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Perturbing anti-apoptotic proteins to develop novel cancer therapies

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by

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Abstract:

The apoptotic pathway involves a tightly regulated network of proteins which respond to various stimuli. Previous studies have indicated Mcl-1 and Bcl-xL are intimately involved in determining cell fate, and if both are concurrently neutralized, it activates the apoptotic pathway. The inactivation of Bcl-xL and Mcl-1 as a mechanism to trigger the intrinsic apoptotic response can be used as a platform to develop therapeutic strategies to target cancer cells. The apoptotic pathway is largely dysregulated and often leads to therapy resistance in cancer cells. Although direct inhibitors of Bcl-xL have been developed and have advanced to clinical trials, development of direct Mcl-1 inhibitors have been elusive. Therefore, we aim to develop small molecule inhibitors (SMIs) that target signal transduction pathways that attenuate Mcl-1 function, expression, and stability. Our ultimate goal is to use these SMIs in combination with existing Bcl-xL inhibitors to achieve synergistic effects. Furthermore, we aim to expand our evaluation to include existing pre-clinical kinase inhibitors and identify synergistic combinations of inhibitors that target either the Mcl-1 arm or the Bcl-xL arm of the apoptotic pathway, with the rationale that their combined inhibition is necessary for the initiation of programmed cell death (PCD). To develop SMIs we have evaluated compounds using the aminopyrazole scaffold which targets cyclin dependent kinases (CDKs). CDKs have been shown to positively regulate Mcl-1 and, to some extent, Bcl-xL. The aim of this dissertation is to develop CDK inhibitors which indirectly target Mcl-1 and identify kinase inhibitor combinations which target Mcl-1 and Bcl-xL.

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List of Abbreviations

Akt- protein kinase B

Apaf-1- apoptotic peptidase activating factor 1

ATP- adenosine tri-phosphate

BAD- Bcl-2-associated death promoter

BAK- Bcl-2 homologous antagonist killer

BAX- Bcl-2-associated X

Bcl-2 B-cell lymphoma 2

Bcl-w- Bcl-2 like protein 2

Bcl-xL- B-cell lymphoma extra large

BH- Bcl-2 homology domain

BID- BH3 interacting-domain death agonist

BIM- Bcl-2 interacting mediator of cell death

BTK- Bruton's tyrosine kinase

CDK- cyclin dependent kinase

CI- combination index

CLL- chronic lymphocytic leukemia

CRC- colorectal cancer

CRISPR- clustered regularly interspaced short palindromic repeats

DMEM- Dulbecco's Modified Eagle's Media

ESCC- squamous esophageal cell carcinoma

FBS- fetal bovine serum

FOXO- forkhead box

GFP- green fluorescent protein

GI- growth inhibition

HSP- heat shock protein

IKKβ- Inhibitor of κB kinase

IκBα- inhibitor of κB

JAK- Janus kinase

KRAS- Kirsten rat sarcoma

Mcl-1- myeloid leukemia cell differentiation protein

MOMP- mitochondrial outer membrane permeabilization

mTOR- mammalian target of rapamycin

NFκB- nuclear factor κB

NMR- nuclear magnetic resonance

NOXA- NADPH oxidase activator

OMM- outer mitochondrial membrane

PCD- programmed cell death

PDAC- pancreatic ductal adenocarcinoma

PEST- proline, glutamic acid, serine, threonine sequence

PI3K- phosphatidylinositol-4,5-bisphosphate 3 kinase

RPMI- Roswell Park Memorial Institute media

SAR- structure activity relationship

SMI- small molecule inhibitor

STAT- signal transducers and activators of transcription

STR- short tandem repeats

TALENS- transcription activator-like effector nucleases

TRAIL- TNF-related apoptosis-inducing ligand

Chapter 1: Introduction

Pancreatic cancer remains the fourth leading cause of cancer related deaths in America with annual deaths exceeding 40,000 from an estimated 53,000 new cases while colorectal cancer remains the fourth deadliest in men and women [1]. Poor clinical outcome is observed in many patients with pancreatic cancer with the five year survival rate remaining at 8%. Pancreatic ductal adenocarcinoma (PDAC) is the most common and deadliest form of pancreatic cancer. Colorectal cancer (CRC), although more promising in the earlier detectable stages, poses more of a threat when diagnosed in the later stage with a five year survival rate of 66% [1]. Limited therapeutic options and late stage diagnosis has contributed to poor clinical outcome in both diseases. This has encouraged the development of multiple approaches to reduce tumor burden in late stage and suppress metastasis. Both diseases follow a relatively similar progression model with p53 mutation occurring early in the disease with subsequent KRAS mutation leading to more aggressive tumors which then metastasize [2, 3]. As a result of the mutations, deregulation of the apoptotic pathway is often observed [4, 5]. *This dissertation will discuss therapeutic strategies that target the apoptotic pathway*.

The apoptotic pathway relies on a tightly regulated network of proteins that determine the fate of the cell. Cell fate is determined at the mitochondria by Bcl-2 homologous antagonist killer (BAK) and Bcl-2-associated X protein (BAX) [6]. Recent studies have suggested that the conformational status of BAK and BAX are ultimately responsible for their localization to the outer mitochondrial membrane (OMM) [7]. However, changes in their conformation can be facilitated by binding of pro-apoptotic proteins [8]. Various stimuli prompt the oligomerization of BAX and BAK at the OMM and subsequently lead to mitochondrial outer membrane permeabilization (MOMP).

Perforation of the OMM by these complexes leads to the secretion of apoptotic stimuli from the mitochondria, specifically cytochrome-c [6]. Cytochrome-c leads to the formation of the apoptosome complex which ultimately initiates PCD [9]. The apoptosome consists of cytochrome-c, Apaf-1 and Caspase-9, an initiator caspase. Formation of the apoptosome leads to caspase 9 activation by self-cleavage and ultimately the cleavage and activation of effector caspases, caspases 3 and 7. Caspases 3 and 7 then cleave their downstream targets to trigger PCD. Oligomerization of BAK and BAX is the irreversible step to PCD. In summary, PCD is triggered as a result of the perforation of the OMM.

Multiple proteins are responsible for the assembly or inhibition of BAK and BAX oligomerization and can be classified to two distinct groups: anti-apoptotic (prevent BAK and BAX oligomerization) or pro-apoptotic (trigger BAK and BAX oligomerization) [9]. The family of proteins which are involved in PCD contain distinct α-helical domains which are termed Bcl-homology (BH) domains. Pro-apoptotic proteins contain either a single BH domain (BH3) or multiple BH domains (BAK and BAX). BH3-only proteins can be further sub-divided into two groups: activators and sensitizers. The specified designation depends on the role the BH3-only protein plays in the initiation of PCD. Activators directly bind and trigger BAK and BAX oligomerization while sensitizers weaken the effects of anti-apoptotic proteins. Anti-apoptotic proteins contain multiple BH domains (BH 1-4) and can prevent the oligomerization of BAK and BAX by directly binding to and preventing their oligomerization or by inhibiting the activator BH3-only proteins.

The rate-limiting step in the initiation of PCD is the oligomerization of BAK and BAX, which is regulated by the levels of anti-apoptotic proteins. Although all anti-apoptotic proteins can hinder the events leading up to PCD, previous studies have identified Mcl-1 and Bcl-xL as crucial inhibitors of PCD. Concurrent knock-down of Mcl-1 and Bcl-xL led to robust apoptosis in HeLa cells and later these findings were verified in HCT-116 cells,

suggesting Mcl-1 and Bcl-xL are the essential anti-apoptotic proteins [7, 10]. The BH-3 only proteins, which bind to either Bcl-xL or Mcl-1, were determined by BH-3-profiling [9]. BH3-profiling studies suggest NOXA selectively binds to Mcl-1 and BAD binds to Bcl-xL (as well as Bcl-2 and Bcl-w). BH3-only proteins BAD and NOXA that inhibit Bcl-xL and Mcl-1, respectively, can be used to design methods to either develop therapeutic strategies or identify novel inhibitors that preferentially attenuate either anti-apoptotic protein. These studies indicate that there are two arms that regulate the apoptotic pathway (NOXA-Mcl-1 and BAD-Bcl-xL). We can develop therapeutic strategies to target each one and when combined will result in synergistic induction of apoptosis (Figure 1A). The overall goal of this dissertation project is to identify SMIs that induce apoptosis by triggering either arm of the apoptotic pathway. Subsequently, we will identify combinations that exploit these two arms of the apoptotic pathway as novel therapeutic strategies.

Tools to Probe Mcl-1 and Bcl-xL in the Apoptotic Pathway

BH3 profiling is a tool to help delineate the role of BH3-only proteins as well as anti-apoptotic protein binders [11]. This method utilizes the BH3 domain of activator and sensitizer BH-3 only proteins in the form of peptides. These peptides are exposed to purified mitochondria and their ability to induce cytochrome-c release is gauged by colorimetric assays such as ELISA. These assays have helped describe the role of Mcl-1 and Bcl-xL and their binding BH3-only proteins. BH3 profiling suggests BH3-only proteins, which are activators are necessary for the induction of apoptosis [9]. Also, through BH3-profiling, several patterns can be identified that are specific to cell lines and can identify dependence of Bcl-2 family anti-apoptotic proteins. Finally, BH3-profiling established the “primed” state of cells in which their dependence on Bcl-2 family anti-apoptotic proteins such as Bcl-2, Bcl-xL, Mcl-1, Bfl-1 and Bcl-w is necessary for their survival [9]. The premise of this theory is that Bcl-2 family proteins sequester BH3-only activator proteins and

therefore BAK and BAX remain inactive. Blocking these interactions frees BH3 only activators such as BIM and BID (tBID) and the activators are free to induce BAK and BAX oligomerization. BH3-profling using BAD and NOXA in CLL cell lines in the absence of activators show that although all the Bcl-2 family proteins are blocked, little apoptosis by cytochrome-c release is observed. Only when activators are present, is apoptosis observed. This suggests that there have to be activators present to induce BAK and BAX oligomerization.

In contrast, siRNA knockdown of anti-apoptotic proteins shows that concurrent knockdown of Mcl-1 and Bcl-xL is enough to induce robust apoptosis, suggesting the anti-apoptotic proteins are the gatekeepers of BAK and BAX oligomerization. DNA-damaged cells were prevented from undergoing apoptosis by the presence of Bcl-xL, suggesting Bcl-xL is an essential block to apoptosis when DNA damage occurs. This result prompted the siRNA knockdown of other members of the anti-apoptotic Bcl-2 family proteins with Bcl-xL. Interestingly, Bcl-xL and concurrent Mcl-1 knock-down induced spontaneous apoptosis. The same study showed knockdown of BAD, BIM and NOXA protected cells against apoptosis in contrast to the previously mentioned BH3-profiling results where the same BH3 domains of each, failed to induce apoptosis. Also contrary to the previous study these studies show NOXA bound to Bcl-xL under the circumstance of DNA damage [10]. BH3 profiling used only peptides derived from the BH3-only proteins whereas these studies used siRNA knockdown and doxycycline inducible cell lines and looked at whole protein complexes. The difference observed are a testament to the differences observed when using two different approaches. However, both emphasize the same axis of Mcl-1 and Bcl-xL arms of the apoptotic pathway and how they clearly are essential blocks to its initiation.

To determine the absolute minimal requirement for BAK and BAX localization and oligomerization at the OMM, gene editing tools such as CRISPR and TALENS were used to generate octa-knockout HCT116 cells which lacked all eight BH3-only proteins [7]. This cell line was used to probe different anti-apoptotic proteins for their ability to block initiation of apoptosis. Interestingly, in the absence of all eight BH3-only proteins, knock down of Mcl-1 and Bcl-xL was still sufficient to induce apoptosis, suggesting the presence of pro-apoptotic BH3-only proteins is secondary to the neutralization of Bcl-xL and Mcl-1 in the induction of apoptosis. Furthermore, activators are not necessary for the oligomerization and localization of BAK and BAX to the mitochondria. More importantly, this study identified the reason for BAK and BAX localization and oligomerization at the mitochondria is motivated by changes in their conformation, particularly through α helix 9 (α9) interaction with the OMM. This study supports the need to target anti-apoptotic proteins, specifically Bcl-xL and Mcl-1, to induce apoptosis.

A tool that will be extensively used in the following studies were a kind gift from Dr. Luo. They developed doxycycline inducible HeLa cell lines, which overexpress either BAD3SA, NOXA, or GFP (Figure 1B). These cell lines were used to probe the two arms of the apoptotic pathway. HeLa Dox-NOXA cell line expressed NOXA when stimulated with doxycycline, NOXA then binds to and inactivated Mcl-1. This cell line can be used to identify inhibitors, which preferentially target Bcl-xL or upstream kinases that may control Bcl-xL activity, expression, and stability. HeLa Dox-BAD3SA expresses a mutant form of BAD in which three serine residues (S112, S136, S155) are mutated into alanine residues. These specific residues are crucial for the interaction of BAD with Bcl-xL [12, 13]. The dox-induced expression of BAD3SA leads to the inactivation of Bcl-xL, the second arm of the apoptotic pathway. HeLa Dox-BAD3SA cells are sensitive to SMIs, which preferentially target Mcl-1 directly or upstream kinases, which control Mcl-1 function, stability, or

expression. HeLa Dox-GFP are a control cell line which express GFP when stimulated with doxycycline. These cell lines can be used as tools to identify SMIs, which affect the apoptotic pathway. Although not as clean as gene editing methods such as siRNA knock-down or more novel methods such as CRISPR, using BH3-only proteins to inactivate Mcl-1 or Bcl-xL leaves their participation in protein complexes intact. We can then observe a more realistic effect when probing for SMIs which target either the Bcl-xL or Mcl-1 arm of the apoptotic pathway.

Other means of directly inhibiting Mcl-1 and Bcl-xL have been developed such as BH3 mimetic compounds or peptides [14-16]. Upregulation of anti-apoptotic proteins, specifically Bcl-xL and Mcl-1, is observed in cancer cells and is often observed when resistance to standard chemotherapy arises [17]. Although Mcl-1 and Bcl-xL inhibitors are designed to be used as treatment for leukemias, these direct inhibitors may be used as tools to probe the apoptotic pathway. Abbott laboratories has successfully developed small molecule BH3-mimetics to target Bcl-xL, Bcl-2 and Bcl-w [16, 18, 19]. These compounds were designed using the natural α-helical BH3-domain pro-apoptotic sensitizers as templates. BH3-only proteins can inhibit the function of anti-apoptotic proteins by competitively binding a groove that is occupied by the BH3 domain of BAK and BAX. BH3-only proteins can bind multiple anti-apoptotic proteins and their α-helical domain has been a model for the development of small molecule inhibitors. Fragment based SAR studies was used for the development of several lead compounds. ABT-737 and its orally bioavailable counterpart, ABT-263 as well as Bcl-2 inhibitor, ABT-199 have all stemmed from the BH3 domain of the BH3-only protein BAD. ABT-199, under the clinical name Venetoclax, has been approved for clinical use for the treatment of chronic lymphocytic leukemia.

Fragment based screening methods have been applied to identify SMIs that directly target Mcl-1 [14]. Although this method has been used successfully to develop Bcl-xL and Bcl-2 inhibitors, Mcl-1 direct inhibitors are more challenging due to the relatively shallow Mcl-1 hydrophobic cleft to which the BH3 α-helix binds [20]. As a result, few inhibitors have been developed to directly inhibit Mcl-1. One particular inhibitor, A-1210477, has been developed by Abbvie using fragment based screening methods [21]. A-1210477 displaces the activator BH3-only protein, BID as well as sensitizer NOXA from Mcl-1 and induces apoptosis. However, A-1210477 has yet to advance to clinical trials. Other direct inhibitors have been developed that aim to mimic the BH3 α helix [22]. However, other means of inhibiting Mcl-1 have been explored, for example stapled peptides derived from its binding partner NOXA have been explored as possible inhibitors Mcl-1 [23]. Obstacles in delivery have hindered the advancement of stapled peptides to the clinics. Finally natural product derivatives, which target Mcl-1 have also been explored [24, 25].

Resistance to the Bcl-xL inhibitors has been observed in several cancer types that over-express Mcl-1 [26-29]. Furthermore, Mcl-1 upregulation occurs in cells treated with ABT-737, Navitoclax (ABT-263) and Venetoclax, indicating the need for Mcl-1 inhibitors. Unlike Bcl-xL, Mcl-1 has a short half-life [30, 31]. Phosphorylation of the N-terminal PEST domain in Mcl-1 enhances its stability. Targeting upstream kinases that stabilize Mcl1, namely CDKs, has been a successful means of perturbing Mcl-1 activity. Rapid expression of Mcl-1 can also be targeted to disable its function. RNA polymerase II is a phosphorylation substrate of CDK9, CDK9 phosphorylation of the carboxyl-terminal domain (CTD), which is required for transcription elongation. Inhibition of CDK9 has been shown to limit Mcl-1 expression indicating CDK inhibition is a viable means to attenuate

Mcl-1 [32, 33]. CDK inhibition and its effects on Mcl-1 have been widely studied and the combination of pan-CDK inhibitors and Bcl-xL inhibitors are well documented [31, 34, 35].

Combinatorial Therapeutic Strategies that Target Mcl-1 and Bcl-xL

Combination strategies using targeted therapeutics is becoming an increasingly popular choice when searching for options to treat multiple cancer types [36]. Two reasons for the shift towards using targeted agents in combination is the availability of a wide variety of drugs that target oncogenic proteins and well-defined methods to stratify patient groups [37]. Synergistic combinations of drugs aim to have a greater anti-tumor effect at lower doses which results in reduced toxicity [38]. We can expect combination strategies which use targeted therapy to grow as a result of personalized medicine. With the increase in availability and variety of potent SMIs, and the advances in bioinformatics, catering to smaller cohorts of patients is becoming more of a possibility.

The most straightforward approach to exploiting the apoptotic pathway would be to use direct Bcl-xL inhibitors in combination with Mcl-1 inhibitors. ABT-263 is a BH3-mimetic SMI that targets Bcl-xL, and A-1210477 is a recently developed Mcl-1 SMI. A-1210477 shifted the sensitivity of Mcl-1 dependent cell lines to ABT-263 and Bliss additive analysis suggests that this combination is synergistic [39]. A similar study used A-1210477 to treat Venetoclax (ABT-199) resistant cell lines and sensitize them to Bcl-2/Bcl-xL inhibition [35]. These studies are the most recent which show that the combinatorial approach to directly inhibit Bcl-xL and Mcl-1 results in synergism. It is important to note, that a similar result observed when Flavopiridol was combined with Venetoclax in Non-Hodgkin's Lymphoma [35], wherein Mcl-1 down regulation was a result of CDK inhibition.

