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Chemical Genetic Screens Identify Kinase Inhibitor Combinations that Target Anti-Apoptotic Proteins for Cancer Therapy

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Abstract

The study presented here provides a framework for the discovery of unique inhibitor combinations that target the apoptosis network for cancer therapy. A pair of doxycycline (Dox) -inducible cell lines that specifically report on the ability of an inhibitor to induce apoptosis either by targeting the Mcl-1 arm or the Bcl-2/Bcl-xL/Bcl-w arm were used. Cell-based assays were optimized for high throughput screening (HTS) with caspase 3/7 as a read out. HTS with a 355-member kinase inhibitor library and the panel of Dox-inducible cell lines revealed that cyclin dependent kinase (CDK) inhibitors induced apoptosis by targeting the Mcl-1 arm whereas PI3K inhibitors induced apoptosis by targeting the Bcl-2/Bcl-xL/Bcl-w arm. Validation studies identified unique combinations that synergistically inhibited growth and induced apoptosis in a panel of cancer cell lines. Since these inhibitors have been or are currently in clinical trials as single agents, the combinations can be rapidly translated to the clinics.

Mounting incentives have prompted the pursuit of combination treatment to counter resistance mechanisms of cancer development.¹ The advent of personalized medicine and increasing resistance observed in single agent therapy are among the many factors advocating for combination treatments. The increasing number and variety of clinical

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ASSOCIATED CONTENT

Supporting Information Available: This material is available free of charge via the Internet. Methods section, Supplementary figures 1-2; Supplementary tables 1-6.

Notes

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Cell fate is determined by a delicate balance between two classes of regulatory apoptotic proteins, *viz.*, anti- and pro-apoptotic proteins. Anti-apoptotic proteins include, Bcl-2, Bcl-w, Bcl-xL, Mcl-1, and BFL-1, inhibit apoptosis by either: (a) directly binding to and inhibiting BAK and BAX oligomerization or (b) by binding to and sequestering BH3-only activator proteins. On the other hand, pro-apoptotic proteins are sub-divided into two groups, *viz.*, multi-domain and BH3 only pro-apoptotic proteins. BAK, BAX, and BOK (expressed in reproductive cells) are members of the multi-domain pro-apoptotic proteins. The BH3-only pro-apoptotic proteins are further sub-divided into two groups, activators and sensitizers. BH3-only proteins BID, BIM and PUMA are called activators as they induce BAK and BAX oligomerization at the outer mitochondrial membrane (OMM). BH3-only proteins BAD, NOXA, HRK, BMF, and BIK are classified as sensitizers as they inhibit anti-apoptotic protein function. Oligomerization of BAK and BAX at the OMM commits cells to apoptosis.

Mcl-1 is a short-lived protein and its rapid expression is regulated by RNA polymerase II.¹³ The stability of Mcl-1 is regulated by phosphorylation of an N-terminal PEST-like sequences.¹⁴ NOXA (BH3-only protein) forms multiple complexes with Mcl-1 to regulate its function in a context dependent manner.⁹ For example, Ser13 on NOXA is phosphorylated in the presence of glucose which suppresses apoptosis by sequestering the NOXA-Mcl-1 complex to the cytoplasm.¹⁵ Phosphorylation of the C-terminal domain (CTD) of RNA polymerase II by CDK9 induces expression of Mcl-1. Phosphorylation of Mcl-1 by CDK2/cyclin E triggers binding to the BH3 only pro-apoptotic protein, Bim,

which is another phosphorylation mediated regulation of Mcl-1 (Supplementary Table 1 and Table 2). Moreover, CDK inhibitors have been well documented as Mcl-1 attenuators.^{13, 16–19,20, 21} Unlike Mcl-1, Bcl-xL has a longer half-life and fewer phosphorylation sites, which regulate its stabilization. On the other hand, its endogenous binding partner Bad has several phosphorylation sites. Bcl-2/Bcl-xL/Bcl-w is regulated at multiple levels by several signal transduction pathways. These include IKK β /NF κ B, PI3K/Akt, and MAPK pathways, which have been extensively studied and found to influence Bad levels and/or function of Bcl-2/Bcl-xL/Bcl-w.^{22–25}

Since Bcl-2/Bcl-xL/Bcl-w/Bad and Mcl-1/NOXA are regulated by a number of kinases, we set out to identify inhibitors that selectively block Bcl-2/Bcl-xL/Bcl-w or Mcl-1 function. The broader goal of our study was to test the hypotheses that combining kinase inhibitors that selectively disrupt Bcl-2/Bcl-xL/Bcl-w or Mcl-1 arms will inhibit growth and induce apoptosis in cancer cells (Figure 1A). We employed a panel of Dox-inducible cell lines, which were previously developed¹¹ that are adapted to identify inhibitors that induce apoptosis by targeting either the Bcl-2/Bcl-xL/Bcl-w arm or Mcl-1 arm (Figure 1B).

