the peptide target molecule [29–32] (Figure 3.1A, Figure 3.7). Since these fragments contain functionality to chemically ligate them to the truncated peptide, peptide-small molecule hybrids (FLIPs, Fragment Ligated Inhibitory Peptides) are generated with the aim of recapitulating native binding of the peptide while converting it into a more drug-like compound. Phosphopeptides from Cdc25C (LLCSpTPNGL) and PBIP, Polo Box Domain-Interacting Protein, (PLHSpTAI) substrates of PLK1 were used as the basis for generating a library of FLIPs. Specifically, the N-terminal tripeptide of the Cdc25C sequence was replaced with drug-like capping groups resulting in partially peptidic potent and selective PLK1 inhibitors [30]. These compounds blocked PLK1 localization to centrosomes, displayed phenotypes consistent with PLK1 knockdown, and possessed significant antitumor activity [29-30]. Here, further application of REPLACE generated non-peptidic compounds named abbapolins, based on their chemical name (2-(4-AlkylBenzamido)
Benzoic Acid) and their action on PLK1. Abbapolins described here exhibit good affinity for the PLK1 PBD, engage endogenous PLK1, and show anti-proliferative activity against cells resistant to ATP-based inhibitors. They also reduce phosphorylation of TCTP, translationally controlled tumor protein, a direct marker of PLK1 kinase activity and block cell cycle progression. Strikingly, abbapolins also induce loss of PLK1 protein. These results demonstrate that targeting the PBD through the abbapolins is a novel approach for inhibiting PLK1.

3.2 METHODS

Fluorescent Polarization (FP) Binding Assay

In vitro binding activities of abbapolins for the PLK1 PBD (367-603) were analyzed essentially as described in: McInnes et al., 2012 (PMC3711794). Fluorescein-tagged tracer peptides MAGPMQS[pT]PLNGAKK for PLK1 were used at a final concentration of 10 nM. Tracers and PBD (#50301 BPS Bioscience Inc. San Diego, CA, USA) were titrated to generate a binding curve that was used to determine binding affinities. The PLK1 PBD bound to the PLK1 tracer with a dissociation constant (Kd) of 4.6 nM. The binding curve was used to determine EC90 values that were subsequently used for the abbapolin competition assays. FP values were measured using a DXT 880 plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Half Maximal Inhibitory Concentration (IC50) was calculated for each abbapolin by a nonlinear regression of inhibition (tracer displacement) vs. log concentration plot using GraphPad Prism 8 (GraphPad Software Inc.).
Cell Culture

HeLa cervical cancer cells (ATCC, Manassas, VA, USA) were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) Nu-serum (BD Bioscience, Franklin Lakes, NJ, USA) and 1% (v/v) penicillin/streptomycin (pen/strep) (#15140122, Thermo Fisher, Waltham, MA, USA) at 37°C, 5% CO2, and 95% humidity. PC3 prostate cancer cells (ATCC, Manassas, VA, USA) were maintained in Hams F12K (Cellgro, Manassas, VA, USA) supplemented with 10% Nu-serum and 1% pen/strep. RPE cells expressing wildtype or C67V PLK1 were kindly provided by Dr. Prasad V. Jallepalli (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The RPE cells were grown in 1:1 DMEM/Hams F-12 and supplemented with Nu-serum and 1% pen/strep at 37°C, 5% CO2, and 95% humidity.

Cell Viability Assay

Anti-proliferative activity of the test compounds was determined by MTT assay in human HeLa, PC3 and RPE cells expressing wildtype and C67V PLK1. Exponentially growing cells in 96 well plates were treated with the test compounds for 72 h then the cells were incubated with MTT (#158990050, Acros Organics, Morris Plains, NJ, USA) for 4 h. Absorbance of the metabolized MTT product was measured at 570 nm. IC50 values were determined by plotting the data relative to control-treated cells.

Immunoblotting

Cells were suspended in RIPA buffer (#N653, VWR, Solon Ohio, USA) supplemented with protease and phosphatase inhibitors and then sonicated on ice. The resulting cell lysate was shaken for 20 min at 4 °C and centrifuged at 12,000 rpm for 20
min. Supernatant was aspirated then transferred into fresh ice chilled tubes and total protein quantity was determined using the BCA protein assay (#23225, Pierce, Rockford, IL, USA (ordered via Thermo Fisher). Proteins (20 µg) were separated by 4-15% SDS-PAGE. Immunoblotting analysis was done using following antibodies: anti-PLK1 (#05-844, Millipore Sigma, Temecula, CA, USA), anti-phospho-TCTP-Ser46 (#5251, Cell Signaling Technology, Danvers, MA, USA), anti-TCTP (#8441, Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Histone H3-Ser10 (#05-806, Millipore Sigma), anti-β tubulin (#NB600-936, Novus Biologicals, Centennial, CO, USA), mouse HRP-conjugated secondary antibody (#NA931, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and mouse HRP-conjugated secondary antibody (#NA934, GE Healthcare). Immunoblotting was performed following manufacturer instructions. ECL Western Blotting Substrate and SuperSignal (#34095, Thermo Fisher) were used for detection.

**Flow cytometry analysis**

Exponentially growing PC3 cells were treated with abbaolins for 23 h. The cells were harvested, centrifuged, rinsed with ice-cold PBS/1% BSA, fixed with 70% ethanol and kept at -20 °C overnight. The fixed cells were centrifuged, rinsed with ice-cold PBS/1% BSA, centrifuged again, treated with 50 µg/ml propidium iodide supplemented with 100 µg/ml RNAse (#EN0531, Thermo Fisher) and incubated for 24 h at room temperature. Cell cycle quantification was performed using a FACS instrument (BD LSR II Franklin Lakes, NJ, USA).
**Cellular Thermal Denaturation Assay**

Thermal denaturation was performed with PC3 cells. Cells dispensed into 96-well PCR plates in growth medium (6000 cells/well/50 µl) for each experiment. Heat gradients were first performed to determine \( \text{Tagg}(50) \) values. Cells were subjected to a temperature gradient (38-60°C) for 10 min. Cold non-denaturing lysis buffer (PBS supplemented with 0.1% TritonX-100 and 1X protease inhibitors) was added to wells, the plate was rocked, then incubated for 15 min on ice. Subsequently, centrifugation was performed at 14000 rpm to sediment the insoluble protein content. Supernatant was collected, SDS-PAGE was performed, and immunoblotting was carried out using anti-PLK1 and anti-PLK3 antibodies. Bands were quantified on LI-COR C-Digit Blot Scanner. PLK1 and PLK3 displayed temperature-dependent decreases in solubility with \( \text{Tagg}(50) \) values of 56.2 and 48°C respectively. In subsequent experiments with small molecule inhibitors, cells were treated with increasing doses (0.03-3.0 µM) of inhibitors or with DMSO control for 2 h. Cells were then subjected to heat shock at the respective \( \text{Tagg}(50) \) for PLK1 or PLK3 for 10 min, and insoluble protein was removed by centrifugation. Following SDS-PAGE and immunoblotting, soluble PLK1 or PLK3 were quantified, normalized to loading control and plotted using GraphPad Prism software. EC\(_{50}\) values for inhibitors were calculated from the plots.

**PLK1 degradation and PLK1 inhibition effect on TCTP Ser46 phosphorylation**

Exponentially growing PC3 cells were treated with test compounds prepared in F12K/10% Nu and 1% pen/strep for 23 h. Cells were harvested, centrifuged, and rinsed with 1X PBS supplemented with protease and phosphatase inhibitors. Protein isolation,
quantitation and western blotting were carried out as described above under immunoblotting section. PLK1 protein bands were quantified using software (Biorad) and normalized to the β-tubulin loading control. Half maximal degradation concentration (DC_{50}) of abbpolins was determined by a non-linear regression of PLK1 degradation versus log concentration plot using GraphPad Prism 8 software. To determine TCTP phosphorylation, immunoblotting was carried out with the same protein lysate used to determine PLK1 degradation. Immunoblotting was performed with anti-TCTP, TCTP Ser46 and β-tubulin antibodies.

3.3 RESULTS

Identification of non-peptidic small molecule inhibitors of the Polo-Box Domain

Initial hits obtained through REPLACE contained a 4-ethylbenzamide capped peptide and recapitulated the PLK1 knockdown phenotype when transfected into cells [30]. These FLIPs were then optimized for binding to the PLK1 PBD, minimized in sequence, shown to engage PLK1 in cells, and possess anti-proliferative activity despite their partial peptidic character, which included the negatively charged phosphothreonine (Figure 3.1A) [29]. Crystal structures of peptides in complex with the PBD were used to propose SAR studies by derivatization of the 4-substituent. An enhancement of potency was observed with increasing chain length and a 4-octyl group was approximately 50-fold more potent than the initial ethyl substituent (Figure 3.1A) [29].

As a next step in the conversion of peptidic inhibitors into more drug-like molecules, REPLACE was further applied to accelerate conversion of FLIPs into non-peptidic PBD inhibitors (Figure 3.1A, Figure 3.9). Because 4-alkyl substituted benzoic acids were
effective replacements for the N-terminal tripeptide [29], this fragment was used as a template to identify compounds containing alternatives for the C-terminal residues, including the critical phosphothreonine (‘C-caps’). Substructure searching for 4-alkylbenzamide derivatives in commercial libraries revealed several thousand such compounds. A subset was then prioritized for computational prediction of binding if possessing a carboxylate moiety on the C-cap as a potential mimic for the phosphothreonine. After docking and scoring several hundred compounds, a set of ~20 were tested for PBD binding in a FP, fluorescence polarization assay, for their ability to compete with a fluorescently labeled PBD peptide. Abbapolin 1 was identified as a lead with reasonable PBD binding activity ($IC_{50} = 60.9 \mu M$) (Table 1) and possessed a 4-hexylbenzamide N-cap and 2-aminobenzoic acid as a potential mimic for the C-terminal peptide residues.