Mechanisms to perturb Mcl-1

IKKβ inhibition attenuated Mcl-1 expression

Previous studies have shown IKKβ inhibitors induce Mcl-1 down regulation [40-42]. These and other studies show that Mcl-1 levels are regulated by NF-κB. Studies performed in squamous esophageal cell carcinoma (ESCC) reveal an increase in Mcl-1 mRNA and protein levels when compared to immortalized esophageal cell line [42]. Furthermore, inhibition of the NF-κB pathway and mutation of NF-κB binding site resulted in decreased Mcl-1 expression [42]. Quinoxaline urea analogs that targeted IKKβ induced apoptosis in HeLa cells where Bcl-xL was inactivated by over-expression of BAD [41]. This Mcl-1 dependent mechanism of inducing apoptosis by IKKβ SMIs also suggests regulation of Mcl-1 by NF-κB. In subsequent studies evaluating the lead quinoxaline urea compound (13-197) in MiaPaCa-2 cells, Mcl-1 levels were shown to decrease in a time and dose dependent manner following treatment [40]. These results further demonstrated Mcl-1 is regulated by this pathway.

Mcl-1 regulation by cyclin dependent kinases (CDKs)

It is well known that Mcl-1 is regulated by cyclin dependent kinases [31, 32, 43]. Cyclin dependent kinases (CDKs) are dysregulated in multiple cancers and contribute to transformation [44, 45]. Hyper-activation of CDKs drive the transcription of genes necessary for proliferation and have been shown to contribute to enhanced migration. CDKs 1, 2, 3, 4, and 6 regulate cell cycle and if over-activated contribute to proliferation [44]. CDK 5 has been implicated in migration and has been studied extensively in neuronal diseases, although it has been gaining traction as a target in pancreatic and colorectal cancer [46-48]. CDK 7, 8, 9, and 11 regulate transcription and if dysregulated may lead to overexpression of oncogenic proteins [49]. Of these CDKs, CDK2, CDK5, and CDK9 has been implicated in the regulation of Mcl-1 expression, function and stability [31, 32, 43]. CDK2 phosphorylates Mcl-1 at T163 and stabilizes it by preventing ubiquitination. CDK9 regulates transcription of Mcl-1, which is vital to maintain the levels of short-lived proteins

in cells. Finally, CDK5 phosphorylates the negative regulator of Mcl-1, NOXA at S13 and prevents its anti-apoptotic function. These studies strongly support the attenuation of Mcl-1 also occurs through CDKs.

Combination therapy strategies, which incorporate Mcl-1 inhibition

Mcl-1 inhibition has been found to sensitize melanoma cancer cells to MEK1/2 inhibitors, lymphoid malignancies to PI3K/Akt/mTOR inhibitors and multiple cancers to Navitoclax [27, 50-53]. These responses are generally observed because overexpression of Mcl-1 has been attributed to resistance in multiple cancers. Mcl-1 overexpression has also been attributed to resistance to standard of care therapies such as gemcitabine, vincristine, and paclitaxel (Table 1). As a result, different combinations are currently under investigation that incorporate Mcl-1 inactivation, specifically with novel Mcl-1 direct inhibitor, A-1210477.

An alternative method to target Mcl-1 would be through CDK inhibition or through the NF-κB pathway as mentioned previously. These pathways contribute to Mcl-1 expression, function and stability through various mechanisms. By targeting these regulatory pathways in combination with pathways that regulate Bcl-xL or direct Bcl-xL inhibitors, we may identify novel synergistic combinations.

Mcl-1 attenuation through the HSP90/ JAK-STAT regulatory pathway has been shown to potentiate TRAIL-induced apoptosis in CRC [54]. This study demonstrates Mcl-1 regulation has a profound impact on the apoptotic pathway and may be targeted by indirect mechanisms. Direct mechanisms can also be used to target Mcl-1 and these direct inhibitors can synergize with Bcl-2 targeting BH3 mimetics. Non-Hodgkin's Lymphoma cells were sensitized to ABT-199 when Mcl-1 was inactivated by the direct inhibitor A-1210477 [35]. Mcl-1 indirect inhibition can also synergize with BH3 mimetics already in

clinical trials. mTOR inhibition has also been found to synergize with BH3 mimetic compound, ABT-263 (Navitoclax) in colorectal cancer by suppressing Mcl-1 [55]. Similarly, in lymphoid malignancies, Mcl-1 suppression by PI3K/Akt/mTOR inhibitors has been shown to potentiate Bcl-2 inhibitor ABT-199 [51]. These studies demonstrate the potential of identifying novel synergistic combinations that target the apoptotic pathway.

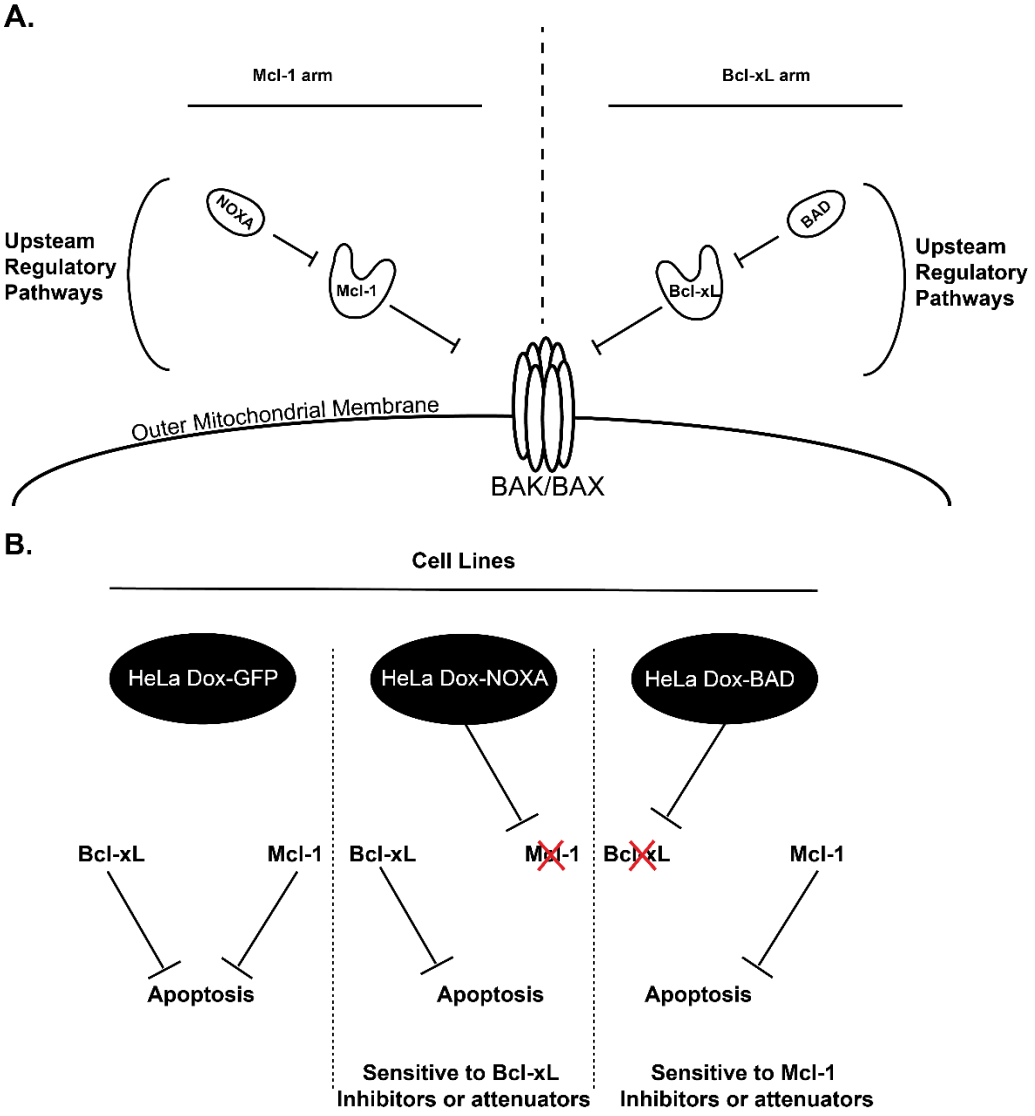


Figure 1. Strategies to probe the apoptotic pathway. **A,** the two arms of the apoptotic pathway. **B,** HeLa doxycycline inducible cell lines designed by Dr. Luo and his laboratory.

Table 1. Mcl-1 Overexpression and Resistance

Cancer Type	Reference
Lung	Hauck *et al*., 2009
Colon	Belmar, J, Fesik, S.W., 2014
Ovarian	Belmar, J, Fesik, S.W., 2014
Lymphoma	Konopleva *et al*., 2006; Moulding et al., 2000
Melanoma	Qin et al., 2006; Thallinger et al., 2003

Drug Resistance	Reference
ABT-263 (Navitoclax) ABT-737	Konopleva et al., 2006; van Delft et al., 2006; Tahir et al., 2007
Paclitaxel	Wertz et al., 2011
Vincristine	Wertz et al., 2011
Gemcitabine	Wertz et al., 2011

Chapter 2: Leveraging the Achilles heel in CDK drug discovery to target Mcl-1 for pancreatic cancer therapy

Introduction:

A common hallmark of cancer is evasion of cell death, which contributes to the persistence of pancreatic cancer and its resistance to chemotherapy [56]. Elevated expression of anti-apoptotic proteins observed in PDAC suggests the apoptotic pathway is dysregulated [57, 58] and targeting these proteins is a viable therapeutic strategy for PDAC [59, 60]

Apoptosis naturally obstructs cancer development under normal cellular conditions [6]. However, resistance to apoptosis is increasingly observed in cancer [61]. In many cases, the imbalance of pro-apoptotic and anti-apoptotic signaling drives malignant transformation [9]. The apoptotic pathway contains a tightly regulated network of proteins that control cell fate. A crucial event in the initiation of apoptosis is mitochondrial outer membrane permeabilization (MOMP) mediated by Bcl-2 homologous antagonist killer (BAK) and Bcl-2-associated X protein (BAX) [62]. Oligomerization of BAK and BAX at the mitochondrial membrane is the earliest, most critical and irreversible step in apoptosis. BAK and BAX activation is prevented by Bcl-2 family anti-apoptotic proteins [63]. Specifically, Bcl-xL and Mcl-1 have been shown to be essential for inhibition of apoptosis [7, 10]. Importantly, concurrent inactivation of both Bcl-xL and Mcl-1 resulted in robust induction of apoptosis in many cancer cells [7, 10, 41]. As a result, Bcl-xL and Mcl-1 are attractive therapeutic targets for inhibitor design [18, 39, 64].

Abbott Laboratories has successfully developed direct inhibitors of Bcl-xL/Bcl-2/Bcl-w (ABT-737 and ABT-263), the first drugs to target Bcl-2 family proteins [18, 64]. However, resistance to Bcl-xL inhibition has been observed and the resistance has been attributed to compensatory activity by Mcl-1 [29, 65]. Consistent with the above observations, studies have shown Mcl-1 inactivation sensitizes cancer cells to Bcl-xL inhibitors [53, 66, 67]. Although direct Mcl-1 inhibitors are currently in preclinical development, none have been approved by the FDA for clinical use [66].

Several members of the cyclin dependent kinases (CDKs) regulate the stability and expression of Mcl-1. An alternate strategy to target Mcl-1 would be through modulation of certain members of the CDK family [31, 32, 43]. Specifically, CDK2, CDK5, and CDK9 control Mcl-1 function and its levels through phosphorylation. For example, (a) Mcl-1 is stabilized through direct phosphorylation at Thr92, while phosphorylation of Thr163 by CDK2/cyclin E inhibits its degradation. Thr70 phosphorylation also induced ubiquitination and subsequent degradation [31, 68]; (b) CDK5 has been shown to phosphorylate the specific Mcl-1 antagonist, NOXA, thus allowing Mcl-1 to retain its anti-apoptotic activity [43] and (c) CDK9 phosphorylates RNA polymerase II carboxyl-terminal domain which activates transcription of Mcl-1 [32]. A testable hypothesis derived from the above observations is that a polypharmacological approach that targets CDKs, specifically CDK2, 5, and 9, will disable Mcl-1 function and serve as promising therapeutic strategy for pancreatic cancer. The polypharmacological

approach the targets multiple CDKs to indirectly inhibit Mcl-1 turns the Achilles heel of CDK drug discovery into an advantage.

Small molecule kinase inhibitors containing the aminopyrazole core have shown promise as CDK inhibitors [69, 70]. The aminopyrazole core is considered a privileged scaffold that targets the ATP binding site of CDKs by forming a triad of hydrogen bonds with the hinge region residues of the kinase. Recent work from our lab, characterized an aminopyrazole analog (CP-668863) and showed its anti-tumor efficacy in a colon cancer mouse model [71]. Using CP-668863 as a guide, we designed, synthesized, and evaluated a focused library to optimize two functional groups on the aminopyrazole core. This resulted in the identification of a potent polypharmacological agent, **24**, that targets CDK2, 5 and 9. The ability of **24** to inhibit CDK2, CDK5, and CDK9 was characterized using cell-free and cell-based assays. Follow up studies showed that **24** induced apoptosis in a Mcl-1 dependent manner. Combination studies with **24** and Bcl-xL inhibitors (ABT-737 or ABT-263) resulted in strong synergism. Importantly, in a PDAC xenograft model, the combination of **24** and ABT-737 was remarkably potent. Collectively, these findings support the development / evaluation of polypharmacological agents that target CDK2, 5, and 9 as indirect Mcl-1 modulators for pancreatic cancer therapy.

Materials and Methods

Pancreatic Cell Lines. S2-013 cells were culture in RPMI-1640 medium (HyClone #SH30027.01). AsPC1, BxPC3, MiaPaca2, and SUIT2 cells were cultured in DMEM high glucose medium (HyClone #SH30022.01). All cell lines were supplemented with 10% FBS (Gibco by LifeTechnologies #26140-079) and 1%

Penicillin-Streptomycin (HyClone # SV30010) and cultured at 5% CO_2 at 37°C. STR profiling has been performed and compared to ATCC to confirm validity of each cell line .

Hela Cell Lines. Inducible Hela Dox, Hela BAD3SA and Hela Noxa cells were a gift from Xu Luo (University of Nebraska Medical Center) [10]. Cells were cultured in DMEM high glucose medium (HyClone #SH30022.01) supplmenented with 10% FBS (Gibco by LifeTechnologies #26140-079) and 1% Penicillin-Streptomycin (HyClone # SV30010) and cultured at 5% CO_2 at 37°C. Cells were treated with 1µg/mL of doxycycline to induce the expression of GFP, BAD3SA, or Noxa,

Western Blot Analyses. Cells were washed with cold 1xPBS and scraped before being lysed by a buffer comprised of 50mM Tris, 100mM NaCl, 1% NP-40, 2nM EDTA, 20% SDS, 20xPPI (Na_3VO_4, NAF, β-glycerophosphate) and 1mmol/L PMSF. Samples were incubated on ice for 30 minutes and vortexed in 15 minute increments. Samples were centrifuged at 14,000rpm for 10 minutes at 4°C and supernatant was collected. Protein quantification was determined by BCA Protein Assay (Pierce #23225). 20-40ug protein samples were run on 4-15% gradient gels (BioRad) in 1x TRIS-Glycine-SDS Buffer (Research Products International Corporation #T32080) at 90V for ~90 minutes and separated by SDS-page electrophoresis. Samples were transferred to a PVDF membrane by semi-dry transfer method (ThermoScientific #35035) run at 18V for ~35 minutes. Membranes were blocked in 5% milk diluted in 1x-Tris Buffered Saline with 0.1% Tween (1xTBST) for 1 hour at room temperature rocking at low speed. Primary antibodies were diluted in 5% milk in 1xTBST and were rocked gently overnight in

4°C. Membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature while gently rocking. 3 washes (10 minute) with 1xTBST occurred before and after secondary antibody. ECL Prime (GE Healthcare #RPB2236) was used to detect protein expression.

Cell Viability. Cells were plated at 4,000 cells/well in a 96-well plate and allowed to adhere overnight. Next day, cells were treated with compounds (21, 24, and 25) using 2-fold dilutions starting at 2μM. PrestoBlue reagent (Invitrogen #A13262) was added to cells after 72 hour drug incubation to assess the growth inhibition. Fluorescence excitation/emission was measured at 560/590nM using SpectraMax M5^{e} instrument. Growth inhibition was calculated using 100-[100*(Samples-T0)/(T100-T0)]. T0 is the vehicle control reading immediately following drug addition and T100 is the control reading at the end of 72 hour incubation.

Statistical Analyses. Graphs were generated using SigmaPlot 11.0. Student's t-tests were used to determine significance between two groups. $p<0.05$ was considered significant. Combination Index (CI) values were determined by CalcuSyn 2.11.

