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An investigation into the efficacy of SVC112, a translation elongation inhibitor, in colorectal carcinoma and acute myeloid leukemia

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Abstract:

Translation is a cellular process frequently dysregulated in cancer (Silvera et al., 2010). There are translation inhibitors currently in the clinic, homoharringtonine (omacetaxine mepesuccinate), or in clinical trials, MNK1/2 inhibitors, however, translation remains an underutilized target for oncology. Here, I report the study of a proprietary translation inhibitor SVC112 in colorectal carcinoma (CRC) and acute myeloid leukemia (AML). These cancers were selected because of their dependence on short half-life proteins such as proto-oncprotein c-Myc or anti-apoptotic protein Mcl-1, which are predicted to be sensitive to inhibition of translation. I found that CRC cells as a group are more resistant to SVC112 than AML cells as a group; the median IC50 for 3-day growth assays for CRC cell lines was 482 nM but 95 nM for AML cell lines. Within each cancer type, cell lines showed IC50s that differed by 1000-fold. Therefore, I set out to understand the basis for differential sensitivities of cancer cell lines to SVC112. I identified gene expression signatures in CRC cells that correlate with sensitivity/resistance to SVC112. In addition, I found that reduction of protein synthesis of c-Myc, p53 and survivin after drug treatment correlates with sensitivity to SVC112. In AML cells, I found that basal, pre-treatment level of anti-apoptotic protein BCL-xL correlates with resistance to SVC112. These findings could be useful in optimizing clinical strategies and developing biomarkers for SVC112.
Introduction:
SVC112 Discovery and Development

SVC112 is a novel anti-cancer agent that is being evaluated for efficacy as a treatment for various cancer types. SVC112 is a synthetic derivative of the compound Bouvardin, which is extracted from the plant Bouvardin ternifolia and has been utilized by indigenous peoples in Mexico as a treatment for various ailments (Jolad et al, 1977). Bouvardins’ anticancer mechanism is to inhibit polypeptide elongation by inhibiting GTP and eEF-2 dependent translocation of the peptidyl tRNA and inhibiting binding of the aminoacyl-tRNA to the ribosome (Zalacain et al., 1982; Stickle et al. 2015). Bouvardin was discovered to be a potential radiation modulator through a proprietary screen developed by the Su lab in which Drosophila Chk1 and p53 homozygous null mutant larvae were irradiated and then given drug treated food. Drugs were determined to be efficacious if percent survival to adulthood with drug was greater than two standard deviations of the population (Gladstone, 2012). This screen was uniquely suited for identifying agents that would inhibit the regeneration and survival of various tissues post irradiation. The key similarity between the organ primordial of Drosophila larvae and mammalian tumors is that they both regenerate through “accelerated repopulation”, where cells that survive irradiation then proliferate at an accelerated rate (Jaklevic and Su, 2004). The proprietary Drosophila screen identified the potential anti-tumor agent Bouvardin as synergizing with radiation.

Figure 1: The proposed mechanism of action for SVC112. The unique ability of SVC112 to block the elongation step of translation which bypasses problem of cap-independent or cap-dependent translation. Adapted from Cox, Doudna, and O’Donnell, 2015.
Bouvardin’s ability to modulate cells response to radiation has been demonstrated in human cancer cell lines. The mechanism of action for Bouvardin as locking eEF2 onto the 80S ribosome complex was confirmed in fractionated HeLa cells through ribosome profiling (Stickel et al., 2015). The Bouvardin derivative developed by the Su lab, SVC112, has a proposed mechanism that inhibits both cap-independent and cap-dependent translation. Most mRNAs are post-transcriptionally modified with a 5' terminal m\(^7\)GpppG cap that is a binding site for eIF-4 which recruits ribosomal subunits to initiate “cap-dependent translation” (Jackson et al., 1995). There are examples of mRNAs that have not been modified to contain a 5' terminal m\(^7\)GpppG cap, or whose caps do not effectively bind to the initiation protein eIF-4. Despite the lack of the 5' G cap, these proteins are still translated in what has been deemed “cap-independent translation” (Jackson et al. 1995). Cancer cells under stress have been demonstrated to switch between cap-dependent and cap-independent translation for the same mRNA, introducing a new challenge to translation inhibition drug development (Silvera et al., 2010; Trivigno et al., 2013).