Based on SAR information established for the benzamide capping group in the FLIP context [29], further SAR exploration was undertaken (Table 3.1). The importance of the carboxylate as a phosphate mimic was confirmed when the methyl ester in two different abbapolins were synthesized (9 and 17) and found to be completely inactive for PBD binding (Table 3.1). Modeling of 1, 9 and 12 with the PBD was undertaken to investigate how the abbapolins successfully mimicked the interactions of the phosphopeptide (green, Figure 3.1B) and validated the structural hypothesis that the ortho-carboxylate forms complementary ion pairing interactions with Lys540 (residues interacting with phosphothreonine) confirming its role as a bioisostere for the phosphate group (Figure 3.1B, Figure 3.8). Moreover, docking studies suggested that the ester 9 could not occupy
the binding site as the binding mode could not accommodate the ester due to steric
lashes. The binding mode of the abbapolins is also further indicated by the similar
interactions of the peptides obtained from crystallography and the modeled FLIPs
previously studied. For the abbapolin series, a diverse set of analogs has been synthesized
and the resulting SAR describing investigation of alkyl chain tail length at R^5 (see Table
3.1) among other modifications to the C-cap will be described elsewhere. This included
analogs with meta and para carboxylates that were shown to be inactive (>600 μM),
进一步指示了正交羧酸酯与氮的必要贡献。在本研究中，进一步的SAR研究探索了
heteroatom
connections of the alkyl tail at R^5, homologation of this group and substitution of the C-
cap in these contexts, were carried out. Firstly, modification of the 2-aminobenzoic acid
C-cap by addition of a methyl group at the 4 or 5 position was found to significantly
increase binding activity in the FP assay and antiproliferative activity in two cell lines.
Abbapolin 7 methylated at the 5-position (R^3) had a 2-fold increase in cellular activity
relative to 4. Extending the alkyl tail from C_9 to C_10 improved the affinity for the PLK1 PBD
from 33.8 μM down to 23.8 μM; however, increasing chain length to C_12 (15) provided no
further benefit (Table 3.1). The equipotency of 14, 15, 16 and 18 suggest that the
hydrophobic groove of the PBD is fully occupied by the 10-carbon alkyl chain.
Confirmation of the critical role of the alkyl tail was further demonstrated by the loss of
all binding for the analog with the free amino group at the 4-position of the benzamide
N-cap in the 4-methyl, 2-aminobenzoic acid C-cap context (22, >600 μM). To explore
synthetic versatility and improve solubility based on prior experience in the FLIP context
[29], the heteroatom linking the alkyl tail was varied. Thioether, ether and alkylamino derivatives were generated (in addition to the straight alkyl chain) with both 7 and 8 carbon groups. The potency order for these in binding to the PBD was S>C, O>N. (compare 2-4, 5-7, 8, 10-12, 16, 19-21). The thioalkyl containing compounds had improved binding and demonstrated an almost 2-fold increase in cellular activity relative to the others (e.g., 12, 15 μM). To lower the pKa of the carboxylate group and potentially enhance its ionic interactions with the side chain of Lys540 (Figure 3.8), the CH\textsubscript{3} group at R\textsuperscript{3} (IC\textsubscript{50} = 34.5 ± 3.7 μM) was replaced with an electron withdrawing CF\textsubscript{3} group (13, IC\textsubscript{50} = 23.5 ± 4.9 μM), which resulted in an improved affinity and cellular activity (Table 3.1).

**Cellular engagement of PLK1 by abbapolins**

Having confirmed that abbapolins interact with the PLK1 PBD as measured by the FP competitive binding assay, engagement of cellular full length PLK1 by these inhibitors was assessed using a cellular thermal denaturation assay [33]. Thermal melting curves were first generated for cellular PLK1 from PC3 tumor cell extracts and an T\textsubscript{agg}(50), aggregation temperature, of 56.2°C for PLK1 was calculated. Asynchronous PC3 cells were treated with increasing concentrations of abapolin 12 and the inactive ester 17 for 2 h followed by heat shock at the T\textsubscript{agg}(50) (Figure 3.2 and Figure 3.6A and B). Abbapolin 12 bound to PLK1 with an EC\textsubscript{50} 0.65 μM to PLK1 (Figure 3.2). Compound 17 showed no significant effect on PLK1 thermal solubility (Figure 3.6B), consistent with the reduced affinity for the PBD determined by the FP assay. These results provide evidence that abapolin 12 binds to full length PLK1 in its cellular context, thus corroborating the data obtained for binding to the PBD domain *in vitro.*
**Abbapolins block PLK1 phosphorylation of TCTP**

The phosphorylation status of TCTP, translationally controlled tumor protein, a direct PLK1 substrate, was evaluated in cells treated with abbapolins. TCTP is phosphorylated by PLK1 and immunodetection of p-TCTP on Ser46 is a validated marker of PLK1 kinase activity in cells \[^{34}\]. Treating asynchronous PC3 cells with increasing doses of 12 for 23 h eliminated detectable p-TCTP, while not affecting the total amount of TCTP (Figure 3.3, left four lanes). TCTP phosphorylation reaches maximal levels in mitosis, so a colcemid co-treatment was also performed to determine the effect of the abbapolin on the ability of PLK1 to phosphorylate TCTP in mitotic cells.

In the presence of colcemid, treatment with 12 abolished p-TCTP (Figure 3.3). In contrast, p-TCTP levels were unaffected by treatment with the ester 17 (data not shown), which did not detectably bind to PLK1 in the FP assay (Table 3.1). Collectively, the results demonstrate that abbapolins can prevent PLK1 from phosphorylating one of its direct downstream targets, thus providing corroborating evidence of PLK1 inhibition in cells.

**Abbapolins induce PLK1 degradation**

In determining the phosphorylation status of TCTP, we noticed a striking, dose dependent loss of PLK1 protein. A hydrophobic tagged PLK1 inhibitor was recently described that caused degradation of PLK1 protein in cells \[^{35-36}\]. Since abbapolins contain a hydrophobic alkyl tail, their effects on PLK1 degradation were examined through western blotting. Treatment of asynchronous PC3 cells with 12 led to a striking, dose dependent reduction in PLK1 protein levels (Figure 3.5A, B) whereas the inactive ester analog 17 did not reduce PLK1 protein (Figure 3.5C). To determine whether the loss
of PLK1 occurred in mitosis, colcemid co-treatment was performed. In colcemid-treated cells, higher PLK1 protein is seen (Figure 3.5A, rightmost lane) compared to asynchronous cells (leftmost lane), but again 12 caused a dose dependent loss of PLK1 protein down to almost undetectable levels at a dose of 45 µM (quantitation shown in Figure 3.5B). A DC$_{50}$, 50% degradation concentration, value of 13 ± 1.7 µM for 12 was determined, whereas 17 caused no detectable loss of PLK1 up to 60 µM. It is noteworthy that the concentration of 12 required for 50% cell death and 50% intracellular PLK1 degradation are remarkably similar (PC3 IC$_{50}$ = 15 ± 2 µM, PLK1 DC$_{50}$ = 13 ± 1.7 µM). As further validation, treatment of a different prostate cancer cell line, DU145, with 12 also led to a dose dependent reduction in PLK1 protein levels (Figure 3.5D).

The finding that abbaopolins cause a striking decrease in PLK1 protein levels suggests that the alkyl tail on the abbaoplin is responsible for induction of proteasomal degradation similar to previously described hydrophobic tagging [35-36]. To explore the mechanism of PLK1 loss caused by abbaopolins, co-incubation with the proteasome inhibitor MG132 was carried out. Figure 3.5E shows that MG132 co-treatment increases observed PLK1 by approximately half. Specifically, PLK1 levels in PC3 cells treated with 10, 30, and 45 µM of 12 were 30, 5 and 7% (compared to untreated) respectively, and when combined with 15 µM MG132 were 64, 25 and 16% respectively. The data demonstrate that the proteasome is at least partially responsible for degradation of PLK1 following abbaoplin treatment. The observation that the uncharged ester 17 had no effect on PLK1 levels demonstrates that PLK1 loss requires a binding interaction with the PBD and excludes the possibility of a non-specific effect resulting from the alkyl tail alone.
Anti-proliferative and cell cycle effects of abbapolins

Cancer cells harboring mutations in p53, RAS, and/or PTEN overexpress PLK1 for survival and inhibition of PLK1 induces apoptosis in these cancer genetic backgrounds [14-15,37]. The anti-proliferative activity of abbapolins was determined in PC3 (p53 and PTEN mutant) and HeLa (inactivated p53) cell lines (Table 3.1). Abbapolins showed somewhat better anti-proliferative activity against HeLa cells compared to PC3 cells and the activity was highly correlated between the two cell lines (Pearson r: 0.96, P value: <0.0001) (Figure 3.54A). Similarly, abba
polin in vitro binding affinities for the PLK1 PBD in the FP assay were highly corelated with anti-proliferative activities in PC3 (Pearson r: 0.74, P value: 0.0001) and HeLa (Pearson r: 0.71, P value: 0.0094) cells (Figure 3.11B and 3.11C). Collectively the results from measuring cellular activity agree with the modeling and in vitro PBD binding results, namely that increasing the length of the N-cap 4-position alkyl group length led to improved anti-proliferative activity (compare abbapolins 8, 14 and 15). Evaluation of the heteroatom series indicated that the thioalkyl derivatives have the best cellular potency. Importantly, two uncharged ester derivatives tested (9 and 17) were inactive in both the FP and cellular assays, providing evidence for on target inhibition of PLK1 through the PBD.

PLK1 is required for cell cycle progression [38]. Cell cycle profiles of asynchronous PC3 cells treated with 12 and 17 alone were first analyzed (Figure 3.10). Cells treated with increasing doses of 12 (Figure 3.10, columns 2-5) showed a very similar profile to the untreated cells, initially indicating that 12 treatment did not induce a striking cell cycle phase-specific arrest. This result was unexpected, given the anti-proliferative activity of
the abba-polins and the anticipation of a mitotic specific effect reminiscent of catalytic inhibition. To further explore the effect of abba-polins on the cell cycle, cells were treated in combination with colcemid. The combined treatment resulted in a substantial decrease in the G2/M population from 67% for colcemid alone down to 20% at the highest dose of 12 (Figure 3.10, columns 6-10), indicating that the abba-polin treatment halted progression throughout the cell cycle. In contrast, the inactive ester analog 17 did not affect cell cycle progression at the highest dose of 60 µM tested regardless of colcemid addition (Figure 3.10, rightmost columns). The data suggest that loss of PLK1 protein caused by abba-polin treatment, at least in this cell line, has effects distinct from the mitotic arrest seen with catalytic inhibitors of PLK1 that stabilize the protein.

