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Evaluation of Aminopyrazole Analogs as Cyclin-Dependent Kinase Inhibitors for Colorectal Cancer Therapy

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EVALUATION OF AMINOPYRAZOLE ANALOGS AS CYCLIN-DEPENDENT KINASE INHIBITORS FOR COLORECTAL CANCER THERAPY

by

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DEDICATION

To the cancer patients of the past, present, and future. You are the reason I get up each and every day and go to work. I hope you know that I will gladly dedicate my career to doing whatever I can to make your lives a little easier.

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EVALUATION OF AMINOPYRAZOLE ANALOGS AS CYCLIN-DEPENDENT KINASE INHIBITORS FOR COLORECTAL CANCER THERAPY

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University of Nebraska, 2017

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Colorectal cancer (CRC) remains one of the leading causes of cancer related deaths in the United States. Currently, there are limited therapeutic options for CRC patients, none of which focus on the cell signaling mechanisms controlled by members of the cyclin dependent kinase (CDK) family. CDK5 has been implicated in a variety of cancers, and most recently as a tumor promoter in CRC.

As such, we evaluated a compound developed by Pfizer, CP-668863 (a.k.a. 20-223), that inhibits CDK5 in neurodegenerative disorders. In our CRC xenograft model, 20-223 reduced tumor growth and tumor weight, indicating its value as a potential anti-CRC agent. We subjected 20-223 to a series of cell-free and cell-based studies to understand the mechanism of its anti-tumor effects. Profiling the CDK family revealed that 20-223 was most potent against CDK2 and CDK5 in cell-free and cell-based systems. The clinically used CDK inhibitor AT7519 and 20-223 share the aminopyrazole core. 20-223 was comparable, or in some cases better, than clinically used AT7519, proving it to be a suitable lead compound.

Next we utilized the new PRoteolysis TArgeting Chimera (PROTAC) strategy to develop CDK5 degraders. Synthesis and evaluation revealed that the heterobifunctional aminopyrazole-based PROTAC capable of cereblon-mediated proteasomal degradation targeted CDK9 while sparing CDK2 and CDK5. While the degrader (**3**) did in fact bind to

CDK5 and inhibit its kinase activity, it was unable to trigger its degradation, likely due to differentially exposed lysine residues. This is the first report of a PROTAC capable of degrading a member of the oncogenic CDK family.

Overall, these studies demonstrate that inhibition of CDK5 is a promising therapeutic strategy and warrants further evaluation. 20-223 is a favorable lead compound for CRC therapy as it exhibits anti-cancer activity both *in vitro* and *in vivo*. Additionally, the PROTAC strategy can be applied to develop selective CDK degraders.

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LIST OF ABBREVIATIONS

ADME	Absorption, distribution, metabolism, and excretion
AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
AR	Androgen receptor
ATP	Adenosine triphosphate
ATM	Ataxia-telangiectasia mutated kinase
Bcl-xL	B-cell lymphoma-extra large
BET	Bromodomain and extraterminal domain
BRD	Bromodomain
CDK	Cyclin-dependent kinase
CDK1	Cyclin-dependent kinase 1
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDK5	Cyclin-dependent kinase 5
CDK6	Cyclin-dependent kinase 6
CDK7	Cyclin-dependent kinase 7
CDK9	Cyclin-dependent kinase 9
CNS	Central nervous system
CRBN	Cereblon
CRC	Colorectal cancer
CRISPR	Clustered regularly interspaced short palindromic repeats
CTD	C-terminal domain

DCM	Dichloromethane
DDB1	DNA damage-binding protein 1
DDR	DNA-damage repair
DIEA	N, N-diisopropylethylamine
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
E2F	E2 factor family
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GFP	Green fluorescent protein
GSK-3β	Glycogen synthase kinase-3 beta
HCEC	Human colon epithelial cells
HRP	Horseradish peroxidase
HTS	High throughput screen
IACUC	Institutional Animal Care and Use Committee
IHC	Immunohistochemistry
IKKβ	Inhibitor of Nuclear Factor Kappa-B kinase subunit beta
IR	Ionizing radiation
LC-MS	Liquid chromatography mass spectrometry

MetAP2	Methionine aminopeptidase 2
Mcl-1	Myeloid cell leukemia-1
MTC	Medullary thyroid cancer
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PK	Pharmacokinetic
PD	Pharmacodynamic
PDK1	Phosphoinositide-dependent kinase-1
PD-L1	Programmed-death ligand 1
PMSF	Phenylmethylsulfonyl fluoride
PP1α	Protein phosphatase PP1-alpha 1 catalytic subunit alpha
PROTAC	PRoteolysis TArgeting Chimera
P-TEFB	Positive transcription elongation factor B
PVDF	Polyvinylidene difluoride
RB	Retinoblastoma
RNA	Ribonucleic acid
RNAi	RNA inhibition
RNAPII	RNA polymerase II
RPA-32	Replication protein A-32
SAR	Structure-activity relationship
SCF	Skp1-cullin-F box
SDS	Sodium dodecyl sulfate
shRNA	short hairpin ribonucleic Acid
siRNA	short interfering ribonucleic Acid
STAT3	Signal transducer and activator of transcription 3

STR	Short tandem repeats
TCGA	The Cancer Genome Atlas
TBST	Tris-buffered saline + Tween
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau

CHAPTER 1: INTRODUCTION

Colorectal Cancer

Stages and Survival Rates

Colorectal cancer (CRC) remains one of the deadliest forms of cancer. In 2017, approximately 135,430 individuals will be diagnosed with CRC, making it the fourth most commonly diagnosed cancer in the United States. Unfortunately, nearly 50,260 individuals will die a CRC-related death this year, making it the second deadliest form of cancer [1]. Similarly to other cancers, CRC develops from the accumulation of mutations over time. As mutations are acquired, the abnormal growth of cells increases and generates a polyp on the innermost lining of the colon wall, known as the mucosa, and is classified as Stage 0. Fortunately, through routine colonoscopies for individuals over 50 years of age, polyps can be discovered early and removed before they are able to progress to later stage disease. Gone unnoticed, continued polyp growth facilitates the progression from stage 0 to stage 1, when the cancer enters the mucosa layer. Stage 2 CRC occurs when the malignant cells have entered the submucosal layer of the colon/rectum. CRC is classified as stage 3 when the cancerous cells expand through all layers of the colon/rectum wall and invade the regional lymph nodes. Finally, the most severe stage of CRC is stage 4, wherein the cancer has metastasized to other organs within the body, most often the lung or liver [2, 3].

The prognosis for patients diagnosed with CRC is often determined by the staging at diagnosis. Survival rates for patients diagnosed with local (stage 1 and stage 2) and regional disease (stage 3) remain high at 89.9% and 71.3% respectively [1]. The high 5-year survival rate observed in these patients, which accounts for approximately 74% of CRC patients, is likely a result of routine screening that allows physicians to discover the

cancer early. Unfortunately, not all patients receive an early diagnosis. Approximately 21% of CRC patients are diagnosed with metastatic disease and the predicted survival for these individuals decreases considerably. Those diagnosed with metastatic CRC have a 13.9% chance to reach the 5-year survival mark [1].

CRC Treatment

Treatment options for CRC patients are determined by the staging of the disease at diagnosis. There are four traditional treatments that are considered when an individual is diagnosed with colorectal cancer: surgery, chemotherapy, radiation, and targeted therapy [4]. Surgical resection with suitable distal and circumferential boundaries is common for stages 0-2. In some cases, patients presenting with mid-stage disease (stage 2 or 3) may require standard of care chemotherapy or radiotherapy to debulk the tumor before surgeons can safely remove the mass [5]. Additionally, adjuvant chemotherapy may also be administered to “high risk” patients in order to eliminate lingering malignant cells at the margins following surgery, which is determined on a case-by-case basis [4, 6]. Presently, the standard cocktail of chemotherapy consists of various regimens and cycles of fluoropyrimidines (5-fluorouracil) plus leucovorin combined with oxaliplatin or irinotecan-based therapies [7]. 5-fluorouracil (5-FU) is an antimetabolite chemotherapy that prevents new DNA synthesis specifically by inhibiting thymidylate synthase. Leucovorin is often administered with 5-FU, as it has been shown to elongate the effects. Patients are typically given a 5-day bolus of 425 mg/m^2 5-FU and 20 mg/m^2 of leucovorin every 4 weeks. Alternatively, patients may instead be given 200 mg/m^2 of leucovorin in addition to a bolus of 5-FU at 400mg/m^2 and a 22-hour 5-FU infusion at 600mg/m^2 for 2 straight days. This cycle is repeated every 2 weeks [7]. Oxaliplatin or irinotecan therapies have been shown to enhance the effect of 5-FU. Oxaliplatin is a cytotoxic agent that induces DNA damage and it usually given in a dosage of either 85 mg/m^2 (FOLFOX-4) or 100 mg/m^2 (FOLFOX-

6) [7, 8]. Irinotecan chemotherapies inhibit topoisomerase I which stops the unwinding of DNA [7]. Irinotecan therapies are typically added to 5-FU regimens at 180 mg/m2 (FOLFIRI) [8]. A diagnosis of stage 4 CRC complicates the treatment options for patients. Individuals with metastatic CRC will often receive a cocktail of therapy, including surgical resection, combination chemotherapy, and targeted therapy when applicable [6].

Targeted Therapy

Targeted therapy has quickly gained recognition as a way of treating cancer from a personalized medicine perspective. Deviating from the original one-size-fits-all approach to cancer therapies, this advancement takes the specific genetics of a patient's tumor and uses them to directly target factors that may be driving malignant transformation. Thus, targeted therapy relies on defining the molecular drivers found in the diseased tissue [6]. Increased understanding of the underlying molecular biology and genetic makeup of each individual tumor gives clinicians the opportunity to tailor treatment to that specific patient [6, 7]. As basic cancer research has continued to improve target identification, the medicinal chemistry field has been able to develop drugs that selectively disrupt target function.

In recent years, the use of targeted therapy has helped patients with late stage CRC. Including targeted therapy as part of the treatment regimens has not only prolonged patient survival, but has also improved quality of life [6, 7]. In fact, the combination of chemotherapy plus target therapy has proven to give metastatic CRC patients the greatest chance of survival. An increase in overall survival of 14 months was observed with the addition of targeted therapies [7]. Currently, the mainstays of targeted therapy are directed at inhibition of the vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR). Bevacizumab is a monoclonal antibody that targets VEGF, and in doing so disrupts the formation of new vascularization in CRC tumors. Cetuximab, another

monoclonal antibody, is used to block the epidermal growth factor (EGF) ligand from binding to the EGFR, which halts downstream signaling that is important for cell growth, differentiation, and angiogenesis. Both agents were approved by the FDA in 2004 and have been used frequently in combination with cytotoxic chemotherapy [9]. Despite the success of these targeted therapies, the mortality rate amongst CRC patients remains high. Consequently, there is continued need for additional therapeutic options that address other biologic factors found within CRC tumors. A greater understanding of CRC biology will open the door to the development of new therapeutic options to combat CRC [7].

Cyclin-Dependent Kinases

The family of serine/threonine kinases known as cyclin-dependent kinases (CDKs) has received much attention as therapeutic targets since its discovery in the 1980s [10]. Similarly to all kinases, CDKs are structurally comprised of N- and C-terminal lobes linked by a hinge region that encompasses the ATP (adenosine triphosphate)-binding pocket [10, 11]. The activation domain (T-loop) of CDKs is housed in the C-terminal portion of the molecule. CDKs are inactive as monomers and require the binding of activator subunits, typically cyclins, to form heterodimer complexes and initiate catalytic activity [10, 12]. In some cases, phosphorylation of the T-loop is also required in order to displace the autoinhibitory segment that blocks the catalytic cleft. Once phosphorylated, the T-loop opens to allow activator binding [10, 13]. The 20 kinases in this family function as catalytic enzymes that facilitate signaling by removing the γ-phosphate from ATP and facilitating its transfer to the hydroxyl group on a Ser or Thr of a downstream substrate. CDKs recognize a particular consensus sequence (S/T-P-X-K/R) in downstream substrates which helps them to identify and phosphorylate their specific targets [14]. CDKs are localized throughout the cell and the localization of specific CDKs is dependent on their function

[15]. Most CDKs are found in the nucleus, as they play an important role in controlling cell cycle transitions or transcriptional regulation [15, 16]. However, a select few members of the CDK family are found in the cytoplasm where they are important for cytoskeletal regulation [10, 15]. CDKs contribute to a variety of functional roles, such as cell cycle control, cell proliferation, transcriptional elongation, cell death, cell migration, and DNA damage response [16-18]. Due to their influential role in multiple signaling events, CDKs are often deregulated in diseased states [16]. As such, all 20 members of the CDK family have been shown to contribute to cancer and therefore are considered potential therapeutic targets.

CDK Inhibitors

ATP-competitive Inhibitors

Kinases have become increasingly popular as oncology drug targets [19]. As protein phosphorylation is a crucial regulatory event in many cellular processes, medicinal chemists have developed drugs to disrupt these signaling pathways [20]. Thus far, the easiest and most effective way to modulate kinase activity is through the use of small molecule kinase inhibitors that mimic ATP, also referred to as ATP-competitive inhibitors [11]. The ATP binding site is sandwiched between the N- and C-terminal lobes of kinases [21]. Once the kinase is active, ATP recognizes the change in conformation and is able to bind through a network of hydrogen bonds formed between its adenine ring and the hinge region of the kinase. The triphosphate portion of the molecule points toward the substrate-binding site, enabling catalysis. ATP-competitive inhibitors act in a similar fashion to ATP. They bind within the ATP binding site and imitate the hydrogen bond network [11]. Inhibitor association with the kinase disrupts the binding of ATP and subsequent transfer of the γ-

phosphate to downstream substrates, and thus perturbs the downstream signaling cascade [11].

First-generation CDK Inhibitors

As mentioned earlier, the original approach to CDK inhibition, which remains popular today, is through the use of ATP-competitive small molecule kinase inhibitors [22, 23]. First-generation CDK inhibitors were discovered more than 20 years ago. At the time, these agents were examined for efficacy in a variety of cancers and considered promising CDK inhibitors [24]. A few examples of first-generation CDK inhibitors are olomoucine, roscovitine, and flavopiridol [25-27].

A panel of CRC cell lines was subjected to treatment with olomoucine and growth inhibitory effects were examined. Doses ranging from 17-32 μM were required to reach 50% growth inhibition. Furthermore, olomoucine proved to have a maximum tolerated dose of 100 mg/kg compared to other CDK inhibitors [28]. Roscovitine proved to be slightly more effective than olomoucine, as evident by IC_{50} values ranging from approximately 10 to 20 μM in CRC cells [29]. Treatment with roscovitine resulted in approximately 68-80% reduction in tumor growth in CRC models [28, 30] and the efficacy of roscovitine was enhanced when combined with conventional CRC chemotherapies [29]. Flavopiridol showed initial promise in cellular assays, but it was ineffective as an individual agent in CRC animal studies [31]. Interestingly, similar to roscovitine, it did prove to be more beneficial when combined with cytotoxic compounds, such as 5-FU, irinotecan (SN-38), or oxaliplatin [32-34]. While the studies outlined above touch briefly on first-generation CDK inhibitors in CRC, a majority of the research has been focused on other tumor types.

Several of the first-generation agents showed good anti-cancer properties, such as decreasing cell proliferation while inducing cell death, however, the concentrations

required to achieve substantial response were considered too high (IC_{50} values > 1μM in cellular systems). Furthermore, many of these compounds failed in clinical trials as single agents [35]. The failure was attributed to the lack of selectivity among the first generation CDK inhibitors and these are often referred to as pan-CDK inhibitors [26]. The lack of selectivity in a subset of studies resulted in dose-limiting toxicity. While the need to inhibit members of the CDK family was clearly recognized as a viable therapeutic option, to be successful, substantial improvements were necessary with the next generation of CDK inhibitors.

In recent years, progress in medicinal chemistry has expanded our knowledge of not only the chemical structures of inhibitors, but also on the structure of CDKs themselves. Together, these two advancements have made it easier to develop new or modify pre-existing inhibitors in order to improve the selectively and potency of CDK inhibitors.

Aminopyrazole Analogs

One scaffold that has been shown to have anti-CDK inhibitory activity is the aminopyrazole core. ATP-competitive aminopyrazole analogs have shown success both in pre-clinical and clinical cancer studies [36-38]. In 2004, this new class of CDK inhibitors, containing an aminopyrazole core, was identified from a high throughput screen (HTS) guided toward CDK2 inhibition [36]. Due to the small nature of the core and the ability to easily modify the substituents, structure-activity relationship (SAR) studies were used to generate the CDK2 inhibitor PNU-292137. X-ray crystallographic studies revealed the potential for the formation of a trio of hydrogen bonds (donor-acceptor-donor) between the nitrogen atoms on the aminopyrazole core and hinge region residues Glu81-Leu83 of CDK2 [36]. The substituents flanking the aminopyrazole core occupy the shallow hydrophobic pocket on one end and solvent exposed on the other end. Having shown that

the aminopyrazole core is a viable scaffold to build CDK inhibitors, medicinal chemists have developed an array of aminopyrazole analogs that have been evaluated for efficacy as anti-CDK therapeutics.

AT7519

After the initial discovery of the aminopyrazole analog PNU-292137, others sought to study and modify the structure to improve potency and selectivity. As such, Astex Therapeutics performed a series of SAR studies and identified AT7519, a 4-aminopyrazole analog [39]. Preliminary *in vitro* kinase profiling and growth inhibition studies showed AT7519 was a promising lead compound for continued evaluation. While the development of AT7519 was aimed at the inhibition of CDK2, kinase profiling revealed AT7519 was a potent inhibitor of multiple members of the CDK family, likely due to the high homology found within the ATP-binding pocket of CDKs. Cell-free IC_{50} values were derived for the family of CDKs. While AT7519 was indeed most potent against CDK2 (IC_{50} = 47 nM), other members, such as CDK1, CDK4 and CDK5, were also potent targets of AT7519 (190 nM, 67 nM, and 18 nM, respectively). At the same time, AT7519 was less effective against non-CDK kinases, such as Aurora A, IR kinase, MEK, PDK1, and c-abl (IC_{50} values > 10μM), perhaps suggesting that the aminopyrazole core is ideal to optimize to develop CDK inhibitors [39]. Initial anti-proliferative effects of AT7519 were analyzed with the CRC cell line, HCT116. A cell-based IC_{50} value of 82 nM was observed. Having confirmed clinically relevant growth inhibition in HCT116 cells, a panel of approximately 100 cell lines was subjected to treatment with AT7519. Analyses of the growth inhibitory effects showed approximately 75% of cell lines had IC_{50} values in the nanomolar range [39]. Follow-up *in vivo* studies using an A2780 ovarian cancer xenograft model examined the efficacy of AT7519 in BALB/c mice. A dosage of 7.5 mg/kg, administered intraperitoneally, resulted in approximately 86% reduction in tumor growth compared the

vehicle control [39]. Collectively, this study demonstrated the promise of aminopyrazole analogs as potential anti-cancer agents.

Subsequent studies were carried out to further characterize the mechanism of action elicited by AT7519. Phosphorylation levels were examined to evaluate kinase inhibition in HCT116 cells. Treatment with AT7519 at low concentrations (0.05 μM, 0.25 μM and 1 μM) resulted in a reduction of pPP1a (Thr320) and pRB(Thr821) levels suggesting inhibition of CDK1 and CDK2, respectively. Additional evaluation of the effects of AT7519 found a decrease in RNA pol II phosphorylation, indicating inhibition of CDK9 [40]. Interestingly, while AT7519 was shown to be the most potent against CDK5 in cell-free systems, the authors did not evaluate AT7519 on CDK5 in cells. As AT7519 was identified as a CDK inhibitor, it was also evaluated for its ability to arrest the cell cycle. Treatment with AT7519 resulted in accumulation of cells in both the G0-G1 and G2-M phases of the cell cycle. To determine if cell growth inhibition was a result of increased cell death, AT7519 was investigated for its effect on apoptosis. Colony formation and TUNEL staining assays showed induction of apoptosis only after 24-hour exposure to AT7519 [40]. Next, a colorectal cancer xenograft model was used to assess efficacy of AT7519 in a different model system. Once again, AT7519 proved to be a promising anti-cancer agent *in vivo*, as evident by reduction in both early (100 mm^3) and late stage (400 mm^3) tumor growth at 4.6 mg/kg and 9.1 mg/kg doses, respectively [40].

The potent and successful anti-tumor activity of AT7519 ultimately led to evaluation of this aminopyrazole compound in clinical trials. A phase I pharmacokinetic (PK) and pharmacodynamic (PD) dose-escalation study was carried out for patients with refractory solid tumors to determine whether AT7519 would be a safe and effective cancer treatment [38]. The 28-patient study included individuals with colorectal, non-small cell lung, pancreatic, breast, gastroesophageal, hepatocellular, and skin cancers. Dose-escalation

revealed 14.4 mg/m^2/day and 28.8 mg/m^2/day were, for the most part, well tolerated [38]. Unfortunately, two patients presented with severe adverse effects, including hypertension and cardiac events, when treated with 40 mg/m^2/day of AT7519. This resulted in termination of the study prior to determining the maximum tolerated dose. Other patients experienced less severe side effects, including nausea, fatigue, vomiting and anorexia, constipation, peripheral edema, and hypotension. The symptoms are consistent with those seen with other anti-cancer agents. CDK inhibition was observed at doses exceeding 1.8 mg/m^2/day, however the anti-proliferative and apoptotic markers were only altered at 28.8 mg/m^2/day, thus suggesting that 28.8 mg/m^2/day was the minimum threshold for a biologically effective dose [38]. Consistent monitoring of tumor diameters revealed various response rates with the most promising being a partial response. Of the five patients that completed at least eight cycles of AT7519, the average increase in survival was ~ 6.6 months (ranging from 2-13 months) [38]. Overall, AT7519 presented an encouraging clinical profile and warranted continued evaluation. Furthermore, it paved the way for exploration into similarly structured compounds as potential cancer therapies.

CP-668863

The success of AT7519 in pre-clinical and clinical studies, as anti-cancer therapy, has made it a benchmark for comparison of new therapies. We set out to evaluate another aminopyrazole analog as a potential CRC therapy. In 2007, Pfizer first reported the development of an aminopyrazole analog (CP-668863) for the selective inhibition of CDK5 in neurodegenerative disorders [41]. Initial studies performed by Pfizer determined CP-668863 has a Ki value of 2.9 nM against CDK5, making it more selective than CDK2, and a non-CDK family member, glycogen-synthase kinase-3beta (GSK-3β) (approximately 5.7-fold and 103-fold, respectively). CP-66883 had a cell-based IC_{50} value of 470 nM for Chinese hamster ovary (CHO) cells. Co-crystallization studied performed with CDK2 and

CP-668863 revealed the aminopyrazole analog was indeed an ATP-competitive inhibitor, as it sits within the ATP-binding pocket [41]. While Pfizer found that the *in vitro* and cellular responses to CP-668863 were encouraging, they reported lack of *in vivo* efficacy. The compound showed no signs of CDK5 inhibition in their murine central nervous system (CNS) model. Specifically, the treatment with CP-668863 did not reduce the phosphorylation of the CDK5 target, tau, in the brains of mice. As a consequence, Pfizer discontinued development of the compound [41]. Although CP-668863 did not live up to Pfizer's expectations as a drug for neurodegenerative disorders, we wondered if it would show efficacy against CDK5 in other cellular contexts or diseased states. With this in mind, we asked whether CP-668863 may have promise as an anti-cancer compound, as CDK5 has recently been recognized as an emerging oncogenic target [42].

Cyclin-dependent Kinase 5

Cyclin-dependent kinase 5 (CDK5) is often considered the peculiar member of the CDK family [43, 44] for a variety of reasons. First, while CDK5 is ubiquitously expressed, it is best known for its role in the CNS where it is essential for neuronal development, neuronal migration, synaptogenesis, pain signaling, and other additional functions [43]. Second, CDK5 was originally thought to have no obvious role in cell cycle regulation; however, it's contributions to the cell cycle have been recently discovered and are discussed later on in this dissertation. Third, CDK5 requires the binding of a regulatory subunit to assume its active conformation, similar to other members of the family, but the regulatory subunit is not a cyclin. Instead, CDK5 requires the binding of p35 or p39 [43, 45-47]. Activators p35 and p39 are anchored to the cell membrane by a myristoylation sequence and thus are typically localized in the cytoplasm of the cell [48]. Nevertheless, upon calcium-mediated activation of calpain, p35 and p39 are cleaved into their respective

counterparts, p25 and p29. These truncated versions of CDK5 activators are then free to move about the cell and stimulate CDK5 activity in other cellular locations [49-52]. These activators (p35, p39, p25, and p29) are thought to be the limiting factors of CDK5 activity, as CDK5 remains inactive in its monomeric form. Fourth, even though almost all members of the CDK family require phosphorylation of the T-loop to make the activator binding site accessible, CDK5 again is the odd man out and does not follow this general rule [43].

Despite these unique characteristics, CDK5 still remains a recognized member of the CDK family for multiple reasons. First, CDK5 shares high sequence homology and key structural features with many members of the CDK family [43, 45, 46]. Second, while p35 and p39 do not share sequence homology with their cyclin equivalents, they do adopt a similar tertiary protein structure, which may suggest that similar protein-protein interactions occur between CDKs and their activating subunits [43]. Finally, like other members of the CDK family, CDK5 recognizes the same substrate consensus sequence (S/T-P-X-K/R) in neuronal and non-neuronal cell types [43].

