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The Chemotherapeutic Agent CX-5461 Affects Cell Viability Through a Topoisomerase II Poison-Like Mechanism

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ABSTRACT

Small molecule inhibitors have astounding potential for treating cancer, but it has become increasingly clearer that they can produce many off-target effects within a cell. Having a proper understanding of a drug's mechanism of cytotoxicity is critical for its informed use in the clinic and for basic science research. The anti-cancer chemotherapeutic, CX-5461, was initially developed as an RNA Polymerase I inhibitor, but recent evidence has surfaced showing its capability to induce G-quadruplex formation and cause DNA damage. To further explore this controversy and properly identify CX-5461's mechanism of cytotoxicity, we performed a validated short hairpin RNA signature drug classification assay, where we identified CX-5461's mechanism of action as a topoisomerase II poison. Additional genetic and biochemical assays, both *in vitro* and in a purified system, were able to confirm our findings by comparing to a known topoisomerase II poison, doxorubicin. These findings highlight the importance of using multiple methodologies to confirm a drug's true mechanism of action, as the misguided usage of the drug can have far reaching impacts. This misclassification could account for CX-5461's mediocre efficacy against hematologic malignancies in clinical trials and its usage as an RNA Polymerase I functional probe in research labs. Future work could aim to further confirm its classification via a CRISPR knockout drug screen, identify biomarkers for CX-5461 sensitivity, test for its effect on other cellular mechanisms, and re-evaluate other RNA Polymerase I inhibitors for a similar misconception. The work completed here contributes to the field of biochemistry and molecular biology, as it exemplifies the importance of proper identification of drug interactions within cells and the implications of this in the clinic and lab.

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- Figure 1: Peter Bruno
- Figure 2: Mengrou Lu
- Figure 3: Mengrou Lu
- Figure 4: Mengrou Lu, Peter Bruno
- Figure 5: Scott Leighow

Chapter 1

Introduction

Understanding a drug's mechanism of action (MOA) is critical for its informed use in the clinic. This is so physicians can use the most appropriate drugs to treat a patient's cancer. Recently, cancer therapy has begun to rely on targeting the specific molecular mechanism contributing to the cancer cell's growth. This has led to the development of small molecule inhibitors focused on targeting oncogene addictions. As per Bernard Weinstein, an oncogene addiction is a cancer cells' dependence on a single oncogenic pathway to sustain proliferation and growth (Sharma & Settleman 2007). Normal cells that may rely on this pathway to function are spared from the targeted effect, as these cells are not as dependent as a cancer cell on one particular pathway for survival. Protein kinases are a common oncogenic addiction as they participate in normal signal transduction pathways but are typically dysregulated in cancers. As of 2017, there were over 250 kinase inhibitors in clinical trials with 37 approved for clinical use (Klaeger et al. 2017). An example of an approved tyrosine kinase inhibitor is cabozantinib, which targets the oncogenes MET/VEGFR for the treatment of medullary thyroid cancer and advanced renal cell carcinoma. Klaeger et al., though, showed that this approved therapeutic has additional potency against FLT3-ITD, a mutated kinase in acute myeloid leukemia (AML). Their data goes on to show additional, unknown druggable targets for both established drugs and other clinically evaluated kinase drugs. It has become increasingly clearer that a drug is capable of targeting more than one compound or producing off-target effects. This creates ambiguity about

how a drug is truly killing the cell, creating an issue for cancer therapy, where patient's lives depend on the use of an appropriate drug to kill a their molecularly specific cancer cell type.

Clinical trials fail for a multitude of reasons, including factors such as poor study design, poor recruitment and execution, ineffective site selection and more, but the largest source of failure stems from a lack of efficacy. It was reported that out of 640 phase III clinical trials, over half failed due to inadequate efficacy (Fogel 2018). While it is not clear how much of this is attributable to other factors, misidentification of a drug target could be contributing to this limited efficacy, especially if the drug is not actually targeting an oncogene addiction, resulting in potential off-target patient toxicity (Lin et al. 2019). For example, Lin et al. found that the small molecule inhibitor, OTS167, which is currently in phase II clinical trials and was designed to target MELK, was still capable of killing MELK knockout cancer cells, suggesting that it likely was killing cells via interactions with proteins other than its designed target. Similarly, Bruno et al. discovered that oxaliplatin, a derivative of cisplatin, killed cells via induction of ribosome biogenesis stress rather than the suspected DNA damage response (Bruno et al. 2017). A benefit to correct identification of MOA is that clinical trials could be designed based on biomarkers, such that patients only received a drug known to target their cancer's molecular signature. It's previously been shown that clinical trials based on biomarker selection are twice as likely to succeed than those without (Wong & Siah 2019). It's possible this value could be depressed as well if the drugs administered during these are targeting superfluous proteins or killing via off-target effects, resulting in less efficacy. By properly identifying drugs' MOA, we can improve the rate of clinical trial success and bring better, more personalized medicine to cancer patients through the approval of efficacious therapeutics.

Another way the correct identification of drug MOA can improve cancer treatment is through combination therapy. Combination therapy is becoming increasingly more common since the success of Emil Frei's 1958 clinical trial where he showed the increase in efficacy when using a combination therapy (Frei et al. 1958). Combination therapy has the added benefit of targeting two or more survival pathways necessary for the cancer cell's survival, leading to use of potentially lower doses of both drugs to kill the cancer cells. By targeting two different pathways, there is also a reduction in drug resistance (Mokhtari et al. 2017). Yet, if two drugs are used in combination that target the same pathway, we loss the advantage of a combination therapy, as there is now the potential for syngeneic toxicity and increased likelihood of drug resistance (Pritchard et al. 2012). Therefore, identification of drug MOA is critical to the beneficial use of combinatorial therapy.

Scientists have proposed a few different ways to identify drugs' MOA over the years, including biochemical drug-target identification and a variety of functional genomic assays. One biochemical drug-target identification method is affinity-based target isolation, whereby a small molecule ligand is covalently linked to a solid support or bound to beads and then cell lysates are passed through via affinity chromatography. The isolated proteins can then be identified via mass spectrometry, but the difficulty with this method is the potential for nonspecific binding proteins (Sato et al. 2010, Bantscheff et al. 2007). This method was recently used by Klaeger et al when examining clinically relevant kinase drugs true interactions. Another common biochemical method of classifying drug class is through systematic measurements of kinase inhibitors' K_d across a panel of potential kinase targets (Karaman et al. 2008). These methods were the "typical" way of characterizing a drug's target, but these assays can lack the identification of cytotoxicity in the cell. This is when genetic signatures were introduced, to begin to elucidate

more about drug's MOA. The first *in vitro* method of drug characterization occurred in yeast cells, but the results from these screens were not clinically relevant due to the lack of conservation between yeast and human drug targets (Jiang et al. 2011). Pathing the way for drug screening in mammalian cells was the NCI-60 human tumor drug screen in the 1980s (Shoemaker 2006).