PBD-targeted abba-polins overcome resistance to ATP competitive inhibitors in a PLK1 point mutant cell line

A point mutation in the kinase domain of PLK1 conferred substantial resistance to several structurally unrelated kinase inhibitors including BI2536 [22]. The previously described retinal pigment epithelial cells were obtained and used to test abba-polin activity in this model of resistance to catalytic inhibitors of PLK1. RPE cells expressing WT PLK1 were sensitive to BI2536 ($IC_{50} = 27.1 \pm 1.8$ nM) while cells expressing mutant PLK1 (C67V) showed striking resistance to the catalytic inhibitor ($IC_{50} > 600$ nM) consistent with prior observations (Figure 3.4A) [22]. In contrast, RPE cells expressing WT or C67V PLK1 showed equal sensitivity to the PBD-targeted abba-polins 7 and 15 (Figure 3.4B). The inactive ester 17 had no anti-proliferative effect at concentrations up to 120 µM when tested in RPE cells expressing WT or mutant PLK1 (Figure 3.4B).
3.4 DISCUSSION

Small molecule inhibitors targeting the PBD of PLK1 and thus non-ATP competitive are a potentially compelling alternative to catalytic-based inhibitors. REPLACE, a strategy for targeting protein-protein interactions, has been successfully applied to iteratively convert potent phosphopeptides into non-peptidic and drug-like PBD inhibitors. Abbapolins do not contain reactive groups capable of covalent modification and do not share chemical features with any previously reported PLK1 PBD inhibitors [27]. In addition to the pThr binding site, the PBD also possesses a cryptic hydrophobic pocket that can act in concert with the pThr site to specifically recognize a subset of PLK1 substrates [39-40]. For certain substrates, Trp414 and Leu490 of the cryptic pocket are critical for substrate recognition via nonpolar interactions whereas His538 and Lys540 of the pThr binding cavity directly interact with phosphates on Thr residues of its substrates [41-42].

Interpretation of structure-activity data, known peptide and peptidomimetic crystallographic structures and computational modeling of the abbapolins indicate that the 2-amino benzoic acid C-cap effectively mimics the interaction of the phosphate (Figure 3.1B) thereby demonstrating its isosteric role in interacting with the positively charged side chain of Lys540 of the PBD. Docked structures do not, however, show evidence of interactions with His538 as observed in the phosphopeptide bound structures, potentially explaining the reduced affinity of the abbapolins compared to these. Stabilizing the carboxylate by introducing an electron withdrawing CF₃ group (13) improved affinity for the PLK1 PBD, allowing more favorable ion-pairing interactions as expected (Table 3.1). Replacing the carboxylate with a methyl ester abolished the
interaction with the PLK1 PBD and all cellular activity (9 and 17, Table 3.1). Adding a methyl substituent to the 2-aminobenzoic acid C-cap at the 4 (R^4) and 5 (R^3) position resulted in a significant potency increase (compare 4 with 7) through improving complementarity with pocket unoccupied in the PBD peptide – PLK1 complex (Figure 3.1B).

Computational and structural analyses demonstrate that the hydrophobic alkyl tail of abbbapolins effectively interacts with the cryptic pocket (Figure 3.1B) in line with the increased affinity and cellular potency obtained by extending its length from up to C_{10} (10 carbons). Further lengthening the alkyl chain to C_{12} did not enhance affinity (Table 3.1), an observation consistent with modeling showing that the tail exits the hydrophobic groove beyond 10 carbons in the chain. The increased cellular activity of this analog most likely results from enhanced cell permeability. Similar results were obtained in the FLIP context (potency: C_{9}>C_{8}>C_{6}>C_{4} for PLK1 PBD binding) [29] providing further confidence that the abbbapolins recapitulate binding of phosphopeptides and partially peptidic PBD inhibitors.

Introduction of heteroatoms connecting the hydrophobic tail to the 4-substituted benzamide Ncap was undertaken to provide synthetic versatility and improve solubility. The order of potency for the PLK1 PBD was S>C=O>N (Table 3.1) in the abbbapolin series, where the potency of 12 both in the binding assay and in cells increased compared to its C_{9} analog 8. The interactions of the heteroatom are similar in each context suggesting that different conformational preferences lead to increased activity.
Abbapolins bound to full-length cellular PLK1 as determined by cellular thermal denaturation (Figure 3.2) in a pattern consistent with binding affinities measured by FP assay *in vitro*. Considering potency for the PLK1 PBD in the FP assay and anti-proliferative activities in cells, abbaopolin 12 (PLK1 PBD FP IC_{50} = 26.8 ± 5 µM, PC3 IC_{50} = 15 ± 2 µM) was chosen for detailed phenotypic studies in cells. The PLK1 PBD recognizes TCTP prior to the phosphorylation event, primarily at Ser46 [34]. P-TCTP was shown to appear at the G1/S transition, peak during G2 and then decline after telophase, which mirrors PLK1 levels [43]. Overexpression of TCTP mutants incapable of being phosphorylated caused mitotic catastrophe-associated phenotypes [34] consistent with phenotypes observed in mitotically arrested cells injected with anti-PLK1 antibody [44]. Abbapolin 12 produced a dose dependent reduction in p-TCTP signal without affecting total levels of TCTP (Figure 3.3). These observations confirm that abbaopolin treatment disrupts cellular PLK1 activity and moreover that p-TCTP can be used as a direct marker for evaluating PLK1 inhibition in cells and in xenograft tumors [43,45].

Abbapolin 12 led to a significant, dose dependent reduction in PLK1 protein levels irrespective of cell cycle status, a result further validated by lack of this effect with the inactive ester analog 17 (Figure 3.5). Striking loss of PLK1 protein may be related to incompletely understood mechanisms through conformational changes in PLK1 that influence its activity and stability. PLK1 is regulated by autoinhibition through reduction in flexibility of the IDL, inter domain linker, or sequestration of the activation loop [46]. In mitosis, phosphorylation at Thr210 or Ser137 of PLK1 by Aurora A kinase is believed to relieve the autoinhibition between the KD, kinase domain, IDL and PBD through
conformational effects. Binding of a phosphopeptide substrate to the PBD can also open
the autoinhibited state of PLK1 [47]. Recent studies have suggested that the PBD and the
kinase domain allosterically regulate each other [46,48]. Studies by Zhu et al. [49]
established that the drosophila protein PON, adaptor Partner of Drosophila Numb, in its
phosphorylated state induces human PLK1 PBD dimerization upon binding and this
relieves PLK1 autoinhibition. Evidence for dimeric PLK1 includes a crystal structure of a
PLK1 homodimer and where Phe60 of Pon interacts with Leu505 of the PLK1 PBD. This
interaction stabilizes dimeric PLK1 (Figure 3.7) and a point mutation at position 505
(L505E) decreased PLK1 PBD dimerization induced by the phospho Pon peptide [49].

Docking studies of abba polins with the PBD putatively suggest that the
hydrophobic tail would block this PLK1 PBD dimerization (Figure 3.7). In the dimer
structure, L505 interacts with the same cryptic hydrophobic groove into which the 4-
position alkyl chain of the abba polin fits. In fact, the abba polin binding mode is
incompatible with dimer formation because the L505 of the dimeric PLK1 PBD occludes
the last three carbons of the alkyl tail from inserting into the cryptic pocket (Figure 3.7).

Therefore, dramatic loss of PLK1 induced by abba polin treatment is hypothesized
to occur because an abba polin-bound PLK1 monomer would not be able to dimerize. Also,
monomeric PLK1 may be less stable since the destruction box motif (R337 located
between the PBD and KD, which is not visible in the dimer crystal structure) is likely to be
more exposed. Data in this study supports this hypothesis as MG132 partially blocks the
loss of PLK1 caused by the abba polins. It was reported that a PBD-targeting compound,
Poloxin-2 coupled to an adamantane group, induced PLK1 proteasomal degradation and
resulted in PLK1-deficient cellular phenotypes and anti-proliferative activity [35-36]. These molecules are more complex than the abbbapolins and also are based on a redox active PBD inhibitor shown to non-specifically alkylate proteins. Hydrophobic tagged small molecule inhibitors that bind to a target protein induce a partially unfolded state of the target protein or can mimic a partially denatured protein and the resulting increase in hydrophobicity can make proteins unstable and targets of proteasomal degradation [50]. Data from treatment with abbbapolins alone and in combination with MG132 suggest that abbbapolins are promoting the degradation of PLK1 by one or more potential mechanism: 1) by interfering with dimer formation, 2) by inducing a partially unfolded state, 3) by interfering with intramolecular interactions between the PBD and its catalytic domain [46,48].

A major issue with clinically evaluated kinase inhibitors is that of drug resistance through point mutation [51-52]. Burkard et al. demonstrated that a single point mutation (C67V) within the ATP-binding domain of PLK1 confers resistance to several structurally unrelated inhibitors, including BI2536 [22]. Abbbapolins possessed similar levels of anti-proliferative activity against cells expressing the catalytically active C67V PLK1 as with cells expressing wild-type PLK1 (Figure 3.4B), despite the mutant-expressing cells being dramatically resistant to BI2536 (Figure 3.4A). These results suggest that PLK1 PBD inhibitors can be used against tumors that acquire resistance to catalytic inhibitors and as combination therapy to prevent the emergence of resistant tumors.

Since ATP competitive inhibitors of PLK1 have underperformed in clinical trials, new therapeutic options for blocking activity of this validated mitotic target are required. The
ability of abbapolins to engage cellular PLK1, to exert potent anti-proliferative activity, and to degrade PLK1 suggest that these are mechanistically unique and drug-like compounds. Future studies will further investigate the mechanism of degradation, and in vivo studies will provide insights into their pharmacokinetic and pharmacodynamic properties. As such the abbapolins have great potential for not only therapeutic development but also as chemical biology probes to better elucidate the roles of PLK1 and its substrates.
### 3.5 TABLE

**Table 3.1 Structure activity of 2-(4-alkylbenzamido) benzoic acid PBD Inhibitors.** PLK1 PBD IC$_{50}$ = IC$_{50}$ value calculated from *in vitro* FP assay. PC3 MTT IC$_{50}$ = IC$_{50}$ value for viability in PC3 prostate cancer cells. HeLa MTT IC$_{50}$ = IC$_{50}$ value for viability in HeLa cervical cancer cells.

![Chemical structure](image)

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3.6 FIGURES

Figure 3.1 Conversion of phosphopeptides into non-peptidic inhibitors by REPLACE (A) An iterative process used to identify abbapolins. The N-terminal capping group replacing Leu-Leu-Cys was identified by applying REPLACE to this tripeptide. After optimization of the Ncap, a substructure search for 4-alkyl benzamide containing molecules with carboxylate groups was used to identify non-peptidic PBD inhibitors. The 2-aminobenzopic acid C-cap was further optimized by addition of a methyl or trifluoromethyl group at the 5-position (R³, Table 3.1). (B) Comparison of the PBD binding mode of 12 (modeled using 3RQ7) with that of the Cdc25C phosphopeptide (3BZI).
Figure 3.2 Abbapolin 12 binds to full length cellular PLK1 in PC3 cells. Asynchronous PC3 cells were treated with abbrev 12 for 2 h and then subjected to heat shock at the Tag (50) (56.2°C for PLK1) for 10 min. (A) Soluble proteins were collected via centrifugation and then analyzed via Western blotting. (B). Quantification of gels plotted as fold change in stabilization compared to DMSO vehicle control-treated cells.