CDK5 in the Central Nervous System

CDK5 was originally discovered in 1992 within the CNS due to it's ability to phosphorylate a serine motif similar to CDKs and also due to it's high homology with members of the family [53]. In 1993, Kobayashi *et al.* identified it as a 30 kDa protein and named it CDK5 [54]. The early investigations of CDK5 focused predominately on roles within the CNS. Nevertheless, continued efforts to understand all the roles of CDK5 have uncovered multiple non-neuronal functions. Through downstream substrate phosphorylation, CDK5 has been linked to angiogenesis [55], apoptosis [56-58], proliferation [59-62], migration [63-67], and gene expression [59, 68, 69]. Interestingly, these processes have all been linked to tumorigenesis and may serve as potential routes for CDK5 to play the role of an oncogene [42, 43].

CDK5 in Cancer

To date, CDK5 has been implicated in a variety of cancers, including those of the pancreas [70, 71], thyroid [72, 73], prostate [59, 67], breast [64, 74], lung [75], liver [76], skin [77], ovary [78], brain [79, 80], and most recently as a tumor promoter in CRC [81]. Data accumulated at the messenger RNA and proteomic levels point to CDK5 as an important oncogenic target [42]. Furthermore, CDK5 expression may be indicative of disease severity. CDK5 levels positively correlate with disease progression in cancers of the brain [79, 80], breast [64, 74], and lung [75]. Consequently, CDK5 expression may serve as a promising biomarker of patient survival.

While CDK5 was largely ignored in the cancer field until recently, many studies have since focused on elucidating the molecular contributions of CDK5 to carcinogenesis. CDK5 activity is tightly regulated, thus keeping its downstream signaling in check. However, in the case of cancer, increased expression or aberrant CDK5 activity drives tumor development through the hyper-phosphorylation of downstream substrates. Substrates often serve as the bridge connecting dysregulated CDK5 activity to its oncogenic function, as these functions are guided by specific substrates downstream of CDK5 signaling. These substrates contribute to a variety of cellular functions depending on their phosphorylation status, which is managed by CDK5. While this CDK5 signaling is important during normal physiological conditions, CDK5 signaling is often overactive which causes hyperphosphorylation of substrates and thus drives disease states.

CDK5 was originally classified as the non-traditional member of the CDK family predominately due to the fact that it was not thought to be involved in cell cycle regulation (**Figure 1**). Contrary to earlier speculations, CDK5 has been shown to regulate the cell cycle in neuronal [82, 83] and non-neuronal cells [84-86]. In the last decade, our knowledge of CDK5 regulation of the cancer cell cycle has evolved, yet little effort has

been made to fully characterize CDK5's cell cycle role. CDK5 was first shown to phosphorylate and inactivate Cdh1, a co-factor of the anaphase-promoting complex (APC). The APC is an E3 ubiquitin ligase that tags cell cycle proteins for degradation [84]. CDK5 phosphorylation of Chd1 inactivates APC, therefore allowing premature S-phase entry of proliferating cells. Additionally, CDK5 phosphorylation of Cdh1 also resulted in decreased expression of p27, an important intrinsic CDK4 inhibitor [85]. Loss of p27 activates CDK4-CyclinD1 phosphorylation of, which also pushes the neuronal cells into S-phase [85]. Huang *et al.* also identified a role for CDK5 in cancer cell cycle regulation by finding a direct link between CDK5 and $p21^{CIP1}$ [86]. $p21^{CIP1}$ is another important intrinsic CDK inhibitor that binds to CDK2 and blocks the progression through the cell cycle at inappropriate times [87]. The loss of $p21^{CIP1}$ expression and activity has been linked to cancer [88]. CDK5 phosphorylates $p21^{CIP1}$ at Ser130 which triggers its proteasomal degradation, thus removing a critical cell cycle regulator from the tumor environment. CDK5-driven loss of $p21^{CIP1}$ results in increased CDK2 activity, cell cycle progression, and tumor growth [86]. Finally, through phosphorylation of focal adhesion kinase (FAK), CDK5 was shown to be important for mitosis. FAK is localized at the microtubules, and its phosphorylation at Ser732 results in mitotic spindle assembly, thus promoting chromosomal segregation [89].

CDK5 also appears to regulate the growth and proliferation of cancer cells through the phosphorylation of transcription factors or their negative regulators (**Figure 1**). CDK5 is responsible for the phosphorylation of signal transducer and activator of transcription 3 (STAT3) at Ser727, which initiates the signaling cascade required for transcription of critical signaling molecules such as cyclin D1 and c-Fos [69]. STAT3 phosphorylation by CDK5 has been shown to contribute to the proliferation of prostate and thyroid cancers [90, 91]. Additionally, CDK5 stabilizes the androgen receptor (AR) through phosphorylation at Ser81, which results in the proliferation of prostate cancer *in vitro* and

in vivo [90]. Studies have also shown CDK5 directly phosphorylates the retinoblastoma (Rb) protein at multiple residues (Ser780, Ser795, and Ser807/811) [61, 62, 72]. Hyperphosphorylation of Rb results in the release of transcription factor E2F which can then freely bind to the promoter regions and drive transcription of genes necessary to transition from the G0/G1 to S phase of the cell cycle [92]. Specifically, in medullary thyroid cancer (MTC), CDK5 phosphorylation of Rb at Ser807/811 contributes to the release of E2F thus allowing for the transcription of cell cycle proteins (CDK2, $p15^{INK4b}$, $p21^{CIP/}$WAF1) necessary for MTC proliferation [72].

Another way in which CDK5 is likely contributing to cancer is through its control of cancer cell motility (**Figure 1**). First, transforming growth factor beta (TGFβ) stimulation increases the expression of p35 and CDK5 which drives epithelial-to-mesenchymal transition (EMT) [64]. Second, CDK5 is responsible for the phosphorylation of multiple cytoskeletal proteins such as FAK [63], Talin [65], PIKE-A [93], and PAK1 [94]. CDK5 phosphorylation of FAK at Ser732 plays an important role in regulating migration of cells. Mutation of Ser732 to the nonphosphorylatable Ala732 resulted in a loss of cell migration, signifying this residue is crucial for cell migration [63]. Additionally, Ser425 of Talin is a direct substrate of CDK5 [65] and has been implicated in cancer cell migration through association with β1-integrins [95]. Prostate cancer migration and metastasis were reduced in the presence of an S425A mutation, thus demonstrating the importance of Ser425 phosphorylation [95]. In addition to these two examples, CDK5 has been shown to regulate the migration of lung [66], prostate [67], melanoma [77], and breast cancers [64]. Disruption of CDK5 activity, either through the use of chemical inhibitors or genetic modifications, resulted in decreased cell migration both *in vitro* and *in vivo,* thus confirming a direct link between CDK5 and cancer cell motility [64, 66, 71].

Finally, CDK5 has recently been shown to play a role in the deoxyribonucleic acid (DNA)-damage response (DDR) and DNA repair (**Figure 1**). These two cellular processes are vital to maintain genomic integrity and repair DNA damage [96, 97]. Failure of these critical repair mechanisms has been shown to contribute to carcinogenesis [98, 99]. In cancer, many therapeutic options, such as ionizing radiation (IR) or chemotherapy, are aimed at introducing DNA damage to elicit a cytotoxic response [100, 101]. Of late, multiple studies have speculated that CDK5 may serve as a potential resistance mechanism to DNA damaging therapies through phosphorylation of ataxia-telangiectasia mutated kinase (ATM), replication protein A-32(RPA-32) or STAT3 [74, 102, 103]. Increased expression of p35, p25, and CDK5 have been observed following ionizing radiation in glioblastoma and neuroblastoma cells [79, 104, 105]. Moreover, there was a marked increase in double strand breaks (DSB) upon blockade of CDK5 activity, which suggests that CDK5 contributes to DNA damage repair mechanisms and thus may be aiding in cancer cell survival [76, 102, 103]. Furthermore, treatment with several DNA-damaging agents such as Poly (ADP-ribose) polymerase (PARP) and topoisomerase inhibitors resulted in an increase in CDK5 expression and activity [74, 76, 106, 107]. Lastly, multiple reports suggest that treatment with a CDK(5?) inhibitor, roscovitine, sensitized cancer cells to DNA-damaging chemotherapeutics both *in vitro* and *in vivo* [76, 108]. While the exact contributions of CDK5 to the DDR and DNA repair mechanisms are not yet fully understood, researchers have identified CDK5 as an emerging player in DNA repair and drug resistance, thus further validating CDK5 as a critical oncogene.

Collectively, the studies outlined above describe some of the functional roles that CDK5 plays in cancer development and progression. Furthermore, they support the notion that inhibition of CDK5 could be a promising therapeutic avenue to explore. Of note, it is critical that we understand CDK5's contributions to specific tumor types, prior to utilizing anti-CDK5 therapies.

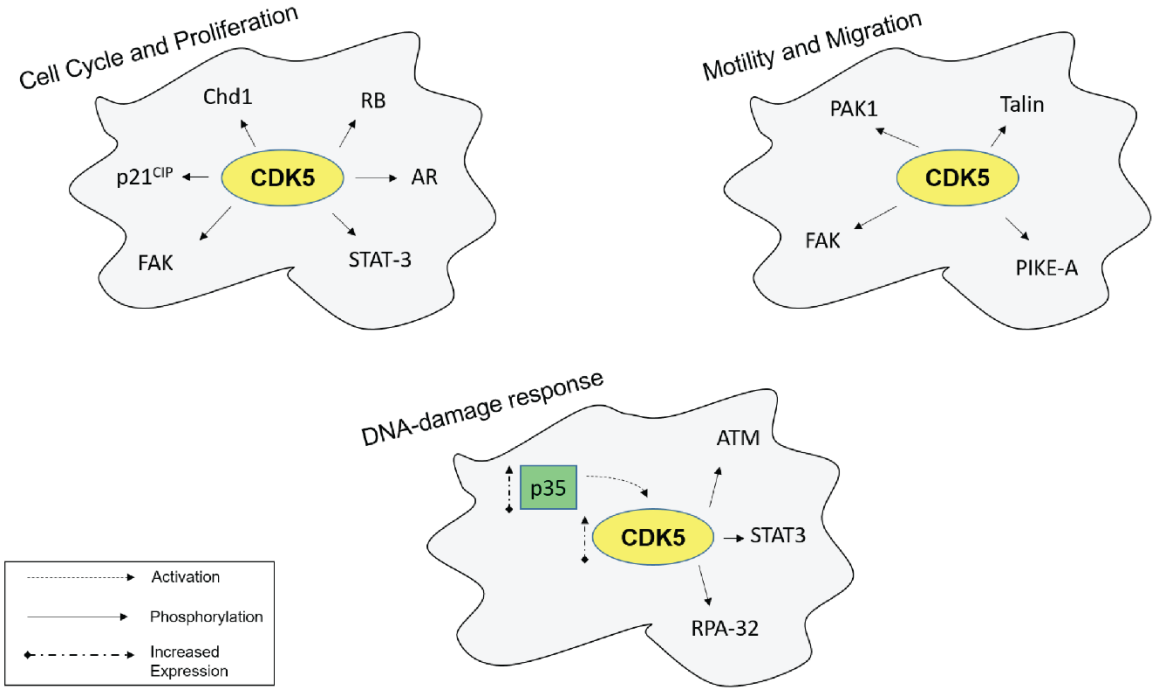


Figure 1: CDK5 in Cancer. CDK5 regulates cancer cell cycle and proliferation, migration and motility, and the DNA-damage response through phosphorylation of downstream substrates. * Modified from Pozo and Bibb (2016) Trends Cancer.

CDK5 in Colorectal Cancer

As mentioned previously, CDK5 was recently identified as an oncogene in CRC, thus CRC may serve as a good model system to test inhibition of CDK5 as a therapeutic strategy. Zhuang *et al.* [81] began their study by examining CDK5 expression in CRC. Profiling a cohort of cell lines revealed increased expression of CDK5 and p35 in seven CRC cell lines. To relate expression to activity, the phosphorylation levels of FAK and PAK1 at CDK5-specific residues were also examined. Phosphorylation of FAK(Ser732) and PAK1(Thr212) was observed, which is indicative of functional CDK5 activity. Notably, the expression and activity of CDK5 were higher in more aggressive CRC cell lines, such as HCT116 and SW480. CDK5 and p35 expression was also analyzed in patient samples by western blot and immunohistochemistry (IHC). Data from ten normal and corresponding tumor samples showed upregulation of CDK5 and p35 in both tumor lysates and tumor tissue sections compared to corresponding normal tissue. CDK5 expression also positively correlated with disease progression, as evident by the increase in CDK5 expression in later stage CRC tissue samples. Further analysis of clinical significance found CDK5 expression correlated with tumor differentiation, tumor size and metastasis. Of importance, CRC patients who overexpressed CDK5 had shorter overall survival compared to individuals with low CDK5 expression (44 vs. 54 months respectively)[81].

The data described above suggest CDK5 as a critical tumor promoter in CRC. Follow-up studies, using genetic silencing and overexpression models, have characterized the biologic function of CDK5 in CRC. Knockdown of CDK5 reduced proliferation, colony formation, migration, and invasion of CRC cells. Treatment with roscovitine mimicked the effects of the CDK5 knockdown. Conversely, overexpression of CDK5 increased these processes. Evaluation of these genetic silencing and overexpression systems *in vivo* showed CDK5 is important for CRC tumorigenesis and

metastasis. CRC cells that expressed CDK5 promoted CRC carcinogenesis while those lacking CDK5 expression and activity had the opposite effect [81]. Together, these data clearly highlight the oncogenic role of CDK5 in CRC.

Summary

In the United States, the number of annual CRC diagnoses and deaths remain high. As such, there is a continued need for the development of new therapeutic options for the treatment of CRC. The studies presented above demonstrate the particular role of CDK5 in the pathogenesis of cancer. Zhuang *et al.* and many others provide evidence that CDK5 expression and activity is an important component of tumorigenesis and cancer progression. Although extensive knowledge of the contributions of CDK5 to cancer has steadily increased in recent years, the identification and application of CDK5 inhibitors has not followed suit. With the latest discovery of CDK5 as a tumor promoter in CRC, we hypothesize that CDK5 is an ideal therapeutic target for CRC and inhibition of CDK5 may therefore be an effective strategy to combat CRC.

CHAPTER 2: MATERIALS AND METHODS

Chemical Inhibitors

20-223

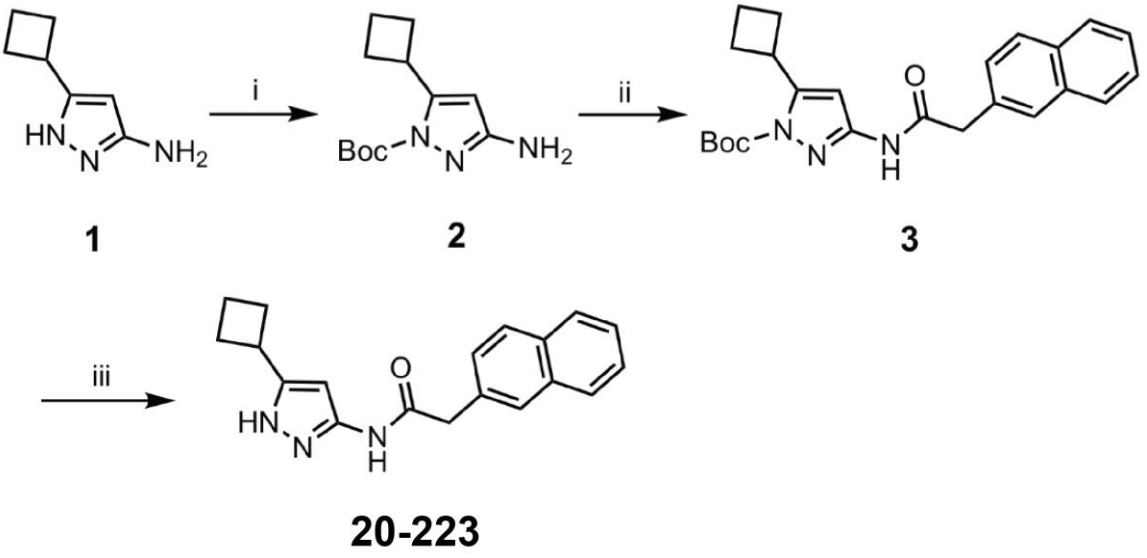
CP-668863 was originally designed, synthesized, and reported by Pfizer Pharmaceutical Company [41]. For this project, CP-668863 was synthesized in house by Drs. Sandeep Rana and Yogesh Sonawane using a modified scheme (**Scheme 1**) and will be identified as 20-223 from this point forward. AT7519 (SelleckChem #S1524) and roscovitine (Apex BioTech #A1723) were used in head-to-head comparison studies with 20-223. All three inhibitors were dissolved in 100% dimethyl sulfoxide (DMSO) (FisherBioreagents CAS # 67-68-5) to a final stock concentration of 10 mM. Chemical inhibitors were stored at -20 °C and gradually brought to room temperature before being used.

PROTAC

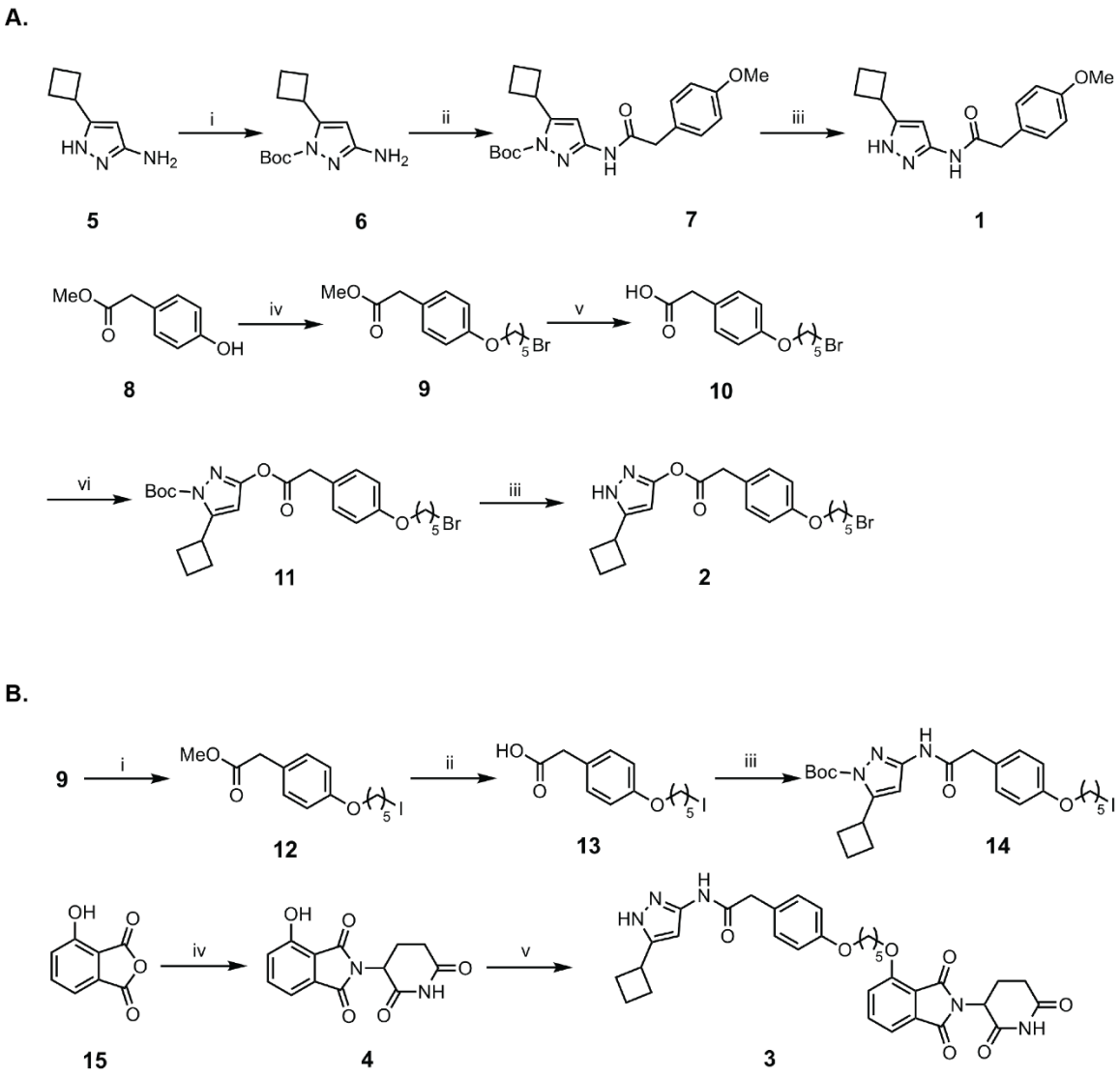
All compounds were designed and synthesized in house by Sandeep Rana, Ph.D. All inhibitors were dissolved in 100% DMSO to a final stock concentration of 10 mM. Inhibitors were stored at -20 °C but used at ambient temperatures for drug studies. The synthetic route to compound **1** and **2** are summarized in **Scheme 2A**. Regioselective *t*-butoxycarbamate (Boc) protection of the ring nitrogen atom in the aminopyrazole **5** was accomplished following a reported procedure, to yield **6**. Coupling of **6** with commercially available 4-methoxyphenylacetic acid using propylphosphonic anhydride solution (T3P, 50% in DMF) and *N*,*N*-Diisopropylethylamine gave **7**. Removal of Boc protecting group under acidic condition resulted in **1**. Alkylation of hydroxyl group in **8** with 1,5-dibromopentane yielded **9**. Compound **10** was obtained by hydrolysis under basic

conditions, which was then subjected to amide coupling with **6** using T3P to yield **11**. Removal of the Boc group under acid condition yielded **2**.

The PROTAC **3** was synthesized following the route described in **Scheme 2B**. A Finkelstien reaction with compound **9** resulted in **12** and a base-catalyzed hydrolysis yielded fragment **13**. Coupling of aminopyrazole **6** and **13** using T3P yielded **14**. Condensation of 3-hydroxyphthalic anhydride (**15**) with 3-aminopiperidine-2,6-dione hydrochloride afforded the intermediate **4**. Alkylation of the hydroxyl group on **4** with **14** an intermediate**,** which was subjected to Boc group deprotection under acidic condition to yield degrader **3**.



Scheme 1: (i) $(BOC)_2O$, KOH, DCM:H_2O (1:1), RT, (88%); (ii) 2-(naphthalen-2-yl)acetyl chloride, DIPEA, DCM, 0 °C, (78%); (iii) CF_3CO_2H, RT, DCM, (92%).



Scheme 2: A) (i). $(Boc)_2O$, KOH, DCM: water, 3 h. (ii). 4-methoxyphenylacetic acid, 50% T3P in DMF, DIEA, 50 °C, 3 h. (iii). TFA, DCM, 0 °C, 3 h. (iv). 1,5-dibromopentane, K_2CO_3, acetone reflux, 72 h. (v). LiOH, ethanol: water, 16 h. (vi). **6**, 50% T3P in DMF, DIEA, 50 °C, 3 h. B) (i). NaI, acetone, reflux, 16 h. (ii). LiOH, ethanol: water, 16 h. (iii). **6**, 50% T3P in DMF, DIEA, 50 °C, 3 h. (iv). 3-aminopiperidine-2,6-dione hydrochloride, KOAc, Acetic acid, reflux, 24 h. (v). (a) $NaHCO_3$, DMF, 70 °C, 6 h; (b). TFA, DCM, 0 °C, 3 h.

CRC Cell Lines and Culture Conditions

Cancer cell lines used in this dissertation are CRC cell lines. FET, CBS, and GEO cells were cultured in serum-free medium (McCoy's 5A medium with sodium bicarbonate, L-serine, asparagine, sodium pyruvate, MEM vitamins, growth factors (4 µg/mL transferrin, 20 µg/mL insulin, and 10 ng/mL EGF), and 1x Penicillin-Streptomycin. SW620, DLD1, and HT29 cells were cultured in DMEM high glucose medium (HyClone #SH30022.01) supplemented with 10% fetal bovine serum (FBS) (Gibco by Life Technologies #26140-079) and 1x Penicillin-Streptomycin (HyClone #SV30010). HCT116 cells were cultured in RPMI-1640 Medium (HyClone #SH30027.01) supplemented with 10% FBS and 1x Penicillin-Streptomycin (HyClone #SV30010). All CRC cell lines were cultured in 5% CO_2 at 37 °C.

Short Tandom Repeat (STR) Profiling

$1x10^6$ cells were collected during routine passage. Cells were washed 1x with 1xPBS and centrifuged at 2,500 rpm for five minutes to generate a cell pellet. Pellets were stored on ice and delivered to the University of Nebraska Medical Center Human DNA Identification Laboratory where they performed STR profiling (**Figure 2**). The DNA report generated by the Forensic Lab contained the information for 13 genetic loci, which were then cross checked with ATCC to confirm validation. Cell lines exceeding an 80% match with the online ATCC database (www.atcc.org) were considered valid [109, 110].

Genetic Locus	HCT116	HT29	DLD1	SW620	FET*	CBS*	GEO*
D7S820	10,11,12	10	10,12	8,9	8,11	8,12	10,11,11.1
CSF1PO	7,10	11,12	11,12	13,14	12	12	13
THO1	8,9	6,9	7,9.3	8	7	8	8
D13S317	10,11,12,13	11,12	8,11	12	11,12	11,12	8,12
D16S539	11,12,13,14	11,12	12,13	9,13	12	9,11	12
vWA	17,18,21,22,23	17,19	18,19	16	16,17	16,17	15
TPOX	8	8,9	8,11	11	8	10,11	8
Amelogenin	X	X	XY	X	X	X	X
D5S818	10,11	11,12	13	13	13,14	12	12
% Match to ATCC database	89%	100%	88%	100%			

*first time STR profile characterization in previously used cell lines

Figure 2: STR profiling for cell line validation.