Since then, genetic assays for drug MOA have become increasingly more common. As technology and science have continued to grow, so have the options for these genetic assays. In 2006, Lamb et al created the Connectivity Map which consisted of a reference collection of gene expression profiles produced by human cells treated with small molecules. The hope was to create a reference that scientists could compare signatures against to try to examine a small molecule drug's MOA. Another method is using RNA interference (RNAi), where the response to short hairpin RNAs and chemotherapeutics is used to establish biochemical modes of action. Similar response to drugs, assessed via a probability-based nearest-neighbors approach, could then group similar drug classes together (Jiang et al. 2011). RNAi interference, though, has been shown to potentially kill cells via off-target effects (Putzbach et al. 2017), leading to possible questions about the reliability of this method for accurate assessment of a drug's MOA. CRISPR-Cas9 mutagenesis is another potential method for evaluating a drug's MOA, whereby targets can be knocked out of the cell and the effect of the drug can be assessed, either via a genetic de-convolution strategy (Lin et al. 2019) or a whole genome level by analyzing the frequency of guide RNAs left in a cell population after treatment with a drug. Lin et al used the CRISPR-Cas9 mutagenesis genetic de-convolution method to determine that the protein targets of clinically relevant anti-cancer drugs were killing via off-target effects, rather than through their protein target. All these methods hold their own merit, but to gain the best understanding of

a drug's MOA, both biochemical and genomic assays should be done to verify a drug's true mechanism of cytotoxicity to a cell.

Recently, the small molecule, CX-5461, MOA has become a source of controversy. Initially designed as an RNA Polymerase I inhibitor (Drygin et al. 2011, Haddach et al. 2012, Bywater et al. 2012), CX-5461 is currently in phase I clinical trials for hematologic malignances (Khot et al. 2019). Targeting RNA Polymerase I has potential as a therapeutic strategy for cancer treatment due to the interruption of nucleolar transcription of ribosomal DNA, which can activate p53 dependent apoptosis or cell cycle arrest (Ferreira et al. 2020). Bywater et al showed that inhibition of RNA Polymerase I via CX-5461 could be used to kill lymphoma cells *in vivo*, as well as in human leukemia and lymphoma cell lines, indicating its potential efficacy for cancer treatment (2012). It has been shown to have therapeutical efficiency in a variety of other models as well, including breast cancer, small cell lung cancer, ovarian cancer, neuroblastoma and more (Ferreira et al. 2020). Thus far, the results of the phase I clinical trial for advanced hematologic cancers have been mediocre, with only one out of sixteen patients seeing a partial response and five others only achieving stable disease (Khot et al. 2019). Additionally, despite its use clinically and scientifically as an RNA Polymerase I inhibitor, recent data has come out suggesting that the small molecule may be cytotoxic to cells via a different mechanism – as a DNA G-quadruplex (G4) stabilizer (Xu et al. 2017).

These discrepancies about CX-5461's MOA sparked our interest in what its true target was in the cell and how this small molecule was actually killing cancer cells. We propose that CX-5461 is killing cells via a topoisomerase II poison-like mechanism. Topoisomerase enzymes are essential for altering the topological structure of DNA, to prevent DNA knots, formed due to the dense compression of the genetic material in the nucleus, from being lethal. Type II

topoisomerases are responsible for cleaving both strands of DNA to regulate DNA winding and remove knots that form. They function by generating a double-stranded break, passing a separate double helix through, and resealing the break. This ability to cleave the DNA intrinsically makes this enzyme a potential danger to a cell. Normally, the cleavage complex formed during the enzyme's activity is maintained to be balanced, but when too many of these complexes are stabilized, this results in DNA breaks that can no longer be ligated back together by the topoisomerase and must rely on other recombination/repair pathways to be fixed. This property of topoisomerase makes it a promising target for anticancer drugs, as one only needs to manipulate the enzyme such that more complexes are formed for cancer cells to die from the DNA damage. This method is especially promising as many cancer cells have decreased or non-functional DNA repair systems, making them more susceptible to the breaks. Targeting topoisomerase II can be done either by inhibiting the enzyme or poisoning it. Inhibitors stop the catalytic activity of the enzyme, causing cells to die due to the dysregulation of DNA topology. On the other hand, topoisomerase II poisons, such as doxorubicin and etoposide, are commonly used to treat cancers due to their ability to increase the levels of DNA cleavage complexes in a cell, leading to a lethal level of DNA damage. Currently, six topoisomerase II poisons are approved for use in the clinic (Vann et al. 2021).

To elucidate CX-5461's true MOA, we will use both genetic and biochemical assays, including an shRNA signature assay (Jiang et al. 2011), an RNAi knockdown of the drug's proposed target, a phenotypic analyses of DNA damage, and a biochemical assay to assess drug function independent of a cell. Through all these methods, we aim to show that CX-5461 is functioning as a topoisomerase II-poison, rather than as an RNA Polymerase I inhibitor, to kill

cancer cells. Understanding CX-5461's true MOA is critical for its appropriate use in the clinic and science moving forward.

Chapter 2

Results

Parts of this chapter have been published (Bruno et al. 2020)

The first method employed to study CX-5461's true mechanism of action was an established functional genomic shRNA signature assay (Jiang et al. 2011, Pritchard et al. 2013a). In this assay, eight validated shRNAs targeting TRP53, CHECK2, ATR, SMG1, BOK, BCL2L11 and PRKDC are infected into Eμ-Myc $p19^{arf-/-}$ lymphoma cells. Cells that contain the shRNA, and therefore have that gene knockdown, are labelled with GFP. Then, drug resistance and sensitivity profiles are determined by measuring the percent of green fluorescent protein (GFP) shRNA expressing cells over time following drug treatment via flow cytometry. If the shRNA gene knockdown conferred resistance to drug treatment, the percentage of GFP expressing cells would increase, indicating that the cells containing that shRNA were growing out despite the presence of drug, and therefore that gene must be involved in the drug's MOA somehow. If the shRNA gene knockdown conferred sensitivity, the percentage of GFP expressing cells would decrease, indicating these cells containing that shRNA were dying from the presence of drug, and therefore that gene must not be involved in the drug's MOA. These resistance/sensitivity profiles are then able to predict the drug mechanism of action across 10 drug classes (Jiang et al. 2011, Pritchard et al. 2013a). This model was chosen because across three different machine learning algorithms, this shRNA signature assay showed remarkable promise at accurately predicting Top2 poisons. Using a reference set with 4 different Top2 poisons, the K-nearest neighbors (KNN), linear discriminant analysis (LDA), and logistic regression models predicted drug class from this 8-shRNA signature classification assay with true positive rates of 0.992, 0.992, and 0.977 for doxorubicin replicates and true negative rates of

0.995, 0.987, and 0.995 for non-Top2 drugs, respectively. The KNN algorithm also produced false positive and false negative rates of 0.005 and 0.008 respectively. For this assay, Eμ-Myc $p19^{arf-/-}$ lymphoma cells were used, as these tumors have previously been used to test CX-5461 as a transcription inhibitor (Bywater et al. 2012). The 80-90% lethal dosage (LD80-90) was determined for these cells before beginning the function genomic shRNA signature assay (Fig. 1A). The results from this assay predicted CX-5461 as a topoisomerase II poison, as seen by the resistance and sensitivity signature of the drug as compared to other Top2 poisons and other transcription/translational inhibitors (Fig. 1B). The cells showed resistance to both TRP53 and CHEK2 knockdown, which is characteristic of DNA damaging agents, such as doxorubicin, but not transcription/translation inhibitors, such as actinomycin D. After determining that CX-5461 could be killing cells through a topoisomerase II poison-like mechanism, we compared the chemical structure of CX-5461 to other drugs thought to cause DNA damage through Top2 poisoning, including the quinolone derivatives voreloxin and ciprofloxacin (Hawtin et al. 2010), and noted structural similarities (Fig. 1C). Based on this, we hypothesized CX-5461's true MOA was as a topoisomerase II poison, rather than as an RNA Polymerase I inhibitor.

