2016

Selectivity Targeting Polo-Like Kinase 1 (PLK1) Using Novel Inhibitors of the Polo-Box Domain

Merissa Lynnea Baxter
University of South Carolina

SELECTIVELY TARGETING POLO-LIKE KINASE 1 (PLK1) USING NOVEL INHIBITORS OF THE POLO-BOX DOMAIN

by

Merissa Lynnea Baxter

Bachelor of Science
Elon University, 2008

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Pharmaceutical Sciences

College of Pharmacy

University of South Carolina

2016

Accepted by:

Michael D. Wyatt, Major Professor

Campbell McInnes, Committee Member

Eugenia Broude, Committee Member

Maria M. Pena, Committee Member

Sherine Chan, Committee Member

Lacy Ford, Senior Vice Provost and Dean of Graduate Studies

DEDICATION

I would like to dedicate this work to my parents, Dennis A. Baxter and JoAn D. Baxter. Everything that I have accomplished is due to their prayers, unwavering support, and total commitment to my success. They sacrificed in many ways to ensure that my goal would be achieved and taught me that I could do all things through Christ (Philippians 4:13). Their labors of love have not gone unnoticed. I owe them everything. I know that they are proud.

I would also like to dedicate this work to my sisters, Jessica N. Baxter and Joy D. Baxter. They encouraged me to continue moving forward and reminded of that light at the end of the tunnel. Thank you.

ACKNOWLEDGEMENTS

I would first like to acknowledge my advisor and mentor, Dr. Michael D. Wyatt for his guidance, training, and support over the past five years. I am grateful to have had the opportunity to learn and grow as a young scientist under his tutelage. His knowledge of the field and integrity as a scientist has been a great example to me. The guidance that he has provided has been priceless and second to none. I can truly say that I have grown as a result of his mentorship. Thank you.

Secondly, I would like to acknowledge my co-advisor, Dr. Campbell McInnes. I never expected that my graduate training would include medicinal chemistry, but I am grateful to have worked with him on the 'PLK1 Project'. His guidance has helped me to grow in my understanding of the drug development and synthesis process, and has challenged me to consider pharmacology from the perspective of a chemist. Thank you.

I would also like to acknowledge my committee members, Dr. Eugenia Broude (chair), Dr. Maria Pena, and Dr. Sherine Chan for their helpful insight and suggestions, and their constructive criticism and encouragement throughout the course of this work.

Thank you, as well, to the faculty, staff, and graduate students within the Department of Drug Discovery and Biomedical Sciences that offered suggestions, support, guidance, and motivation throughout the course of this work. In addition, I must acknowledge my numerous family members and friends that have been extremely supportive of my endeavors from the beginning.

ABSTRACT

Polo-like Kinse 1 (PLK1) is a serine/threonine protein kinase that is expressed in dividing cells. It is a widely studied protein that plays an important role in regulating mitotic progression. PLK1 is over-expressed in several tumor types, and studies have shown that down-regulating PLK1 expression inhibits cancer cell proliferation, validating PLK1 as an oncotarget. Efforts to target this protein therapeutically has led to the development of several ATP-based inhibitors, although these are not exclusively specific. Because other PLK family members are tumor suppressors, it is important that PLK1 inhibitors selectively bind to PLK1 and not the other PLKs, particularly PLK3. Moreover, resistance is always a problem with single agent therapies. PLK1 is comprised of two structural domains, the N-terminus catalytic kinase domain, and the C-terminus polo-box domain (PBD). The PBD is a phospho-peptide binding motif that determines substrate recognition and sub-cellular localization. By targeting the PBD, we can develop selective PLK1 inhibitors to inhibit cancer cell proliferation. REPLACE (Replacement with Partial Ligand Alternatives for Computational Enrichment) uses the structure activity relationships of peptide binding fragments to generate pharmaceutically acceptable lead molecules. We began by replacing the N-terminus tripeptide sequence of known PLK1 substrates like Cdc25C (LLCSpTPNGL) with molecular fragment mimics. This generates partially peptidic compounds termed FLIPs (Fragment Ligated Inhibitory Peptides). FLIPs were analyzed by fluorescence polarization (FP) to determine PLK1 and PLK3 PBD binding. Our most promising FLIPs were analyzed in cellular

assays. N-terminal FLIP SAR was also used to develop non-peptidic small molecules by carrying out a compound library search. This led to the development and characterization of more than 40 small molecules from a lead structure that was tested as part of a structure activity relationship program. In summary, the work described advances our understanding of the binding determinants for the PBD of PLK1 and produced lead molecules worthy of further exploration in their ability to target PLK1 in cancer.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP ..... Adenosine Triphosphate

CDK ..... Cyclin Dependent Kinase

Cys .....Cysteine

DMEM .....Dulbecco's Modified Eagle Medium

DMSO .....Dimethyl Sulfoxide

DNA .....Deoxyribonucleic Acid

EC_{50} .....50% of maximal effective concentration

FACS.....Fluorescence-Activated Cell Sorting

FBS .....Fetal bovine serum

FLIP .....Fragment Ligated Inhibitory Peptide

FP .....Fluorescent Polarization

G1, G2 phase.....Gap 1, Gap 2 phase

IC_{50} ..... 50% of maximal inhibitory concentration

mP ..... milli-Polarization

M phase.....Mitosis Phase

MPF..... Mitosis Promoting Factor

MTT ..... 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBD.....Polo Box Domain

PI ..... Propidium Iodide

PLK .....Polo-like Kinase

PPI..... Protein-Protein Interactions

REPLACE....Replacement with Partial Ligand Alternative for Computational Enrichment

S phase ..... Synthesis phase

SAR.....Structure Activity Relationship

Val.....Valine

WT .....Wild type

CHAPTER ONE

INTRODUCTION

1.1 GENERAL OVERVIEW

Cancer is a disease of the genome. Research has revealed tumorigenesis to be a multistep process resulting from genetic mutations that occur, leading to a gain of function of oncogenes, and a loss of function of tumor suppressors. The processes that drives the transformation of normal cells to a malignant state are considered the six hallmarks of cancer. They include: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of programmed cell death, 4) limitless replicative potential, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (1). These hallmarks acquired by cancer cells allow them to survive, proliferate, and disseminate when they otherwise would not. Cancer cells are able to acquire these changes because of two 'enabling characteristics' recently described by Hanahan and Weinberg (2011). The two characteristics they describe are 1) the development of genomic instability in cancer cells, and 2) immune system cells that drive the inflammatory state of premalignant and malignant lesions. Genomic instability drives the random mutations expressed in cancer cells while an inflammatory state promotes tumor progression by a variety of ways (2). They also argue that two additional hallmarks contribute to the development of cancer – the first being the reprogramming of cellular energy

metabolism, and the second being the cancer cell's avoidance from attack by immune cells (2). Normal cells tightly regulate cellular growth and division, and properly maintain homeostasis of cell number through careful production and release of growth-promoting signals. However, cancer cells deregulate this process resulting in an overgrowth of cells (2).

It is well established that several kinases regulate the progression of both normal and cancer cells through the cell cycle. Polo-like kinases are a subfamily of Serine/Threonine protein kinases that are involved in cell cycle regulation (3). This family of kinases was first discovered during the 1980s through a series of genetic screens in yeast (Cdc5), Xenopus (Plxs), Drosophila (polo), and mammals (PLKs). Polo-like kinases are evolutionarily conserved, having a conserved N-terminal catalytic domain – responsible for substrate phosphorylation; and a C-terminal polo-box domain (PBD) – responsible for substrate recognition and binding (4).

Genetics studies in Drosophila and yeast indicated a variety of mitotic roles for PLK1 in that mutants of Polo, CDC5, and PLO1 exhibited mitotic abnormalities and spindle defects (5-7). It was in 1994 that human cDNAs (homologous to the Drosophila polo gene) were cloned and named PLK (8). *D. melanogaster, S. cerevisiae*, and *S. pombe* only have one PLK (Polo, Cdc5, Plo1, respectively), but vertebrate species have several PLKs (9).

There are four mammalian PLKs functioning in cell cycle regulation including, PLK1, PLK2 (a.k.a. SNK), PLK3 (a.k.a. PRK, FNK and CNK) and PLK4 (a.k.a. SAK). PLK5, also a member of the PLK family, is not involved in cell cycle regulation and does not have a functioning catalytic domain (10). PLK1 is the most widely studied among all

PLK family members. Moreover, cell division was impaired in proliferating cells with over- and under-expression of PLK1 (11, 12) – suggesting an important role PLK1 plays in cell division of normal cells.

1.2 Function of PLK Family Members

PLK1

Polo-like kinase 1 (PLK1) is the most widely studied among all of the PLK family members. It is only expressed in proliferating cells, having its maximum activity during mitosis. PLK1 expression is low in G_0, G1, and S, with expression beginning to rise in G2, and peak in M phase (13-15).

Interphase

During interphase, PLK1 localization occurs at the cytoplasm and centrosomes (16). Centrosome maturation occurs prior to mitosis and PLK1 regulates this process by recruiting γ-tublin ring complexes (γ-TuRCs) to the centrosomes, and by phosphorylating ninein-like protein (NLP) and translationally controlled tumor protein (TCTP) – proteins involved in microtubule dynamics (17, 18). A mutation at the PLK1 phosphorylation site within NLP results in improper formation of the mitotic spindle (18).

Synthesis (S Phase)

PLK1 is known for its role in mitosis, but studies have shown that PLK1 inhibition can slow DNA replication and even activate the DNA damage checkpoint, suggesting a need for it in replication (19-21). PLK1 was shown to phosphorylate Hbo1 both in vitro and in cells at Ser-57 (19) . Hbo1 is responsible for facilitating the assembly of the pre-replicative complex (pre-RC) on replication origins at the end of mitosis and continuing through G1. Overexpressing mutant Hbo1 (S57A) at the PLK1

phosphorylation site lead to G1/S cell cycle arrest, decreased the rate of DNA replication, and inhibited chromain loading of the Mcm complex (19). It has been shown by co-immunoprecipitation that PLK1 and all members of the minichromosome maintenance complex (Mcm2-7) bind (22). PLK1-dependent phosphorylation of Hbo1 is believed to be necessary for pre-RC formation and DNA replication licensing, and is believed to occur primarily at the end of mitosis (19).

Mitosis

PLK1 regulates mitotic entry (23), progression through mitosis (24-27), cytokinesis (28), and is involved in mitotic exit (29). During mitosis, a cell's genetic material and its centrosomes are duplicated and equally distributed between the two daughter cells that form to complete the process of cell division. In prophase, the chromatin condenses and the nuclear envelope breaks down. During prometaphase, the bipolar spindle forms, and in metaphase the chromosomes are aligned at the metaphase plate and bound, via the kinetochores, to the microtubules. The two sets of chromosomes are separated during anaphase, and during telophase – the chromosomes de-condense and two nuclei are formed. After this, cytokinesis takes place to seperate the two daughter cells (30).

Mitotic Entry

PLK1 regulates the G2 to M phase transition through the mitosis promoting factor (MPF) – a complex of cyclin-dependent kinase- 1 (CDK-1) and cyclin-B (31). During S and G2 phase, CDK-1 is phosphorylated and cyclin B accumulates. They form a heterodimer resulting in a conformational change that activates CDK-1. WEE1 and MYT1 are enzymes that phosphorylate and inhibit this complex. PLK1 is involved with

cyclin B localization and WEE1 phosphorylation (32). During G2, the MPF is dephosphorylated by Cdc25C – a phosphatase that is a substrate of PLK1 – activating the MPF. This activation is what causes mitotic entry (33). PLK1 recognizes a specific sequence on Cdc25C and binds to it during prophase. Once bound by PLK1, Cdc25C is translocated to the nucleus allowing it to be phosphorylated and activated by PLK1 (32). PLK1's involvement in mitotic entry has been clearly documented, however, PLK1's role is not essential because PLK1 depleted cells are still able to enter mitosis. Problems arise as these cell progress through mitosis (34).

Mitotic Progression

PLK1 promotes mitotic entry, although its primary role is to regulate mitotic progression and exit (7, 35, 36). Once a cell enters mitosis the centrosomes migrate to opposite poles to form the spindles needed for cell division. During mitosis, PLK1 localizes to the centrosomes, kinetochores, and equatorial spindle mid-zone (37, 38). Bipolar spindle formation does not occur when PLK1 is inhibited by RNAi or small molecules, resulting in monopolar spindle formation and prometaphase arrest (39). In addition, kinetochores fail to properly attach to microtubules upon PLK1 depletion (16, 40).

PLK1 plays a vital role in the metaphase to anaphase transition, in that it helps to regulate chromosome alignment at the metaphase plate. PLK1 localizes to the kinetochores where it provides stability by creating tension between the kinetochores and microtubules (34). To transition from metaphase to anaphase PLK1 phosphorylates EMI1, resulting in its inactivation. This allows the anaphase promoting complex/cyclosome (APC/C) to become activated (41).

During telophase PLK1 localizes to the mid-zone and mid-body (42). Studies have shown that cells overexpressing PLK1 are multinucleated, indicating that appropriate PLK1 levels are necessary for proper completion of cytokinesis (11). In addition, studies have shown PLK1 phosphorylation of Golgi-associated protein GRASP65, leads to Golgi stack fragmentation. This also suggests an indirect role of PLK1 in cytokinesis via Golgi-associated proteins, since many of the proteins that dissociate from the Golgi during fragmentation help to regulate cytokinesis (43).

Therapeutic Target

Efforts to target PLK1 therapeutically are due to its over-expression in a number of cancers including colorectal, prostate, and non-small cell lung cancer (44-46). Over-expression of PLK1 correlates to poor prognosis in some cancers (3, 47, 48). Many ATP-competitive inhibitors have been developed, and some have advanced to clinical trials; however, these inhibitors are not selective for PLK1 and target other kinases. Because the catalytic domain is highly conserved among all kinases, it is important to target a non-catalytic domain of PLK1 to achieve selectivity. PLK1 is a unique target for drug development in that, due to its highly conserved domains, there are two target sites within the molecule that have differing functions and properties (discussed later) (3).

PLK2

PLK2 (SNK; serum inducible kinase) was first identified as an early-growth response gene having a role in early mitogenic response (49), but was later found to be involved in centriole duplication and cell proliferation (50-53). There is also evidence suggesting PLK2 is a tumor suppressor, being downregulated in a variety of cancer cells (54, 55).

PLK2 expression and activity increases as cells enter S phase (49, 53), localizing to the centrosomes. Although studies have shown that PLK2 inhibition leads to an aberrant number of centrioles (50, 51, 56), PLK2 is not believed to be essential due to the viability of PLK2 knockout mice (53). PLK2 transcripts are expressed in both proliferative and non-proliferative tissues, including the testis, mammary gland, spleen, uterus, trachea and cardiomyocytes. PLK proteins are known for their role in the cell cycle, but PLK2 is also expressed in the central nervous system – playing a role in synaptic homeostasis and neuronal differentiation. It is expressed in the cerebral cortex, hippocampus, occipital, temporal and parietal lobes, putamen and trigeminal ganglion, and it is minimally expressed at the cerebellum (4). PLK2 is also involved in neuronal cell differentiation (57). PLK2 levels are significantly increased in the brains of patients with Alzheimer's and Lewy Body disease (58).

There is high sequence homology between PLK1 and PLK2. There is nearly 50% homology between their kinase domains and about 30% homology between their PBD's, however, PLK1 and PLK2 have opposing functions in tumor development (59).

PLK3

PLK3 (Fnk or Prk) was first identified as an early response gene product (60, 61), and was later shown to regulate mitosis and DNA damage response (62-64). PLK3 protein levels are constant throughout the cell cycle, having increased mRNA levels during G1 (61, 65), and kinase activity peaking during late S and G2 (66, 67). PLK3 localizes to the nucleolus (68), centrosomes, spindles, microtubules (66, 69), and the Golgi apparatus where it is involved in regulating golgi dynamics (70). During interphase, PLK3 localizes around the nuclear membrane (66). PLK3 is required for

transition from G1 to S phase, through interacting with Cdc25A and indirectly impacting cycle E accumulation (68). PLK3 becomes phosphorylated and activated when a cell is stressed due to DNA damage. When this occurs, PLK3 binds to and activates p53 (65), resulting in p53 stabilization (63).

Gene expression studies show that PLK3 levels are decreased in tumors (71), while overexpression inhibits cellular growth (72). PLK3- deficient mice develop tumors, suggesting that is protein acts as a tumor suppressor (72). Ectopic expression of PLK3 disturbs microtubule integrity, causes G2/M arrest, and leads to apoptosis (66, 73). In addition, PTEN – a known tumor suppressor – is a substrate of PLK3 (74). Taken together, these results confirm PLK3's role as a tumor suppressor.

PLK4

PLK4 (SAK) is the most divergent PLK family member, having a C-terminus that contains only one polo-box. It is not believed to function as a binding domain. Instead it functions as a homo-dimerization domain (75). PLK4 expression increases during mitosis and has been shown to be an important regulator of centriole duplication in both Drosophila and mammals (76-79). Overexpressing SAK/PLK4 in Drosophila embryos induced centriole amplification (76). Altered PLK4 levels results in irregularities in centriole biogenesis and cell division. This leads to mitotic disruptions and aneuploidy (80). Specifically, downregulation of PLK4 in Drosophila results in a loss of centrioles leading to the formation of disorganized spindle poles (78, 79, 81). PLK4 has been shown to interact with proteins involved in cell proliferation including p53 (82) and Cdc25C (83).

PLK5

PLK5 is the most recently identified PLK family member. It has a stop codon within the catalytic domain and is highly expressed in the central nervous system. PLK5 is only expressed in non-proliferative tissues and is mostly transcribed in the cerebellum (84). PLK5 is also in the cytoplasm of neurons and glia. PLK5 over-expression results in G1 cell cycle arrest (84). Hyper-methylation and silencing of PLK5 was observed in human brain tumors at the mRNA and protein level. Re-expressing PLK5 in this environment resulted in kinase-dependent apoptosis. Taken together, this suggests that PLK5 may function as a tumor suppressor in the brain (84). PLK5 was also implicated in DNA damage responses (85).

1.3 PLK1 AS VALID THERAPEUTIC TARGET

Many mitotic proteins have been targeted for cancer therapeutic development (30, 86, 87), and anti-mitotic drugs have proven to be valid and effective anticancer therapies (88). PLK1 is only expressed in proliferating cells (11) and, as previously stated, PLK1 is over-expressed in many cancers, including – breast, colorectal, non-small cell lung cancer, prostate, pancreatic, papillary thyroid, ovarian, Non-Hodgkins lymphoma, and head and neck cancer (71, 89-93). Overexpression has been associated with poor prognosis and lower survival rates (48, 91, 94). In fact, the Kaplan-Meier-Plotter online tool was used to create a survival plot for patients with lung and gastric cancer based on PLK1 expression (95). This meta-analysis based biomarker assessment shows decreased patient survival when tumors overexpress PLK1 in lung and gastric cancers (Figure 1.1).

PLK1 has been evaluated as an anti-cancer therapeutic target because of the critical role that PLK1 plays throughout the cell cycle (86). PLK1 is a valid

chemotherapeutic target and many PLK1 inhibitors have been developed and evaluated in preclinical and clinical studies (3, 88, 96).

PLK1 AND ITS ROLE IN CANCER

PLK1 promotes the proliferation of both normal and cancer cells. However, when PLK1 is mutated or over-expressed, mitotic cell cycle checkpoints are bypassed, leading to tumorigenesis. PLK1 is overexpressed in many cancers, including breast, ovarian, and colorectal, and its over-expression correlates with poor prognosis in some cancers (97). Microinjected PLK1 mRNA into quiescent NIH 3T3 cells induced cell cycle progression from G_0 to mitosis. In addition, constitutively expressed PLK1 in NIH 3T3 cells resulted in anchorage-independent growth of cells in soft agar and tumor formation in nude mice (12). Moreover, elevated PLK1 mRNA levels have been detected in numerous tumor types (3).

Tumor suppressors like p53 (98) and PTEN (74) , and oncogenes like Kras (99) are frequently mutated in cancer. Synthetic lethality has been demonstrated when knocking down or inhibiting PLK1 in cancer cells lacking p53, expressing Kras mutations, or when PTEN deficient. The following section briefly discusses the interaction between PLK1 and p53, Kras, and PTEN.

PLK1 and p53:

p53 is a tumor suppressor that is involved in many important cellular processes including differentiation, senescence, cell cycle arrest, DNA repair, inhibition of angiogenesis, and apoptosis (100). p53 induced cell cycle arrest allows for DNA repair or recovery to occur prior to replication events. This process prevents the replication of damaged DNA. If damage is too extensive and repair cannot occur, p53 induced

programmed cell death occurs. This process prevents tumorigenesis in normal cells, but very often cancer cells have lost p53 function.

The association between p53 and PLK1 has been confirmed by many studies. These proteins negatively regulate each other in that p53 stability increased in PLK1 deficient HeLa cells while p53 levels remained low in PLK1 proficient HeLa control cells (36). PLK1 interacts with p53, inhibiting transcription and proapoptotic mechanisms (101, 102). In addition, p53 depleted cancer cells are more sensitive to PLK1 loss than p53 proficient cells (102).

PLK1 and Kras:

The Ras family of GTPases are responsible for transmitting proliferative signals from membrane growth factor receptors to the cell nucleus. Numerous tumors – including lung, liver, and colon – have been found to express KRAS mutations (103). Efforts to develop drugs that target Ras were marginal therefore, studies were carried out to determine what synthetic lethal interactions exist in cancers expressing mutant KRAS (99). Results showed that Ras mutant cells were sensitive to knockdown of mitotic genes (99). More specifically, mutant Ras tumor cells were found to be more sensitive than wild-type cells when PLK1 was knocked down by siRNA or inhibited by BI-2536. In addition, the cellular phenotypes of these cells were consistent with mitotic stress, including prometaphase cell cycle arrest. It was suggested that Ras mutations led to increased mitotic stress. This in turn causes the cells to have a greater dependence on PLK1 for mitotic progression (99).

PLK1 and PTEN:

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor that negatively regulates the PI3K/Akt pathway by removing a phosphate group from phosphoinositide 3,4,5-trisphosphate, blocking the recruitment and activation of PKB/Akt kinases responsible for signal transduction (104). Loss of PTEN function leads to excessive signaling of this pathway and promotes tumorigenesis (105). PTEN is a substrate of PLK3 and it is phosphorylated by PLK3 at Thr-366 and Ser-370. Xu et. al. (2010) showed that PLK3-mediated phosphorylation regulates PTEN stability (74). PTEN is also involved in mitosis, playing a role in PLK1 degradation and helping to facilitate centromere integrity (106, 107). PTEN depleted prostate cancer cells have elevated PLK1 expression and undergo mitotic stress. PLK1 was shown to play an important role in the survival of PTEN depleted cancer cells. PTEN depleted cells were more sensitive to PLK1 inhibition compared to wild-type cells, and underwent greater levels of apoptosis (108).

Taken together, this information validates PLK1 as a feasible oncotarget. Several ATP-based inhibitors of PLK1 have been developed as potential therapeutics, and some have reached clinical trials. Non-ATP-competitive inhibitors have also been developed. The section below will summarize information for some of the PLK1 inhibitors found in literature.

1.4 Catalytic Inhibitors of PLK1

BI2536

BI 2536 (developed by Boehringer Ingelheim Pharma, Ingelheim, Germany) is a small molecule inhibitor of PLK1 that works by targeting the catalytic domain via the

ATP-binding site. This molecule has a sub-nanomolar binding affinity to PLK1 in vitro and was shown to be 1000-fold more selective for PLK1 compared to other kinases, however, it does also inhibit the function of PLK2, PLK3, and PLK4 (109). This drug has been studied in cancer cells, providing valuable insight into the effects of PLK1 inhibition. Cells treated with BI2536 experienced prometaphase arrest, lacked microtubule asters, and formed monopolar spindles (110). BI2536 also prevented localization of PLK1 to the centrosomes and kinetochores. Studies with this drug indicate that PLK1 is not necessary for entry into prophase, but it does cause cells to accumulate in prophase, delaying entry into prometaphase (16). Anti-tumor activity was also observed in xenograft mouse models of human cancer (93), with side effects occurring in the bone marrow and other organs with high cell proliferation (111).

BI2536 entered phase I and II clinical trials where the activity and safety of BI-2536 in patients with a variety of advanced tumors – including head and neck, breast, ovarian, soft tissue sarcoma, and melanoma, were examined. BI-2536 was well tolerated, with neutropaenia and thrombocytopenia being the primary adverse side effect, but they observed insufficient anti-tumor activity due to poor pharmacokinetics (110, 112-114). Additionally, its potency toward PLK3 might account for its poor anti-tumor effects since PLK3 has opposing functions to PLK1.

