UNIVERSITY OF CALIFORNIA SAN DIEGO

Study of Anti-Cancer Drugs and their Relationship to Autophagy-Driven Chemotherapy Resistance

A thesis submitted in partial satisfaction of the requirements
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by

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The thesis of Lily Rajaee is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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Professor Trey Ideker, Chair
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Chemotherapies are systemic treatments designed specifically to target various cancer types. However, there are still unknowns about the effectiveness of different chemotherapy drugs in individual cancer patients. Here, we study a mechanism of chemotherapy resistance related to autophagy, a cellular process involving degradation of cellular components through a lysosome-dependent mechanism. We design our experiment by using DrugCell, a visible neural network created by the Ideker lab, which models the hierarchical organization of human cancer

cells and predicts the responses to 216 anti-cancer drugs. More specifically, we leverage DrugCell's visible neural network data to identify top-candidate autophagy-modulating drugs: Vitamin A derivatives, ROS-p38 MAPK-1 Activator, AMPK activators, BL-2 Activators/Inhibitors, CDK Inhibitors, HDAC Inhibitors, microtubule depolymerization, PIK Inhibitors, PLK1 Inhibitors, and Replicative Stress Inducers. Since replicative stress drugs are commonly used to treat head and neck cancers, we characterize this drug group by studying the dose-dependent sensitivities to various head and neck cancer cell-lines. We also test the replicative stress response through western blot analyses checking for the phosphorylation status of ATR and Chk1. To identify the genetic components which dictate the autophagy-drug interactions, we conduct a pilot CRISPR chemogenetic screen and establish the experimental parameters for conducting a screen using autophagy-modulating drugs.

INTRODUCTION

Chemotherapies are widely used around the world as a systemic treatment designed to fight against various cancer types. There are many different types of individual drugs which can be used in combination to create an effective chemotherapy therapy targeting a patient's specific cancer type[1]. In addition to carefully choosing what drug combinations to use in a chemotherapy treatment, there exists the risk for chemotherapy resistance [2]. Chemotherapy resistance is the event where the cancer cells prove to be unresponsive to the effects of chemotherapeutic agents, leading to undesirable patient outcomes. One promising explanation for why a patient would experience chemotherapy resistance can be attributed to the induction of autophagy.

Autophagy is a natural cellular process which leads to the degradation of unwanted foreign components through a lysosome-dependent mechanism [12]. Experimental studies have shown how induction of autophagy can lead to chemotherapy resistance for the patient, leading to cancer cell survival [14]. Introduction of anti-cancer drugs into the cells is often recognized by the cell as foreign substances, and the cell might attack or respond to the introduction of these foreign materials, activating the process of autophagy [13]. Therefore, administration of anti-autophagy drugs may aid in the efficacy of the chemotherapy drugs; autophagy's complex relationship with cancer has recently been studied in the form of combining chemotherapies with anti-autophagy drugs in efforts to provide novel drug synergy [15]. One study using chloroquine, a known autophagy inhibitor, showed how anticancer activity was increased in lymphoma models upon chloroquine administration [15]. Other studies show how induction of autophagy in certain cancer types can actually aid preventative chemotherapy drugs [14]. Thus, autophagy-modulating drugs' ability to enhance the effects of classical chemotherapy drugs that directly target cancer cells offers significant clinical relevance.

Thus, our interest in studying the relationship between cancer and autophagy began with needing to identify a focused list of autophagy-modulating drugs, which we acquired by utilizing a visible neural network, DrugCell [16]. Unlike other autophagy screens, which typically select drugs at random or purely on experimental observation, we utilized this artificial intelligence technology to strategically enrich for autophagy-modulating drugs[17]. In identifying and grouping our chosen autophagy-modulating drugs, we selected two chemotherapy drugs falling under the category of replicative stress drugs. This study focuses on characterizing the behavior of these replicative stress drugs by testing the sensitivities of different cell lines to Hydroxyurea and 5-Fluorouracil, as well as testing for downstream effects in the DNA Repair Response Pathway.

Replicative stress inducing drugs are agents which physically inhibit the natural process of DNA replication [3]. Replicative stress occurs when there is disruption to the processes of DNA unwinding, replicative fork formation, or nucleotide incorporation [4,5], and the induction of replicative stress results in the activation of kinase checkpoints such as ATR and Chk1 [6]. These DNA damage checkpoints respond to stress by activating dormant origins of replication in order to restore genomic stability [7]. Thus, a classic metabolic response to the induction of replicative stress is a signaling cascade which involves the phosphorylation of the ATR substrate [8]. ATR, in turn, activates its downstream Chk1 protein, and phosphorylated Chk1 effectively arrests cell cycle progression [4]. Thus, replicative stress-inducing drugs are agents which can help inhibit the growth of malignant tumor growth [9][10], making the study of these drugs particularly interesting to characterize.