Results

Bcl-xL inhibition upregulates Mcl-1 in PDAC cells

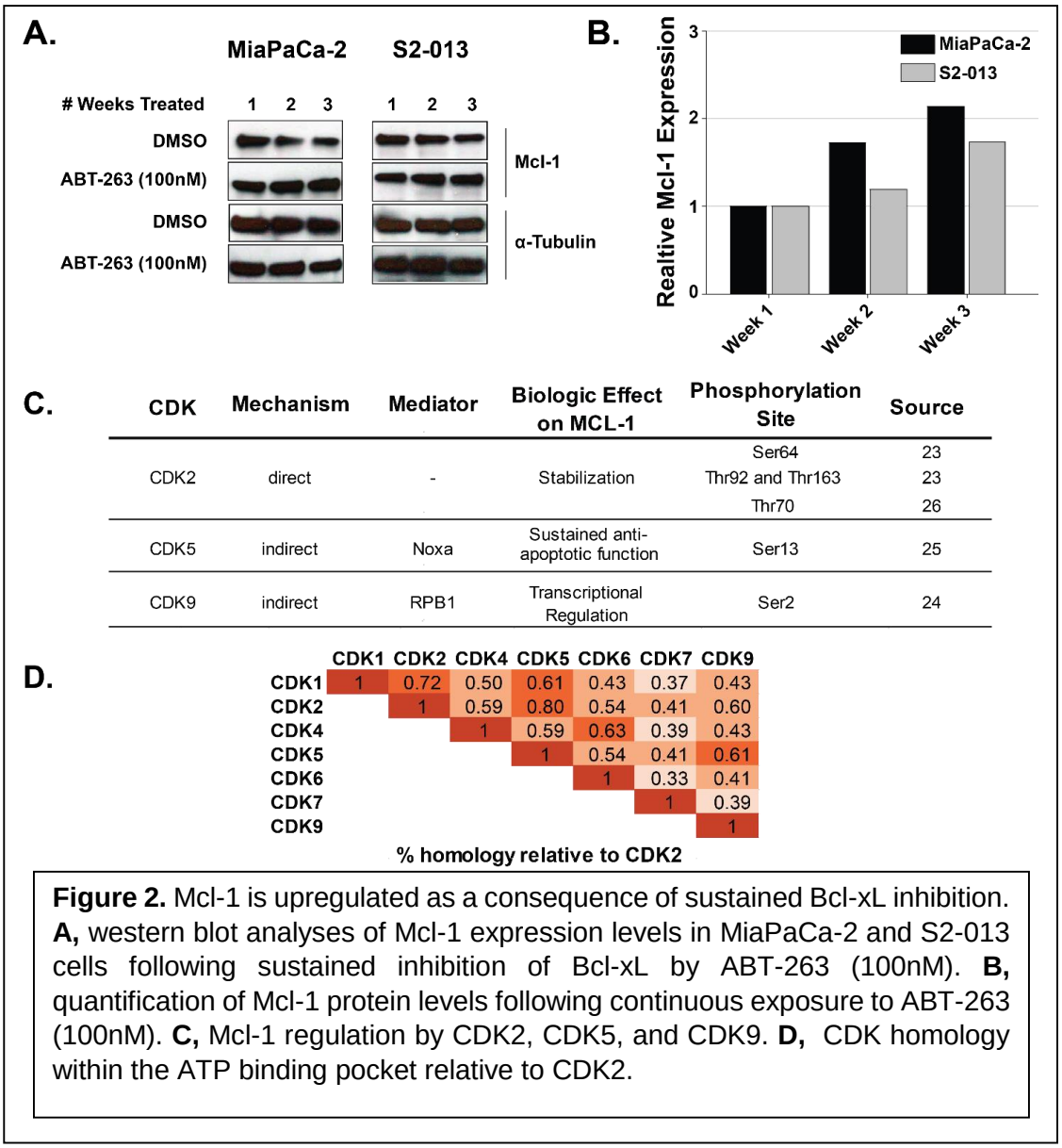
Previous studies have shown that Mcl-1 compensates for the loss of Bcl-xL to circumvent apoptosis. For example, in colorectal cancer, treatment with Bcl-xL inhibitor, ABT-737, resulted in increased Mcl-1 expression [53]. To determine whether this holds true in PDAC, we treated pancreatic cell lines, MiaPaCa-2 and S2-013, with Bcl-xL inhibitor ABT-263 (at > IC_{75} = 100nM) for three-weeks. Western

blot analyses after sustained exposure to ABT-263 showed an incremental increase in Mcl-1 expression over the three-week period (Figure 2A). Quantification of Mcl-1 levels revealed ~2-fold increase in Mcl-1 levels by week 3 (Figure 2B). These results are consistent with the compensatory activation of Mcl-1 observed in other systems as response to sustained Bcl-xL inhibition. This suggests that inhibition of Mcl-1 will sensitize PDAC cells to Bcl-xL inhibition. Since CDK2, CDK5, and CDK9 regulate the stability and expression of Mcl-1 through various mechanisms at various stages (Figure 2C).

Analyses of the residues in the ATP binding pocket of the CDKs

We conducted an informatics study to determine the homology among residues that interact with ATP within the CDK2, CDK5 and CDK9 binding pockets. A high homology would indicate that we could develop a polypharmacological agent that is selective for CDK2, CDK5 and CDK9. Using CDK2 as a reference, we aligned the residues within this conserved region to build a homology model of residues proximal to the ATP binding pocket. CDK2/ATP co-crystal structure (pdb: 1FIN) allowed us to identify all the CDK2 residues < 8Å away from atoms in the ATP molecule. This resulted in 46 residues spanning the ATP binding pocket that could potentially make contact with the small molecule. Next, we overlaid the equivalent residues in CDK1, CDK4, CDK5, CDK6, CDK7, CDK9 to identify residues that overlapped with 46 residues proximal (<8Å) to the atoms in the ATP molecule crystallized with CDK2. Based on the overlay, we determined % homology (Figure 2D). Analyses of this data revealed that the small molecule binding pocket of CDK2 and CDK5 showed the highest homology at 80% among

these residues. Interestingly, CDK1, CDK2, CDK5 and CDK9 shared higher (>60%) with each other when compared to the other CDKs (Figure 2D). This suggests that a polypharmacological agent that selectively targets CDK2, CDK5 and CDK9 can be developed.



C.

CDK	Mechanism	Mediator	Biologic Effect on MCL-1	Phosphorylation Site	Source
CDK2	direct	-	Stabilization	Ser64	23
				Thr92 and Thr163	23
				Thr70	26
CDK5	indirect	Noxa	Sustained anti-apoptotic function	Ser13	25
CDK9	indirect	RPB1	Transcriptional Regulation	Ser2	24

D.

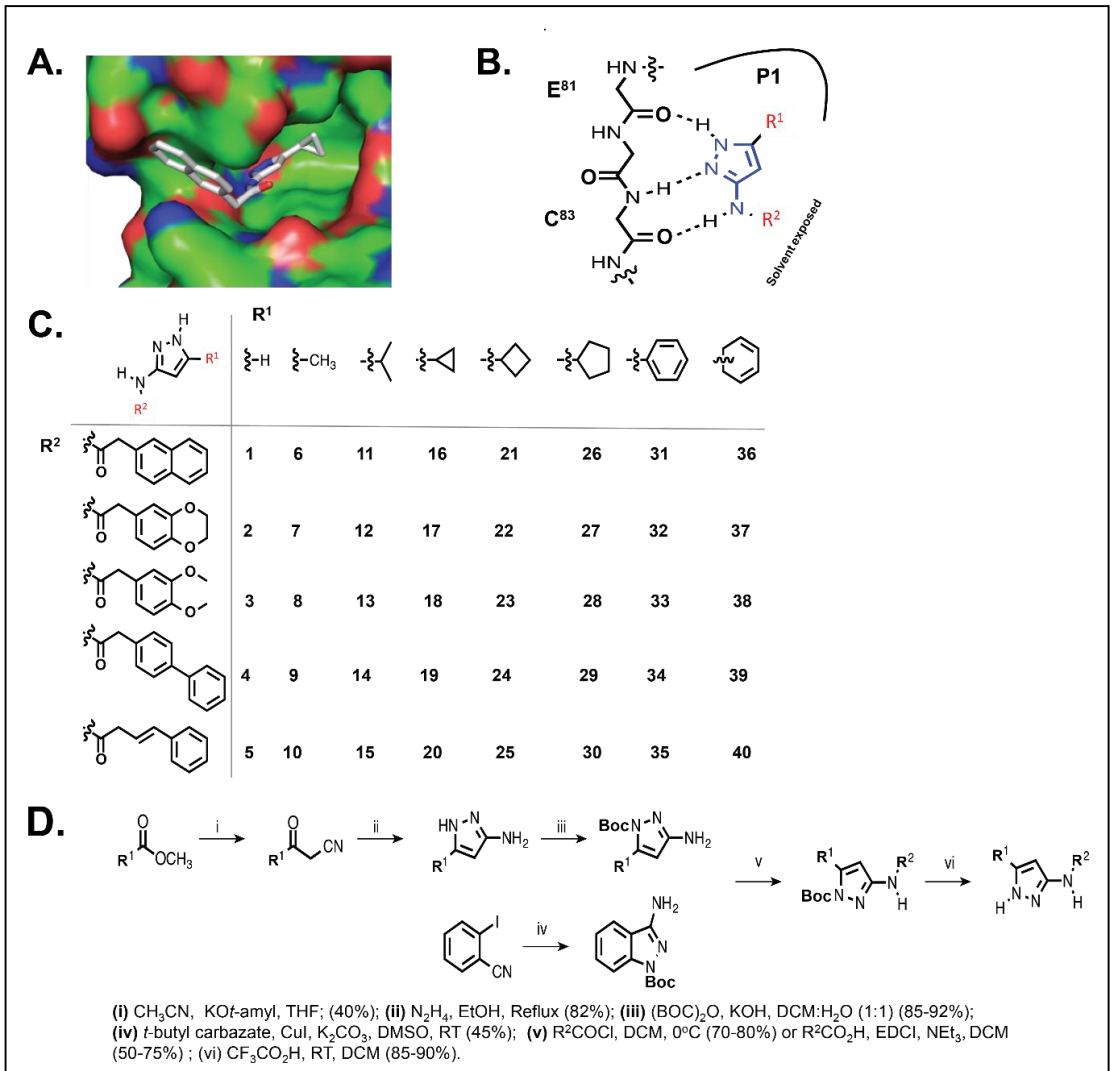
	CDK1	CDK2	CDK4	CDK5	CDK6	CDK7	CDK9
CDK1	1	0.72	0.50	0.61	0.43	0.37	0.43
CDK2		1	0.59	0.80	0.54	0.41	0.60
CDK4			1	0.59	0.63	0.39	0.43
CDK5				1	0.54	0.41	0.61
CDK6					1	0.33	0.41
CDK7						1	0.39
CDK9							1

% homology relative to CDK2

Figure 2. Mcl-1 is upregulated as a consequence of sustained Bcl-xL inhibition. **A,** western blot analyses of Mcl-1 expression levels in MiaPaCa-2 and S2-013 cells following sustained inhibition of Bcl-xL by ABT-263 (100nM). **B,** quantification of Mcl-1 protein levels following continuous exposure to ABT-263 (100nM). **C,** Mcl-1 regulation by CDK2, CDK5, and CDK9. **D,** CDK homology within the ATP binding pocket relative to CDK2.

Design and synthesis of a focused library of aminopyrazole analogs

The aminopyrazole core was originally investigated as a CDK2 inhibitor that occupies the ATP binding pocket [69, 72]. Structural studies indicate that the aminopyrazole core in CDK2 interacts with the hinge region residues (Figure 3A). The substituent at the 5-position (R^1) of the aminopyrazole is embedded in a shallow hydrophobic pocket (P1) while the substituents at the R^2 position are solvent exposed (Figure 3B). Docking and structural studies reveal that the hinge region residues of the CDKs (CDK2 = Glu^{81} and Leu^{83}, CDK5 = Glu^{81} and Cys^{83} and CDK9 = Asp^{104} and Cys^{106}) are involved in hydrogen bonding with the nitrogen atoms of the aminopyrazole core (Figure 3B). The substituents at the R^1 and R^2 positions on the aminopyrazole were systematically varied to explore the size of the P1 pocket and hydrophobicity of the solvent exposed site, respectively. This led to the design of a focused library of aminopyrazole analogs (Figure 3C). The R^1 substituted aminopyrazoles were either commercially available or synthesized in two steps from the corresponding R^1-esters [69, 73]. The internal nitrogen on the pyrazole was more nucelophillic and was therefore Boc-protected [69]. The Boc protected phenyl-fused aminopyrazole was synthesized in a single step by a copper mediated condensation of t-butyl carbazate and 2-iodobenzonitile [74]. The Boc-protected aminopyrazoles were condensed with various R^2 substituted acids or acid chlorides and the removal of the Boc group on the resulting amides yielded the 40 analog aminopyrazole library [41, 70, 75-79] (Figure 3D).



	R1 = H	CH_3						
R2	1	6	11	16	21	26	31	36
	2	7	12	17	22	27	32	37
	3	8	13	18	23	28	33	38
	4	9	14	19	24	29	34	39
	5	10	15	20	25	30	35	40

Figure 3. Design and synthesis of a 40- analog aminopyrazole library. **A,** aminopyrazole analog docked into the ATP binding pocket of CDK5. **B,** aminopyrazole core forms a triad of hydrogen bonds with the hinge region residues of CDK5. The R^1 portion of the compound occupies a shallow hydrophobic pocket while R^2 is solvent exposed. **C,** R^1 and R^2 substituents in the 40-member library. **D,** synthesis scheme used to generate 40 analog library.

Cell-free screen identified nine aminopyrazole analogs as potent inhibitors of CDK2, CDK5, and CDK9

The aminopyrazole library was screened against a panel of CDKs at 100 nM in a cell-free kinase assay (Figure 4A). Irrespective of the CDK, the activities of the compounds increased when the substituent at R^1 (across the row – Figure 4D) was larger than isopropyl or smaller than cyclopentyl. Surprisingly, analogs with more hydrophobic substituents at R^2 (rows 1, 4 and 5 - Figure 4D) were more active than analogs with more hydrophillic substituents at the R^2 position (Figure 4A). This indicates that the substituents are not truly surface exposed. To narrow down our library to the most potent inhibitors of CDK2, CDK5 and CDK9, we set a threshold of 90% inhibition against all three kinases, which resulted in the identification of 9 aminopyrazole analogs (Figure 4B). Demonstrating a clear bias, five (**21**-**25**) of the nine analogs identified as the top hits had a cyclobutyl substituent at the R^1 position and no such clustering was observed for the R^2 position.

Aminopyrazole analogs induce apoptosis through caspase activation

Since our objective was to identify polypharmacological CDK inhibitors that indirectly inhibited Mcl-1 to induce apoptosis, we used activation of caspase 3/7, which is considered a viable surrogate to assess induction of apoptosis [80], as a secondary screen. Pancreatic cancer cells were subjected to the nine aminopyrazole analogs and a caspase activity assay was performed following a six hour incubation. The short incubation period ensured that the caspase activation was a direct result of inhibition of the intended targets. This secondary

screen identified three aminopyrazole analogs (**21**, **24**, **25**) that induced greater than two-fold increase in caspase activation (Figure 4C). It is important to note that all three analogs (**21**, **24**, **25**) have a cyclobutyl substituent at the R^1 position and a hydrophobic substituent at the R^2 position.

Next, to determine if the induction of apoptosis was Mcl-1 dependent, we employed a pair of doxycycline (Dox) inducible HeLa cell lines [10, 41, 81]. These cell lines overexpress BH3-only proteins, BAD3SA and Noxa, which are negative regulators of Bcl-xL or Mcl-1 respectively. Treatment with Dox results in the induction of BAD3SA or Noxa expression leading to inhibition of Bcl-xL or Mcl-1 respectively. We utilized these cell lines in a caspase activity assay to study the ability of the aminopyrazole analogs to induce apoptosis in either a Bcl-xL or Mcl-1 dependent manner. Remarkably, all three analogs (**21**, **24**, **25**) induced robust apoptosis in a dose-dependent manner only in HeLa Dox-BAD3SA cells and not in HeLa Dox-Noxa cells (Figure 4D). This is because in HeLa Dox-BAD3SA cells, Dox induction results in BAD3SA expression, which inactivates Bcl-xL and the CDK inhibitors target the Mcl-1 arm of the apoptotic pathway thus creating the concurrent inactivation of Bcl-xL and Mcl-1 to induce apoptosis (Figure 4D). On the other hand, in HeLa Dox-Noxa cells, Dox induction results in Noxa expression, which inactivates Mcl-1 and the CDK inhibitors also target the same pathway allowing functional Bcl-xL to block induction of apoptosis (Figure 4E). These results are consistent with genetic studies that show concurrent knock down of Bcl-xL and Mcl-1 is required for the induction of apoptosis [7, 10]. Together, these results

demonstrate that CDK2, CDK5, and CDK9 inhibition by **21**, **24** and **25** induced apoptosis in a Mcl-1 dependent manner.

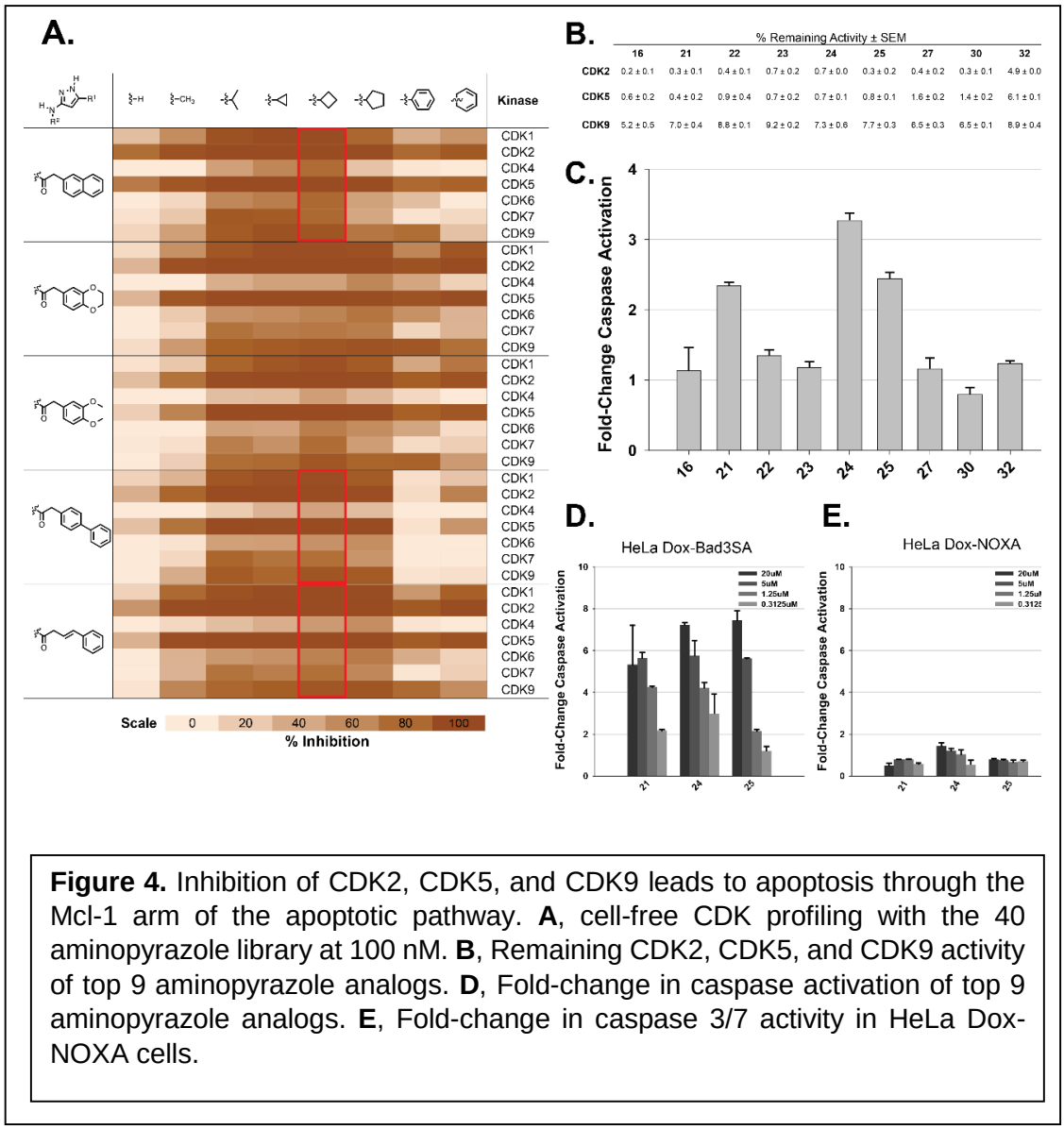


Figure 4. Inhibition of CDK2, CDK5, and CDK9 leads to apoptosis through the Mcl-1 arm of the apoptotic pathway. **A**, cell-free CDK profiling with the 40 aminopyrazole library at 100 nM. **B**, Remaining CDK2, CDK5, and CDK9 activity of top 9 aminopyrazole analogs. **D**, Fold-change in caspase activation of top 9 aminopyrazole analogs. **E**, Fold-change in caspase 3/7 activity in HeLa Dox-NOXA cells.