Figure 3.3 TCTP phosphorylation in cells treated PLK1 inhibitors. Asynchronous PC3 cells were treated with 12 alone, and in combination with colcemid to induce a metaphase arrest, for 23 h. A clear reduction of TCTP-pSer46 was observed while no loss of TCTP or Beta-tubulin protein occurred.

Figure 3.4 Cell viability for RPE cells expressing WT and C67V PLK1. (A) RPE cells treated with PLK1 kinase inhibitor BI2536. (B) RPE cells treated with abbrevs that target the PLK1 PBD.
Figure 3.5 PLK1 degradation in prostate cancer cell lines treated with abba polins (A). PC3 cells were treated with 12 alone and in combination with colcemid for 23 h. (B) Quantitation of data shown in A. (C) PLK1 degradation was not seen after treatment with 17 in PC3 cells. (D) DU145 cells were treated with 12 alone and in combination with colcemid for 23 h. Mean values and standard deviations were from three independent experiments. (E) PLK1 protein degradation induced by abba polins is partially blocked by MG132. PC3 cells treated with 12 for 23 h followed by immunoblotting with anti-PLK1 antibody. In the right-hand lanes, PC3 cells were treated with 15 µM MG132 for 1 h prior to 23 h co-treatment with 12 and MG132.

Figure 3.6 Affinity of abba polins 17 with full length cellular PLK1. Asynchronous PC3 cells were incubated with abba polins 17 for 2 h and then subjected to heat shock at the $T_{agg}$ (50) (56.2°C for PLK1) for 10 min. (A). Soluble proteins were collected via centrifugation and then analyzed via Western blotting. (B). Quantification of gels plotted as fold change in stabilization compared to DMSO vehicle control-treated cells.
Figure 3.7 Abbapolins could prevent formation of the putative PLK1 dimer. Results shown for docking of abbapolin 12 with the PLK1 monomer which was then overlaid on the PLK1 dimer structure. One monomer is shown as a ribbon diagram and the other as a molecular surface representation. The side chain of L505 is highlighted in blue, abbapolin 12 is colored with green carbon atoms and the phospho PON peptide is shown with orange carbons.

Figure 3.8 Binding mode obtained by docking abbapolin 12 into the PBD. The interacting residues of PLK1 are numbered and the non-bonded interactions are color coded.
Figure 3.9 Schematic illustration of REPLACE used for discovery of the Abbapolins

Figure 3.10 Flow cytometry analysis of PC3 cells treated with abbapolins 12 or 17. Mean values and standard deviations were from three independent experiments. The cell cycle distribution of untreated asynchronous cells was G1= 63 ± 7 %, S = 16 ± 4 %, G2/M=19 ±2% and Sub G1= 0.3 ± 0.1 % and served as the comparison for all treated samples.
Figure 3.11 Correlation of *In vitro* PBD binding with anti-proliferative activities. In each plot, a Pearson correlation coefficient was calculated. (A) Plot of anti-proliferative activities of individual abbapolins in HeLa cells (y-axis) versus in PC3 cells (x-axis). Pearson correlation coefficient $r$: 0.96, $p$ value: <0.0001. (B) Plot of anti-proliferative activities of individual abbapolins in PC3 cells (y-axis) versus *in vitro* PLK1 PBD binding affinities (x-axis). Pearson correlation coefficient $r$: 0.74, $p$ value: 0.0001. (C) Plot of anti-proliferative activities of individual abbapolins in HeLa cells (y-axis) versus *in vitro* PLK1 PBD binding affinities (x-axis). Pearson correlation coefficient $r$: 0.71, $p$ value: 0.0094. Anti-proliferative activities of abbapolins in PC3 and HeLa cells were highly correlated. PLK1 PBD binding affinities in the FP assay were highly correlated with anti-proliferative activities in PC3 and HeLa cells.
3.7 CHAPTER 3 REFERENCES


CHAPTER 4
DIFFERENTIAL CHANGES IN PLK1 CONFORMATION AND INTRACELLULAR STABILITY INDUCED BY ATP COMPETITIVE AND NOVEL NON-COMPETITIVE ABBAPOLIN INHIBITORS

ABSTRACT

Polo Like Kinase 1 (PLK1) is an essential protein kinase with multiple roles in mitotic progression, and more recently with non-mitotic roles. PLK1 consists of its kinase domain (KD) and the polobox domain (PBD), which is a phosphopeptide-binding domain unique to the PLK family and responsible for protein substrate recognition and subcellular localization. Early models of PLK1 regulation proposed an autoinhibitory conformation in which the KD and PBD interact, and that phosphorylation of a hinge region to produce mitotically active PLK1. Subsequent proposed models invoke PLK1 dimer formation in the presence of different protein partners that inhibit or promote PLK1 activity. Our previous work identified PBD binding molecules termed abbpapolins, which bind to PLK1 in vitro and intracellularly, and inhibit cellular phosphorylation of a known PLK1 target. Moreover, abbpapolins uniquely induce the loss of intracellular PLK1. Here, we explored the binding of PBD-targeted abbpapolins compared to ATP-catalytic molecules with in vitro and cellular assays to gain better insight into the regulation of PLK1.

3 Danda Chapagai, Campbell McInnes and Michael D. Wyatt, “Differential changes in PLK1 conformation and intracellular stability induced by ATP competitive and novel non-competitive abbpapolin inhibitors”. (Manuscript In preparation).
Utilizing a thermal shift assay to measure intracellular PLK1 binding, abبابولینs produce the expected increase in soluble PLK1, suggesting a stabilization of the PLK1 structure. In contrast, KD-binding inhibitors produce an unexpected decrease in soluble PLK1, suggesting that ATP binding causes a less thermally stable PLK1 conformation. In vitro binding measurements with purified full length PLK1 were consistent. Interestingly, the cellular consequences of ATP versus PBD binding dramatically contrast, with ATP-binding causing an increase accumulation of intracellular PLK1 over 24 hours, whereas PBD-binding produces a striking loss of nuclear PLK1 as measured by blotting and fluorescence. Collectively, the results highlight a previously under appreciated aspect of targeting PLK1, the consequences of conformational perturbations induced by PBD versus ATP binding.
4.1 INTRODUCTION

Mammalian Polo Like Kinase 1 (PLK1) is a Serine/Threonine kinase that regulates several key components of the cell cycle and proliferation [1, 2]. PLK1 performs multiple roles including regulating entry into mitosis, centrosome maturation, bipolar spindle formation, separation of sister chromatids, activation of the anaphase-promoting complex/cyclosome (APC/C) and cytokinesis (1, 3). PLK1 is overexpressed in many tumor types compared to normal controls and its overexpression correlates with poor prognosis. Accordingly, there has been much interest in developing inhibitors of PLK1, predominantly by targeting the highly conserved Kinase domain (KD) of PLK1 [3, 4]. The most advanced molecule in the clinical setting is volasertib (BI6727), which did not meet the primary endpoint of objective response in a Phase III study for adult acute myeloid leukemia, but more recently received orphan drug designation from the U.S. FDA as a potential treatment for rhabdomyosarcoma. The equivocal activity of ATP-competitive catalytic inhibitors has spurred interest in alternative means to target PLK1 therapeutically.

The substrate specificity of PLK1 for its protein targets is mediated by the C-terminal Polo Box Domain (PBD), which is a Ser-pThr phospho-recognition motif. In the time since the crystal structure of the PBD domain was published [5], there has been much interest in the PBD to better understand its subcellular localization and binding partners, and as a means to improve selectivity in targeting PLK1 compared to the highly related PLK2-4 family members [6, 7]. There has also been interest in understanding intramolecular interactions between the KD and PBD related to the regulation and
stability of PLK1 [8-10]. For example, *in vitro* biochemical and biophysical studies showed that the Drosophila protein phospho-Pon (adaptor Partner of Drosophila Numb), induces human PLK1 PBD dimerization upon binding [11]. An interaction between Phe60 of Pon and Leu505 of the PLK1 PBD was proposed to stabilize dimeric PLK1, and the dimerization induced by the phospho-Pon peptide was lost by a single point mutation at position 505 (L505E) [11]. With regards to PLK1 recruitment to kinetochores, a recent study indicated that phospho CENP-U (the PLK1 receptor in the kinetochore core) promotes PLK1 docking through PLK1 dimerization [12]. However, the mechanism of PLK1 dimerization and its contribution to the spatiotemporal regulation of PLK1 in G2 and mitosis remains somewhat controversial.

PLK1 kinase activity is tightly controlled during the cell cycle, and its expression is almost completely absent in non-cycling cells [13]. PLK1 protein levels increase in G2 and peak in mitosis. During G2, PLK1 localizes to centrosomes and kinetochores. One model proposes that in G2 the Aurora-A kinase co-ordinates with its co-factor Bora to phosphorylate centrosome-localized PLK1 in its activation loop (T210), which is important for timely entry into mitosis [14]. Yet interestingly, Bora exclusively localizes in the cytoplasm throughout interphase and its degradation is induced approximately two hours before cells enter mitosis [15]. Other studies have implicated monomeric PLK1 as the active mitotic form. Raab *et al.* performed elegant experiments with chromosomally integrated (non-ectopically expressed) PLK1 to demonstrate that Bora transiently promotes PLK1 dimerization early in G2, whereas T210 phosphorylation by Aurora-A initiates dissociation of the dimer [16].
The interactions between the PBD and KD are implicated in a mechanism of autoinhibition of PLK1 that produces a closed/inactive conformation. Phosphorylation at T210 or Ser137 of PLK1 by Aurora-A coordination with its cofactors disrupts the intramolecular interactions between the PBD and KD, resulting in an opened/active conformation [9, 10, 17, 18]. PBD binding to a phosphorylated target substrate can also relieve the autoinhibition [5, 9]. One study measured the allostery communication between the PBD and KD, notably by using small molecule probes with different PLK1 binding interactions [9]. One observation of particular interest was that small molecules that bind to the KD domain, including the catalytic inhibitor BI6727, also appeared to promote an open conformation of PLK1 in vitro. KD domain binders that did not bind to the active site thus relieved the autoinhibition of the KD, whereas small molecule binders of the PBD domain stabilized the PBD-KD interaction and thus inhibited PLK1 activity [9].

We recently reported the development of a unique class of small molecule inhibitor of the PBD domain, termed abbpapolin [19, 20]. Abbpapolins were found to specifically bind to the PBD of PLK1 in biochemical and cellular assays. We also made the novel observation that abbpapolins upon binding to PLK1 induced its intracellular loss in a mechanism at least partially dependent on the proteasome [19]. Spurred on by this unique mechanism of action, we initiated studies on the interactions of catalytic inhibitors and PBD-binding abbpapolins with PLK1 in vitro and in a cellular context. Here, we report novel findings regarding the intracellular conformational changes of PLK1 during mitosis, namely that catalytic binding by BI2536 or BI6727 induce an unanticipated conformational change in PLK1 that destabilizes the PLK1 structure as measured by a
cellular thermal shift assay ([21]) and causes PLK1 to accumulate in mitotic cells. In contrast, abbbapols stabilizes the structure of PLK1 \textit{in vitro} and as measured by thermal shift, in a manner that ultimately results in its cellular degradation.