After studying the behavior of these specific replicative stress drugs, we designed a screening experiment using Hydroxyurea, one of our replicative stress drugs, in order to study the genetic components responsible for autophagy's synergistic effects. Clinical trials testing

combinations of chemotherapy with hydroxychloroquine, an autophagy inhibitor, have led to mixed results, with only some patients demonstrating successful responses [15]. This challenge is at least partly due to a lack of prognostic biomarkers, gene sequences that would predict a patient would experience a synergistic drug interaction [14]. These difficulties stem from an incomplete understanding of which chemotherapies activate autophagy, and in which patients. The drug combinations that work for one patient might not work for the next patient. Therefore, understanding which drugs have what effects on autophagy can lead to the creation of better predictive power models that can aid physicians and scientists to develop more specialized, personalized chemotherapy plans. Thus, we address this gap in knowledge by designing a CRISPR chemogenetic screen to identify the biomarkers most important to the process of autophagy modulating drugs in cancer cell-lines [18]. By characterizing autophagy-modulating drugs and optimizing the experimental parameters to conduct a genetic screen, we aim to improve predictive power associated with what cancer types can benefit from autophagy-modulating drug combinations.

RESULTS

In choosing which drugs to use in our study, we performed an expansive literature search for autophagy-modulating drugs and combined that knowledge with DrugCell to further decide which drugs showed the best autophagy-modulating behavior. One way we used DrugCell was by ranking the GO_term RLIPP (Relative Local Improvement in Predictive Power) values for each drug. Out of all the GO_terms, we looked at the value of the highest autophagy-related GO_term RLIPP score and graphed each drug's results side by side. The hypothesis was that the best autophagy-modulating drugs should be the ones with the highest RLIPP value for autophagy_GO_terms. The drug which had the highest RLIPP score for autophagy-modulating GO_terms was PIK-93 (Figure 1a), which we made sure to include in our final drug list. Additionally, we wanted to include autophagy-modulating drugs from different classes of drug mechanisms in order to see if the drugs behaved differently by group. With this in consideration, we considered the drugs which had autophagy-modulation ranked highly on their RLIPP scores, and we chose 22 drugs (highlighted in red) which have high RLIPP scores relative to others (Figure 1a). Altogether, this analysis led to the creation of our hypothesis which tests the autophagy-modulating behavior of these drugs, including drugs from the following categories: Vitamin A derivatives, ROS-p38 MAPK 1 Activator, AMPK activators, BL-2 Activators, BCL-2 Inhibitors, CDK Inhibitors, HDAC Inhibitors, microtubule depolymerization, PIK Inhibitors, PLK1 Inhibitors, and Replicative Stress Inducers (Figure 1b).

Focusing on one class of autophagy-modulating drugs, we first sought out to analyze the behavior of replicative stress inducing drugs. In order to compare the drug sensitivities to replicative stress drugs, compared between different cell lines, a series of drug titers were performed using replicative stress drugs, Hydroxyurea and 5-Fluorouracil. The top dose for each drug was adjusted based on the observed sensitivity each cell line experienced throughout the

course of the experiment. Each of the 5 head and neck cancer cell lines (RPE1, Cal-27, Cal-33, SCC-9, and SCC-25) were treated with both hydroxyurea and 5-fluorouracil independently, and cell viability was measured on a microscope using a phenoxazine dye, Resazurin. The observed IC50 values for Cal-27, Cal-33, SCC-9, SCC-25, and RPE1, and with treatment with Hydroxyurea were 0.67 uM, 50.3 uM, 1000 uM, 628.9 uM, and 4.08 uM, respectively (Figure 2a-e). The observed IC50 values for Cal-27, Cal-33, SCC-9, SCC-25, and RPE1 with treatment with 5-Fluorouracil were 8.79 uM, 1.76 uM, 28.4 uM, 24.3 uM, and 500uM, respectively (Figure 2a-e). After the drug dose curves were completed and generated, we tested the effect of the replicative stress drug on the DNA repair pathway by checking for the phosphorylation status of proteins ATR and Chk1. For all five cell-lines, we did not observe an increase in phosphorylation status for ATR after the addition of replicative stress drugs (Figure 2a-e). We did, however, for all five cell-lines, observe an increase in phosphorylated Chk1 protein expression after the administration of both replicative stress drugs (Figure 2a-e). Cal-27, SCC-9, and RPE1 saw an increase in pChk1 expression level for both hydroxyurea and 5-fluorouracil treatments (Figure 2a,b,e). Cal-33 only saw an increase in pChk1 expression with Hydroxyurea treatment, not with 5-Fluorouracil (Figure 2b). Conversely, SCC-25 only saw an increase in pChk1 expression with 5-Fluorouracil treatment, not with Hydroxyurea. For the SCC-9 cell-line specifically, there was an increase in expression of phosphorylated Chk1 with the introduction of 5-Fluorouracil; however, the phosphorylation status in addition to Hydroxyurea was inconclusive since there was not a uniform expression level of total-Chk1 with the addition of Hydroxyurea (Figure 2c).