Aminopyrazole analogs inhibit cell growth in a panel of PDAC cell lines

Next, we subjected a panel of PDAC cell lines to the top three compounds (**21**, **24** and **25**) to identify the most potent inhibitor of cell growth. AsPC1, BxPC3, MiaPaca2, SUIT3, and S2-013 cells were treated with **21**, **24**, or **25**, for 72 hours and then analyzed for inhibition of cell proliferation using the PrestoBlue assay. Dinaciclib, AT7519, and Roscovitine, three CDK inhibitors that are in the clinics were used in the same assay as control compounds to benchmark the potency of our inhibitors. This screen revealed that analog **24** had nanomolar potency in all five cell lines and was less potent than Dinaciclib but more potent than AT7519 and Roscovitine (Figure 5A). Collectively, our studies revealed that analog **24** is a promising polypharmacological CDK inhibitor that induced apoptosis in a Mcl-1 dependent manner and possessed nanomolar potency against a panel PDAC cell lines.

*Dose response studies to validate **24** as a CDK2, CDK5, and CDK9 inhibitor in cell-free and cell-based systems.*

To determine selectivity of **24**, we performed cell-free screen with a small panel of kinases and compared **24** to known non-selective (Staurosporine) and selective (Ibruitinib, ML-120B, and AT7519) kinase inhibitors. Staurosporine displayed little to no selectivity as it had nM to sub-μM potency against the entire panel (CDK2, CDK5, CDK9, IKKβ, and BTK). Conversely, ML-120B, Ibrutinib, and AT7519 were selective for their targets IKKβ, BTK, and CDKs respectively. Likewise, **24** effectively inhibited CDK2 (24nM), CDK5 (23nM), and CDK9 (911nM) and was inactive (>10,000nM) towards IKK□β and BTK (Figure 5B).

We next evaluated **24** for its ability to disrupt CDK2, CDK5 and CDK9 in PDAC cells. MiaPaca2 and S2-013 cells were treated with increasing concentrations (0 - 20□M) of **24** for 6, 12, and 24 hours. The efficacy of cell-based kinase inhibition can be estimated by Western blot analyses of the phosphorylation states of the corresponding substrates. We used previously reported CDK2, CDK5 and CDK9 substrates, i.e., pRB (Ser807/811), pFAK (Ser732) and pRPB1 (Ser2) respectively [48, 82-85] as read outs to assess the ability of **24** to inhibit the corresponding CDKs in PDAC cells. PDAC cells treated with **24** showed a dose- and time-dependent decrease in the levels of pRB (Ser807/811), pFAK (Ser732) and pRPB1 (Ser2) suggesting effective inhibition of the kinase activity of CDK2, CDK5, and CDK9, respectively (Figure 5C). While **24** did not affect the total levels of RB or FAK, we did observe a decrease in total RPB1 at higher concentrations and longer time points. Together, these results showed that **24** is a potent inhibitor of CDK2, CDK5, and CDK9 in cell-based systems.

A.

Cell line	IC_{50} (nM) ± SEM					
	21	**24**	**25**	**Dinaciclib**	**AT7519**	**Roscovitine**
AsPC1	1163 ± 105	315 ± 17	> 2000	15.3 ± 0.6	533 ± 33	17767 ± 1284
BxPC3	> 2000	924 ± 62	> 2000	< 10	640 ± 55	15284 ± 633
MiaPaCa-2	861 ± 49	247 ± 26	1559 ± 62	< 10	411 ± 37	18214 ± 1148
SUIT2	> 2000	209 ± 16	> 2000	12.6 ± 1.8	557 ± 148	14801 ± 2515
S2-013	875 ± 120	192 ± 20	1468 ± 264	17 ± 1.5	2770 ± 558	21700 ± 3500

B.

Kinase →	**CDK2/cyclinE**	**CDK5/p25**	**CDK5/p35**	**CDK9/cyclinT1**	**IKKβ**	**BTK**
Inhibitor ↓	IC_{50} (nM) ± SEM					
Ibrutinib	N/D	> 10,000	N/D	N/D	> 10,000	0.21 ± 0.01
ML-120B	N/D	> 10,000	N/D	N/D	465 ± 74	> 10,000
Staurosporine	2.6 ± 0.3	13 ± 2	3.8 ± 0.1	6.6 ± 0.8	2500 ± 589	43 ± 3
AT7519	388 ± 72	N/D	32 ± 2	4.5 ± 0.3	N/D	N/D
24	24 ± 3	77 ± 3	23 ± 2	911 ± 148	> 10,000	> 10,000

C.

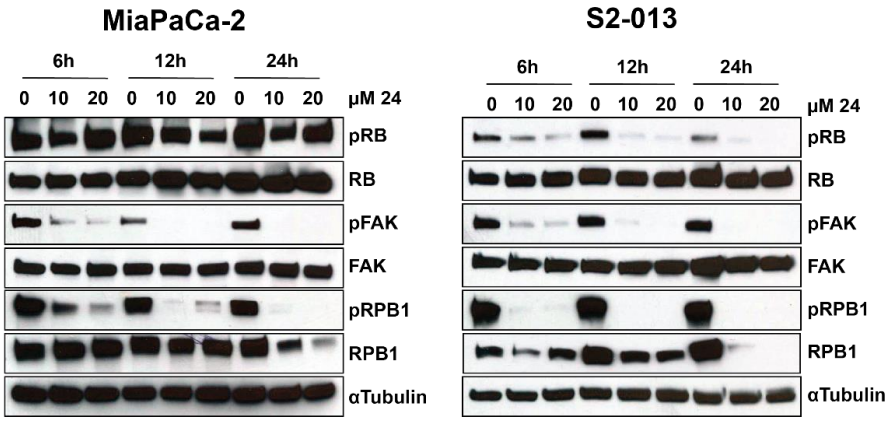


Figure 5. **24** inhibits cell growth in a panel of pancreatic cancer cell lines and selectively inhibits CDK2, CDK5, and CDK9 activity. **A**, IC_{50} values from 72 hour growth inhibition assays with **21, 24, 25** and clinically used CDK inhibitors: Dinaciclib, AT7519 and Roscovitine. **B**, cell-free kinase screen of Ibrutinib, ML-120B, Staurosporin, AT7519, and **24** for their effects against CDK2, CDK5, CDK9, IKKβ, and BTK. **C**, dose- and time-dependent studies with **24** in MiaPaCa-2 and S2-013 cells to assess its activity against CDK2, CDK5, and CDK9 using western blot analyses of pRB/RB, pFAK/FAK, and pRPB1/RPB1.

***24** induces apoptosis in a Mcl-1 dependent manner*

The design, synthesis and screening funnel led us to **24**, which inhibits CDK2, CDK5, and CDK9, in cell-free and cell-based assays. Next, we wanted to evaluate the effect of **24** on apoptosis, in dose- and time-dependent studies using a panel of Hela Dox cell lines (Figure 6A). Briefly, the HeLa-Dox-GFP cell line in the presence of Dox expressed GFP therefore has functional Bcl-xL and Mcl-1; the HeLa-Dox-BAD3SA cell line in the presence of Dox expressed BAD3SA which binds to and inactivates Bcl-xL therefore has only functional Mcl-1; and the HeLa-Dox-Noxa cell line in the presence of Dox expressed Noxa which binds to and inactivates Mcl-1 therefore has only functional Bcl-xL. There are three possible outcomes of the screen: (a) if we observe caspase 3/7 activation in HeLa Dox-GFP cell line it would indicate non-selective induction of apoptosis as it has to hit both the Bcl-xL and Mcl-1 arms; (b) if we observe caspase 3/7 activation in HeLa-Dox-BAD3SA cell line it would indicate Mcl-1 dependent apoptosis as the inhibitor disables functional Mcl-1; and (c) if we observe caspase 3/7 activation in HeLa-Dox-Noxa cell line it would indicate Bcl-xL dependent apoptosis as the inhibitor disables functional Bcl-xL. Treatment of the above three cell lines with **24** in the presence of Dox resulted in a dose- (Figure 6B) and time- (Figure 6C) dependent increase in caspase 3/7 activity only in the HeLa-Dox-BAD3SA cell line and not the HeLa-Dox-GFP or HeLa-Dox-Noxa. This result clearly demonstrates that **24** induces apoptosis in a Mcl-1 dependent manner. To confirm that the apoptosis is a result of Mcl-1 down regulation, we performed a dose-response study with **24** in all three HeLa-Dox cell lines. We observed a dose-dependent decrease in Mcl-1

levels in each of the three HeLa-Dox cell lines. However, PARP cleavage, a hallmark of apoptosis, was only observed in the BAD3SA cell line, consistent with reported studies that show that apoptosis only occurred when both the Mcl-1 and Bcl-xL arms are concurrently disabled (Figure 6D).

Concurrent pharmacological disruption of Mcl-1 and Bcl-xL results in synergism

Because **24** decreases the expression of Mcl-1 and **24** combined with the genetic disruption of Bcl-xL results in robust apoptosis, we next sought to examine the effects of the combined pharmacologic inhibition of Mcl-1 and Bcl-xL. To accomplish this we used two specific BH3 mimetic inhibitors of Bcl-xL, ABT-737 and ABT-263 developed by Abbott Laboratories [16, 18]. The panel of HeLa-Dox cell lines were treated with increasing doses ABT-737 or ABT-263 for 6 hours and evaluated for changes in caspase 3/7 activity. Not surprisingly, with both ABT-737 and ABT-263, we observed dose-dependent increases in caspase 3/7 activities (~3-fold) only in the HeLa-Dox-Noxa cell line and not in the HeLa-Dox-GFP or HeLa-Dox-BAD3SA cells (Figure 6E and 6F). Given the robust caspase activation observed only in HeLa-Dox-Noxa with ABT-263 and since **24** induced caspase activation only in HeLa Dox-BAD3SA; we anticipated that concurrent inactivation of Mcl-1 and Bcl-xL by **24** and ABT-263 would lead to synergistic caspase activation and apoptosis in the HeLa-Dox-GFP cell line. Indeed, caspase activation assays confirmed that only the combination of ABT-263 and **24** robustly induced apoptosis in HeLa-Dox-GFP cell line (Figure 6G). Importantly neither treatment individually at the reported concentrations and time points induced apoptosis.

Next we determined if this observed synergism would extend to pancreatic cancer cell lines. S2-013 cells were treated individually with ABT-737/ABT-263, **24**, or the combination and the levels of Mcl-1, PARP and cleaved PARP were monitored by Western blot analyses. Treatment with **24** alone decreased Mcl-1 levels while ABT-737/ABT-263 did not. Consistent with the data from the HeLa-Dox-GFP cells, induction of PARP cleavage was only observed with the combined treatment of **24** and ABT-737/ABT-263 (Figure 6H). These results suggest that the combined pharmacological inhibition of Mcl-1 (**24**) and Bcl-xL (ABT-737/ABT-263) induced robust apoptosis in pancreatic cancer cell lines.

Finally, to determine whether the combination of Mcl-1 and Bcl-xL pharmacological inhibition will by synergistic, we performed a growth inhibition study with either S2-013 or MiaPaCa-2 cells using increasing concentrations of ABT263 alone or ABT-737 alone, or **24** alone, or the combinations of **24** and ABT compounds. IC_{50} values were derived from growth curves and used to calculate combination index (CI) values using Calcusyn. CI values < 1 indicates synergism, CI = 1 indicates additive effects, and CI > 1 indicates antagonism [38]. For cancer therapies synergism at high effect levels is therapeutically relevant. Therefore CI values were determined for effective dose (ED) when greater than 75%, 90% and 95% of cells are affected by the treatment (Figure 6I). The synergism study revealed that treatment with **24** and ABT-737 was strongly synergistic with CI values of 0.19 (S2-013) and 0.38 (MiaPaca-2).

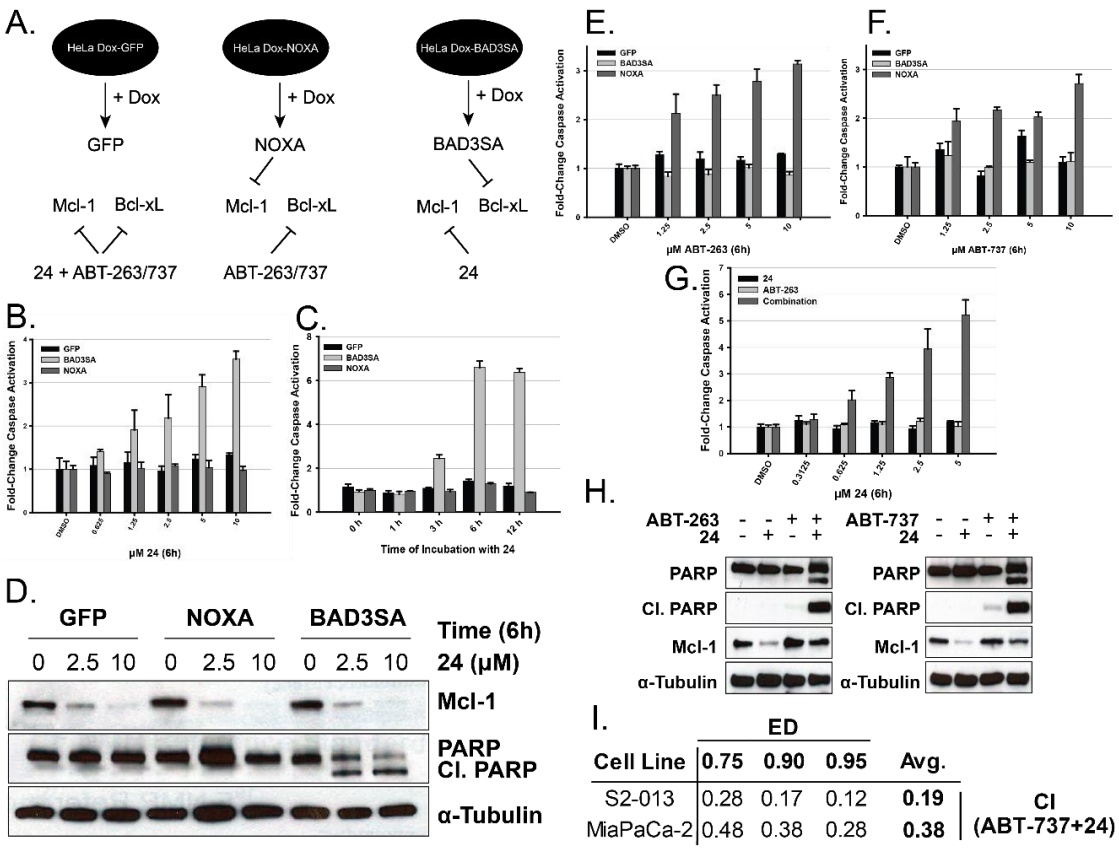


Figure 6. Synergism studies with 24 and BH3-mimetic Bcl-xL inhibitors. **A**, validation strategy with the inhibitors and HeLa Dox-GFP, HeLa Dox-NOXA, and HeLa Dox-BAD3SA cell lines. **B,C,** fold-change in caspase 3/7 activation in dose-response and time-course studies with **24** and HeLa Dox-cell lines. **D,** western blot analyses of Mcl-1, PARP, and cleaved PARP levels in HeLa Dox-cell lines following **24** treatment. **E,F,** dose response studies in Hele dox-cell lines with ABT-263 and ABT-737. **G,** dose response studies in HeLa Dox-GFP cells with **24** alone, ABT-263 alone, and the combination. **H,** western blot analyses of PARP, cleaved PARP, and Mcl-1 levels in S2-013 cells treated with ABT-263 or ABT-737, **24** (5μM), or a combination of ABT-263 or ABT-737 and **24**. **I,** combination index (CI) values derived from growth inhibition studies with ABT-737 and **24** in S2-013 and MiaPaCa-2 cell lines.

Collectively, our data thus far shows that in pancreatic cancer cell lines (a) polypharmacological inhibition of CDK by analog **24** results in the down regulation of Mcl-1 and (b) concurrent pharmacological inactivation of Mcl-1 and Bcl-xL is strongly synergistic.