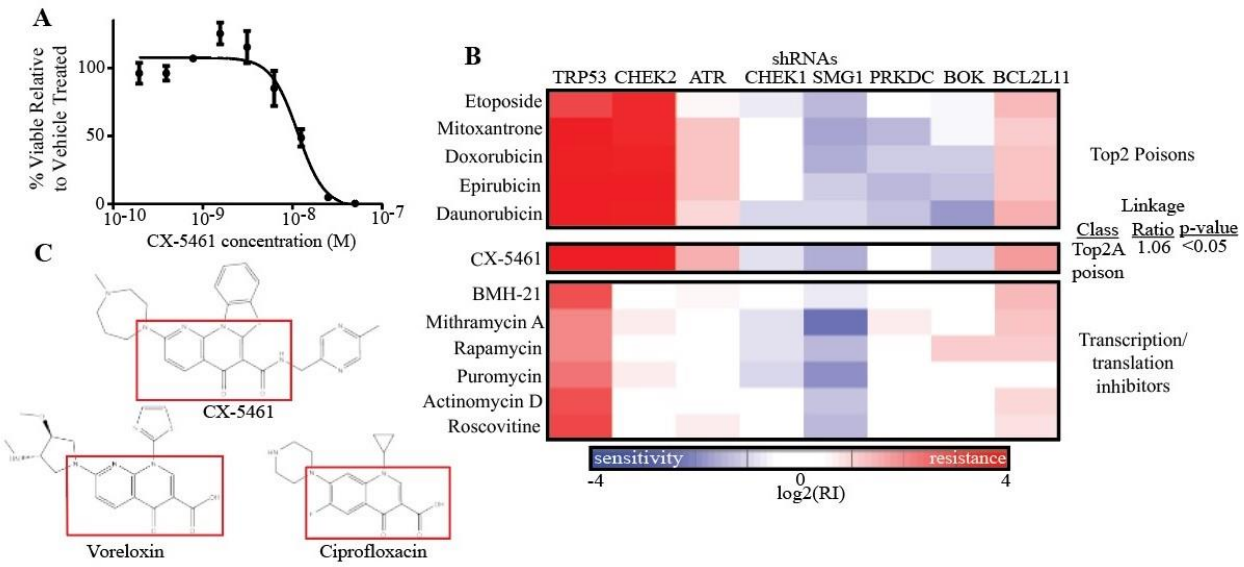

Figure 1: CX-5461 has a predicted MOA as a Top2 poison. Results of the functional genomic shRNA signature assay: A) Eμ-Myc dose response to CX-5461 as determined by flow cytometry analysis of propidium iodide exclusion as a live/dead marker. Data represented as mean +/- SEM relative to vehicle treatment with three technical replicates. (B) RNAi signatures of CX-5461 (middle) as compared to other Top2 poisons (top) and transcription/translation inhibitors (bottom), with the K-nearest neighbors algorithm results to the right. (C) Quinolone structural similarities between CX-5461 (top) and other DNA damaging Top2 poisons, voreloxin (bottom left) and ciprofloxacin (bottom right), outlined in red. This data was generated by Peter Bruno and has been published (Bruno et al. 2020).

The next methodology employed to determine if CX-5461 was functioning as a topoisomerase II poison was to observe the phenotypic effects of a DNA damaging drug. To do this, we analyzed the levels of γH2AX, which is a common biomarker for intracellular double-stranded DNA damage (Mah et al. 2010), via immunofluorescent staining and analysis by flow cytometry and microscopy imaging of foci. We began by analyzing the levels of γH2AX in Eμ-Myc cells, to confirm the results seen via the shRNA signature assay. Doxorubicin was used as a topoisomerase II poison positive control, while actinomycin D was used as the transcription inhibitor control. After treating the cells with the IC50 of each drug (Fig. 2), we saw that the levels of γH2AX observed between CX-5461 and doxorubicin were significantly higher than the DMSO drug control, while those for actinomycin D where not (Fig. 3A). This indicated that CX-

5461 was able to phenotypically cause similar amounts of DNA damage as topoisomerase II poisons, and not transcriptional inhibitors. To ensure these results were not cell type specific, we also analyzed the levels of γH2AX in human embryonic kidney 293T, colon HCT116, lung A549 and melanoma A375 cells. Again, the levels of γH2AX for CX-5461 and doxorubicin proved to be significantly different from the DMSO control, yet similar to each other and dissimilar to actinomycin D (Fig. 3B). These results were all seen via flow cytometry analysis of the immunofluorescent staining of γH2AX, but to recapitulate the results with a different method, microscopic analysis of γH2AX foci was performed on A375 cells. The results aligned with the flow cytometry data – with CX-5461 causing similar foci counts to doxorubicin (Fig. 3C), further proving that CX-5461 was causing levels of DNA damage similar to a topoisomerase II poison, and not a transcription inhibitor. Interestingly, the foci observed for CX-5461 were more punctate than those observed for doxorubicin. Additionally, actinomycin D produced significantly fewer foci than even DMSO, which could be explained by it causing cell cycle arrest early in G1 (Kim et al. 2005), which has less basal DNA damage compared to cells that progress through the cell cycle (McManus & Hendzel 2005).

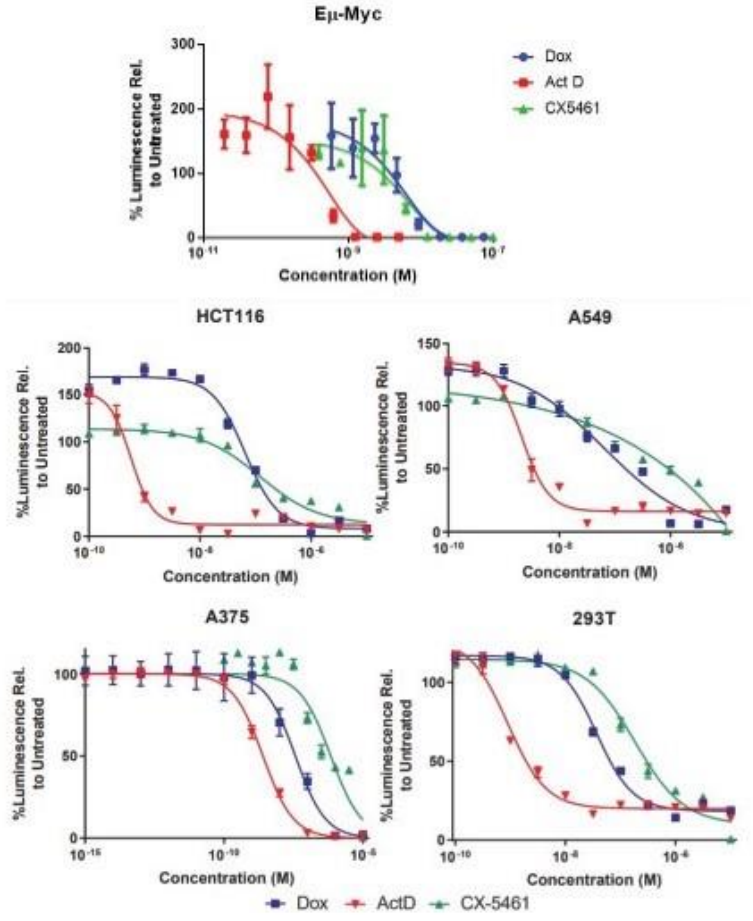

Figure 2: Cell Line IC50 Curves. Relative viability of the indicated cell lines following treatment with the indicated drugs after 72 hours, as assessed by Cell Titer Glo. Each dot is representative of the mean +/- SEM of three technical replicates. This data has been published (Bruno et al. 2020).