Next, we planned to study the genetic component of autophagy-modulating drugs by performing a genetic screen to identify important biomarkers. However, this required first optimizing our CRISPR screening protocol to account for establishing the experimental parameters. The first step in designing this experiment involved creating a specific virus that we

would use in the screen; we started from cloning a gRNA viral library into Lenti-Guide Puro Plasmid backbone, transformed the viral plasmid, and continued to create the virus solution with that transformed plasmid (Figure 3a). Before making a virus with the Lipofectamine 3000 Protocol, we checked for the quality of the transformed plasmid through both PCR and sequencing methods. Of the two DNA purification methods, both the mini-prepped and mxi-prepped samples showed PCR bands at the expected size of ~148bp (Figure 3b). However, only the mini-prepped sample had successful sequencing results (Figure 4c), while the maxi-prepped sample did not show successful integration of the gRNA library (Figure 4d).

We conducted a pilot screen in order to optimize this screening protocol, using home-made RPE1-reporter cell-lines and Hydroxyurea as our drug of choice. After the 28-day CRISPR screen procedure was concluded, the cell pellets were collected, extracted for DNA, and amplified using PCR primers. We studied and optimized each step of the protocol while conducting this CRISPR screen, and each step required extensive troubleshooting before we were able to make improvements to the experimental workflow (Figure 4a). While the positive control lanes had strong bands at 148 bp for all 3 cell cycle numbers (32, 34, 36), the four samples only showed similar bands at 34 and 36 cycles, while showing a slightly lower size band at 32 cycles (Figure 4b).