During cell proliferation signals from growth factors, hormones, and cytokines stimulate signaling cascades that activate protein kinase AKT, which phosphorylates and activates mTOR. Activated mTOR inactivates inhibitory binding proteins for eukaryotic initiation factor 4E (eIF4E) enabling eIF4E to promote cap-dependent translation. However, during times of cellular stress such as hypoxia and nutrient deprivation, mTOR is downregulated, increasing the binding of eIF4e inhibitory proteins to switch the predominant mode of protein translation from cap-dependent to cap-independent (Silvera et al. 2010). The ability for cancers to switch between cap-independent and cap-dependent translation illustrates the need for a drug that will inhibit both modes of translation.

Three cancer types were identified as potential models for studies to assess the efficacy of SVC112. Head and neck squamous cell carcinomas (HNSCC) were targeted because radiation therapy is standard of care and pairing standard of care with a radiation modulator such as SVC112 would be a new potential treatment strategy (Cognetti et al, 2009). Colorectal Carcinoma (CRC) and Acute Myeloid Leukemia (AML) were selected based on their reliance on the short half-life proteins c-Myc and Mcl-1. A
key characteristic of c-Myc and Mcl-1 that make them possible targets for SVC112 is their ability to be translated by cap-dependent and cap-independent mechanisms, meaning a translation inhibitor that blocks both forms of translation is necessary to fully deplete cancer cells of these oncoproteins (Trivigno et al., 2013). A translation inhibitor that targets the elongation step of translation thus inhibiting cap-dependent and independent translation would be a novel way to deplete these cancers of driver proteins. Evaluating the efficacy of SVC112 as a treatment for CRC and AML was the focus of this thesis.

Efficacy of SVC112 in treating Colorectal Cancer (CRC)

Colorectal Carcinoma (CRC) is the third most commonly diagnosed cancer for both men and women in the United States (Siegel et al., 2017). Resistance to standard-of-care is a persistent problem in patients with CRC with 90% of patients diagnosed with metastatic disease developing resistance to therapy (Hammond et al., 2016). The five-year survival rate for patients diagnosed with metastatic CRC is 12.5%, demonstrating a need for the development of new therapeutics (Hammond et al., 2016). The proto-oncoprotein Myc plays a role as a transcription factor activating numerous growth pathways and is tightly regulated in normal cells. Deregulation of Myc is a common driver of CRC carcinogenesis (Weigering et al., 2015). Myc has been identified as one of the most commonly amplified oncogenes in human cancers and is translated in both a cap-dependent and cap-independent manner (Wiegering et al., 2015; Dang, 2012).
From a translation perspective Myc overexpression promotes increased cap-dependent mRNA translation and decreased cap-independent mRNA translation, enabling a switch between these two forms of translation (Silvera et al., 2010). By inhibiting translation of internal ribosome entry site (IRES) dependent (cap independent) CDK11 expression, Myc increases mitotic cytokinesis defects and subsequently increases genetic instability, furthering tumorigenesis (Barna et al., 2008). The identification of Myc and the powerful role it plays in tumorigenesis has highlighted it as a potential target for future cancer therapies (Wiegering et al., 2015). Because the Myc protein has a half-life of 15-20 minutes and excess Myc protein expression is a consequence of Myc gene amplification or deregulation; it shows potential as a protein whose depletion through SVC112 blocked translation could inhibit CRC growth (Murphy, 2008). Perhaps more importantly is that Myc can be expressed through cap-dependent and cap-independent mechanisms meaning a translation inhibitor that can block both forms of translation is necessary to properly deplete cells of the myc oncoprotein (Barna et al., 2008). Myc is one of only 10% of proteins that can be translated through cap-dependent translation and also has an internal ribosome entry site (IRES) and so can also be translated in a cap-independent manner (Trivigno et al., 2013). It is necessary to inhibit cells ability to translate in both a cap-dependent and a cap-independent manner to ensure that Myc and other proto-oncoproteins like Mcl-1 are fully depleted.