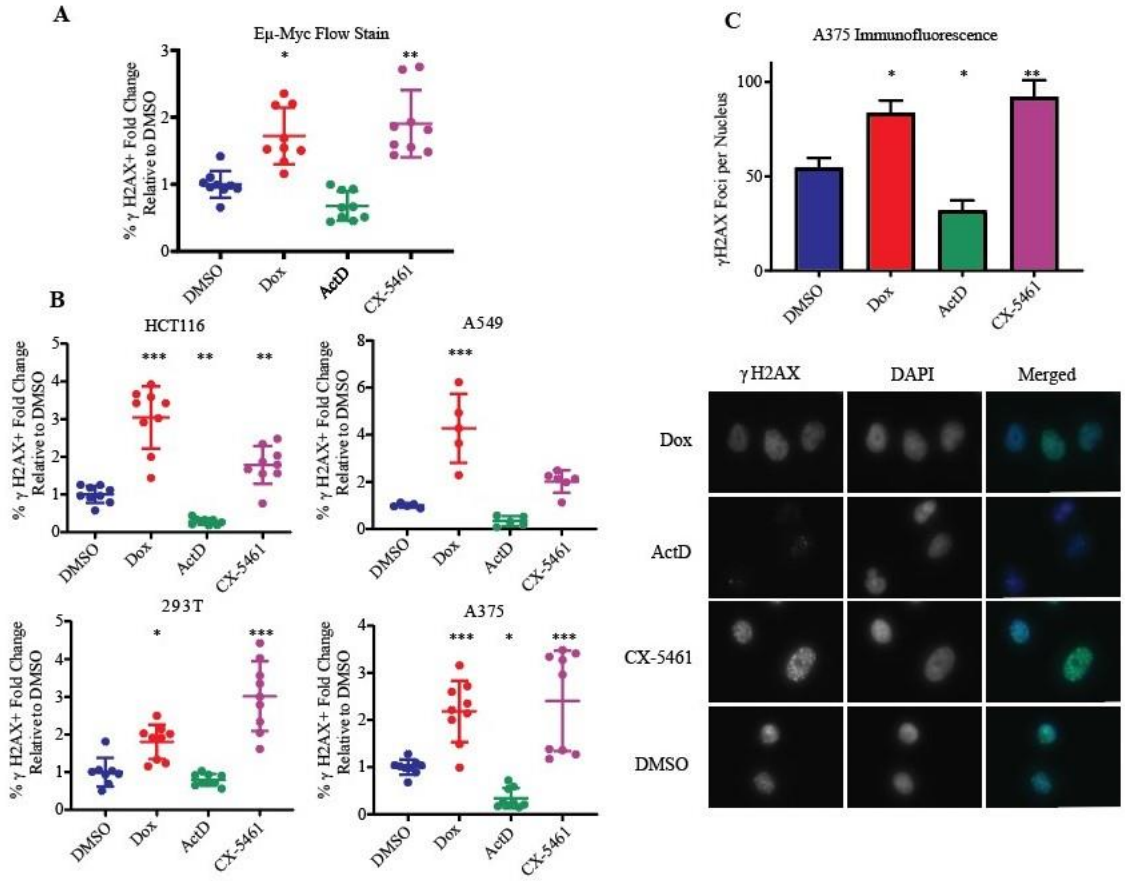

Figure 3: CX-5461 causes levels of DNA damage similar to topoisomerase II poisons. Quantification of DNA damage via immunofluorescent staining of γH2AX via flow cytometry (A,B) and microscopy (C) 12 post-treatment with the IC50 of each respective drug in each respective cell line. (A,B) Each dot is representative of a different replicate with each cell line containing three biological and technical replicates each, except A549 which only has two biological replicates. Data is given as the relative fold change in % γH2AX positive compared to the DMSO control. P-values calculates via ANOVA with Holm-Sidak's multiple comparison test (*P<0.05, **P<0.01, or ***P<0.001). (C) γH2AX foci/nucleus represented as the mean +/- the SEM. Images of individual and merged channels for γH2AX (green) and DAPI (blue). P-values calculated via Kruskal-Wallis with Dunn's multiple comparison's test (*P<0.05, **P<0.01). This data has been published (Bruno et al. 2020).

A phenotypic correlation, though, does not prove that it is functioning as a topoisomerase II poison, therefore, additional genetic and biochemical assays were conducted. First, an shRNA enrichment assay was conducted. In this assay, four previously validated shRNAs targeting topoisomerase IIA (Gobble et al. 2011) were used to knockdown the intracellular levels of

topoisomerase IIA in A375 cells, and the relative resistance to each drug at their IC40-50 was quantified via a GFP competition assay, where cells infected with one of the shRNAs were labelled with GFP, allowing for quantification of the fold change of the percentage of live GFP positive cells following drug treatment via flow cytometry. Multiple hairpins were used to ensure the results seen were not due to an off-target effect of a singular hairpin, and a shFirefly hairpin was used as a negative hairpin control. Relative to the DMSO control, CX-5461 caused significant resistance to the knockdown of Top2A, as did doxorubicin, but not actinomycin D (Fig. 4A). This suggested that the DNA damage observed was likely to do CX-5461 acting as a topoisomerase II poison. Despite all this cellular and genetic data, it still did not prove CX-5461's direct ability to interfere with topoisomerase II. Perhaps it was inhibiting an inhibitor of Top2A, and this is why these correlational results were seen.

To prove direct association with topoisomerase II activity, a biochemical kinetoplast DNA decatenation assay was performed in a purified system. During this assay, Top2A should be able to catalyze the decatenation of DNA with ATP, resulting in lower molecular weight bands, while Top2 poisons which trap the Top2 on the DNA will inhibit its ability to do this, resulting in higher molecular weight bands (Nitiss et al. 2012). Since there is an excess amount of Top2 used in this assay compared to within a cell, the concentration of drug used was multiplied 500x the LC80-90 of the Eμ-Myc $p19^{arf-/-}$ as this was determined to be the minimum concentration required to see a positive result for doxorubicin, a known topoisomerase II poison. The results of the assay showed that both doxorubicin and CX-5461 had significantly higher levels of catenated DNA relative to the DMSO control, while actinomycin D did not (Fig. 4B and 4C) – indicating that CX-5461 is acting directly on topoisomerase II.