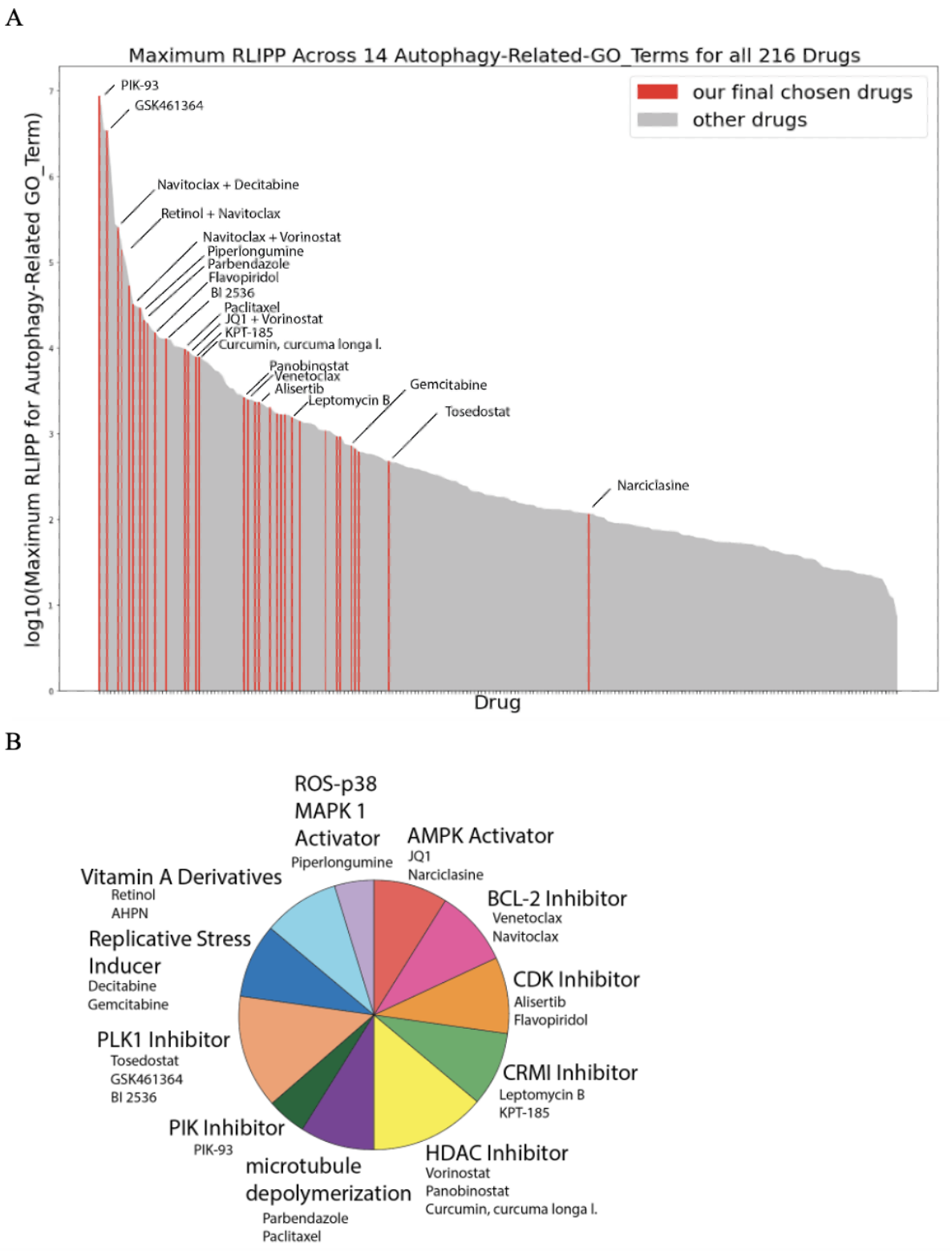


Figure 1: **DrugCell and Selection of Autophagy-Interacting Drugs.** A. Using DrugCell to visualize maximum RLIPP(Relative Local Improvement in Predictive Power) scores for autophagy-specific GO_terms across each of our chosen drugs (red) and other highly trained drugs (training_correlation >0.5). A. Pie Chart highlighting the 22 chosen autophagy-interacting drugs categorized by drug class/ mechanism of action.