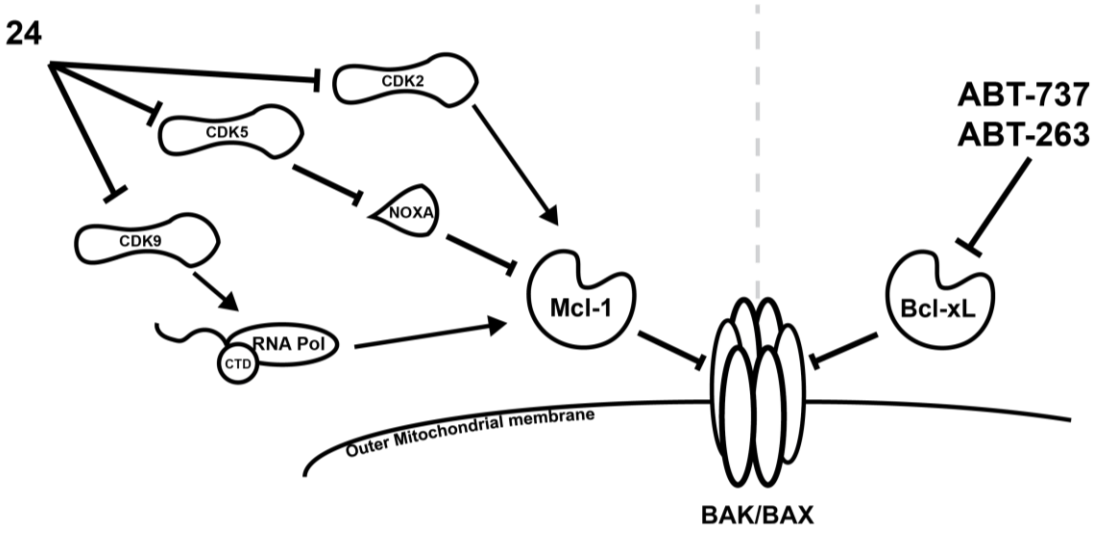


Figure 7. 24 inhibits Mcl-1 through CDKs. Model of compound 24 inhibition of Mcl-1 through CDK2, CDK5, and CDK9.

Discussion:

Genetic and pharmacological evidence clearly demonstrate that concurrent inactivation of Bcl-xL and Mcl-1 is a viable therapeutic strategy for cancers [7, 10, 41, 81]. Although potent direct inhibitors of Bcl-xL have been clinically validated, direct Mcl-1 inhibitors are currently in preclinical development. Mcl-1 is regulated at multiple levels and by varied mechanisms. For example, (a) the STAT family of transcription factors regulate Mcl-1 levels, (b) a network of kinases through phosphorylation of specific residues on Mcl-1 modulate its stability, and (c) the activity and stability is regulated by BH3 mimetics such as Noxa [86]. This diversity in its regulation provides a unique opportunity to indirectly target Mcl-1.

Several members of the CDK family of kinases are known to regulate Mcl-1 through different mechanisms. CDK2 is a direct and a major regulator of Mcl-1. Context dependent phosphorylation of Mcl-1 residues by CDK2 (Ser^{64}, Thr^{70}, Thr^{92} and Thr^{163}) has been previously shown to stabilize Mcl-1 [31]. Noxa is known to bind to and regulate Mcl-1 and phosphorylation of Ser^{13} on Noxa by CDK5 results in survival and proliferation by activating aerobic glycolysis [43]. CDK9, another member of the CDK family, activates transcription of Mcl-1 by phosphorylation of Ser^{2} on DNA directed RNA polymerase 1 (RPB1) [32]. This suggests disabling these phosphorylation events on Mcl-1 by CDK2, CDK5 and CDK9 will likely destabilize Mcl-1 and reduce its levels in cells.

Indirect perturbation of Mcl-1 function, stability, and expression is feasible through a polypharmacological approach to CDK inhibition. Polypharmacology is a growing paradigm in drug design which uses a single molecule to disrupt multiple

targets [87, 88]. When considering the promiscuity of CDK inhibitors, a polypharmacology approach may be adapted to design a single molecule which is capable of disrupting kinases essential to Mcl-1 function. Although multiple CDK inhibitors have been shown to attenuate Mcl-1 activity, this has largely been a desirable trademark of CDK inhibitors and has widely been considered their mechanism of action. To our knowledge, this is the first focused approach to develop a polypharmacologic agent that targets CDK2, CDK5, and CDK9 with the specific objective of disrupting Mcl-1 and potentiating Bcl-xL direct inhibitors. Understanding the structural homology of the CDK hinge region/ATP binding site allowed us to bypass reverse docking methods. Additionally, aminopyrazole analogs are known to bind hinge region residues of CDKs. Therefore, our goal became to design a compound which would selectively and potently disrupt CDK2, CDK5, and CDK9 with the aim of attenuating Mcl-1.

Developing selective CDK inhibitors has been an Achilles heel in the CDK drug development field [89]. This problem presents a unique opportunity when it comes to developing indirect Mcl-1 inhibitors. We hypothesized that polypharmacological CDK (2, 5 and 9) inhibition will lead to degradation of Mcl-1 and combining that with a Bcl-xL inhibitor is a viable strategy for pancreatic cancer. To test this we used the aminopyrazole core, which has been previously explored for the development of CDK2 inhibitors. Analyses of CDK residues that interact with ATP revealed that among the CDKs, CDK2, CDK5 and CDK9 shared high homology between each other. The structural comparison between these particular regions was performed around the ATP binding site and therefore, for

the aminopyrazole analog. This structural based approach allowed us to design a focused library of analogs that would probe the hydrophobic pocket within the ATP binding site and bypass reverse docking techniques which use a larger library of compounds against a larger cohort of kinases. This focused approach facilitated the funneling process and allowed us to identify compounds which would selectively attenuate Mcl-1 through CDK inhibition. These analyses led us to design and synthesize a focused library of aminopyrazole analogs and conduct CDK profiling to identify inhibitors that target CDK2, CDK5 and CDK9. Using secondary screens, which included an assay to identify inhibitors that induce apoptosis in a Mcl-1 dependent manner, we identified analog **24** as a polypharmacological CDK (2, 5 and 9) inhibitor. We showed **24** induced apoptosis as indicated by caspase activation in a Mcl-1 dependent manner. Cell-free and cell-based studies demonstrated that **24** indeed inhibited CDK2, 5 and 9.

Chemical genetic screens with a panel of Dox inducible HeLa cell lines revealed that **24** selectively induced apoptosis in a Mcl-1 dependent manner and was synergistic with Bcl-xL inhibitors. We also showed **24** reduced Mcl-1 levels in multiple pancreatic cancer cell lines and induced apoptosis when combined with Bcl-xL inhibitors. Synergism studies in pancreatic cancer cell lines showed that the combined treatment of **24** and ABT-737 resulted in remarkably low CI values at therapeutically relevant ED^{75}, ED^{90} and ED^{95}. The combination was validated in an *in vivo* pancreatic cancer model. In conclusion, our studies suggest that polypharmacological CDK inhibition down regulates Mcl-1 and when combined with Bcl-xL inhibitors is a viable therapeutic option for pancreatic cancer.

Chapter 3: A chemical genetic approach to target anti-apoptotic proteins by profiling the functional kinome network to identify novel combinations as cancer therapeutics

Introduction

Mounting incentives have prompted the pursuit of combination treatment to counter resistance mechanisms of cancer development [36]. The advent of personalized medicine and increasing resistance observed in single agent therapy are among the many factors advocating for combination treatments. Furthermore, the increasing number and variety of clinical candidate drugs that target specific proteins warrants an effort to repurpose these compounds for combination therapy [90]. As such, an effort is being made to repurpose clinical candidate drugs to combination treatments with existing pre-clinical and clinically approved drugs. To streamline the search for viable combination treatments, novel screening methods are being developed to identify potent combinations [37, 91]. Here, we present a novel chemical genetic screening strategy to identify novel combinations of inhibitors which target the apoptotic network proteins.

Cell fate is determined by a delicate balance between two classes of regulatory apoptotic proteins: anti- and pro-apoptotic proteins. Anti-apoptotic proteins, which include Bcl-2, Bcl-w, Bcl-xL, Mcl-1, and BFL-1, inhibit apoptosis by either: (1) directly binding to and inhibiting BAK and BAX oligomerization or (2) by binding to and sequestering BH3-only activator proteins. Pro-apoptotic proteins can further be sub-divided into two groups: multi-domain pro-apoptotic proteins, which include BAK, BAX, and their counterpart BOK which is expressed in reproductive cells; the second class of pro-apoptotic proteins include BH3-only proteins. BH3 only proteins are small proteins which are sub-divided further into two groups: activators and sensitizers. Activators, which include BID, BIM and PUMA directly induce BAK and BAX oligomerization at the outer mitochondrial membrane

(OMM). Sensitizers, which include BAD, NOXA, HRK, BMF, and BIK inhibit anti-apoptotic protein function. Commitment to apoptosis is dependent on the oligomerization of BAK and BAX and the perforation of the OMM [6].

One of the hallmarks of cancer is evasion of apoptosis [61]. Malignant cancer cells may accomplish this by overexpressing certain proteins which serve as blocks to the naturally occurring apoptotic pathway [58, 92, 93]. BH3-profiling studies have revealed an overexpression of a heterogeneous combination of anti-apoptotic proteins may prevent cells from entering apoptosis and these cells are “primed” for apoptosis [9]. In a primed state, anti-apoptotic proteins carry activator BH3-only members such as BID and BIM, which when freed may induce BAK and BAX oligomerization. Inactivation of these proteins by synthetic means may be enough to push these cells into apoptosis. These observations bear in mind the question of which anti-apoptotic proteins are necessary for induction of apoptosis. Recent studies have suggested an intimate association of Bcl-xL and Mcl-1 in the apoptotic pathway [7, 10, 41]. Concurrent knockdown of Mcl-1 and Bcl-xL in HeLa cells induced robust apoptosis without any additional stimuli. These studies identified Mcl-1 and Bcl-xL as the only two proteins which, when inactivated concurrently, induced apoptosis. Additional studies sought to identify the role of BH3-only proteins in the apoptotic by using elaborate gene editing techniques to knockout all eight BH3-only proteins. Under these conditions, concurrent Bcl-xL and Mcl-1 knockdown persisted in inducing apoptosis, even in the absence of activator BH3-only proteins, validating their role as essential blocks to apoptosis.

Naturally, Mcl-1 and Bcl-xL are overexpressed in multiple cancers and their role in resistance to standard chemotherapy is well documented [58, 94, 95]. As a result, small molecule inhibitors (SMIs) are being developed to directly target both proteins [96, 97]. Bcl-xL inhibitors are currently being developed and are advancing into clinical trials for the

treatment of Chronic Lymphocytic Leukemia, Non-Hodgkin's Lymphoma, and Non-Small-Cell Lung Cancer. Similarly, Mcl-1 inhibitors are being developed using the same fragment based screening technique [14]. However, due to differences in the hydrophobic binding groove of Mcl-1, direct inhibitors of Mcl-1 have been more challenging to develop [20]. Ultimately, direct Mcl-1 inhibitors have yet to be developed for clinical trials.

Mcl-1 is a short-lived protein that relies heavily on its expression, which is mediated by RNA Polymerase II [32]. Unlike Bcl-xL, Mcl-1 is also stabilized on its N-terminus and relies heavily on phosphorylation of PEST and PEST-like sequences [98]. Stabilization or degradation is dependent on the phosphorylation status of these sequences. Additionally, NOXA, the BH3-only regulator of Mcl-1, shares an intricate relationship with Mcl-1 forming multiple complexes which may or may not induce apoptosis depending on the cellular environment [9]. The phosphorylation status of NOXA determines the apoptotic function of Mcl-1 in different cellular environments [43]. Therefore, we may conclude phosphorylation events that control Mcl-1 function, stability and expression may be used to target Mcl-1 as an alternative to direct means of inhibition. Indeed, CDK inhibitors have been well documented as Mcl-1 attenuators [32, 99, 100]. Inhibition of CDK9 leads to loss of phosphorylation of RNA polymerase II carboxyl-terminal domain which leads to failure of transcription initiation, ceasing the transcription of Mcl-1 and eventually leading to loss of expression [32]. Mcl-1 is also stabilized at its PEST sequence by CDK2/cyclin E [31]. Phosphorylation by CDK2/cyclin E also leads to its binding to the BH3 only pro-apoptotic protein Bim, adding to its anti-apoptotic function [101]. Finally, CDK5 has been shown to phosphorylate NOXA, the BH3-only regulator of Mcl-1, at Ser 13 under high glucose cellular conditions [43]. As a result leading to loss of its pro-apoptotic activity, CDK inhibitors have been a well characterized attenuator of Mcl-1, however, they do not follow a uniform axis of inhibition as a single kinase inhibitor may inhibit multiple CDKs and even

the most potent inhibitors, such as Dinaciclib, exhibit toxic effects [102]. Therefore, combination strategies are being sought to offset toxic effects observed in pre-clinical drugs.

The availability of Bcl-xL direct inhibitors offers a unique opportunity to develop combinatorial therapeutic strategies that target the apoptotic pathway using the Mcl-1/Bcl-xL axis of regulation. Although Bcl-xL inhibitors have been developed and are currently undergoing clinical trials, the regulation of Bcl-xL involves multiple signal transduction pathways to which SMIs have already been developed [13, 16, 103, 104]. IKKβ/NFκB, PI3K/Akt, and MAPK pathways all have been extensively studied and found to influence expression of Bcl-xL. Unlike Mcl-1, Bcl-xL has limited phosphorylation sites which influence its stabilization and is a relatively long-lived protein. Based on these observations, is there a pair of kinase inhibitors that can be identified which trigger the apoptotic pathway by synergistically attenuating Bcl-xL and Mcl-1?

To answer this question we developed a screen to identify synergistic combinations of kinase inhibitors which trigger either the Mcl-1 arm of the apoptotic pathway or the Bcl-xL arm of the apoptotic pathway. We used three HeLa cell lines developed by Dr. Luo and his group which overexpress NOXA, BAD, and GFP under doxycycline control. NOXA and BAD inactivate Mcl-1 and Bcl-xL respectively while GFP serves as a negative control in which neither protein is inactivated. These cell lines allow us to probe both arms of the apoptotic pathway similar to the way an inhibitor would. By overexpressing the natural BH-3 only regulators of Mcl-1 and Bcl-xL, we can recapitulate the conditions of a free inhibitor. Both proteins are still expressed and translated and are free to form their natural complexes. In contrast, gene editing techniques such as CRISPR or siRNA may not fully recapitulate the effects of inhibition of either protein.

Using these three cell lines, we optimized a high-content screen of 355 kinase inhibitors and sought kinase inhibitors which targeted each individual cell line with a high confidence interval determined by previously developed statistical methods. We did not expect the hits to cluster around a uniform class of kinases. However, kinase inhibitors which clustered around HeLa Dox-NOXA, or kinase inhibitors which attenuate Bcl-xL, were primarily PI3K/mTOR inhibitors. Cyclin dependent kinase (CDK) inhibitors clustered as hits to the HeLa Dox-BAD3SA cell line, were deemed kinase inhibitors which induce apoptosis through the Mcl-1 arm of the apoptotic pathway. Interestingly, Pelitinib, an EGFR inhibitor, was identified as a hit for the HeLa Dox-GFP cell line indicating that it probably affects both arms of the apoptotic pathway or is non-specific. We validated the hits using western blot to identify compounds which induced PARP cleavage as well as caspase 3 cleavage. We then evaluated the compounds in a pancreatic and colorectal cancer cell lines using growth inhibition assays. From growth inhibition assays, we calculate IC_{50} values and calculated fold change in IC_{50} shift. CalcuSyn software was then used to calculate combination Index (CI) values in for all combinations in the different cell lines.

Materials and Methods

Pancreatic Cell Lines. Maintenance of pancreatic cancer cell lines was as previously described. S2-013 cells were cultured in RPMI-1640 medium (HyClone #SH30027.01). MiaPaca2 cells were cultured in DMEM high glucose medium (HyClone #SH30022.01). All media were supplemented with 10% fetal bovine serum (FBS) (Gibco by LifeTechnologies #26140-079) and 1% Penicillin-Streptomycin (HyClone # SV30010). All cell lines were kept in culture at 5% CO_2 at 37°C.

Hela Cell Lines. HeLa Dox-NOXA, HeLa Dox-BAD3SA and HeLa Dox-GFP cell lines were a gift from Dr. Xu Luo (University of Nebraska Medical Center [10]. Cells were cultured in DMEM high glucose medium (HyClone #SH30022.01) and supplemenented with 10% FBS (Gibco by LifeTechnologies #26140-079) and 1% Penicillin-Streptomycin (HyClone # SV30010). Cells were cultured at 5% CO_2 at 37°C. Cells were treated with 1µg/mL of doxycycline for 3 hours to induce the expression of GFP, BAD3SA, or NOXA.

Western Blot Analyses. Western blot analyses was performed as previously described. Cells were washed with cold 1xPBS 3 times and scraped before being lysed by a buffer containing the following: 50mM Tris, 100mM NaCl, 1% NP-40, 2nM EDTA, 20% SDS, 20xPPI (Na_3VO_4, NAF, β-glycerophosphate) and 1mmol/L PMSF. After collection, samples were incubated on ice for 30 minutes and vortexed in 15 minute intervals. Samples were then centrifuged at 14,000rpm for 10 minutes at 4°C and supernatant was collected. Protein quantification was determined by BCA Protein Assay (Pierce #23225). 20-40ug protein samples were run on 4-15% gradient gels (BioRad) in 1x TRIS-Glycine-SDS Buffer (Research Products International Corporation #T32080) at 120V for ~60 minutes and separated by SDS-page electrophoresis. Samples were transferred to a PVDF membrane by semi-dry transfer method (ThermoScientific #35035) run at 18V for ~35 minutes. Membranes were blocked in 5% milk diluted in 1x-Tris Buffered Saline with 0.1% Tween (1xTBST) for 1 hour at room temperature rocking at low speed. Primary antibodies were diluted in 5% milk in 1xTBST and were rocked gently overnight in 4°C. Membranes were incubated with the appropriate HRP-

conjugated secondary antibody for 1 hour at room temperature while gently rocking. 3 washes (10 minute) with 1xTBST occurred before and after secondary antibody. ECL Prime (GE Healthcare #RPB2236) was used to detect protein expression.

Cell Viability. Cell viability studies were conducted as previously described. Cells were plated at 4000 cells/well in a 96-well plate and allowed to adhere overnight. The following day, cells were treated with compounds (21, 24, and 25) using 10-fold dilutions starting at 1000nM. PrestoBlue reagent (Invitrogen #A13262) was added to cells after 72 hour drug incubation to assess the growth inhibition. Fluorescence excitation/emission was measured at 560/590nM using SpectraMax M5^{e} instrument. Growth inhibition was calculated using 100-[100*(Samples-T0)/(T100-T0)]. T0 is the vehicle control reading immediately following drug addition and T100 is the control reading at the end of 72 hour incubation.