A common characteristic of CRC are mutations in the epidermal growth factor receptor (EGFR) pathway. Overexpression of GTPases or kinases in the EGFR pathway is characteristic of CRC and has also been a general marker of poor prognosis (Spano et al., 2005). Because of the role of EGFR pathway mutations in the oncogenesis of colorectal cancer, there has been a surge in new targeted therapies attempting to target mutations in kinases and GTPases in the EGFR pathway. BRAF is a kinase in the EGFR pathway that is commonly mutated to become constitutively activated through a valine to glutamate mutation at the 600th peptide. Vemurafenib is a targeted agent developed as a small molecule inhibitor that binds to the ATP-binding domain of mutant V600E BRAF (Luke and Hodi, 2012). While vemurafenib has proven to be a largely effective treatment in metastatic melanoma it has demonstrated limited
efficacy as a viable single agent treatment for V600E BRAF mutant colorectal carcinoma (Korphaisam and Krittiya, 2016). This is likely because of the ability of colorectal cancers to activate secondary pathways, for example, the PI3 Kinase pathway to evade the anti-tumor effects of vemurafenib (Luke and Hodi, 2012). However, there is significant potential for vemurafenib if the implementation of these compensatory pathways is blocked, thus driving the search for agents to combine with vemurafenib in the treatment of CRC.

A second combination choice for SVC112 is MLNO128. MLNO128 is a dual mTORC1/mTORC2 inhibitor which makes it a more potent treatment than first generation mTOR inhibitors like rapamycin which inhibit mTORC1, neglecting mTORC2 and its ability to phosphorylate downstream proteins such as AKT, which plays an important role in growth and proliferation pathways (Sun, 2013). Cap-dependent protein expression is inhibited by mTORC1 inhibitors which decreases some protein expression including cap-dependent Myc expression (Wiegering et al., 2015). The role that mTORC1/mTORC2 inhibitor MLNO128 plays in translation regulation means that MLNO128 and SCV112 may both target Myc expression and turnover mechanisms and could make efficacious combination partners.

**Efficacy of SVC112 in treating Acute Myeloid Leukemia (AML)**

Acute Myeloid Leukemias (AML) are an age-related leukemia that is the most common acute leukemia in U.S. adults, accounting for 10,000 U.S. deaths in 2015 (De Kouchovsky et al., 2016). AML is characterized by the rapid expansion of clonal immature hematopoietic stem cells in the bone marrow (Gores and Kaufmann, 2012). The standard of care for AML is cytarabine and an anthracycline (i.e. doxorubicin or daunorubicin), with stem cell transplantation. This treatment is highly toxic and not a viable treatment option for most elderly patients, therefore there is a demonstrated need for new, less toxic therapeutics (De Kouchovsky et al., 2016). AML was evaluated as a cancer with potential for treatment with SVC112 because of evidence demonstrating that AML cell lines, are dependent on the Bcl-2 family protein Mcl-1 (Bose and Grant, 2012; Gores and Kaufmann, 2012; Gong et al., 2016). Bcl-2 family proteins include Bcl-2, Bcl-Xₐ, Mcl-1, A1, Bax, and Bak. All Bcl-2 proteins play a key role in mitochondrial
outer membrane permeabilization (MOMP) (Bose and Grant, 2013). Bcl-2 proteins share homologous regions termed BH (bcl-2 Homology) 1-3. BH3-only proteins are pro-apoptotic proteins (Bad, Bid, Bim, Puma, etc) that bind the hydrophobic groove on the surface of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1). Binding of the pro-apoptotic BH3-only protein to the BH3 groove of the anti-apoptotic Bcl-2 proteins prevents the anti-apoptotic Bcl-2 proteins from engaging with Bax and Bak, both pro-apoptotic effector proteins that trigger apoptosis through permeabilization of the mitochondria (Bose and Grant, 2013). Mcl-1 is an example of one of these anti-apoptotic Bcl-2 proteins that AML cell lines and MM cell lines over produce in order to evade apoptosis (Bose and Grant, 2013; Derenne, Monia, Dean, Taylor, and Rapp, 2002; Gores and Kaufmann, 2012).

Mcl-1 has a half-life of 2-4 hours and cells are reliant on active transcription and translation to maintain levels (Bose and Grant, 2013). A general translation inhibitor is in a unique position to deplete AML lines of Mcl-1 as opposed to a specific inhibitor like S63845, which specifically binds with high-affinity to the BH3-binding groove of Mcl-1. An enormous number of pathways regulate Mcl-1 transcription, translation, and degradation, demonstrating a need for a general protein inhibitor that can inhibit both cap-dependent and cap-independent translation of Mcl-1.