BI6727

BI6727 (Volasertib) is a second-generation drug from BI2536 (115). Although it is a potent PLK1 inhibitor (IC_{50} of 0.87 nM), it also has an affinity for two other PLK family members – PLK2 (IC_{50} of 5 nM) and PLK3 (IC_{50} of 56 nM). Like BI2536, this compound works by targeting the catalytic domain. It binds to the ATP- binding pocket

and causes monopolar spindles to form (116). This compound was found to be active against many cancer cell lines, with cell proliferation EC_{50} values between 10-40 nM. It was also shown to be effective in human cancer xenograft models (116). The sensitivity of colorectal tumor cells to BI6727 was found to be independent of p53 status, however, cells having defective p53 experienced a greater incidence of multipolar spindles. It is suggested that this is because cells having wild-type p53 undergo apoptosis, while p53-defective cells undergo cell cycle arrest in mitosis with multipolar spindles (117).

BI6727 advanced to clinical trials (118, 119), and was well tolerated in patients. Like BI2536, phase I clinical trials determined that the most common side effects were neutropenia or thrombocytopenia, but the conditions were treatable and reversible. BI6727 also demonstrated favorable pharmacology having a MTD of 400 mg and a half-life of 110 hours. The anti-tumor activity was also encouraging, being effective in patients that had poor responses with other treatments (111). BI6727 progressed to phase II clinical trials and was granted breakthrough status by the FDA after significant results were observed when used in combination with cytarabine for Acute Myeloid Leukemia treatment.

GSK461364A

GSK461364A is another catalytic inhibitor that was developed by Glaxo Smith Kline (Middlesex, UK) (120, 121). It inhibits PLK1 with a Ki of 2.2 nM and is 390-fold more selective for PLK1 over PLK2, and 1000-fold selective for PLK1 than PLK3. When compared to other PLK1 inhibitors that have also reached clinical trials, GSK461364A shows greater specificity to PLK1 then to other PLKs. GSK461364 induced mitotic arrest is concentration dependent, with concentrations >300 nM causing a

delay in G2. Concentrations ranging between 10-30 nM induced mitotic arrest which led to severe micronucleation and cell death. Concentrations < 10 nM resulted in modest mitotic effects and moderate cytokinesis inhibition (122).

This compound was shown to be active against many cancer cell lines (123). Cell lines having non-functional p53 were shown to be most sensitive to PLK1 inhibition, however phenotypic results in lung and pancreatic tumor cell lines suggested a p53 independent effect. Following treatment with this catalytic inhibitor, phenotypic results showed that both Cyclin B1 and phospho-histone H3 levels accumulated, and mitotic slippage occurred (123). In vivo studies investigating the efficacy of this compound in preventing brain metastases of breast cancer showed that it was successful in inhibiting brain metastases by 60% in a xenograft model system (124).

Phase 1 clinical trials were carried out in patients with advanced solid malignancies (125). These patients were split into two groups that were administered the drug once a week (schedule A) or two times a week (schedule B). PLK1 was effectively targeted in tumors, however, thromboembolism occurred twenty percent of the time, with mild hematologic toxicity also taking place. Eighteen percent of patients developed neutropenia. This is a significant improvement from the 45% of patients that developed neutropenia in the BI 2536 clinical trial (125). The overall best response of stable disease (>16 weeks) occurred in 15% of patients. Although GSK461364 targeted PLK1 and anti-tumor activity it was recommended to be used in conjunction with a prophylactic anticoagulant (125).

GW843682X

GW843682X – a derivative of GSK461364A – causes mitotic arrest, spindle defects, and aberrant cytokinesis – phenotypes that are consistently observed with catalytic inhibition of PLK1 (120). Lung adenocarcinoma (H460) cells displayed aberrant mitotic events, including multipolar spindle formation, underwent cell cycle arrest, and died via mitotic catastrophe following treatment with this compound (120).

TAK-960

TAK-960 was developed by Takeda Pharmaceuticals. It binds to PLK1, PLK2, and PLK3 to varying degrees (IC50 = 0.8 nM, 17 nM, 50 nM, respectively). Cell cycle analysis results in HT-29 cells showed an accumulation of cells in G2/M phase following treatment with TAK-960. Fluorescence microscopy results showed monopolar spindle formation and increased levels of phosopho-histone H3 (126). Anti-proliferative activity was shown to be p53 independent in a panel of 18 cell lines. In addition, a reduction in tumor volume was observed in 10 xenograft mouse models (126). This compound is currently in clinical trials.

ZK-THIAZOLIDINONE

Like the previously mentioned catalytic PLK1 inhibitors, ZK-Thiazolidinone (TAL; Bayer Schering Pharma AG, Berlin, Germany) prevents the proliferative activity of a number of cancer cell lines, causes G2/M arrest, increases phosphoHistone-H3 levels, and causes cell death by mitotic catastrophe (91). In addition, TAL treatment resulted in monopolar spindle formation, impaired recruitment of γ-tublin to the centrosomes, and failed completion of cytokinesis (91). TAL also impaired recruitment of Aurora A kinase to the centrosomes.

ON 01910.Na

ON 01910.Na is a small molecule that was developed by Onconova Therapeutics. It inhibits PLK1 in substrate-dependent and ATP-independent manner (127). This compound primarily active against PLK1 but it is not selective, inhibiting other kinases like PLK2. ON 01910.Na causes mitotic arrest, induces multipolar spindles, causes abnormal centrosome localization and fragmentation, chromosome misalignment and detachment from spindles. These aberrant mitotic events leads to apoptosis (127). ON 01910.Na was active against many cancer cell lines, including drug-resistant cell lines. It also showed activity in vivo as a single agent, and when used in combination with other therapies like paclitaxel, vincristine, and doxorubicin (127).

ON 01910.Na went through phase I trials. Twenty patients having solid tumors were administered the drug at two hour infusions. It was determined that the maximum tolerated dose was 3120mg with skeletal and abdominal toxicity. Patients also experienced nausea and fatigue. Overall, ON 01910.Na was found to show moderate toxicity (128).

HMN-214

HMN-214 is an oral prodrug of HMN-176 with cytotoxic effects against several tumor types (89). HMN-176 indirectly inhibits PLK1 by altering is spatial distribution. This leads to cell cycle arrest, destruction of spindles, and DNA fragmentation (89, 129, 130). HMN-176 was active against a verity of human tumors in vivo, demonstrating antitumor activity in an exposure-dependent manner. HMN-214 in vivo antitumor activity was found to be comparable or greater to that of known anti-cancer agents like doxorubicin (89, 130).

HMN-214 was tested in phase I clinical trials to determine its phramacokinetic properties. HMN-214 was found to be rapidly converted to HMN-176 and patients developed neutropenia with modest anti-tumor activity (89, 130).

1.5 NON-ATP COMPETITIVE PBD INHIBITORS OF PLK1

POLOXIN

Poloxin was the first small molecule shown to bind to the polo-box domain (PBD) in vitro, by fluorescence polarization assay, and in cancer cells (131). This compound is a non-peptidic inhibitor of PLK1, having an IC_{50} value of 4.8 μM. Poloxin also binds to the PBD of PLK2 and PLK3 with IC_{50} values of 18.7 μM and 53.9 μM, respectively. It displays anti-proliferative activity in a variety of cancer cell lines with IC_{50} values ranging between 15-35 μM (132). Mitotic phenotypes associated with Poloxin treatment were found to include mislocalization of PLK1, fragmented and dissociated centrosomes, abnormal mitotic spindles, and misaligned chromosomes at the metaphase plate (132). These defects resulted in mitotic catastrophe leading to apoptosis. Unlike catalytic inhibitors that caused mono and multipolar spindle formation, bipolar spindles were present upon treatment with Poloxin (132).

More recently an optimized analogue of Poloxin, Poloxin-2, has been shown to have increased potency and selectivity over Poloxin. It induces mitotic arrest and apoptosis in human cancer cells at low micromolar concentrations, and is a promising candidate for future studies in cancer cells (133).

PURPUROGALLIN (PPG)

Purpurogallin (PPG) is a small molecule identified by high-throughput screening that binds to PLK1 with an IC_{50} value of 0.3 μM (134). PPG-treated HeLa cells

displayed reduced PLK1 locatization to the centrosomes and kinetochores, and cell accumulation in G2/M. Consistent with Poloxin – another PBD inibitor – PPG did not induce monopolar spindle formation.

1.6 Our Approach to Targeting the Polo-Box Domain (PBD)

PLK1 is just one of more than 500 protein kinases contained in the human proteome (135). This fact only emphasizes the complexity involved in developing a selective PLK1-inhibitor. While catalytic inhibitors have proven potent, there is still an issue of selectivity. Due to the non-selective attributes of catalytic inhibitors, there are off-target affects that cause dangerous clinical side effects. The kinase domain of the PLK family members contain many of the conserved features of other protein kinases, and they are highly similar to one another. This makes it difficult for most catalytic inhibitors to discriminate between the PLK family members (136). The catalytic domains of PLK2 and PLK3 are most alike while PLK1 and PLK4 are least alike (135, 137, 138).

There is an additional challenge when considering intracellular ATP concentrations. ATP is typically at a millimolar concentration within a cell, making it difficult to develop a small molecule that can compete for the ATP-binding site. To develop a useful ATP-inhibitor, the compound must be both selective and have a high binding affinity.

Because many ATP- competitive inhibitors have issues with specificity (139), there are efforts to develop inhibitors that target different domains. The Polo-box domain (PBD) is a highly conserved C-terminal domain among PLK family members. It is a phospho-dependent docking motif that brings the protein – having a phospho-threonine

or phospho-serine – into close proximity to its substrates (140). Structural analysis of the two polo-boxes (PB) that form the PBD were shown to have similar tertiary structures. The boxes fold together and form the interface for ligand binding (140). The PBD serves a regulatory function in that when the PB is bound to the kinase domain, it blocks the docking site within the kinase domain. When the PBs (forming the PBD) are bound to their phosphoprotein substrate, the PBD releases from the kinase domain, allowing it to bind to its target substrate (11, 141, 142).

Overexpressing the PLK1-PBD domain causes mitotic arrest and abnormal spindle formation (143). Catalytic activity of PLK1 also decreases when peptides bind to the PBD (144). Additionally, there are significant structural and functional differences between the PBD's of the PLK family members (145). Taken together, this suggests that PBD-inhibition could mimic the phenotypes observed with ATP-inhibition and provide significant target selectivity (86).

Lastly, a recent chemical genetics study has demonstrated that cells containing a single point mutation within the ATP-binding site of PLK1 (C67V) are resistant to several structurally unrelated ATP-inhibitors, while retaining the catalytic activity for its substrates (146). Such a mutation is likely to be observed clinically, eventually rendering the ATP-inhibitors ineffective. For these reasons, it is important to take an alternative approach in targeting PLK1 in cancer cells. We are interested in developing non-catalytic PBD-inhibitors to selectively target PLK1 to avoid off-target side effects that result from non-selective ATP-inhibition and to circumvent resistance observed with catalytic inhibitors.

REPLACE METHOD

This study examined the use of a systematic approach to examine the structure activity relationship (SAR) of peptide inhibitors to generate pharmaceutically acceptable small molecules. REPLACE (Replacement with Partial Ligand Alternatives through Computational Enrichment) uses the SAR of known peptide inhibitors to generate lead molecules. Individual amino acid residues – or groups of amino acid residues – from truncated substrate peptides were replaced with non-peptidic small molecular fragments. This is a structure guided drug discovery process that is a validated and more cost effective alternative to high-throughput screening (147, 148).

We exploit the truncated phosphopeptide sequence of known PLK1 substrates Cdc25C (LLCSpTPNGL) and PBIP (PLSHpTAI) (140, 149, 150). These truncated peptides are known to selectively bind to the PBD of PLK1. Although peptides are non-drug like, they are able to discriminate between the PLK family members. We can exploit the substrate selective differences of these peptides to develop selective PLK1 inhibitors (147).

We used the REPLACE method to determine the structure activity relationship of the sequence, and replace portions of the peptide – starting with the N-terminal fragment (LLC) – with small molecule fragments. The most promising partially peptidic molecules (FLIPs; Fragment Ligated Inhibitory Peptides) will be synthesized and tested biochemically to determine potency and selectivity for PLK1. The most promising FLIPs will then be tested in cells to determine activity and mechanism of action.

A biochemical and cellular analysis of truncated peptides and FLIPs will be discussed in Chapter 3. Here we analyze binding to PLK1-PBD and determine the

selectivity relative to PLK3-PBD. For the most promising FLIPs, we measure efficacy in cancer cell lines. Chapter 4 will discuss PLK1-PBD and PLK3-PBD binding of small molecule (non-petidic) PLK1 inhibitors synthesized by Dr. Sandra Craig (McInnes Lab). In addition, cellular analysis PBD-inhibitors will also be discussed. The overall conclusions, clinical implications of these findings, and the future directions of this work will be discussed in Chapter 5.

FIGURES

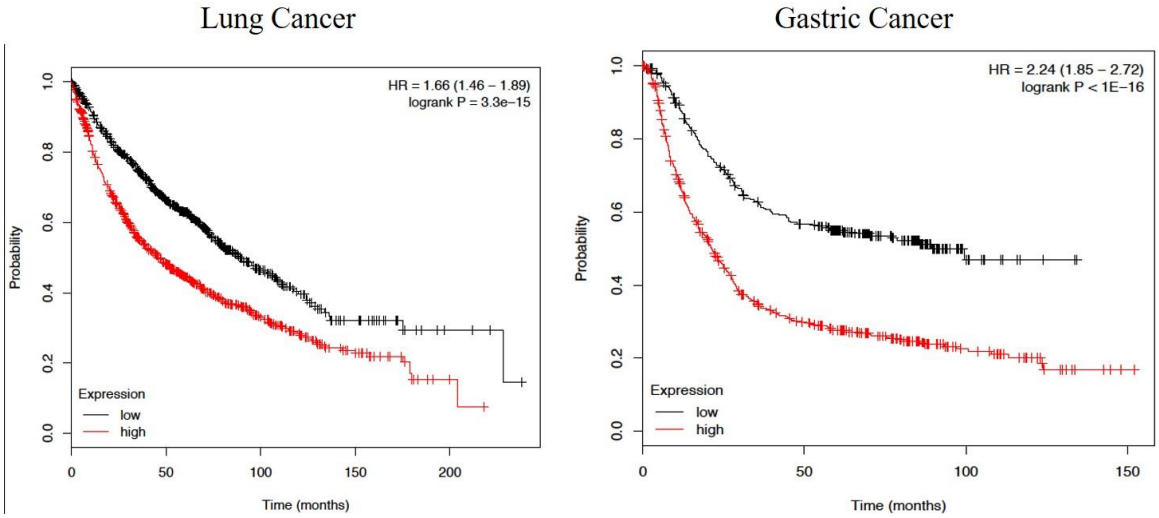


Figure 1.1 Kaplan Meier survival curve analysis of PLK1expression. Plots were generated using KM-plotter's online analysis software. Gene expression and clinical data from >1900 lung and >870 gastric cancer patients were split into two cohorts and compared by a Kaplan-Meier survival plot.

CHAPTER ONE REFERENCES

1. Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000;100:57-70.

2. Hanahan D, Weinberg RA. Hallmarks of Cancer: The next generation. Cell. 2011;144:646-74.

3. Strebhardt K, Ullrich A. Targeting polo-like kinase 1 for cancer therapy. Nature Reviews. 2006;6:321-30.

4. de Carcer G, Manning G, Malumbres M. From Plk1 to Plk5: Functional evolution of polo-like kinases. Cell Cycle. 2011;10(14):2255-62.

5. Sunkel CE, Glover DM. polo, a mitotic mutant of Drosophila displaying abnormal spindle poles. Journal of cell science. 1988;89(1):25-38.

6. Kitada K, Johnson A, Johnston L, Sugino A. A multicopy suppressor gene of the Saccharomyces cerevisiae G1 cell cycle mutant gene dbf4 encodes a protein kinase and is identified as CDC5. Molecular and Cellular Biology. 1993;13(7):4445-57.

7. Ohkura H, Hagan IM, Glover DM. The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. Genes & development. 1995;9(9):1059-73.

8. Hamanaka R, Maloid S, Smith MR, O'Connell CD, Longo DL, Ferris DK. Cloning and characterization of human and murine homologues of the Drosophila polo serine-threonine kinase. Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research. 1994;5(3):249-57.

9. Barr FA, Silljé HH, Nigg EA. Polo-like kinases and the orchestration of cell division. Nature reviews Molecular cell biology. 2004;5(6):429-41.

10. McInnes C, Wyatt MD. PLK1 as an oncology target: current status and future potential. Drug Discovery Today. 2011;16(13):619-25.

11. Mundt KE, Golsteyn RM, Lane HA, Nigg EA. On the regulation and function of human polo-like kinase 1 (PLK1): effects of overexpression on cell cycle progression. Biochemical and Biophysical Research Communication. 1997;239(2):377-85.

12. Smith MR, Wilson ML, Hamanaka R, Chase D, Kung H, Longo DL, et al. Malignant transformation of mammalian cells initiated by constitutive expressin of polo-like kinase. Biochemical and Biophysical Research Communication. 1997;234(2):397-405.

13. Golsteyn RM, Schultz SJ, Bartek J, Ziemiecki A, Ried T, Nigg EA. Cell cycle analysis and chromosomal localization of human Plk1, a putative homologue of the

mitotic kinases Drosophila polo and Saccharomyces cerevisiae Cdc5. Journal of Cell Science. 1994;107:1509-17.

14. Golsteyn RM, Mundt KE, Fry AM, Nigg EA. Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle formation. The Journal of Cell Biology. 1995;129:1617-28.

15. Lee KS, Yuan YO, Kuriyama R, Erikson RL. Plk is an M-phase specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. Molecular and Cellular Biology. 1995;15(12):7143-51.

16. Lenart P, Petronczki M, Steegmaier M, Fiore BD, Lipp JJ, Hoffmann M, et al. The small molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. Current Biology. 2007;17:304-15.

17. Yarm FR. Plk phosphorylation regulates the microtubule-stabilizing protein TCTP. Molecular and Cellular Biology. 2002;22:6209-21.

18. Casenghi M, Meraldi P, Weinhart U, Duncan PI, Korner R, Nigg EA. Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. Developmental Cell. 2003;5:113-25.

19. Wu Z-Q, Liu X. Role for Plk1 phosphorylation of Hbo1 in regulation of replication licensing. Proceedings of the National Academy of Sciences. 2008;105(6):1919-24.

20. Trenz K, Errico A, Costanzo V. Plx1 is required for chromosomal DNA replication under stressful conditions. The EMBO journal. 2008;27(6):876-85.

21. Lei M, Erikson R. Plk1 depletion in nontransformed diploid cells activates the DNA-damage checkpoint. Oncogene. 2008;27(28):3935-43.

22. Tsvetkov L, Stern DF. Interaction of chromatin-associated Plk1 and Mcm7. Journal of Biological Chemistry. 2005;280(12):11943-7.

23. Cogswell JP, Brown CE, Bisi JE, Neill SD. Dominant-negative polo-like kinase 1 induces mitotic catastrophe independent of cdc25C function. Cell Growth & Differentiation. 2000;11:615-23.

24. Qian YW, Erikson E, Li C, Maller JL. Activated Polo-like kinase Plx1 is requried at multiple points during mitosis in Xenopus laevie. Molecular and Cellular Biology. 1998;18(7):4262-71.

25. Smits VAJ, Klompmaker R, Arnaud L, Rijksen G, Nigg EA, Medema RH. Polo-like kinase 1 is a target of the DNA damage checkpoint. Nature Cell Biology. 2000;2:672-6.

26. Kotani S, Tugendreich S, Fujii M, Jorgensen PM, Watanabe N, Hoog C, et al. PKA and MPF-activated Polo-like kinase regulate anapahse-promoting complex activity and mitosis progression. Molecular Cell. 1998;1:371-80.

27. Dai W, Wang Q, Traganos F. Polo-like kinases and centrosome regulation. Oncogene. 2002;21:6195-200.

28. Burkard ME, Randall CL, Larochelle S, Zhang C, Shokat KM, Fisher RP, et al. Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. PNAS. 2007;104(11):4383-8.

29. Moshe Y, Boulaire J, Pagano M, Hershko A. Role of Polo-like kinasein the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. PNAS. 2004;101(21):7937-42.

30. de Castro P, de Carcer G, Malumbres M. A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy. Carcinogenesis 2007;28(5):899-912.

31. Hunt T. Cyclins and their partners: from a simple idea to complicated reality. Seminars in Cell Biology. 1991;2(4):213-22.

32. Toyoshima-Morimoto F, Taniguchi E, Nishida E. Plk1 promotes nuclear translocation of human Cdc25C during prophase. EMBO. 2002;3(4):341-8.

33. Coleman TR, Dunphy WG. Cdc2 regulatory factors. Current opinion in cell biology. 1994;6(6):877-82.

34. van Vugt MA, van de Weerdt BC, Vader G, Janssen H, Calafat J, Klompmaker R, et al. Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. The Journal of Biological Chemistry. 2004;279(35):36841-54.

35. Lee KS, Erikson RL. Plk is a functional homolog of Saccharomyces cerevisiae Cdc5, and elevated Plk activity induces multiple septation structures. Molecular and cellular biology. 1997;17(6):3408-17.

36. Liu X, Erikson RL. Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells. PNAS. 2003;100(10):5789-94.

37. Arnaud L, Pines J, Nigg EA. GFP tagging reveals human polo-like kinase 1 at the kinetochore/centromere region of mitotic chromosomes. Chromosoma. 1998;107:424-9.

38. Wianny F, Tavares A, Evans MJ, Glover DM, Goets MZ. Mouse polo-like kinase 1 associates with the acentriolar spindle poles, meiotic chromosomes and spindle midzone during oocyte maturation. Chromosoma. 1998;107:430-9.

39. Lane HA, Nigg EA. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturtion of mitotic centrosomes. The Journal of Cell Biology. 1996;135(6 Pt2):1701-613.

40. Liu X, Erikson RL. Activation of Cdc/cyclin B and inhibition of centrosome amplification in cells depleted of Plk1 by siRNA. PNAS. 2002;99(13):8672-6.

41. Miller JJ, Summers MK, Hansen DV, Nachury MV, Lehman NL, Loktev A, et al. Emi1 stably binds and inhibits the anaphase-promoting complex/cyclosome as a pseudosubstrate inhibitor. Genes and Development. 2006;20(17):2410-20.

42. van de Weerdt BC, Medema RH. Polo-like kinases: a team in control of the division Cell Cycle. 2006;5(8):853-64.

43. Lin CY, Madsen ML, Yarm FR, Jang YJ, Liu X, Erikson RL. Peripheral Golgi protein GRASP65 is a target of mitotic polo-like kinase (Plk) and Cdc2. PNAS. 2000;97(23):12589-94.

44. Takahashi T, Sano B, Nagata T, Kato H, Sugiyama Y, Kunieda K, et al. Polo-like kinase 1 (PLK1) is overexpressed in primary colorectal cancers. Cancer Science. 2003;94(2):148-52.

45. Yuan J, Horlin A, Hock B, Stutte HJ, Waigmann HR, Strebhardt K. Polo-like kinase, a novel marker for cellular proliferation. American Journal of Pathology. 1997;150(4):1165-72.

46. Wolf G, Elez R, Doermer A, Holtrich U, Ackermann H, Stutte HJ, et al. Prognostic significance of polo-like (PLK) expression in non-small cell lung cancer. Oncogene. 1997;14:543-9.

47. Degenhardt Y, Lampkin T. Targeting Polo-like kinase in cancer therapy. Clinical Cancer Research. 2010;16(2):384-9.

48. Eckerdt F, Yuan J, Strebhardt K. Polo-like kinases and oncogenesis. Oncogene. 2005;24:267-76.

49. Simmons DL, Neel BG, Stevens R, Evett G, Erikson RL. Identification of early-growth-response gene encoding a novel putative protein kinase. Molecular and Cellular Biology. 1992;12(9):4164-9.

50. Warnke S, Kemmler S, Hames RS, Tsai HL, Hoffmann-Rohrer U, Fry AM, et al. Polo-like kinase-2 is required for centriole duplication in mammalian cells. Current Biology. 2004;14(13):1200-7.

51. Cizmecioglu O, Warnke S, Arnold M, Duensing S, Hoffmann I. Plk2 regulated centriole duplication isi dependent on its localization to the centreoles and a functional polo-box domain. Cell Cycle. 2008;7(22):3548-55.

52. Matthew EM, Yen TJ, Dicker DT, Dorsey JF, Yang W, Navaraj A, et al. Replication stress, defective S-phase checkpoint and increased death in Plk2-deficient human cancer cells. Cell Cycle. 2007;6(20):2571-8.