4.2 METHODS

Cell Culture and Immunoblotting

PC3 prostate cancer cells (ATCC, Manassas, VA, USA) were maintained in Hams F12K (Cellgro, Manassas, VA, USA) supplemented with 10% Nu-serum and 1% pen/strep. Immunoblotting was carried out as described before [19]. Briefly, protein was quantified using the BCA protein assay (#23225, Pierce, Rockford, IL, USA). Proteins (20 µg) and separated by 4-15% SDS-PAGE. Immunoblotting utilized the following antibodies: anti-PLK1 (#05-844, Millipore Sigma, Temecula, CA, USA), anti-Phospho-PLK1 (Thr210) Antibody (#5472, Cell signaling, Danvers, MA, USA), anti-GAPDH (#5174, Cell signaling, Danvers, MA, USA), mouse HRP-conjugated secondary antibody (#NA931, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and mouse HRP-conjugated secondary antibody (#NA934, GE Healthcare). Immunoblotting was performed following manufacturer instructions. ECL Western Blotting Substrate and SuperSignal (#34095, Thermo Fisher) were used for detection.

Cellular Thermal Denaturation Assay

Thermal shift was performed with PC3 cells cultured in F12K medium supplemented with 10% NU and 1 % penicillin/streptomycin (pen/strep). To determine the melting profile of PLK1, asynchronous and colcemid blocked cells dispensed into PCR tubes in the above medium were subjected to temperature gradient (38-60°C) for 10 min.
Cold non-denaturing lysis buffer (PBS supplemented with 0.1% TritonX-100 and 2X protease and phosphatase inhibitors) was added to wells, and the plate was rocked for 20 min. Lysis of cell and nuclear membranes was confirmed by looking under microscope. Subsequently, centrifugation was performed at 16000 rcf to sediment the insoluble proteins. After collection of the supernatant and SDS-PAGE, immunoblotting was performed using anti-PLK1 and anti-GAPDH antibodies. PLK1 protein was quantified using Image Lab software, normalized to the GAPDH loading control, and plotted over time to calculate the $T_{agg50}$ was calculated for mitotic and non-mitotic PLK1 using GraphPad Prism software. In subsequent experiments, the $T_{agg50}$ 50 was utilized for dose response curves for compounds by treating mitotically arrested and asynchronous cells with increasing doses of abbbapolin, BI2536 and BI6727 (Volasertib) along with a DMSO control for 2 h followed by incubation at the respective $T_{agg50}$ 50 for 10 min. Cells were then lysed, insoluble protein removed by centrifugation, SDS-PAGE and immunoblotting performed. Soluble PLK1 was normalized and quantified as mentioned above.

**Nuclear and cytoplasmic fractionation**

Exponentially growing PC3 cells treated with abbbapolin for 23 h were harvested, centrifuged, and rinsed with 1X PBS supplemented with protease and phosphatase inhibitors. The nuclear and cytoplasmic fractionation of the resulting pellet was carried out using the NE-PER extraction kit (#78833, Thermo Fisher, Waltham, MA, USA) following manufacturer instructions. Briefly, the pellet was suspended in 100 µl of cytoplasmic extraction reagent-I by vertexing and incubated on ice for 10 min. The suspension was mixed with 5.5 µl of the cytoplasmic extraction reagent-II by vertexing, incubated on ice
for 1 min, vortexed again for 5 s, and centrifuged for 5 min at 16,000 g. The resulting supernatant (cytoplasmic extract) was transferred to a pre-chilled eppendorf tube. The insoluble (pellet) fraction, which contains nuclei, was suspended in 50 µl of ice-cold nuclear extraction reagent by vertexing. Then, tubes were placed on ice and continued vertexing for 15 s every 10 min, for a total of 40 min, then centrifuged at 16,000 g for 10 min. The resulting supernatant (nuclear extract) was transferred to a pre-chilled eppendorf tube, and immunoblotting was carried out as described above. The nuclear and cytoplasmic fractions were prepared, and immunoblotting performed as described above.

Immunofluorescence staining and quantitation

PC3 cells (10,000 per coverslip) were grown in F12K/10% NU and treated with abbpapolins for 23 h. Cells were fixed with ice cold 4% formaldehyde solution for 30 min at room temperature (RT), then permeabilized with 0.2 % Triton X-100 (v/v) for 15 min at RT. The permeabilized cells were washed with PBS and blocked in DMEM/10% NU overnight at 4°C. The cells were incubated with anti-PLK1 antibody (1:500) for 1 h at RT. Cells were washed with PBS, followed by 0.1 % Triton X-100 (v/v) then with PBS a 1 min each. The coverslips were incubated with Alexa Fluor 561–conjugated anti-mouse secondary IgG (1:800) (Invitrogen) for 1 h at RT to visualize PLK1. Cells were washed again and incubated with DAPI for 10 min to stain nuclei. Cells were washed again before mounting on slides with Prolong Gold antifade (Life Technologies) and examined using a Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss). The nuclear, cytoplasmic,
and total PLK1 levels were separately quantitated using Image J software. The results are presented as mean ± SD (n = 100–200 cells).

**Fluorescent Polarization (FP) Binding Assay**

*In vitro* binding activities were analyzed as described before with little modification [19, 20, 22]. The fluorescein-tagged tracer peptide MAGPMQS[pT]PLNGAKKK was used at a final concentration of 10 nM. Tracer and full-length PLK1 (FL PLK1) (#10676-H07B Sino Biological US Inc, Wayne, PA) were titrated to generate a binding curve that was used to determine binding affinities. Additionally, tracer and FL PLK1 were titrated in the presence of 50 nM BI2536. Fluorescence mili polarization (mP) and fluorescence values were measured using a SpectraMax i3 plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

4.3 RESULTS

**Different thermal stability of non-mitotic and mitotic PLK1 proteins**

To determine whether endogenous PLK1 exists in different conformations depending on the stage of the cell cycle, thermal shift analysis of PLK1 was performed on both asynchronous and mitotically arrested cells. In this assay, protein solubility and by inference its conformational stability is measured by treating cells or cell lysates with an increasing temperature gradient and plotting the loss of soluble protein as a function of temperature. Asynchronous and colcemid-blocked cells were harvested, subjected to heat shock (a range from 37 to 60 °C) for 10 min. After electrophoresis of the soluble fractions for each temperature point, immunoblotting was performed to detect PLK1 and p-T210 PLK1, the latter phosphorylated by Aurora A and presumed to relieve the autoinhibition of the KD by the PBD [16, 18]. Indeed, pT210 PLK1 was only observed in
mitotically arrested cells and was undetectable in the soluble fraction above 39.5 °C (Figure 4.1A, lower panel). The thermal melting profile was calculated for PLK1 from asynchronous and colcemid-blocked cells. The data shows that mitotic PLK1 has a lower aggregation temperature, $T_{agg50}$ of 45.5 ± 0.9°C compared to 49.3 ± 0.4°C for non-mitotic PLK1. The results suggest that because of its lower $T_{agg50}$, the presumably monomeric, mitotic PLK1 has a less stable thermal conformation.

**Differential effects of catalytic and PBD inhibitors on the thermal stability of PLK1**

Thermal shift analysis was used to compare the thermal stability of PLK1 from both asynchronous and mitotic cells and carried out in the presence of two classes of small molecule binders to PLK1, namely ATP-competitive molecules and a representative PBD-competitive abbapolin from our previous study (Compound 12 in [19], hereafter referred to as “abbapolin”). A thermal shift assay has been utilized to demonstrate on target binding of small molecules with proteins in the cellular context for multiple molecules, including our recent work demonstrating interactions of peptidic molecules and abbapolins with PLK1 [19, 20, 22]. In this application of the thermal shift assay, small molecule binding to a target protein has been shown to stabilize the protein to thermal melting and produce a “right shift” to the curve plotting the loss of soluble protein. Examples of intracellular small molecule-protein interactions shown include classic competitive (non-covalent) inhibitors such as methotrexate for its target dihydrofolate reductase, raltitrexed for its target thymidylate synthase, and several BRAF V600E inhibitors [21].
We performed thermal stability assay analysis of PLK1 in the presence of BI2536 and BI6727 (volasertib) individually, which are well-characterized small molecules that competitively bind to the ATP pocket of PLK1 and inhibit its catalytic activity. In the first set of experiments, asynchronous arrested cells were treated with small molecule inhibitors for 2 h, then subsequently processed and analyzed as above, except by holding the temperature constant at the $T_{agg50}$ of 49.5°C for asynchronous PLK1 (Figure 4.2 A-B). Surprisingly, the presence of either BI compound produced a striking dose dependent decrease in the amount of soluble PLK1, which suggests that their binding to the ATP pocket has resulted in a conformational shift and a form less stable to temperature change. The same experiment was performed in mitotically arrested cells at the $T_{agg50}$ of 45.5 °C for mitotic PLK1, and the results with BI2536 (Figure 4.2C, 4.2E) and BI6727 again showed a dose dependent reduction in soluble PLK1 that plateaued at approximately 80%. Although somewhat surprising, these results agree with the in vitro observations by Raab et al., in that the binding of catalytic inhibitors including BI6727 decrease the thermal stability of purified, full length PLK1 (FL PLK1) [9]. In contrast to the inhibitors that bind to the PLK1 KD and in agreement with our prior work, abbapolin binding to the PBD of mitotic PLK1 produced the expected right shift in the thermal melting curve, similar to the shift observed with abbapolins and PLK1 from asynchronous cells [19, 20].

**Thermal stability of mitotic PLK1 co-treated with catalytic and PBD inhibitors**

Because BI2536 and the abbapolin bind to the KD and PBD respectively, the effect on PLK1 thermal stability was measured after co-incubation of both inhibitors. We co-treated mitotically arrested cells with 30 nM BI2536 and increasing concentrations of
abbapolin for 2 h. The dose of 30 nM BI2536 was chosen because as a concentration that reduces around 40-50% of soluble PLK1 at the T_{agg} of 45.5°C (Figure 4.3A and 4.3B) and is pharmacologically relevant in the range of its growth inhibitory properties in the PC3 cells. The presence of BI2536 alone (Figure 4.3A, 3^{rd} lane from left) caused a loss of soluble PLK1 that was not affected by the addition of abbapolin at all doses tested (Figure 4.3A). Co-treatment of BI2536 and abbapolin slightly further destabilized the PLK1 compared to 30 nM BI2536 alone (P=0.039, comparing BI with BI plus 3 µM abbapolin (Figure 4A-B). Figure 4B shows the quantitation of the blots from Figure 4.2D and Figure 4.3A.