The current studies evaluate the efficacy of SVC112 in the treatment of CRC and AML through the successful depletion of short half-life proteins that cancers are reliant on for growth and survival. A secondary goal was to search for potential mechanisms of resistance to SVC112 in colorectal cancer cell lines based on previously known genetic characteristics of each cell line. In addition, I also evaluated the ability to SVC112 to act in combination with inhibitors of major signaling pathways in CRC cells, Sensitivity to
SVC112 was evaluated across a panel of 44 CRC cell lines and 10 AML cell lines. SVC112 depletes the CRC cell line WIDR of Myc when treated at concentrations as low as 50 nM. Further, there is a correlation between BRAF mutation and sensitivity to SVC112 in CRC cell lines. Synergy was observed between SVC112 and a variety of drugs when combined drug 3-day growth assays were performed. SVC112 depletes AML cell lines of Mcl-1, though Mcl-1 expression is not an effective predictor for sensitivity to SVC112. When the Bcl-2 family protein Bcl-X\textsubscript{L} was investigated as a predictive marker for sensitivity to SVC112, there was an observed correlation between resistance and higher production of Bcl-X\textsubscript{L} protein. This indicates that Bcl-X\textsubscript{L} expression may be a marker for sensitivity to SVC112 in AML cell lines.
Materials and Methods:

Cell Culture

Forty-four human colon carcinoma cell lines and 10 human acute myeloid leukemia cell lines were cultured and evaluated for sensitivity and response to treatment with the anti-cancer agent SVC112. CRC cell lines were obtained from G. Eckhardt (University of Colorado, Aurora, CO) and AML cell lines were obtained from the J. DeGregori lab (University of Colorado, Aurora, CO). CRC cell lines were authenticated through short tandem repeat (STR) examination by the Eckhardt lab. AML cell lines were authenticated through STR examination by the DeGregori lab (Gregory et al., 2016). All CRC cell lines were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% antibiotic/antimitotic and maintained in a 37°C in an atmosphere containing 5% CO₂. All AML cell lines, with the exception of Kasumi-3 and KG-1a, were cultured in RPMI 1640 supplemented with 10% FBS and 1% antibiotic/antimycotic. Kasumi-3 and KG-1a were cultured in RPMI 1640 supplemented with 20% FBS and 1% antibiotic/antimycotic.

3-day cell growth assays

CRC cell suspensions of 1500-5000 cells (depending on the cell line) per 100μL were plated into flat-bottomed 96 well plates and allowed to incubate overnight. AML cell suspensions of 7000 cells per 100μL were plated into flat-bottomed 96 well plates and allowed to incubate overnight. SVC112 was maintained in a 10mM stock suspended in DMSO at -25°C. Cells were then treated with SVC112 in 100μL of media to reach 10 final concentrations ranging from 3 nM to 10 μM. Cells were allowed to grow for 72 hours from drug addition, and after which cells were treated with 95μL of CellTiter-Glo reagent (Promega) for ATP quantification. Fluorescence was quantified using a Biotek Synergy 2 Plate Reader. Inhibitory concentration 50% (IC50) values were then calculated by plotting the logarithmic value of fraction of cells affected vs the logarithmic value of the dose SVC112 used. This plot yielded a linear line that was used to calculate the point at which 50% of cells were affected with SVC112. CRC and AML cell lines were determined to be sensitive if they had an IC50 < 200 nM.
**Drug combinations**

The effect of drug combinations were studied in two CRC cell lines WIDR and MIP101, using 96-well assays as described above. SVC112 was tested in combination with vemurafenib (BRAF inhibitor) provided by the Eckhardt lab (Fig. 6), PF-04601502 (mTOR/PI3 Kinase inhibitor) (SFig. 2) provided by the Eckhardt lab, MLNO128 (mTORC1/ mTORC2 inhibitor) (Fig.6) provided by the Eckhardt lab, trametinib (MEK inhibitor) (SFig. 2) provided by the Eckhardt lab, verapamil (calcium channel inhibitor) (Fig. 8) purchased from Selleck Chemical, MG-132 (proteasome inhibitor) (SFig. 3) purchased from Selleck Chemical, and ICG-001 (Wnt/ β-catenin inhibitor) (Fig.8) provided by the Eckhardt lab. These drugs were added to the cells at the same time as SVC112. Combination Index (CI) values were calculated utilizing Calcusyn™ software where the entire growth inhibition curve is considered and then synergistic, additive, or antagonistic interactions are identified (Bjinsdorp et al., 2011).