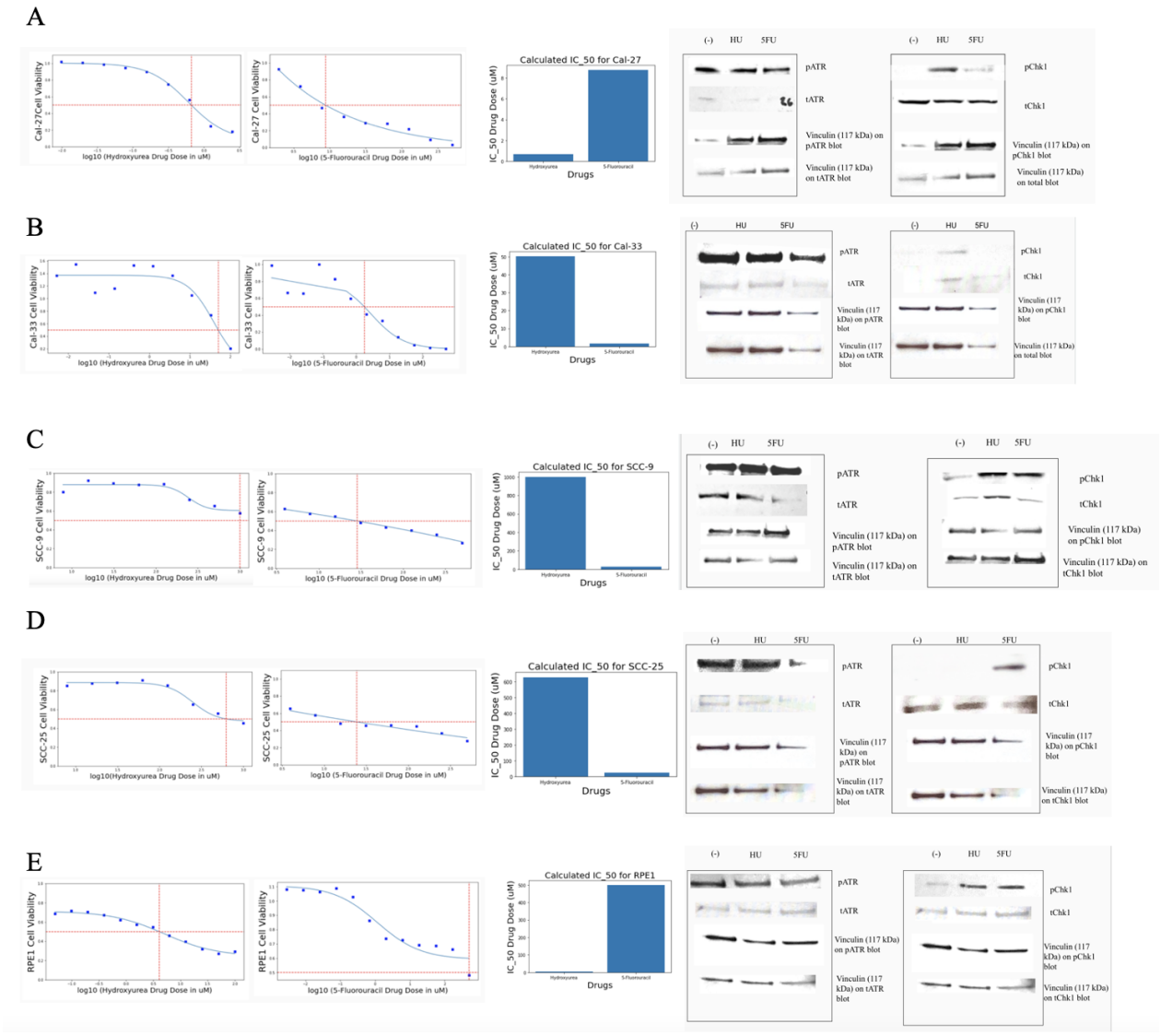


Figure 2: **Dose-Response Curves and Western Blot Analysis: Testing the Effects of Replicative Stress Drugs on the DNA Repair Pathway in Various Head and Neck Cancer Cell-Lines**. (**A**) Cal-27 Cells with Hydroxyurea and 5-Fluorouracil. (**B**) Cal-33 Cells with Hydroxyurea and 5-Fluorouracil. (**C**) SCC-9 Cells with Hydroxyurea and 5-Fluorouracil. (**D**) SCC-25 Cells with Hydroxyurea and 5-Fluorouracil. (**E**) RPE1 Cells with Hydroxyurea and 5-Fluorouracil.