**BI2536 modulates conformation of PLK1, enhancing the interaction with peptide ligands**

The thermal shift analysis intriguingly suggested that the binding of catalytic inhibitors to PLK1 produced a less thermally stable form, likely resulting from a conformational change or change in state from dimeric to monomeric. However, in the cellular milieu, many of the PLK1 binding partners are also present, which would confound such simplistic conclusions. We therefore performed a fluorescence polarization (FP) assay *in vitro* with purified PLK1. In this assay, a fluorescently tagged peptide ligand of the PBD (MAGPMQS[pT]PLNGAKK) was used at a concentration of 10 nM. Increasing the concentration of FL PLK1 (20-460 nM) increased the polarization and when plotted generates a binding curve. In the absence of small molecule inhibitor, the binding of PLK1 to the tracer peptide produced a modest mP change of 68.5 ± 0.9 at the highest PLK1 concentration. In contrast, the presence of BI2536 at a concentration of 50 nM increased the relative mP values by more than 2-fold to 136.2 ± 5.3, demonstrating an increased binding of the tracer to the BI2536-bound PLK1. Note the change in polarization was
independent of fluorescence intensity at the tracer concentration tested, which essentially remained constant across the range of protein concentration and irrespective of BI2536. Next, the PLK1 concentration (230 nM) phosphopeptide tracer (10 nM) was held constant, and a concentration range of BI2536 from 0 to 130 nM was tested. Increasing the BI2536 concentration increased the relative mP values and thus the affinity of PBD to the phosphopeptide tracer (Figure 4.4B). Note in these experiments the total fluorescence value remained unchanged within experimental error at the fixed concentration of tracer, indicating that there was no interference with fluorescence excitation or emission. Collectively, these findings are in good agreement with a previous study, in which it was shown that binding of BI6727 to PLK1 enhanced the interaction between PLK1 and a very similar peptide (MAGPMQS[pT]PLNGAK) in an in vitro alphascreen assay [9].

*Abbapolins preferentially decrease nuclear PLK1 protein levels*

As reported previously, abbapolins bind to the PBD of PLK1 in vitro and in the cellular context, as demonstrated by FP assay and thermal denaturation analysis [19]. Note it was also observed that abbapolin binding to PLK1 in the cellular context caused the loss of PLK1 protein in a manner reminiscent of a report that a PBD-targeting compound coupled to an adamantane group induced proteasomal degradation of PLK1 [23, 24]. We speculated that the striking loss of PLK1 protein is likely related to poorly understood mechanisms of conformational changes in PLK1 that might also affect its subcellular localization [19]. Here, immunofluorescence staining was undertaken to visualize the subcellular localization and level of PLK1 in abbapolin-treated cells to further
investigate their effects on cellular PLK1. PC3 cells were treated with increasing concentrations of abbpapol for 23 h, followed by staining for PLK1 (Red) and DNA (blue). An inactive abbpapol structural analog with an ester instead of the phosphomimetic carboxylic acid functional group was included as a negative control [19]. In agreement with the measurement of PLK1 levels by western blotting, increasing concentration of abbpapol caused a dose-dependent reduction in total PLK1 as measured by immunofluorescence (Figure 4.5). Mitotic cells were not observed after abbpapol treatment, in agreement with previous cell cycle and gene expression studies demonstrating that abbpapolins do not induce a mitotic cell cycle arrest [19, 20]. Treatment with the inactive abbpapol did not affect PLK1 levels (Figure 4.5). The amount of nuclear and cytoplasmic PLK1 was also quantified, and in untreated cells, nuclear PLK1 protein was approximately 2-fold higher than cytoplasmic PLK1. After abbpapol treatment, there was a striking, dose dependent loss of nuclear PLK1 (P<0.05 at 10 and 20 mM abbpapol compared to control), whereas cytoplasmic levels were not reduced significantly (P > 0.05) (Figure 4.5A and C). At a dose of 20 µM abbpapol, near the anti-proliferative IC₅₀ value of 15 mM ± 2 in PC3 cells [19], there was a near complete (89%) loss of nuclear PLK1, while cytoplasmic PLK1 levels remained equivalent to that seen untreated cells. BI2536 is well known to induce a prometaphase arrest [25], and at a dose of 30 nM for 23 h in PC3 cells, the vast majority of cells are mitotic. Therefore, total PLK1 was quantified in the cells treated with BI2536. Treatment of the PC3 cells with BI2536 induced a >2.5-fold increase in cellular PLK1 (Figure 4.8).
The analysis of cytoplasmic and nuclear PLK1 was also performed by subcellular fractionation and immunoblotting following treatment of cells with increasing doses of abbbapolin. Nuclear PLK1 levels were higher than cytoplasmic PLK1 in the untreated samples, in agreement with the immunofluorescence studies. Abbbapol treatment caused a steeper loss of nuclear PLK1 detected by blotting (Figure 4.6A and 4.6B). Quantitation of a 50% degradation concentration (DC\textsubscript{50}) revealed a DC\textsubscript{50} of 8.92 ± 0.16 µM abbbapol dose for nuclear PLK1, whereas a DC\textsubscript{50} of 15.01 ± 0.74 µM was observed for cytoplasmic PLK1 (Figure 4.6B). The nuclear PLK1 significantly reduced compared to cytoplasmic PLK1 (P<0.05).

4.4 DISCUSSION

There is much interest in the conformational features of PLK1 that influence its cellular regulation, subcellular localization, and activity. In this study, the thermal and cellular stability of PLK1 was measured in the absence and presence of ATP-competitive and non-competitive inhibitors. The observations utilizing thermal shift analysis that mitotic PLK1 is less stable compared to non-mitotic PLK1 upon thermal stress support a proposed conformational change of PLK1 between interphase and mitosis. These observations are consistent with the idea that phosphorylation on T210 of PLK1 by Aurora A relieves an autoinhibition of the KD by the PBD [18, 26, 27]. The T210-phosphophorylated PLK1 (pT210 PLK1) was only observed in mitotic cells and only at the lowest temperature of 39.5°C tested (Figure 4.1A, lower blot). These observations are also consistent with the recently proposed model whereby dimeric PLK1 bound by Bora remains in an inactive conformation until late G2, when phosphorylation of PLK1 at T210
releases monomeric PLK1 for entry into the nucleus [16]. In untreated PC3 cells the percentage in G2/M as measured by flow cytometry is 21 ± 2% [19]; thus only a small fraction of asynchronous cells would be in late G2 when analyzed by thermal shift. In mitosis, Aurora A in co-ordination with WAC (WW-domain-containing adaptor protein with a coiled-coil region) phosphorylates PLK1 at T210 [18, 27].

It was somewhat surprising to find that incubation with the catalytic inhibitors BI2536 and BI6727 caused an apparent conformational change that decreased thermal stability of PLK1 in a 2 h incubation regardless of cell cycle, when the presumption is that small molecule binding to a target protein would stabilize its conformation [21]. Yet, it is not without precedent. Raab et al., measured the thermal stability of purified FL PLK1 and the KD alone by differential scanning fluorimetry in the presence of BI6727. The presence of BI6727 strongly stabilized the PLK1 KD, but partially destabilized FL PLK1 between 40 to 50 °C in a biphasic curve before stabilizing FL PLK1 at higher temperatures [21]. Here, results from the FP binding assay showed in vitro that FL PLK1 binds more efficiently to a phosphopeptide ligand in the presence of BI2536 (Figure 4.4), potentially by relieving an intermolecular interaction between the KD and PBD. Intriguingly, incubation with BI2536 reduced soluble mitotic PLK1 in a concentration dependent manner as measured by a cellular thermal denaturation assay, indicating that ATP-competitive catalytic inhibitors have the same effect on PLK1 in biochemical and cellular contexts. Our studies also reveal that non-ATP competitive PBD binders produce a distinctly different cellular phenotypic response from the mitotic arrest associated with catalytic inhibition [19, 20]. Abbapolins halted cell cycle progression broadly, and gene expression studies revealed that
Abbapolin treatment induced a distinct gene expression pattern that only partially overlapped with that seen for BI2536 [20]. Related, it was reported that BI2536 and GSK461364 activated the BubR1 mitotic assembly checkpoint kinase and consequently blocked cells in mitosis [28]. In contrast, two reported PBD binders, poloxin and thymoquinone, caused an increase in p21 and an S-phase arrest, suggesting that PBD-based inhibitors have broader effects on PLK1 beyond mitosis [28].

Thermal shift analysis of mitotic PLK1 revealed that PBD-binding abbapolins increased the thermal stability of PLK1, in a similar manner to that seen in asynchronous cells [19]. Yet this increase in thermal stability produces a seemingly paradoxical loss of intracellular PLK1 over time. In cells treated with abbapolins, nuclear PLK1 levels were depleted compared to cytoplasmic PLK1 as determined by immunofluorescence and nuclear-cytoplasmic fractionation. This may be explained by one or more of the following: 1) the import of nuclear PLK1 may be disrupted by abbapolin binding to the PBD. In support of this, it was reported that mutations within the nuclear localization sequence (a.a. 396 to 433) within PB1 of the PBD resulted in the cytoplasmic accumulation of PLK1 [29]. Nuclear, monomeric PLK1 is proposed to have an open conformation whereas the cytoplasmic PLK1 proposed have a closed conformation [18, 26]. In this regard, abbapolin binding to the PBD in interphase promotes an abnormal conformation that promotes the intracellular instability and degradation of PLK1. Abbapolins arrest cell cycle progression in a manner not specific for a phase of the cell cycle and induce a gene expression pattern distinct from that seen for BI2536 [19, 20]. 3) Nuclear and cytoplasmic PLK1s are degraded by different ubiquitin proteasome systems and might therefore be differentially affected.
by abbpolin treatment. Specifically, nuclear PLK1 is degraded by SCF^{FBXW7}, whereas cytoplasmic PLK1 is degraded by SCF^{β-TrCP} [26, 30]. Because our experimental approaches could not distinguish between monomeric and dimeric forms of PLK1, we cannot distinguish between abbpolin binding that either disrupts the closed conformation (auto-inhibited) monomeric PLK1 or disrupts PLK1 dimerization interactions via the key PBD contacts described above. However, the data presented here is quite consistent with the in vitro, the cellular observations, and the model of PLK1 conformational changes proposed by Raab et al [9, 16].
4.5 FIGURES

Figure 4.1 Thermal melting curves of non-mitotic and mitotic PLK1. PLK1 isolated from mitotic cells displayed a lower $T_{agg 50}$ than PLK1 from asynchronous cells. Asynchronous cells and colcemid (Colc)-blocked cells were subjected to a temperature gradient (37-60°C). A) Representative immunoblots. B) Quantitation of PLK1 levels as a function of temperature.