**Clonogenic assays**

CRC cell lines were seeded 2,000 cells per well into 6 well plates and cultured overnight. The next day cells were treated with 0 μM (DMSO control), 0.0 5μM SVC112, or 0.5 μM SVC112 and allowed to incubate for 72 hours. Drug was then removed from the cells, cells were washed with 1 mL of phosphate buffer solution (PBS) and 3 mL of untreated media was added to each well. Cells were incubated for one week and then fixed with 100% methanol. Cells were then stained with 1x crystal violet for 30 minutes. Crystal violet and methanol were removed from wells and cells were washed with water three times and then allowed to air dry. Pictures of wells were taken using a digital camera and total colony area was quantified using ImageJ (Public Domain Software, National Institutes of Health) total colony area software plug in.

**Immunoblotting**

CRC and AML cell lines were seeded into six-well plates at a density of 500,000 cells/well and cultured in 2 mL of RPMI. Cells incubated overnight and SVC112 in equal volume of media was added to reach the final concentrations of 0 μM (DMSO control), 0.05 μM, and 0.5 μM. Cells were harvested at 2,4, and 24 hours after drug treatment.
CRC cells were harvested by removing the media and lysing cells in 100μL lysis buffer composed of NaCl, Tris-Base buffer 7.4 pH, Triton-X, protease inhibitor, and sodium orthovanadate. Cells were then frozen at -20°C in 1.5 mL microcentrifuge tubes until needed for DC protein analysis or immunoblotting. AML cells were treated with 200nM of SVC112 and cultured for two hours. AML cells were then centrifuged at 1500 rpm for 5 minutes and lysed in 100μL lysis buffer. AML cells were stored at -20°C in 1.5 mL microcentrifuge tubes until needed for DC protein analysis or immunoblotting. Total protein content of the extracts was quantified using Bio Rad DC Protein Assay. For immunoblotting cell extracts were mixed with 5x loading buffer containing crystal violet, ethidium bromide, and SDS, and lysis buffer to standardize the amount of protein loaded per well to 25 μg per 20 μL. Samples were then boiled at 95°C for 5 minutes and mixed thoroughly before loading into 10% polyacrylamide gels, electrophoresed, and then transferred overnight onto polyvinylidiene difluoride (PVDF) membranes. The blots were then blocked for one hour at room temperature in 5% nonfat dry milk in Tris-buffered saline with Tween-20 (TBST). Blots were then incubated overnight at 4°C with primary antibodies for β-actin, PARP, mcl-1, bcl-xl, bcl-2, p53, and c-myc at a dilution of 1:500 in blocking solution. After incubation with the primary antibody, blots were washed three times with TBST for five minutes each and then probed with an anti-rabbit or anti-mouse secondary antibody that is linked to horseradish peroxidase at 1:1000 dilution in blocking solution at room temperature for one hour. Blots were then washed three times with TBST for five minutes and then developed using Immobilon Western Chemoluminescent horseradish peroxidase substrate (Millipore).

**Flow Cytometry**

CRC cell lines were plated in 2 mL RPMI at a density of 2x10⁵ cells per well. The cells were incubated overnight and then treated with SVC112 at the concentrations of 0 μM, 0.05 μM, and 0.5 μM for 24 hours. Cells were then treated with trypsin and centrifuged at 1500 rpm for 5 minutes. Cells were then washed three times with phosphate buffered saline (PBS) and stained with annexin-5 FITC conjugate and propidium iodide (PI) as instructed in the Sigma Aldrich Annexin 5 apoptosis detection kit. Cells were then
analyzed through flow cytometry and cells positive for Annexin-5 FITC and PI were determined to be actively undergoing apoptosis.