53. Ma S, Charron J, Erikson RL. Role of Plk2 (Snk) in mouse development and cell proliferation. Molecular and Cellular Biology. 2003;19:6936-43.

54. Syed N, Smith P, Sullivan A, Spender LC, Dyer M, Karran L, et al. Transcriptional silencing of Polo-like kinase 2 (SNK/PLK2) is a frequent event in B-cell malignancies. Blood. 2006;107(1):250-6.

55. Pellegrino R, Calvisi DF, Ladu S, Ehemann V, Staniscia T, Evert M, et al. Oncogenic and tumor suppressive roles of polo-like kinases in human hepatocellular carcinoma. Hepatology. 2010;51(3):857-68.

56. Chang J, Cizmecioglu O, Hoffmann I, Rhee K. Plk2 phosphorylation is critical for CPAP function in procentriole formation during the centrosome cycle. The EMBO Journal. 2010;29(14):2395-406.

57. Draghetti C, Salvat C, Zanoguera F, Curchod M-L, Vignaud C, Peixoto H, et al. Functional Whole-genome Analysis Identifies Polo-like Kinase 2 and Poliovirus Receptor as Essential for Neuronal Differentiation Upstream of the Negative Regulator αB-crystallin. Journal of Biological Chemistry. 2009;284(46):32053-65.

58. M.K. M, Paleologou KE, Boucharaba A, Oueslati A, Schell H, Founier M, et al. Phosphorylation of synucleins by members of the polo-like kinase family. The Journal of Biological Chemistry. 2010;285(4):2807-22.

59. Kim JH, Ku B, Lee KS, Kim SJ. Structural analysis of the polo-box domain of human Polo-like kinase 2. Proteins: Structure, Function, and Bioinformatics. 2015.

60. Li B, Ouyang B, Pan H, Reissmann PT, Slamon DJ, Arceci R, et al. Prk, a cytokine-inducible human protein serine/threonine kinase whose expression appears to be down-regulated in lung carcinomas. Journal of Biological Chemistry. 1996;271(32):19402-8.

61. Donohue PJ, Albets GF, Guo Y, Winkles JA. Identification by targeted differential display of an immediate early gene encoding a putative serine/threonine kinase. The Journal of Biological Chemistry. 1995;270(17):10351-7.

62. Ouyang B, Pan H, Lu L, Li J, Stambrook P, Li B, et al. Human Prk is a conserved protein serine/threonine kinase involved in regulating M phase functions. Journal of Biological Chemistry. 1997;272(45):28646-51.

63. Xie S, Wu H, Wang Q, Cogswell JP, Husain I, Conn C, et al. Plk3 functionally links DNA damage to cell cycle arrest and apoptosis at least in part via the p53 pathway. The Journal of Biological Chemistry. 2001;276(46):43305-12.

64. Xie S, Wang Q, Wu H, Cogswell J, Lu L, Jhanwar-Uniyal M, et al. Reactive oxygen species-induced phosphorylation of p53 on serine 20 is mediated in part by polo-like kinase-3. Journal of Biological Chemistry. 2001;276(39):36194-9.

65. Bahassi EM, Conn CW, Myer DL, Hennigan RF, McGowan CH, Sanchez Y, et al. Mammalian Polo-like kinase 3 (Plk3) is a multifunctional protein involved in stress response pathways. Oncogene. 2002;21:6633-40.

66. Wang Q, Xie S, Chen J, Fukasawa K, Naik U, Traganos F, et al. Cell cycle arrest and apoptosis induced by human Polo-like kinase 3 is mediated through perturbation of microtubule integrity. Molecular and cellular biology. 2002;22(10):3450-9.

67. Ouyang B, Wang Y, Dai W. Caenorhabditis elegans contains structural homologs of human prk and plk. Mitochondrial DNA. 1999;10(2):109-13.

68. Zimmerman WC, Erikson RL. Polo-like kinase 3 is required for entry into S phase. PNAS. 2007;104(6):1847-52.

69. Holtrich U, Wolf G, Yuan J, Bereiter-Hahn J, Karn T, Weiler M, et al. Adhesion induced expression of the serine/threonine kinase Fnk in human macrophages. Oncogene. 2000;19(42):4832-9.

70. Xie S, Wang Q, Ruan Q, Liu T, Jhanwar-Uniyal M, Guan K, et al. MEK1-induced Golgi dynamics during cell cycle progression is partly mediated by Polo-like kinase-3. Oncogene. 2004;23(21):3822-9.

71. Winkles JA, Alberts GF. Differential regulation of polo-like kinase 1, 2, 3, and 4 gene expression in mammalian cells and tissues. Oncogene. 2005;24(2):260-6.

72. Yang Y, Bai J, Shen R, Brown SAN, Komissarova E, Huang Y, et al. Polo-like kinase 3 functions as a tumor suppressor and is a negative regulator of hypoxia-inducible factor-1alpha under hypoxic conditions. Cancer Research. 2008;68(11):4077-85.

73. Conn CW, Hennigan RF, Dai W, Sanchez Y, Stambrook PJ. Incomplete cytokinesis and induction of apoptosis by overexpression of the mammalian polo-like kinase, Plk3. Cancer research. 2000;60(24):6826-31.

74. Xu D, Yao Y, Jiang X, Lu L, Dai W. Regulation of PTEN stability and activity by Plk3. The Journal of Biological Chemistry. 2010;285(51):39935-42.

75. Lowery DM, Lim D, Yaffe MB. Structure and function of Polo-like kinases. Oncogene. 2005;24(2):248-59.

76. Rodrigues-Martins A, Riparbelli M, Callaini G, Glover DM, Bettencourt-Dias M. Revisiting the role of the mother centriole in centriole biogenesis. Science. 2007;316(5827):1046-50.

77. Kleylein-Sohn J, Westendorf J, Le Clech M, Habedanck R, Stierhof YD, Nigg EA. Plk4-induced centriole biogenesis in human cells. Developmental Cell. 2007;13(2):190-202.

78. Bettencourt-Dias M, Rodrigues-Martins A, Carpenter L, Riparbelli M, Lehmann L, Gatt MK, et al. SAK/PLK4 is required for centriole duplication and flagella development. Current Biology. 2005;15(24):2199-207.

79. Habedanck R, Stierhof YD, Wilkinson CJ, Nigg EA. The Polo kinase Plk4 functions in centriole duplication. Nature Cell Biology. 2005;7(11):1140-6.

80. Hudson JW, Kozarova A, Cheung P, Macmillan JC, Swallow CJ, Cross JC, et al. Late mitotic failure in mice lacking Sak, a polo-like kinase. Current Biology. 2001;11(6):441-6.

81. Hatch EM, Kulukian A, Holland AJ, Cleveland DW, Stearns T. Cep152 interacts with Plk4 and is required for centriole duplication. The Journal of Cell Biology. 2010;191(4):721-9.

82. Swallow CJ, Ko MA, Siddiqui NU, Hudson JW, Dennis JW. Sak/Plk4 and mitotic fidelity. Oncogene. 2005;24(2):306-12.

83. Bonni S, Ganuelas ML, Petrinac S, Hudson JW. Human Plk4 phosphorylates Cdc25C. Cell Cycle. 2008;7(4):545-7.

84. de Carcer G, Escobar B, Higuero AM, Garcia L, Anson A, Perez G, et al. Plk5, a polo-box domain only protein with specific roles in neuron differentiation and glioblastoma suppression. Molecular and Cellular Biology. 2011;31(6):1225-39.

85. Andrysik Z, Bernstein WZ, Deng L, Myer DL, Li YQ, Tischfield JA, et al. The novel mouse Polo-like kinase 5 responds to DNA damage and localizes in the nucleolus. Nucleic Acids Research. 2010;38(9):2931-43.

86. de Castro P, de Carcer G, Montoya G, Malumbres M. Emerging cancer therapeutic opportunities by inhibiting mitotic kinases. Current Opinion in Pharmcology. 2008;8(4):375-83.

87. de Carcer G, de Castro P, Malumbres M. Targetig cell cycle kinases for cancer therapy. Current Medicinal Chemistry. 2007;14(9):969-85.

88. Lens SM, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nature Reviews Cancer. 2010;10(12):825-41.

89. Garland LL, Taylor C, Pilkington DL, Cohen JL, Von Hoff DD. A phase I pharmacokinetic study of HMN-214, a novel oral stilbene derivative with polo-like kinase-1–interacting properties, in patients with advanced solid tumors. Clinical cancer research. 2006;12(17):5182-9.

90. Holtrich U, Wolf G, Bräuninger A, Karn T, Böhme B, Rübsamen-Waigmann H, et al. Induction and down-regulation of PLK, a human serine/threonine kinase expressed in proliferating cells and tumors. Proceedings of the National Academy of Sciences. 1994;91(5):1736-40.

91. Santamaria A, Neef R, Eberspächer U, Eis K, Husemann M, Mumberg D, et al. Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis. Molecular biology of the cell. 2007;18(10):4024-36.

92. Fisher R, Ferris D. The functions of Polo-like kinases and their relevance to human disease. Current Medicinal Chemistry-Immunology, Endocrine & Metabolic Agents. 2002;2(2):125-34.

93. Steegmaier M, Hoffmann M, Baum A, Lenart P, Petronczki M, Krssak M, et al. BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. Current Biology. 2007;17:316-22.

94. Weichert W, Ullrich A, Schmidt M, Gekeler V, Noske A, Niesporek S, et al. Expression patterns of polo-like kinase 1 in human gastric cancer. Cancer science. 2006;97(4):271-6.

95. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer [Internet]. 2013.
96. Strebhardt K. Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. Nature Reviews: Drug Discovery. 2010;9(8):643-60.

97. Wei L, Efferth T. Polo-like kinase 1 as a target for cancer therapy. Experimental Hematology and Oncology. 2012;1(38).

98. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science. 1991;253(5015):49-53.

99. Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF, et al. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell. 2009;137:835-48.

100. Bates S, Vousden KH. Mechanisms of p53-mediated apoptosis. Cellular and Molecular Life Sciences. 1999;55(1):28-37.

101. Ando K, Ozaki T, Yamamoto H, Furuya K, Hosoda M, Hayashi S, et al. Polo-like kinase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation. Journal of Biological Chemistry. 2004;279(24):25549-61.

102. Liu X, Lei M, Erikson RL. Normal cells, but not cancer cells, survive severe Plk1 depletion. Molecular and Cellular Biology. 2006;26(6):2093-108.

103. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nature reviews Molecular cell biology. 2008;9(7):517-31.

104. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the pten tumor suppressor Cell Biology. 2012;13:283-96.

105. Jiang BH, Liu LZ. PI3K/PTEN signaling in angiogenesis and tumorigenesis. Advances in cancer research. 2009;102:19-65.

106. Song MS, Carracedo A, Salmena L, Song SJ, Egia A, Malumbres M, et al. Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. Cell. 2011;144:187-99.

107. Shen WH, Balajee AS, Wang J, Wu H, Eng C, Pandolfi PP, et al. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell. 2007;128:157-70.

108. Liu XS, Song B, Elzey BD, Ratliff TL, Konieczny SF, Cheng L, et al. Polo-like kinase 1 facilitates loss of Pten tumor suppressor-induced prostate cancer formation. The Journal of Biological Chemistry. 2011;286(41):35795-800.

109. Lu B, Mahmud H, Maass AH, Yu B, van Gilst WH, de Boer RA, et al. The Plk1 inhibitor BI 2536 temporarily arrests primary cardiac fibroblasts in mitosis and generates aneuploidy in vitro. PLOS One. 2010;5(9):e12963.

110. Mross K, Frost A, Steinbild S, Hedbom S, Rentschler J, Kaiser R, et al. Phase I dose escalation and pharmacokinetic study of BI 2536, a novel Polo-like kinase 1 inhibitor, in patients with advanced solid tumors. Journal of Clinical Oncology. 2008;26(34):5511-7.

111. Schöffski P. Polo-like kinase (PLK) inhibitors in preclinical and early clinical development in oncology. The oncologist. 2009;14(6):559-70.

112. Sebastian M, Reck M, Waller CF, Kortsik C, Frickhofen N, Schuler M, et al. The efficacy and safety of BI 2536, a novel Plk-1 inhibitor, in patients with stage IIIB/IV non-small cell lung cancer who had relapsed after, or failed, chemotherapy: results from an

open-label, randomized phase II clinical trial. Journal of Thoracic Oncology 2010;5(7):1060-7.

113. Schoffski P, Blay JY, De Greve J, Brain E, Machiels JP, Soria JC, et al. Multicentric parallel phase II trial of the polo-like kinase 1 inhibitor BI 2536 in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma. European Journal of Caner. 2010;46(12):2206-15.

114. Hofheinz RD, Al-Batran SE, Hochhaus A, Jager E, Reichardt VL, Fritsch H, et al. An open-label, phase I study of the polo-like kinase- 1 inhibitor, BI 2536, in patients with advanced solid tumors. Clinical Cancer Research. 2010;16(18):4666-74.

115. Rudolph D, Steegmaier M, Hoffmann M, Grauert M, Baum A, Quant J, et al. BI 6727, a Polo-like kinase inhibitor with improved pharmacokinetic profile and broad antitumor activity. Clinical Cancer Research. 2009;15:3094-102.

116. Rudolph D, Steegmaier M, Hoffmann M, editors. Characterization of BI 6727, a novel Polo-like kinase inhibitor with a distinct pharmacokinetic profile and efficacy in a model of taxane-resistant colon cancer. 20th European Organization for Research and Treatment of Cancer–National Cancer Institute–American Association for Cancer Research Symposium on Molecular Targets and Cancer Therapeutics, Geneva, Switzerland; 2008.

117. Sanhaji M, Kreis NN, Zimmer B, Berg T, Louwen F, Yuan J. p53 is not directly relevant to the response of Polo-like kinase 1 inhibitors. Cell Cycle. 2012;11:543-53.

118. Stadler WM, Vaughn DJ, Sonpavde G, Vogelzang NJ, Tagawa ST, Petrylak DP, et al. An open-label, single-arm, phase 2 trial of the polo-like kinase inhibitor volasertib (BI 6727) in patients with locally advanced or metastatic urothelial cancer. Cancer. 2014;120(7):976-82.

119. Craig SN, Wyatt MD, McInnes C. Current assessment of polo-like kinases as anti-tumor drug targets. Expert Opinion Drug Discovery. 2014;9(7):773-89.

120. Lansing TJ, McConnell RT, Duckett DR, Spehar GM, Knick VB, Hassler DF, et al. In vitro biological activity of a novel small-molecule inhibitor of polo-like kinase 1. Molecular cancer therapeutics. 2007;6(2):450-9.

121. Emmitte KA, Adjebang GM, Andrews CW, Alberti JGB, Bambal R, Chamberlain SD, et al. Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & medicinal chemistry letters. 2009;19(6):1694-7.

122. Laquerre S, Sung C-M, Gilmartin A, Courtney M, Ho M, Salovich J, et al. A potent and selective Polo-like kinase 1 (Plk1) inhibitor (GSK461364) induces cell cycle arrest and growth inhibition of cancer cell. Cancer Research. 2007;67(9 Supplement):5389-.

123. Degenhardt Y, Greshock J, Laquerre S, Gilmartin AG, Jing J, Richter M, et al. Sensitivity of cancer cells to Plk1 inhibitor GSK461364A is associated with loss of p53 function and chromosome instability. Molecular Cancer Therapeutics. 2010;9(7):2079-89.

124. Palmieri D, Hua E, Qian Y, editors. Preclinical studies investigating the efficacy of GSK461364A, an inhibitor of Polo-like kinase-1, for the prevention of breast cancer brain metastases. American Association for Cancer Research 2009 Annual Meeting, Denver, CO; 2009.

125. Olmos D, Barker D, Sharma R, Brunetto AT, Yap TA, Taegtmeyer AB, et al. Phase I study of GSK461364, a specific and competitive Polo-like kinase 1 inhibitor, in patients with advanced solid malignancies. Clinical Cancer Research. 2011;17(10):3420-30.

126. Hikichi Y, Honda K, Hikami K, Miyashita H, Kaieda I, Murai S, et al. TAK-960, a novel, orally available, selective inhibitor of polo-like kinase 1, shows broad-spectrum preclinical antitumor activity in multiple dosing regimens. Molecular cancer therapeutics. 2012;11(3):700-9.

127. Gumireddy K, Reddy MVR, Cosenza SC, Nathan RB, Baker SJ, Papathi N, et al. ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. Cancer Cell. 2005;7:275-86.

128. Jimeno A, Li J, Messersmith WA, Laheru D, Rudek MA, Maniar M, et al. Phase I study of ON 01910. Na, a novel modulator of the Polo-like kinase 1 pathway, in adult patients with solid tumors. Journal of Clinical Oncology. 2008;26(34):5504-10.

129. Tanaka H, Ohshima N, Ikenoya M, Komori K, Katoh F, Hidaka H. HMN-176, an active metabolite of the synthetic antitumor agent HMN-214, restores chemosensitivity to multidrug-resistant cells by targeting the transcription factor NF-Y. Cancer research. 2003;63(20):6942-7.

130. Takagi M, Honmura T, Watanabe S, Yamaguchi R, Nogawa M, Nishimura I, et al. In vivo antitumor activity of a novel sulfonamide, HMN-214, against human tumor xenografts in mice and the spectrum of cytotoxicity of its active metabolite, HMN-176. Investigational new drugs. 2003;21(4):387-99.

131. Reindl W, Yuan J, Krämer A, Strebhardt K, Berg T. Inhibition of polo-like kinase 1 by blocking polo-box domain-dependent protein-protein interactions. Chemistry & biology. 2008;15(5):459-66.

132. Yuan J, Sanhaji M, Krämer A, Reindl W, Hofmann M, Kreis N-N, et al. Polo-box domain inhibitor poloxin activates the spindle assembly checkpoint and inhibits tumor growth in vivo. The American journal of pathology. 2011;179(4):2091-9.

133. Scharow A, Raab M, Saxena K, Sreeramulu S, Kudlinzki D, Gande S, et al. Optimized Plk1 PBD inhibitors based on Poloxin induce mitotic arrest and apoptosis in tumor cells. ACS chemical biology. 2015;10(11):2570-9.

134. Watanabe N, Sekine T, Takagi M, Iwasaki J-i, Imamoto N, Kawasaki H, et al. Deficiency in chromosome congression by the inhibition of Plk1 polo box domain-dependent recognition. Journal of Biological Chemistry. 2009;284(4):2344-53.

135. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298(5600):1912-34.

136. Johnson EF, Stewart KD, Woods KW, Giranda VL, Luo Y. Pharmacological and functionl comparison of the polo-like kinase family: insight into inhibitor and substrate specificity. Biochemistry. 2007;46(33):9551-63.

137. McInnes C, Mezna M, Fischer PM. Progress in the discovery of polo-like kinase inhibitors. Current topics in medicinal chemistry. 2005;5(2):181-97.

138. Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science. 1988;241(4861):42-52.

139. Fedorov O, Marsden B, Pogacic V, Rellos P, Muller S, Bullock AN, et al. A systematic interaction map of vlidated kinase inhibitors with Ser/Thr kinases. PNAS. 2007;104(51):20523-38.

140. Elia AEH, Rellos P, Haire LF, Chao JW, Ivins FJ, Hoepker K, et al. The molecular basis for phosphodependent substrate targeting and regulation of plks by the polo-box domain. Cell Press. 2003;115:83-95.

141. Song S, Grenfell TZ, Garfield S, Erikson RL, Lee KS. Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. Molecular and Cellular Biology. 2000;20(1):286-98.

142. Jang Y-J, Lin C-Y, Ma S, Erikson RL. Functional studies on the role of C-terminal domain of mammalian polo-like kinase. PNAS. 2002;99(4):1984-9.

143. Pinsky BA, Biggins S. The spindle checkpoint: tension versus attachment. Trends in cell biology. 2005;15(9):486-93.

144. Garcia-Alvarez B, Carcer G, Ibanez S, Bragado-Nilsson E, Montoya G. Molecular and structural basis of polo-like kinase 1 substrate recognition: Implications in centrosomal localization. PNAS. 2007;104(9):3107-12.

145. van de Weerdt BC, Littler DR, Klompmaker R, Huseinovic A, Fish A, Perrakis A, et al. Polo-box domains confer target specificity to the Polo-like kinase family. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2008;1783(6):1015-22.

146. Burkard ME, Santamaria A, Jallepalli PV. Enabling and disabling Polo-like kinase 1 inhibition through chemical genetics. ACS Chemical Biology. 2012;7:978-81.

147. McInnes C, Estes K, Baxter M, Yang Z, Farag DB, Johnston P, et al. Targeting subcellular localization through the polo-box domain: Non-ATP competitive inhibitors recapitulate a PLK1 phenotype. Molecular Cancer Therapeutics. 2012;11(8):1683-92.

148. Andrews MJ, Kontopidis G, McInnes C, Plater A, Innes L, Cowan A, et al. REPLACE: A strategy for interative design of cyclin-binding groove inhibitors. ChemBiochem. 2006;7:1909-15.

149. Elia AEH, Cantley LC, Yaffe MB. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science. 2003;299:1228-31.

150. Yun SM, Moulaei T, Lim D, Bang JK, Park JE, Shenoy SR, et al. Structural and functional analyses of minimal phosphopeptides targeting the polo-box domain of polo-like kinase 1. Nature Structural and Molecular Biology. 2009;16(8):876-82.

CHAPTER TWO

RE-OPTIMIZING THE PLK1 AND PLK3 POLO BOX DOMAIN (PBD) COMPETITIVE BINDING FLUORESCENCE POLARIZATION (FP) ASSAY

ABSTRACT

The fluorescence polarization (FP) assay is a competitive binding assay that we use as our primary screen to measure compound binding to PLK1 and PLK3. We began using low volume 384 well plates to reduce the amount of reagents used per assay. As a result, new assay conditions had to be determined to accurately measure binding. This chapter explains the process by which PLK1 and PLK3 FP assay conditions were determined for optimum detection by the plate reader.

2.1 INTRODUCTION

We determined the relative affinities of peptides, Fragment Ligated Inhibitory Peptides (FLIPs) and compounds for the PBD of PLK1 and PLK3 using a fluorescence polarization (FP) assay. This is a competitive binding assay that allows us to indirectly measure compound binding by quantifying the compound's ability to displace a fluorescein labelled tracer peptide from its PBD binding site. This is an equilibrium reaction with the equation:

$$[\text{Tracer}] + [\text{PBD Protein}] \rightleftharpoons [\text{Tracer} - \text{PBD Protein}]$$

When tracer molecules are unbound to the PBD protein, they freely rotate in solution resulting in low fluorescence polarization. Conversely, PBD-bound tracer rotates

$$p = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + GI_{\perp}}$$

slowly in solution and yields high fluorescence polarization values. Polarization values are generated from parallel and perpendicular fluorescence intensities.

The inhibitor will compete with the fluorescein tracer for PBD binding, displacing it from the PBD if it has an affinity for the protein. We are able to 'visualize' this competition by measuring the polarization of bound tracer relative to unbound tracer.

$$[\text{Tracer}] + [\text{PBD Protein}] \leftrightarrow [\text{Tracer} - \text{PBD Protein}]\ [\text{Inhibitor}] \leftrightarrow [\text{Tracer}] + [\text{PBD Protein} - \text{Inhibitor}]$$

Therefore, to accurately measure binding the assay conditions must be properly optimized for optimum detection. First, the tracer concentration must be selected that provides an excellent signal to noise ratio and does not saturate detection limits. Next, the concentration of fluorescent tracer is held constant and a concentration curve with the PBD protein must be analyzed to determine the concentration at which at least 80% of the tracer is bound to the PBD. Once the proper concentrations are determined, increasing concentrations of the compound can be added to the reaction, and compound binding affinity for the PBD can be determined by measuring fluorescence polarization in millipolarization (mP) units (1).

The FP assay is our primary screen to determine the binding affinity of our compounds to the PBD of PLK1 and PLK3. Molecules of interest are quantitated by a reduction in millipolarization (mP) values that occur as the tracer is displaced from the PBD, and plotted as an IC_{50}. The 2012 MCT paper (2) details the results of the first utilization of this assay on the prior series of peptides and FLIPs that formed the starting point of this thesis. During the course of this work, the assay conditions were further

optimized as part of a regular quality control process and after switching to low-volume 384-well plates to save on reagents. This chapter explains the process to optimize the FP assay and discusses why this process is so important for accurately measuring binding affinity.