Human Colon Epithelial Cell Line

Immortalized non-transformed human colonic epithelial cell lines (HCEC) were kindly provided by Dr. Rob Lewis' lab in which he had received them as a gift from Dr. J. Shay (University of Texas Southwestern) [111]. HCECs were grown in medium composed of 4 parts DMEM to 1 part media 199 (Sigma-Aldrich) with 2% Cosmic Calf Serum (GE Healthcare), 25 ng/mL EGF, 1 µg/mL hydrocortisone, 10 µg/mL insulin, 2 µg/mL transferrin, 5 nM sodium selenite, and 50 µg/mL gentamycin sulfate. HCECs were grown in a hypoxia chamber with 2% O_2 and 5% CO_2 at 37 °C.

Xenograft Studies

All animal studies were performed following approval of the Institutional Animal Care and Use Committee (# 07-047-08FC) and in accordance with facility guidelines. This xenograft model has been used previously in our lab [112]. Briefly**,** GEO-GFP cells ($7x10^6$) were subcutaneously injected into the flank of athymic nude mice [112]. Xenograft tumors were allowed to grow until reaching a tumor volume of approximately 100-200 mm^3, at which point they were separated into two treatment groups: I) DMSO vehicle control or II) 8 mg/kg 20-223. Each group contained 7 animals (n = 7). Drug or vehicle treatments were given by subcutaneous injections daily for the first week and every other day for two more weeks for a total of 14 injections. (**Figure 3**) Throughout the study, animal weight and tumor volume were recorded regularly. Tumor volume was measured using calipers and calculated using the $l^2 \times h \times \pi/6$ equation. The study was concluded when control tumors reached maximum size according to facility guidelines (approximately 2000 mm^3). Mice were euthanized and then full body and excised tumor images were taken using Near-IR enhanced Macro Imaging System Plus Cooled with the LT-99D2 with the Dual Tool

excitation upgrade. Tumor samples were flash frozen on dry ice and preserved in liquid nitrogen prior to western blot analyses (see below for western blot protocol).

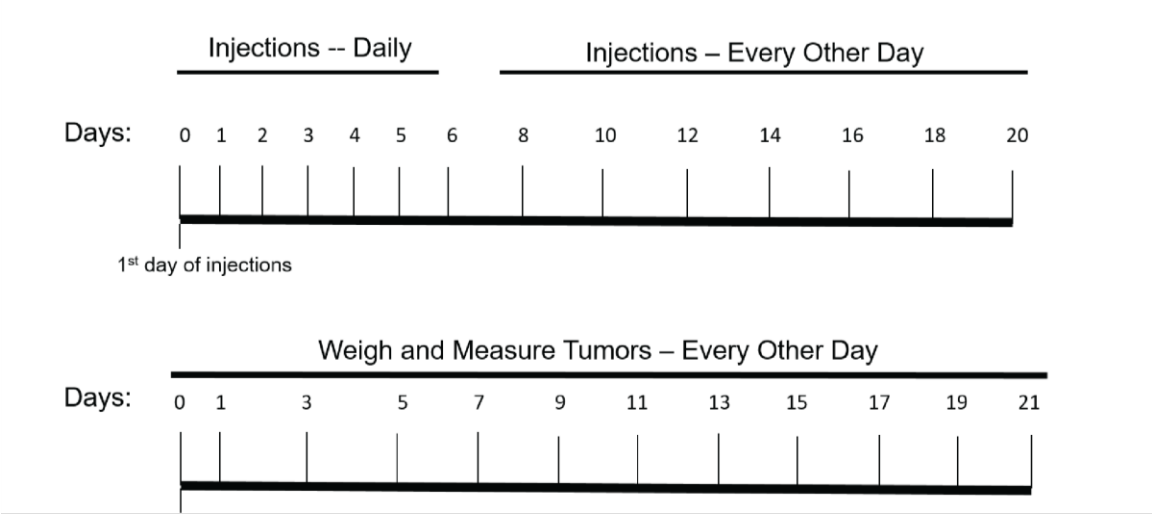


Figure 3: 20-223 treatment schedule

The Cancer Genome Atlas (TGCA) Analyses

TCGA provisional data was retrieved from cBioPortal on January 19, 2017. CRC sample type and mRNA expression (RNA-seq) were downloaded from UCSC Xena (https://genome-cancer.soe.ucsc.edu/proj/site/xena/heatmap/). All provisional cancer datasets were analyzed for CDK5 mutation. The genomic profile of CDK5 was further analyzed in the CRC (Colorectal Adenocarcinoma - TCGA Provisional) dataset for putative somatic copy-number alterations from GISTIC, using Onco Query Language (OQL), and mRNA expression (RNA-seq). GISTIC predicts copy number alterations according to sample specific thresholds generated by comparing chromosomal segments with median chromosomal arm copy numbers. All parameters were set at default.

Cell-Free System Analyses

Samples were sent to Reaction Biology Corporation for cell-free analysis. Kinase profiling of 20-223 was carried out in duplicates with a panel of CDKs (CDK1/Cyclin B, CDK2/cyclin E, CDK4/cyclin D1, CDK5/p35, CDK6/cyclin D1, CDK7/cyclin H, and CDK9/cyclin T1) at a single dose (0.1 μM or 0.01 μM) in the presence of 30 μM ATP. The enzymatic activity was determined by measuring of ATP hydrolysis. Dose-response studies containing 10 concentrations (beginning at 5 μM at 3-fold dilutions) of 20-223 or AT7519 were used. IC_{50} values were generated through fitting the dose response curves.

Western Blot Analyses

Cells were lysed using a buffer containing 50 mM Tris, 100 mM NaCl, 1% NP-40, 2 mM EDTA, 20% SDS combined with 20xPPI (Na_3VO_4, NAF, β-glycerophosphate), and 1 mmol/L PMSF. Samples were kept on ice and vortexed prior to centrifugation at 14,000 rpm for 10 minutes in the 4 °C cold room. Supernatant was collected and protein was

quantified using BCA Protein Assay (Pierce # 23225). 40 µg of protein were run on 4-15% gradient gels (BioRad) in 1x TRIS-Glycine SDS (sodium dodecyl sulfate) (Research Products International Corporation #T32080) at 120 V for approximately 90 minutes and separated by SDS-PAGE electrophoresis. Proteins were transferred to a PVDF membrane using a Semi-dry transfer method (ThermoScienctific, #35035) at 18 V for 35 minutes. The membrane was blocked in 5% milk in 1X Tris Buffered Saline with 0.1% Tween (1xTBST) for 1 hour at room temperature while gently rocking. Primary antibodies (**Figure 4**) were incubated in 5% milk in 1x TBST and rocked overnight at 4°C. Complimentary HRP(horseradish peroxidase)-conjugated secondary antibodies were incubated in 5% milk in 1xTBST and rocked for 1 hour at room temperature. Protein expression was detected using ECL Prime (GE Healthcare #RPN2236) and developed on film (ThermoScientific #34090) using the KODAK X-OMAT 2000 Processor system. Developed films were scanned, and the images were saved as jpg images and processed using the 2017 Adobe Creative Cloud software (Photoshop and Illustrator). Kinase activity was measured by changes in substrate phosphorylation. Quantification of phosphorylation levels representative of the western blots shown were generated using ImageJ. Western blots were performed in triplicate (n = 3).

Antibody	Company	Catalog #	Lot #	Dilution of 1* Antibody	Source
α-Tubulin	Cell Signaling	13499	10	1 : 10,000	Mouse
CDK2	Cell Signaling	2546	6	1 : 2,000	Rabbit
CDK5	Cell Signaling	2506	2	1 : 2,000	Rabbit
CDK9	Cell Signaling	2316	5	1 : 2,000	Rabbit
pRB (S807/811)	Cell Signaling	9308	12	1 : 2,000	Rabbit
pFAK (S732)	Abcam	ab4792	GR2406125	1 : 2,000	Rabbit
pRPB1 (S2)	Cell Signaling	13499	1	1 : 5,000	Rabbit
total RB	Cell Signaling	9309	9	1 : 2,000	Mouse
total FAK	Cell Signaling	3285	9	1 : 2,000	Rabbit
total RPB1	Cell Signaling	2629	3	1 : 5,000	Mouse
total PARP	Cell Signaling	9542	13	1 : 10,000	Rabbit
cleaved PARP	Cell Signaling	9541	15	1 : 10,000	Rabbit
GAPDH	SantaCruz	sc-25778	C2415	1 : 4,000	Rabbit
BCL-XL	Cell Signaling	2762	7	1 : 2,000	Rabbit
MCL-1	Cell Signaling	5453	4	1 : 2,000	Rabbit

Figure 4: Antibody validation.

Wound Healing Migration.

HCT116 cells were plated at $1.25x10^6$ cells in 2 mL medium in a 6-well plate and allowed to adhere and grow overnight to reach 90% confluency. Cell cultures were scratched using a sterile 10 µL pipette tip down the middle of the well to create a “wound”. Scratched cells were washed gently with 1x PBS before being stimulated with a final concentration of 100 ng/mL of EGF (Invitrogen # PHG0311L) and immediately treated with either 1.5 µM 20-223 or DMSO control. Directly after the start of treatment, cells were taken to the live cell imaging facility where they were imaged every 15 minutes over a 36-hour time course (only the first 24 hours were considered for migration purposes to exclude any chance of proliferation). Migration assays were performed in triplicate (n = 3).

Cell Viability

CRC cells were plated at 4,000 cells/well in a 96-well plate. Cells were treated with 20-223, AT7519 or roscovitine at 4-fold dilutions starting at 10 µM (20-223 and AT7519) or 100 µM (roscovitine) and incubated at 37 °C for 72 hours. The ability of these compounds to inhibit cell growth was assessed using the dye PrestoBlue. Following a 15-minute incubation with PrestoBlue reagent (Invitrogen #A13262), fluorescence was measured at 560 nM excitation and 590 nM emission using SpectraMax M5*e*. Growth inhibition was calculated using 100-[100*(Sample – T0)/(T100-T0)] equation, where T0 is the control reading immediately following treatment and T100 is the control reading at the end of a 72-hour incubation. Each assay was performed in triplicate (n = 3).

DNA-Cell Cycle Analyses

CRC cells were plated at $1x10^6$ cells in a 10-cm plate and allowed to adhere overnight. Cells were starved for 24 hours prior to treatments at 2x the growth inhibition IC_{50} values with 20-223, AT7519, or DMSO and were incubated for 24 and 48 hours before cell cycle analyses. $1x10^6$ cells were collected and pelleted by centrifugation at 2,000 rpm for 1 minute at 4 °C. Supernatant was decanted and pellets were resuspended in 1 mL of 70% ethanol and incubated at 4 °C for 1 hour. Samples were centrifuged at 2,000 rpm for 1 minute at 4 °C and ethanol was removed. Pellets were washed 1x with 1 mL of 1xPBS then centrifuged. PBS was removed and samples were resuspended in 1 mL of Telford Reagent (115 µM EDTA, 27 µg/mL RNAseA, 50 µg/mL Propidium Iodide, 0.1% Triton X-100, made in 1xPBS) and incubated at 4 °C for 1 hr. Cells were analyzed for DNA content by flow cytometry in the UNMC Flow Cytometry Core Facility. % of cells in the G1, G2, and S phases were determined for each treatment. (n = 2).

Statistical Analyses

Graphs and figures were generated using SigmaPlot 11.0 and Graphpad Prism statistical software (GraphPad Software, Inc). Student's t-test was used to compare differences between means between two groups. One-way analyses of variance (ANOVA) with a post-test for linear tend was used to compare two or more groups. For all analyses, significance was inferred at $P < 0.05$ and P values were two-sided.

CHAPTER 3: CHARACTERIZATION OF 20-223

Introduction

CRC continues to be a major health concern in the United States, where it accounts for 8% of all newly diagnosed cancer cases and is responsible for 8.4% of cancer related deaths. In the year 2014, approximately 1.4 million individuals were living with CRC [113]. Despite extensive research and numerous attempts at developing promising therapies for CRC, few drugs have successfully improved patient outcome.

The oncogenic family of CDKs have been extensively studied and characterized for their roles in cancer. There are 20 members of the CDK family, all of which have been linked to cancer. CDKs are often categorized into two major groups, those that contribute to tumorigenesis through cell cycle control and those that regulate transcription [114, 115]. One peculiar member of the CDK family that does not regulate transcription, and only recently has been shown to contribute to cell cycle progression, is CDK5. Uniquely, CDK5 is not activated in typical CDK fashion *i.e.*, through binding of cyclins, but instead is activated by regulatory proteins p35 and p39 or their cleaved counterparts, p25 and p29 [47]. CDK5 is best known for its role in the central nervous system where it regulates development, axon elongation, synaptogenesis and neuronal migration. Recently, reports have identified CDK5 as a key player in non-neuronal functions, including apoptosis, senescence, angiogenesis, insulin secretion, wound healing, and adhesion/migration [43]. These functions associated with CDK5 are believed to contribute to its role in tumorigenesis. CDK5 is quickly gaining recognition for its role in a variety of cancers, including those of the pancreas [70, 71], thyroid [72, 73], prostate [59, 67], breast [64], lung [75], liver [76], and most recently as a tumor promoter in CRC [81].

CDKs have received considerable attention as potential targets for cancer therapy. The traditional approach to targeting CDKs, which still remains popular, is through the use of ATP-competitive inhibitors that bind within the catalytic sites of CDKs and outcompete the binding of ATP. The earliest CDK inhibitors were pan-CDK inhibitors that often targeted several members of the family. While they showed promise in targeting CDKs, they often required high doses, which resulted in off-target effects and significant toxicity in preclinical animal trials [26]. To address these issues, substantial efforts have been made to improve upon the potency and selectivity of CDK inhibitors. While CDK inhibitors are currently being used to treat a variety of malignancies, few are currently being tested for CRC [116].

ATP competitive inhibitors typically form hydrogen bonds with the residues in the hinge region of the kinase. Aminopyrazole is a privileged scaffold that forms a network of hydrogen bonds between 3 nitrogen atoms of the scaffold and the hinge region residues of the kinase [36, 37]. AT7519, a well-characterized pan-CDK inhibitor built on a 4-aminopyrazole core, has shown promise in pre-clinical and clinical studies [38-40].

Herein, we take a 3-aminopyrazole analog, previously reported by Pfizer (CP-668863 a.k.a. 20-223) [41], beyond its intended context and describe our findings in colorectal cancer model systems. Preliminary xenograft studies showed 20-223 reduced tumor growth and tumor weight *in vivo*, indicating that 20-223 is a suitable lead compound for CRC therapy. We subjected 20-223 and AT7519 to a series of cell-free and cell-based assays to understand the mechanistic basis of the observed 20-223 anti-tumor effects. Docking studies suggested both 20-223 and AT7519 are ATP competitive inhibitors. The 2 aminopyrazole analogs were compared head-to-head in cell-free kinase assays which demonstrated 20-223 was more potent than AT7519. Contrary to a previous report, we found 20-223 was equipotent against CDK2 and CDK5 and was selective against other

members of the CDK family. Examination of downstream substrate phosphorylation showed 20-223 inhibited the kinase activity of CDK2 and CDK5. Migration studies utilizing a wound-healing assay showed that 20-223 decreased CRC cell migration. 20-223 was a nanomolar inhibitor of cell growth in a panel of CRC cell lines and was more potent than AT7519. Finally, 20-223 phenocopied cell cycle effects associated with AT7519. Together, our studies suggest 20-223 is a CDK 2/5 inhibitor, an effective anti-CRC agent, and a suitable lead for pre-clinical development.

Results

TCGA analyses reveal CDK5 is upregulated in primary colorectal tumors as a result of increased copy number

With increasing evidence suggesting a role for CDK5 in a variety of malignancies, we turned to The Cancer Genome Atlas (TCGA – http://cancergenome.nih.gov/) database to gain insight into CDK5 expression in patient populations. We found the colorectal cancer cohort in the TCGA online database consisted of 50 samples of normal mucosa and 347 primary colorectal tumor samples. The mRNA profiles of these samples were examined for CDK5 expression. As seen in **Figure 5A**, CDK5 mRNA levels were significantly higher in primary tumor compared to normal colon. Additional analyses that compared normal tissue with corresponding primary tumor revealed that of the 31 patients examined, all but 2 showed a significant increase in CDK5 levels in primary tumors when compared to normal colon tissue (**Figure 5B**). Next we examined the CDK5 copy number to determine whether increased CDK5 levels correspond to increased copy number. Of the 616 sequenced CRC samples, few exhibited homozygous deletion or heterozygous loss of CDK5 (0.3% and 1.9% respectively). Interestingly, 46.0% of individuals were

diploid for CDK5 while 51.9% of individuals had a copy number gain for CDK5 (**Figure 5C**). Additionally, we found that across all 4 groups, there is a significant linear trend. As copy number of CDK5 increases, there is a corresponding increase in mRNA expression (**Figure 5D**) thus suggesting that copy number is a contributing factor to the increased CDK5 mRNA expression that we observed in CRC. Next, to investigate whether CDK5 mutation could possibly be contributing to its activity in CRC, we examined the mutational frequency of CDK5 in all TCGA cancers. We found that CDK5 is rarely mutated across cancers and, more relevantly, is not mutated in CRC (**Figure 5E**). Collectively, these data suggest that CDK5 activity is a consequence of increased expression that results from an increase in copy number. Furthermore, it is the increase in CDK5 expression, not a mutation, which is likely responsible for its contributions to CRC. These data are consistent with a recent report implicating CDK5 as a tumor promoter in CRC, and thus warrant investigation into inhibition of CDK5 as a potential therapeutic option for CRC [81].

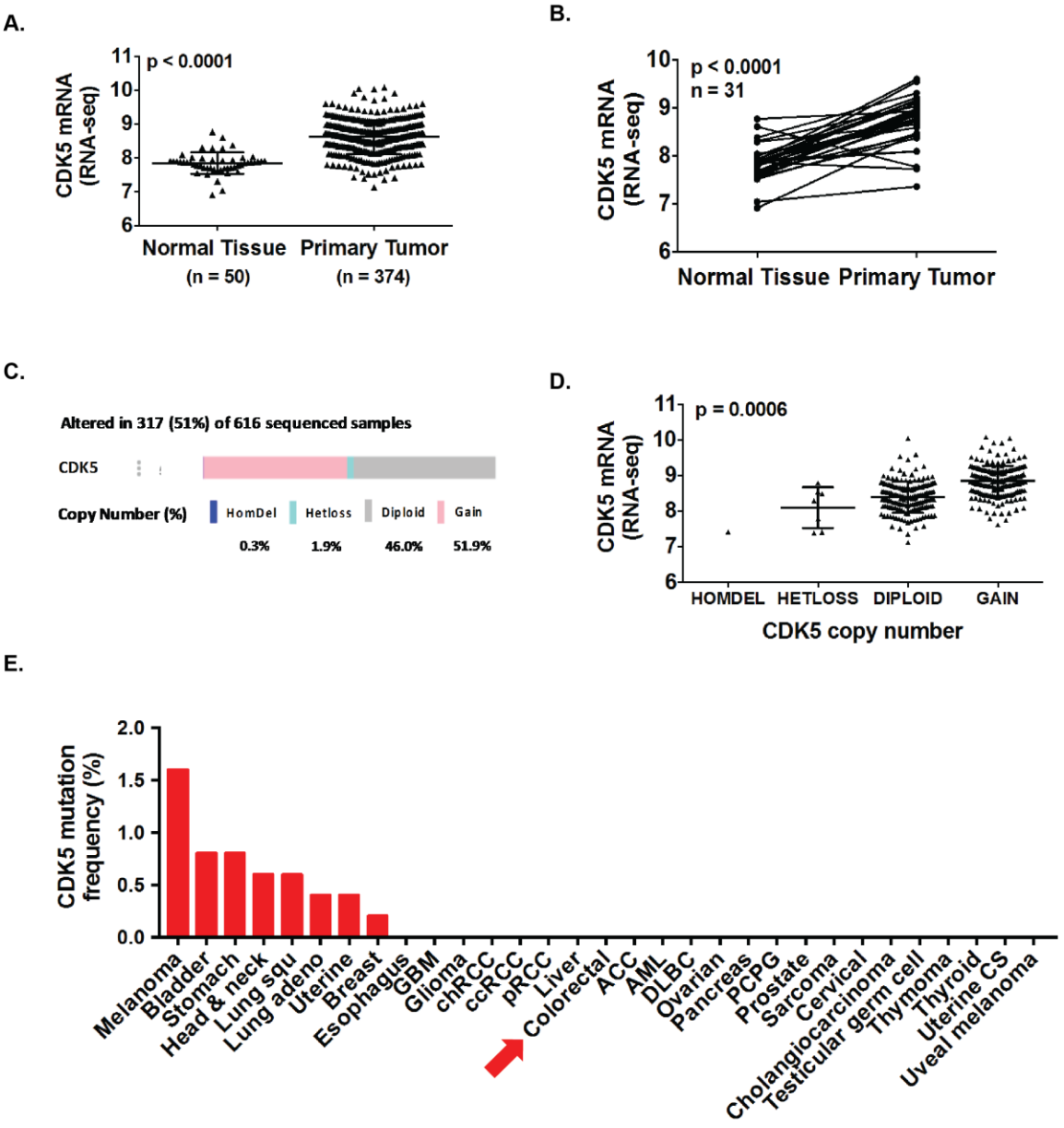


Figure 5: CDK5 expression in TCGA patient samples. A) CDK5 mRNA expression in normal CRC tissue (n=50) and primary tumor tissue (n=374). B) Paired analysis of CDK5 mRNA expression of CRC patient (n=31) normal tissue and corresponding tumor samples. C) CDK5 copy number status in 616 CRC samples. This diagram shows the % of homozygous deletion (dark blue – 0.3%), heterozygous loss (light blue – 1.9%), diploid (grey - 46%), and copy number gain (pink – 51.9%) of CDK5 in CRC patients. D) CDK5 mRNA expression compared to CDK5 copy number. E) CDK5 mutation frequency across TCGA cancers. Arrow indicates CRC.

20-223 shows anti-tumor activity in human CRC xenograft tumors

CP668863, a substituted 3-aminopyrazole analog, was first reported by Pfizer as an ATP-competitive CDK5 inhibitor that was explored for the treatment of neurodegenerative disorders [41]. With increasing evidence that CDK5 activity contributes to CRC tumorigenesis, we synthesized CP-668863 (a.k.a. 20-223) to screen for its efficacy against CRC. We utilized our well established CRC xenograft model [112] to determine the effects of 20-223 *in vivo*. As our xenograft model uses GEO cells, we performed an initial growth inhibition study to show efficacy of 20-223 in this cell line. We found 20-223 to have an IC_{50} value of 79nM in GEO cells (**Figure 6A**). In preliminary PK studies, mice were dosed with 8 mg/kg of 20-223. The plasma concentration was greater than 79 nM for 24 hours as determined by LC-MS (**Figure 6B**). We used a GEO cell line in which GFP is stably expressed for our xenograft model. GEO-GFP cells were subcutaneously injected into the flank of athymic nude mice and tumors allowed to grow to ~100 mm^3. Animals with tumors were then randomly divided into two treatment groups (I) DMSO or (II) 8mg/kg 20-223 (**Figure 6C**). Subcutaneous treatment injections were given in the shoulder area of each mouse daily for the first week and every other day for the following two weeks. The mice were weighed and tumor volumes were measured every other day. At the end of the three-week treatment period the mice were euthanized and the tumors excised, weighed and imaged (**Figure 6D**).

Average changes in tumor volume for both treatment groups are summarized in **Figure 7A**. At the end of the first week of treatment, average tumor volume in the DMSO-treated group was approximately 2-fold greater than average tumor volume in the 20-223-treated group (~429 mm^3 vs. ~197 mm^3). The tumor volumes of DMSO-treated animals continued to grow rapidly, while the tumor progression in 20-223-treated animals was slower (**Figure 7A**). At the conclusion of the study, tumors from the DMSO-treated mice

were significantly larger (~3-fold: 1138 mm^3 vs. 386 mm^3) than the tumors from the 20-223-treated mice. We also found a decrease in GFP fluorescence in 20-223-treated tumors compared to DMSO-treated tumors, which is consistent with the tumor volume trends (**Figure 7B**). The average tumor weights from DMSO-treated mice were also ~2-fold greater than tumors from 20-223-treated mice (0.7 g vs. 0.3 g) (**Figure 7C**). Of note, 20-223 treated animals did not exhibit any overt signs of toxicity, as there was no change in animal weight or behavior (**Figure 7D**).

To confirm inhibition of CDK5 *in vivo*, we performed western blot analyses on the tumor lysates from three representative animals from each treatment group (vehicle or 20-223-treated). 20-223-treated tumors showed a decrease in the pFAK levels, a phosphorylation site specific to CDK5 [63], (**Figure 7E**) suggesting inhibition of CDK5 *in vivo*. In summary, these studies suggest that 20-223 treatment results in anti-tumor activity in a CRC xenograft model.