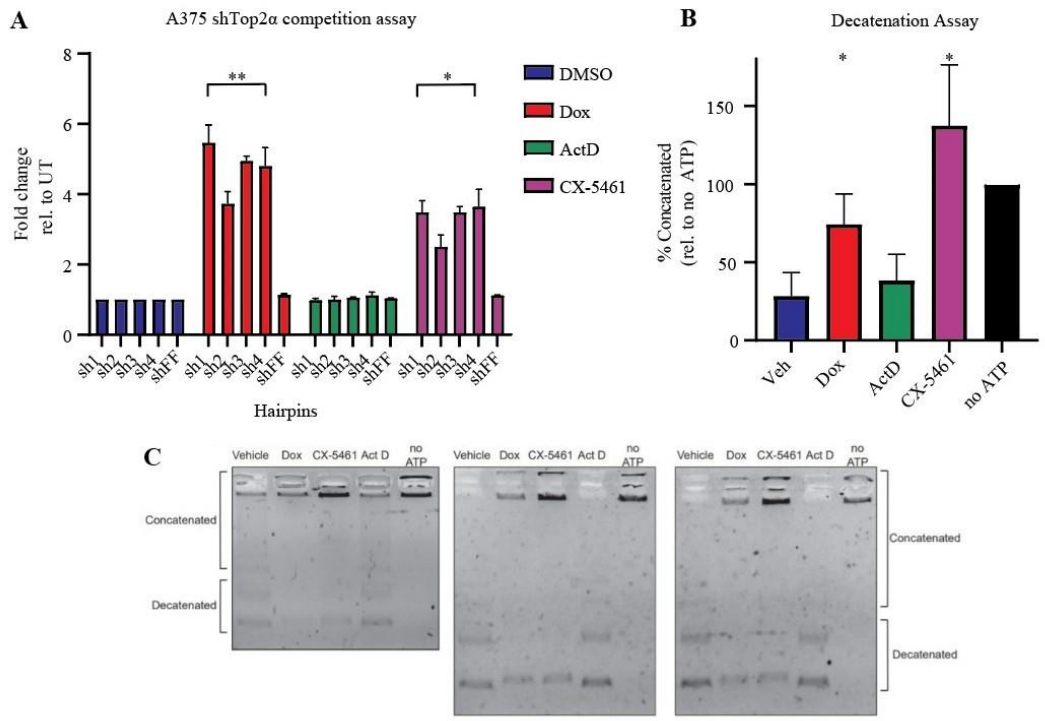

Figure 4: CX-5461 genetically and biochemically resembles a Top2A poison. A) Results of the GFP competition assay done 72 hours after treatment with the IC50 of each drug with four shTop2A hairpins and an shFirefly hairpin control in A375 cells, as analyzed by flow cytometry. Data represented as mean +/- SEM of three biological replicates of each condition. P-values calculated via Kruskal-Wallis with Dunn's multiple comparison's test (*P<0.05, **P<0.01). This data has been published (Bruno et al. 2020). B) Results of the kinetoplast DNA decatenation assay with the indicated inhibitors. Data represented as mean +/- SEM of each condition. P-values calculated via two-tailed Welch's t test (*P<0.05). C) Kinetoplast DNA, indicated agents and recombinant human Top2A enzymes were incubated for 30 minutes and then run on 1% agarose gels, stained with ethidium bromide. This data was generated by Peter Bruno and has been published (Bruno et al. 2020).

Together these results support that CX-5461's main cytotoxicity comes from a topoisomerase II poison-like mechanism. This is supported with our 8-shRNA signature drug classification assay, a phenotypic analysis of DNA damage levels, genetic *in vitro* assays, and biochemical assays in a purified system, where it was observed that CX-5461 produces data similar to another topoisomerase II poison and not a transcription/translation inhibitor. Additionally, its structural similarity to other quinolones and quinolone derivatives strengthens our hypothesis on its true cellular effect.

Chapter 3

Discussion

Proper identification of drug mechanism of action is critical for proper use of a compound in the clinic as well in basic science research. Misunderstandings of a drug's cytotoxicity could be contributing to the primary source of clinical trial failure - lack of efficacy (Fogel 2018). With a proper understanding of a drugs MOA, we could work to improve the efficacy of clinical trials by stratifying trials based on biomarkers known to be sensitive to a drugs specific MOA. It can also help reduce the effects of off-target toxicity. Additionally, we can improve the combination therapy patients receive by avoiding targeting the same molecular mechanisms, which has the potential to create syngeneic toxicity in the patient and create additional selective pressure for resistance (Pritchard et al. 2012). In addition to the clinical benefit of proper drug MOA, there also is a basic science benefit. Drugs with the potential to target new mechanisms can quickly become the exciting new basis of much scientific research, which in turn, generates more scientific insights, but if initial drug MOA is not properly understood, then the new discoveries coming to light could all be faulty as the basis of the work is incorrect. This could be potentially catastrophic to a field of scientific research, if not caught and corrected early.

The results presented here support the hypothesis that CX-5461, a drug initially designed as an RNA Polymerase I inhibitor and recently progressed through phase I clinical trials treating hematologic malignancies (Haddach et al. 2012, Bywater et al. 2012, Drygin et al. 2011, Khot et al. 2019), is actually killing cells via a topoisomerase II poison-like mechanism. We predicted this using an established and reliable 8-shRNA signature drug classification assay (Jiang et al. 2011), and then compared its structure to known quinolones and quinolone derivatives to see if it

was structurally similar to other drugs known to cause DNA damage through a Top2 mechanism. Following this, we observed similar levels of DNA damage between CX-5461 and a known topoisomerase II poison, doxorubicin, and not actinomycin D, a known transcription/translation inhibitor, in five different cell lines. Next, we genetically knocked down the levels of Top2A and observed resistance in these cells to CX-5461 and doxorubicin, with four different, previously validated shRNAs (Gobble et al. 2011), indicating that the effect is specific to the target and not an off-target effect of the shRNAs. Finally, we showed in a purified system that CX-5461 inhibited Top2 ability to catalyze the decatenation of kinetoplast DNA in the presence of ATP.

All this data has already been published (Bruno et al. 2020), but there is also additional data published in this paper to support this hypothesis, including computational analyses, additional genetic knockdown assays, and resistant clone generation. This shows that with multi-modal analyses of drug MOA, we can more accurately determine a drug's true cytotoxicity.

Since publishing our results, our paper has been cited by 49 additional papers, which according to Dimensions, is 22 times more citations that the average citation in the same field (Digital Science & Research Solutions, Inc 2022). Based on this, we know this work has had some astounding effects on the field of drug discovery - especially RNA Polymerase I inhibitors. When examining the literature, though, it is obvious that there are still discrepancies about its MOA. The latest paper published on CX-5461 still referred to it as an RNA Polymerase I inhibitor (Pan et al. 2022), but there is another paper published just from last year that agrees with our conclusion that is mischaracterized and targeting Top2 (Pan et al. 2021). It will take time for the drug's true MOA to gain recognition, but we can see that the new classification is beginning to hold merit with other scientists in the field.

While we have shown that CX-5461 is killing cells through a topoisomerase II poison, we want to address that it is likely also inhibiting RNA Polymerase I. Burger et al has shown previously that topoisomerase II poisons frequently are also capable of inhibiting rRNA transcription (Burger et al. 2010). They analyzed the inhibitory effects of chemotherapeutics by culturing human 2fTGH fibrosarcoma cells, with drug, phosphate-depleting the media, and then labeling with [^{32}P]orthophosphate. After extracting the RNA, separating it via gel electrophoresis and transferring it to Whatman paper to visualize via phosphorimager analysis, the level of phosphate label incorporated was used to determine the amount of 47S primary transcript, 32S major intermediate form, and mature 18S and 28S rRNAs present, and as such the stage of rRNA inhibition. Burger et al. observed reduced incorporation into 47S rRNA, and therefore rRNA transcription inhibition, for multiple topoisomerase II poisons, including doxorubicin, mitoxantrone, and oxaliplatin (Burger et al. 2010). Oxaliplatin was actually found to be killing cells via the ribosome biogenesis stress it causes rather than the DNA damage it induces (Bruno et al. 2017), which is in contrast to our findings for CX-5461. This just further exemplifies the ongoing need to deconvolute a drug's MOA, as it can be extremely complicated – where sometimes drugs are targeting more than one thing, yet each drug is inducing cell death in differing ways because of this.