2.2 METHODS

Optimization of Fluorescence for the Peptide Tracer

To determine the optimal tracer concentration that provides excellent signal to noise ratio without saturating the detection signal, a tracer titration curve was carried out. Increasing concentration (0.1 – 300 nM) of tracer was added to the 384 well plate in triplicate. Because the concentration will be diluted by one-third once all solutions are added to the well, tracer was made up at a concentration 3 times the desired final concentration, such that 0.1 nM final concentration was made up at 0.3 nM concentration. Dilutions were made using a BSA containing buffer (10 mM Tris HCl pH 8, 1.0 mg/mL BSA). 5 µL tracer was added to each well along with 10 µL of 1X buffer (50 mM Tris HCl, 200 mM NaCl, 0.005% Tween, 20 mM DTT). 1X buffer is used to dilute the PBD protein in a typical FP assay. The plate was then spun down using a centrifuge (Eppendorf 5804 R) and analyzed for fluorescence values using the Beckman Coulter DTX 880 multimode detector plate reader.

PBD Protein Optimization

After confirming the appropriate fluorescent tracer concentration needed for the assay (typically no less than 10-fold signal above background) (1), a protein dilution curve was carried out. Briefly, using a 384 well plate, 5 µL of tracer was added to the appropriate wells, keeping the concentration constant. Increasing concentration (0.5

ng/well – 250 ng/well) of PBD protein was also added to the 384 well plate in triplicate. PBD protein dilutions were made using a 1X buffer (50 mM Tris HCl, 200 mM NaCl, 0.005% Tween, 20 mM DTT). The plate was then spun down using a centrifuge (Eppendorf 5804 R), incubated on a shaker at room temperature for 45 minutes, and spun down once more before analyzing using the Beckman Coulter DTX 880 multimode detector and multimode analysis software. Binding was measured by calculating millipolarization (mP) values. The optimum PBD concentration was determined to be when 90% of the fluorescent tracer was bound (EC_{90}).

Determining the Dissociation Constant (K_d)

Fluorescence polarization, in millipolarizaton (mP) units, was measured at an excitation wavelength of 488 nm and an emission wavelength of 535 nm, using the DTX 880 plate reader and multimode analysis software (Beckman Coulter). We determined the dissociation constant (K_d) of the PLK1 fluorescent tracer (MAGPMQS[pT]PLNGAKK) to the PLK1 PBD (amino acids 367-603) and PLK3 fluorescent tracer (GPLATS[pT]PKNG) to the PLK3 PBD (amino acids 335-646). A K_d measurement for the PBD of PLK3 was determined in order to establish a counter screening assay. This allows us to accurately determine the selectivity of our compounds for PLK1 relative to PLK3.

2.3 RESULTS

PLK1 and PLK3 Fluorescent Peptide Titration Curve

Using the raw fluorescent values, a fold fluorescent curve was plotted for the PLK1 and PLK3 tracer dilutions. It is recommended to use the concentration that provides at least 10-fold signal above background (1), however, the concentration

equivalent to 10-fold fluorescent signal (~0.1 nM) was not sufficient for detecting binding when doing the protein dilution curve. We determined that a 10 nM concentration provided a strong fluorescent signal and we went on to use this concentration for the PLK1 and PLK3 PBD binding curves (Figure 2.1 and 2.2).

PLK1 PBD Binding Curve

We used a 612 nM concentration of PLK1 PBD (250 ng/well) under previous assay conditions. We determined that 41.4 nM (17 ng/well) of PLK1 PBD should be used in the re-optimized FP assay. This represents the concentration at which 90% of the tracer is bound to the PBD (Figure 2.3).

PLK3 PBD Binding Curve

Under previous assay conditions we used PLK3 PBD at 487.4 nM (250 ng/well). New assay conditions require 245 nM (125.6 ng/well) to be used. This concentration represents 90% binding of the tracer to the PBD (Figure 2.4).

Fold Selectivity

The PLK1 PBD fluorescent tracer binds to the PLK1 PBD with a K_d of 4.6 nM (Figure 2.3), while the PLK3 PBD fluorescent tracer binds to its PBD with a K_d of 27.2 nM (Figure 2.4). The PLK1 fluorescent tracer binds with a ~ 5-fold higher affinity to its PBD. This difference in binding affinity is taken into account when calculating the fold selectivity index of our compounds.

2.4 DISCUSSION

We began using low volume 384 well plates to analyze the binding affinity of our compounds to the PBD of PLK1 or PLK3. In doing so, the volume contained in the wells (15μL) were brought much closer to the PMT detector of the plate reader, thereby

increasing the intensity of the fluorescence signal being read by the plate reader. Using the tracer concentration from previous FP assay conditions (100 nM) caused signal saturation in the low volume plates. As a result, we re-optimized the assay conditions by determining the new concentration of fluorescent peptide, and the concentration of PBD protein needed when using low volume 384 well plates.

Upon switching to low volume 384 well plates (Greiner Bio-one), we determined from fluorescent tracer titration curves that the tracer concentration could be reduced 10-fold yet maintained optimum fluorescent signal. The PLK1 and PLK3 tracer could be used at 10 nM, therefore working stocks of each tracer peptide was made up at a 30 µM concentration and stored at -20˚C. We found that 30 µM tracer stocks could undergo 5-7 freeze – thaw cycles before the fluorescent signal began to weaken, presumably because of degradation. When the signal weakens we are no longer able to accurately detect PBD binding, so the stock was discarded after 5 – 7 uses.

Under prior assay conditions the PLK1 and PLK3 PBD was used at a 250 ng/well (50 ng/ µL) concentration. New protein dilution curves were used to calculate the concentration of PBD needed for the competition assay in which 90% of the tracer is bound. Optimized assay conditions revealed that the PLK1 PBD could now be used at a 17 ng/well (3.4 ng/µL) concentration and the PLK3 PBD concentration at 125 ng/well (25 ng/µL).

Importance of Quality Control Processes

Although new assay conditions have been determined, it is important to confirm the assay conditions by repeating a tracer titration curve and PBD binding curve anytime a new stock or lot of PBD protein or fluorescent tracer is used. The FP assay is a

competitive binding assay that allows for an indirect measure of compound binding to the PBD by measuring the polarized fluorescence of bound tracer relative to unbound tracer. In other words, we are able to measure the binding affinity of our compounds to the PBD of PLK1 and PLK3 by measuring the displacement of the tracer molecule from the PBD, because the compounds are competing for the same binding site.

If too high a concentration of tracer is used relative to PBD protein, the signal from the unbound excess tracer will saturate the detection limits of the machine. This, in turn, results in an inaccurate measure of the binding affinity of a compound.

Also, too low of a tracer concentration relative to PBD protein will result in an underestimation of compound binding. We are only able to detect a compound's binding affinity by measuring the displacement of the tracer molecule from the binding site. An excess of unbound PBD protein leads to an underestimation of compound binding because the compound will bind to the excess PBD without displacing a tracer molecule. Therefore, more compound will be needed to detect binding, resulting in an inaccurately high IC_{50} value.

Note that, as part of this process, several key peptides and FLIPs originally published in the 2012 MCT paper (2) were re-tested in the FP assays optimized for 384 well low volume plates. In the subsequent chapters, the values for these previously tested peptides and FLIPs are from the more newly optimized assay. These values are more appropriately (directly) compared to the IC_{50} values of all peptides, FLIPs, and non-FLIPs in the following chapters, which are all determined in the low volume plate assays.

FIGURES

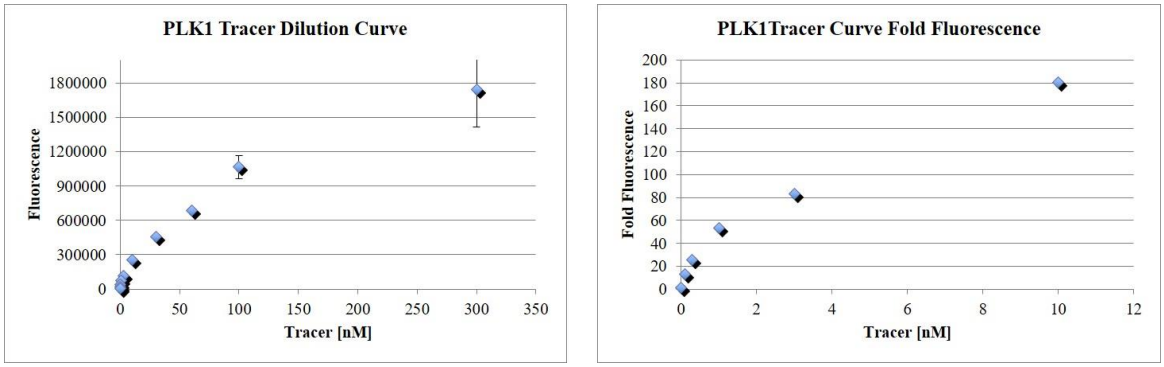


Figure 2.1 PLK1 tracer dilution curve and fold-fluorescence tracer curve

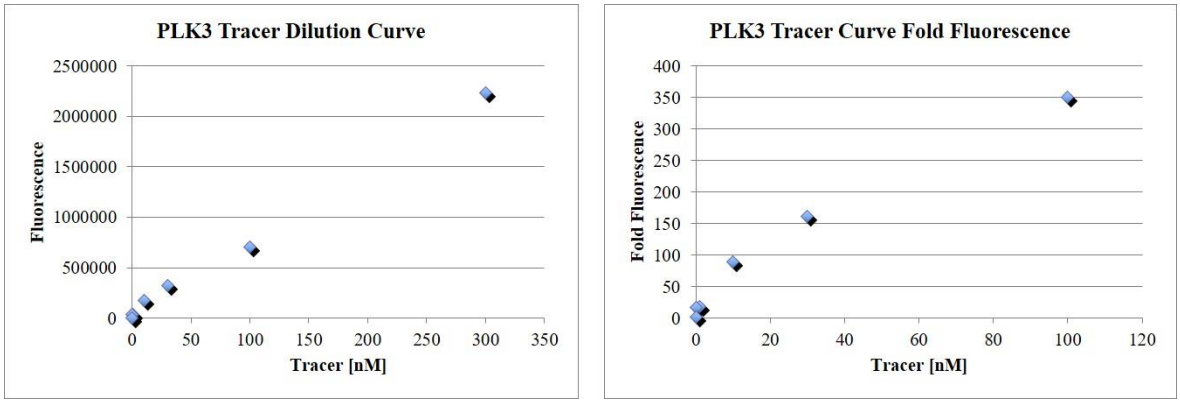


Figure 2.2 PLK3 tracer dilution curve and fold-fluorescence tracer curve

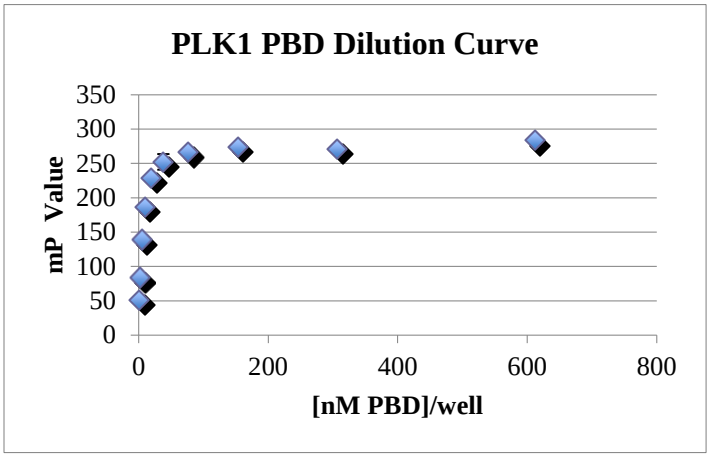


Figure 2.3 PLK1 PBD dilution curve. The dissociation constant (K_d = 4.6 nM) and EC_{90} (41.1 nM) was determined from the curve.

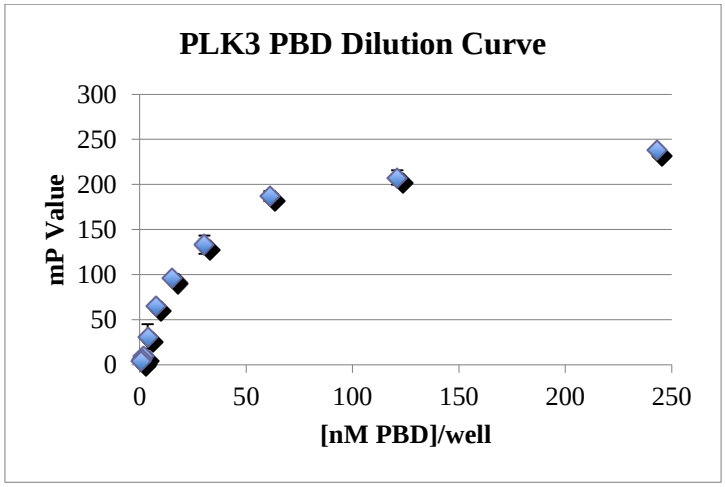


Figure 2.4 PLK3 PBD dilution curve. The dissociation constant (K_d = 27.2 nM) and EC_{90} (245 nM) was determined from the curve.

CHAPTER TWO REFERENCES

1. Arkin MR, Glicksman MA, Fu H, Havel JJ, Du Y. Inhibition of Protein-Protein Interactions: Non-Cellular Assay Formats In: Sittampalam GS, Coussens NP, Nelson H, al. e, editors. Assay Guidance Manual [Internet]. Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2012.

2. McInnes C, Estes K, Baxter M, Yang Z, Farag DB, Johnston P, et al. Targeting subcellular localization through the polo-box domain: Non-ATP competitive inhibitors recapitulate a PLK1 phenotype. Molecular Cancer Therapeutics. 2012;11(8):1683-92.

CHAPTER THREE

BIOCHEMICAL AND CELLULAR ANALYSIS OF PEPTIDES AND FLIPS[*]

ABSTRACT

Polo-like Kinase 1 (PLK1) performs critical roles in the coordination of mitosis and is also an oncogene over-expressed in many cancer types. ATP-binding site inhibitors of PLK1 have progressed to clinical trials, and one was recently given FDA breakthrough status to treat acute myeloid leukemia. However, recent data suggests that there are major disadvantages to blocking the kinase activity of PLK1. We have validated the innovative REPLACE strategy by discovering non-ATP competitive and PLK1-selective inhibitors as an alternative therapeutic strategy for this important target. The Polo-box domain (PBD) of each PLK is a phospho-peptide binding motif that determines substrate recognition and subcellular localization. Our approach targets the PBD of PLK1 to achieve desired selectivity and improve efficacy. Through modeling and design we produced a series of fragment-ligated inhibitory peptides (FLIPs). We report three FLIPs with an octyl-benzamide group, having the following different amino acid C-terminus sequences: -PNGL, -AI, and –PL. In vitro binding to the PBDs of PLK1 and PLK3 was measured by a fluorescence polarization assay. The IC_{50} values for PLK1 calculated from the competition assay are 0.36 μM, 0.41 μM, and 1.2 μM, respectively, and all three are at least >1800-fold more selective for PLK1 compared to PLK3. Initial

[*] Merissa Baxter, Sandra Craig, Michael D. Wyatt, Campbell McInnes. 2016. In preparation for submission.

testing in two cancer lines revealed respectable anti-proliferative activity for two of the FLIPs. In addition, we report the investigation of our PBD-inhibitors in cells expressing the mutant C67V PLK1 that is resistant to ATP-based inhibitors. We find that our compounds are equally active in cells expressing wild-type or C67V PLK1, whereas cells expressing C67V PLK1 are dramatically resistant to the ATP-based inhibitor, BI 2536. These exciting developments demonstrate the validity of our approach to produce drug-like lead PBD-inhibitors that are PLK1 selective and are active against tumors resistant to ATP-inhibitors. PBD-inhibitors could ultimately be used in combination therapy regimens with ATP-based inhibitors for a dual attack on this important, clinically validated oncology target.

3.1 INTRODUCTION

The polo-like kinases are central players in regulating entry into and progression through mitosis (1). A significant body of literature has determined that profound anti-proliferative activity is achieved through selective inhibition of PLK1 functions (2). The four known human PLKs have non-redundant and non-overlapping functions. Overexpression of PLK1 is frequently observed and PLK1 expression is a prognostic indicator for outcome of patients suffering from various tumors (3-5). More than half of prostate cancers overexpress PLK1 and this expression is positively correlated with tumor grade (6). PLK1 is extensively overexpressed in colorectal cancer (7) and recently was demonstrated to be a potential therapeutic target in such tumors with inactivated p53 (8). Moreover, it has been reported that p53 transcriptionally regulates PLK1 expression, providing direct evidence that PLK1 is oncogenic when p53 is mutated (9). Thus, there is a strong rationale for pursuing PLK1 as an anti-tumor drug target. Indeed, the

therapeutic rationale for PLK inhibition has been validated through several studies and shown to profoundly inhibit cancer cell proliferation both *in vitro* and *in vivo* (10, 11). 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. At least two compounds have been evaluated in phase I clinical trials. Results from two compounds suggest acceptable toxicity profiles warranting further investigation in phase II trials (12, 13). Recently BI6727 (volasertib) was granted FDA breakthrough therapy designation for Acute Myeloid Leukemia. Unfortunately, however, there are numerous drawbacks to targeting the ATP cleft, including prominently the inhibition of the 3 other known members of the mammalian PLKs (14). Because PLK3 has been reported to have opposing functions to PLK1, PLK3 inhibition may lead to diminution of the anti-tumor effect mediated by blocking PLK1 (15-17). These issues were revealed after the initial clinical development of ATP-competitive PLK inhibitors and strongly suggest that inhibiting PLK3 would not a desirable feature of a clinical candidate. In addition, ATP competitive inhibitors will only block the enzymatic functions of PLK1 and will not necessarily affect its critical non-catalytic functions. Therefore, alternative approaches to develop potent and highly selective PLK1 inhibitors are required. Studies with peptides provide evidence that the substrate and sub-cellular targeting binding site in the Polo Box Domain (PBD) forms a compact and druggable interface amenable to small molecule inhibitor development (18-21). Although high-throughput screening approaches have identified small molecule inhibitors of the PBD-peptide interaction, these either are weakly binding or non-drug-like in nature (22, 23). In addition, these inhibitors display a contrasting phenotype to PLK1 knockdown and to cellular treatment with inhibitors of

catalytic activity (14, 24, 25). Recently progress has been reported in the generation of derivatized peptides that occupy a novel site in the PBD binding groove (26, 27).

Peptides, while also non-drug-like, can discriminate between the PLK family members and can therefore provide a structural basis for the development of selective PLK1 inhibitory compounds. Herein, REPLACE, a validated strategy for the iterative discovery of non-peptidic protein-protein interaction inhibitors, has been applied to discover fragment alternatives for the N-terminal hydrophobic motif in a Cdc25C PBD substrate peptide. Furthermore, using transfected PBD binding peptides and Fragment Ligated Inhibitory Peptides (FLIPs), a workflow for phenotypic and PLK1 specific cellular effects has been established. Results demonstrate that PBD-targeted inhibitors replicate a PLK1 phenotype, in contrast to the partial phenotype obtained with PBD dominant negative and small molecule inhibitors, suggesting that they inhibit both subcellular localization and substrate phosphorylation.

3.2 Materials and Methods

Peptide and FLIP Synthesis

The McInnes group generated fragment ligated inhibitory peptides (FLIPs) and small molecule (non-peptidic) inhibitors through a validated strategy called REPLACE (Replacement with Partial Ligand Alternatives through Computational Enrichment). This is a computational and synthetic approach that uses structure activity relationships of peptide inhibitors to generate pharmaceutically acceptable lead molecules. Fragments are docked into the crystal structure of a truncated peptide/receptor complex, synthesized, and subsequently tested in an *in vitro* binding assay. While retaining the peptide core structure, the N-terminal tripeptide sequence was removed and replaced by molecular

fragments that mimic the binding contributions of the amino acids in the PBD binding pocket. Replacing both the N and C- terminal peptide sequences with small molecular fragments generate small molecules.

Fluorescence Polarization Assay

FLIPs and peptides to be tested were dissolved in DMSO (10 mM), and diluted from 600 μM (maximum of 6% DMSO tolerance in the assay) to 10 nM. The PLK1 PBD (367-603) and PLK3 PBD (335-646) proteins were obtained from BPS Bioscience Inc. (San Diego, CA); 17 ng PLK1 and 156 ng 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 minutes while shaking. Fluorescence was measured using a DTX 880 plate reader and Multimode Analysis software (Beckman Coulter, 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 linear regression analysis of the plots.

PLK1 Kinase Inhibition Assay

The CycLex™ Polo-like kinase 1 Assay/Inhibitor Screening Kit was used to measure catalytic inhibition of peptides and FLIPs (CycLex Co, Ltd., Nagano, Japan). This ELISA assay measures the catalytic activity of full length PLK1 for a defined substrate, which is detected by an anti-phospho-threonine antibody (PPT-07) and peroxidase coupled secondary antibody. Plates pre-coated with a Threonine containing

substrate were incubated with PLK1, kinase buffer containing 7.5 μM ATP (modification from recommended concentration), in the absence or presence of increasing concentration of peptides or FLIP molecules. After incubation, the phosphorylated substrate resulting from PLK1 kinase activity was detected using the PPT-07 antibody, and horseradish peroxidase conjugated anti-rabbit IgG antibody. Peroxidase catalyzes the conversion of the colorless solution to yellow, which was quantified using a DTX 880 plate reader. Absorbance measurements (450 nm) were plotted to calculate activity in each sample, and % inhibition was calculated in the FLIP containing wells relative to activity in the absence of inhibitor.

Cell Culture

HeLa cervical cancer cells were obtained from ATCC (Manassas, VA) and were not authenticated by the authors for this study. Histone H2B GFP-labeled HeLa cells (HeLa-H2B-GFP) were kindly provided by Dr. Geoffrey Wahl (Gene Expression Laboratory, Salk Institute) [29], and were confirmed as >95% GFP positive by FACS (data not shown) but were not otherwise authenticated. Cells were maintained in DMEM (Invitrogen, Carlsebad, CA) supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

PC3 prostate cancer cells were kindly provided by Dr. Shafiq Khan (Clark Atlanta University, Atlanta, GA) and were not authenticated by the authors for this study. Cells were maintained in DMEM (Invitrogen, Carlsebad, CA) supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

A-549 lung cancer cells were obtained from ATCC (Manassas, VA) and were not authenticated by the authors for this study. Cells were maintained in Hams F-12 1X with glutamine (Cellgro, Manassas, VA), and supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

Retinal Pigment Epithelial (RPE) cells were kindly provided by Dr. Prasad V. Jallepalli (Memorial Sloan-Kettering Cancer Center, New York, NY) Cells were maintained in 1:1 DMEM/Hams F-12 (Invitrogen, Carlsebad, CA; Cellgro, Manassas, VA), and supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

Cell Viability Assay

Exponentially growing cells were plated in 96- well dishes. Dose response curves were used to treat cells for 72 hours with FLIPs. Following the three-day treatment, cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. Cells were incubated for 3 hours with MTT allowing viable cells to metabolize the tetrazolium dye to a purple colored solution. Absorbance measurements (595 nm) were quantified using a DTX 880 plate reader and IC_{50} values were generated for FLIPs.

Serum Starved Cell Synchronization

Cells were synchronized in G1 by serum starvation. Briefly, exponentially growing cells were plated overnight. Media (DMEM, 10% FBS, 1% penicillin/streptomycin) was then removed and replaced with serum free media for 72

hours. Cell were subsequently treated for 24 hours with FLIP inhibitors. Following treatment, cells were collected, fixed with 70% ethanol, and processed by flow cytometry.

3.3 RESULTS

Structure-activity relationship of peptidic inhibitors for the polo-box domains of PLK1 and PLK3.

Initially, a titration curve of the PLK1 and PLK3 PBDs with their respective fluorescent tracers was performed to determine a K_d value (binding affinity). The PLK1 PBD fluorescent tracer binds to the PLK1 PBD with a K_d of 4.6 nM (Chapter 2, Figure 2.3), while the PLK3 PBD fluorescent tracer binds to its PBD with a K_d of 27.2 nM (Chapter 2, Figure 2.4). These curves were also used to calculate the concentration of PBD for the competition assay in which 90% of the tracer is bound. The PLK1 fluorescent tracer binds with a 5- fold better affinity to its PBD, which is taken into account to calculate a fold selectivity index.