To verify the expression of GFP, NOXA and BAD the Dox-inducible cell lines were treated with doxycycline (1 μ g mL⁻¹, 3h), and the lysates subjected to Western blot analyses. As expected, in the presence of doxycycline, HeLa-BAD cells expressed BAD while HeLa-NOXA expressed NOXA and the HeLa-GFP cells expressed neither (Figure 2A).

To assess the function of these proteins in the Dox-inducible cell lines we used camptothecin¹¹ and ABT-263 as control compounds, which are known to perturb Mcl-1 and Bcl-2/Bcl-xL/Bcl-w arms, respectively. The three Dox-inducible cell lines were subjected to either vehicle or Dox (1 μ g mL⁻¹ – 3h) in the presence or absence of camptothecin (50 μ M – 6h) or ABT-263 (5 μ M – 6h). We assessed the ability of these 12 variations to induce apoptosis in the three cell lines using caspase 3/7 assay (Figure 2B).

As expected, in the HeLa-GFP cells, the 4 conditions (camptothecin $\pm$ Dox and ABT-263 $\pm$ Dox) showed minimal induction of apoptosis. In the HeLa-NOXA cells, we observed ~4-fold induction in caspase 3/7 activity only in wells treated with ABT-263 and Dox. In contrast, we observed ~4-fold induction in caspase 3/7 activity only in the wells with HeLa-BAD cells treated with camptothecin and Dox. Under these conditions we obtained a Z-score of 0.61 and 0.64 with HeLa-BAD and HeLa-NOXA cell lines indicating that the assay is suitable for high throughput screen (HTS) (Supplementary Figure 1).²⁶

Next, we screened a 355-member kinase inhibitor library in the panel of Dox-inducible HeLa cell lines and measured induction of apoptosis using the caspase 3/7 activation assays. The kinase inhibitor library is composed of inhibitors that target a variety of kinases that regulate various signal transduction pathways (Supplementary Table 3). The optimized assay conditions *i.e.*, Dox for 3 hours at 1 μ g mL⁻¹ followed by 1 μ M kinase inhibitor treatment for 6 hours, was used for the HTS. We binned the hits into three groups: (1) Mcl-1 pathway inhibitors; these are inhibitors that induce apoptosis selectively in the HeLa-BAD cells, (2) Bcl-2/Bcl-xL/Bcl-w pathway inhibitors; these are inhibitors that induce apoptosis selectively

in the HeLa-NOXA cells, and (3) non-selective inhibitors; these are inhibitors that induce apoptosis equally in more than one cell line (Supplementary Table 4).

The results from the screen are summarized in Figure 3A. Remarkably, CDK inhibitors clustered as Mcl-1 pathway inhibitors and PI3K inhibitors clustered as Bcl-2/Bcl-xL/Bcl-w pathway inhibitors. Three inhibitors induced caspase 3/7 activation in more than one cell line. Unexpectedly, the non-selective EGFR inhibitor Pelitinib had a unique profile in that it was selective for HeLa-GFP cells (Figure 3B).

The 10 hits identified from the HTS were validated by PARP cleavage in the Dox-inducible HeLa cell lines (Figure 4A). Among the Mcl-1 pathway inhibitors (AT7519, Barasertib, Dinaciclib, Flavopiridol and P276-00), only AT7519 and P276-00 induced PARP cleavage in HeLa-BAD cells and not in HeLa-NOXA or HeLa-GFP cells. Barasertib on the other hand did not show PARP cleavage in any cell line and Dinaciclib and Flavopiridol induced PARP cleavage in all three cell lines. Among the Bcl-2/Bcl-xL/Bcl-w pathway inhibitors (BGT226, GSK126458, PF04691502, PF05212384 and Torin-2), only the two PF compounds induced PARP cleavage in HeLa-NOXA cells and not in HeLa-BAD or HeLa-GFP cells. The three other inhibitors induced PARP cleavage in both HeLa-BAD and HeLa-NOXA cells. There are 4 inhibitors (1 CDK – Flavopiridol and 3 PI3K/mTOR – BGT226, GSK126458 and Torin 2) induce PARP cleavage in both HeLa-NOXA and HeLa-BAD cells but not in HeLa-GFP. This indicates that the inhibition of the individual pathways by the corresponding Dox induced BH3 only proteins are more effective than the small molecule inhibitors. As a result partial inhibition of the Mcl-1 and Bcl-2/Bcl-xL/Bcl-w arms by these inhibitors do not result in robust PARP cleavage in the HeLa-GFP cells. Validation studies show that these 4 inhibitors are false positives, which is one of the limitations of the screens.