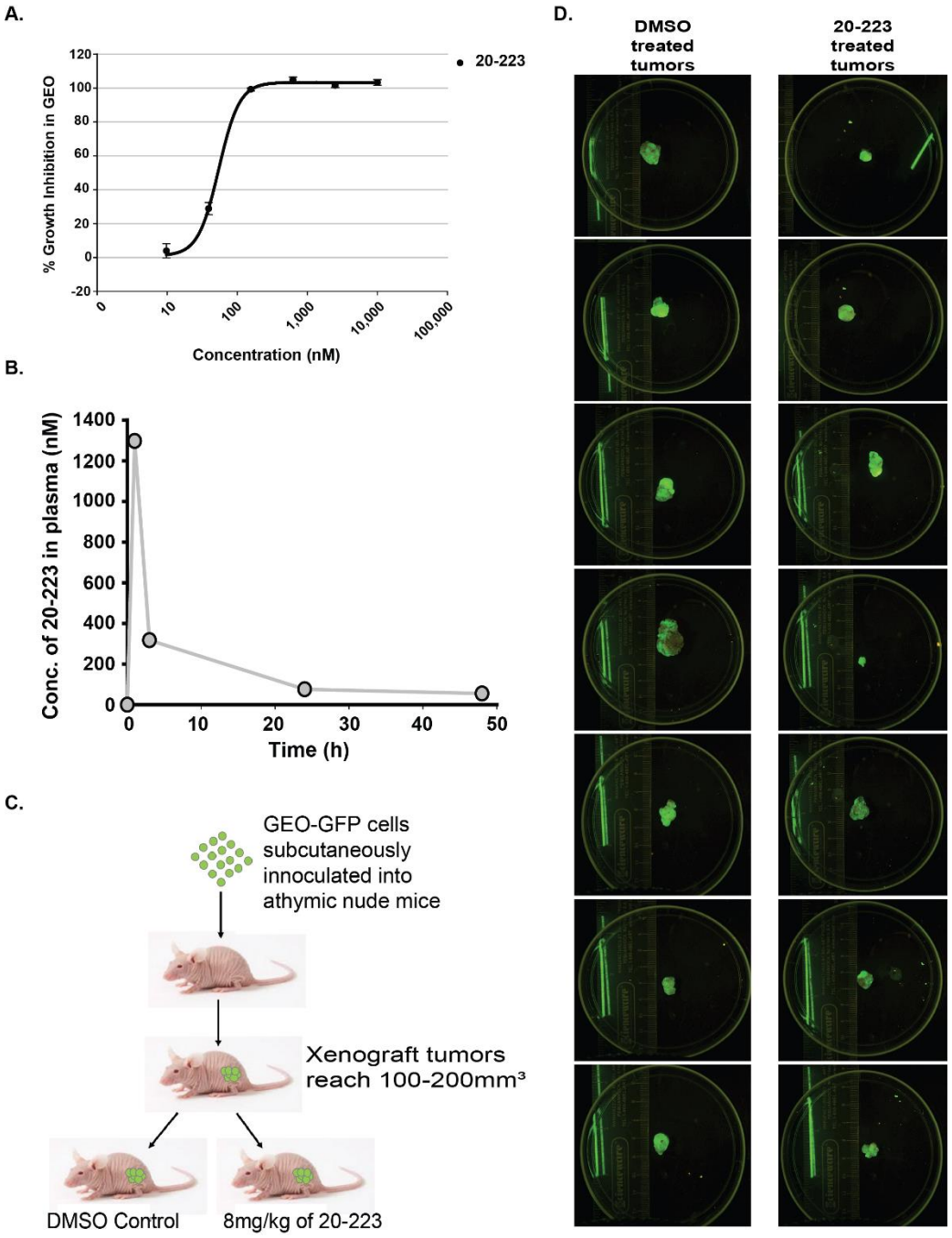


Figure 6: Preliminary in vivo studies with 20-223. A) Growth Inhibition of GEO cells after 72-h treatment with 20-223. B) PK studies after mice were dosed with 8 mg/kg 20-223. C) Schematic representation of CRC xenograft model using GEO-GFP cells. D) Tumor images from DMSO- and 20-223-treated mice.

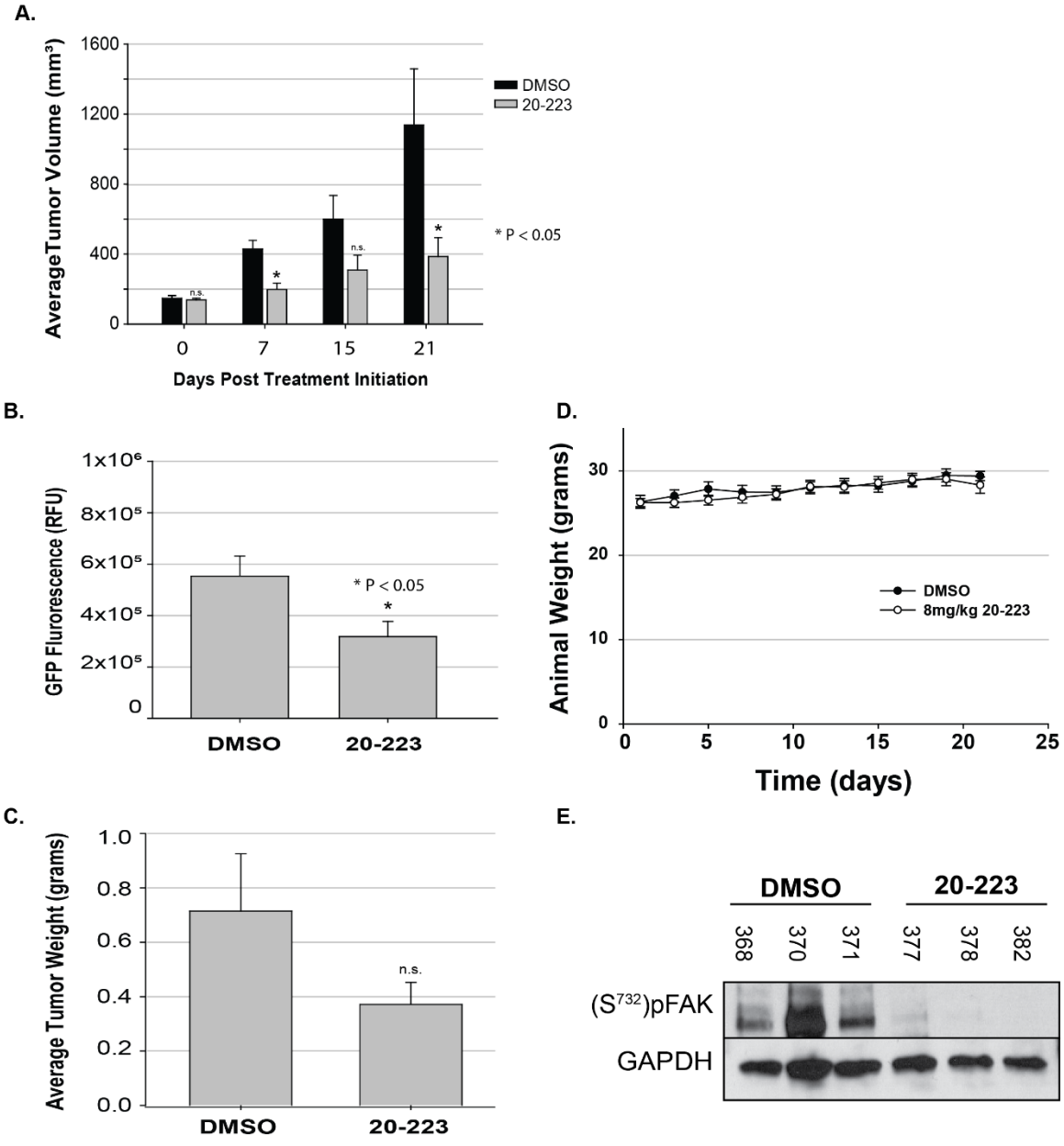


Figure 7: 20-223 anti-tumor activity in CRC xenograft model. A) Average tumor volume comparison of DMSO- and 20-223-treated tumors throughout the study. B) Average GFP fluorescence in DMSO- and 20-223-treated tumors. C) Average tumor weight of DMSO- and 20-223-treated tumors. Average animal weight throughout the xenograft study. E) Western blot analysis of pFAK(S732) levels in DMSO- or 20-223-treated tumor lysates.

20-223 is an ATP-competitive inhibitor

Our *in vivo* data suggest 20-223 may be a promising therapeutic agent for CRC, therefore we began to evaluate and characterize its mechanism of inhibition in cell-free and cell-based studies. We started with docking studies that compared 20-223 to another known CDK inhibitor, AT7519, which is currently in clinical trials and shares the aminopyrazole core structure with 20-223 [38, 39].

X-ray crystallographic studies of reported aminopyrazole analogs and CDKs showed that they occupy the ATP binding site in the CDKs [36, 37]. Since there is no co-crystal structure of 20-223, we docked 20-223 into CDK5 using Autodock Vina to explore its binding mode. Our docking studies revealed that 20-223 indeed occupied the ATP binding site of CDK5 and the 3 nitrogen atoms of the 3-aminopyrazole core are involved in a donor-acceptor-donor hydrogen bond triad with Glu81 and Cys83 of the hinge region. The cyclobutyl ring occupied a narrow hydrophobic pocket formed by Phe80, Leu55 and Val64 and the naphthalene ring of 20-223 is directed towards the solvent-accessible region of the kinase (**Figure 8A**). Since CDK2 and CDK5 share sequence homology of ~60% [43], we overlaid the co-crystal structure of an aminopyrazole analog PNU-181227-CDK2 with our docked 20-223-CDK5 and observed similar binding mode (**Figure 8B**). X-ray crystallographic studies demonstrated AT7519 to be an ATP-competitive CDK inhibitor [39]. Overlay of AT7519 complexed with CDK2 and docked 20-223-CDK5 showed a similar mode of binding with similar hydrogen bonding interactions anchoring the molecules to the hinge region **(Figure 8C**). The chemical structures of all three of these compounds are compared in **Figure 8D**

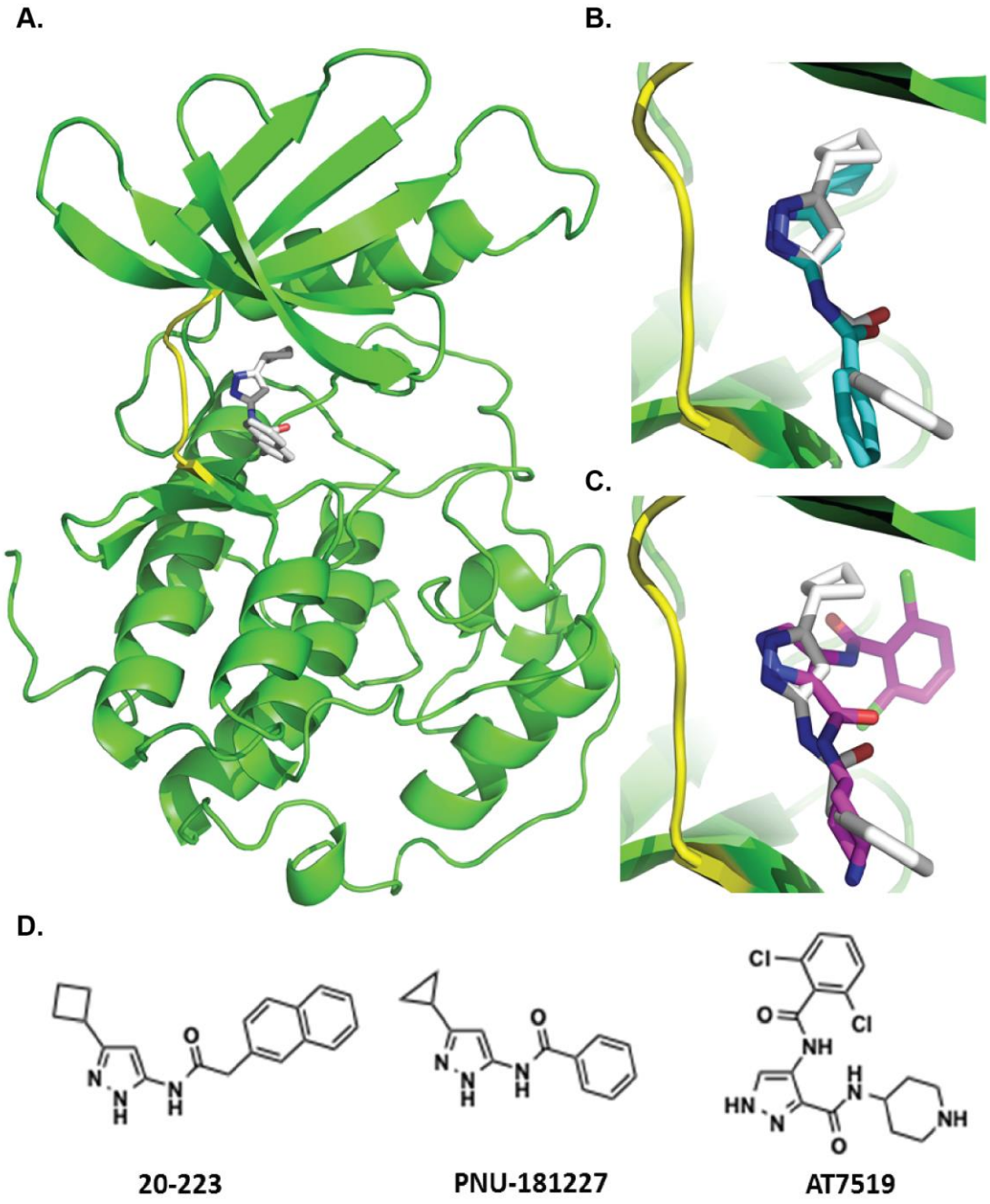
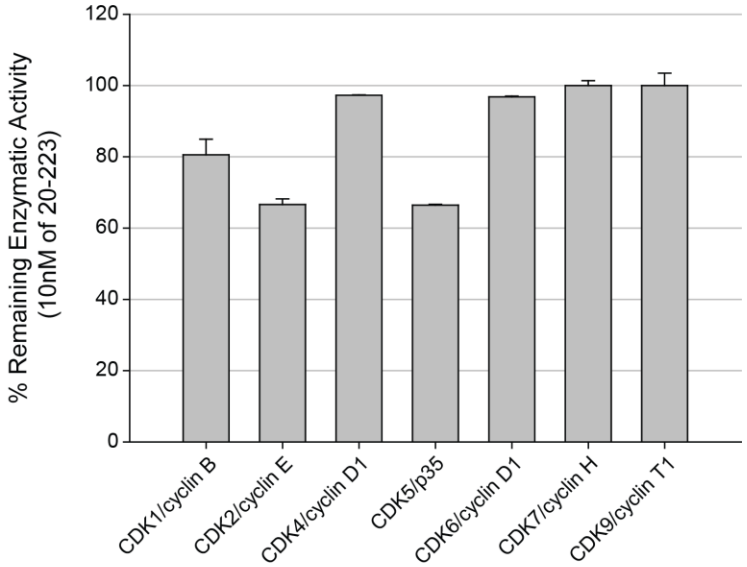


Figure 8: Docking studies with 20-223. A) Docking of 20-223 into CDK5 using AutoDock Vina software. B) Overlay of 20-223 and PNU181227 in the hinge region of CDK5. C) Overlay of 20-223 and AT7519 in the ATP binding pocket. D) Chemical structures of 20-223, PNU-181227, and AT7519.

Cell free kinase assays reveal 20-223 is a CDK2/5 inhibitor

In order to determine the selectivity profile of 20-223 for various CDKs, we conducted a single dose kinase screen with a panel of CDKs. Members of the CDK family bound to their respective activators were incubated with 0.01 μM or 0.1 μM of 20-223 and 30 μM ATP. The percentage remaining enzymatic activity was determined for each of the examined CDKs after inhibition by 20-223. At 0.01 μM 20-223, all members of the family still had a substantial amount of functional activity (approximately 60-100%) (**Figure 9A**). However, incubation with 20-223 at 0.1 μM markedly inhibited the enzymatic activity of CDK2 and CDK5 with only 0.26% and 0.39% enzymatic activity remaining (**Figure 9B**). At this concentration, 20-223 was less effective against the enzymatic activity of CDK1, CDK4, CDK6, CDK7, and CDK9. These results show that 20-223 is most effective against CDK2 and CDK5 in a cell-free system. To determine cell-free IC_{50} values of 20-223 against CDK2/5, we performed a dose-response study. CDK2/CyclinE and CDK5/p35 were incubated with 20-223 at various concentrations and IC_{50} values of 6.0 nM for CDK2 and 8.8 nM for CDK5 were derived from curve fitting the data (**Figure 9C**). Similar studies were also carried out in the presence of AT7519. IC_{50} values of 392 nM for CDK2 and 32.8 nM for CDK5 were obtained. Results from the dose-response study show that 20-223 is equipotent against CDK2 and CDK5 in a cell-free system and is more potent than a comparable CDK inhibitor, AT7519.

A.



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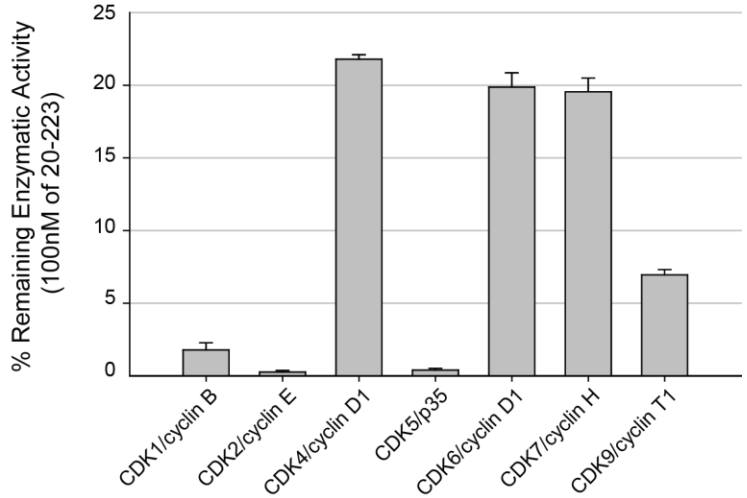


C.

	Cell Free IC_{50} (nM)	
	CDK2	**CDK5**
20-223	6.0 ± 1.0	8.8 ±1.6
AT7519	392 ± 75.2	32.8 ± 1.8

Figure 9: Evaluation of 20-223 and AT7519 in cell-free kinase assays. A-B) % of remaining enzymatic activity of a panel of CDKs after incubation with 0.01 μM (A) or 0.1 μM (B) 20-223 and 30 μM ATP. C) IC_{50} values (nM) of CDK2 and CDK5 after incubation with 20-223 or AT7519 in cell free dose-escalation study.

CDK2 and CDK5 expression and phosphorylation activity in a panel of human CRC cell lines

Having determined that 20-223 targets CDK2 and CDK5, we next examined the basal levels of these kinases in a cohort of colorectal cancer cell lines which includes seven CRC cell lines and one normal human colon epithelial cell line (HCEC). All the cell lines expressed CDK2 and CDK5, albeit at different levels. HCEC cells also expressed CDK2 and CDK5 but at lower levels than many of the CRC cell lines (**Figure 10**). This observation is consistent with the TCGA data.

As a measure of CDK2 and CDK5 activity, we examined basal phosphorylation levels of substrates specific to CDK2 and CDK5. Phosphorylation levels of RB (Ser807/811) were used as a readout for CDK2 kinase activity. While CDK4 has also been shown to phosphorylate RB at Ser807/811 [117, 118], our kinase profile screen showed 20-223 targets CDK2/5 more effectively than CDK4/6. Phosphorylation levels of FAK (Ser732) were used as a readout for CDK5 kinase activity [63]. We observed differential phosphorylation of RB (Ser807/811) and FAK (Ser732), indicating both CDK2 and CDK5 are active in each of the cell lines (**Figure 10**). Of importance, the activity as a function of RB(Ser807/811) or FAK(Ser732) phosphorylation levels did not correlate with CDK2 or CDK5 expression.

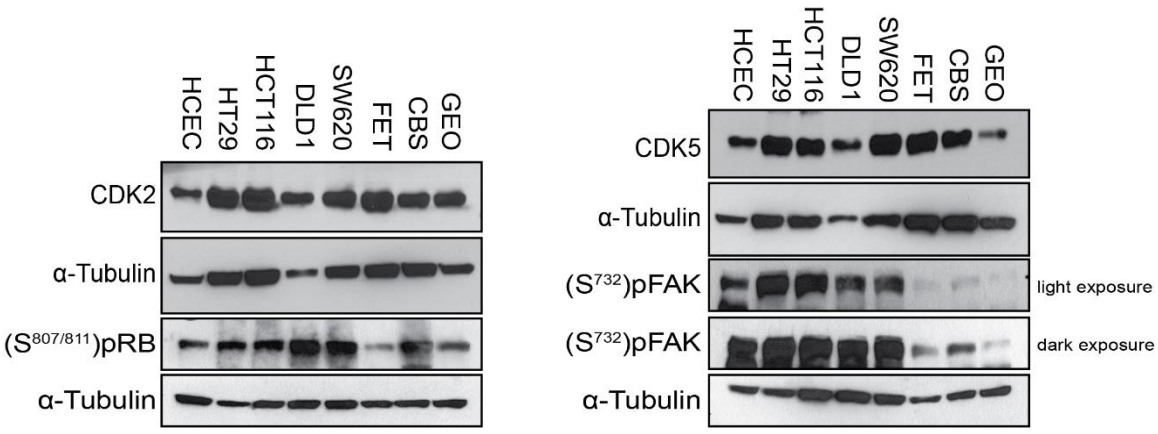


Figure 10: Basal expression and activity of CDK2 and CDK5 in CRC Cells. A) Baseline expression of CDK2 and pRB (Ser807/811) (left), CDK5 and pFAK (Ser732) (right), in untreated CRC cells.

20-223 disrupts CDK2 and CDK5 kinase activity in cell-based studies

Since 20-223 was shown to most potently inhibit CDK2 and CDK5 in a cell-free system, we next explored the ability of 20-223 to target CDK2 and CDK5 in a cellular setting. To characterize the effects of 20-223 on substrate phosphorylation, three CRC cell lines were chosen: GEO, HCT116 and HT29. CRC cells were incubated with DMSO or various concentrations (20 μM – 2-fold dilutions, 7 doses) of 20-223 for 6 hours prior to western blot analyses. In the dose response study, 20-223 did not affect the total levels of CDK2 or CDK5 (**Figure 11A**). As expected, 20-223 induced a dose-dependent decrease in pRB (Ser807/811) and pFAK (Ser732) levels in each of the three CRC cell lines (**Figure 11A**). Of note, treatment with 20-223 did not affect the total levels of RB or FAK (**Figure 11B**). Quantification of phosphorylated RB and FAK levels was performed to reveal the effect of the inhibitor on CDK2 and CDK5 kinase activity, respectively. As the concentration of 20-223 increased, there was a corresponding increase in percent kinase inhibition for CDK2 and CDK5 (**Figure 11C**). This pattern was consistent for each of the three cell lines. Quantification was also used to assess the fold selectivity of 20-223 in each of the three cell lines. **Figure 11D** summarizes the cell-based IC_{50} values for each cell line. 20-223 was ~10 fold more selective for CDK5 over CDK2 in GEO cells (1.44 μM vs 15.79 μM) and ~8-fold more selective for CDK5 over CDK2 in HCT116 cells (1.08 μM vs 8.76 μM), However, in HT29 cells, 20-223 was equally potent against CDK5 and CDK2 (2.45 μM vs. 2.25 μM). While the generated IC_{50} values are based on a qualitative observation, these results demonstrated that 20-223 effectively blocks the kinase activity of CDK2 and CDK5 in multiple CRC cell lines.

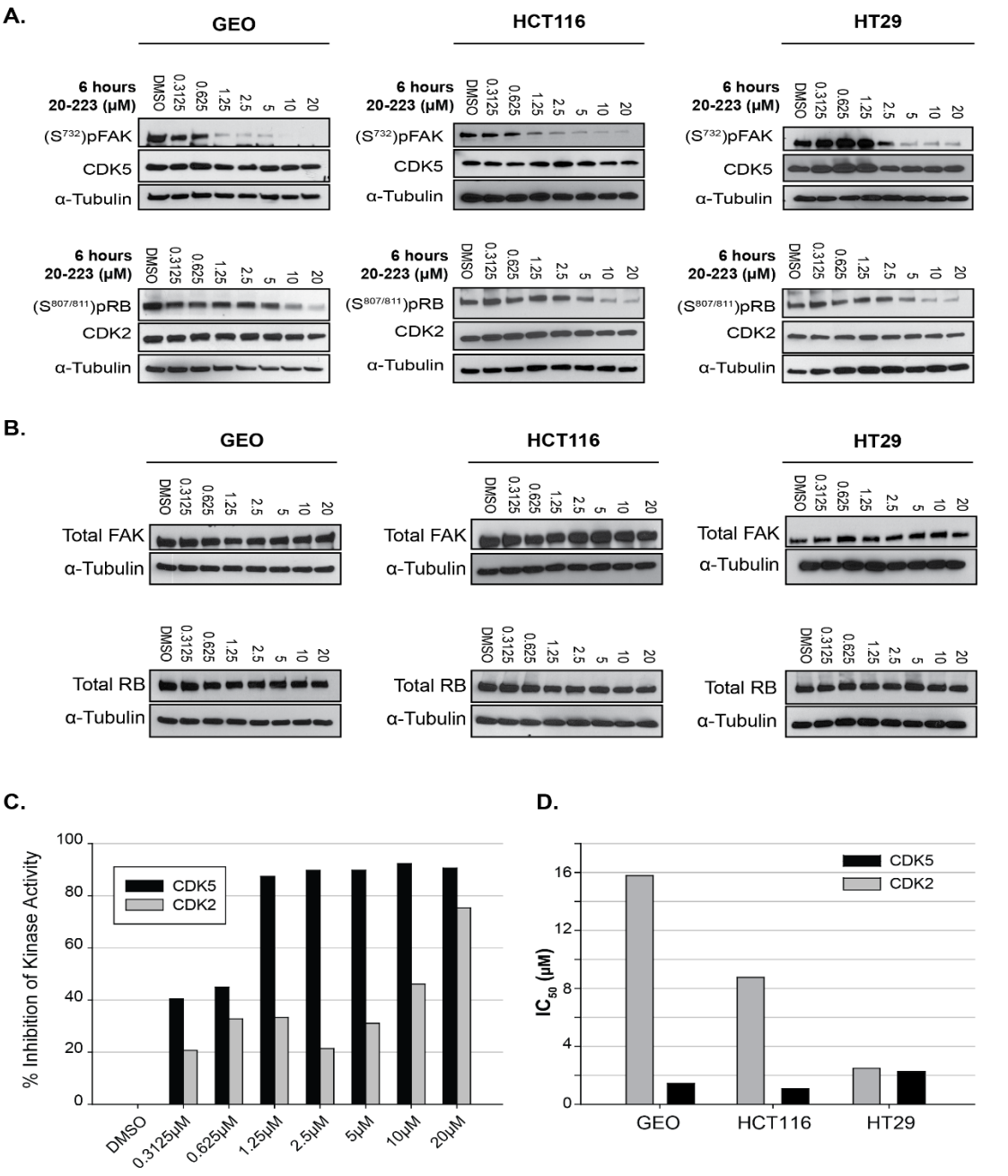


Figure 11: 20-223 inhibition in CRC cells. A) Representative western blots of target substrate pRB and pFAK phosphorylation levels in GEO (left), HCT116 (middle) and HT29 (right) cell lines after 6 hour incubation with 20-223. B) Quantification of % inhibition of CDK2 and CDK5 kinase activity in GEO cells found in Figure 3B. C) Cell-based IC50 values generated from phosphorylation levels in Figure 3B of CDK2 and CDK5 in three CRC cell lines. D) Western blot analyses of total RB or FAK levels after GEO (left), HCT116 (middle), and HT29 (right) cells were treated with varying concentration of 20-223 for 6 hours.