The peptide structures were designed to probe the contributions of the N- and C-terminal residues of the recognition sequences from Cdc25c (LLCSpTPNGL) and PBIP (PLHSpTAI) (18-20), two known substrates of PLK1. The affinities of the peptides and FLIPs for PLK1 or PLK3 were measured by plotting the loss of polarization against increasing concentration of competitor peptide. C-terminal truncations were previously shown to retain binding affinity to PLK1 (28). We also showed that acetylation of the N-terminal Pro positively contributes to binding (28). The results in Table 3.1 shows that combining portions of the two naturally occurring substrates created a peptide (**5987**) that bound with high affinity (IC_{50} = 0.06 ± 0.04 μM) for PLK1. Importantly, these peptides

retain strong selectivity for PLK1 over PLK3 (Fold Selectivity >13,000). We also examined **5902**, which is the consensus recognition sequence for PLK3 reported in the literature (22). Interestingly, **5902** has a high affinity for the PLK1 PBD (IC_{50} = 0.0057 ± 0.002 μM), higher than that seen for PLK3 (PLK3 IC_{50} = 2.2 μM). However, it should be pointed out that **5902** and **6023**, with the N-terminal GPLATS sequence, possess significant affinity for PLK3 compared to the other peptides with the N-terminal PLHS sequence (Table 3.1).

Optimization of 4-alkylbenzamido N-cap and truncation studies on residues C-terminal to the phosphothreonine.

Our previous report identified an N-terminal benzamide capping group with an alkyl tail as a promising starting structure, and identified a butyl-benzoic containing FLIP (**5827**) with low μM affinity for the PLK1 PBD and undetectable binding to PLK3 (28). FLIPs containing N-terminal benzamide capping groups with increasing length of alkyl tail were designed to exploit a hydrophobic pocket in the PBD of PLK1 (Table 3.2). With FLIPs containing the C-terminal portion of the Cdc25c sequence (PNGL), a clear structure activity relationship (SAR) was observed with lengthening the alkyl tail from butyl (**5827**), to hexyl (**5956**), then octyl (**5954**), and improved affinity to PLK1 (IC_{50} = 2.5 ± 0.96; 1.06 ± 0.51; 0.36 ± 0.16 μM, respectively) while maintaining excellent selectivity. Although **5954** (octylbenzoic-FLIP) measurably bound to the PBD of PLK3, it remains over 2000-fold more selective for PLK1 (Table 3.2).

Based on the results from the PNGL FLIPs, C-terminal modifications were explored with the octyl-benzamide FLIPs (Table 3.3). When the C-terminal PNGL sequence was replaced with alanine, the resulting FLIP (**5988**) bound to the PLK1 PBD

with a 42-fold weaker affinity (comparing **5954**, 0.36 ± 0.16 μM to **5988**, 15.2 ± 4.74 μM). Although PLK3 binding (IC_{50} = 201.9 μM) also decreased, it was not by as much, which dramatically reduced the selectivity (compare >2400-fold selectivity to 66-fold selectivity). Adding an isoleucine to the alanine (**5996**) improved PLK1 PBD binding (IC_{50} = 0.41 ± 0.14 μM) by 37-fold relative to **5988**, while only marginally changing PLK3 binding (IC_{50} = 152.8 μM). This substantially improved selectivity. When the C-terminus was replaced with proline and leucine, the resulting FLIP (**6026**) bound to the PLK1 PBD with a 3-fold weaker affinity (IC_{50} = 1.2 ± 0.18 μM), compared to **5996** but remained selective for PLK1 (Table 3.3). These results indicate the importance of the C-terminal interactions in PLK1 affinity and selectivity. Future iterations of REPLACE are focused on C-terminal substitutions with drug like structures that retain PLK1 binding.

Select FLIPs were also tested in a commercially available *in vitro* kinase assay that utilizes full-length PLK1 (Materials and Methods). We tested BI-2536 in the assay at a range of ATP concentrations up to 50 μM (the manufacturer's recommended concentration) and observed a decrease inhibition with increasing ATP concentration as is expected for a competitive inhibitor. Utilizing an ATP concentration of 7.5 μM reported by Steegmaier et al in their characterization of BI-2536 (25), we calculated for BI-2536 an IC_{50} value of 2.8 nM (not shown), which is within good range of the prior report but ~3-fold lower. Table 3.2 and Table 3.3 shows that the FLIPs (**5954**, **5996**, **6026**) tested can inhibit the catalytic activity of PLK1, demonstrating that targeting the PBD can inhibit substrate recognition and kinase activity.

PLK1-inhibition by FLIPs is synthetic lethal with PLK1 and PTEN deficient or mutant Ras cancer cells

Cell viability was measured to determine the anti-proliferative activity of **5996** and **6026** (Table 3.4). Despite their peptidic composition, the FLIPs possessed modest cellular activity with no delivery agent. The IC_{50} of **5996** in HeLa cells was 128.1 μM. PTEN deficient prostate cancer (PC3) cells and Kras mutant (A-549) lung cancers cells also showed sensitivity to PLK1 inhibition. While **6026** possessed cellular activity in Hela cells is it less potent than **5996** so its effect in PC3 and A-549 cells was less dramatic relative to **5996**. These results are consistent with the concept that inhibiting PLK1 is synthetic lethal with PLK1 and PTEN deficient or mutant Ras cancer cells (Table 3.4).

Targeting the Polo-Box Domain of PLK1 circumvents resistance to catalytic inhibitors in cells expressing mutant (C67V) PLK1

Burkard *et al.* showed that a single point mutation (C67V) within the ATP-binding domain of PLK1 confers resistance to several structurally unrelated ATP-binding site inhibitors, including BI-2536 (29). We obtained the RPE cells generated by the Burkard group and confirmed the resistant phenotype by cell viability assay (Table 3.5). Our results confirm that WT RPE cells are sensitive to BI-2536 (IC_{50} = 21.2 nM) while mutant RPE cells are dramatically resistant to BI-2536 (> 2.5 μM). The PBD-targeted FLIPs are growth inhibitory in the PLK1 C67V mutant-expressing cells that are resistant to catalytic inhibitors (Table 3.5).

G2/M Cell Cycle Arrest

PLK1 catalytic inhibition induces prometaphase arrest (14, 30, 31), and we have previously demonstrated that PBD- inhibition induces a consistent mitotic phenotype (28). Here we show that synchronized (G1) PC3 cells treated for 24 hours with **6026** accumulate in G2/M. G1 arrest is observed when the cells are treated using a higher dose, which suggests off target or additional effects (Table 3.6; Figure 3.1).

3.4 DISCUSSION

Our goal was to build on the finding of our previous study (28) and further explore the development of PBD- inhibitors of PLK1 through the use of REPLACE. By exploiting the protein-protein interactions of known substrates we applied the REPLACE method to discover new fragment alternatives for the N-terminal hydrophobic motif of Cdc25C and PBIP. In addition, we characterized the efficacy of these inhibitors and determined their mechanism of action. Our results demonstrate that PBD- targeted PLK1 inhibitors can be developed through REPLACE. Peptide structures were probed to determine the N- and C-terminal contributions within the PBD binding pocket. Although peptides are not useful drug molecules, the SAR helped to identify important residues for substrate binding and selectivity. These amino acid contributions were used to generate FLIP molecules and can be further explored and used to develop non-peptidic drug-like small molecules.

We previously reported the importance of N-terminal acetylation to improve the binding affinity of peptides for the PLK1 PBD (28). Here we report acetylated PIBP binding to PLK1 with an IC_{50} = 0.064 μM (**6025**). Although there is measureable binding to PLK3, **6025** is more than 20,000 fold more selective for PLK1. Removal of

the isoleucine from the C-terminus of this sequence resulted in a 7-fold decrease in PLK1 binding (compare **6025** to **5743**). This suggests an important contribution of isoleucine in the binding pocket of PLK1 PBD. A decrease in PLK3 binding was also observed, further suggesting that isoleucine is contributing to PLK1 selectivity. Removal of the N-terminal proline from **6025** (and acetylated leucine, **5781**) results in an even greater loss of binding to PLK1 (~24-fold).

Combining the amino acid sequences of two substrates (N-terminal sequence of PBIP with C-terminal sequence of Cdc25C) led to one of our most promising peptide inhibitors (**5987**) – having high affinity and selectivity for PLK1. After it was determined that **5902**, the consensus recognition sequence for PLK3, was more selective for PLK1, we combined the acetylated N-terminal amino acid sequence of PBIP (**5743**) with the C-terminal amino acid sequence of **5902**. This also resulted in a structure (**6024**) with high affinity and selectivity for PLK1. Differences in C-terminal amino acid sequence between **5902** and **6023** (compare -PNGL with -PKNG) resulted in modest differences in PLK3 binding, suggesting the C-terminus is playing a greater role in PLK1 selectivity. Further study of the C-terminal SAR could provide more insight into generating a PLK1 selective small molecule.

We previously report an N-terminal benzamide capping group with an alkyl tail as a promising starting structure for the development of PBD-inhibitors. We identified a butyl-benzoic containing FLIP (**5827**) with low μM affinity for the PLK1 PBD and undetectable binding to the PLK3 PBD (28). We observed increased binding to the PLK1 PBD with lengthening the alkyl tail, however PLK1 selectivity was reduced due to measurable PLK3 PBD binding. We also observed measurable catalytic inhibition with

5954. This demonstrates that targeting the PBD not only inhibits substrate recognition, but it also interferes with the catalytic function of the protein.

We went on to develop FLIPs to investigate the C-terminal amino acid contributions to the PBD binding pocket by replacing the –PNGL sequence with alanine (**5988**). We observed a decrease in PLK1 PBD binding and an even greater decrease in PLK1 selectivity. Upon the addition of an isoleucine (**5996**), PLK1 binding increased relative to **5988**, while PLK3 binding changed moderately, resulting in a substantial increase in PLK1 selectivity. Lastly, we replaced the C-terminal amino acids with –PL (**6026**). While this change only moderately impacted PLK1 binding, we observed decreased binding to PLK3, thereby further increasing PLK1 selectivity. Taken together, these results suggests that the C-terminal amino acids do contribute more to PLK1 selectivity – as previously suggested from the peptide SAR – while the N-terminal amino acids contribute more significantly to PLK1 binding affinity. Future studies should further explore C-terminal SAR and develop molecular fragment replacements that mimic the amino acid contributions.

Peptides are typically not effective drugs due to their poor permeability and stability. However, we were able to measure cellular activity with our most potent FLIPs, **5996** and **6026**. These compounds are believed to be cell permeable due to the hydrophobic alkyl tail. Although partially peptidic, we observed a measurable decrease in cellular proliferation without the use of a drug delivery agent. Literature indicates a synthetic lethal interaction between PLK1 and PTEN deficient and Kras mutant cancer cells (32-34). Our results are consistent with these findings when treating PTEN

deficient PC3 and Kras mutant A-549 lung cancer cells with **5996**. PC3 and A-549 cancer cells were also sensitive to PLK1 inhibition.

Cell having a single point mutation (C67V) within the catalytic domain of PLK1 were greater than 110-fold more resistant to inhibition with BI-2536 than cells expressing wild type PLK1 (Table 3.3). These results are consistent with the study by Burkard et al (29). We have demonstrated that both wild type and mutant (C67) PLK1 expressing cells are sensitive to PBD inhibition. This is a very encouraging result because the issue of resistance is always a concern with cancer therapeutics. Future studies could explore the effectiveness of combination therapy using ATP-based and PBD-targeted inhibitors.

This study further validates the use of the REPLACE method to develop small molecule PBD-inhibitors. Our PBD inhibitors have a high binding affinity for PLK1 and are also selective for PLK1. Through REPLACE we have identified a promising drug-like molecular fragment that replaces the three N-terminal amino acids of the original peptide sequence (Cdc25C; -LLC). In addition, we have identified the importance of the C-terminal contacts that contribute to PLK1 binding and selectivity. Moreover, despite their peptidic composition, our FLIPs inhibit the catalytic activity of PLK1, are active in a cell viability assay, and circumvent resistance to ATP-based inhibitors observed in mutant PLK1 cells. Future studies should further explore the potency of our PBD inhibitors in several cancer cells models using a drug delivery system. Although the FLIPs were permeable, a drug delivery system will allow for a more efficacy, and better studies of on target activity in cells. One approach is to develop a prodrug. This would protect the amino acids until the compound penetrates the cell membrane and is metabolized to its active form. Additionally, a continued study of the C-terminal SAR

would provide additional insight into the binding contributions of the C-terminal amino acids. This information could then be used with REPLACE to generate acceptable lead molecular fragments as we work toward developing a non-peptidic small molecule.

Table 3.1 Structure Activity Relationship of Peptidic Inhibitors. Values indicate binding affinity for PLK1 and PLK3. PLK1 selectivity is also indicated.

SCCP ID#	Sequence	PLK1 IC_{50} [μM]	PLK3 IC_{50} [μM]	Selectivity Index
292	LLCS[pT]PNGL	0.17 ± 0.03	>600	>17,000
293	LLCSTPNGL	>600	ND	ND
5743	Ac-PLHS[pT]A	0.46 ± 0.12	>600	>6500
5987	Ac-PLHS[pT]PNGL	0.06 ± 0.04	163.6	13,633
6025	Ac-PLHS[pT]AI	0.064 ± 0.01	270.5	21,133
5781	Ac-LHS[pT]AI	1.57± 0.47	>600	>1900
5902	GPLATS[pT]PKNG	0.0057 ± 0.002	2.2	1928
6023	GPLATS[pT]PNGL	0.011 ± 0.003	6.3	2864
6024	Ac-PLHS[pT]PKNG	0.032 ± 0.012	133.8	20,906

Table 3.2 Biochemical Analysis of FLIP Inhibitors with N-terminal benzamide capping group. Compound structure, binding affinities, PLK1 selectivity, and catalytic inhibition are indicated.

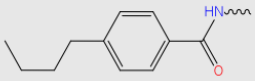
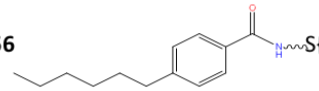
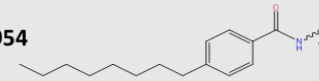
SCCP ID #	STRUCTURE	C-terminal Sequence	PLK1 IC_{50} [μM]	PLK3 IC_{50} [μM]	Selectivity Fold Index	PLK1 Kinase Inhibitory Activity IC_{50} [μM]
5827		S{pT}PNGL	2.5±0.96	>600	>1200	ND
5956		S{pT}PNGL	1.06±0.51	>600	>2830	>400
5954		S{pT}PNGL	0.36±0.16	178.5	2479	288.4 ± 33.9

Table 3.3 Biochemical analysis of octyl-benzamide FLIP Inhibitors. Compound structure, binding affinities, PLK1 selectivity, and catalytic inhibition are indicated.

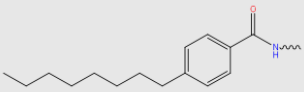
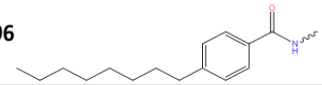
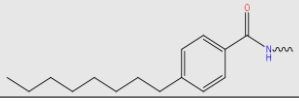
SCCP ID#	STRUCTURE	C-terminal Sequence	PLK1 IC_{50} [μM]	PLK3 IC_{50} [μM]	Selectivity Fold Index	PLK1 Kinase Inhibitory Activity IC_{50} [μM]
5988		S{pT}A	15.2 ± 4.74	201.9	66	ND
5996		S{pT}AI	0.41 ± 0.14	152.8	1863	174.4
6026		S{pT}PL	1.2 ± 0.18	537.5	2240	265.5 ± 40

Table 3.4 Anti-proliferative activity in PTEN deficient and Kras mutant cancer cells. Values were generated from three independent cell proliferation experiments.

SCCP ID#	HeLa IC_{50} [µM]	PTEN Deficient PC3 IC_{50} [µM]	Kras Mutant A-549 IC_{50} [µM]
5996	128.1	55.7 ± 2.7	79.45 ± 16.6
6026	142.3 ± 14.4	102.7 ± 27.3	160.2 ± 60.5

Table 3.5 Anti-proliferative activity in mutant PLK1 Retinal Pigment Epithelial (RPE) cells. Values were generated from three independent cell proliferation experiments

SCCP ID#	WT PLK1 RPE Cells IC_{50} [µM]	C67V RPE Cells IC_{50} [µM]
BI-2536	0.021	>2.5
5996	298.6	136.8
6026	158.5 ± 8.8	86.8 ± 33.8

Table 3.6 Cell Cycle Analysis of PC3 cells. Cells were treated with 6026 (24 hours) after being serum starved (SS) for 72 hours. The untreated group represents SS cells (72 hours) and released into drug free media (24 hours). Numbers are bold and underlined to emphasize cell cycle population comparisons.

	Untreated	SS+200μM 6026	SS+400μM 6026
Sub-G1	1.3	0.6	0.7
G1	**73**	38	**73.7**
S	4.5	7.4	9.5
G2/M	**19.9**	**53**	**15.5**

FIGURES

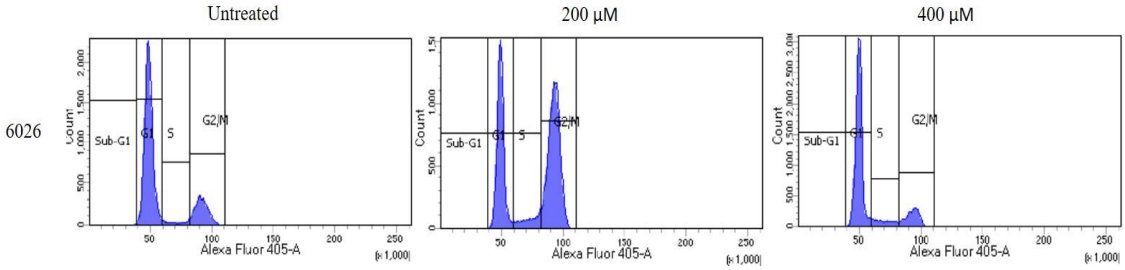


Figure 3.1. Cell cycle analysis of PC3 cells following 24 hour treatment with 6026. Cell were synchronized in G1 by 72 hour serum starvation before treatment. Untreated cells were serum starved then released into drug free media for 24 hours.

CHAPTER THREE REFERENCES

1. Xie S, Xie B, Lee MY, Dai W. Regulation of cell cycle checkpoints by polo-like kinases. Oncogene. 2005;24(2):277-86.

2. McInnes C, Wyatt MD. PLK1 as an oncology target: current status and future potential. Drug Discovery Today. 2011;16(13):619-25.

3. Yuan J, Horlin A, Hock B, Stutte HJ, Waigmann HR, Strebhardt K. Polo-like kinase, a novel marker for cellular proliferation. American Journal of Pathology. 1997;150(4):1165-72.

4. Wolf G, Elez R, Doermer A, Holtrich U, Ackermann H, Stutte HJ, et al. Prognostic significance of polo-like (PLK) expression in non-small cell lung cancer. Oncogene. 1997;14:543-9.

5. Tokumitsu Y, Mori M, Tanaka S, Akazawa K, Nakano S, Niho Y. Prognostic significance of polo-like kinase expression in esophageal carcinoma. International Journal of Oncology. 1999;15(4):687-92.

6. Weichert W, Schmidt M, Gekeler V, Denkert C, Stephan C, Jung K, et al. Polo-like kinase 1 is overexpressed in prostate cancer and linked to higher tumor grades. The Prostate. 2004;60(3):240-5.

7. Takahashi T, Sano B, Nagata T, Kato H, Sugiyama Y, Kunieda K, et al. Polo-like kinase 1 (PLK1) is overexpressed in primary colorectal cancers. Cancer Science. 2003;94(2):148-52.

8. Sur S, Pagliarini R, Bunz F, Rago C, Diaz Jr. LA, Kinzler KW, et al. A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. PNAS. 2009;106(10):3964-9.

9. McKenzie L, King S, Marcar L, Nicol S, Dias SS, Schumm K, et al. p53-dependent repression of polo-like kinase-1 (PLK1). Cell Cycle. 2010;9(20):4200-12.

10. Elez R, Piiper A, Kronenberger B, Kock M, Brendel M, Hermann E, et al. Tumor regression by combination antisense therapy against Plk1 and Bcl-2. Oncogene. 2003;22(1):69-80.

11. Spankuch-Schmitt B, Wolf G, Solbach C, Loibl S, Knecht R, Stegmuller M, et al. Downregulation of human polo-like kinase activity by antisense oligonucleotides induces growth inhibition in cancer cells. Oncogene. 2002;21(20):3162-71.

12. Jimeno A, Li J, Messersmith WA, Laheru D, Rudek MA, Maniar M, et al. Phase I study of ON 01910. Na, a novel modulator of the Polo-like kinase 1 pathway, in adult patients with solid tumors. Journal of Clinical Oncology. 2008;26(34):5504-10.

13. Mross K, Frost A, Steinbild S, Hedbom S, Rentschler J, Kaiser R, et al. Phase I dose escalation and pharmacokinetic study of BI 2536, a novel Polo-like kinase 1 inhibitor, in patients with advanced solid tumors. Journal of Clinical Oncology. 2008;26(34):5511-7.

14. Lenart P, Petronczki M, Steegmaier M, Fiore BD, Lipp JJ, Hoffmann M, et al. The small molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. Current Biology. 2007;17:304-15.

15. Yang Y, Bai J, Shen R, Brown SAN, Komissarova E, Huang Y, et al. Polo-like kinase 3 functions as a tumor suppressor and is a negative regulator of hypoxia-inducible factor-1alpha under hypoxic conditions. Cancer Research. 2008;68(11):4077-85.

16. Wang Q, Xie S, Chen J, Fukasawa K, Naik U, Traganos F, et al. Cell cycle arrest and apoptosis induced by human Polo-like kinase 3 is mediated through perturbation of microtubule integrity. Molecular and cellular biology. 2002;22(10):3450-9.

17. Xu D, Yao Y, Jiang X, Lu L, Dai W. Regulation of PTEN stability and activity by Plk3. The Journal of Biological Chemistry. 2010;285(51):39935-42.

18. Yun SM, Moulaei T, Lim D, Bang JK, Park JE, Shenoy SR, et al. Structural and functional analyses of minimal phosphopeptides targeting the polo-box domain of polo-like kinase 1. Nature Structural and Molecular Biology. 2009;16(8):876-82.

19. Elia AEH, Cantley LC, Yaffe MB. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science. 2003;299:1228-31.

20. Elia AEH, Rellos P, Haire LF, Chao JW, Ivins FJ, Hoepker K, et al. The molecular basis for phosphodependent substrate targeting and regulation of plks by the polo-box domain. Cell Press. 2003;115:83-95.

21. Garcia-Alvarez B, Carcer G, Ibanez S, Bragado-Nilsson E, Montoya G. Molecular and structural basis of polo-like kinase 1 substrate recognition: Implications in centrosomal localization. PNAS. 2007;104(9):3107-12.

22. Reindl W, Yuan J, Krämer A, Strebhardt K, Berg T. Inhibition of polo-like kinase 1 by blocking polo-box domain-dependent protein-protein interactions. Chemistry & biology. 2008;15(5):459-66.

23. Watanabe N, Sekine T, Takagi M, Iwasaki J-i, Imamoto N, Kawasaki H, et al. Deficiency in chromosome congression by the inhibition of Plk1 polo box domain-dependent recognition. Journal of Biological Chemistry. 2009;284(4):2344-53.

24. van Vugt MA, van de Weerdt BC, Vader G, Janssen H, Calafat J, Klompmaker R, et al. Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for

anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. The Journal of Biological Chemistry. 2004;279(35):36841-54.

25. Steegmaier M, Hoffmann M, Baum A, Lenart P, Petronczki M, Krssak M, et al. BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. Current Biology. 2007;17:316-22.

26. Liu F, Park JE, Qian WJ, Lim D, Scharow A, Berg T, et al. Identification of high affinity polo-like kinase 1 (Plk1) polo-box domain binding peptides using oxime-based diversification. ACS Chemical Biology. 2012;7(5):805-10.

27. Liu F, Park JE, Qian WJ, Lim D, Graber M, Berg T, et al. Serendipitous alkylation of Plk1 ligand uncovers a new bindng channel. Nature Chemical Biology. 2011;7:595-601.

28. McInnes C, Estes K, Baxter M, Yang Z, Farag DB, Johnston P, et al. Targeting subcellular localization through the polo-box domain: Non-ATP competitive inhibitors recapitulate a PLK1 phenotype. Molecular Cancer Therapeutics. 2012;11(8):1683-92.

29. Burkard ME, Santamaria A, Jallepalli PV. Enabling and disabling Polo-like kinase 1 inhibition through chemical genetics. ACS Chemical Biology. 2012;7:978-81.

30. Lane HA, Nigg EA. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturtion of mitotic centrosomes. The Journal of Cell Biology. 1996;135(6 Pt2):1701-613.

31. Lu B, Mahmud H, Maass AH, Yu B, van Gilst WH, de Boer RA, et al. The Plk1 inhibitor BI 2536 temporarily arrests primary cardiac fibroblasts in mitosis and generates aneuploidy in vitro. PLOS One. 2010;5(9):e12963.

32. Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF, et al. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell. 2009;137:835-48.

33. Jiang BH, Liu LZ. PI3K/PTEN signaling in angiogenesis and tumorigenesis. Advances in cancer research. 2009;102:19-65.

34. Liu XS, Song B, Elzey BD, Ratliff TL, Konieczny SF, Cheng L, et al. Polo-like kinase 1 facilitates loss of Pten tumor suppressor-induced prostate cancer formation. The Journal of Biological Chemistry. 2011;286(41):35795-800.

CHAPTER FOUR

BIOCHEMICAL AND CELLULAR ANALYSIS OF SMALL MOLECULE INHIBITORS[*]

ABSTRACT

Polo-like kinase 1 (PLK1) is a serine/threonine protein kinase involved in cell cycle regulation and mitotic progression. Studies have shown that PLK1 is upregulated in several tumors, and in some cases its overexpression is adversely related to prognosis. Studies have also shown synthetic lethality knocking down or inhibiting PLK1 in PTEN deficient prostate tumors and in Kras mutant colorectal tumors, further validating PLK1 as an oncotarget. PLK1 is comprised of two structural domains. The highly conserved kinase domain, and the Polo-Box Domain (PBD). The PBD is phospho-peptide binding motif that is responsible for substrate recognition and binding, and subcellular localization. An effort to target this protein therapeutically has led to the development of several ATP-binding site inhibitors, some of which have advanced to clinical trials. However, there is concern about the selectivity of these compounds for PLK1, as these inhibitors have been shown to inhibit three of the four PLK family members. This is problematic because PLK3 has been shown to act as a tumor suppressor. Moreover, it has been shown that a point mutation in PLK1 (C67V) confers dramatic resistances to ATP-binding site inhibitors. An alternative approach to developing potent and selective PLK1 inhibitors is to target the PBD. Through our validated REPLACE strategy we have

[*] Merissa Baxter, Sandra Craig, Michael D. Wyatt, Campbell McInnes. 2016. In preparation for submission.

developed PLK1 inhibitors that target the PBD. We report the structure activity relationship (SAR) of our first generation small molecules. *In vitro* binding to the PBDs of PLK1 and PLK3 was measured by fluorescence polarization (FP) assay, and we confirmed PLK1 binding in an orthogonal *in vitro* ELISA based PLK1 kinase assay. We also demonstrate that our compounds inhibit cancer cell proliferation and are effective in cells expressing mutant (C67V) PLK1.

4.1 INTRODUCTION

Polo-like kinase 1 (PLK1) is a mitotic protein that is only expressed in dividing cells (1). It plays an important role in cell cycle regulation and mitotic progression (2-4). PLK1 is overexpressed in a number of tumors (5-9), and its over-expression correlates with poor prognosis in some cancers (10-12). In addition, studies have demonstrated a synthetic lethal interaction when knocking down or inhibiting PLK1 in Pten deficient prostate tumors and Kras mutant colorectal tumors (13-15). Studies have also shown that inhibiting PLK1 expression results in cancer cell death, further validating PLK1 as an oncotarget.

PLK1 is comprised of two structural domains (16). The catalytic kinase domain is highly conserved among PLK family members, and is the target site of many catalytic inhibitors – several of which have advanced to clinical trials (17-22). The polo-box domain (PBD) of each PLK is a phospho-peptide binding motif that is responsible for substrate recognition and sub-cellular localization (23). Although several catalytic inhibitors have advanced to clinical trials, there is concern about the selectivity of these compounds for PLK1. ATP-based compounds inhibit three of the four known mammalian PLKs to various degrees (24-26). This is problematic because PLK3

functions as a tumor suppressor, therefore inhibiting its activity could counteract the antitumor effect of blocking PLK1. Moreover, a recent study demonstrates that a single point mutation within the catalytic domain of PLK1 (C67V) confers dramatic resistance to several structurally unrelated ATP-binding site inhibitors (27). Such a mutation is likely to be observed clinically, eventually rendering the ATP-inhibitors ineffective.

An alternative approach to developing potent and selective PLK1 inhibitors is to target the PBD. While the PBD is a conserved domain among PLK family members, each PBD is individually distinct (28). We generated fragment ligated inhibitory peptides (FLIPs) through a strategy called REPLACE (29).

Taking into account the N-terminus FLIP SAR (Chapter 3), a virtual screening of small molecules was performed by Dr. McInnes. Specifically, because 4-alkyl substituted benzoic acids were effective replacements for the N-terminal tripeptide (LLC), this fragment was used as a template for the identification of compounds containing alternatives for the C-terminal residues, including the critical phosphothreonine. Substructure searching for 4-alkylbenzamide derivatives in commercial libraries and subsequent testing identified one lead molecule, **5881**. From this lead, an SAR program was built, which included the synthesis, characterization, and analysis of over 40 non-peptidic small molecules. The compounds were synthesized and characterized by Dr. Sandra Craig in the McInnes lab. This chapter reports the biochemical and cellular characterization of compounds from this program.

4.2. MATERIALS AND METHODS

Fluorescence Polarization Assay

FLIPs and peptides to be tested were dissolved in DMSO (10 mM), and diluted from 10 nM to 600 μM (maximum of 6% DMSO tolerance in the assay). The PLK1 PBD (367-603) and PLK3 PBD (335-646) proteins were obtained from BPS Bioscience Inc. (San Diego, CA); 17 ng PLK1 and 156 ng 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 minutes. Fluorescence was measured using a DTX 880 plate reader and Multimode Analysis software (Beckman Coulter, 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 linear regression analysis of the plots.

PLK1 Kinase Inhibition Assay

The CycLex™ Polo-like kinase 1 Assay/Inhibitor Screening Kit was used to measure catalytic inhibition of peptides and FLIPs (CycLex Co, Ltd., Nagano, Japan). This ELISA assay measures the catalytic activity of full length PLK1 for a defined substrate, which is detected by an anti-phospho-threonine antibody (PPT-07) and peroxidase coupled secondary antibody. Plates pre-coated with a Threonine containing substrate were incubated with PLK1, kinase buffer containing 7.5 μM ATP (modification from recommended concentration), in the absence or presence of increasing

concentration of peptides or FLIP molecules. After incubation, the phosphorylated substrate resulting from PLK1 kinase activity was detected using the PPT-07 antibody, and horseradish peroxidase conjugated anti-rabbit IgG antibody. Peroxidase catalyzes the conversion of the colorless solution to yellow, which was quantified using a DTX 880 plate reader. Absorbance measurements (450 nm) were plotted to calculate activity in each sample, and % inhibition was calculated in the FLIP containing wells relative to activity in the absence of inhibitor.

Cell Culture

HeLa cervical cancer cells were obtained from ATCC (Manassas, VA) and were not authenticated by the authors for this study. Histone H2B GFP-labeled HeLa cells (HeLa-H2B-GFP) were kindly provided by Dr. Geoffrey Wahl (Gene Expression Laboratory, Salk Institute) [29], and were confirmed as >95% GFP positive by FACS (data not shown) but were not otherwise authenticated. Cells were maintained in DMEM (Invitrogen, Carlsebad, CA) supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

PC3 prostate cancer cells were kindly provided by Dr. Shafiq Khan (Clark Atlanta University, Atlanta, GA) and were not authenticated by the authors for this study. Cells were maintained in DMEM (Invitrogen, Carlsebad, CA) supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

A-549 lung cancer cells were obtained from ATCC (Manassas, VA) and were not authenticated by the authors for this study. Cells were maintained in Hams F-12 1X with

glutamine (Cellgro, Manassas, VA), and supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

HCT-116 (p53 +/+ and -/-) colon cancer cells were obtained from ATCC (Manassas, VA) and were not authenticated by the authors for this study. Cells were maintained in DMEM (Invitrogen, Carlsebad, CA) supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

Retinal Pigment Epithelial (RPE) cells were kindly provided by Dr. Prasad V. Jallepalli (Memorial Sloan-Kettering Cancer Center, New York, NY). Cells were maintained in 1:1 DMEM/Hams F-12 (Invitrogen, Carlsebad, CA; Cellgro, Manassas, VA), and supplemented with 10% FBS or Nu-serum (BD Bioscience, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Invitrogen) in a humidified incubator and 5% CO_2 at 37°C.

Cell Viability Assay

Exponentially growing cells were plated in 96- well dishes. Dose response curves were used to treat cells for 72 hours with FLIPs. Following the three-day treatment, cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. Cells were incubated for 3 hours with MTT allowing viable cells to metabolize the tetrazolium dye to a purple colored solution. Absorbance measurements (595 nm) were quantified using a DTX 880 plate reader and IC_{50} values were generated for FLIPs.

Serum Starved Synchronization

Cells were synchronized in G1 by serum starvation. Briefly, exponentially growing cells were plated overnight. Media (DMEM, 10% FBS, 1% Pen. Strep.) was then removed and replaced with media containing no FBS. Cells were starved for 72 hours, then treated for 24 hours with small molecule. Following treatment, cells were harvested and processed for endpoint experimental analysis.

BrdU Labeling/ FACS

Ethanol fixative was removed by centrifugation and the cells were washed with 1mL ice-cold PBS/1%BSA. Cells were denatured by resuspending in 0.2 mg/mL pepsin in 2N HCl and incubated in 37º C water bath for 15 minutes. Hydrolysis was then terminated by adding 1 M Tris-Glycine. Cells were then washed with PBS/1%BSA then the primary anti-BrdU antibody (1:100 dilution in TBFP; 0.5% Tween-20, 1%BSA, 1%FBS, PBS) was added and cells were incubated for 25 minutes in the dark, at room temperature. Cells were subsequently washed with PBS/1%BSA then allowed to incubated for 25 minutes in the secondary fluorescent antibody (1:200 dilution Alexa Fluor® 488 $F(ab')_2$ fragment of goat anti-mouse IgG). Cells were washed once more with PBS/1%BSA and re-suspended in DAPI for 30 minutes before being analyzed by flow cytometry.

Cell Viability, Apoptosis and Cytotoxicity Assay

The ApoTox Glo ™ Triplex Assay (Promega) was used to measure cell viability, cytotoxicity, and apoptosis in PC3 cells following treatment with **6037**. Briefly, PC3 cells were plated at 5,000 – 10,000 cells per well (96 well plate) and attached overnight. The PBD-inhibitor was then added for a 24 hours treatment, then the results were

analyzed by fluorescence and luminescence. Cell viability fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm, cytotoxicity fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, and apoptosis was determined with the luminescence measurement.

4.3. RESULTS

PBD-Inhibitors Bind Potently to the PLK1-PBD and are Moderately Selective

Our previous report identified an N-terminal benzamide capping group with an alkyl tail as a promising starting structure, and identified a butyl-benzoic containing FLIP (**5827**) with low µM affinity for the PLK1 PBD and undetectable binding to PLK3 (29). We also found that increasing the length of the alkyl tail led to increased binding to PLK1 while maintaining over 2000-fold selectivity **(5954)** (Chapter 3). Substructure searching for 4-alkybenzamide derivatives in commercial libraries and subsequent testing identified one lead molecule, **5881**. From this lead, the structure activity relationship (SAR) of more than forty small molecules was analyzed. The total of these compounds can be found in Table 4.1. Specifically, we analyzed compound binding to PLK1 (discussed first) and PLK3 (discussed second) *in vitro*. We also measured ATP inhibition and analyzed compound activity in cancer cells.

Structure Activity Relationship of C-capping group on Benzamide Small Molecules

Compound **5880** (butyl-benzamide) did not bind to the PBD of PLK1, however **5905** (pentyl-benzamide) bound to the PLK1 PBD with weak affinity (IC_{50} = 223.6 ± 4.88 µM). When the alkyl tail length was increased from pentyl to hexyl (**5881)**, PLK1 binding increased 12-fold (IC_{50} = 18.4 ± 5.3 µM). This result is consistent with FLIP

SAR which also demonstrated that increasing the length of the alkyl tail improved PLK1 binding (Chapter 3). **5881** also binds to PLK3 (IC_{50} = 18.04 ± 5.18 µM), but remains 5-fold selective for PLK1. We observed an increase in binding for PLK1 by further lengthening (octyl) the alkyl tail (**5912**) (IC_{50} = 11.27 ± 2.7 µM). Selectivity was also marginally improved (PLK3 IC_{50} = 15.48 ± 0.01 µM), relative to **5881** (compare **5881** fold selectivity = 5, to **5912** fold selectivity = 6.9). We continued to lengthen the alkyl tail even further, to determine if PLK1 binding would be further increased **(6037)**. Increasing the carbon chain to 12 carbons resulted in the generation of our least selective small molecule (PLK1 IC_{50} = 5.97 ± 0.38; PLK3 IC50 = 1.99 ±0.69; Fold selectivity = 1.6) (Table 4.2)

Structure Activity Relationship of C-capping group on Hexyl-Benzamide Small Molecules

Several derivatives of **5881** were synthesized and an analysis of the SAR of substituents on the C-terminal replacement was analyzed (Table 4.3). When the position of the carboxylic acid was moved from ortho to meta (from the amide), we observed a loss of PLK1 binding affinity (**5903** IC_{50} = 129.8± 3.6 µM) with minimal PLK1 selectivity (PLK3 IC_{50} = 43.62 ± 1.69 µM, Fold selectivity =1.6). No binding was observed with a sulfonamide phospho-mimic (**5908**), or when using a phosphate (**5940**) or hydroxyl (**5943**) group (Table 4.3). Interestingly, when a methyl group was positioned meta to the carboxylic acid (**5924**), we observed PLK3 PBD binding (IC_{50} = 24.5 µM), but no binding to PLK1.

Structure Activity Relationship of C-capping Group on Octyl-Benzamide Small Molecules

Several derivatives of **5912** were synthesized and an analysis of the SAR of substituents on the C-terminal replacement was analyzed. As expected due to the

putative contribution of the negatively charged carboxylate group, PLK1 binding was lost when the carboxylic acid was converted to an ethyl ester (**5914;** IC_{50} = >600 μM) (Table 4.4A). This compound was used as a negative control for cellular analysis. When compound **5932** (Table 4.4A) was synthesized containing a fluorine ortho to the carboxylic acid, PLK1 PBD binding affinity did not improve (relative to **5912**), nor was PLK1 selectivity increased. When fluorine was added at both the ortho and para positions (**5938**) (Table 4.4B) PLK1 PBD binding decreased (IC_{50} = 30.6 ± 3.2 μM) as did PLK3 binding (IC_{50} = 39.7 μM). Improved PLK1 binding (IC_{50} = 2.16 ± 0.01 μM) and selectivity (PLK3 IC_{50} = 7.68 ± 2.41 μM, Fold Selectivity = 18) was observed upon addition of a methyl group meta to the carboxylic acid (**5915,** Table 4.4A). It is important to note that the only difference between this compound and **5924 (hexyl)** is the length of the alkyl tail. Increasing the length from hexyl **(5924,** Table 4.3**)** to octyl **(5915)** dramatically improved PLK1 binding and produced the most selective compound in this series (Table 4.4A). When the methyl group is positioned para to the carboxylic acid (**5937,** Table 4.4A), PLK1 selectivity decreased 2-fold relative to **5915** (compare **5915** fold selectivity = 18 with **5937** fold selectivity = 9.3). The compound containing a methoxy group positioned meta to the carboxylic acid **(5935)** was 11.5-fold selective for PLK1, binding with an IC_{50} = 5.89 ± 1.25 μM (Table 4.4B). **5939** contains a nitro group ortho to the carboxylic acid (Table 4.4B). It is 8-fold selective for PLK1, binding to the PLK1 PBD with an IC_{50} = 6.05 ± 1.9 μM (PLK3 PBD IC_{50} = 9.96 ± 1.52 μM).

Structure Activity Relationship of C-capping Group with Flexible Octyl Benzamine Small Molecules

Small molecules containing a flexible alkyl benzamine structure were also synthesized to explore the SAR of compounds with less rigidity with respect to the two benzyl rings (Table 4.5). Accordingly, **5953** was synthesized with a methylamino instead of the amide linkage found in **5912**. Binding to PLK1 was slightly improved (**5953** IC_{50} = 9.57 ± 2.03), relative to **5912**. Next, **5961** contains the methylamino linker and a methyl group meta to the carboxylic acid – the same substitution at the R4 position as **5915**. Binding of **5961** to the PLK1 PBD was undetectable, but it did have weak affinity for the PLK3 PBD (IC_{50} = 156.7 μM) (Table 4.5). Positioning of a methyl group para to the carboxylic acid (**5971**), resulted in improved PLK1 binding (IC_{50} = 21.69 ± 2.8 μM) relative to **5961.** This represents more than 27-fold increase in PLK1 binding relative to **5961**, however there was a 2-fold loss in binding relative to **5953** (Table 4.5).

PBD- Inhibitors reduce the catalytic activity of PLK1

The ability of the most promising compounds to inhibit the catalytic activity of full length PLK1 was investigated using an *in vitro* ELISA based PLK1 kinase assay. The PBD-based PLK1 inhibitors (**5912**, **5932**, and **5915**) inhibited the catalytic activity of the protein (IC_{50} = 42.3 μM, 48.9 μM, and 24.3 μM, respectively) (Table 4.4A). The results from this assay were an important confirmation of compound binding to PLK1 in an orthogonal assay.

PBD-Inhibitors decrease Cancer Cell Proliferation

Our most promising compounds were tested in multiple cancer cell lines, including HeLa cervical cancer cells, PTEN deficient prostate cancer (PC3) cells, and

Kras mutant lung cancer (A-549) cells (Table 4.6). Each of these cancer cells lines were sensitive to the lead PBD-inhibitors in a cell viability assay with the A-549 lung cancer cells being the most sensitive overall. This is consistent with literature stating a synthetic lethal interaction exists with PLK1 mutant Ras cancer cells (13). A synthetic lethal interaction has also been demonstrated between PLK1 and PTEN deficient prostate cancer cells (14, 15). We also observed a differential sensitivity depending on p53 status. Specifically, a greater anti-proliferative effect was observed on p53 null HCT 116 colon cancer cells compared to p53 proficient HCT 116 cells (Table 4.6).

Targeting the Polo-Box Domain of PLK1 circumvents resistance to catalytic inhibitors in cells expressing mutant (C67V) PLK1

Burkard et al (2012) demonstrated that a single point mutation within the catalytic domain of PLK1 conferred resistance to ATP-based inhibitors while retaining full catalytic activity (27). We obtained the Retinal Pigment Epithelial (RPE) cells generated by the Burkard group and confirmed the resistant phenotype by cell viability assay (Figure 4.1). Wild Type (WT) RPE cells are sensitive to BI-2536 (IC_{50} = 21.2 nM) while mutant RPE cells are dramatically resistant to BI-2536 (> 2.5 μM) (Chapter 3). We found that non-peptidic PBD-inhibitors were able to circumvent the resistance observed by ATP-based inhibitors using the RPE cell line generated by the Burkard group. Cells containing wild type and mutant (C67V) PLK1 were sensitive to our lead PBD-inhibitors (5912 and 5932) while C67V mutant cells were resistant to BI-2536 – an ATP-competitive PLK1 inhibitor (Figure 4.1).

Cell cycle analysis reveals dose-dependent phenotype

Our PLK1 PBD-inhibitors were analyzed further by cell cycle analysis to determine if PBD-inhibition induces a similar phenotype as ATP-inhibition (G2/M arrest) (Figure 4.2). PC3 cells were synchronized in G1 by serum starvation then treated for 24 hours with PBD inhibitor (see methods). At a concentration of 5 µM, **5912** shows moderate (13% increase relative to control) increase in G2/M population, suggesting PLK1 is being inhibited (Figure 4.3). This result is modest when compared to those obtained using an ATP-competitive inhibitor (BI-2536), which shows nearly 80% of cells accumulate in G2/M. A higher concentration of **5912** (15 µM) results in a phenotype that suggests other targets are being engaged (Figure 4.2). **5912** shows S phase delay (most likely entry into S phase) (Figure 4.2 and 4.3) – implicating PLK3 inhibition at 15 µM; whereas **5915** and **5935** shows G1 delay (delayed exit) or arrest (cells no longer cycling) at 15 µM which could imply PLK2 or PLK3 inhibition. To quantify the percentage of cells actively proliferating (synthesizing their DNA) we carried out Bromodeoxyuridine (BrdU) pulse labeling and analyzed these by flow cytometry. Results showed that 28.3% of cells are replicating their DNA when treated with 5 µM 5915, however, cells are arrested in G1 (BrdU + = 3.6%) following treatment with 15 µM **5915**.

After treating PC3 cells with **6037** – our least selective inhibitor (Fold Selectivity = 1.6) – we observed G1 accumulation at both the lower (5 µM) and higher (15 µM) dose (Figure 4.4). Upon low dose (5 µM) treatment, BrdU labeling showed that 75.7% of the cells are replicating their DNA. This suggests that the lower dose observations result from either a delay of cells exiting G1 or delayed progression through S phase, as opposed to an S-phase arrest. Like **5915**, a G1 arrest is observed following the higher

dose (15 μM) treatment with **6037** (%G1 = 31.2; BrdU + = 1.9%). Interestingly, 40% of the cells were in sub-G1, indicating death is occurring at the higher (15 μM) dose (Figure 4.4). Cell death and cytotoxicity analysis showed that **6037** induces apoptosis at 15.0 ± 5.6 μM in PC3 cells (Figure 4.5).

4.4. DISCUSSION

Development of small molecules that bind to the PBD of PLK1 and inhibit catalytic function

Through the use of REPLACE we were able to successfully develop non-peptidic small molecules that bind to the PBD of PLK1. N-terminal SAR developed from the study of peptides and FLIPs in Chapter 3 was useful for the discovery and optimization of our small molecule PBD-inhibitors. The development of a lead series of compounds was a big leap forward. An *in vitro* catalytic inhibition assay showed that in addition to PBD binding, our small molecules also inhibit the catalytic activity of the protein. These results from an orthogonal assay provide reassurance that the PBD binding observed in the FP assay for the small molecules accurately reflects compound activity for PLK1 despite only using the PBD fragment in the FP Assay.

Although these first generation compounds are modestly selective for PLK1, they provide a good starting point for the development of more selective compounds in the future. FLIP results indicate that the C-terminal amino acids contribute to selectivity within the PLK1 binding pocket (Chapter 3). Additional analysis of FLIP C-terminal SAR will allow the application of those parameters in a compound library search, and will lead to the development of more selective second generation small molecules.

Small molecule SAR remained consistent with results observed in Chapter 3, in that lengthening the alkyl tail resulted in increased binding to PLK1 (Table 4.2). The PBD is a phospho-peptide binding motif, so it recognizes and binds to the phosphorylated serines and threonines of its substrates. The carboxylic acid serves as a phosphate mimic to which both the PLK1 and PLK3 PBD bound (**5912**) (PLK1 IC_{50} = 11.27 ± 2.7 μM, PLK3 IC_{50} = 15.48 ± 0.01 μM). Although **5912** bound to the PLK3 PBD it remained 7-fold selective for PLK1. A fluorine group was positioned ortho to the carboxylic acid (**5932**) to make the carboxylic more phosphate like (and increase the bulk of the negative charge). The fluorine substituent did not improve PLK1 binding or selectivity. Octyl-benzamide SAR shows methyl group substituents (meta or para to the carboxylic acid) (**5915** and **5937**) provided the greatest contribution to PLK1 binding and selectivity in this series. The methyl group positioned meta to the carboxylic acid (**5915**) makes the greatest contribution to PLK1 selectivity (Fold selectivity = 18), however, PLK1 binding and selectivity was completely lost when the octyl benzamide was replaced with an octyl benzamine (**5961**). Although PLK3 PBD binding also decreased with the flexible amine, binding was still detectable, resulting in a PLK3 selective compound. The same trend was observed (loss in PLK1 PBD binding) when comparing **5937** (octyl benzamide with methyl group para to the carboxylic acid) with **5971** (octyl benzamine with methyl group para to the carboxylic acid). Binding was not completely lost, like we observed with the methyl group in the meta position, but there was 5.5-fold decrease in PLK1 PBD binding with loss of rigidity when the methyl is in the para position (compare **5937** with **5971**). This suggests an important conformational role in that the loss of PLK1 selectivity observed with **5961** and **5971** may result from additional flexibility which makes PLK1

PBD binding entropically unfavorable, and PLK3 PBD binding favorable. In addition, the methyl group is a hydrophobic residue that could be removing water from the proteins binding site. This could also explain its contribution to PLK1 PBD binding and selectivity

Cancer cells are sensitive to PBD-inhibitors

HeLa, PC3, A-549, and HCT-116 cancer cells were sensitive to our PBD-inhibitors. Cell viability decreased in a dose-dependent manner in all cell lines, but A-549 (Kras mutant) lung cancer cells were most sensitive. This supports research findings showing a synthetic lethal interaction exist between cells expressing Kras mutations and PLK1. Synthetic lethality also exist between PTEN deficient prostate cancer cells and PLK1. However, we did not rigorously test this possibility in isogenic PTEN proficient and deficient prostate cancer cell lines (15). The literature also reports a sensitivity of p53 defective cancer cells to PLK1 inhibition (30). p53 is a tumor suppressor that is frequently mutated in cancer (31). p53 and PLK1 negatively regulate one another (32). Interestingly, when testing our compounds in an isogenic colon cancer cell line (HCT-116), we found that p53 null cells were more sensitive than p53 wild type (WT) cells. Sur et al (2009) showed that the viability of stressed cells was dependent on PLK1 when those cells lack wild type (WT) p53. Specifically, it was shown that a G1 arrest was induced in cells expressing WT p53 upon treating with ionizing radiation. This G1 arrest protected the cells from the toxic effects of BI-2336 – a PLK1 inhibitor – while cells expressing mutant p53 did not arrest in G1 and were thus sensitized to BI-2536 (33). They demonstrated that combining PLK1 inhibitors with ionizing radiation can induce a p53 dependent G1 cell arrest that will protect normal cells from the toxic effects of PLK

inhibition (33). The observed p53 dependent effect with our small molecules has encouraging clinical implications and future work could explore the potential protective effects of ionizing radiation (or other therapies that induce p53-dependent G1 arrest) with PBD inhibitors.