In parallel, we conducted growth inhibition studies wherein we evaluated the 25 combinations of Bcl-2/Bcl-xL/Bcl-w pathway inhibitor and Mcl-1 pathway inhibitor in three cancer cell lines (Figure 4B and Supplementary Table 5). For cancer therapies only combination index (CI) values at high effect levels are therapeutically relevant.²⁷

Data from dose response studies with individual inhibitors and combinations were analyzed using CalcuSyn and CI values were estimated for each combination in each cell line (MiaPaCa2, S2013 and HCT116) at ED90, ED95 and ED99 (Supplementary Table 5). We observed >80% of the combinations to be synergistic (avg. CI < 1.0) in each of the three cell lines. 11/25, 7/25 and 3/25 combinations showed strong synergism (avg. CI < 0.3) in S2013, MiaPaCa2 and HCT116 cell lines respectively. Only one combination (P276-00 + PF05212384) showed strong synergism in all three cell lines (Supplementary Table 5).

For these two inhibitors (P276-00 and PF05212384) we determined cellular selectivity for the corresponding pathways by immunoblot analyses (Supplementary Fig 2). Our screen indicated that PI3K inhibitor (PF05212384) induced PARP cleavage by targeting the Bcl-2/Bcl-xL/Bcl-w arm, while the CDK inhibitor (P276-00) induced PARP cleavage by targeting the Mcl-1 arm (Figure 4A). Consistently we observed a decrease in Mcl-1 levels in cells treated with P276-00 alone (0.5μM for 6h) but not in cells treated with PF05212384 alone

(Figure 4C). However, a previous study with PI3K/mTOR inhibitor (NVP-BEZ235) showed down regulation of Mcl-1 in lung and breast cancer cell lines when treated with 1 μ M for 30h.²⁸ The longer incubation time suggests that the Mcl-1 down regulation in the Faber et al, study could be due to secondary effects.

Next, we evaluated P276-00 + PF05212384 combination for PARP cleavage in three cell lines (MiaPaCa2, S2013, and HCT116). In all cell lines, there was robust PARP cleavage in the combination samples as compared to the individual treatments indicating synergism. This finding can be rapidly translated to the clinics because a phase II clinical trial (NCT01903018) with P276-00 in head and neck cancer has been completed and PF05212384 is in phase II clinical trials for the treatment of Acute Myeloid Leukemia (NCT02438761) (Supplementary Table 6).

In summary, the clustering of PI3K inhibitors and CDK inhibitors as Bcl-2/Bcl-xL/Bcl-w pathway inhibitors and Mcl-1 pathway inhibitors respectively is novel, considering CDK and PI3K inhibitor combinations are seldom explored,²⁹ let alone in the context of pursuing effects on apoptosis. Our results further support the simultaneous targeting of Bcl-2/Bcl-xL/Bcl-w and Mcl-1 as a viable therapeutic approach. The strategy presented here offers an unbiased approach to identify synergistic combinations that perturb the apoptotic pathway for cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A strategy to identify kinase inhibitor combinations to target proteins in the apoptotic pathway. (A) Overview of the approach. (B) Dox-inducible cell lines to identify Bcl-2/Bcl-xL/Bcl-w pathway and Mcl-1 pathway inhibitors.

Figure 2.

Validation of the Dox-inducible cell lines for (A) the expression of Dox-inducible gene products and (B) the induction of apoptosis through chemical inactivation of Mcl-1 and Bcl-2/Bcl-xL/Bcl-w using camptothecin and ABT-263 respectively.

Figure 3. Summary of the HTS. (A) Venn diagram representation of the clustering of hits from the screen. (B) Fold change caspase activation normalized to DMSO.

Figure 4.

Validation of hits. (A) Western blot analyses for PARP cleavage in the Dox-inducible HeLa cell lines treated with 0.5 μ M for 6h. (B) Average combination index (CI) values at effective dose (ED)90, ED95 and ED99 in three cancer cell lines. (C). Western blot analyses of PARP cleavage and Mcl-1 after individual (P276–00 and PF05212384) and combination treatment.