**Amino Acid Incorporation**

Inhibition of protein synthesis by SVC112 was assayed using a Click-it amino acid incorporation kit (Thermo Fischer). The head and neck cancer cell line known to be sensitive to SVC112, Det562, was used as a positive control while CRC cell lines HCT116 (S) and HCT15 (R) were used to test the ability of SVC112 to inhibit amino acid incorporation in CRC cell lines. Cells were treated with DMSO or 0.5 μM of SVC112, and allowed to incubate for 24 hours in the presence of the amino acid analog, L-azidohomoalanine. Cells were then processed for detection of AHA using a chemoselective (click) ligation and electrophoresed. The gel was then visualized under UV to determine if AHA incorporation was lower in treated cells than control cells.
Results: 

Single Drug Treatment of CRC cells with SVC112

SVC112 was evaluated in a panel of forty-four different CRC cell lines by Kelli Robertson from the Eckhardt lab at University of Colorado, Aurora, CO. After treatment with SVC112 cell lines were determined to be sensitive if they had an Inhibitory concentration 50% (IC50) less than 200 nM, between 200 nM and 500 nM cell lines were determined to be moderately resistant and above 500 nM cell lines were determined to be resistant (Figure 4a). Based on these guidelines 13 CRC cell lines were determined to be sensitive, 10 were determined to be moderately resistant, and 21 were determined to be resistant (Figure 4a). Mutation status of these cell lines was then collected and analyzed through a Fisher exact test by Kelli Robertson. Genetic mutations that may play a role in determining sensitivity to SVC112 are V600E BRAF, and K177N kRAS (Figure 4a). The genetic amplification of cMyc may also play a role in the sensitivity of CRC cell lines to SVC112. Genetic status of CRC cell lines was provided by the Eckhardt lab. The p-values for the statistical likelihood that these mutations are correlated to sensitivity or resistance to SVC112 were determined through Fisher exact test, and are kRAS K177N p-value = 0.0056 (**), BRAF V600E p-value = 0.0046 (**), and cMyc p-value = 0.0154 (*). This initial evaluation of a panel of 44 CRC cell lines and their subsequent genetic profile provides a foundation to determine the genetic and molecular characteristics of resistance to SVC112 in cancer.

One resistant cell line (MIP101) and one sensitive cell line (WIDR) were selected for the remainder of experiments involving the single agent effects of SVC112. Relative response to SVC112 was confirmed through clonogenic assays, where long term recovery potential of cells was evaluated after treatment with SVC112 (Figure 4b). In clonogenic assays drug treatment is transient and so conclusions about long-term effects of SVC112 can be made. The results of this assay demonstrate that sensitivity to SVC112 in WIDR cells is not transient. When they are plated at single cell densities and then treated with SVC112 their ability to proliferate and form colonies is severely hampered, at both 50nM and 500nM concentrations. SVC112 does not impact the ability of MIP101 cells to form colonies after treatment, affirming that they are resistant in a long-term context at both 50nM and 500nM concentrations (Figure 4b).
Apoptosis in response to SVC112 was evaluated through annexin-5 staining and flow-cytometry. Results of this assay are shown in Figure 4c and demonstrate that cell death through apoptosis does not occur in response to SVC112 in cell lines WIDR or MIP101. The results of this experiment match preliminary data demonstrating that another derivative of Bouvardin, SVC101, does not induce apoptosis in HNSCC cell lines. The results of this assay imply that SVC112 does not induce apoptosis in CRC cell lines.

The final evaluation of the single agent activity of SVC112 in WIDR and MIP101 cells was through immunoblotting of cells treated at 50nM and 500nM with SVC112 and treated for 2, 4, and 24 hours (Figure 4d). The cell lines were tested for protein expression of p53, survivin, and β-actin. The transcription factor p53 is a tumor suppressor that plays a role in induction of cell cycle arrest, apoptosis, and senescence when cells are under cellular stress (Li et al., 2015). While inactivating mutations of p53 are some of the most common mutations in cancer, occurring in 34% of proximal colon tumors and 45% of distal colon tumors, there are also mutant forms of the p53 protein that can gain novel oncogenic functions (Li et al., 2015). Additionally, p53 has a short half-life ranging from 5 to 20 minutes, making it a protein that is likely to be quickly and effectively depleted by SVC112 (Giaccia and Kastan, 1998). When p53 protein levels were analyzed through immunoblotting, the levels decreased with treatment of SVC112 at 4 and 24 hours in WIDR (S) cells. Total levels of p53 protein appeared to increase across time points specifically in the 24 hour sample in MIP101 (R), however there is no variation in this sample with increasing concentrations of SVC112, demonstrating that SVC112 may not be a cellular stressor that causes increased p53 expression over time (Figure 5d).