Cell Cycle Analysis Suggest Dose-Dependent Phenotype

Treating PC3 cells with **5912** at a lower dose leads to an accumulation of cells in G2/M. This phenotype is consistent with ATP-competitive PLK1 inhibition. At a higher dose, we observe a G1 arrest. One of our compounds (**6037**) induced a G1 arrest at all doses examined. **6037** is our least selective compound in this series, with almost equipotent binding to PLK1 and PLK3. It has been shown that depleting PLK3 by siRNA results in a G1 arrest, and the authors concluded that PLK3 is required for entry into S phase (34). We therefore speculate that **6037**, and other compounds in the series at higher doses, might be inhibiting PLK3, which would invoke a G1 arrest and preclude the inhibition of PLK1 in mitosis. However, additional investigation is needed to confirm that PLK3 is being targeted since there are other possible explanations.

Cell cycle analysis confirmed that cells were synthesizing their DNA when treated with the lower (5 μM) dose of **6037**, suggesting the compound induces a G1 delay. However, at the higher dose (15 μM) cells were arrested in G1 and began to undergo apoptotic cell death. Taken together the cellular analysis supports the FP data showing low PLK1 selectivity for **6037**.

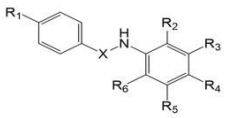
There is approximately 30% sequence homology between the PBD's of PLK1 and PLK2, however, these proteins have opposite functions in tumor development (35). PLK2 expression and activity has been shown to increase as cells enter S phase (36, 37).

We observe a delay in cell cycle progression through S phase when treating cells with 15 μM dose of our PBD inhibitors. It is possible that we could be targeting PLK2 when treating cancer cells at a higher dose. Notably, one other possibility is that targeting the PBD of PLK1 (as opposed to catalytic inhibition) in cancer cells with elevated PLK1 expression in interphase might produce a novel phenotype.

PBD-inhibitors circumvent resistance to ATP-competitive inhibitors in cells expressing mutant PLK1

A chemical genetics study from 2012 has shown that cells containing a point mutation within the ATP-binding site of PLK1 (C67V) are resistant to several structurally unrelated ATP-inhibitors. In addition to being resistant, these cells also retain the catalytic function of the PLK1 protein (27). Although this mutation has not yet been reported clinically, the likelihood of such a mutation occurring is possible, and would render ATP-inhibitors ineffective in a relapsed tumor. Our PBD inhibitors were equally effective in cells expressing the C67V PLK1 mutation as they were in cells expressing the wild type form or the protein.

Table 4.1 SAR of 40 PBD Inhibitors. Compounds are organized numerically and the IC_{50} values for PLK1 and PLK3 are indicated. ND indicates values that were Not Determined.



Structure activity of 2-(4-alkylbenzamido)benzoic acid PBD Inhibitors

SCCP ID	R1	R2	R3	R4	R5	R6	X	PLK1 PBD IC_{50} (mM)	PLK3 PBD IC_{50} (mM)
5880	butyl	H	H	COO^-	H	H	C=O	>600	ND
5881	hexyl	COO^-	H	H	H	H	C=O	18.4 ± 5.3	18.04 ± 5.18
5903	hexyl	H	COO^-	H	H	H	C=O	129.8± 3.6	43.62 ± 1.69
5905	pentyl	COO^-	H	H	H	H	C=O	223.6 ± 4.88	ND
5908	hexyl	H	SO_2NH_2	H	H	H	C=O	>600	ND
5910	hexyl	COO^-	H	H	OCH_3	OCH_3	C=O	107.4	ND
5911	octyl	COO^-	H	H	OCH_3	OCH_3	C=O	119.5	ND
5912	octyl	COO^-	H	H	H	H	C=O	11.27 ± 2.7	15.48 ± 0.01
5914	octyl	$COOC_2H_5$	H	H	H	H	C=O	>600	ND
5915	octyl	COO^-	H	CH_3	H	H	C=O	2.16 ± 0.01	7.68 ± 2.41
5916	$Ph(CH_2)_3CH_2O$	COO^-	H	H	H	H	C=O	140	ND
5917	$Ph(CH_2)_4CH_2O$	COO^-	H	H	H	H	C=O	11.7 ± 3.2	ND
5924	hexyl	COO^-	H	CH_3	H	H	C=O	>600	24.5
5928	octyl	NO_2	H	H	H	H	C=O	>600	ND
5929	octyl	SO_2NH_2	H	H	H	H	C=O	259.1 ± 24.6	ND
5932	octyl	COO^-	F	H	H	H	C=O	11.1 ± 1.1	13.24 ± 0.46
5934	octyl	COO^-	H	OH	H	H	C=O	29.62	11.75
5935	octyl	COO^-	H	OCH_3	H	H	C=O	5.89 ± 1.25	13.6 ± 6.3
5936	octyl	$B(OH)_2$	H	H	H	H	C=O	>600	ND
5937	octyl	COO^-	H	H	CH_3	H	C=O	3.99 ± 2.3	7.42 ± 1.89
5938	octyl	COO^-	F	H	F	H	C=O	30.6 ± 3.2	39.7
5939	octyl	COO^-	NO_2	H	H	H	C=O	6.05 ± 1.9	9.96 ± 1.52
5940	hexyl	PO_4	H	H	H	H	C=O	>600	ND
5943	hexyl	OH	H	H	H	H	C=O	>600	ND
5944	octyl	H	OH	H	H	H	C=O	>600?	ND
5945	octyl	COO^-	CF_3	H	H	H	C=O	29.36 ± 8.2	22.86 ± 3.62
5946	octyl	COO^-	OH	H	H	H	C=O	7.92 ± 2.9	12.12 ± 5.06
5948	octyl	COO^-	H	H	H	OH	C=O	12.03 ± 2.7	ND
5949	octyl	COO^-	Cl	H	H	H	C=O	25.3 ±3.4	ND
5950	octyl	COO^-	OCH_3	H	H	H	C=O	29.6 ± 3.4	ND
5951	octyl	H	PO_4	H	H	H	C=O	98.3 ± 2.4	ND
5953	octyl	COO^-	H	H	H	H	CH_2	9.57 ± 2.03	ND
5955	octyl	N	PO_4	H	H	H	CH_2	>600	ND
5961	octyl	COO^-	H	CH_3	H	H	CH_2	>600	156.7
5971	octyl	COO^-	H	H	CH_3	H	CH_2	21.69 ± 2.8	ND
5972	octyl	COO^-	H	H	H	H	CH2CH3	117.5 ± 3.11	ND
5973	octyl	COO^-	F	H	H	H	CH_2	18.8 ± 3.31	ND
5974	octyl	H	CH_2CH_3	H	H	H	CH_2	>600	ND
5999	$Ph(CH_2)_7$	COO^-	H	H	H	H	C=O	>600	ND
6037	dodecyl	COO^-	H	H	H	H	C=O	5.97 ± 0.38	1.99 ±0.69

Table 4.2 Biochemical analysis of C-capping group on benzamide small molecules. Compound structure, binding affinities, PLK1 selectivity, and catalytic inhibition are indicated.

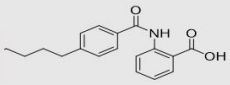
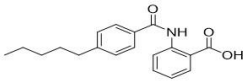
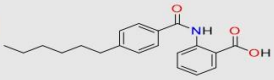
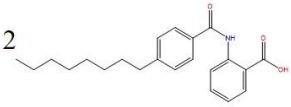
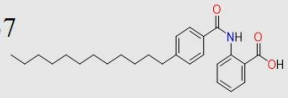
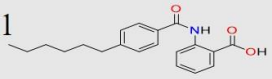
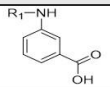
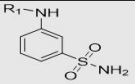
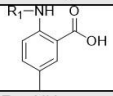
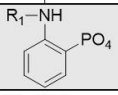
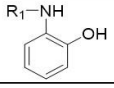
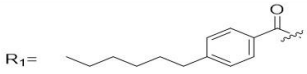
SCCP ID#	Structure	PLK1 IC_{50} [µM]	PLK3 IC_{50} [µM]	Selectivity Fold Index	PLK1 Kinase Inhibitory Activity IC_{50} [µM]
5880		>600	ND	ND	ND
5905		223.6 ± 4.88	ND	ND	ND
5881		18.4 ± 5.3	18.04 ± 5.18	5	ND
5912		11.27 ± 2.7	15.48 ± 0.01	6.9	42.36
6037		5.97 ± 0.38	1.99 ±0.69	1.6	ND

Table 4.3 Biochemical analysis of C-capping group on hexyl benzamide small molecules. Compound structure, binding affinities, and PLK1 selectivity are indicated.

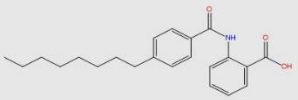
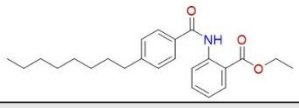
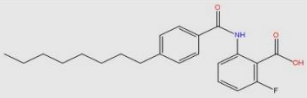
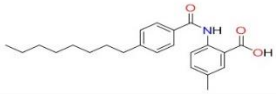
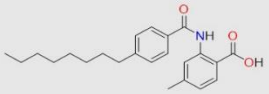
SCCP ID#	Structure	PLK1 IC_{50} [µM]	PLK3 IC_{50} [µM]	Selectivity Fold Index
5881		18.4 ± 5.3	18.04 ± 5.18	5
5903		129.8± 3.6	43.62 ± 1.69	1.6
5908		>600	ND	ND
5924		>600	24.5	ND
5940		>600	ND	ND
5943		>600	ND	ND



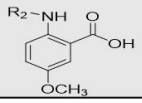
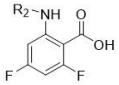
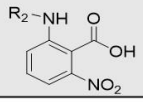
(alkyl-benzamide)

Table 4.4 Biochemical analysis of C-capping group with Rigid Octyl Benzamide. Compound structure, binding affinities, PLK1 selectivity, and catalytic inhibition are indicated.

A.

SCCP ID#	Structure	PLK1 IC_{50} [µM]	PLK3 IC_{50} [µM]	Selectivity Fold Index	PLK1 Kinase Inhibitory Activity IC_{50} [µM]
5912		11.27 ± 2.7	15.48 ± 0.01	6.9	42.36
5914		>600	ND	ND	ND
5932		11.1 ± 1.1	13.24 ± 0.46	6	48.9
5915		2.16 ± 0.01	7.68 ± 2.41	17.8	24.3
5937		3.99 ± 2.3	7.42 ± 1.89	9.3	ND

B.

SCCP ID#	Structure	PLK1 IC_{50} [µM]	PLK3 IC_{50} [µM]	Selectivity Fold Index
5935		5.89 ± 1.25	13.6 ± 6.3	11.5
5938		30.6 ± 3.2	39.7	6.5
5939		6.05 ± 1.9	9.96 ± 1.52	8.2

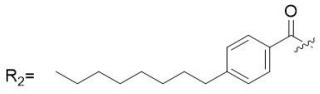
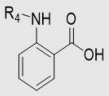
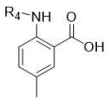
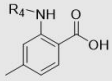
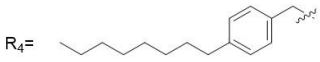


Table 4.5 Biochemical analysis of C-capping group with Flexible Octyl Benzamine. Compound structure, binding affinities, and PLK1 selectivity are indicated.

SCCP ID#	Structure	PLK1 IC_{50} [µM]	PLK3 IC_{50} [µM]	Selectivity Fold Index
5953		9.57 ± 2.03	ND	ND
5961		>600	156.7	ND
5971		21.69 ± 2.8	ND	ND



(alkyl-benzamine)

Table 4.6 Cancer cell viability analysis of small molecules. Numbers represent IC_{50} values generated from three independent 72 hour cell proliferation (MTT) assays.

5912 Derivatives	HeLa [µM]	PC-3 [µM]	A-549 [µM]	HCT116 (p53-/-) [µM]	HCT116 (p53+/+) [µM]
5912	15.2±1.4	15.1±1.7	2.8±0.1	8.0±1.7	14.8±6.4
5914	>100	>100	ND	ND	ND
5915	15.5±1.7	16.3±2.1	5.1±1.6	10.0±1.0	17.4±5.6
5932	12.7±2.3	11.6±1.2	2.9±0.5	10.0±2.4	12.4±2.7
5937	17.9±2.1	18.8±1.7	3.5±1.2	11.4±1.6	24.2±7.1
5945	20.1±3.1	27.9±2.6	13.3±0.8	24.9±2.0	ND
5946	33.6±2.6	34.2±2.5	ND	ND	ND
6037	14.0±3.5	9.4±0.8	ND	ND	ND

FIGURES

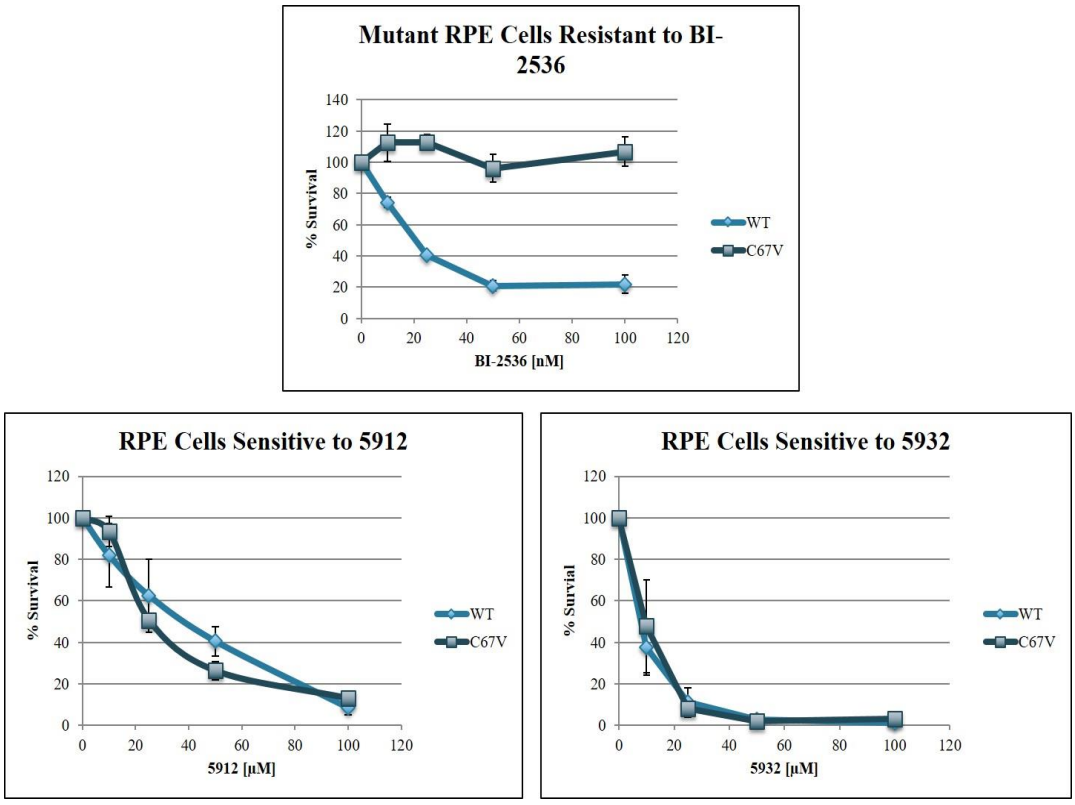


Figure 4.1 Retinal Pigment Epitelial (RPE) cells are sensitive to 5912 and 5932. RPE cells expressing C67V PLK1 are resistant to BI-2536, an ATP-competitive inhibitor. Dose response curves generated from three cell proliferation assays. Standard Deviation (SD) used to generate error bars.

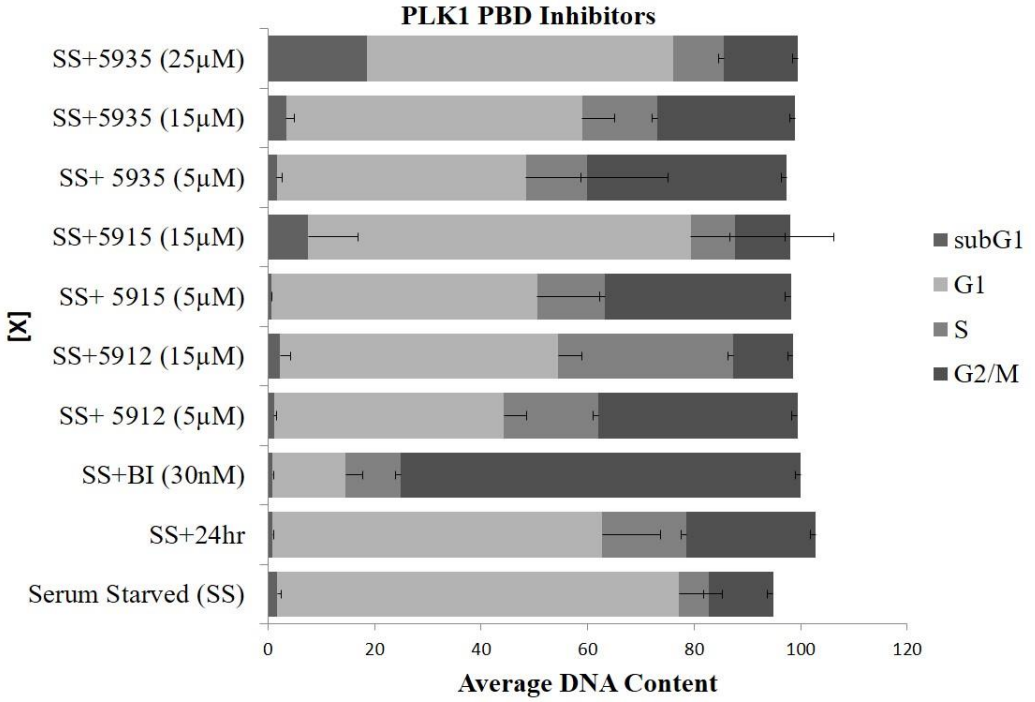


Figure 4.2 Cell cycle analysis of PBD inhibitors in prostate cancer (PC3) cells. Cells were synchronized in G1 by 72 hr serum starvation, followed by 24 hr treatment with PBD inhibitor. DNA content was averaged across three or more independent experiments and standard deviation was calculated to generate error bars.

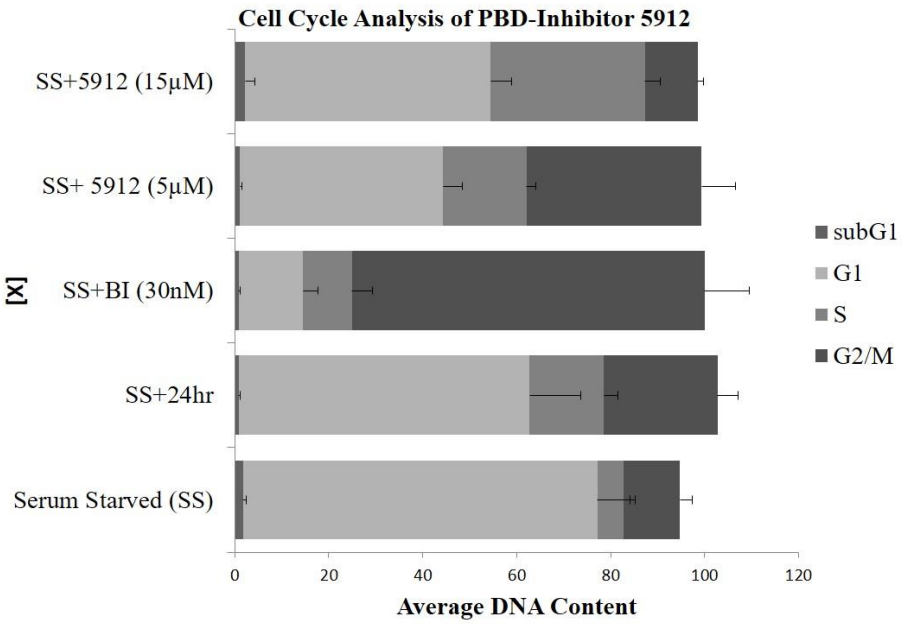


Figure 4.3 Cell cycle analysis of 5912 in prostate cancer (PC3) cells. Cells were synchronized in G1 by 72 hr serum starvation, followed by 24 hr treatment with PBD inhibitor. DNA content was averaged across three or more independent experiments and standard deviation was calculated to generate error bars.

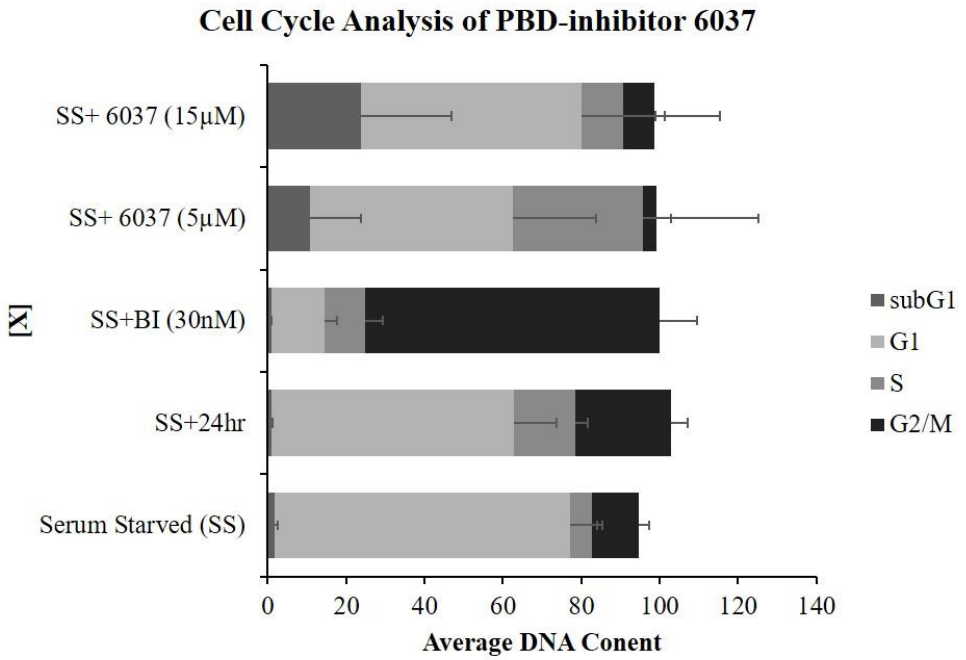


Figure 4.4 Cell cycle analysis of 6037 in prostate cancer (PC3) cells. Cells were synchronized in G1 by 72 hr serum starvation, followed by 24 hr treatment with PBD inhibitor. DNA content was averaged across two independent experiments and standard deviation was calculated to generate error bars.