20-223 reduces migration of CRC cells

Since 20-223 effectively inhibits CDK2 and CDK5, both of which have previously been shown to regulate cell motility [63, 119], we next examined its ability to disrupt CRC cell migration. Wound-healing scratch assays are routinely used to assess the effect of small molecule inhibitors on the ability of cells to migrate [120]. EGF-stimulated wound healing has previously been shown to enhance migration of cells; therefore, we used this ligand to stimulate CRC cells for migration [121]. We checked protein levels of CDK2/5 and their substrates after EGF stimulation (100 ng/mL) to ensure that treatment with EGF would not affect their basal levels or activity. Upon treatment with EGF, no changes in the levels of CDK2/5 or pRB/pFAK were observed, indicating that EGF is not affecting the expression or activity of these kinases (**Figure 12**). HCT116 cells were used to model cell migration because they have been used previously in wound-healing scratch assays [122]. To assess the ability of 20-223 to inhibit migration, HCT116 cells were stimulated with EGF and treated with DMSO or 1.5 μM of 20-223. Live cell imaging was utilized to monitor cell motility through the 24-hour incubation period at 15 minute intervals. Still images and higher magnification regions of the images emphasize the ability of 20-223 to inhibit cell migration (**Figure 13A**). HCT116 cells treated with DMSO had greater ability to migrate into the open wound areas compared to cells treated with 20-223. While cells treated with DMSO were able to close approximately 40% of the wound area, cells treated with 20-223 only closed approximately 10% of the wound (**Figure 13B**). To confirm that the reduced migration was a result of CDK2/5 inhibition, corresponding western blots were performed under the same conditions as the migration experiment and pFAK and pRB levels determined after treatment with EGF and 1.5 μM of 20-223. Although treatment with 20-223 effectively reduced the phosphorylation levels of both FAK (Ser732) and RB (Ser807/811) (**Figure 13C**) the effects were more pronounced on the FAK phosphorylation

over RB phosphorylation. Collectively, these results suggest that inhibition of CDK2/5 by 20-223 disrupts CRC cell migration.

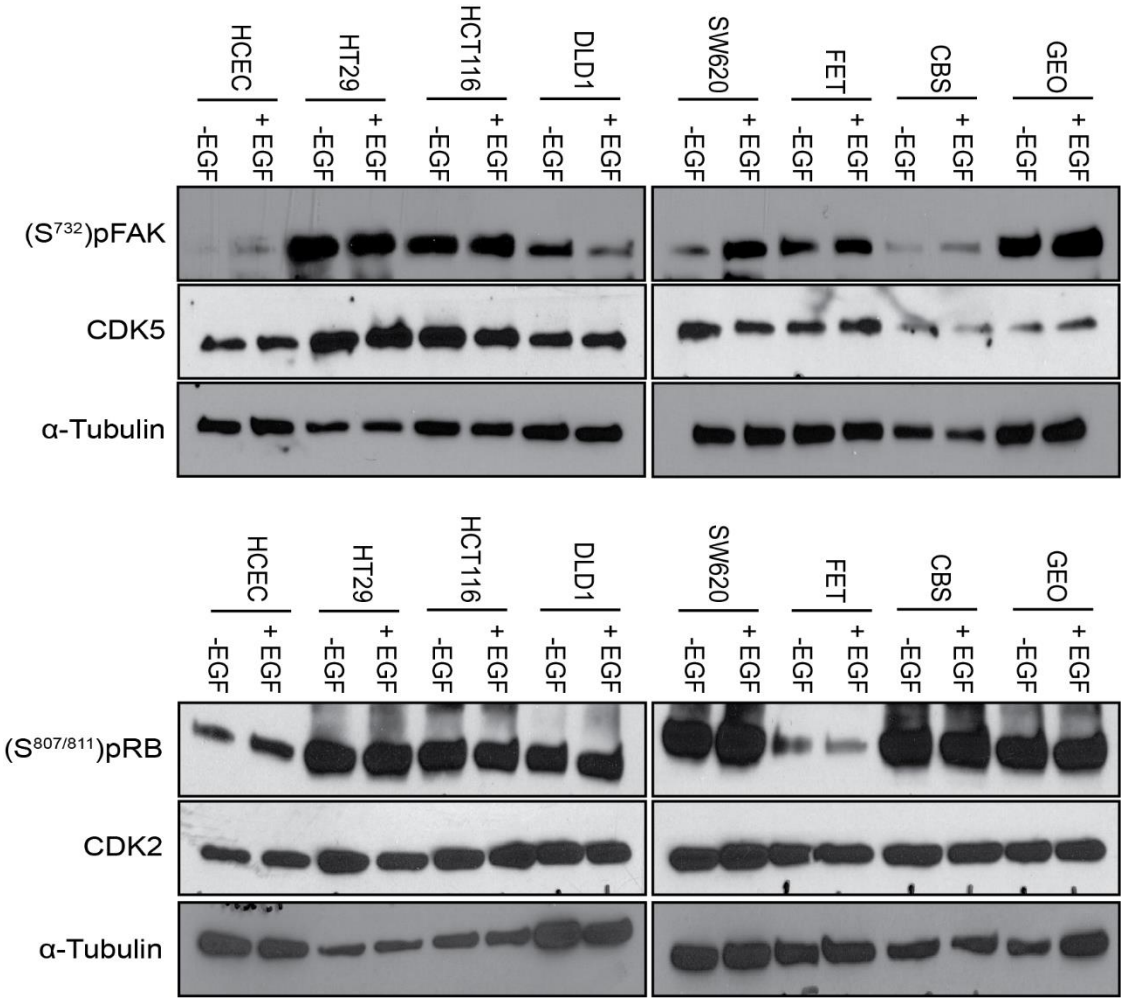


Figure 12: Effect of EGF stimulation on CDK2 and CDK5. Western blot analyses of CRC cells treated -/+ 100 ng EGF.

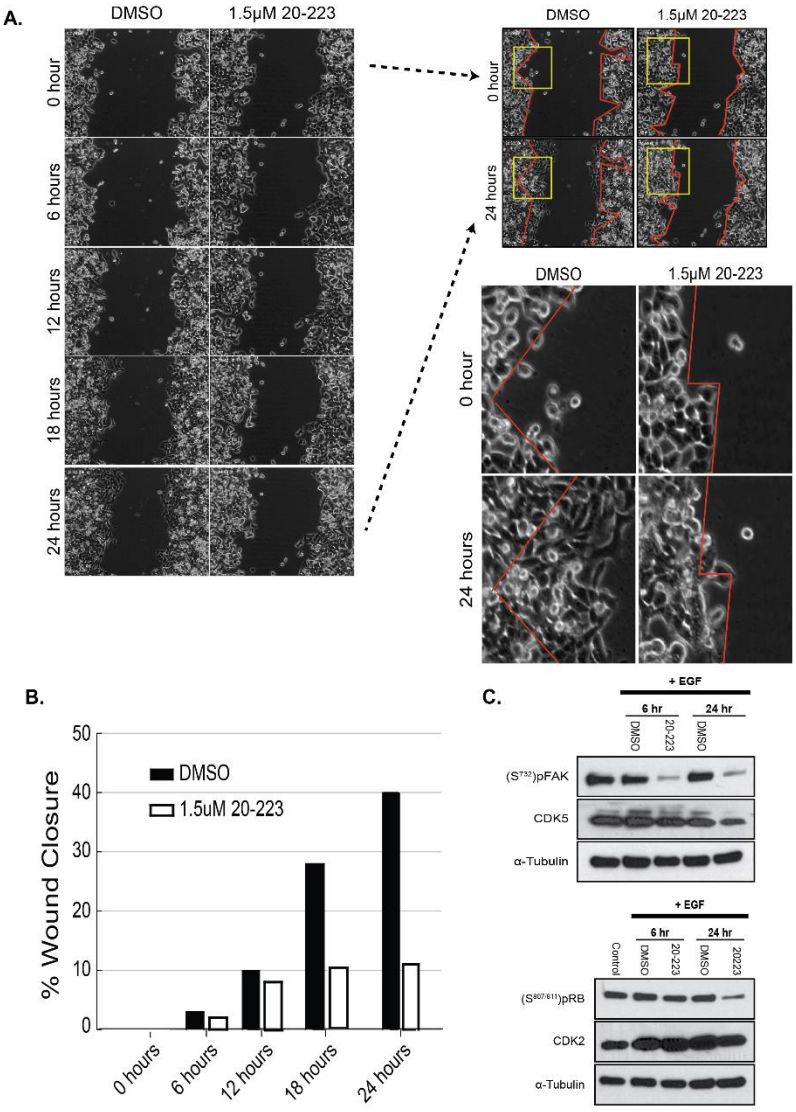


Figure 13: Effect of 20-223 on CRC cell migration. A) Wound gap images taken during the 24-hour incubation of HCT116 cells with DMSO or 1.5 μM 20-223. Zero- and 24-hour images were further evaluated by outlining the wound area (red lines) and zooming in on the wound boundaries (yellow box). B) Quantification of % wound closure after treatment of HCT116 cells with DMSO of 1.5-μM 20-223 C) Western blot analyses at 6 and 24 hours after stimulation with EGF and treatment with either DMSO or 1.5 μM 20-223.

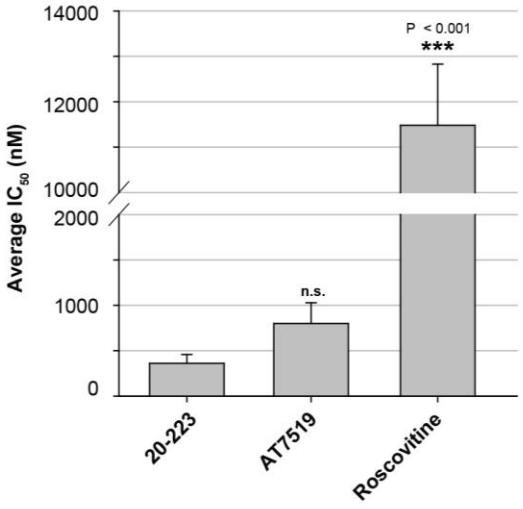
20-223 reduces cell growth in a panel of human CRC cell lines

Since 20-223 effectively targets CDK2 and CDK5, we next examined its effect on cell growth. We subjected a panel of CRC cell lines to treatment with three CDK inhibitors (20-223, AT7519 or Roscovitine). Roscovitine, which contains a purine core, was one of the first CDK inhibitors to enter clinical trials. CRC cells were treated with 20-223, AT7519 and Roscovitine at four-fold dilutions starting at 10 μM (20-223 and AT7519) or 100 μM (Roscovitine). Among the three inhibitors, 20-223 had lower IC_{50} values when compared to the clinically used CDK inhibitors, AT7519 and Roscovitine (**Figure 14A**). Among the CRC cell lines, SW620, GEO and FET cells were the most sensitive to 20-223, whereas HCT116 and HT29 were more responsive to AT7519 treatment as evident by lower IC_{50} values. It is important to note that a ~10-fold higher dose of Roscovitine was required to observe similar growth inhibitory effects. Average IC_{50} values were calculated across cell lines to determine the overall efficacy for each compound (**Figure 14B**). 20-223 had an overall average IC_{50} value of 362 nM across seven cell lines, while AT7519 and Roscovitine had overall average IC_{50} values of 799 nM and 11481 nM respectively, thus suggesting 20-223 is a more potent inhibitor of cell growth compared to the clinical compounds. CRC mutational profiles [123-128] (**Figure 14C**) were examined to determine if the presence of any particular mutations made any cell line more or less responsive to treatment with 20-223. We did not find any obvious correlation between IC_{50} values and the mutational profiles. Based on these findings, we conclude that 20-223 is a sub-μM inhibitor of CRC cell growth. Specifically, these data show that 20-223 is ~2.2 fold and ~31.7 fold more potent than AT7519 and Roscovitine, respectively. Therefore, 20-223 is comparable to, or marginally better, than the CDK inhibitors advanced to the clinics.

A.

Cell Line	IC_{50} (nM) ± SEM		
	20-223	**AT7519**	**Roscovitine**
SW620	168 ± 20	280 ± 61	17239 ± 2239
DLD1	480 ± 41	501 ± 169	13111 ± 2738
HT29	360 ± 72	184 ± 15	12418 ± 1019
HCT116	763 ± 92	318 ± 51	10665 ± 558
FET	117 ± 49	1510 ± 487	13076 ± 6237
CBS	568 ± 49	1464 ±149	9101 ± 1538
GEO	79 ± 31	1336 ± 253	5756 ± 1538

B.



C.

Cell Line	APC	βCatenin	KRAS	BRAF	PIK3CA	TP53
DLD1	Mutant	WT	G13D	WT	E545K ; D549N	S241F
HCT116	WT	Mutant	G13D	WT	H1047R	WT
HT29	Mutant	WT	WT	V600E	P449T	R273H
SW620	Mutant	WT	G12V	WT	WT	R273H;P309S
FET	Mutant	NA	G12D	NA	WT	Mutant
CBS	Mutant	NA	G12D	NA	WT	Mutant
GEO	Mutant	NA	G12A	NA	WT	Mutant

Figure 14: Effect of 20-223 on CRC cell proliferation. A) IC_{50} values from growth inhibition studies after CRC cells were treated with 20-223, AT7519, or Roscovitine for 72 hours. B) Average IC_{50} values across all seven CRC cell lines after treatment with 20-223, AT7519, or Roscovitine ($P < 0.001$). C) Panel containing the seven CRC cell lines used in this study and mutational status of important regulatory genes.

Reduced CRC cell growth and tumor growth induced by 20-223 is probably not due to the induction of apoptosis

To determine if induction of apoptosis was responsible for potent CRC cell growth inhibition, we examined the effect of 20-223 on poly(ADP-ribose)polymerase (PARP) cleavage. PARP cleavage is one of the hallmarks of cell death and is widely used as a read-out of apoptosis in cancer research. To determine the effect of 20-223 on cell death, we evaluated the panel of CRC cells treated with 20-223 for PARP cleavage. CRC cell lines treated with 20-223 for 24 hours all exhibited PARP cleavage (**Figure 15A**). Next, we performed a dose-response (**Figure 15B**) and a time-course (**Figure 15C**) study in GEO cells and found that μM concentrations of 20-223 and long exposure were required to induce apoptosis. This suggests that the reduced tumor growth observed in the mouse model is not due to induction of apoptosis.

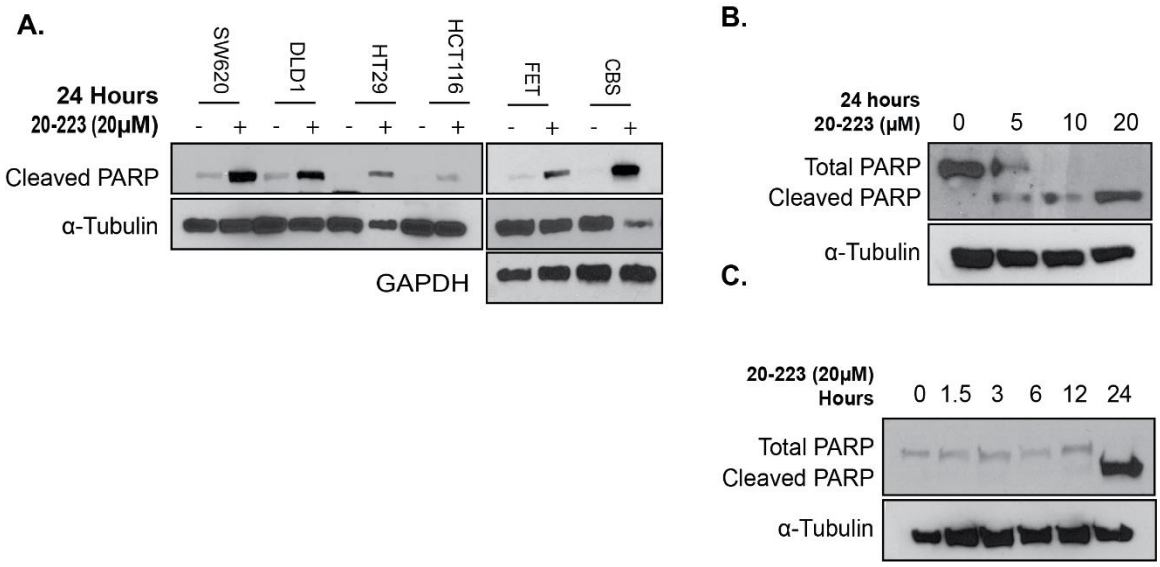


Figure 15: Effect of 20-223 on cell death. A) PARP cleavage in a panel of CRC cells treated with 20 μM of 20-223 for 24 hours. B) Dose-response studies examined after 24-hour treatment with 20-223 in GEO cells. C) Time-course studies to examine PARP cleavage after treatment of GEO cells with 20 μM 20-223.

Reduced CRC cell growth and tumor growth in mice is probably due to the induction of cell cycle arrest by 20-223

The CDK family has been extensively studied for its regulation of all phases of the cell cycle. This kinase family is essential for normal cells to proliferate and divide. CDK2 has been shown numerous times to be required for progression from G1 and S phase of the cell cycle [129]. The role of CDK5 in cell cycle is less understood; however, recent reports suggest that it regulates the cell cycle through mitotic control and dysregulation of the cell cycle inhibitors $p21^{CIP1}$ and p27 [85, 86, 89]. Having shown that 20-223 effectively targets CDK2 and CDK5 and also decreases cell growth, we sought to understand how it may alter cell cycle progression. GEO and HCT116 cells were treated with DMSO, AT7519 or 20-223 for 24 and 48 hours and then analyzed for DNA content by flow cytometry. The results from the above experiment are summarized in **Figure 16A**. 20-223 and AT7519 both effectively arrested the CRC cells in either the G2 or S phase of the cell cycle. GEO cells treated with either 20-223 or AT7519 arrested in G2 phase of the cell cycle. This is consistent with the previous findings that CDK2 regulates the G2/M checkpoint in the absence of functional p53 [130]. Profiling of the GEO cell line indicates that GEO cells carry a p53 mutation, therefore the G2/M arrest seen in GEO cells may be due to CDK2 inhibition. Alternatively, the G2 arrest could also be attributed to CDK1 inhibition, as it was the third CDK inhibited in our profiling. On the contrary, HCT116 cells treated with either 20-223 or AT7519 resulted in S-phase arrest at the 24- and 48-hour time points. **Figure 16B** shows representative traces from the cell cycle analyses. The data clearly show that 20-223 mimics the effects observed with AT7519. These data suggest that the observed CRC cell growth inhibition and the tumor growth in mice induced by 20-223 were due to cell cycle arrest.

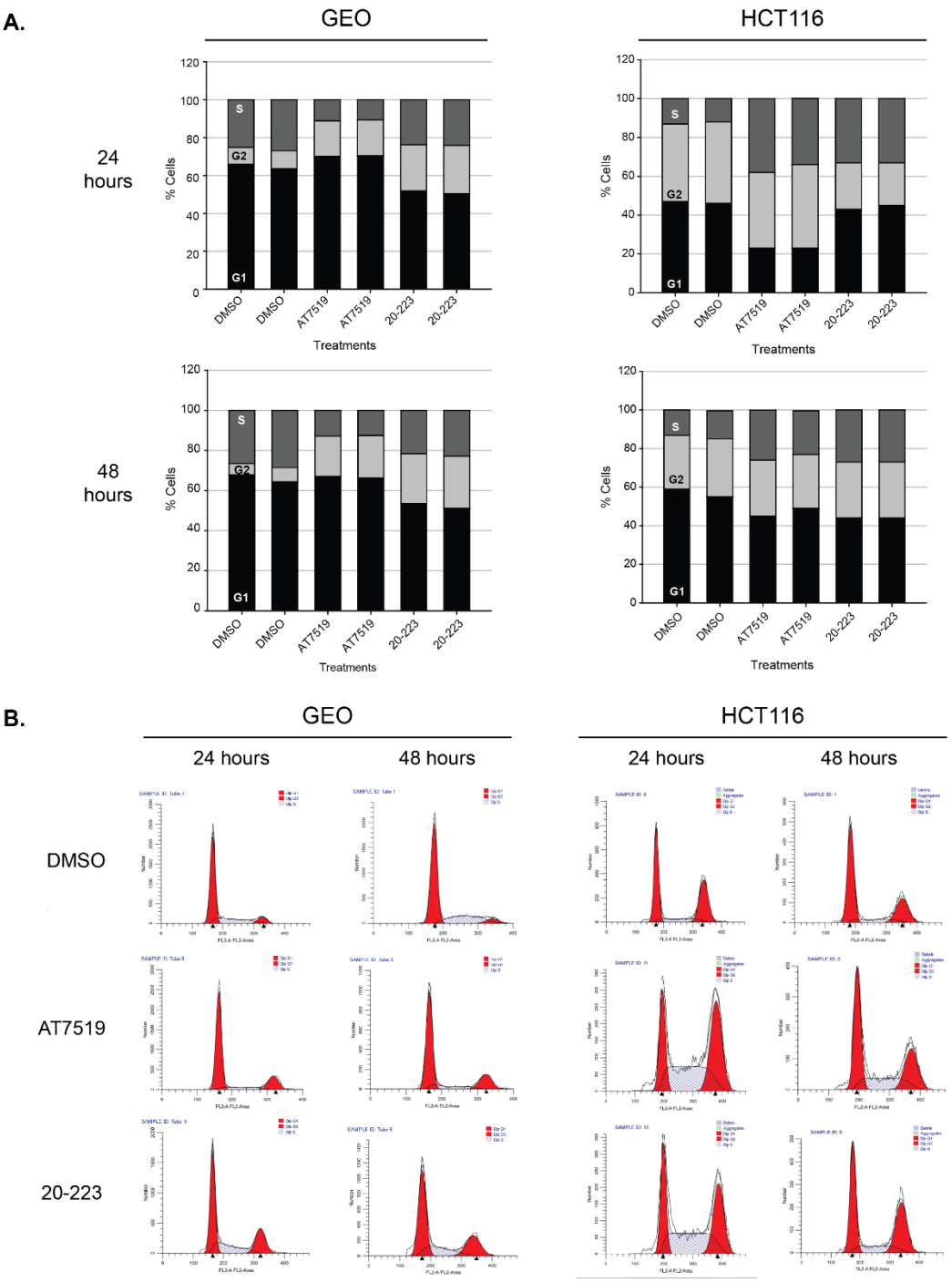


Figure 16: Effect of 20-223 on cell cycle. A) % of cells in each phase of the cell cycle after treatment with DMSO, AT7519, or 20-223 for 24 (top) and 48 (bottom) hours in GEO (left) and HCT116 (right) cells. B) Traces representative of cell cycle analysis in GEO (left) and HCT116 (right) cells after treatment with DMSO, AT7519, or 20-223 after 24 or 48 hours.

Discussion

In the present study, we evaluated 20-223 (CP-668863), a previously identified CDK5 inhibitor, for its potential as an anti-CRC agent. In a proof of concept study, we used an established CRC xenograft model to show that 20-223 effectively slowed tumor progression. Tumors in mice treated with 20-223 had reduced tumor volumes and tumor weights compared to the tumors in vehicle-treated mice. Moreover, we observed lower levels of phosphorylated FAK, a well-characterized target of CDK5, in 20-223-treated tumors as compared to vehicle-treated tumors. These results are consistent with the studies reported with a neurodegenerative model [41].

Having successfully shown that 20-223 slows tumor progression *in vivo*, we followed up with characterization of the mechanistic basis for the observed anti-CRC effects in cell-free and cell-based studies. For these studies, we used AT7519 and roscovitine, both CDK inhibitors previously explored as anti-cancer CDK inhibitors in clinical trials. Of importance, AT7519 and 20-223 share the same core structure, which makes it an optimal compound to benchmark the potency of 20-223.