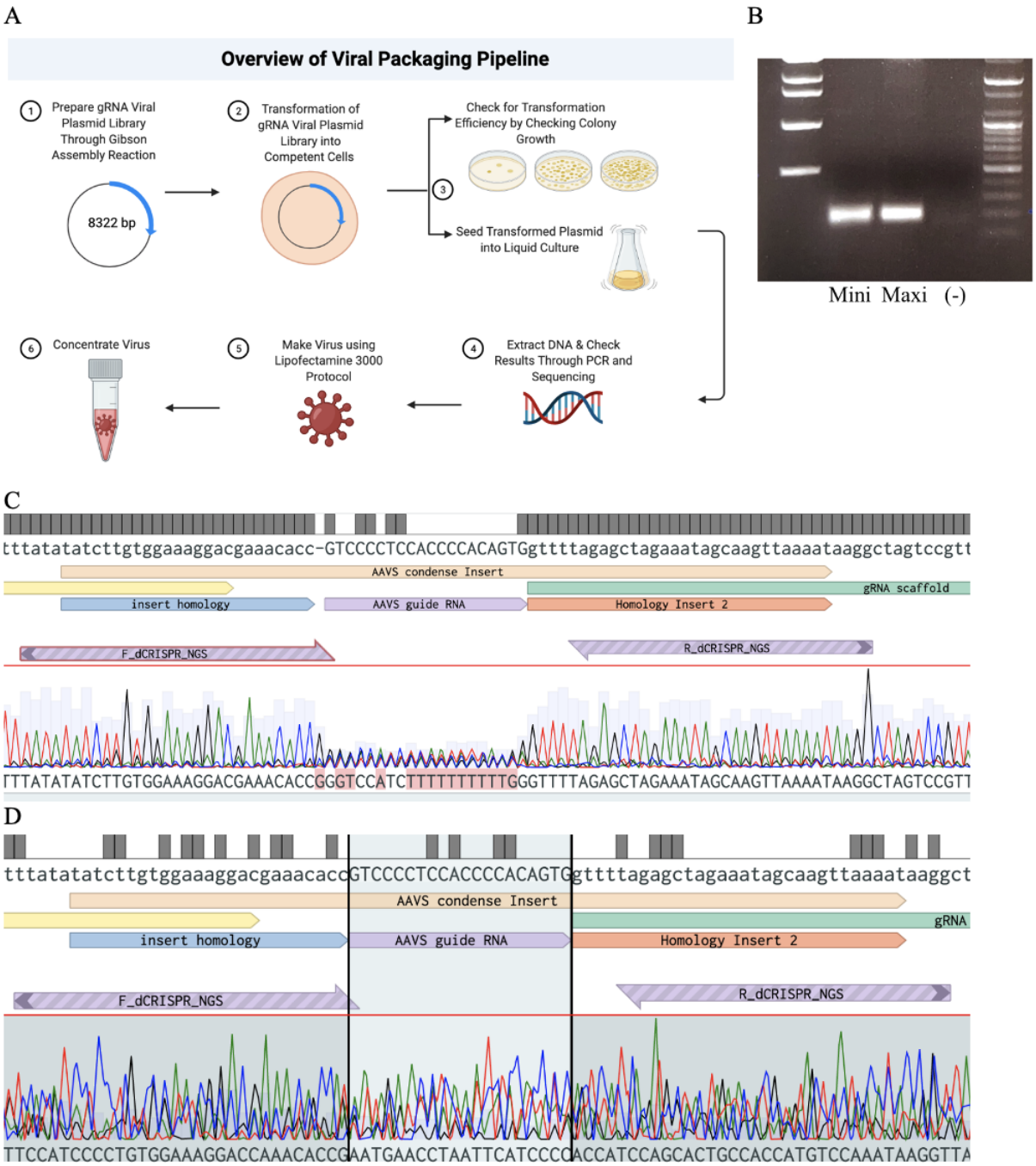
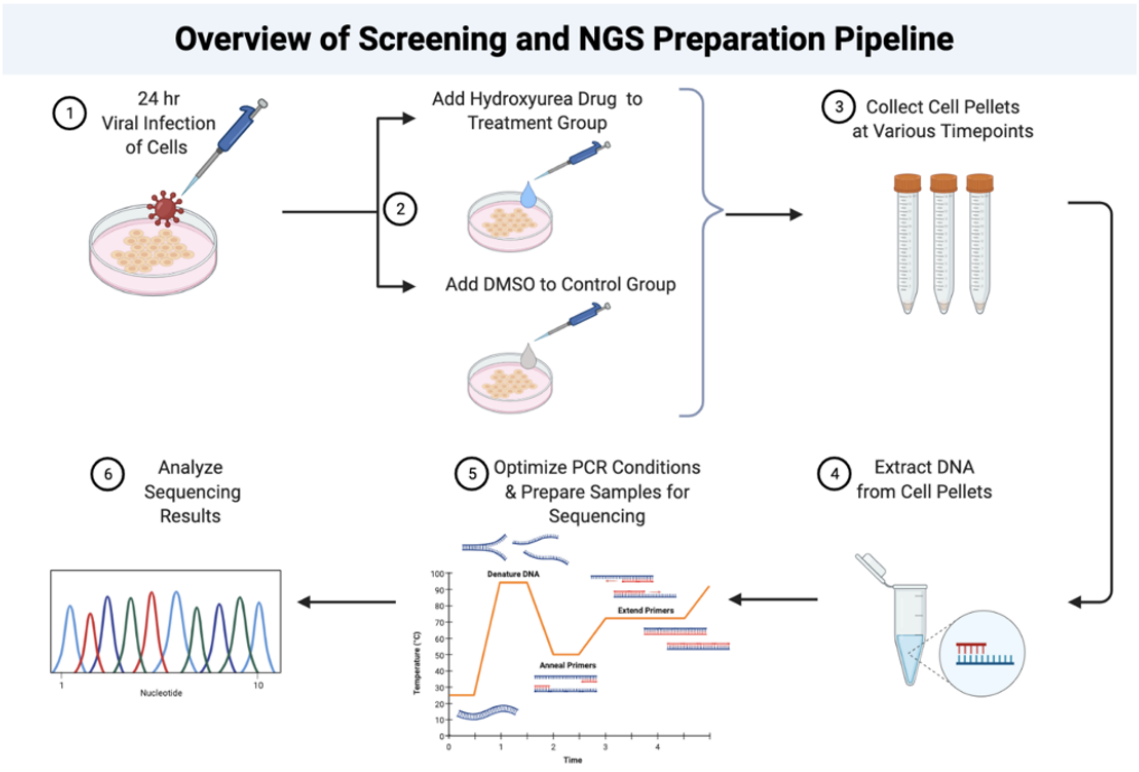
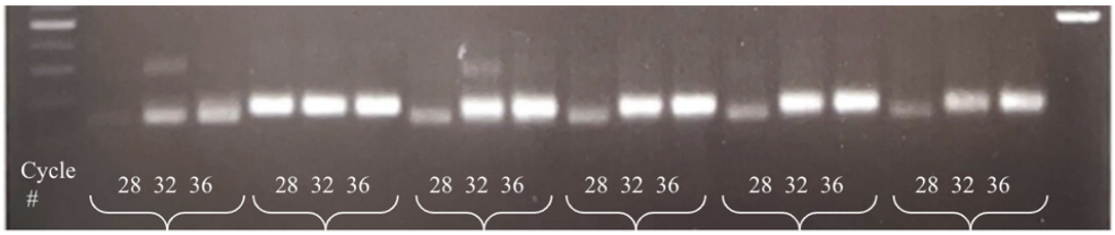


Figure 3**. Virus Design and Preparation**. **(A)** Schematic showing the overview of the Viral Packaging pipeline. Image created using BioRender. **(B)** PCR Results from both Mini-Prepped and Maxi-Prepped DNA extracts from Transformed Plasmids. **(C)** Mini-Prepped Sample Sanger Sequencing Results. **(D)** Maxi-Prepped Sample Sanger Sequencing Results.

A



B



Negative Control | Positive Control | A: Drug(+) | AC: Drug(-) | B: Drug(+) | BC: Drug(-)

Figure 4**. Establishing a Comprehensive CRISPR Chemogenetic Screen Protocol**. (**A**) Schematic showing the overview of the CRISPR screen pipeline. Image created using BioRender. (**B**) PCR1 Results from two pellets which received Hydroxyurea drug Treatment ("A" and "B") and two pellets which did not receive Hydroxyurea drug treatment ("AC" and "BC").