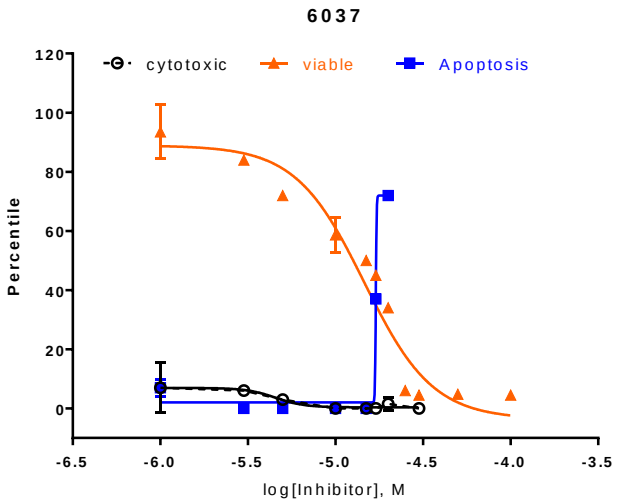


Figure 4.5 Viability, cytotoxicity, and apoptotic analysis of 6037 in PC3 cells. Cells were analyzed using the ApoTox-Glo ™ Triplex Assay (Promega). Dose-response curves were measured in triplicate. Viable Concentration = 15.6 ± 3.4 µM, Apoptosis Concentration = 15.0 ± 5.6 µM

CHAPTER FOUR REFERENCES

1. Mundt KE, Golsteyn RM, Lane HA, Nigg EA. On the regulation and function of human polo-like kinase 1 (PLK1): effects of overexpression on cell cycle progression. Biochemical and Biophysical Research Communication. 1997;239(2):377-85.

2. Qian YW, Erikson E, Li C, Maller JL. Activated Polo-like kinase Plx1 is requried at multiple points during mitosis in Xenopus laevie. Molecular and Cellular Biology. 1998;18(7):4262-71.

3. Kotani S, Tugendreich S, Fujii M, Jorgensen PM, Watanabe N, Hoog C, et al. PKA and MPF-activated Polo-like kinase regulate anapahse-promoting complex activity and mitosis progression. Molecular Cell. 1998;1:371-80.

4. Dai W, Wang Q, Traganos F. Polo-like kinases and centrosome regulation. Oncogene. 2002;21:6195-200.

5. Winkles JA, Alberts GF. Differential regulation of polo-like kinase 1, 2, 3, and 4 gene expression in mammalian cells and tissues. Oncogene. 2005;24(2):260-6.

6. Garland LL, Taylor C, Pilkington DL, Cohen JL, Von Hoff DD. A phase I pharmacokinetic study of HMN-214, a novel oral stilbene derivative with polo-like kinase-1–interacting properties, in patients with advanced solid tumors. Clinical cancer research. 2006;12(17):5182-9.

7. Holtrich U, Wolf G, Bräuninger A, Karn T, Böhme B, Rübsamen-Waigmann H, et al. Induction and down-regulation of PLK, a human serine/threonine kinase expressed in proliferating cells and tumors. Proceedings of the National Academy of Sciences. 1994;91(5):1736-40.

8. Fisher R, Ferris D. The functions of Polo-like kinases and their relevance to human disease. Current Medicinal Chemistry-Immunology, Endocrine & Metabolic Agents. 2002;2(2):125-34.

9. Steegmaier M, Hoffmann M, Baum A, Lenart P, Petronczki M, Krssak M, et al. BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. Current Biology. 2007;17:316-22.

10. Eckerdt F, Yuan J, Strebhardt K. Polo-like kinases and oncogenesis. Oncogene. 2005;24:267-76.

11. Santamaria A, Neef R, Eberspächer U, Eis K, Husemann M, Mumberg D, et al. Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis. Molecular biology of the cell. 2007;18(10):4024-36.

12. Weichert W, Ullrich A, Schmidt M, Gekeler V, Noske A, Niesporek S, et al. Expression patterns of polo-like kinase 1 in human gastric cancer. Cancer science. 2006;97(4):271-6.

13. Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF, et al. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell. 2009;137:835-48.

14. Jiang BH, Liu LZ. PI3K/PTEN signaling in angiogenesis and tumorigenesis. Advances in cancer research. 2009;102:19-65.

15. Liu XS, Song B, Elzey BD, Ratliff TL, Konieczny SF, Cheng L, et al. Polo-like kinase 1 facilitates loss of Pten tumor suppressor-induced prostate cancer formation. The Journal of Biological Chemistry. 2011;286(41):35795-800.

16. Strebhardt K, Ullrich A. Targeting polo-like kinase 1 for cancer therapy. Nature Reviews. 2006;6:321-30.

17. Mross K, Frost A, Steinbild S, Hedbom S, Rentschler J, Kaiser R, et al. Phase I dose escalation and pharmacokinetic study of BI 2536, a novel Polo-like kinase 1 inhibitor, in patients with advanced solid tumors. Journal of Clinical Oncology. 2008;26(34):5511-7.

18. Sebastian M, Reck M, Waller CF, Kortsik C, Frickhofen N, Schuler M, et al. The efficacy and safety of BI 2536, a novel Plk-1 inhibitor, in patients with stage IIIB/IV non-small cell lung cancer who had relapsed after, or failed, chemotherapy: results from an open-label, randomized phase II clinical trial. Journal of Thoracic Oncology 2010;5(7):1060-7.

19. Schoffski P, Blay JY, De Greve J, Brain E, Machiels JP, Soria JC, et al. Multicentric parallel phase II trial of the polo-like kinase 1 inhibitor BI 2536 in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma. European Journal of Caner. 2010;46(12):2206-15.

20. Stadler WM, Vaughn DJ, Sonpavde G, Vogelzang NJ, Tagawa ST, Petrylak DP, et al. An open-label, single-arm, phase 2 trial of the polo-like kinase inhibitor volasertib (BI 6727) in patients with locally advanced or metastatic urothelial cancer. Cancer. 2014;120(7):976-82.

21. Lansing TJ, McConnell RT, Duckett DR, Spehar GM, Knick VB, Hassler DF, et al. In vitro biological activity of a novel small-molecule inhibitor of polo-like kinase 1. Molecular cancer therapeutics. 2007;6(2):450-9.

22. Olmos D, Barker D, Sharma R, Brunetto AT, Yap TA, Taegtmeyer AB, et al. Phase I study of GSK461364, a specific and competitive Polo-like kinase 1 inhibitor, in

patients with advanced solid malignancies. Clinical Cancer Research. 2011;17(10):3420-30.

23. de Carcer G, Manning G, Malumbres M. From Plk1 to Plk5: Functional evolution of polo-like kinases. Cell Cycle. 2011;10(14):2255-62.

24. Lu B, Mahmud H, Maass AH, Yu B, van Gilst WH, de Boer RA, et al. The Plk1 inhibitor BI 2536 temporarily arrests primary cardiac fibroblasts in mitosis and generates aneuploidy in vitro. PLOS One. 2010;5(9):e12963.

25. Rudolph D, Steegmaier M, Hoffmann M, editors. Characterization of BI 6727, a novel Polo-like kinase inhibitor with a distinct pharmacokinetic profile and efficacy in a model of taxane-resistant colon cancer. 20th European Organization for Research and Treatment of Cancer–National Cancer Institute–American Association for Cancer Research Symposium on Molecular Targets and Cancer Therapeutics, Geneva, Switzerland; 2008.

26. Rudolph D, Steegmaier M, Hoffmann M, Grauert M, Baum A, Quant J, et al. BI 6727, a Polo-like kinase inhibitor with improved pharmacokinetic profile and broad antitumor activity. Clinical Cancer Research. 2009;15:3094-102.

27. Burkard ME, Santamaria A, Jallepalli PV. Enabling and disabling Polo-like kinase 1 inhibition through chemical genetics. ACS Chemical Biology. 2012;7:978-81.

28. Clay FJ, McEwen SJ, Bertoncello I, Wilks AF, Dunn AR. Identification and cloning of a protein kinase-encoding mouse gene, Plk, related to the polo gene of Drosophila. PNAS. 1993;90:4882-6.

29. McInnes C, Estes K, Baxter M, Yang Z, Farag DB, Johnston P, et al. Targeting subcellular localization through the polo-box domain: Non-ATP competitive inhibitors recapitulate a PLK1 phenotype. Molecular Cancer Therapeutics. 2012;11(8):1683-92.

30. Liu X, Lei M, Erikson RL. Normal cells, but not cancer cells, survive severe Plk1 depletion. Molecular and Cellular Biology. 2006;26(6):2093-108.

31. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science. 1991;253(5015):49-53.

32. Liu X, Erikson RL. Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells. PNAS. 2003;100(10):5789-94.

33. Sur S, Pagliarini R, Bunz F, Rago C, Diaz Jr. LA, Kinzler KW, et al. A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. PNAS. 2009;106(10):3964-9.

34. Zimmerman WC, Erikson RL. Polo-like kinase 3 is required for entry into S phase. PNAS. 2007;104(6):1847-52.

35. Kim JH, Ku B, Lee KS, Kim SJ. Structural analysis of the polo-box domain of human Polo-like kinase 2. Proteins: Structure, Function, and Bioinformatics. 2015.

36. Simmons DL, Neel BG, Stevens R, Evett G, Erikson RL. Identification of early-growth-response gene encoding a novel putative protein kinase. Molecular and Cellular Biology. 1992;12(9):4164-9.

37. Ma S, Charron J, Erikson RL. Role of Plk2 (Snk) in mouse development and cell proliferation. Molecular and Cellular Biology. 2003;19:6936-43.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

This study builds upon the work published by our group in 2012 (1) and further validates our approach to developing PBD targeted PLK1 inhibitors by the REPLACE (Replacement with Partial Ligand Alternatives through Computational Enrichment) method. This structure guided drug discovery process was further validated by the successful development of potent and selective peptide and FLIP PBD inhibitors. In addition, synthesis of the first generation of small molecules represents an important leap forward, and provides a good starting point for the development of more selective compounds in the future. This chapter will provide suggestions for experimental approaches to move this project forward.

5.1 CHAPTER TWO SUMMARY

In chapter two we demonstrate the importance of developing a fluorescent polarization (FP) assay that will accurately measure the binding of our peptides, Fragment Ligated Inhibitory Peptides (FLIPs), and compounds to the PBD of PLK1 and PLK3. To accurately measure binding the assay conditions must be properly optimized and routinely confirmed. We were able to determine the tracer concentration that provides adequate signal above background without saturating the detection limits of the machine. Both the PLK1 and PLK3 FP assays required a 10 nM tracer concentration. We also identified the Polo-Box Domain (PBD) concentration at which 90% of the tracer was bound. Proper assay development has allowed for the continued use of the PLK1 FP

assay as our primary screen, and the PLK3 FP assay as a counter screen to detect compound binding.

In addition to continued quality control processes, future studies should include optimizing assay conditions with the full length PLK1 and PLK3 protein. Doing so would more accurately reflect compound binding in a system, in that cells express the full length protein and not the PBD alone. Additionally, an FP assay could be developed for PLK2 and PLK4 as additional counter screening tools.

Future studies should also include the use of a more stable fluorophore for the tracer peptide. For example, BODIPY dyes remain in the excited state longer than fluorescein – the dye we currently use. This results in an increased sensitivity to binding interactions over a broader molecular weight range (2). The use of red-shifted probes are also recommended to reduce fluorescence interference from test compounds (2).

5.2 Chapter Three Summary

Chapter three demonstrates the successful development of highly potent and selective peptide and FLIP PBD inhibitors through the use of REPLACE. By truncating the N-terminal amino acids of known PLK1 substrates and replacing them with small molecular drug-like fragments we were able to exploit the PBD binding pocket and investigate the Structure Activity Relationship (SAR) of a series of compounds containing alkyl benzamide N-terminal replacements. The peptides and FLIPs bound both potently and selectively to PLK1, providing the basis for the development of non-peptidic small molecules (discussed in Chapter 4). FLIPs were also able to inhibit the catalytic activity of PLK1 *in vitro*, and cause a decrease in prostate (PC3), cervical (HeLa), and lung (A-549) cancer cell proliferation. Both wild type and mutant (C67V)

PLK1 Retinal Pigment Epithelial (RPE) cells were sensitive to **5996** and **6026** RPE cells expressing mutant PLK1 were resistant to ATP-based PLK1 inhibitors.

The results demonstrated that the N-terminal amino acids contributed to compound potency while the C-terminal amino acids contributed to compound selectivity (Table 3.1). This was further demonstrated from the SAR of the octyl-benzamide FLIPs (Table 3.3) in which C-terminal amino acids were analyzed (**5988**, **5996**, **6026**). Future studies should include a more in depth study of C-terminal amino acid SAR with the octyl-benzamide N-capping group. Doing so will provide valuable information about the PLK1 PBD binding pocket. That information can then be used to generate a drug-like molecular fragment that will mimic the intermolecular contributions of the amino acid sequence that produces the most selective FLIP.

Peptides are typically not useful as drugs because they get degraded or cleaved before reaching their target. Despite the peptidic nature of our compounds, we were able to detect cellular activity (Table 3.4) in HeLa, PC3, and A-549 cancer cells. While this was encouraging, the calculated IC_{50} values were in the higher microMolar range. This is due, in part, to the degradation that occurs with all peptides. A drug delivery system should be used in the future, for a more accurate IC_{50} measurement in cells because it will ensure that the compound reaches its target without degrading. I expect that the cellular IC_{50} values will decrease as a result of the compound reaching its target. A drug delivery system will also allow additional mechanistic and phenotypic questions about PBD-inhibition to be answered. We cannot assume that PBD inhibition will result in phenotypes consistent with ATP-inhibition. To determine similarities or differences with

PBD inhibition, future studies should include immunofluorescence microscopy, cell cycle analysis, and western blotting

Our efforts to carry out PLK1 localization studies were unsuccessful due to inconsistencies with our drug delivery system. Immunofluorescence microscopy will allow for the visualization of aberrant mitotic phenotypes (i.e. PLK1 localization, spindle pole formation, and the number of centrosomes).

Future studies should build upon the cell cycle analysis results for **6026** (200 μM) (Table 3.6, Figure 3.1) and use a mitotic marker to identify whether cells are in G2 or Mitosis by flow cytometry. This will allow for a better understanding of the affect FLIPs have on the cell cycle, and it will provide a starting point for investigation into mechanistic studies. If cells are in G2, it can be assumed that PBD inhibition affects PLK1's role in mitotic entry, therefore, further investigation into CDK1 and cyclin B should take place. Recall that PLK1 phosphorylates and activates the phosphatase Cdc25C in G2 (Chapter 1). Cdc25C then dephosphorylates CDK1, allowing it to form a complex with cyclin B, thereby initiating mitotic entry. If cells are in mitosis, mitotic phenotypes should be further explored.

Efforts to determine apoptotic induction by **6026** with flow cytometry were also unsuccessful. Asynchronous HeLa cells were treated for 24 and 48 hours with 250 μM of **6026**. We failed to see an induction of apoptosis, but additional analysis using a drug delivery system is needed (Table 5.1). Future work could also explore apoptosis by western blot analysis, probing for cleaved PARP, or caspase 3. Western blot analysis could also be used to directly measure PLK inhibition by probing for PLK1 and its substrates after treating cells with FLIPs.

Building upon the cell proliferation data is also an important initial step in determining the mechanism by which cells are dying when treated with FLIPs (Table 3.4).

Lastly, cells expressing mutant (C67V) PLK1 were sensitive to our PBD inhibitors (Table 3.5). Future studies should include establishing a cancer cell line expressing the C67V mutation and cell lines expressing mutation(s) within the PBD. This will be discussed further in section 5.3.

5.3 Chapter Four Summary

Chapter 4 discusses the development of 40 small molecules synthesized by the McInnes group (Dr. Sandra Craig). These compounds bind to the PLK1 PBD and inhibits the catalytic activity of the protein (Table 4.1). Our small molecules also cause a decrease in cancer cell proliferation (Table 4.6), and **5912** and **5932** where shown to circumvent the resistance observed in cells expressing mutant (C67V) PLK1 when treated with catalytic inhibitors (Figure 4.1). Lastly, our results suggests PLK1 is being targeted when treating PC3 cells with a lower dose (5 μM), and our compounds are targeting a different protein (possibly PLK3) when treating with a higher dose (15 μM). Our least selective compound (**6037**) has off target effects at both the lower and higher doses.

N-terminus FLIP SAR was used to identify replacements for the C-terminus amino acid residues in a virtual compound library screen. This was done in parallel with the development of the FLIPs. We developed a lead molecule (**5881**) and went on to generate forty small molecules that have modest PLK1 selectivity. Future studies should take into account the C-terminus SAR when carrying out the next compound library

screen to develop second generation small molecules. Doing so could provide replacements that result in a lead molecule with increased PLK1 selectivity.

The C67V RPE cells were sensitive to our non-peptidic compounds while remaining resistant to BI-2536 (ATP- inhibitor) (Figure 4.1). We established a transient resistance model to test the efficacy of our PBD based inhibitors. We obtained GFP-fusion expression constructs for wild-type PLK1 and PLK3 (a gift from Dr. Wei Dai, New York University School of Medicine, NY). The PLK1 construct was transfected into PC3 cells to ensure cancer cells would take up the plasmid (Figure 5.1). Once confirmed the C67V mutant PLK1 construct was created by site-directed mutagenesis (Figure 5.2). We confirmed the mutation by sequencing. Transient transfections of GFP only (control), WT-PLK1, and C67V PLK1 into HeLa cells were carried out (Figure 5.3) by electroporation. We were able to confirm the results of Burkard *et al.* (3) in our system, showing that HeLa cells transiently overexpressing the C67V mutant PLK1 are more resistant than cells overexpressing WT-PLK1, as evidenced by the number of colonies in the respective wells treated with 1 nM BI-2536 (Figure 5.3).

Future studies should build upon this work and establish an isogenic cancer cell line stably expressing mutant PLK1. Our efforts to establish a stable cancer cell line were unsuccessful. We found that cells expressed our GFP fusion constructs for 72 – 96 hours, with expression peaking at 48 hours (Figure 5.4). This was observed with HeLa, PC3, DU-145, A-549, and Daudi cancer cells (not shown). After 48 hours, there was a decrease in plasmid expression (as evidenced by GFP) in all cell lines tested. Decreased expression after 48 hours was also observed when cells were placed under selection and we were unsuccessful in isolating any stable colonies.

Future work should include knocking out endogenous PLK1 and adding back the PLK1 plasmid constructs, as described in Burkard et al, (2012) (3). This would allow for mechanistic investigation of our small molecules in a cancer cell resistance model.

It would be interesting to determine if a mutation within the Polo-box Domain would 1) allow the protein to retain its substrate specificity and catalytic function while, 2) generating resistance to PBD-inhibition. This is important because drug resistance is always a concern with cancer therapeutics because cancer cells will generate mutations to survive. Potential mutations could be determined by exposing cancer cell lines to a low dose of our PBD inhibitors over time. The DNA of any surviving cells could be sequenced and then compared to untreated cells. This would allow for the discovery of any acquired genetic mutations as a result of PBD inhibition.

Future studies should also include a direct measure of PLK1 inhibition in cells by western blot analysis. Probing for PLK1 and its substrates will directly confirm that PLK1 is being targeted in cancer cells when treated with our small molecules.

Because of the concern with resistance developing as a result of single agent therapy, future studies could also investigate the potential for a synergistic affect when treating cancer cells with PBD and ATP-based inhibitors. It will be important to take the next step and carry out *in vivo* studies once there is a compound that is both potent and selective *in vitro* and in cells.

TABLES

Table 5.1 HeLa cell death analysis following treatment with 6026. HeLa cells were treated for 24 and 48 hours with 250 μM **6026**. BI-2536 is an ATP-inhibitor of PLK1 and serves as the positive control. Values represent cell percentages.

	Untreated	BI-2536 48hr	**6026** 24hr	**6026** 48hr
Necrotic	**4.3**	**15.6**	9.1	**11.9**
Late Apoptotic	1.3	24.3	2.7	3.4
Normal	92.7	37.5	86.4	81.2
Early apoptotic	**1.7**	22.6	1.9	**3.5**

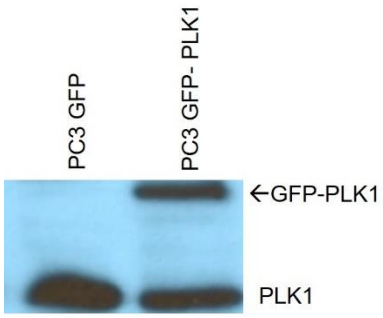


Figure 5.1 Western Blot analysis of GFP and GFP-PLK1 fusion construct expression in PC3 cells. The lower band shows endogenous PLK1 expressed in PC3 cells. The left lane shows extracts from cells electroporated with the GFP only expression construct. The right lane shows that the GFP-labeled PLK1 is expressed in cells electroporated with the expression construct. The data shows that the expression construct works and that PC3 cells are capable of over-expressing PLK1.

DNA sequence for PLK1 from 5' to 3'

ATG AGT GCT GCA GTG ACT GCA GGG AAG CTG GCA CGG GCA CCG GCC GAC CCT GGG AAA GCC
GGG GTC CCC GGA GTT GCA GCT CCC GGA GCT CCG GCG GCG GCT CCA CCG GCG AAA GAG ATC
CCG GAG GTC CTA GTG GAC CCA CGC AGC CGG CGG CGC TAT GTG CGG GGC CGC TTT TTG GGC
AAG GGC GGC TTT GCC AAG TGC TTC GAG ATC TCG GAC GCG GAC ACC AAG GAG GTG TTC GCG
GGC AAG ATT GTG CCT

TGC = Cys67

Need to change TGC (Cys) to GTC (Val)

Primers used for Site Directed Mutagenesis

- 5'-G GGC GGC TTT GCC AAG GTC TTC GAG ATC TCG GAC GCG
- 3'-C CCC CCC AAA CGG TTC CAG AAG CTC TAG AGC CTG CGC-5'

Figure 5.2 Primers used for C67V site directed mutagenesis. These primers generated the C67V mutation in the GFP-PLK1 fusion construct. Mutation was confirmed by sequencing

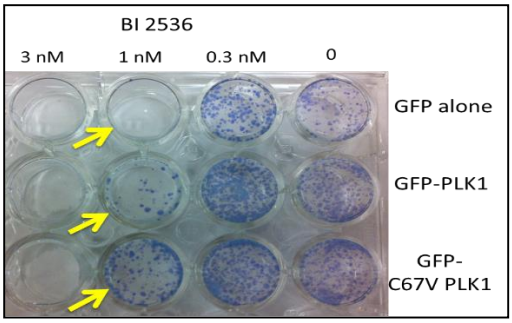


Figure 5.3 C67V PLK1 resistance model in HeLa cells. HeLa cells transiently expressing mutant PLK1 are resistant to BI-2536.

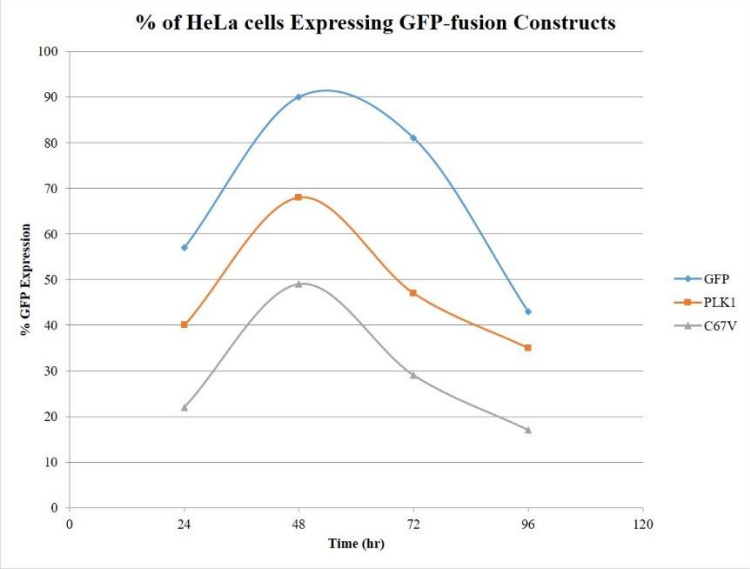


Figure 5.4 Percentage of HeLa cells expressing the GFP-fusion constructs. Expression 24 hours following electroporation. Expression peaked 48 hours after electroporation for all constructs.

CHAPTER FIVE REFERENCES

1. McInnes C, Estes K, Baxter M, Yang Z, Farag DB, Johnston P, et al. Targeting subcellular localization through the polo-box domain: Non-ATP competitive inhibitors recapitulate a PLK1 phenotype. Molecular Cancer Therapeutics. 2012;11(8):1683-92.

2. Arkin MR, Glicksman MA, Fu H, Havel JJ, Du Y. Inhibition of Protein-Protein Interactions: Non-Cellular Assay Formats In: Sittampalam GS, Coussens NP, Nelson H, al. e, editors. Assay Guidance Manual [Internet]. Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2012.

3. Burkard ME, Santamaria A, Jallepalli PV. Enabling and disabling Polo-like kinase 1 inhibition through chemical genetics. ACS Chemical Biology. 2012;7:978-81.