We performed a series of studies to gain insight into the mechanism associated with the anti-tumorigenic properties elicited by 20-223. The aminopyrazole core found in CDK inhibitors has proven successful due to the flat heterocyclic core and a series of hydrogen bond donors and acceptors. The positioning of nitrogen atoms in the aminopyrazole core enables them to compete with ATP. A hydrogen bond donor acceptor donor triad within the aminopyrazole core targets the hinge region residues of the kinase and blocks the binding of ATP [130]. Docking studies suggested that aminopyrazole analogs 20-223 and AT7519 interact with Glu81 and Cys83 within the hinge region of CDK5. Profiling 20-223 against a panel of CDKs revealed that it most potently inhibits

CDK2 and CDK5 over other CDKs. Importantly, 20-223 is more potent than the clinically used 4-aminopyrazole analog AT7519 in cell-free kinase assays.

Cell-based studies corroborated cell-free kinase assays as 20-223 effectively disrupted the kinase activity of CDK2 and CDK5 in CRC cells. In two of the three cell lines' profiles, 20-223 was selective for CDK5 over CDK2. The observed differential sensitivity/selectivity associated with 20-223 in three different CRC cell lines suggests that the functional misregulation of CDKs is probably not the same across the cell lines. The cell-free and cell-based IC_{50} values were approximately two orders of magnitude apart. This loss of potency when going from cell-free to cell-based activity assays is commonly observed in drug discovery programs. For example, Palbociclib, the recently approved CDK4/6 inhibitor, has single digit nM potency in cell-free assays and has a single digit μM potency in cell-based assays [131]. One possible explanation for this observed difference is the emerging view that kinases are part of larger protein complexes and evaluating selectivity in cell-free conditions does not always reflect the effects observed in the cellular context [132].

Since 20-223 showed ~8-fold selectivity for CDK5 over CDK2 in HCT116 cells, we evaluated its efficacy in inhibiting migration of HCT116 cells in a wound-healing scratch assay. 20-223 treated HCT116 cells showed reduced cell migration when compared to vehicle-treated HCT116 cells. This is consistent with reported literature that shows that CDK5 plays an important role in regulating the migration of cells by phosphorylation of Ser732 on FAK [63]. Studies with a CDK2 inhibitor also showed it blocked EMT and subsequent cell migration; however, in that study the effect of the inhibitor on CDK5 was not determined [119].

As CDK2/5 have been known to drive proliferation of cancer cells, we investigated the effect of 20-223 on cell growth in a larger panel of CRC cell lines. Among these CRC

cell lines, 20-223 not only proved to be a nanomolar inhibitor of cell growth across the panel, but it was also more potent when compared to AT7519 and Roscovitine. These results suggest that 20-223 is comparable to, or in some cases more potent than, the benchmark clinical aminopyrazole analog, AT7519.

Broadly the cause of CRC cell growth inhibition or tumor growth inhibition could be either due to induction of apoptosis or due cell growth arrest. A dose and time dependent study with 20-223 revealed that CRC cells required μM concentrations to induce PARP cleavage, a hallmark for the induction of apoptosis. However, at high nM to low μM concentrations of AT7519 or 20-223, we observed cell cycle arrest. Together our data show that 20-223 phenocopies the cell cycle effects of AT7519 in CRC cell lines. The observed CRC growth inhibition can be largely attributed to inhibition of proliferation and to a lesser extent on the induction of apoptosis.

It is important to note that our study also unveiled that 20-223 is a potent inhibitor of CDK2. As such, this study shed light on the importance of understanding the true mechanism of action of small molecule kinase inhibitors. Too often, patients are given therapeutic drugs that have not been fully characterized. This is likely a reason we commonly see adverse side effects within patients, as we do not know what the drug is binding to or interacting with. In taking the time to fully understand the true mechanisms of action for 20-223, we found it to not only potently inhibit CDK5, but also CDK2. While this was hinted at previously [41], there are no published reports that confirmed this mechanism, particularly outside of the CNS. While we did indeed confirm 20-223 is a potent inhibitor of CDK5, we also observed potent inhibition of CDK2. Fortunately, the fact that 20-223 does disturb CDK2 kinase activity is not detrimental to our studies. In fact, CDK2 has also been shown to be a critical oncogenic player [133]. Specifically, CDK2 is amplified in CRC [134] and it's overexpression increases with disease progression [135].

Furthermore, inhibition of CDK2, using a small molecule kinase inhibitor, resulted in cell cycle arrest and increased apoptosis *in vitro* and *in vivo* [136]. This data suggest that the inhibition of CDK2 by 20-223 could prove to be beneficial to CRC patients. More importantly, efforts to characterize the full mechanism of action of anti-cancer agents should be pursued more often before compounds are pushed toward the clinic.

In summary, our study argues for the continued preclinical development of 20-223 for CRC therapy (**Figure 17**). Collectively, our results reveal that 20-223 exhibits anti-cancer properties in a CRC mouse model. Mechanism studies indicate that it inhibits CDK2/5 in both cell-free kinase assays and in CRC cell lines. Migration of CRC cells was inhibited by 20-223, which targeted CDK5 and as a consequence inhibited Ser732 phosphorylation, a key event in the migration of cells. 20-223 inhibits proliferation of CRC cell lines by inducing cell cycle arrest. A recent review article outlined in detail the contributions of CDK5 to many types of cancer, supporting its potential as a novel target for cancer therapy across many tumor types [42]. While we demonstrated 20-223 is not selective for CDK5, it does indeed inhibit CDK5 *in vitro* and *in vivo*. 20-223 had comparable, or in several assays better, potency than the clinically used aminopyrazole CDK inhibitor, AT7519, which is a good benchmark for advancing a compound through development. In order to explore this core for improved selectivity, structure-activity relationship studies are currently underway in our lab and will be reported in due course.

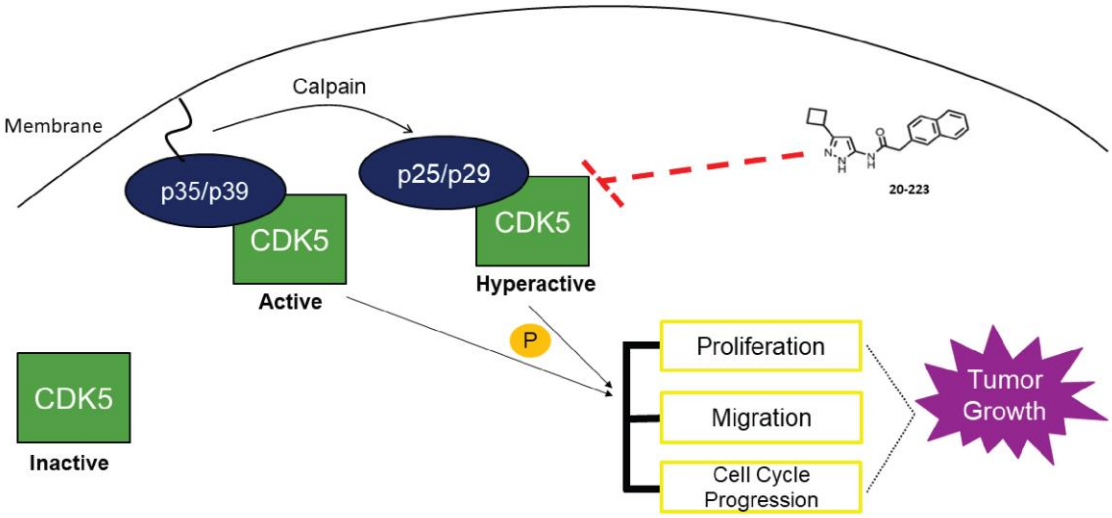


Figure 17: CDK5 and 20-223 activity in CRC.

CHAPTER 4: CHEMICALLY INDUCED DEGRADATION OF CDK9 BY A PROTEOLYSIS TARGETING CHIMERA (PROTAC)

Introduction

The previous chapter demonstrates the use of an aminopyrazole analog (20-223) to inhibit CDK5 as a viable option for CRC therapy. Our work and that of others [36, 37, 39, 40, 137, 138] have proven this scaffold targets CDKs' selectivity and can be optimized for selectivity among the CDKs. In an attempt to continue our efforts to inhibit CDK5, we utilized a new strategy aimed at regulating its protein levels within cells. The PRoteolysis TArgeting Chimera (PROTAC) concept has exploded with interest and gained significant attention over the last few years. This new technology uses chemical probes to exploit the intrinsic cellular mechanisms of the ubiquitin protease system (UPS) to degrade malicious proteins that are disease drivers.

Ubiquitin Proteasome System

The UPS system plays an important regulatory role in maintaining protein homeostasis within cells [139, 140]. This system controls normal protein turnover and oversees the destruction of misfolded and damaged proteins that pose a threat [141]. This regulatory process requires two critical steps – I) the covalent linking of ubiquitin tags on lysine residues of a target protein and II) the degradation of the target protein facilitated by the 26S proteasome [139, 142]. Cells have abundant free-floating ubiquitin molecules, however, they are unable to attach them to target molecules. Therefore, the ubiquitination process requires a series of enzymes to facilitate protein ubiquitination. The activating enzyme (E1) snatches a floating ubiquitin and activates it before transferring it to the carrier enzyme (E2). The main function of the E2 enzyme is to escort the ubiquitin to the ubiquitin ligase (E3). E3 is conveniently bound to the target protein and facilitates the easy

transfer of the ubiquitin tag from the E2 to the target protein. When the E2 arrives carrying the ubiquitin tag, the E3 ligase can catalyze the covalent binding of the ubiquitin onto the lysine residue on the target protein [139, 143]. This ubiquitination process is only able to transfer one ubiquitin molecule at a time, therefore the process is repeated numerous times to achieve poly-ubiquitination of the target protein, followed by proteasomal degradation by the 26S proteasome [143]. This large protease complex sequesters the ubiquitinated target protein and chops it into small peptides. The 26S proteasome houses two separate subunits. The 19S subunit is believed to be responsible for poly-ubiquitin recognition, while the 20S subunit fulfills the catalytic role and cuts the target protein into small pieces [139].

Proteolysis Targeting Chimera

The UPS system described above can be used by the PROTAC technology to tag disease-causing proteins for intracellular protein degradation [144-146]. PROTACs are heterobifunctional small molecules that are comprised of two separate ligands that are connected by a linker. One ligand is required to bind the target protein, while the other is necessary to bind the E3 ubiquitin ligase. If successful, this will form a ternary complex in which the target protein and E3 ligase are linked via the PROTAC molecule (**Figure 18**). By “hijacking” the E3 ubiquitin ligase, the PROTAC molecule brings the target protein in close proximity to the necessary cellular machinery to initiate protein degradation [145].

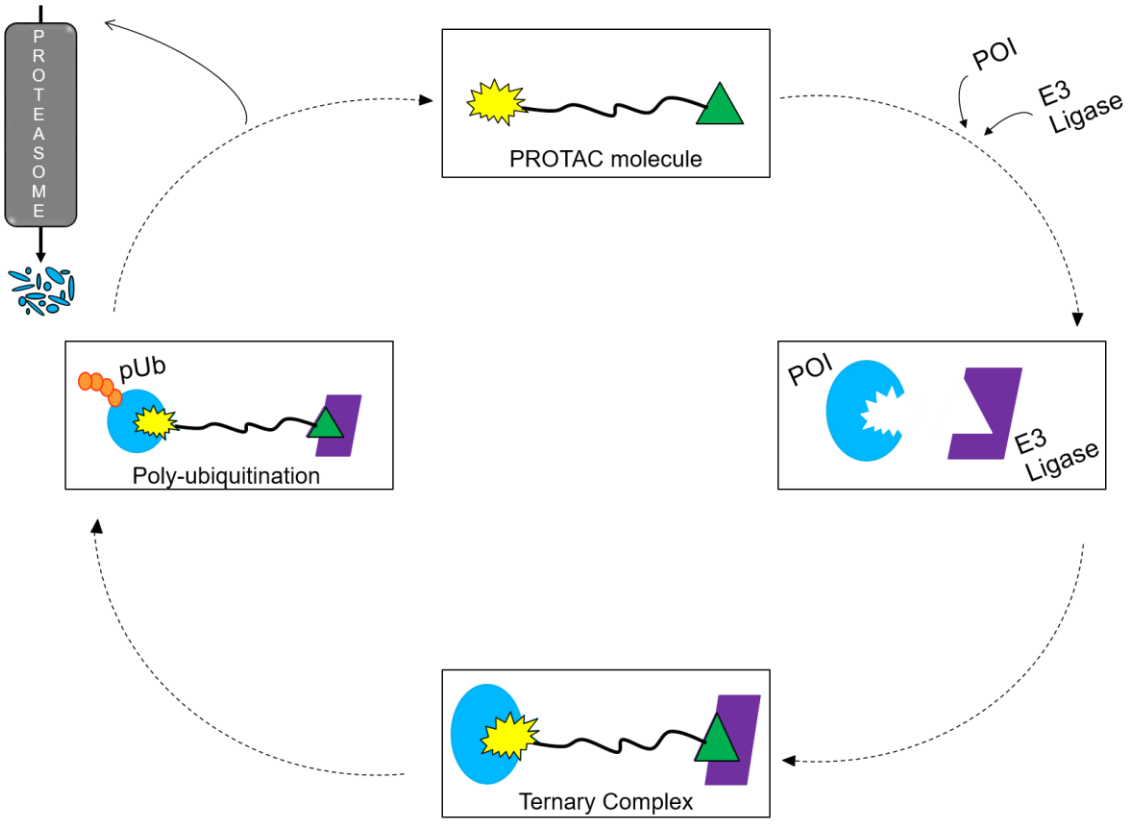


Figure 18: PROTAC concept. *Modified from Toure and Crews (2017) Angewandte Chemi International Edition.

This method of drug development puts a new twist on the common use of small-molecule kinase inhibitors that pharmaceutical companies and medicinal chemists have perfected over time. The widely used occupancy-driven pharmacology has long since focused on the concept that drugs are more effective the longer they sit in the catalytic site and block kinase activity [145]. While this statement holds true, it is not without its limitations or challenges. To achieve efficacy, it requires binding to the catalytic site to block function and may often require large quantities of drug. Furthermore, the traditional small molecule kinase inhibitors are non-covalent compounds, meaning they can dissociate, which returns function to the target protein [147]. Interestingly, the PROTAC strategy instead utilizes an event-driven model, which results in the irreversible inhibition of target function by destroying the protein of interest. Importantly, the degradation of the target protein does not result in degradation of the PROTAC molecule itself. Instead, the newly released PROTAC is able to find another target and E3 ligase to engage. This means the PROTAC mechanism is a catalytic system (**Figure 18**). That allows for multiple rounds of target degradation and thus may require less quantity of drug. Importantly, the only way to counteract this inhibition is for new protein synthesis to occur [145, 147, 148].

While substantial efforts have been made to identify promising therapeutic targets, there are still many targets that we are unable to chemically inhibit. In fact, approximately 85% of the human proteome remains “undruggable” [149]. Not surprisingly, kinases are the most commonly inhibited proteins due to the ease of targeting the ATP-binding pocket [19, 20]. However, the PROTAC strategy has opened doors to the inhibition of “undruggable” targets [147, 148, 150]. As mentioned previously, the target ligand on the PROTAC molecule is only required to bind to the protein of interest; it does not necessarily have to bind within a catalytic site. This allows researchers to identify small molecules that may have alternative binding sites on the protein of interest [147]. This concept opens the

door to investigate the inhibition of other influential disease drivers such as non-enzymatic proteins, transcription factors, and scaffolding proteins [149, 151].

Researchers have long since utilized other approaches to study “undruggable” targets, such as siRNA or shRNA. While RNAi has been a useful tool to study protein modulation and the subsequent cellular effects, investigators have struggled to apply it therapeutically [152]. The PROTAC approach provides a similar means to studying protein function, such as can be done with siRNA- or shRNA-mediated knockdown.

There are multiple advantages to using the PROTAC strategy as opposed to RNAi. First, PROTACs allow for modifications to be made at the protein level, not just the genetic level, thus allowing for modifications to be made to the basal levels of existing proteins. Additionally, the amount of degradation can be controlled by the drug concentration, (*e.g*. higher concentrations would result in greater % of degradation) while RNAi lacks this type of protein level control. Thus, this allows for direct regulation of protein levels within cells. Furthermore, this effect of the PROTAC is seen very rapidly (as early as 15 minutes), whereas it takes longer for RNAi to take effect. Finally, PROTAC molecules are now proving to be highly permeable and have quick effects on the existing protein of interest [152]. Not surprisingly, the evidence presented above has driven researchers to investigate the use of PROTACs as therapeutic interventions for a broad range of targets.

While the PROTAC technology has provided researchers with a new and exciting drug discovery platform, it is not without its limitations. First, PROTAC molecules defy “Lipinski’s Rule of 5” [147] which predicts that any molecule with more than 5 hydrogen-bond donors and 10 hydrogen-bond acceptors will have poor absorption and permeability. Furthermore, it also states that any molecule which is greater than 500 Da will also have difficulty entering cells [153]. Thalidomide alone is approximately 260 Da. The addition of a linker as well as a target ligand will certainly exceed the 500 Da rule. Those using the

PROTAC strategy have already witnessed some indications of permeability issues mainly with their *in vivo* models and as such, have resorted to administering the PROTAC molecules intravenously. However, in the last few months, Arvinas Inc., a pharmaceutical company founded by PROTAC pioneer Craig M. Crews (Yale University), was the first to generate a potent orally bioavailable PROTAC molecule which exhibited a promising absorption, distribution, metabolism, and excretion (ADME) profile and diminished tumor growth *in vivo* [154]. Another limitation of PROTACs that could cause difficulties is the specificity and selectivity profiles of the drugs. While PROTACs rely heavily on the surface-exposed lysine residues to generate selectivity, there are other ways to potentially add an extra layer. Currently, our knowledge of available E3 ubiquitin ligases remains small, however, with the discovery of the PROTAC technology, it is likely that exploration into new E3 ubiquitin ligases could increase. As such, one way in which to improve the selectivity would be to utilize E3 ubiquitin ligases that themselves are unique, for example, tissue- or disease- specific E3 ubiquitin ligases, or those with certainly subcellular localization. This allows researchers to conditionalize the degradation of target proteins [147]. While the PROTAC industry has only recently been conceived and applied, the studies thus far regarding potency and efficacy, both in cell culture and *in vivo*, are encouraging. With a few limitations that should be easily overcome, it seems as though the PROTAC strategy will continue to have a large impact on the field of drug discovery and therapeutics.

PROTAC applications

The PROTAC technology has been applied to multiple proteins of interest (**Figure 19**) [145, 147]. The ground-breaking proof of concept PROTAC study conjugated a 10-amino acid peptide of IκBα to a methionine aminopeptidase-2 (MetAp2) inhibitor, ovalicin, for the purpose of recruiting MetAP-2 to SCF (Skp1-Cullin-F box). Through a series of

immunoprecipitation and ubiquitination assays *in vitro*, Sakamoto *et al*., successfully showed that the PROTAC molecule recruits MetAP2 to SCF, and that the PROTAC molecule mediates the ubiquitination and eventual degradation of MetAP2 [155]. While first-generation peptide PROTACs proved to have difficulties with cell permeability and lacked typical drug-like properties (*e.g.* stability, potency, biodistribution), the overall concept proved to be promising [145]. The discovery of improved E3 ligands, particularly those aimed at interacting with von Hippel-Lindau (VHL) and cereblon (CRBN), was monumental for the broader applicability of the PROTAC technology.

Cereblon is an important part of a larger E3 ubiquitin ligase complex (**Figure 20**), which is essential for a variety of cellular processes, including cell cycle regulation, DNA repair, cell survival, proliferation, and metabolism [156]. The E3 ubiquitin ligase complex consist of four interacting proteins: (I) Cullin4 (Cul4), a scaffolding protein that interacts with the receptor protein to engage substrate recognition, (II) adaptor protein, DNA damage-binding protein 1 (DDB1), (III) ring finger E3 ligase, (Roc1) that binds to the ubiquitin-carrying E2 ligase to catalyze the transfer of the ubiquitin molecule to the substrate, and (IV) cereblon (CRBN) the substrate receptor which binds directly to thalidomide [146, 157].

E3 Ligase	Bifunctional Molecule	Degraded Proteins	Conc. of maximal degradation	Ref.
VHL	ARV-711	BET (BRD2,3,4)	0.011 μM	Raina et al., 2016
	MZ1	BET (BRD2,3,4)	1 μM	Zengerle et al., 2015
	DAS-6-2-2-6-VHL	c-ABL	1 μM	Lai et al., 2016
	PROTAC_RIPK2	RIPK2	0.01 μM	Bondenson et al., 2015
	PROTAC_ERRα	ERRα	1 μM	Bondenson et al., 2015
Cereblon	ARV-825	BRD4	0.1 μM	Lu et al., 2015
	dBET-1	BET (BRD2,3,4)	0.25 μM	Winter et al., 2015
	dFKBP-1	FKBP12	0.5 μM	Winter et al., 2015
	BOS-6-2-2-6-CRBN	c-ABL / BCR-ABL	2.5 μM / 2.5 μM	Lai et al., 2016
	DAS-6-2-2-6-CRBN	c-ABL / BCR-ABL	1 μM / 1 μM	Lai et al., 2016

***Adapted from: Collins et al. (2017) Biochemical Journal & Lai and Crews (2017) Nature Reviews Drug Discovery

Figure 19: Small molecule PROTAC compounds, targets, and concentrations used for maximal degradation. *Modified from Collins et al. (2017) Biochemical Journal & Lai and Crews (2017) Nature Reviews Drug Discovery.

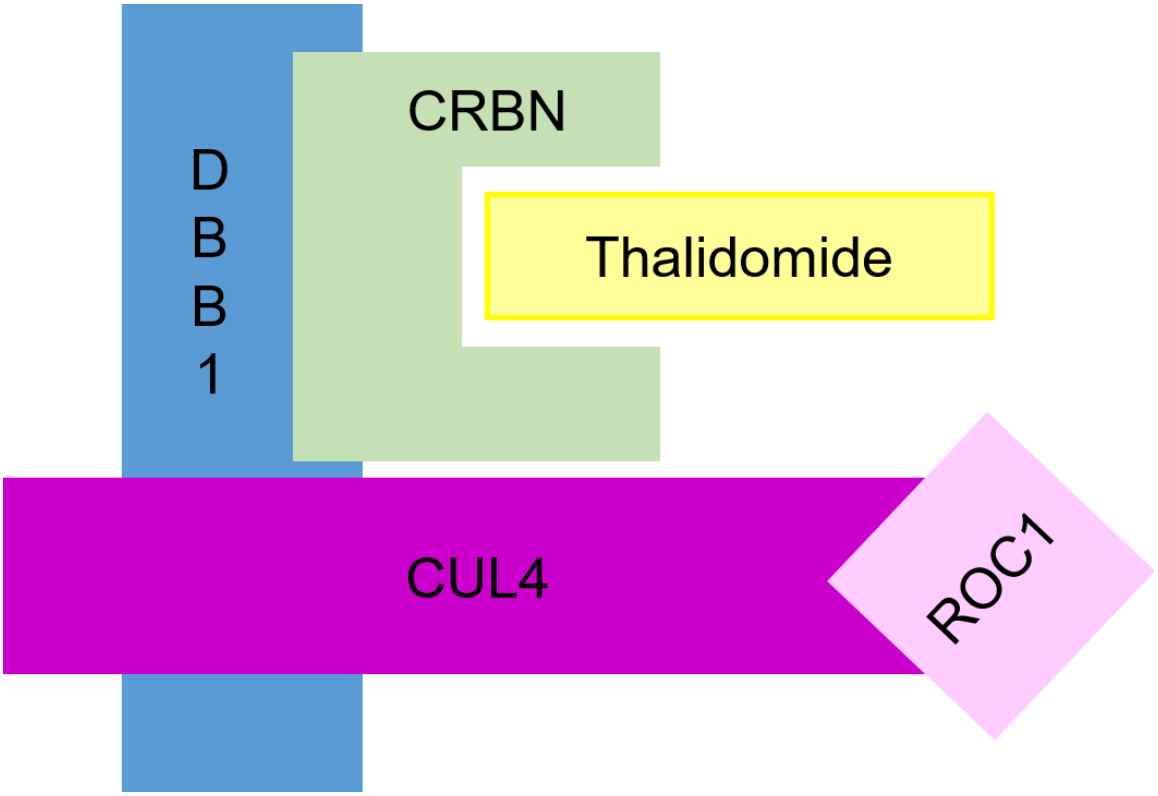


Figure 20: E3 Ubiquitin Ligase Complex – modified from Collins et al., (2017) Biochemical Journal.

In 2010, Ito *et al*. discovered that thalidomide interacts with the E3 ubiquitin ligase, cereblon. Thalidomide is an immunomodulatory drug that has a controversial history. Throughout the 1950s and 1960s, thalidomide was prescribed to pregnant women as a way to combat morning sickness. However, soon after, it was discovered that babies born to women taking thalidomide were born with severe birth defects. While physicians were able to conclude that treatment with thalidomide led to severe birth defects, many questions remained about the true mechanism of action of thalidomide. In 2010, Ito *et a*l. published their work detailing the identification of the primary target of thalidomide. Through affinity purification they were able to elute two polypeptides. Subsequent proteolytic digestion and tandem mass spectrometry revealed the two eluted polypeptides were cereblon and damage DNA binding protein 1 (DDB1). Furthermore, through coprecipitation assays, they were able to show that thalidomide-bound-cereblon was indeed bound to the E3 ligase complex, consisting also of Cul4A and Roc1 [158]. This late-breaking discovery proved thalidomide to be a useful ligand to target E3 ubiquitin ligases.