Though it can come as a shock initially, the correct identification of a drug's MOA can be extremely beneficial. For example, Lin et al. studied OTS964, a PBK inhibitor, and discovered its true target – CDK11 – and is hoping to use the drug for treatment of cancers that have an oncogene addiction to CDK11. Klaeger et al. also studied clinical kinase inhibitors and observed many targeting more than one kinase, but after proper identification of golvatinib and cabozantinib's MOA, they were able to show their potential to treat AML based on their new

knowledge of their targets (Klaeger et al. 2017). While sometimes unwarranted or undesired, proper understanding of a drug's off-target effects is critical to its proper use in the clinic, to prevent toxicity and lead to its use in its most efficacious setting.

Scientifically, correctly identifying a drug's MOA is also important. For example, CX-5461 has been used as chemical genetic probes for RNA Polymerase I complex functionality (Valvezan et al. 2017, Chen et al. 2018) and to provide insight into RNA Polymerase I function (Ray et al. 2013). Interestingly, Ray et al. proposes that inhibition of topoisomerase II can reduce rDNA transcription –supporting the idea that these two functions can go hand-in-hand and lead to convolution of which mechanism is truly killing a cell.

Another key takeaway of this work has been the importance of proving drug MOA using a variety of different methods. No one method is capable of accurately predicting a drug MOA – as evidenced by the initial work identifying CX-5461, where it was only screened for its ability to inhibit rRNA synthesis (Drygin et al. 2011, Haddach et al. 2012). By combining computational, genetic, and biochemical assays, we can more accurately identify a drugs cytotoxic mechanism, even given the off-target effects of many drugs. Moving forward, we would like to use an additional method to further characterize CX-5461 as a topoisomerase II poison – via a CRISPR knockout assay with our lab's compressed gene library (L200). Initial work shows this method having promise for accurately identifying drug class (Supplementary Data, Fig. 5).

Given this new characterization of CX-5461, we hope to provide advice on its future use in the clinic. Specifically, we believe it should no longer be used in patient's refractory to Top2 poisons. Additionally, we believe its toxicity needs to be investigated further, given evidence that doxorubicin, a known topoisomerase II poison, has the potential to cause cardiotoxicity (Zhang

et al. 2012). It's reasonable to believe that if CX-5461 has a molecular mechanism similar to doxorubicin, it has also the potential to induce similar toxicities. Future studies could aim to determine biomarkers that would enhance CX-5461's efficacy in particular cancer cell lines. Currently, there is a clinical trial (NCT04890613) recruiting for patients with solid tumors and BRCA1/2, PALB2, or homologous recombination deficiency (HRD) mutations (NIH U.S. National Library of Medicine 2021), after Xu et al. helped prove that CX-5461 stabilizes the DNA G-quadruplex (Xu et al. 2017). Other possible future directions could include investigating if the drug has other cellular effects, given its ability to affect both RNA Polymerase I and topoisomerase II. The most recent paper published by Pan et al. on CX-5461 describes its ability to contribute to immunosuppression due to inhibition of expression of T-cell activation agonists (Pan et al. 2022). They hope to use this discovery to repurpose CX-5461 in anti-rejection therapies. Based on this, performing RNA-sequencing to analyze the transcriptome of a cell following treatment could potentially aid in the discovery of other possible novel molecular mechanisms CX-5461 affects. Finally, given the convoluted nature and overlapping drugs and mechanisms of RNA Polymerase I inhibitors and topoisomerase II poisons, it would be interesting to re-investigate other drugs in this class using our methods to clarify their true mechanism of cytotoxicity. Overall, by reclassifying CX-5461 as a topoisomerase II poison, we hope to show the significance of proper drug mechanism of action identification, via both biochemical and genetic assays, to improve both its use in the clinic, by guiding its use in the proper patient population and reducing off-target effects in future clinical trials, and its use in the laboratory, to prevent its mis-guided use as an RNA Polymerase I inhibitor before it affects future work.

Chapter 4

Methods

Parts of this chapter have been published (Bruno et al. 2020)

shRNA Constructs

For the initial shRNA signature assay in Eμ-Myc p19$^{arf-/-}$ cells, the shRNAs were in the mir30-LTR-SV40-GFP (MLS) retroviral vector (Dickins et al. 2005). For the human Top2A hairpins used in the genetic shRNA competition assay, the shRNAs were from the pGIPZ Hannon-Elledge shRNA collection (Open Biosystems) (Meerbrey 2011). Transfections were performed by packaging the shRNA plasmids into HEK293T cells. Viral supernatants were collected using the copolymer precipitation method (Landazuri & Doux 2004), which were then used to infect 5 x 10^4 cells to achieve infection of 10-20% of the population. All signatures included replicates from two distinct replicates. Vector controls were included in all shRNA experiments to rule out effects of the vector alone (Pritchard et al. 2013b). Sequences of shRNAs used can be found below.

Table 1: shRNA Sequences

Gene Target	mRNA Target Sequence
TOP2A-1*	ACCAGTAGAGAATACAAGAAA
TOP2A-2*	CATGGAGAAGATTATACATGT
TOP2A-3*	CCAGATAGATGATCGAAAGGA
TOP2A-4*	CTTCGAAAGCAGTCACAAGCA
TRP53**	TGGAGAGTATTTCACCCTCAA
CHEK2**	CACTTTCACTATGTAGAAATA
ATR**	ACCTTTAATGAGTGTCTTAAA
CHEK1**	AAGGGCTTGACCAATTATAAA
SMG1**	CAGGCTGCATTCAATAACTTA
PRKDC**	CTCCAACATGTAGAGAACAAA
BOK**	TCGGTGTCCAGCCCTAGAGAA
BCL2L11**	CACCCTCAAATGGTTATCTTA

*Sequences targeting human genes; published in Bruno et al. 2020. **Sequences targeting mouse genes; published in Jiang et al 2010.

Cell Culture

Eμ-Myc p19$^{arf-/-}$ cells were cultured in 45% Dulbecco's modified Eagle medium (DMEM), 45% Ivesco's modified Dulbecco's medium (IMDM) and 10% fetal bovine serum (FBS). This was also supplemented with 55 μM β-mercapatoethanol and 2 mg/L L-glutamine. All human cell lines, including A375, 293T, HCT116, and A549, were cultured in 90% DMEM and 10% FBS. To minimize contamination, 100 units/mL of penicillin and 100 μg/mL of streptomycin was added. All cells were negative for mycoplasma, as tested by MycoAlert, Lonza. Cell lines were not authenticated.

RNAi signatures

Eμ-Myc p19$^{arf-/-}$ cells were dosed with the LD80-90, as determined by flow cytometry analysis of propidium iodide exclusion 48 hours after treatment. GFP enrichment/depletion was

determined by flow cytometry with a BD FACScan and KSR2 72 hours after drug treatment. The doxorubicin, CX-5461 and actinomycin D LD80-90 values used for treatment where 7-9 nM, 20-28 nM, and 1-2 ng/μL, respectively. Linkage ratios and P values were generated as previously described by Jiang et al (2011) and Pritchard et al. (2013b).