Figure 4.2 Thermal shift analysis of intracellular PLK1 thermal stability. PLK1 catalytic inhibitors BI2536 and BI6727 denatured PLK1 whereas PLK1 PBD inhibitor abbaapolin stabilized PLK1. A-B) Asynchronous PC3 cells were treated with a dose range of BI2536 and BI6727 for 2 h and then subjected to $T_{agg 50}$ of 49.5°C for 10 min. Soluble proteins were collected via centrifugation and analyzed via Western blotting. C-F) Colcemid blocked PC3 cells were treated with increasing doses of BI2536 and abbaapolin 12 for 2 h and then subjected to $T_{agg 50}$ of 45.5°C for 10 min and subsequently analyzed as above.
Figure 4.3 Thermal shift analysis of PLK1 stability after co-treatment of Abbapolin and BI2536. The presence of the abbapolin did not alter the thermal destabilization of PLK1 caused by BI2536. A) Colcemid-blocked cells were treated with increasing doses of abbapolin 12 alone or combination with 30 nM of BI2536 for 2 h then subjected to $T_{agg50}$ of 45.5 °C for 10 min. B) Quantitation of blots from Figure 4.2D and Figure 4.3A.

Figure 4.4 In vitro binding of FL PLK1 with catalytic inhibitor BI2536. BI2536 enhanced the interaction of PLK1 to phosphopeptide ligands. A) An increasing concentration of FL PLK1 (20-460 nM) was incubated with fluorescent-tagged phosphopeptide tracers in the absence or presence of 50 nM BI2536 for 45 min. B) FL PLK1 (230 nM) and tracer (10 nM) with increasing concentrations of BI2536 (0-130 nM) for 45 min.
Figure 4.5 Immunofluorescence analysis of intracellular PLK1. Abbapolin 12 treatment reduces nuclear PLK1 levels. A) Representative images of untreated cells, cells treated with 20 µM active or inactive abbapolin 17. Red represents detection of immunodetection of PLK1 and blue is nuclear DNA (DAPI staining). B) Quantitation of immunostaining images in A.
Figure 4.6 Abbapolin treatment caused a preferential loss of nuclear PLK1. PC3 cells were treated with 10, 30, and 45 μM doses of abbapolin 12. Cytoplasmic and nuclear PLK1 proteins were isolated and detected with anti-PLK1 antibody. N, Nuclear and C, cytoplasmic. A) Representative blot. B) Quantitation of bands shown in A by Image lab software.

Figure 4.7 Proposed model of regulation of PLK1 confirmation. PBD inhibitors Abbapolin first binds to PLK1 PBD that in turn eventually leads to degradation partially via a proteosome [19]. While the catalytic based inhibitor Bi2536 binds and opens up PLK1 structure and hence leads to accumulation. Zhu et al., 2016 [11] mainly based on biochemical assays showed Drosophila protein Pon induces PLK1 dimerization mediated by PBD. Raab et al., 2021 [16] performed elegant experiments with chromosomally integrated PLK1 to demonstrate that Bora transiently promotes PLK1 dimerization early in G2, whereas T210 phosphorylation by Aurora A initiates dissociation of the dimer.
Figure 4.8 Immunofluorescence analysis of intracellular PLK1 treated with BI2536. BI2536 treatment increases PLK1. A) Representative images of untreated cells and cells treated with 30 nM BI2536. Images in B were taken with lower intensity compared to images shown in Figure 4.5A. B) Quantitation of immunostaining images in A. The results are shown in box-and-whisker plots with mean values (n = 100–200 cells per treatment group).
4.6 CHAPTER 4 REFERENCES


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CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CHAPTER 2 SUMMARY

In chapter two we demonstrate the development of FLIPs (Fragment Ligated Inhibitory Peptides) PBD inhibitors that selectively bind to the PLK1 PBD over the PLK3 PBD. It was concluded that N-terminal amino acids of FLIP contribute to potency while the C-terminal amino acids contribute to FLIP selectivity (Table 2.1). Prior work examined the contributions of C-terminal amino acids from two different PLK1 substrates. In this study, the C-terminus was modified by amidation to convert a negatively charged carboxylate to a neutral carboxamide. The modification potentially improves the permeability of FLIPs through the plasma membrane. Adding an isoleucine (23) improved PLK1 PBD binding by 7-fold, while only marginally improving PLK3 binding, which improved selectivity as shown by Fluorescence polarization (FP) assay. When the C-terminus was replaced with proline and leucine, the resulting FLIP (24) bound to the PLK1 PBD with a 3-fold stronger affinity and remained selective for PLK1. These results indicate the importance of the C-terminal interactions in PLK1 affinity and selectivity. When the carboxylate on the C-terminus of 23 was replaced with an amide, the resulting FLIP (25) bound to the PLK1 PBD with similar affinity and remained selective for PLK1. When the length of alkyl group on N-terminus of 25 was increased, the resulting FLIP (26) showed similar PLK1 and PLK3 binding affinity as 25 (Table 2.1).
Cell viability was measured to determine the anti-proliferative activity of octyl and nonyl FLIPs. Despite their peptidic composition, the FLIPs possessed cellular activity. The neutral C-terminal amidated octyl FLIP (25) showed significantly higher anti-proliferative activity than octyl FLIPs (23 and 24). The neutral C-terminal amidated nonyl FLIP (26) showed similar anti-proliferative activity as the neutral C-terminal amidated octyl FLIP (25) (Table 2.1). Moreover, FLIP 25 inhibited TCTP phosphorylation in a dose dependent manner (Figure 2.1) which confirms cellular inhibition of PLK1 activity. Future studies should involve carrying out the FP assay with FL PLK1 to determine if the binding of FLIPs can allosterically modulate the catalytic domain. The FP assay can be carried out with varying FLIP concentrations, a fixed concentration of BODIPY FL ATP-γ-S and FL-PLK1.

Selectivity of FLIPs in terms of inhibition of PLK1 and PLK3 target phosphorylation in cells

PLK1 phosphorylates several target proteins in different phases of the cell cycle. For example, BubR1 and TCTP in mitosis, Cdh1 in G1, and Cdc25C1 in the G2 phase of the cell cycle [1-2]. Following DNA damage, PLK3 is activated in an ATM-dependent manner followed by PLK3 phosphorylation of Chk2, p53 and Cdc25A [4-5,7]. Raab et al., 2018 demonstrated that despite being weak inhibitors of PLK1 in vitro, allosteric inhibitors potently inhibit PLK1 function in cells by affecting its conformation and mechanism of regulation. We measured the selectivity of FLIPs in term of PBD binding as determined by in vitro FP assays with PLK PBD (1&3) (Table 2.1) and PLKs (1&3) binding by a cellular thermal denaturation assay (Figure 2.1). To determine selectivity of FLIPs, future studies should evaluate inhibition of phosphorylation of PLK1 in comparison to PLK3 targets in cellular environment.
5.2 CHAPTER 3 SUMMARY

In chapter three, I described the structure activity relationship of small molecule inhibitor candidates (abbapolins) and the mechanistic study of selected inhibitors. We demonstrated that abbapolins specifically bind to full-length cellular PLK1 over PLK3 as determined by cellular thermal shift assay in a pattern consistent with binding affinities measured by FP assay (Table 3.1, Figure 3.2, and Figure 3.6). Abbapolin 12 caused a striking dose dependent reduction in PLK1 protein levels in two different prostate cancer cell lines (Figure 3.5A, B and D); the loss being partially due to proteasome mediated degradation (Figure 3.5E). Interestingly the degradation of PLK1 caused by abbapolin binding does not require the conjugation of an E3 ligase ligand for ubiquitination as is usually required for PROTAC molecules. Abbapolins produced a dose dependent reduction in p-TCTP (pSer46 TCTP) signal without affecting total levels of TCTP (Figure 3.3). Moreover, the therapeutic potential of abbapolins in RPE cells expressing a PLK1 C67V mutant that are dramatically resistant to PLK1 catalytic inhibitors (C67V PLK1 mutant) was studied. RPE cells overexpressing both mutant and wild type PLK1 showed sensitivity to abbapolins (Figure 3.4B).

Mechanism of loss of PLK1 protein

Modeling of the PBD dimer with abbapolin 12 showed that the abbapolin hydrophobic tail could block PLK1 dimerization (Figure 3.7). The 4-alkyl chain of abbapolin 12 interacts with the same hydrophobic groove where L505 binds in the PBD dimer, suggesting that abbapolin 12 binding to the PBD would block dimerization. It is proposed that the decreased stability of PLK1 and as a result, its degradation at least partially results
from induction of monomeric PLK1 proteins. Interestingly, the destruction box motif (R337 present between the KD and PBD) is exposed more in the PLK1 monomer while it is not exposed in PLK1 dimer crystal structure. Zhu et al., showed PLK1 dimerization induced by phospho Pon (pPon) is lost upon a single point mutation at position L505E [14]. Future studies should examination of PLK1 degradation cells expressing the PLK1 L505E mutant treated with abbapolins. Additionally, future studies should examine cell cycle analysis with cells expressing the PLK1 L505E mutants.

In a recent study by Raab et al., 2022, it was shown that a Bora homodimer transiently promotes dimerization of cytoplasmic PLK1 throughout the G2 phase of the cell cycle. Cdk1 is responsible for a priming phosphorylation of Bora at S252 that in turn promotes homodimerization and enhances the interaction with the PBD [8]. Studies by Chan et al., 2008 and Elia et al., 2003 showed that the motif centered around S252 is a major Cdk1-dependent PLK1 PBD binding site on Bora [24-25]. HeLa cells transiently expressing the Bora S252A mutant reduces the level of PLK1 dimer while the Bora S252E mutant shows the same level of PLK1 dimer compared to Bora WT, further supporting that phosphorylation of S252 promotes PLK1 dimerization [8]. Future studies should compare PLK1 degradation between cells expressing Bora S252A and Bora S252E treated with abbapolins.