In light of this discovery, researchers realized that thalidomide could be repurposed to target the degradation of disease-driving proteins. In 2015, the Bradner lab at Dana-Farber Cancer Institute was the first to demonstrate the use of a thalidomide PROTAC molecule to degrade a particular protein of interest, specifically bromodomain and extra terminal domain 4 (BRD4)[159]. BRD family members are important transcriptional regulators. In cancer, BRD proteins have been shown to regulate the transcription of critical oncogenes, drive the cell cycle, and serve as important scaffolding players, and as such have been implicated in a variety of cancers [160]. The Bradner group took their previously published BET bromodomain inhibitor (JQ1) [161] and conjugated it to thalidomide (dBET1) to test the PROTAC strategy to hijack the cereblon ubiquitin ligase complex [159]. Immunblot studies performed in acute myeloid leukemia (AML) cells revealed a substantial loss (~85%) of BRD4 expression. Additional studies confirmed that neither treatment with JQ1 or thalidomide alone were capable of degrading BRD4. Furthermore, pretreatment with either JQ1 or thalidomide disrupted BRD4 degradation confirming both BRD4 and cereblon were required to form the ternary complex and trigger targeted degradation. Subsequent treatment with the proteasome inhibitor carfilzomib blocked degradation of BRD4, confirming the degradation was indeed caused by the UPS system. Systematic comparison studies performed between JQ1 (BETi) and dBET1 revealed the degrader was a more potent inducer of apoptosis. Additionally, the degrader (dBET1) was also a more potent inhibitor of cell proliferation compared to tradition BET inhibition with JQ1. In an AML xenograft model, animals treated with dBET1 had reduced tumor volumes and tumor weights compared to vehicle control. In summary, this was the first proof of concept in which a thalidomide-conjugated PROTAC molecule successfully showed targeted protein degradation. Furthermore, it brought to light that extension of the PROTAC application could be utilized to regulate the protein stability of numerous disease-driving proteins [159].

Since 2015, multiple groups have conjugated thalidomide, or analogs, lenalidomide and pomalidomide, to various target ligands, with the goal of hijacking cereblon and bringing it in contact with the protein of interest to trigger their degradation.

A study from the Wang group at the University of Michigan Cancer Center was the first group to investigate the degradation of BET proteins as a form of triple-negative breast cancer therapy [162]. Through modifications made to a potent BET inhibitor (BETi-211), Bai *et al.* generated a library of analogs conjugated to thalidomide and identified BETd-246 as a BET degrader. Next, they investigated the mechanism of action and compared the therapeutic potential between a BET inhibitor (BETi-211) and the BET degrader (BETd-246). BETd-246 degraded BRD2, BRD3, and BRD4 at low nanomolar concentrations (30-100 nM) after only 1-hour incubation. Head-to-head comparison of growth inhibitory and apoptotic activity revealed BETd-246 was approximately 50 times more potent than the inhibitor, BETi-211. Global analysis of gene expression determined that BETd-246 and BETi-211 had distinct transcriptional effects. BETi-211 caused equal up- and downregulation of approximately 250-450 genes, while BETd-246 was responsible for the downregulation of roughly 500-800 genes. Interestingly, the transcriptional analysis identified Mcl-1 as a downstream target of BET degradation and a key component responsible for the induction of apoptosis. Not surprisingly then, they found BETd-246 was synergistic with B-cell Lymphoma Extra Large (Bcl-xL) inhibitors, BM-1197, A-1155463, and ABT-263. The final evaluation of BETd-246 and BETi-211 compared efficacy in patient-derived and murine xenograft studies. Once again, BETd-246 (and next generation compound, BETd-260) successfully diminished tumor growth in the breast cancer models [162].

A second study by the Bhalla group at M.D. Anderson Cancer Center performed an in-depth comparison of another BRD4 PROTAC and BET-protein inhibitor [163]. Palmolidomide, a close analog of thalidomide, was used as the E3 ubiquitin ligase target. Like thalidomide, palmolidomide's E3 ubiquitin ligase target is cereblon/CUL4. Palmolidomide was conjugated to OTX015, a small molecule kinase inhibitor of BET proteins. The hetero-bifunctional molecule (ARV-825 – Arvinas, Inc) successfully depleted BRD2 and BRD4, while OTX015 actually caused an increase in BRD4 levels. This result is not overly surprising as inhibitors have been shown to counteract the initial inhibitory response by upregulating target protein levels. As such, this response mechanism is avoided with target degradation. ARV-825 also proved to be a more potent inducer of apoptosis compared to OTX015, as there was an increase in positive annexin V staining after treatment with the PROTAC molecule. Next, RNA sequencing revealed ARV-825 greatly affected the transcription of a much larger population of genes, compared to OTX015. Specifically, treatment with ARV-825 altered the transcription of multiple key oncogenes. Treatment with ARV-825 downregulated Myc, Bcl-xL, and PIM1, and it upregulated p21. Interestingly, washout experiments that compared the sustained activity of ARV-825 and OTX015 found the degrader to have a long lasting effect, while inhibition was short lived. As such, expression of downstream oncogenes Myc, PIM1 and Bcl-xL returned after washout, suggesting that inhibition alone lacks a sustained effect. In this study, *in vivo* analysis was carried out using a second-generation PROTAC molecule, ARV-711, which recruits VHL instead of cereblon by using a VHL ligand. Comparison of ARV-711 and OTX015, in an AML mouse model, found ARV-711 to reduce tumor burden and improve median survival. While OTX015 also decreased tumor burden, toxicity was observed in the form of weight loss. Collectively, this study demonstrated that degradation has a more profound anti-tumor effect compared to simple target inhibition [163].

The two proof of concept studies outlined above, along with many others, portray a common theme in which small molecule degraders outperform small molecule inhibitors. This is likely due to the global effect a PROTAC has on its target of interest. Proteins are part of large complexes within cells. While an inhibitor can bind and transiently disrupt kinase activity, it does not disturb the protein-protein interactions. Alternatively, the destruction of the target protein, using the PROTAC method, means it is no longer present to form the critical protein-protein interactions. Thus it is no surprise that researchers are attempting the degradation of a variety of disease-causing targets.

The emergence of this novel new therapeutic strategy is beginning to cause a transformational shift from traditional inhibition of kinases to now driving their destruction [147]. Therefore, we asked whether the degradation of CDK5 would be more beneficial than the traditional kinase inhibition route that we explored in the previous chapter. Hence, we set out to develop a PROTAC molecule that would degrade CDK5 and thus might serve as an anti-CRC therapy.

Results

Aminopyrazole analogs were synthesized and evaluated as CDK2 inhibitors [36, 37]. A majority of these inhibitors are ATP competitive in nature due to a unique donor-acceptor-donor architecture of nitrogen atoms of the aminopyrazole core, which mimics the ATP adenine core [36, 37, 137]. Analyses of the X-ray crystal structure reveal that the three nitrogen atoms of the aminopyrazole core interact with the hinge region residues of CDK [36, 37]. Substituents at the 3’ position on the aminopyrazole core occupy the adjacent hydrophobic pocket. Substitutions on the exocyclic amine are solvent exposed [36] (**Figure 21**).

The kinase domain and specifically the ATP-binding site of the CDKs are structurally similar, which makes it challenging to develop selective CDK inhibitors [164]. On the other hand, the shape of the surface and the distribution of lysine residues on the surface among the CDKs are different (**Figure 22**). This provides a unique opportunity to develop a selective CDK degrader using the PROTAC strategy because an appropriately placed surface exposed lysine residue is required for ubiquitination and proteaosomal degradation [165]. We hypothesized that a PROTAC utilizing a pan-CDK inhibitor might lead to the development of a selective CDK degrader.

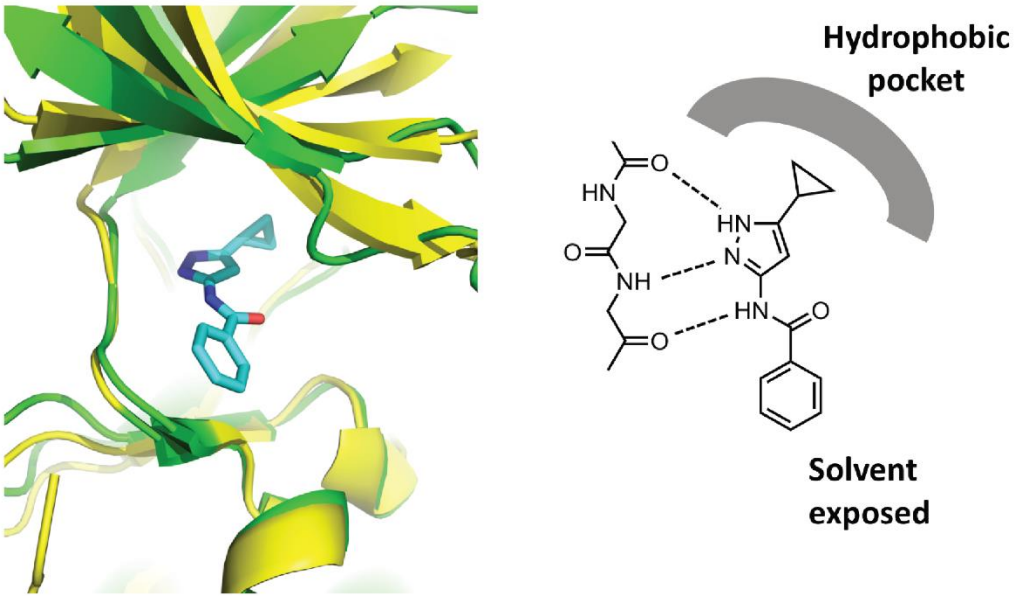


Figure 21: Left: Overlay of CDK2 (PDB id 1VYZ, yellow) and CDK9 (PDB id 4BCG, green). Right: Hinge region residues of CDK2 interacting with the nitrogen atoms of aminopyrazole core. The potential hydrogen bonds between the CDK2 hinge region and the inhibitor are shown as dashed lines.

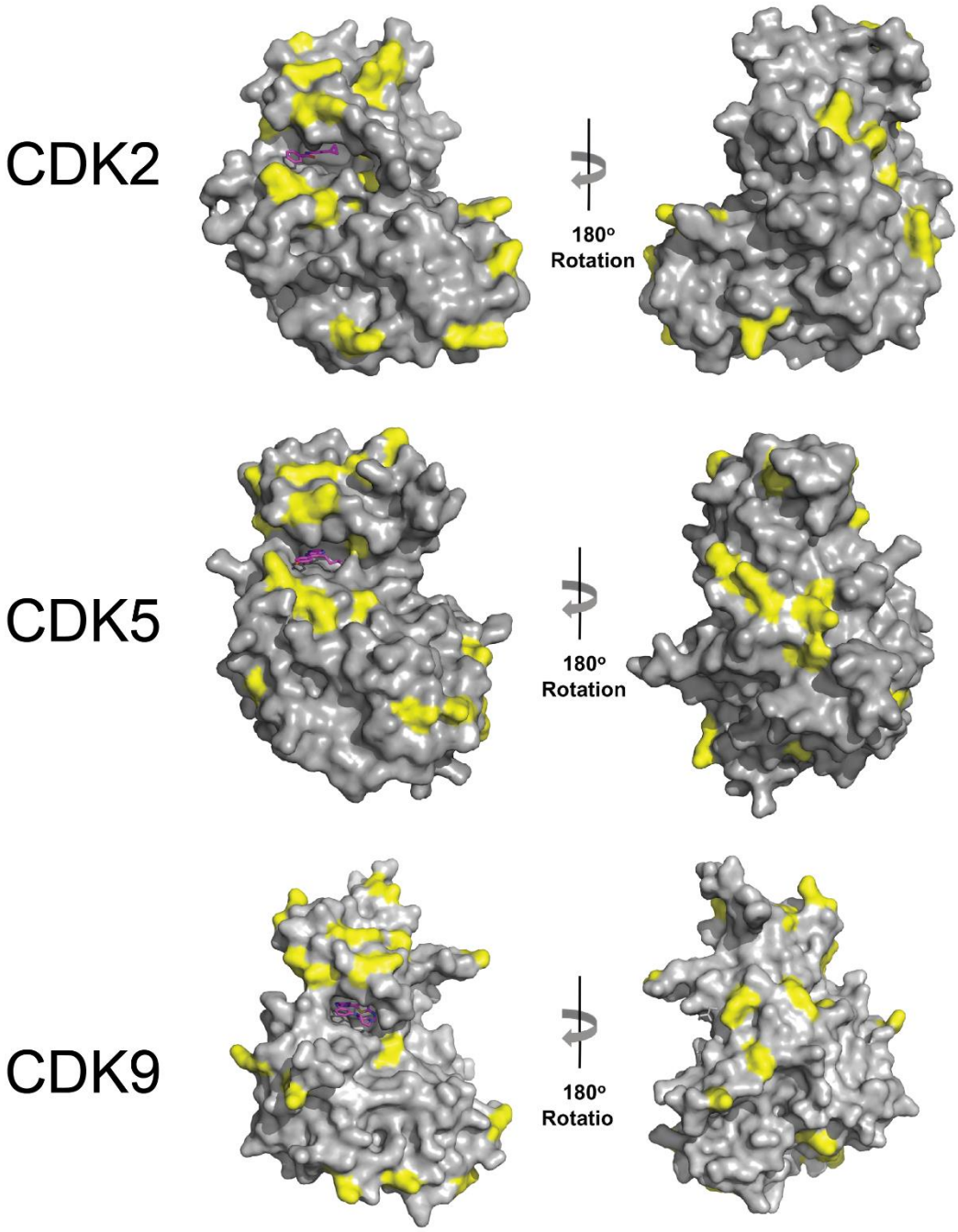


Figure 22: Distribution of surface-exposed lysine residues (yellow) for CDK2 (PDB id 1VYZ), CDK5 (PDB id 1UNG) and CDK9 (PDB id 4BCG). Inhibitor is shown in magenta.

We designed a focused library of aminopyrazole analogs and screened for CDK activities to identify selective inhibitors [138]. Structure-activity relationship (SAR) studies revealed that the para position on the phenyl ring that is surface exposed is amenable to substitution without loss of activity. To explore this position with a linker to conjugate thalidomide, we generated compounds **1** and **2** (**Figure 23**). HCT116 cells were treated with 10 μM of **1** and **2** for 6 hours and probed for CDK2, 5 and 9 levels and the phosphorylation state of their substrates RB, FAK and RPB1 respectively. We observed the inhibition of phosphorylation of FAK and RPB1 indicating selectivity for CDK5 and CDK9 over CDK2 (**Figure 24**). Based on this observation, we synthesized degrader **3** by conjugating **2** and thalidomide (**4**) (**Figure 23**)**,** which was previously demonstrated as a bona fide ligand for cereblon/Cullin4A E3 ubiquitin ligase [157].

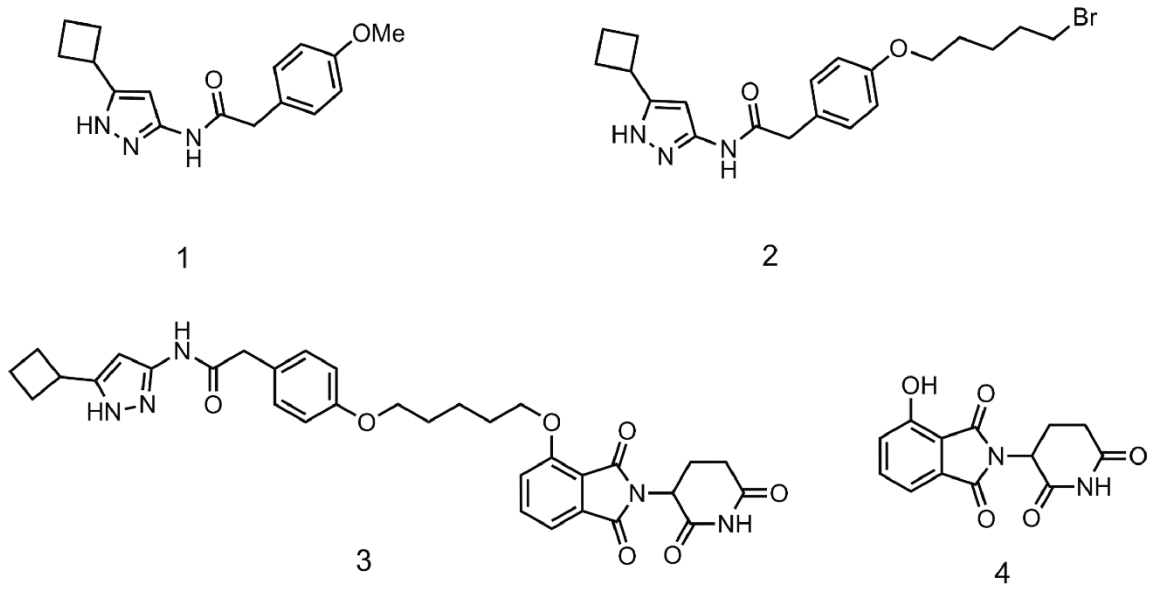


Figure 23: Structures of aminopyrazole inhibitors (**1** and **2**), PROTAC degrader (**3**) and thalidomide (**4**).

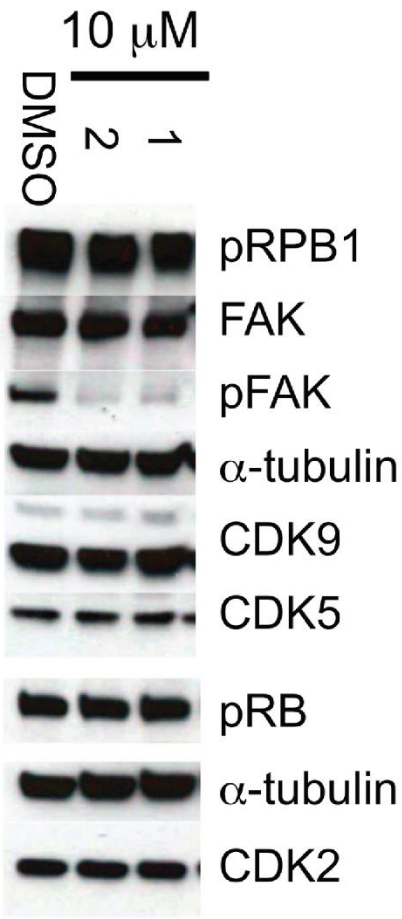


Figure 24: Effect of the aminopyrazole inhibitors **1** and **2** on CDK2, CDK5, and CDK9 expression and activity.

To determine if **3** is a CDK degrader, we treated HCT116 cells with increasing concentrations of **3** for 6 hours and the lysates were subjected to western blot analyses with antibodies for a panel of 6 kinases (**Figure 25A**). Thalidomide analog (**4**) and the inhibitor (**2**) were included as controls in this assay. Remarkably, **3** degraded CDK9 in a dose dependent manner. Inhibitor (**2**) and thalidomide analog (**4**) showed no effect on the levels of any of the kinases (**Figure 25A**). Interestingly, CDK2 and CDK5 levels and the levels of the other kinases (IKKβ, Akt and FAK) were unaltered, suggesting selective CDK9 degradation (**Figure 25A**). Given the high concentrations of **3** used in these studies, the lack of degradation of CDK2, CDK5, FAK, Akt and IKKβ could be attributed either to the inability to form the ternary complex due to steric hindrance or the absence of a proximal lysine residue that can be ubiquitinated.

RPB1 is a direct substrate of CDK9; therefore, we probed the membrane for the phosphorylation status of Ser2 on RPB1 using a phosphospecific antibody. Consistent with CDK9 inhibition and degradation, we observed inhibition of phosphorylation with **2** and **3** respectively (**Figure 25B**). Since CDK9 activity regulates the levels of pro-survival protein Mcl-1 [166, 167], we investigated the effects of **2** and **3** on Mcl-1. As anticipated, we observed a dose-dependent decrease in Mcl-1 levels with **3** and reduction of Mcl-1 levels with **2** (**Figure 25C**). Quantification of the western blots showed that at 10 and 20 μM degrader **3** reduced the levels of CDK9 by ~56% and ~65%, respectively (**Figure 25D**). In order to determine if the degrader inhibits CDK5, we conducted an *in vitro* kinase assay and show that **3** indeed inhibits the kinase activity of CDK5 (**Figure 25E**). It is important to note that although inhibitor **2** and degrader **3** have similar effects on the kinase activity of CDK9 (**Figure 25B**), the effect of the degrader **3** on the Mcl-1 levels is more pronounced than the inhibitor **2** (**Figure 25C**). This suggests the existence of a potential kinase-independent function of CDK9 associated with the regulation of Mcl-1.

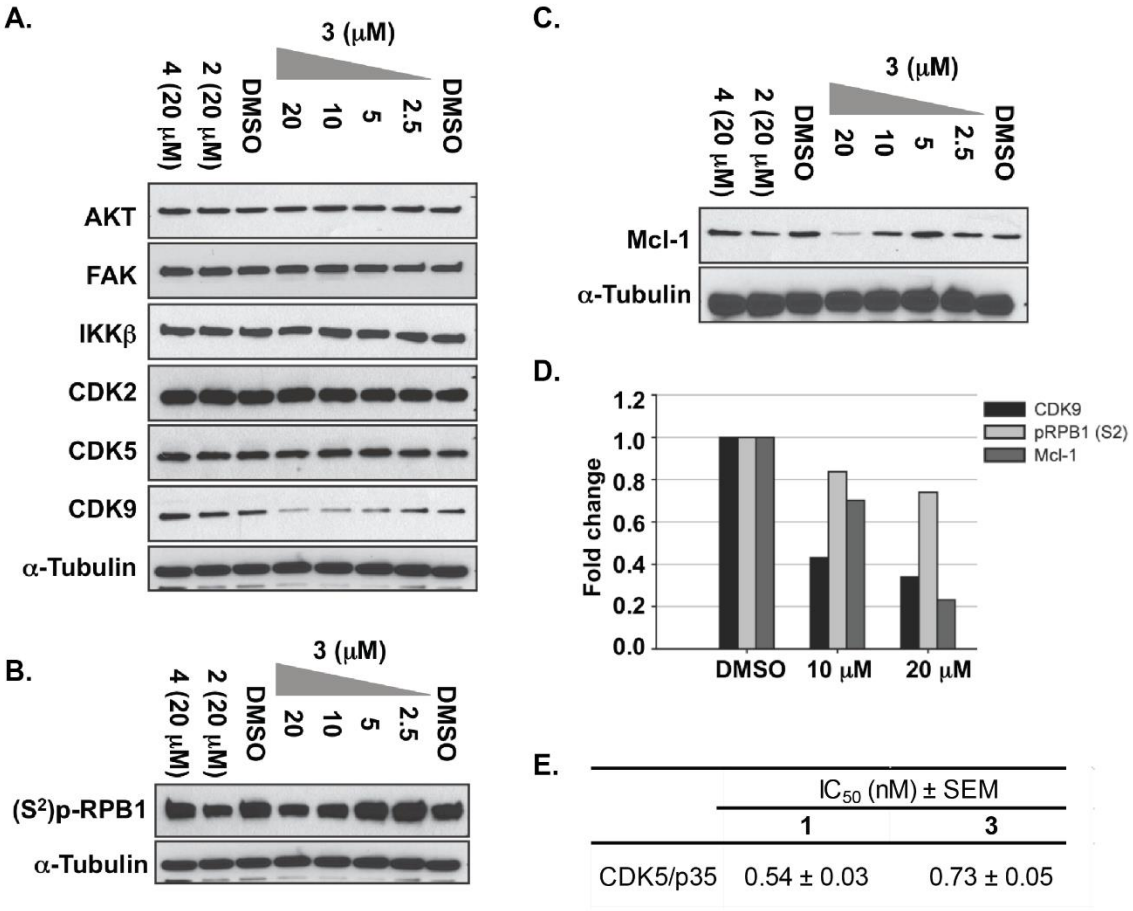


Figure 25: Effect of the aminopyrazole inhibitor (**2**), thalidomide (**4**) and PROTAC (**3**) in CRC cells A) Western blot analyses of a panel of kinases with lysates generated from HCT116 cells treated with **2**, **3** and **4**. B) The effect of **2**, **3**, and **4** on phosphorylation status of RPB1. C) The effect of **2**, **3**, and **4** on Mcl-1 levels. D) Quantification of CDK9, *p*-RPB1 and Mcl-1 levels at 10 and 20 □M. E) *In vitro* kinase assay for CDK5.

Discussion

In the study outlined above, we set out to investigate whether degradation of CDK5 could be a potential therapeutic strategy for the treatment of colorectal cancer. Utilizing the new PROTAC concept, which manipulates the UPS system, we developed a chemical tool comprised of an aminopyrazole ligand that inhibits CDKs, conjugated to a thalidomide ligand that interacts with cereblon. Contrary to our original intent, our heterobifunctional molecule facilitated the formation of a ternary complex, which consisted of a non-covalent linkage between CDK9 and cereblon (**Figure 26**). As such, our efforts resulted in the selective degradation of CDK9. Interestingly, analogs **1** and **2** inhibited CDK5 and CDK9 kinase activity, as evident by decreases in target substrate phosphorylation (pFAK-Ser732 and pRPB-Ser2, respectively). Of note, CDK2 was unaffected by treatment with **1**, **2** or **3.** We also showed that while the degrader (**3**) is capable of binding to and inhibiting the kinase activity of CDK5, it did not result in its degradation (**Figure 27**). These observations led us to multiple theories. First, we postulate that the structure or composition of our PROTAC molecule (**3**) makes it unable to bind or interact with the ATP binding pocket of CDK2, as we saw no signs of inhibition or degradation. Second, CDK5 likely does not have accessible surface-exposed lysine residues that are reachable by the E3 ligase, which is bound by the PROTACT molecule. Thus, when the PROTAC molecule binds to CDK5, the length of the linker is not optimal to bring cereblon into an appropriate position to ubiquitinate CDK5 and trigger its removal. Additionally, CDK5 and CDK9 must have differentially displayed surface lysine residues, as the degrader was capable of selectively degrading only CDK9. Collectively, our study suggests that selective CDK degraders can be developed by exploiting differentially displayed surface lysine residues.