Calcusyn. To determine fraction affected as a decimal of 1, percent growth inhibition data was divided by 100. If a value exceeded 100%, 0.999 was assumed. If a negative value was observed a value of 0.001 was assumed. Using calcusyn software, combination index (CI) values were calculated as a mean of CI values calculated for each clinically relevant effect dose [38]. Clinically relevant effect doses and their corresponding CI values were determined from the following ED values: ED_{75}, ED_{90}, and ED_{90}, where ED_{75} is the dose at which 75% of the cells are affected.

Statistical Analyses. Graphs were generated using SigmaPlot 11.0. Student's t-tests were used to determine significance between two groups. $p<0.05$ was

considered significant. Combination Index (CI) values were determined by CalcuSyn 2.11.

Caspase Assay. Cells were plated in a 384-well black walled clear bottom plate at 12,500 cells per well in 100µL per well with 4.5µM doxycycline. Cells were incubated overnight to adhere to plate. The following day cells were treated with drugs and combinations. Media used to dilute compounds contained 1µg/mL doxycycline. After cells were treated, plate was allowed to incubate for 6h. Caspase-glo (promega) reagent was added and the plate was allowed to incubate for 30min. Plate was then read for luminescence at 100ms. Presto-blu was added at a volume of 1/10 total volume per well (2.5µL) and cells were allowed to incubate for 10min. Plate was then read for fluorescence at $560_{ex}/590_{em}$. Values were calculated by: ([Luminescence*100]/Fluorescence)/$DMSO_{avg}$

Results

Regulation of Mcl-1 and Bcl-xL by Multiple Kinases. Mcl-1 and Bcl-xL are phosphorylated at different sites (Figure 8A). Mcl-1 is phosphorylated at different sites along its N-terminal region directly by different kinases. Particularly the PEST and PEST-like regions which can be phosphorylated at different residues to either stabilize or target Mcl-1 for degradation (Figure 8B). Well documented phosphorylation sites include S64, T92, S121, and T163. S64 phosphorylation results in resistance to TRAIL mediated apoptosis and enhance binding of Mcl-1 to BIM [101]. T92 phosphorylation by CDK1 results in Mcl-1 degradation [105]. In contrast, T92 phosphorylation by CDK2 leads to stabilization [31]. S121 phosphorylation by JNK results in stabilization of Mcl-1 [106]. T163 phosphorylation results in either stabilization (CDK2) of Mcl-1 or inactivation (JNK) of Mcl-1 depending on the state of the cell [30, 31, 106]. T70 phosphorylation by CDK2 results in

ubiquitination and subsequent degradation of Mcl-1 [68]. T68, T156, and S159 all have been characterized but are not well documented [107]. Mcl-1 transcription is mediated by RNA polymerase II. Previous studies have detailed the expression of Mcl-1 by RNA pol II and how it is dependent on phosphorylation of the carboxy-terminal domain of RPB1 by CDK2 [32, 99, 108]. Inhibition of CDK2 leads to decrease in Mcl-1 expression as Mcl-1 is a short-lived protein. Furthermore, NOXA, the inhibitory BH3-only protein of Mcl-1 is also regulated at different sites which determine its pro-apoptotic role. For example, S13 is phosphorylated by CDK5 under high glucose cellular conditions, as a result NOXA no longer retains its pro-apoptotic function [43]. These data indicate an intimate regulation of Mcl-1 through kinases and their network of signaling cascades. Targeting kinases which are responsible for Mcl-1 stability, function, or expression would be a favorable method of targeting Mcl-1.

In contrast, Bcl-xL lacks PEST domains and is not as impacted by phosphorylation as Mcl-1. However, different residues of BclxL have been implicated in Bcl-xL function (Figure 8B). For example, phosphorylation at T47 and T115 by JNK decreases the ability of Bcl-xL to prevent apoptosis [109]. IKKβ/NFκB pathway regulates Bcl-xL and Mcl-1 expression [40, 42, 110]. Both proteins fall under NFκB/p65 control as demonstrated previously. MAPK signaling also leads to Bcl-xL expression through FOXO transcription factors [111-113]. Additionally, BAD, the regulator of Bcl-xL is controlled by multiple pathways and phosphorylation sites. Akt phosphorylates BAD, thus preventing its interaction with Bcl-xL. BAD is also phosphorylated at multiple serine residues which control its stability [12]. In addition to direct phosphorylation of BAD, multiple signal transduction pathways control BAD expression thus Bcl-xL function. For example, Akt is also a well-known negative regulator of FOXO3a, a transcription regulator that controls expression of critical apoptosis regulatory proteins such as BAD, Bim and Fas death ligand

[114]. Finally, it has been shown that Akt regulates survival in macrophages by promoting the expression of Bcl-xL through the NF-κB pathway [103]. These data suggest multiple kinase inhibitors may be used to indirectly target Bcl-xL.

Considering the variety of kinases that modify Mcl-1 and Bcl-xL, and their concurrent inactivation leads to apoptosis, a screening strategy was developed to identify kinases which trip either arm of the apoptotic pathway (Figure 8C). To identify kinase inhibitors which are selective for either arm of the apoptotic pathway, doxycycline inducible HeLa cell lines were used (Figure 8D). HeLa Dox-NOXA over-express the Mcl-1 inhibitor NOXA and are sensitive to kinase inhibitors which target the Bcl-xL arm of the apoptotic pathway. HeLa Dox-BAD3SA overexpress the Bcl-xL inhibitor BAD and would be sensitive to kinase inhibitors which target Mcl-1. HeLa Dox-GFP is the negative control and would be sensitive to non-specific kinase inhibitors, *i.e.* kinase inhibitors which target both Bcl-xL and Mcl-1. In theory, the combination of kinase inhibitor hits from the HeLa Dox-NOXA and HeLa Dox-BAD3SA would be lethal in HeLa Dox-GFP. These combinations can then be further validated in other cancer cell lines.

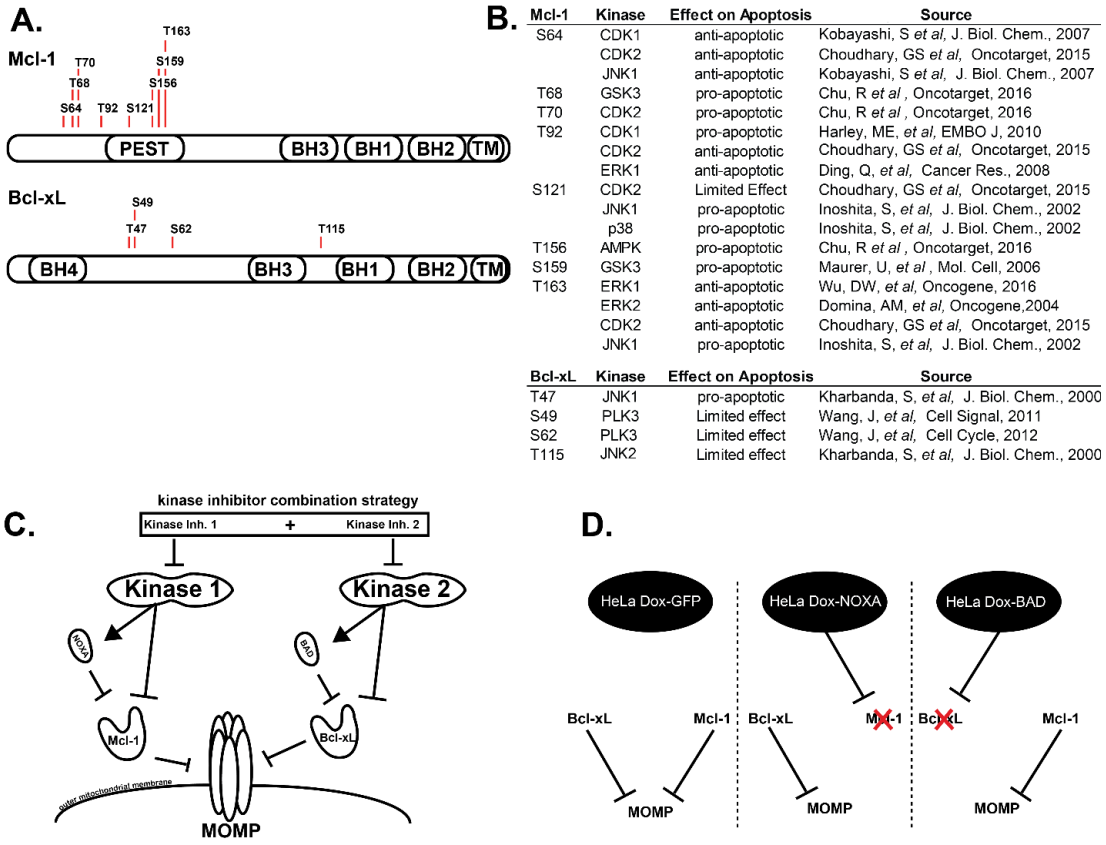


Figure 8. Rational approach to identifying novel combinations that perturb the apoptotic pathway using chemical-genetic screens. **A,** phosphorylation sites on mcl-1 and Bcl-xL. **B,** phosphorylation site on Mcl-1 and Bcl-xL and the resulting effect on apoptosis. **C,** schematic representation of identifying combinations of kinase inhibitors that will induce apoptosis. **D**, schematic of doxycycline inducible cell lines. HeLa Dox-NOXA and HeLa Dox –BAD3SA rely on either Mcl-1 or Bcl-xL for survival. Where HeLa Dox-GFP has both functional Mcl-1 and Bcl-xL.

Validation of Chemical Genetic Screen.

HeLa Dox-inducible cell lines were validated by western blot showing maximal NOXA and BAD3SA expression at 3 hours at 1µg/mL of doxycycline (Figure 9A). After 12 hours, NOXA expression decreased although BAD3SA expression persisted. Based on these results doxycycline concentrations were kept at 1 µg/mL and were incubated for 3 hours. To validate the screen, camptothecin and ABT-263 were used as control compounds to induce apoptosis in either a Mcl-1- or Bcl-xL-dependent manner (Figure 9B). Based on previously described methods, camptothecin, a DNA damaging agent induces NOXA expression which then inhibits Mcl-1 [10]. As a result, camptothecin was used as a control compound to validate selectivity of Mcl-1 attenuators. ABT-263, a direct inhibitor of Bcl-xL was used as a control for inducing apoptosis in a Bcl-xL dependent manner. Camptothecin selectively induced robust apoptosis in HeLa Dox-BAD3SA cells in the presence of doxycycline with a 4-fold increase in caspase activity as determined by caspase activation assay. In contrast, ABT-263 induced apoptosis in HeLa Dox-NOXA cells with similar 4-fold increase in caspase activation. In HeLa Dox-GFP robust apoptosis was not observed to the extent of caspase activation observed in HeLa Dox-NOXA and HeLa Dox-BAD3SA cells.

Z-score is a non-statistical parameter that is used to determine whether an assay is suitable for high content screens[115]. The equation (Figure 9C) uses the standard deviation of the control treated replicates and the treatment group replicates and the mean of the values of replicates to calculate a value between 0 and 1. The larger the value, the more precise an assay is at identifying hits. An optimal value is between 0.5 and 1 which means the assay is optimal and there is enough of a difference between the control and treatment group standard deviations that a hit will be valid. Using 50µM camptothecin and

5μM ABT-263 we treated both HeLa Dox-BAD3SA and HeLa Dox-NOXA respectively at n = 75 and used caspase activation assays to determine the compounds ability to induce apoptosis. (Figure 9D and E). A Z-score of 0.61 and 0.64 was calculated for HeLa Dox-BAD3SA/ 50μM Camptothecin and HeLa Dox-NOXA/5μM ABT-263 respectively. This indicated either cell line would identify kinase inhibitors that would trip either arm of the apoptotic pathway with relatively high precision (Figure 9F).

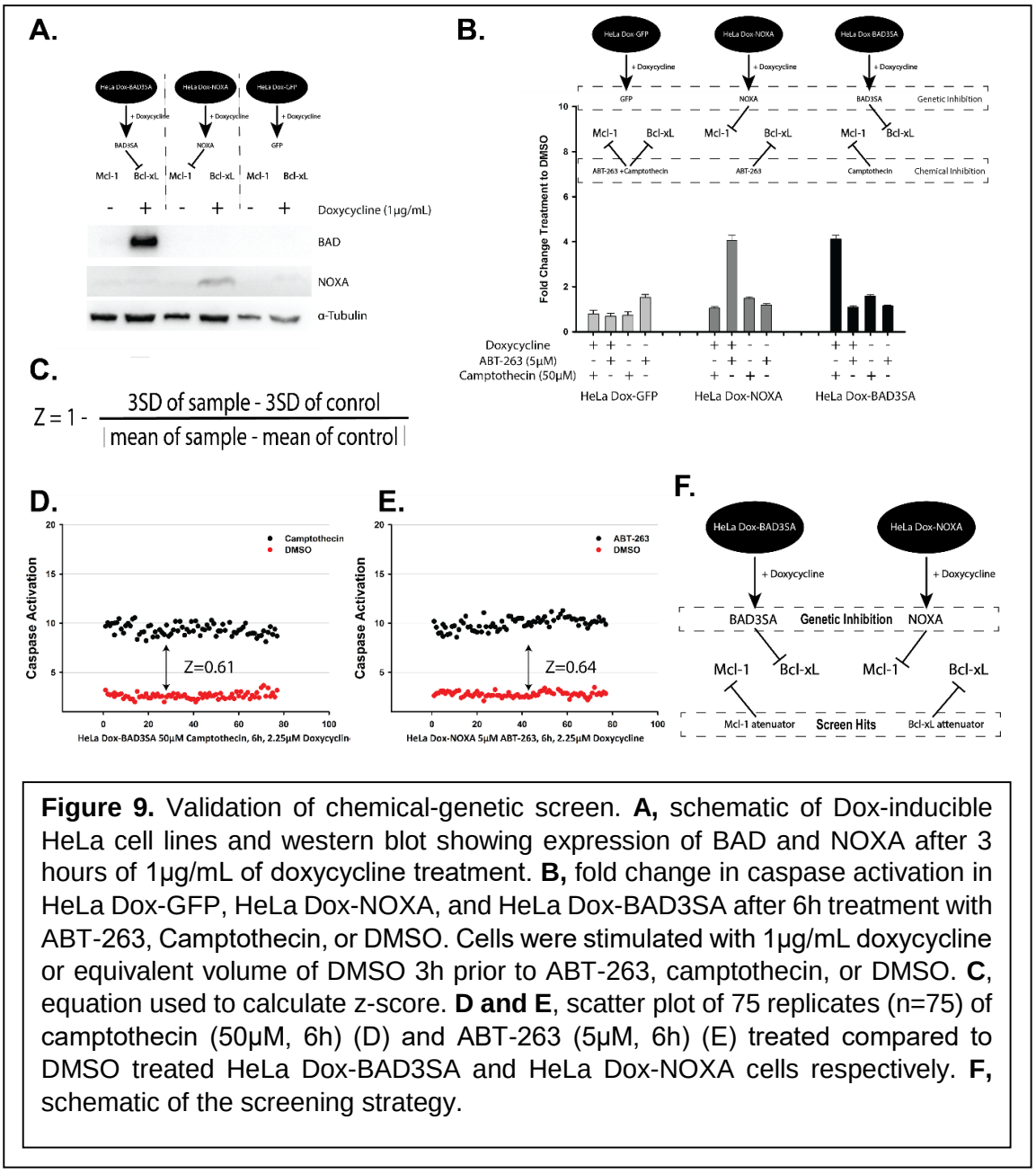


Figure 9. Validation of chemical-genetic screen. **A,** schematic of Dox-inducible HeLa cell lines and western blot showing expression of BAD and NOXA after 3 hours of 1µg/mL of doxycycline treatment. **B,** fold change in caspase activation in HeLa Dox-GFP, HeLa Dox-NOXA, and HeLa Dox-BAD3SA after 6h treatment with ABT-263, Camptothecin, or DMSO. Cells were stimulated with 1µg/mL doxycycline or equivalent volume of DMSO 3h prior to ABT-263, camptothecin, or DMSO. **C**, equation used to calculate z-score. **D and E**, scatter plot of 75 replicates (n=75) of camptothecin (50µM, 6h) (D) and ABT-263 (5µM, 6h) (E) treated compared to DMSO treated HeLa Dox-BAD3SA and HeLa Dox-NOXA cells respectively. **F,** schematic of the screening strategy.

Screen Results.

With the screening method validated, we sought to screen a 355 member kinase library available through SelleckChem (Figure 10A). The kinase library spans a diverse set of inhibitors which target a variety of kinases and signal transduction pathways. We screened the 355-member kinase library against the panel of the dox-inducible HeLa cell lines and measured induction of apoptosis through caspase activation using caspase activation assays. We optimized the assay conditions to 3 hours of 1µg/mL doxycycline treatment followed by 6 hours of 1µM kinase inhibitor treatment. We anticipated three different types of hits that could be identified from the screen: (1) Mcl-1 targeting compounds, (2) Bcl-xL targeting compounds, or (3) compounds which possibly target both arms of the apoptotic pathway (Figure 10B). Screen results show only compounds which would induce caspase activation at a low dose and at an early time-point, therefore there were many inactive compounds (Figure 10C). However, a small subset of compounds were observed to induce apoptosis as indicated by caspase activation in either a Mcl-1 or Bcl-xL dependent manner (Figure 10D). Screen hits were determined by calculating a threshold of 99.7% outside the normal distribution. This was determined by using the statistical equation: $CI_{99.5\%} = mean + 3 \times standard\ deviation$ (Figure 10E). The equation is used to identify kinase inhibitors which induced caspase activation at a high enough value that we can determine with 99.5% confidence interval that the kinase inhibitor is a hit. We calculated fold change in caspase activation relative to DMSO treated cells. We then normalized the data to a value calculated from the equation for each cell line. Therefore, any fold change observed that exceeded 1 selectively in a single cell line was considered a hit. Taken together, hits were identified if the kinase inhibitor met two criteria: [1]If the fold change in

caspase activation observed exceeded the threshold and [2]the kinase inhibitor had to specifically exceed the threshold in a single cell line.