Sensitivity and Specificity Values for Top2 Poison Mechanism Classification

The multiclass drug mechanism predictions were made in R using three machine learning algorithms: KNN, LDA, and logistic regression. To quantify the predictive power of each model and record class predictions or individual drugs in a reference set of 535 (including 131 Top2 poisons and 384 other observations spanning 6 drug classes), leave-one-out cross-validation was used. True positive, false positive, true negative, and false negative results were recorded for the Top2 poison mechanism classification for all three models. A true positive was when Top2 poisons, including the four in the dataset (doxorubicin, etoposide, mitoxantrone, and daunorubicin), were predicted as Top2 poisons. A true negative was when a drug other than a Top2 poison did not predict as a Top2 poison. A false positive was when a drug other than a Top2 poison predicated as a Top2 poison. Finally, a false negative was when one of the 4 Top2 poisons in the set was predicted as something other than a Top2 poison. The true rates were obtained by dividing the count by the total number of Top2 poisons in the reference set (131), and the false rates were calculated by dividing the counts by the total number of drugs belonging to drug classes other than Top2 poison (384). Summary plots were generated using the *ggplot* package in R.

Eμ-Myc and Human Cell Line Dose-Responses

One day before drug treatment, 3,000 cells were seeded per well in a 96-well microplate. Cells were treated with 2-2000 nM doxorubicin, 0.05 to 50 nM actinomycin D, or 4-4,000 nM CX-5461 at half-log dilutions for 72 hours. DMSO was used as the drug control, as all drugs are dissolved in this solvent. After 72 hours of treatment, a Fluoroskan Ascent Microplate Fluorometer and Luminometer from Thermo Fisher Scientific was used to analysis the luminescent signals after adding 30 μL of CellTiter-Glo to each well. Cell viability curves and ICs were graphed and calculated using GraphPad Prism 6.

Staining and Flow Cytometry for γH2AX

Eμ-Myc cells were seeded at a density of 1 x 10^6 per well in a 12-well microplate one day before treatment. A375, 293T, A549 and HCT116 cells were also seeded one day before treatment at the same density in a 10-cm tissue culture dish. Cells were then treated with their respective drug concentrations (Table 2). DMSO was used as the drug control, as all drugs are dissolved in this solvent. Twelve hours after treatment, cells were harvested in PBS, fixed with 4% paraformaldehyde (PFA), and permeabilized with 0.5% Triton X-100 in PBS. Cells were then incubated with an anti-phospho-histone H2A.X [(Ser139) (20E3)] rabbit monoclonal antibody (9718, Cell Signaling Technology) in a staining buffer (1% blood serum albumin (BSA), 0.5% Triton X-100 in PBS) at 1:500 dilution at 4°C overnight. The next day, the cells were incubated with a secondary goat anti-rabbit-Alex Fluor 488 (FITC) fragment conjugation (4412, Cell Signaling Technology) at 1:1500 dilution in staining buffer for 1 hour in the dark,

before being analyzed with an Accuri C6 Plus Flow Cytometer (BD Biosciences) for fluorescence.

Table 2: IC-50 Drug Concentrations used for γH2AX assays

Cell Line	DMSO (nM)	Dox (nM)	CX-5461 (nM)	ACT D (nM)
Eμ-Myc	6	5.5	6	0.4
A375	1000	60	1000	2
293T	500	75	500	2
A549	250	250	250	3
HCT116	350	75	350	1

Immunofluorescent Imaging

First, round cover-slips were presterilized and coated with 2% gelatin. A375 cells were then seeded at a density of 1 x 10^5 in 12-well microplates with coverslips one day prior to drug treatment. The cells were then treated with the respective IC-50 drug dosages for A375 cells (Table 2) for 12 hours before beginning analysis. DMSO was used as a drug control, as all drugs were dissolved in this solvent. Cells were then fixed, permeabilized, and incubated with the same primary and secondary antibodies used in **Staining and Flow Cytometry for γH2AX,** and 1 μg/mL of 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). The coverslips were then mounted onto glass microscope slides. Following this, an Olympus IX-8 inverted microscope was used to image foci, with CellProfiler processed to determine the foci-per-nucleus count.

A375 Competition Assays

A375 cells were infected with shTop2A hairpins and an shFirefly control hairpin so that 10-25% of the cell population was GFP positive. Following infection, 100,000 cells were plated and treated with the IC-40 concentration of each drug: doxorubicin: 24 nM, actinomycin D: 0.8 nM, CX-5461: 400 nM. DMSO, at 400 nM, was used as the drug control, as all drugs were dissolved in this solvent. After drug treatment for 74 hours, cells were analyzed for the percentage of live GFP+ cells left compared to the controls via flow cytometry with an Accuri C6 Plus Flow Cytometer (BD Biosciences).

Top2 Decatenation Assay

A human Top2 assay kit (Topogen) was performed by Peter Bruno according to the manufacturer's instructions. Concatenated kinetoplast DNA was incubated with human Top2 for 30 min at 37°C in the presence or absence of the following drugs: doxorubicin at 3.5 μM, actinomycin D at 400 nM, and CX-5461 at 12 μM. DMSO at 12 μM was the vehicle control, as all the drugs were dissolved in this solvent. These concentrations were chosen as these were 500x the LD80-90 in Eμ-Myc $p19^{arf-/-}$ cells, which was the required minimum multiple of the doxorubicin LD80-90 to achieve a positive result. There is an excess of enzyme in this assay as compared to a typical cell which is why higher concentrations are needed. Following the incubation, the DNA was run on a 1% agarose gel, stained with 0.5 μg/mL of ethidium bromide and briefly washed in water. The gel was imaged and the highest bands, indicative of concatenated DNA, were quantified using ImageJ.

Selection/Infection of L200 Library for CRISPR Knockout Drug Screen

The lossy compression library consists of 200 genes that are meant to capture an entire genome's worth of data. This library was identified as a part of a "public" genetic architecture, accounting for cell context specificity, and was based on a "CRISPR predicts CRISPR" machine learning model, as described in Zhao et al. (2022). To maintain 500-fold coverage of the 850 guides (approximately 4 guides per gene) in the library, 425k cells were always maintained per condition. With 10 drugs at 2 concentrations each plus one DMSO control, and two replicates of each drug each, there were 42 different conditions. Therefore, 18M cells needed to be present post-infection in order to conduct this screen. With an assumed infection efficiency of 10%, 180M Jurkat cells were seeded at 500 k/mL and combined with 8 μg/mL polybrene. Cells were then infected for 1 day with a viral concentration that achieves 15% infection post-selection, before being removed from virus and allowed to recover for 1 day. They were then placed under selection in 0.5 μg/mL puromycin. Fresh puromycin was added after 3 days, and selection was continued for another 3 days, until uninfected control cells had all died.

Drug Passaging and Analysis of L200 CRISPR Knockout Drug Screen

Jurkat cells infected with the L200 library and selected were seeded at 750k/mL in 6-well plates and a range of drug concentrations were added to each well to capture the IC5 to IC85 of each drug (Table 3). Four topoisomerase II poisons (doxorubicin, etoposide, mitoxantrone, CX-5461), three histone deacetylase (HDAC) inhibitors (SAHA, VPA, MS-275), and three DNA damaging agents (cisplatin, carboplatin, chlorambucil) were used. Every five days, whichever well had 30% viability, the cells were collected, re-seeded at 750k/mL in new plates, and dosed

again. After 15 days, cells were collected and frozen into pellets. Genomic DNA was extracted using a Monarch Kit from New England Biolabs. Sequences targeted by the guides were amplified via PCR, purified via AMPure XP for PCR Purification (Beckman Coutler), and then sent for Illumina sequencing. Gene enrichment/depletion was observed as log-fold changes in sgRNA counts between Day 0 and Day 15. These values were batch corrected (using the ComBat algorithm in R) to remove biases from infection. Results were plotted using principal component analysis (PCA) and a combination resistance/sensitivity map, where red indicates resistance and blue indicates sensitivity, and cluster dendrogram by gene and drug.