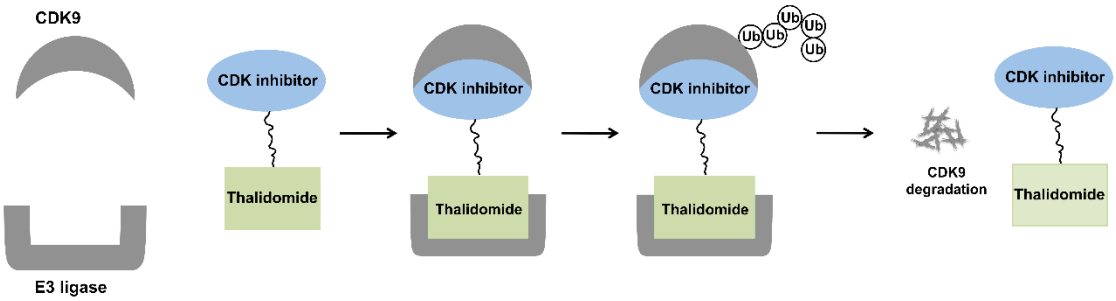


Figure 26: CDK9 PROTAC mechanism of action.

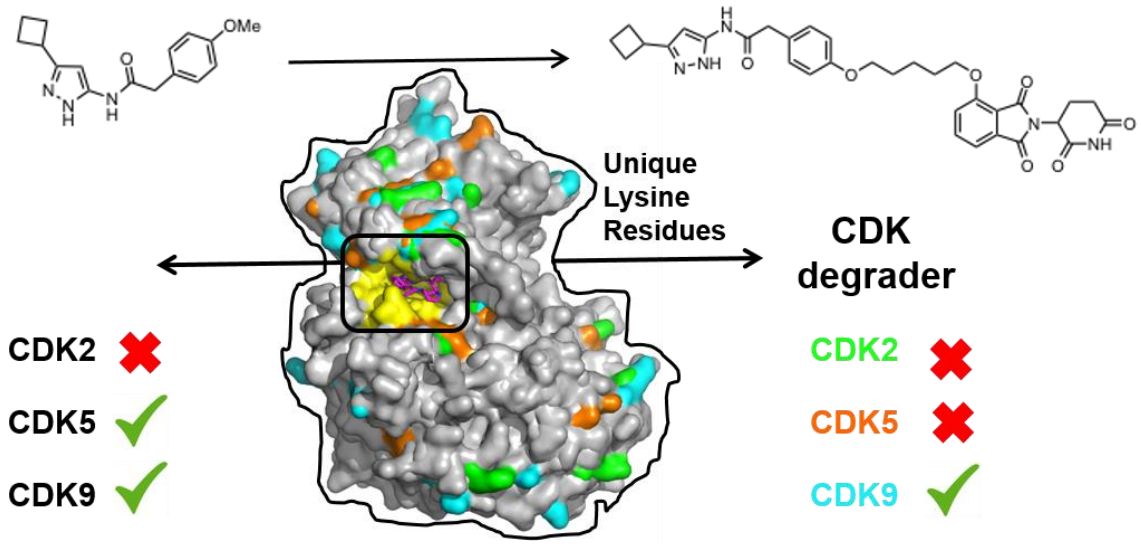


Figure 27: Differentially exposed lysine residues and PROTAC (**3**) mechanism of action.

In this study, we generated a novel tool (**3**) that can be used to dissect the role of CDK9 in various diseased states. CDK9, similar to other members of the family, has also emerged as a potential therapeutic target [115, 168-174]. It is one of the few members of the family that are important for transcriptional control [175, 176]. CDK9 is found in two different isoforms, the major isoform being 42 kDa, while the minor isoform is 55 kDa [177, 178]. These two forms of CDK9 have been shown to have different localization and expression, which suggests the possibility for distinct functions [177, 178]. Both isoforms require the binding of Cyclin T to become active and have full catalytic ability [115, 179]. CDK9/Cyclin T, along with other components, forms the positive transcription elongation factor b (P-TEFb) which is an important mRNA regulatory complex [115, 180]. Once becoming active, CDK9 is able to phosphorylate downstream substrates, particularly the C-terminal domain (CTD) of RNA polymerase II (also known as RNAPII or RPB1), an essential component of transcriptional control [115, 179]. When CDK9 phosphorylates Ser2 on RNAPII, it now allows for the mRNA transcript to be elongated, thus CDK9 is required to create mature mRNA [179]. Importantly, CDK9 has been shown to regulate the transcription of critical regulatory genes, such as Mcl-1 [181]. Mcl-1 is an important anti-apoptotic protein that has also been implicated in a variety of malignancies [182-184]. As such, CDK9 has emerged as a potential therapeutic target. Thus far, the most promising therapeutic strategy to abrogate CDK9 activity has been through the generation of ATP-competitive inhibitors. Multiple CDK9 non-selective inhibitors have shown promise in pre-clinical studies and have progressed into clinical trials [115]. Flavopiridol and dinaciclib are the two inhibitors triggering the best response in clinical trials. While neither compound is considered selective, both are potent inhibitors of CDK9 activity [115, 179]. Unfortunately, both compounds underperformed in clinical trials, as there were few reports of complete response and many patients (approximately 75-95%) suffered from adverse side effects [179, 185-189]. It is believed that the “off-target” effects spanning from

inhibition of other members of the CDK family are likely to blame for the toxic effects [168, 179]. However, both compounds did signify that inhibition of CDK9 is a favorable therapeutic avenue to pursue. Thus, our selective CDK9 degrader could serve as a tool to dissect the role of CDK9 in a variety of malignancies and is a promising lead compound to pursue further.

Moreover, due to the fact that degrader **3** perturbed Mcl-1 levels, we could pursue therapeutic combination strategies that incorporate Bcl-xL inhibitors, such as those developed by Abbot Laboratories (ABT-263 and ABT-737). Multiple reports have shown that the combined loss of Bcl-xL and Mcl-1 activity results in robust apoptosis *in vitro* [190-192]. Additionally, we show blocking these two critical anti-apoptotic components results in synergism in cancer cells and mouse xenograft models [138, 193]. Finally, increases in expression of Mcl-1 have been observed upon blockade of Bcl-xL, thus suggesting that Mcl-1 is a crucial player in resistance [138]. Collectively, these studies support the rationale to combine our degrader (**3**) with Bcl-xL inhibitors.

CHAPTER 5: DISCUSSION

Central Theme

The overall goal of this dissertation was to investigate the inhibition of CDK5, using aminopyrazole analogs, as small molecule kinase inhibitors, as a method for colorectal cancer therapy. As many individuals are diagnosed and succumb to the disease each year, it is vital that the development and investigation of new therapeutic options be at the forefront of CRC research.

Current Standing

The work throughout this dissertation has focused on characterizing inhibitors that perturb CDK5 activity. We validated a selective CDK5 inhibitor (CP-668863) that was originally designed, synthesized, and investigated by Pfizer. CP-668863 was intended to be used against neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, due to the role that CDK5 signaling plays in the central nervous system. While CP-668863 failed to effectively perturb CDK5 in the CNS setting, we chose to repurpose this compound for CRC therapy.

In order to determine whether CDK5 inhibition is a promising therapeutic strategy to combat CRC, we began our studies by characterizing the Pfizer CDK5 inhibitor, with the intent to eventually improve upon the compound (via SAR studies) if proven to be effective. Chemists in the Natarajan lab synthesized CP-668863 (a.k.a. 20-223) and we characterized its efficacy using cell-free kinase assays, human CRC cell lines, and a xenograft mouse model. Currently, we have shown that 20-223 effectively inhibits CDK2 and CDK5 in CRC systems. It reduces CRC cell migration and proliferation and causes cell cycle arrest. 20-223 also slowed tumor progression in an *in vivo* xenograft model. Importantly, 20-223 is comparable to, or in some cases significantly better than, clinically

evaluated CDK inhibitors, AT7519 and roscovitine, which serve as good benchmarks for advancing a compound through pre-clinical investigations. Collectively, we showed that using small molecule aminopyrazole analogs to inhibit the kinase activity of CDK5 is a viable therapeutic strategy.

Subsequently, we attempted to identify a degrader of CDK5 using the novel PROTAC strategy. While our efforts resulted in the selective degradation of CDK9, as opposed to CDK5, our proof of concept study served as a pioneering effort to show that selective degradation of a member of CDK family is indeed possible. We can now improve upon our efforts not only to inhibit the kinase activity of CDK5 using ATP-competitive small molecule aminopyrazole analogs, but we can also continue our efforts to develop CDK5 degraders. Both strategies are potential routes to combat CDK5 activity, and interfering with CDK5 activity is believed to have therapeutic benefit for CRC patients.

Future Directions

Structure Activity Relationship

20-223

As mentioned previously, our intent was to determine whether disrupting CDK5 activity could serve as a viable therapeutic option for CRC patients. Efforts by Zhuang *et al.* uncovered CDK5's contributions to CRC tumorigenesis, thus implicating its role as a therapeutic target in CRC [81]. We have now shown that inhibition of CDK5, using an ATP-competitive small molecule kinase inhibitor, has therapeutic benefit. Our next task would be to perform SAR studies to generate the next generation of small molecule inhibitors to inhibit CDK5. This study would have two main objectives: I) to improve upon the selectivity of 20-223 and II) to improve upon the potency of 20-223. We [137, 138, 194] and others [36, 37, 39, 40] have shown the aminopyrazole core engages in critical hydrogen bonds

with residues in the hinge region of CDK family members, specifically it interacts with Glu81 and Cys83 of CDK5. While these interactions are important to allow the compound to occupy the ATP binding pocket, structural modifications can be made on the ends of the aminopyrazole core (R1 and R2 positions) to improve the potency and selectivity of next-generation CDK5 inhibitors. These two positions extend off of the aminopyrazole core and are either embedded within the shallow hydrophobic pocket (R1) or solvent exposed (R2). Docking studies performed by our lab have shown that the cyclobutyl moiety of 20-223 is in the shallow hydrophobic pocket while the naphthalene is solvent exposed [137]. Follow-up studies would aim to enhance the efficacy of 20-223 while using it as a structural road map. We would work to generate a 3-aminopyrazole analog library with various functional groups at the R1 and R2 positions while keeping the aminopyrazole core constant. Small hydrophobic functional groups, of varying size (such as hydrogen, methyl, isopropyl, cyclopropyl, cyclobutyl cyclopentyl, or phenyl), would be used to probe the biological space of the shallow hydrophobic pocket. Bulky hydrophobic functional groups would be tested at the surface-exposed R2 position. While our goal is to identify novel new compounds, it is possible that we may again run into selectivity issues similar to those encountered with 20-223. The ATP-binding pockets of CDK family members are highly conserved. In fact, we have shown that CDK2 and CDK5 share approximately 80% homology when comparing the ATP-binding pockets [138]. One way to circumvent the selectivity dilemma may be to analyze the components within the ATP-binding pocket of CDK5 and compare it to other members of the family. Using this approach we would aim to identify unique residues that could then be exploited during structure-based design.

PROTAC

While ATP-competitive small molecule drugs have long since been a staple in kinase inhibition, the selectivity still remains an issue. As an alternative, we and others have begun to utilize the PROTAC strategy to facilitate the destruction of target proteins, as opposed to just inhibiting their kinase activity. In doing so, we attempted to generate a selective CDK5 inhibitor, but we ended up synthesizing a selective degrader of CDK9. We believe our degrader (**3**) was unable to access the surface-exposed lysine residues on CDK5. While the outcome did not satisfy our original intent, it gave us great insight into how to make a selective degrader.

We can now use degrader **3** as a lead compound, which can be modified to attempt our original goal of identifying a selective CDK5 degrader. As we reported previously, we know the degrader is fully capable of inhibiting the kinase activity of CDK5, however, binding did not result in destruction of the target protein. This is likely due to the fact that surface-exposed lysine residues are not in close proximity to E3 ligase to drive ubiquitination. Therefore, the PROTAC molecule was unable to bring the thalidomide ligand to the appropriate position to trigger the poly-ubiquitination that is necessary to initiate degradation. Furthermore, in addition to not triggering the degradation of CDK5, we also noticed that high concentrations of degrader **3** were necessary to initiate the degradation of CDK9 and inhibition of CDK5. This suggests there is room to improve the potency of the compound as well. Taken together, these observations suggest we should perform SAR studies to improve the potency and selectivity of the compound. We could potentially make 4 different structural modifications in order to accomplish our goal of generating a potent degrader of CDK5: (I) change the length of the linker; (II) change the composition of the linker; (III) modify the E3 ligase binding moiety; and (IV) modify the aminopyrazole moiety.

As mentioned previously, we believe that the surface-exposed lysine residues on CDK5 are not reachable by our current degrader (**3**). Thus, we can make structural modifications to our compound in order to optimize it for CDK5 degradation. One possible change to help trigger CDK5 degradation would be to modify the linker length to allow the thalidomide component of the PROTAC molecule to reach a shorter or longer distance. Chan *et al.* recently showed that changes in linker length may effect PROTAC efficacy [195]. Currently the linker length of degrader **3** spans 5 carbons. Preparing an analog library with varied linker length (*e.g.* spanning 2 – 20 carbons) may generate an optimal lead compound that now can reach the necessary surface exposed lysines and trigger their polyubiquitination and subsequent proteasomal degradation.

Second, we could modify the composition of the linker to make it more flexible or rigid. Currently, the 5-carbon linker serves as a flexible connection between the aminopyrazole and thalidomide ligands. This flexibility allows the PROTAC molecule to bend around the CDK structure and put itself in the right configuration to facilitate degradation. However, it is possible that changes in the linker composition may make it easier for the PROTAC molecule to bend or twist in a precise way that accommodates formation of the ternary complex.

Next, it is possible that modifying the E3 ligand moiety might enhance the efficacy of the degrader. In our current study, we utilized thalidomide as our E3 ligand of choice, however there are alternative ligands that can be used. Cereblon serves as the E3 ubiquitin ligase of choice for thalidomide [158], but cells also have another E3 ubiquitin ligase, VHL. VHL has been well characterized [196-198] and also used in PROTAC studies [148, 163, 199]. VHL is recognized by the VHL-ligand and facilitates proteasomal degradation similar to the cereblon-thalidomide route [200]. The use of both cereblon and VHL E3 ubiquitin ligands have shown efficacy in PROTAC studies. Lai *et al.* explored the

degradation profiles of several PROTACs by comparing various target ligands and E3 ligands [199]. While they ultimately showed that VHL outperformed cereblon when conjugated to the same BCR-ABL inhibitor [199], there is no evidence suggesting that VHL consistently performs better than cereblon. Therefore, the use of either cereblon or VHL-mediated proteasomal degradation should be determined on a case-by-case basis. As such, we could perform comparison studies using both E3 ligases (cereblon-thalidomide vs. VHL-VHL ligand) to determine which pairing proves to enhance the selectivity or potency of our degrader (**3**) compound.

We and others have shown that the aminopyrazole core is a privileged scaffold. Making modifications to the R1 and R2 substituents of CP-668863 yielded a 40-aminopyrazole analog library which was screened for efficacy against members of the CDK family. That study found that placing a cyclobutyl substituent at the R1 position that would interact with the shallow hydrophobic pocket, and a biphenyl ring at the solvent-exposed position, yielded a potent CDK (2, 5, and 9) inhibitor [138]. Thus, we kept the cyclobutyl of our degrader constant and substituted the biphenyl with a phenyl group attached to an oxygen to connect the 5-carbon linker and generate our PROTAC molecule (**3**). However, this compound (**3**) required μM concentrations to degrade CDK9. Therefore, we could make structural modifications at the R1 and R2 position of the degrader to potentially improve the potency and selectivity of a next-generation degrader.

Collectively, the proposed SAR studies mentioned above aim to generate potent molecules that will perturb CDK5 activity, and thus serve as potential therapeutic options for CRC patients.

Combination Strategies

The next phase of investigating whether the inhibition of CDK5 would be a viable therapeutic strategy for CRC would be to identify potential combination approaches that may enhance the efficacy of CDK5 inhibition. CDK5 inhibition alone is not likely going to serve as a fix all for CRC patients. However, it could serve as an additive target therapy, which acts in combination with other therapeutic options. To start, we could investigate potential combinations of 20-223 or next-generation compounds with chemotherapy. CRC patients often receive a complex cocktail as standard therapy. While standard of care chemotherapy provides a survival benefit to early stage CRC patients, additive therapy is often required for individuals to combat late stage disease [6]. Multiple reports have suggested that CDK5 may contribute to cancer therapy resistance and consequently, the addition of anti-CDK5 therapy enhances the effect of other cytotoxic agents. Multiple groups have observed an increase in CDK5 or activator (particularly p35 and p25) expression following treatment with chemotherapeutic agents [79, 102-104]. This suggests that CDK5 may be a driving force of resistance to cancer therapy, thus abrogating CDK5 activity on top of chemotherapies could have a potentially beneficial effect for cancer patients. In fact, some groups have even shown that inhibition of CDK5 sensitizes cancer cells to chemotherapeutic treatments [76, 102, 106]. Of importance, multiple reports have shown that knockdown of CDK5 enhanced the effects of 5-fluorouracil [74] and irinotecan analogs [76], both of which are part of the standard of care chemotherapeutic regimen that most CRC patients receive [7]. Collectively, these data suggest that inhibition of CDK5 on top of chemotherapeutic agents may prove to be a promising therapeutic strategy that we could explore for CRC patients.

Another prospective route to explore for combination therapies may be to combine immunotherapy with CDK5 inhibition. Recently, CDK5 was shown to serve as a major

roadblock to the immune eradication of medulloblastoma tumor cells [201]. Programmed death ligand-1 (PD-L1) is overexpressed in a variety of cancers [202] and serves as a key facilitator of immune evasion [203]. Expression of PD-L1 allows tumor cells to suppress the anti-tumor immune response and block the eventual cell death cascade that is triggered by T cells [204]. T cells are important surveyors in the tumor microenvironment. When they identify a cell as foreign, they trigger an immune response to destroy the unrecognizable cells. Thus they serve as important players in host immunity [205]. Unfortunately, CDK5 helps tumor cells evade circulating T cells by driving the indirect expression of PD-L1 [201]. CRISPR-Cas9 mediated knockout of CDK5 resulted in decreased PD-L1 expression and tumor incidence [201]. Presumably, the inhibition of CDK5 using small molecule inhibitors should result in a similar response. A critical step would be to first prove that CDK5 does in fact increase PD-L1 expression on CRC cells. Assuming this does occur, inhibition of CDK5 could be an interesting approach to counteract immune evasion. Additionally, CDK5 small molecule inhibitors or degraders may prove to be synergistic with other immunotherapeutic compounds. At this point, the strategy would be to block multiple immune checkpoints with the hopes of inducing a strong immune response [206]. While this strategy may prove to be beneficial for some patients, it is possible that treatment with multiple immunomodulatory agents may be counterproductive and elicit a massive immune response, which could result in adverse effects. As such, it may be a better option to use CDK5 inhibitors in place of anti-PD-L1 therapies, since it will presumably have a similar anti-immune effect, while also disrupting other catastrophic CDK5 functions. Either way, we would anticipate that treatment with CDK5 modulators (such as 20-223, second-generation analogs, or CDK5 degraders) may aid in immune destruction, either as a combination with other immunotherapies or as an additive therapy.

Finally, it is highly likely that we have yet to uncover the multitude of compounds that may prove to synergize with anti-CDK5 therapies. Therefore, it may be wise to perform a synthetic lethality screen to identify other agents that would be effective in combination with CDK5 blockade. This particular tactic has been applied previously in the context of prostate cancer. CDK5 has been shown to be key player in the development and progression of prostate cancer [59, 67, 90, 95]; as such, researchers set out to identify preexisting compounds that would synergize with CDK5-deficient prostate cancer cells [207]. Ultimately, they discovered tilorone analogs exhibited great efficacy in CDK5-deficient prostate cancer cells, thus identifying this particular subset of compounds as agents that may enhance the efficacy of CDK5 inhibition [207]. A similar approach may be used to find compounds that would synergize with CDK5 modulators in CRC. We could screen chemical libraries (*e.g.* Selleckchem, Cloud library, NCI-approved oncology drug set IV, FDA-approved drug collection, NCI-mechanistic set, etc) in genetically modified cell lines that lack functional CDK5 activity. The CRISPR-Cas9 system could be used to develop CDK5 knockout or CDK5 loss-of-function mutant (D144N) [64, 71] cell lines. Any combination in which the chemical inhibitor enhanced the genetic loss of CDK5 would be considered a lead and would warrant further investigation to determine whether it could be synergistic with CDK5 inhibitors (20-223 or second-generation analogs). Of note, one caveat to this approach is that currently there are no specific CDK5 inhibitors, therefore the outcomes from the CDK5 inhibitor screen may differ from the original results observed in the CDK5 knockout screen, *i.e.* the inhibitors may hit additional targets and elicit various responses. Thus, an alternative strategy would be to first identify CDK5 selective inhibitors (i.e 20-223, second-generation inhibitors, *etc.*) and then perform an RNAi screen (Dharmacon, GeneScript, Sigma-Aldrich, *etc.*), to identify potential targets that would be synergistic with CDK5 inhibition. Next, assuming there are specific inhibitors available for

the targets we identified, we could then test these compounds for synergistic effects when used in combination with CDK5 inhibitors.

While our studies were the first to show that inhibition of CDK5 may hold promise as CRC therapy, there is definitely still room to improve upon and enhance the effects of anti-CDK5 therapies. In any of the cases proposed above, extensive preclinical studies are required to understand drug-drug interactions and to ensure they warrant continued pre-clinical and eventually clinical investigation. Additional studies that would identify various patient populations that would benefit from these combinations would also be useful.

Broad Implications

Scientists have dedicated time, energy, and effort to uncover disease drivers that promote tumorigenesis and ultimately validate them as therapeutic targets [208]. Recently, massive commitment to basic cancer research has paved the way for the emergence of individualized or personalized medicine initiatives. As such, the focus of precision medicine is now geared toward understanding the specifics of each individual patient's tumor. The unique genetic signature that each tumor holds can give clinicians great insight into how the patients may benefit from various pharmacological agents. This allows physicians to tailor treatment plans to the specific genomic or proteomic makeup of patients, to maximize the therapeutic benefit [209]. Molecular profiling of CRC patients has improved the understanding of the genetic and epigenetic changes that cause the development and progression of CRC [6]. It is likely that CRC patients respond to therapies differently, due to the distinct molecular signatures of their tumors. With the knowledge that CRC is a very heterogeneous disease, both at the intertumoral and

intratumoral levels, it is increasingly important to identify and understand the specifics of each individual patient's tumor, in order to improve the selection of therapies [6].

Zhuang *et al.* identified CDK5 as a tumor promoter in CRC. Survival analysis revealed that CDK5 expression is inversely related to survival. Thus, those patients with high levels of CDK5 expression had significantly lower overall survival compared to individuals with low CDK5 expression [81]. From a personalized medicine standpoint, it would make sense to identify a specific patient population that would benefit from anti-CDK5 therapy. Ideally, those patients that are overexpressing CDK5, which is then likely contributing to disease progression, would receive additive anti-CDK5 therapy. This concept is not as easy as it sounds. Improvements will need to be made in the form of diagnostic testing and screening that have the capacity to easily understand the genetic makeup of various tumors so we can assure patients are receiving specific therapies for their specific conditions [209].

Importantly, overexpression of CDK5 is not specific to colorectal cancer. In fact, CDK5 has been implicated in a variety of malignancies including those of the breast [64, 74], liver [76], lung [66, 75], ovary [78], pancreas [70, 71], prostate [59, 67, 68, 95], thyroid [72, 73] and skin [77]. Hence, the strategy to inhibit CDK5 can also be applied widely to a variety of tumor types. In an era where personalized therapies are becoming the trend of modernized medicine [209], cancer patients with any form of CDK5-positive malignancy will greatly benefit from CDK5-directed therapy.

Final Conclusions

Shockingly, the World Health Organization predicts that the incidence (77%) and number of CRC-related deaths (80%) will increase dramatically by 2030 [5]. Therefore, there is a dire need to develop novel new therapies for the treatment of CRC. Evidence suggests that CDK5 plays an important role in the progression of CRC [81]. This observation led us to ask whether inhibition of CDK5 would be a promising therapeutic strategy to fight CRC. From the work in this dissertation, we can conclude that inhibition of CDK5 using a 3-aminopyrazole analog (20-223) showed anti-CRC effects both *in vitro* and *in vivo.* Furthermore, the success shown with 20-223 validates it as a promising lead compound and warrants its further investigation and optimization. While our attempts to degrade CDK5 using the novel PROTAC approach fell short, we ultimately demonstrated it is possible to selectively degrade another member of the oncogenic CDK family (CDK9). As with all cancer research, the ultimate goal is to eventually be able to improve patient survival. Our work serves as a foundation on which we can continue to build, in order to chemically inhibit an often overlooked but critical oncogenic player, CDK5. Our efforts to characterize a preliminary CDK5 inhibitor and attempt CDK5 degradation serve as the initial findings needed to encourage the continued investigation into inhibition of CDK5 as a viable method for cancer therapy.

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