Interestingly, the hits for each individual cell line clustered according to their apoptotic protein target (Figure 10F). Remarkably, CDK inhibitors were clear hits against the HeLa BAD3SA cell line with AT7519, Dinaciclib, Flavopiridol, and P276-00. Interestingly, this find is corroborated by many studies supporting Mcl-1 attenuation by CDK inhibitors. This find was a welcome assurance in the reliability of the screen. Barasertib, an Aurora kinase inhibitor was also identified as a hit against HeLa Dox-BAD3SA cells, indicating it may perturb Mcl-1 and induce apoptosis through Mcl-1 inactivation. Unexpectedly, PI3K/mTOR inhibitors clustered as hits against HeLa Dox-NOXA cells, suggesting they induce apoptosis in a Bcl-xL manner. Finally, a non-specific hit, Pelitinib, was identified that induced caspase activation in only the HeLa Dox-GFP cell line.

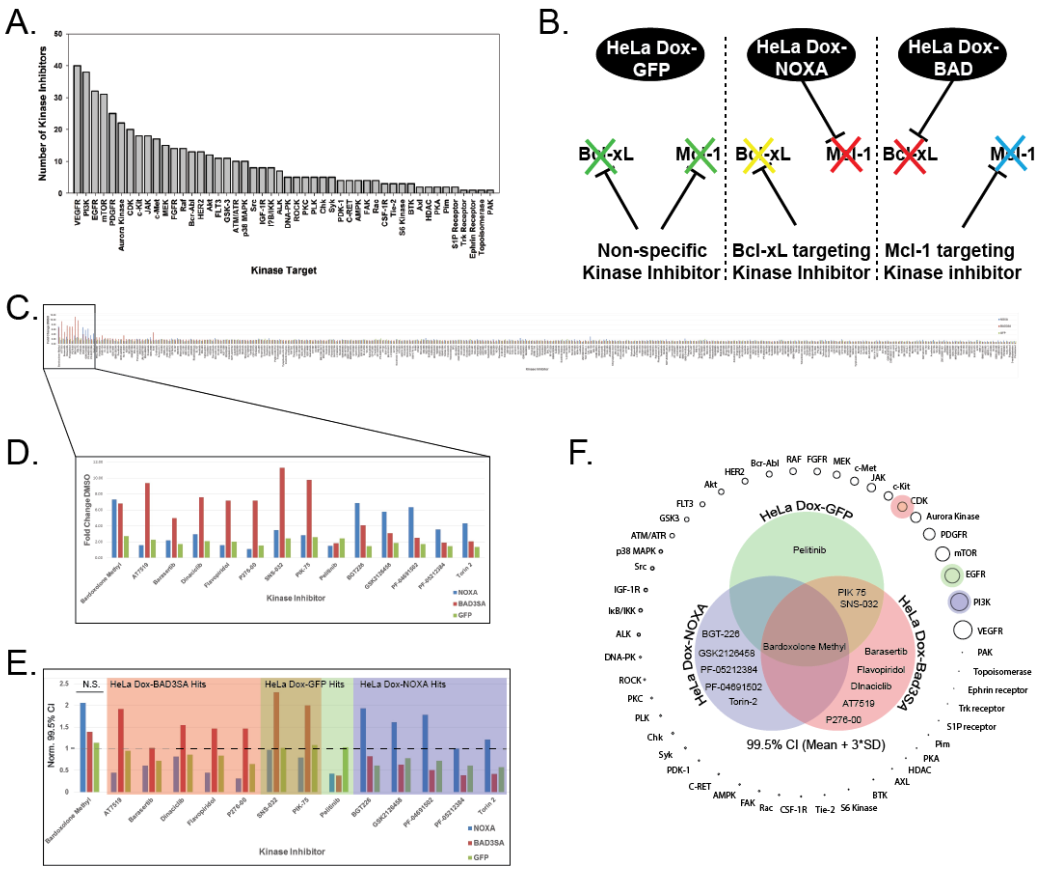


Figure 10. Screen results. **A,** bar chart representation of kinase or protein targets of the SellechChem inhibitor library. **B,** schematic depicting the three possible hits from the screen. **C,** bar chart representing fold change caspase activation in three HeLa Dox-cell lines after 1μM treatment with kinase inhibitor library for 6h. HeLa Dox-cell lines were stimulated with dox. For 3h prior to treatment with kinase inhibitors. **D,** inset of hits expanded from larger bar chart. **E,** bar chart representing normalized screen hits. Hits are normalized to 99.5% confidence interval threshold. **F,** venn diagram representation of the clustering of hits with 99.5% confidence interval.

Validation of Screen Hits. Surprisingly, 10 of the eleven (Torin 2 has not entered clinical trials) hits are in multiple phases of clinical trails (Figure 11A). AT7519 has entered phase II clinical trials for the treatment of Mantle Cell Lymphoma (NCT01652144) and multiple myeloma (NCT01183949). Barasertib which is an Aurora kinase inhibitor, has entered phase I/II clinical trials as well [116, 117]. Dinaciclib has entered phase III clinical trials for treatment of Chronic Lymphocytic Leukemia (NCT01580228) comparing it to Ofatumumab. Flavipiridol has been evaluated in a phase II clinical trial for treatment of Acute Myeloid Leukemia (NCT02520011). Finally, P276-00 has been evaluate in a phase II clinical trail to treat head and neck cancer (NCT01903018). Of note, Dinaciclib and P276-00 have advanced to Phase III clinical trials indicating they are excellent candidates for drug repurposing. Of the PI3K/mTOR inhibitors, most have made it to at least phase I clinical trials. BGT226 has been used in a phase I/II clinical trial to treat breast cancer (NCT00600275). GSK2126458 has entered phase I clinical trials for the treatment of solid tumors (NCT01248858 and NCT00972686). PF04691502 and PF05212384 have both advanced to phase II clinical trials for the treatment of breast cancer and Acute Myeloid Leukemia respectively (NCT01430585 and NCT02438761). Of significance, all compounds with the exception of Torin 2 have advanced to at least Phase I clinical trials. Consequently, the pipeline to optimize the combinations in an *in vivo* model will be facilitated by previous data.

To validate the hits, PARP cleavage as well as caspase cleavage was used as a determining factor of identifying compounds which were specific in inducing apoptosis in select cell lines (Figure 11B). When the concentration of 1μM was kept constant but the time of incubation was decreased to 3 hours. Dinaciclib, Flavopiridol, P276-00, and BGT-226 showed specificity as evidenced by cleaved caspase. PF05212384 showed specificity as evidenced by cleaved PARP. When the time of incubation was kept at 6h and the

concentration decreased to 500nM, AT7519, P276-00, PF04691502, and PF05212384 showed specificity as shown by cleaved PARP (Figure 11C). GSK2126458 and Torin 2 showed limited specificity as they induced PARP cleavage as well as caspase cleavage in both HeLa Dox-NOXA and HeLa Dox-BAD3SA at both time points and at both concentrations. Barasertib showed contradictory results as inducing PARP cleavage selectively in the HeLa Dox-NOXA cell line, since Barasertib is an Aurora Kinase inhibitor it may be a false positive in the screen. These results validate most of the hits as specific to a single cell line. Because of the rational clustering of the hits and the limited number, subsequent studies include only the ten hits which targeted both Hela Dox-NOXA and HeLa Dox-BAD3SA. Pelitinib targeted HeLa Dox-GFP and no rational combinations could be derived from this compound, therefore it is excluded in subsequent studies.

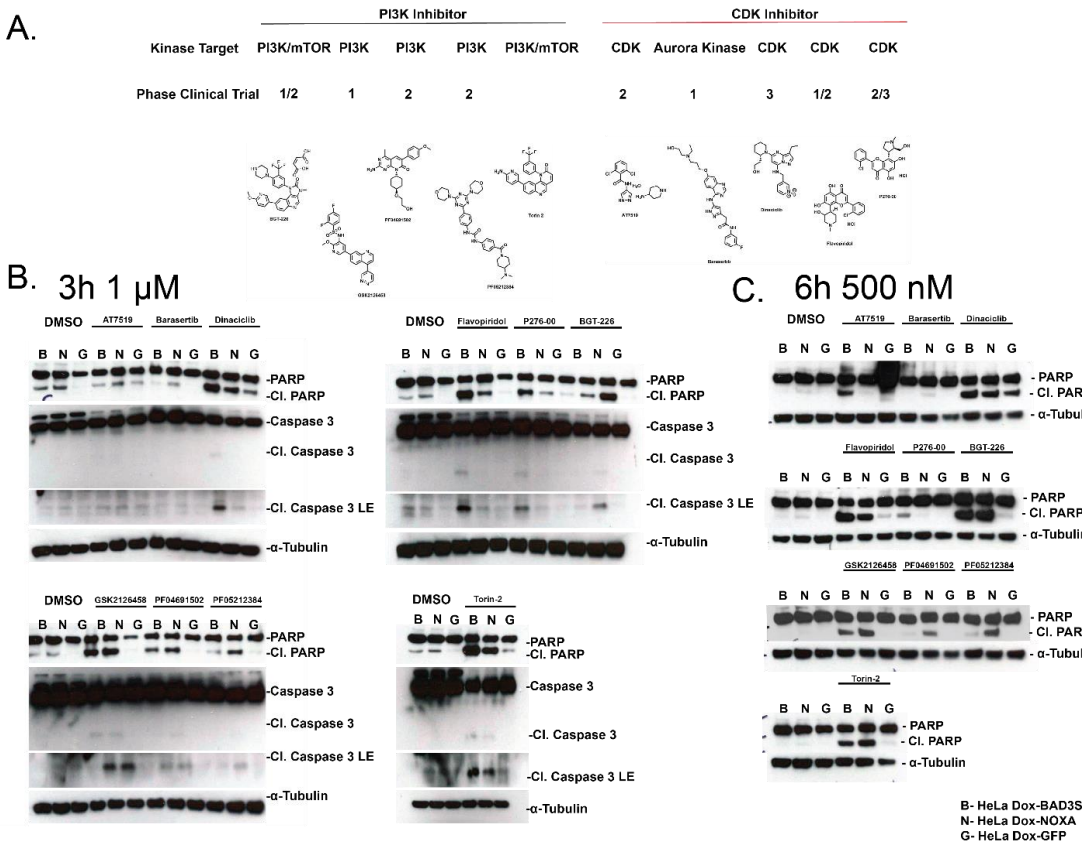


Figure 11. Validation of hits. **A,** table showing structure and clinical phase of inhibitor development. **B,** western blot analyses of PARP, cleaved PARP, and caspase 3 in HeLa Dox-cell lines treated with kinase inhibitors at 1μM for 3h. **C,** western blot analyses of PARP and cleaved PARP in HeLa Dox-cells treated at 500nM for 6h.

Cell Based Studies and Synergism Analysis.

To determine whether the combinations from the screen were synergistic we performed growth inhibition (GI) studies on three different cell lines: S2-013, HCT116 and MiaPaCa-2. GI studies were performed at a broad range of doses, which would accommodate for the diverse compounds treated and ranged from 1000nM to 0.01nM. To determine if the IC_{50} is decreasing when comparing the combination to the single treatment, we plotted the IC_{50} of each drug compared to the single treatment. The fold change difference between the combination and single treatment is plotted as a 3-D bar graph (Figure 12 A-F). Each panel is a representation of the fold change in IC_{50} when normalized to each CDK or PI3K/mTOR inhibitor. To determine whether the combinations were synergistic, the average CI value was calculated from the high effect doses (ED_{90}, ED_{95}, and ED_{99}) (Figure 12 G-I) [38]. Consistent with the fold change in IC_{50}, HCT116 cells had lower CI values. Strong synergism, as indicated by CI values below 0.3, was observed in many combinations, however 80% of the combination CI values were below 1, indicating synergism. AT7519 and PF046915 showed moderate synergism in S2-013 cells, the CI value for their combination was below 1 but above 0.3. AT7519 and PF04691502 induced PARP cleavage in only HeLa Dox-BAD3SA and HeLa Dox-NOXA cells respectively (Figure 11C).

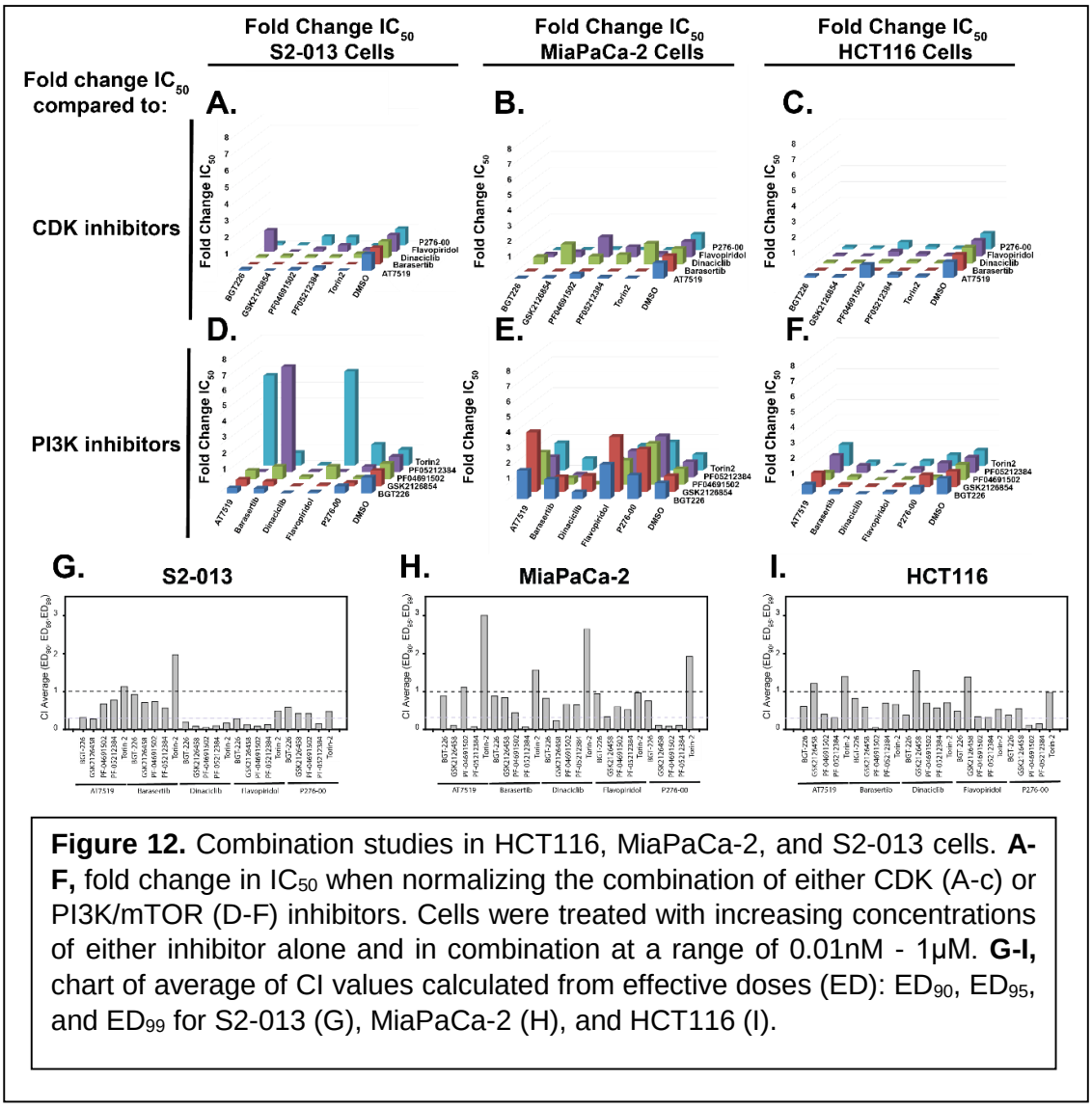


Figure 12. Combination studies in HCT116, MiaPaCa-2, and S2-013 cells. **A-F,** fold change in IC_{50} when normalizing the combination of either CDK (A-c) or PI3K/mTOR (D-F) inhibitors. Cells were treated with increasing concentrations of either inhibitor alone and in combination at a range of 0.01nM - 1μM. **G-I,** chart of average of CI values calculated from effective doses (ED): ED_{90}, ED_{95}, and ED_{99} for S2-013 (G), MiaPaCa-2 (H), and HCT116 (I).

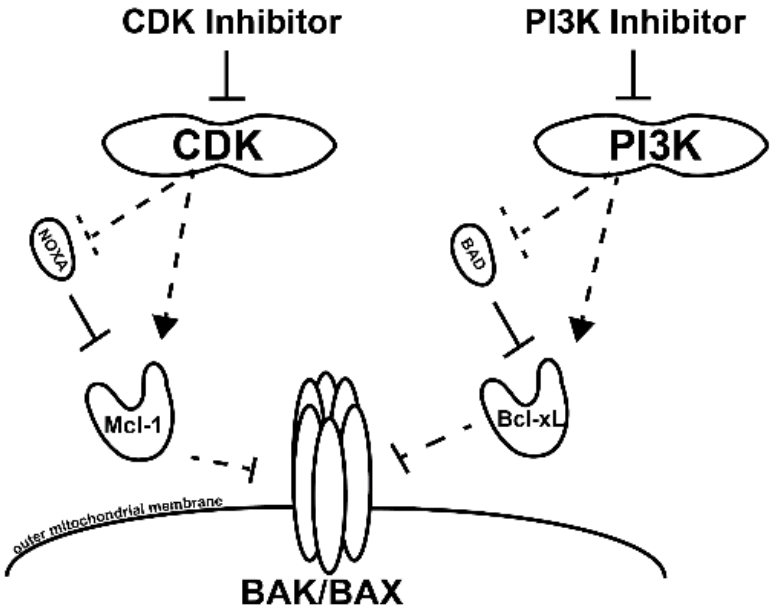


Figure 13. Combination strategy. PI3K/mTOR inhibitors induce apoptosis through Bcl-xL arm of the apoptotic pathway. CDK inhibitors induce apoptosis through the Mcl-1 arm of the apoptotic pathway.