DISCUSSION

We effectively used the information stored in DrugCell to choose which autophagy-interacting drugs to use for our experiments, allowing us to narrow our list of candidate drugs and, as a result, make an informed hypothesis about which drugs to focus our study on. While there have been previous experiments involving the use of screening methods to study autophagy [21], there has been no such study involving the specific list of drugs which we meticulously chose. One way scientists in previous studies picked their autophagy-modulating drugs was by using high-content imaging of LC3B puncta, a cell biological marker of autophagy; thus, they relied solely on these imaging experiments to decide which drugs had sufficient autophagy-modulating abilities [21]. Here, we first filtered through drugs by eliminating drugs with a training correlation <0.5. Then, we took each of the highly trained drugs and ranked the list of go_terms for each drug based on RLIPP (Relative Local Improvement in Predictive Power) scores. More specifically, we looked for the maximum RLIPP value for autophagy-related GO_terms for each drug (Figure 1a). The hypothesis here was that drugs with higher RLIPP numbers would have a greater autophagy-modulating effect, so we narrowed down our drug list by eliminating drugs with low RLIPP values. We then further narrowed that list by doing a literature search of each drug's mechanism of action, and compiled a list of 22 drugs which had high autophagy RLIPP scores and come from a variety of various drug classes (Figure 1b). Thus, this demonstrates how DrugCell can be used as a tool to aid in the design and construction of a screen, allowing for the identification of top drug candidates to use.

In order to compare the drug sensitivities between different cell lines, a series of drug titers were performed using two replicative stress drugs, Hydroxyurea and 5-Fluorouracil. The reason we chose Hydroxyurea is because it is a drug that is already FDA approved for use in treating squamous cell carcinomas of the head and neck [22]. There is less known about

5-fluorouracil and its effects on head and neck cancers; however, its anti-cancer properties have been studied thoroughly in other cancer types and is FDA approved for treatment of colon cancers [23]. The top dose for each drug was adjusted based on the observed sensitivity that each cell line experienced throughout the course of the experiment. Each of the 5 head and neck cancer cell lines (RPE1, Cal-27, Cal-33, SCC-9, and SCC-25) were treated with both hydroxyurea and 5-fluorouracil independently, and cell viability was measured on a microscope using a phenoxazine dye, Resazurin. The observed IC50 values for Cal-27, Cal-33, SCC-9, SCC-25, and RPE1, and with treatment with Hydroxyurea were 0.67 uM, 50.3 uM, 1000 uM, 628.9 uM, and 4.08 uM, respectively (Figure 2a-e). This shows how of the 5 cell-lines, Cal-27 was the most sensitive to treatment with Hydroxyurea because it required a lower dose to achieve the same results as the other 2 drugs. The observed IC50 values for Cal-27, Cal-33, SCC-9, SCC-25, and RPE1 with treatment with 5-Fluorouracil were 8.79 uM, 1.76 uM, 28.4 uM, 24.3 uM, and 500uM, respectively (Figure 2a-e). Here, Cal-33 proved to be the more sensitive drug to 5-Fluorouracil because it required a lower dose than the others to achieve comparable results. After the drug dose curves were completed and generated, we tested the effect of the replicative stress drug on the DNA repair pathway by checking for the phosphorylation status of protein Chk1. For all five cell-lines, we did not observe an increase in phosphorylation status for ATR after the addition of IC20 replicative stress drug doses (Figure 2a-e). An immediate explanation of these results would be that the DNA Repair Pathway was not effectively induced, as we had hypothesized an increase in the phosphorylation of both proteins ATR and Chk1. However, a new hypothesis is that these results are to be expected, since ATR and Chk1 might not have equal sensitivities to Hydroxyurea and 5-Fluorouracil. One study supports this new hypothesis by comparing the basal phosphorylation status to induced phosphorylation status, showing how sensitivity to Chk1 or ATR does not correlate with defects in the DNA damage

response pathway [19]. We did, however, for all five cell-lines, observe an increase in phosphorylated Chk1 protein expression after the administration of both replicative stress drugs (Figure 2a-e). What this means is that the addition of the replicative stress drugs positively activated the DNA Repair pathway, activating the phosphorylation of the Chk1 protein [19]. This explanation is supporting the idea that the IC20 drug dose is sufficient enough to induce a replicative stress response.