Determination of apoptosis induced by abbapolins

Human cancer cells deficient in p53, RAS and PTEN overexpress PLK1 for survival and its inhibition can lead to mitotic arrest followed by apoptosis [17-19]. Mitotic arrest following PLK1 inhibition either by small molecule inhibitors (BI2536) or siRNA leads to
the inactivation of DAPK by autophosphorylation at S308 in HeLa cells. Prolonged mitotic stress induced by PLK1 inhibition results in the dephosphorylation of DAPK. This, in turn, activates the proapoptotic function of DAPK. Apoptosis induced by PLK1 inhibition could be inhibited by siRNA mediated DAPK silencing in various human cancer cell lines. So, DAPK is a critical component of cell death machinery, and it is activated upon mitotic stress induced by PLK1 inhibition [6]. Future studies should investigate how apoptosis is induced in cells treated with abbpapolins 24, 48 and 72 hours and BI2536 (positive control). Also, future studies should analyze cleaved PARP-1, DAPK/pS308, DAPK and CAMKK2/pS511 (DAPK substrate) in cells treated with abbpapolins and BI2536. If PLK1 inhibition leads to DAPK inactivation, cells can be treated with abbpapolins alone or in combination with DAPK inhibitor.

5.3 CHAPTER 4 SUMMARY

Chapter 4 discusses novel finding regarding the intracellular conformational changes of PLK1 in mitosis induced by BI2536 as determined by thermal shift and hence destabilizes the PLK1 structure and causes PLK1 accumulation. In contrast, abbpapolins stabilize PLK1 and that eventually lead its degradation.

Cytoplasmic accumulation of PLK1 induced by Abbpapolins

Abbpapolins preferentially induce nuclear PLK1 loss compared to cytoplasmic PLK1 as determined by immunofluorescence (Figure 4.5) and nuclear-cytoplasmic fractionation (Figure 4.6). It is hypothesized that the import of nuclear PLK1 could be disrupted by abbpapolin binding to the PBD resulting in cytoplasmic accumulation of PLK1. Mutation within the nuclear localization sequence (aa396 to 433) on PB1 of the PBD results in the
cytoplasmic accumulation of PLK1 [12]. Moreover, sumoylation at K492 of PLK1 promotes its nuclear localization in G2/M and suppresses the ubiquitin mediated proteasomal degradation of PLK1 in mitosis by reducing its interaction with APC/Cdh1. PLK1 PBD binding partner Ubc9 is a sole SUMO-conjugating enzyme required for the SUMOylation. Prime phosphorylation on Ubc9 by Cdk1/Cyclin B enhances its sumoylation activity and interaction with PBD in G2/M phase of HeLa cells. K492 is an important regulatory site which is differentially regulated by monoubiquitination and sumoylation resulting in diverse cellular functions. Monoubiquitination at K492 controls the PLK1 association with kinetochore while the sumoylation at this site causes PLK1 nuclear localization, its protein stability and hence its mitotic functions [13]. Future studies should investigate cytoplasmic accumulation of PLK1 caused by abbpapolins treatment and mechanism of PLK1 degradation in cells expressing K492R (deficient in sumoylation) or WT PLK1.

**Bi2536 preferentially destabilizes the nuclear PLK1 compared to the cytoplasmic PLK1**

In addition to PLK1 activity in mitosis, PLK1 activity is required in interphase cells [2,15-16]. For example, PLK1 promotes Cdh1 phosphorylation to trigger its ubiquitination and degradation in the late G1 phase and that in turns allows cells to progress from G1 to S phase in HeLa cells [2]. However, PLK1 activation takes place in the late G2 phase of cell [8-10] cycle. In G2, Aurora-A kinase co-ordinates with its co-factor Bora to phosphorylate centrosome-localized PLK1 in its activation loop (T210). In addition to Bora-dependent activation of cytoplasmic PLK1, Aurora A in co-ordination with WAC (WW-domain-containing adaptor protein with a coiled-coil region) phosphorylates PLK1 at T210 to ensure timely entry into mitosis [9-10]. Since phosphorylation of PLK1 takes place in late
G2 phase of cell cycle, how then is PLK1 mediated phosphorylation of its substrates in G1 and S phases occurring? One possibility is that residual PLK1 after mitosis is complete, can phosphorylate target proteins in interphase (such as Cdh1). 60-75 % of mitotic PLK1 is degraded in anaphase in an APC/C-mediated proteolysis mechanism and this degradation is required for the proper cytokinesis and mitotic exit in HeLa cells [11]. So, interphase cells can therefore have the residual PLK1 not degraded during mitosis. Though PLK1 activity is essential in interphase cells for the G1/S transition, cells treated with the PLK1 catalytic inhibitor, BI2536 blocks cells during mitosis [20]. The unanswered question is therefore why cells treated with catalytic inhibitors piled up in mitosis not in interphase? Future studies should investigate the reasons for this and also should compare thermal destabilization of nuclear and cytoplasmic PLK1 caused by BI2536 in several phases of cell cycle in HeLa, PC3 and other cancer cell lines by cellular thermal shift analysis and other techniques. In addition to that, further studies should explore activity of cytoplasmic and nuclear PLK1 in several phases of cell cycle in several cancer cell lines.

Thermal melting curve of the nuclear and cytoplasmic PLK1 in cells treated with BI2536 and abbapolins

It is generally observed that small molecules binding to a target protein stabilize the conformation against thermal stress. However, catalytic inhibitors BI2536 and BI6727 caused a conformational change that decreased thermal stability of PLK1 from both mitotically arrested and asynchronous cells. In line with this observation, in an in-vitro thermal stability assay by differential scanning fluorimetry, BI6726 strongly stabilizes the catalytic domain (ΔTm = 20.8 ± 0.02 °C) but overall destabilizes the FL-PLK1. The thermal
denaturation curve in presence of BI6727 showed a distinct denaturation pattern. The first portion of curve showed that BI6737 destabilizes PLK1 (ΔTm = −11.1°C) whereas the second portion of curve reveals a stabilization of PLK1 by 6.6°C [7]. Future studies should perform cellular thermal shift analyses to determine melting curve of the nuclear and cytoplasmic PLK1 treated with a fixed dose of BI2536 and BI6727 for 2 hours and heated across a temperature range from 37 to 60°C for 10 minutes. The data from the cellular thermal shift and FP assays suggest that catalytic inhibitors BI2536 and BI6726 open the PLK1 structure. Future studies should investigate whether ATP binding to the catalytic site also causes a similar PLK1 conformational change to the inhibitor binding to the catalytic site. Abbapolin stabilizes the PLK1 as determined by a cellular thermal denaturation assay in a concentration dependent manner (Figure 3.2, and Figure 3.6). Future studies should determine the melting curve of nuclear and cytoplasmic PLK1 treated with fixed dose abbapolin and heated at a temperature range from 37 to 60 °C.

In vivo study of abbapolins

Our preliminary in vivo mouse xenograft study showed that abbapolins were well tolerated by mice as no sign of toxicity was observed and resulted in no loss of body weight, nor was there a change in liver, kidney, and prostate weight observed (Figure 5.1 A-D) (P> 0.05, non-significant). Both analogues inhibited the prostate xenograft tumors growth but only 6369 showed statistically significance (p<0.0476) compared to PBS (Figure 5.1 E-F). This is in agreement with the PLK1 degradation in the xenograft tumor. 6369 caused significantly more PLK1 loss compared to PBS and 6359 (Figure 5.2 A-B). This
indicates that inhibition of the PC3 tumor growth was because of PLK1 degradation caused by abapolins.

PCa is thought to be driven by the activation of PI3K/AKT/mTOR pathway, oxidative stress, lipid metabolism and androgen receptor (AR) signaling. A study by Zhang et al., 2014 demonstrated that PLK1 is elevated in PCa where its expression is linked to high tumor grade. In addition, PLK1 and lipid metabolism are highly upregulated pathways in a mouse xenograft model of human PCa. Interestingly, they found that oxidative stress activates both AR signaling and PI3K/AKT/mTOR pathways in a PLK1 dependent manner in PCa and inhibition of the PI3K/AKT/mTOR pathway prevented oxidative stress induced activation of AR signaling. Most importantly, they observed the inhibition of PLK1 by small molecule inhibitors BI2536 enhanced cellular response to androgen signaling inhibitors (abiraterone) and overcame abiraterone resistance in PCa and patient derived xenograft tumors [22]. Enzalutamide (Enza) is used to treat metastatic castration-resistant prostate cancer (mCRPC) patients. Though the majority of patients benefit from the Enza treatment, the disease progression is inevitable and the mechanism of the resistance to Enza is unknown. Guan et al., 2020 analyzed the genomes and transcriptomes of Enza-naive and Enza-resistant mCRPC patients’ tumors. 16% of the Enza-resistant mCRPC tumors showed focal deletion of chromosome 17q22 while that deletion was not observed in the Enza-naive tumors. The focal deletion of chromosome 17q22 has a negative prognostic value. PLK1, Cdk1/2 and AKT were predicted to be activated in those tumors with the 17q22 loss [23] so suggesting that PLK1 is a relevant target for the treatment of mCRPC. For future xenograft study, first the sensitivity of abapolins alone
or in combination with Enza in prostate cancer cells such as VcaP (p53 deleted, PTEN+, responsive to Enza), 22Rv1 (p53 WT, PTEN WT, non-responsive to Enza) and PC3 (PTEN deleted, AR-, metastatic, non-responsive to Enza) will be analyzed via cell viability assay. In addition, future study should determine the mechanism of cell death mediated by DAPK inactivation following the inhibition/degradation of PLK1 by abbpapolins. Based on the cellular studies, further xenograft studies should be carried out to see whether the abbpapolins re-sensitize the Enza and mechanism of cell death. Moreover, the future study should determine if the pSer46 TCTP can be used as a direct marker of evaluating inhibition of PLK1 activity in cells and xenograft tumors. We observed that synergism between the BI2536 and abbpapolin in vitro and a cellular thermal denaturation assay. In future xenograft study, we should carry out combination treatment of BI6727 and abbpapolins.
5.4 FIGURES

**Figure 5.1. Abbapolins were well tolerated in mice.** PC3 prostate cancer cells were injected into flanks of athymic nude mice and allowed to grow the tumors there for up to 13 days. 5 to 8 mice per group were treated with **6369** (40 mg/kg body weight) via intraperitoneal injection (IP) and **6359** (100 mg/kg per body weight) via oral gavage (OG) every day for up to 15 days. Tumor volume was estimated using the formula: \( V = L \times W^2 / 2 \) (V, mm³; L, mm; W, mm) and measured every three days using digital caliper. Following the treatment, mice were sacrificed, and their body, liver, kidney, prostate and tumor weight was measured.
Figure 5.2. Abbapolins induced PLK1 degradation in PC3 xenograft tumors. Proteins were isolated from xenograft PC3 tumors and PLK1 and GAPDH were detected using the respective antibodies by western blotting. A) Representative blot. B) Quantitation of bands shown in A by Image lab software. IP: Intraperitoneal, OG: Oral gavage.
5.5 CHAPTER 5 REFERENCES


APPENDIX A

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