Discussion

The data suggest CDK inhibitors cluster as hits in the screen with HeLa Dox-BAD3SA. BAD3SA acts as an inhibitor of Bcl-xL and CDK inhibitors P276-00, AT7519, Dinaciclib and Flavopiridol all induce caspase by inactivating the Mcl-1 arm of the apoptotic pathway. Strong evidence lends validity to this find based on studies performed with pan-CDK inhibitors such as Roscovitine and Flavopiridol [32, 99]. As previously mentioned, CDK2, CDK5, and CDK9 regulate Mcl-1 [31, 32, 43]. CDK2 directly phosphorylates Mcl-1 at T163, increasing its stability. CDK5 has been shown to phosphorylate NOXA at S13, which causes it to lose its apoptotic function of inhibiting Mcl-1. Finally CDK9 phosphorylates RNA polymerase II at the carboxyl terminal domain, this phosphorylation event regulates transcription elongation.

The CDK inhibitors identified all have a preference for CDK2 and CDK9. P276-00 inhibits CDK2 and CDK9 at 224nM and 20nM respectively [118]. AT7519 inhibits CDK9, CDK5, and CDK2 with IC50 values less than 50nM [119]. Dinaciclib, the most potent inhibitor of CDKs inhibits CDK9, CDK5, and CDK2 at concentrations less than 10nM [120]. Finally, Flavopiridol inhibits CDK2 and CDK9 with IC50 values of 40nM and 3nM respectively [121, 122]. The finding that CDK inhibitors clustered in this manner suggests targeting these three CDKs is a viable polypharmacological approach to inhibiting Mcl-1.

On the other hand, the clustering of PI3K/mTOR inhibitors: BGT263, GSK2126458, PF05212384, PF04691502, Torin-2 as hits to HeLa Dox-NOXA was unexpected. Direct phosphorylation of Bcl-xL is not as frequent or is known to have an impact on its function. Bcl-xL does not form as many complexes as Mcl-1 and lacks a PEST region. However, phosphorylation events impact BAD, the Bcl-xL regulator. Direct phosphorylation of BAD by Akt at S112 and S136 has been shown to prevent interaction with Bcl-xL [12, 123]. Furthermore, Akt regulates BAD expression be phosphorylating

forkhead transcription factor (FOXO). Phosphorylation of FOXO transcription factors prevents their nuclear translocation and consequent BAD expression [114]. More recently, PI3K/Akt/mTOR/p70S6K signaling has been implicated in BAD phosphorylation and trafficking of BAD, leading to decreased association with Bcl-xL [124]. These studies suggest the PI3K/Akt/mTOR cell signaling cascades may be targeted to facilitate BAD inhibition of Bcl-xL. BGT226 and Torin-2 induced PARP cleavage in both HeLa Dox-NOXA and HeLa Dox-BAD3SA suggests they are non-selective. However, GSK2126458, PF04691502 and PF05212384 showed selectivity in the PARP cleavage studies indicating that they may be suitable for combination studies.

The clustering of PI3K/mTOR inhibitors around HeLa Dox-NOXA and CDK inhibitors around HeLa Dox-BAD3SA cells was an interesting find considering CDK and PI3K combinations are seldom explored, let alone in the context of pursuing effects on apoptosis. One recent study identified Dinaciclib and MK2206 as a promising therapeutic option for pancreatic cancer [125]. They showed the combined treatment of Dinaciclib, a potent inhibitor of CDK 1, 2, 5, and 9, with MK2206, an AKT inhibitor, exhibited potent effects in a pancreatic ductal adenocarcinoma (PDAC) xenograft mouse model. Although Dinaciclib has been widely studied as an anti-cancer agent, this study is unique as the combination of CDK inhibition combined with Akt inhibition is novel, and supports our results, lending validity to the proposed combination of CDK and PI3K/mTOR inhibitors. Akt is a major downstream effector of PI3K and further investigation will have to be performed as to why MK2206 was not identified as a hit in our screen. The model proposed in this particular study stems from previous data. They suggest CDK5 inhibition by Dinaciclib leads to attenuation of RalA activity, which was previously shown to result in loss of migration, proliferation, and metastasis [126]. Simultaneous inhibition of PI3K/Akt and MAPK (through CDK5 dependent RalA activation) pathways resulted in further

inhibition of tumor growth. These findings prompted them to use the combination of Dinaciclib and MK2206. Co-inhibition of Akt and CDK5 results in profound tumor regression in a PDAC xenograft model. Based on these results, a phase I clinical trial has been initiated to explore the combination of Dinaciclib and MK2206 (NCT01783171). Together these studies propose a model for combination therapy of pancreatic cancer, in which inhibition of CDK5 has a synergistic effect when paired with Akt inhibition. In contrast, our results suggest the apoptotic pathway may be the reason for the synergistic response observed between both inhibitors. Further investigation will be required to determine the mechanism of action behind these combinations. However, combining PI3K inhibitors with CDK inhibitors as a therapeutic option to target the apoptotic pathway is novel. Furthermore, this screening system could be used to find novel combinations by screening a larger library.

Chapter 4: Discussion

Summary

Mcl-1 and Bcl-xL play a major role in regulating the apoptotic pathway and are essential proteins that control cell fate. In reported studies, Mcl-1 was targeted through indirect mechanisms and a novel combination of kinase inhibitors was identified that perturb the apoptotic pathway. Consistently our studies suggest there are therapeutic combinations that indirectly target Mcl-1 and Bcl-xL to induce apoptosis.

There have been successful inhibitors developed against Bcl-xL, which have advanced to the clinics, and alternative methods of Mcl-1 inhibition are being explored. So far, the number of kinase inhibitors which have been found to perturb Mcl-1 are few aside from CDK inhibitors. The studies presented in this dissertation *were to develop a polypharmacological approach to target Mcl-1 through CDK inhibition, and to identify novel kinase inhibitor combinations that indirectly target the Mcl-1 and Bcl-xL to induce apoptosis.*

The first study explored CDK inhibition as a mechanism to inhibit Mcl-1. As discussed above, CDKs are involved with the regulation of Mcl-1 function, stability, and expression. We took a poly-pharmacological approach to designing an inhibitor, where in the inhibitor binds to the hinge region of CDK2, CDK5 and CDK9, key regulators of Mcl-1. We first used cell free kinase assays to construct a target profile for each compound then based on the kinase target, select compounds which preferentially inhibit the kinase activity of CDK2, CDK5, and CDK9. After funneling the compounds using a caspase 3/7 activation assay and growth inhibition assays, a single molecule, **24**, was shown to inhibit the selected CDKs and induce apoptosis by targeting Mcl-1. The compound was then paired with a Bcl-xL selective BH3 mimetic to determine if there was synergism. These

studies indicate CDK inhibition can be tailored to perturb Mcl-1 through a poly-pharmacological approach.

The second study aimed to determine whether the Mcl-1 and Bcl-xL apoptotic pathway can be targeted by different kinase inhibitors. Furthermore, would kinase inhibitors, which target each arm of the apoptotic pathways when combined, lead to synergism. To identify kinase inhibitor combinations, we used a chemical genetic screening approach. Cell lines, which express the anti-apoptotic BH3 regulators of Mcl-1 and Bcl-xL, NOXA and BAD respectively were used to identify kinase inhibitors, which selectively inhibit the Bcl-xL and Mcl-1 arm of the apoptotic pathway. We approached the study expecting a diverse representation of kinase inhibitors as hits to either arm of the apoptotic pathway. Surprisingly, we identified a cohort of kinases, which clustered in two distinct categories. Mcl-1 targeting kinase inhibitors were predominantly CDK inhibitors while Bcl-xL inhibitors were predominantly PI3K/mTOR inhibitors. This study identified novel combinations of kinase inhibitors, which will be further validated in future studies.

Mcl-1 regulation by CDKs

CDKs regulate Mcl-1 through multiple mechanisms: direct phosphorylation, which directly impacts Mcl-1 stability; phosphorylation of NOXA, which influences Mcl-1 function; and phosphorylation of RNA polymerase II, which affects Mcl-1 expression. Most studies focus on a single mechanism which perturbs Mcl-1. However, with regulatory proteins affecting Mcl-1, it becomes difficult to determine which ones are most critical for Mcl-1 function, expression, and stability. Mcl-1 is phosphorylated by many proteins, namely GSK, CDKs, and JNK. However, our studies show that CDK inhibitors appear to have the greatest impact on Mcl-1. Previous studies have taken a focused approach to determining the mechanism of action of CDK inhibitors and often attribute the entirety of their activity to a single kinase. CDK9 has been shown to regulate Mcl-1 transcription by

phosphorylation of RNA polymerase II carboxy terminal domain [32, 127, 128]. CDK9, or transcription elongation factor P-TEFb, regulates transcription by phosphorylating the carboxy terminal domain of the large subunit or RNA polymerase II [129, 130]. Phosphorylation of the RNA pol II leads to transcription elongation. When CDK9 is inhibited, Mcl-1 is rapidly degraded. Mcl-1 degradation has been observed upon treatment with other CDK inhibitors including Roscovitine, Flavopiridol, and Dinaciclib [32, 99, 127]. Roscovitine and Flavopiridol are described as CDK 9 inhibitors when considering their effects on Mcl-1 [32, 99]. Roscovitine has a cell free IC_{50} against CDK2 and CDK5 of 700nM and 160nM respectively [131]. Flavopiridol inhibits CDK2 at 40nM [122]. Dinaciclib inhibits CDK1, CDK2, CDK5, and CDK9 [120]. These studies show that the activity of CDK inhibitors against Mcl-1 may not be uniform. Our study leverages the promiscuity of CDK inhibitors to design a single compound, with poly-pharmacological activity against CDKs that regulate Mcl-1.

CDK inhibitors can be divided into two distinct groups: 1st generation CDK inhibitors and 2nd generation CDK inhibitors [132]. 1st generation CDK inhibitors are generally non-specific, an excellent example is Roscovitine, which inhibits multiple CDKs including CDK1, CDK2, CDK5, CDK7, and CDK9 [131]. 2nd generation CDK inhibitors are more selective, such as the clinically approved Palbociclib which inhibits CDK 4 and CDK6 [133], Palbociclib is the first CDK inhibitor to be approved by the FDA for clinical use. Each of these inhibitors targets the ATP binding sites of CDKs making their development as selective kinase inhibitors a challenge. However, more selective CDK inhibitors such as Palbociclib target CDK4 and CDK6. CDK4 and CDK6 are responsible for G1-S transition in the cell cycle, therefore inhibition of both of these kinases results in cell cycle arrest at the G1 phase [133]. Conversely, first generation CDK inhibitors were more akin to polypharmacological agents, targeting multiple CDKs. Roscovitine, a 1st generation CDK

inhibitor, has been successful in clinical studies, advancing to phase II clinical trials (NCT02649751). Dinaciclib is a more potent CDK inhibitor, which has been shown to potently inhibit multiple CDKs [120]. Of the three previously mentioned CDK inhibitors, only Dinaciclib and Roscovitine have been shown to inhibit Mcl-1. This is because they target CDKs, which regulate Mcl-1. This comparison suggests that although CDK inhibitors have different biological effects based on their target profile.

Polypharmacology approach to repurposing drugs

Polypharmacology is gaining traction in the search for cancer therapeutics and is a concept where a single molecule can inhibit multiple targets [87]. The aminopyrazole core is a privileged scaffold, which has been shown to bind to the hinge region of multiple CDKs. In our study we optimized an analog which was previously developed to inhibit CDK5 by Pfizer [134].

Here we used a screening strategy that was intended to identify a polypharmacological agent that selectively targeted CDKs that regulate Mcl-1. We approached the design of this molecule by analyzing the ATP binding pocket in CDK1, CDK2, CDK4, CDK5, CDK6, CDK7, and, CDK9. The observation that CDK1, CDK2, CDK5, and CDK9 had high homology in their ATP binding pocket and that three of these kinases were previously shown to be involved in Mcl-1 regulation prompted us to design a polypharmacological agent to target Mcl-1.

A limitation to this approach is that it would likely also target CDK1 which may lead to undesirable side effects [135]. While CDK1 inhibition has been explored for cancer therapy [136], the inactivation of CDK1 is considered toxic based on knockout studies [137]. However, inhibition of CDK1 may also be playing a role in Mcl-1 inhibition. A recent study suggests inhibition of CDK1 led to the perturbation of the p53-NOXA-Mcl-1 axis [138]. In this study, CDK1 is knocked down in mouse embryonic stem cells (mESC), the

result is decreased Mcl-1 and subsequent apoptosis which was only observed in mESCs. The CDK1 inhibition leads to a DNA damage response by p53, which upregulates NOXA. This study suggests that the aminopyrazole compound may also be functioning through CDK1. However, in pancreatic cancer, p53 is often lost. Despite this, it is interesting that the inhibition of CDK1 and its induction of apoptosis is ESC specific and that it is dependent on Mcl-1. It would be interesting to determine whether these effects may be observed in cancer stem cells, if so, the polypharmacological approach can be taken further to target CDK1 and specifically target differentiated cells with CDK2, CDK5, and CDK9; and target cancer stem cells with CDK1.

Perturbing the apoptotic pathway using kinase inhibitors

We decided to take our studies a step further by asking whether: A) kinase inhibitors perturb either the Mcl-1 or Bcl-xL arm of the apoptotic pathway specifically and B) whether we can design kinase inhibitor combinations that target these two arms of the apoptotic pathway. The series of experiments performed to answer these questions not only yielded various synergistic combinations, it also revealed an interesting pattern of kinase inhibitors which are specific to either arm of the apoptotic pathway: PI3K/mTOR inhibitors target the Bcl-xL arm of the apoptotic pathway and CDK inhibitors target the Mcl-1 arm of the apoptotic pathway. Interestingly, the combination of CDK and PI3K/mTOR inhibitors are seldom tried.

CDK and PI3K inhibitors are increasingly being explored as therapeutics for multiple cancers [139-142]. In breast cancer, resistance to BYL719, a PI3K inhibitor, was overcome by treatment with a CDK4/6 inhibitor LEE011 [139]. The mechanism of action was not apoptotic, instead the combination resulted in cell cycle arrest with accumulation of cells in G1. Conversely, a polypharmacological approach was used to synthesize a dual CDK/PI3K inhibitor. CDK2 inactivation paired with PI3K inhibition by the single dual

inhibitor and the combination of PI3K and CDK inhibitors resulted in cell death in colon cancer cells [141]. Finally, the combination of mTOR inhibitor, rapamycin, as well as CDK inhibitors, roscovitine and purvalanol were found to be a favorable combination against prostate cancer cells [142]. These studies show favorable results when CDK inhibitors are paired with PI3K/mTOR inhibitors and that the mechanism of action may involve apoptosis. In our study we identified four CDK inhibitors and five PI3K inhibitors to be tested in combination.

Although we identified 25 possible combinations, not all were synergistic in the three cell lines we tested them in, HCT116, S2013, and MiaPaCa-2. The four CDK inhibitors, which were identified from the screen are pan-CDK inhibitors. AT7519 and Dinaciclib target CDK2, CDK5, and CDK9, which are regulators of Mcl-1 [119, 120]. However, synergism was observed with P276-00 and Flavopiridol, which are more pan-CDK inhibitors, targeting a wider variety of CDKs [118, 143]. AT7519 and Dinaciclib combinations yielded between 40- 50% strong to moderately synergistic combinations whereas Flavopiridol and P276-00 combinations yielded 60-70% strong to moderately synergistic combinations. All of the PI3K/mTOR inhibitors target P110α with the exception of Torin 2, which inhibits mTOR [144-148]. The mechanism of PI3K inhibition and its effect on Bcl-xL was not investigated in detail by this study. Instead, the discovery that PI3K/mTOR inhibition preferentially induces apoptosis in cell line with functional Bcl-xL is the novel concept. Previous studies have indicated a connection between Mcl-1, Bcl-xL and Bcl-2 to PI3K/mTOR signaling [51, 149, 150]. However, the signaling cascades that determine the apoptotic response to kinase inhibitors is highly convoluted. Here, we simplify the pathway to include two essential arms and classify kinase inhibitors as perturbing one or the other. The fact that these kinase inhibitors clustered to include a specific cohort based on their targets was the most significant find.

PI3K regulates Bcl-xL expression through various mechanisms. In monocyte differentiation, PI3K/Akt signaling is involved in cell resistance to apoptosis by inducing expression of Bcl-xL through the NFκB pathway [103]. When THP1 (monocytes) were stimulated with phorbol 12-myristate 13-acetate (PMA), IκBα levels decreased, indicating activation of the NFκB canonical pathway. When the PI3K inhibitor was added to cells, IκBα levels accumulated. The PI3K/Akt may also be regulating Bcl-xL function through BAD. Akt has been shown to phosphorylate BAD directly and also phosphorylate FOXO transcription factors, which regulate BAD expression [12, 13, 114]. Phosphorylation of BAD at Ser112 and Ser136 leads to binding by 14-3-3 and sequestration from binding to Bcl-xL. Phosphorylation of FOXO transcription factors leads to reduction of levels in the nucleus by nuclear export and sequestration in the cytoplasm. These studies show strong evidence that PI3K/mTOR/Akt pathway may be a viable route to target Bcl-xL. Additional studies will have to be performed to decipher the true mechanism of action as there is overlap between CDK and PI3K functions in regards to the regulation of proteins in the apoptosis pathway.

One combination was strongly synergistic in all three cell lines was P276-00 and PF-05212384. P276-00 and PF-05212384 both were selective in the validation studies, showing PARP cleavage only in HeLa Dox-BAD and HeLa Dox-NOXA respectively. P276-00 is a CDK inhibitor, which inhibits CDK 1, CDK2, CDK4, CDK6, and CDK9 [118]. PF05212384 targets PI3Kα, PI3Kγ, and mTOR [147]. It probably inhibits Mcl-1 through CDK2 and CDK9 however, inhibits the cell cycle through CDK1, CDK4, and CDK6. PF05212384 probably compliments the activity of P276-00 through dual inhibition of PI3K and mTOR, targeting Bcl-xL. Future studies would aim to explore the mechanism of action of these inhibitors and test their efficacy in an *in vivo* model.

In conclusion, we present a novel compound (**24**) that can inhibit Mcl-1 through poly-pharmacological inhibition of CDKs. Moreover, **24** synergizes with selective Bcl-xL inhibitors. Using dox-inducible cell lines we identified a cohort of CDK inhibitors that regulated Mcl-1 function and a cohort of PI3K/mTOR inhibitors that perturbed the Bcl-xL arm of the apoptotic pathway. The combinations of these inhibitors led to synergistic inhibition of cancer cell growth. The identification of novel combination of compounds that are in clinical trails suggests rapid translation to clinical trials.

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