With this characterization of replicative stress drugs completed, we decided to move forward with establishing the experimental parameters for a genetic screen by using Hydroxyurea in a pilot screen (Figure 4a). However, we first had to create our virus which would be used in the screen (Figure 3a). Within the virus-making protocol, we tested the quality of the transformed plasmid by checking for the size of the PCR product on a gel, which positively looked at the correct size (~148bp) for both the mini-prepped and maxi-prepped product (Figure 3b). While these results supported the notion that the transformation had been successfully executed, sequencing results raised doubts about the quality of the maxi-prepped product. The mini-prepped product, when aligned with the lenti-guide Puro backbone plasmid, clearly showed an area of mismatch in the area where the gRNA should have been effectively inserted (Figure 3c); however, this was not observed with the maxi-prepped sample (Figure 3d). What this reveals is that any downstream complications with the processing of the pilot screen pellets could be attributed to the fact that there was a problem with the virus stock quality.

Furthermore, the goal of conducting a pilot screen was to improve and establish a CRISPR screen protocol which we could then replicate with our other selected autophagy-modulating drugs. With the use of homemade RPE1-reporter cell-lines and self-designed pooled CRISPR viral package, we conducted the screen for the entirety of 28 days, collected cell pellets, and extracted the DNA from the pellets. The hypothesis was that if

everything worked correctly, we should be able to obtain PCR products which fall at the right size and we should then be able to analyze the specific DNA sequences through Next Generation Sequencing. Although the PCR bands at 34 and 36 cycles appeared to be the right size in comparison to the positive control lanes (Figure 4b), there were some abnormalities observed on the gel. The first abnormality was that there appeared to be a smaller sized band for all the samples at 32 cycles only, and the size increased for 34 and 36 cycles. One explanation could be that the smaller band is actually evidence of primer dimers, and the band is not actually a PCR product [20]. The second abnormality was that the negative control lanes, which received primers but no DNA, were not empty lanes as expected. An explanation for this occurrence could be that the primer stocks were contaminated in the process of preparing the PCR reactions, or this could be evidence of primer dimer formation. After subsequent PCR optimization experiments, we purified the PCR products using bead column cleanup extraction, and sent the samples for Sanger Sequencing. We were unsuccessful at attempts to sequence the PCR products, and we quickly realized that the problem must be with the virus we used for the screen. Thus, alongside experimenting with technical parameters such as deciding on drug doses, cell counts, and PCR conditions, the most significant conclusion from the pilot screen was that we would need to use a fresh stock of virus for subsequent genetic screening.

Future experimentation would require going through with conducting this genetic screen with various autophagy-modulating drugs in order to nominate biomarkers which show to modulate drug synergy. Although there have been unique cases where autophagy can aid anti-cancer therapies, autophagy's complex relationship with cancer has recently been studied in the form of combining chemotherapies with anti-autophagy drugs in efforts to provide novel drug synergy [15]. The next step for this experiment would be to conduct the chemogenetic screen with all the appropriate experimental changes and new information that was gathered by

the pilot screen. Limitations to this approach might include having non-identical biochemical conditions between tissue culture and a patient's body, which is significant in that there could be more complex drug interactions to consider when moving the drug combination to the clinical trial phase. The identification of the genetic components responsible for aiding/ inhibiting the synergy between autophagy-modulating drugs and chloroquine is significant because we improve the predictive power regarding which cancer patients can benefit from receiving this synergistic drug combination.

‘

MATERIALS AND METHODS

Drug Curve/ Dose-Sensitivity Experiments

Wildtype human cells (RPE1, SCC-9, SCC-25, Cal-27, and Cal-33) were used in these drug assays. Replicative Stress Drugs tested include Hydroxyurea and 5-Fluorouracil. For each replicative stress experiment, the wildtype cells were plated in 96-well plates at 2,000 cells/ well on day 1. On day 2, the drugs were added to each treatment lane. Each duplicate of drug-treated lanes was treated with a top dose (identified on Figure 1) followed by a serial dilution down the wells. There were 2 control groups, one group of cells with no drug, and one group with media only (no cells and no drug). The cells were left under drug treatment until the top-dose wells appeared physically sick under the microscope. At that time, we followed the Resazurin Assay protocol and measured cell viability quantitatively using our Tecan Microplate Reader Software.

Western Blot Experiments

The same wildtype cells (listed above) were plated in 6-well plates on Day 1, each well containing 5,000 cells. On Day 2, drugs were added to the wells using a concentration identified as being the IC20 value (calculated from the dose-response curves (Figure 1). Cell pellets were collected after 24 hour drug treatment period concluded, and cells were lysed and quantified in preparation for western blotting.

Autophagy Project

The pilot screen was performed using housemade RPE1-reporter cell lines which added GFP, a fluorescent marker used to measure autophagy activity. The cells were plated on Day 1, and the Condense Virus (Puro-resistant) was added on Day 2. Puromycin Selection was added on day 3, and cells were kept on selection until the negative control plate was dead. Hydroxyurea drug was added on day 15, and cell pellets were collected and analyzed through PCR and sequencing analysis.

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