Table 3: Drug Concentrations Used for L200 CRISPR Knockout Drug Screen

Drug	**IC5 (μM)**	**IC85 (μM)**
Doxorubicin	0.01	0.142
Etoposide	0.212	2.475
Mitoxantrone	0.006	0.071
CX5461	0.013	0.276
Cisplatin	0.517	1.934
Carboplatin	5.352	21.988
Chlorambucil	0.423	4.701
SAHA	0.01	0.738
VPA	151.379	1988.893
MS-275	0.01	0.835
DMSO		4.701

Data Analysis

All statistical tests were done through GraphPad Prim 8.0, and unless otherwise stated, all other analysis was performed in R.

Appendix A

Supplementary Data

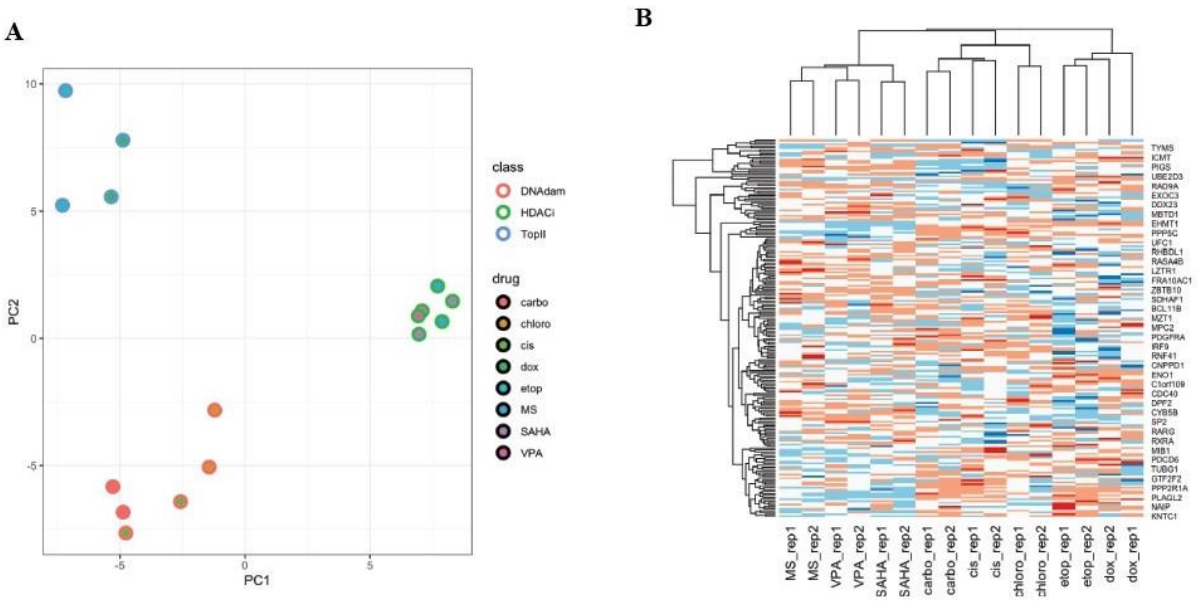

Figure 5: L200 CRISPR Knockout Drug Screen Can Predict Drug Function. Results of the L200 CRISPR knockout drug screen in Jurkat cells depicted as a PCA plot (A) and a combination resistance/sensitivity map (red = resistance; blue = sensitivity) and cluster dendrogram by gene and drug (B), showing drug classes clustering together. Gene enrichment/depletion were observed as log-fold changes in sgRNA counts following drug treatment. Two biological replicates included. Data for CX-5461 and mitoxantrone not included due to sequencing error.

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ACADEMIC VITA

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EDUCATION

The Pennsylvania State University - University Park, PA *Anticipated Graduation: May 2022*
Schreyer Honors College

Bachelor of Science in Biochemistry and Molecular Biology, Molecular and Cellular Biology Option

RESEARCH EXPERIENCE

The Pritchard Lab Spring 2019 – Present

The Pennsylvania State University – University Park, PA

- Research small molecular cancer drug therapeutics and how to combat drug resistance in the clinic using forward engineering
    - Performed immunofluorescence assay to compare the dsDNA break level in drug-treated cells
    - Conducted RNAi knockdown to confer drug resistance to cancer cells
    - Performed meta-analysis on clinical trials to understand re-purposing of approved therapeutics
    - Optimized infection efficiency to develop library of BCR-Abl mutants to detect mutations that confer drug resistance
    - Performed CRISPR knockout screen with compressed gene library to identify drug function

Schreyer MD/PhD Summer Exposure Program - The Moldovan Lab Summer 2021

Penn State Hershey College of Medicine – Hershey, PA

- Studied the effect loss of MED12 has in BRCA-deficient cell lines
    - Conducted short-term Cell Titer-Glo and long-term clonogenic survival assays
    - Ran Western blots to assess protein expression after siRNA knockdown of genes

Vibriophage Genomics-Based Freshmen Research Experience Summer 2018 – Spring 2019

The Pennsylvania State University – University Park, PA

- Isolated and purified Vibriophage before annotating the genome using DNA Master and trying to identify viral lifecycle switch components

TEACHING EXPERIENCE

Undergraduate Teaching Assistant – Organic Chemistry Lab Present

The Pennsylvania State University – University Park, PA

Undergraduate Learning Assistant – Organic Chemistry Lab Fall 2020 – Spring 2021

The Pennsylvania State University – University Park, PA

Undergraduate Teaching Assistant – Vibriophage…Freshmen Research Experience Summer 2019

The Pennsylvania State University – University Park, PA

AWARDS

- NASA PA Space Grant Consortium – WISER (2018-2019)
- The President's Freshmen Award (2019)
- The Sparks Award (2020)
- The Evan Pugh Scholar Senior Award (2021)
- Kevin Daniel Gilmore Memorial Scholarship (2021-2022)

PUBLICATIONS

Liu, C., Leighow, S.M., McIlroy, K., Lu, M., Abello, K., Brown, D.J., **Dennis, K.A**., Moore, C.J., Shah, A., Rivera, V.M., & Pritchard, J.R. "Principled *in vitro* does selection in translational experiments improves clinical drug repurposing." Submitting to *Cancer Research.*

Jackson, L.M., Dhoonmoon, A., Hale, A., **Dennis, K.A**., Schleicher, E.M., Nicolae, C.M., Moldovan, G.L. "Loss of MED12 activates the TGFβ pathway to promote chemoresistance and replication fork stability in BRCA-deficient cells." Accepted at *Nucleic Acids Research,* 2021.

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PRESENTATIONS

Dennis, K.A., Bruno, P.M., Lu, M., Hemann, M.T., & Pritchard, J.R. "CX-5461 Affects Cell Viability Through A Topoisomerase II Poison-Like Mechanism." *Biomedical Engineering Society Annual Conference – Poster Presentation and WISER/MURE/FURP Undergraduate Research Symposium.* Fall 2019.

Dennis, K.A., Jackson, L.M., Dhoonmoon, A., Schleicher, E.M., Nicolae, C.M., Moldovan, G.L. "Loss of MED12 confers chemoresistance via activation of the TGFβ pathway in BRCA-deficient cells." *Penn State College of Medicine Undergraduate Research Symposium.* August 2021.