Survivin is a second cellular protein that was analyzed for changes over time in response to treatment with SVC112. Survivin is a member of the inhibitor of apoptosis protein family (IAP), and is highly expressed in many cancers as a method of evading apoptosis (Kelly et al., 2011). Survivin has been identified as a biomarker for outcome with high levels of survivin expression associated with poor clinical outcome and increased rates of resistance to conventional chemotherapies (Rodel et al., 2012). Additionally, survivin has the potential to be targeted by SVC112 as it also has a short
half-life of approximately 30 minutes (Zhao, 2000). Survivin, similar to p53, decreased with treatment of SVC112 in the sensitive WIDR cells specifically at the 4 and 24 hour time points (Figure 4d). Levels of survivin were unaffected by SVC112 in the resistant MIP101 cell line.

The final protein analyzed in response to SVC112 was cMyc. Myc is also a protein with a short half-life, ranging from 15-20 minutes, and because there is a statistically significant role between sensitivity to SVC112 and insertion of cMyc in the DNA, Myc levels were investigated in response to SVC112 treatment (Murphy, 2008). Similar to all other short half-life proteins analyzed through immunoblotting, cMyc levels decreased after treatment with SVC112 in the sensitive WIDR cell line but not in the resistant MIP101 cell line (Figure 5d). Dissimilar from the other proteins analyzed though, cMyc protein levels are reduced within two hours of treatment with SVC112, demonstrating the ability of SVC112 to deplete sensitive cancer cells of this cancer driving protein in a short period of time (Figure 4d).
SVC112 prevents protein Synthesis in Sensitive Cell Lines

SVC112 was evaluated for its’ ability to effectively prevent protein translation in sensitive cell lines. This was tested through Click-it amino acid incorporation. Two cell lines were tested, HCT116, a sensitive CRC cell line, and GEO, a resistant CRC cell line. The IC50 for HCT116 cells is 21 nM SVC112 and the IC50 for GEO cells is >10,000 nM SVC112. Cell lines were previously determined to be sensitive or resistant to SVC112 based on CellTiter-Glo 3-day growth assays (Figure 4a). The left side of Figure 5a is shown to illustrate even protein loading between protein samples with and without SVC112. The right side of Figure 5a is the same gel visualized under UV, in which incorporation of L-azidohomoalanine (AHA) can be visualized. Relative amounts of AHA incorporation were quantified using ImageJ software and plotted in Figure 5b. The results demonstrate that in HCT116 there is successful prevention of protein synthesis. In the resistant GEO cell line there is no loss of protein synthesis, demonstrating that in some resistant cell lines SVC112 does not inhibit protein synthesis.
The efficacy of SVC112 in combination with various targeted agents for common colorectal pathways was evaluated. Based on the potential relevance for BRAF V600E mutation in sensitivity to SVC112 the BRAF inhibitor vemurafenib was selected to combine with SVC112 and treat WIDR (S) and MIP101 (R) in 3-day combined drug growth assay. The sensitive WIDR cell line has an IC50 of 46 nM and the resistant MIP101 cell line has an IC50 of 4779 nM. Cells were treated at SVC112 concentrations of 7.8nM, 3.9nM, and 0.78nM. The combination index (CI) values were generated through Calcusyn. The results showed that SVC112 in combination with inhibitors of pathways known to be important in CRC resulted in synergistic effects with IC50 values of 46 nM and 4779 nM. The synergy was further confirmed by the ClickiT Amino Acid incorporation assay where Det562 (S), HCT116 (S), and HCT15 (R) were tested. Results demonstrated that in sensitive cell lines amino acid incorporation was prevented and in the resistant cell line amino acid incorporation was not prevented. This shows that SVC112 effectively prevents translation. Figure 5b is the quantification of the gel results from Figure 5a. Data courtesy of Kelli Robinson from the Eckhardt Lab.
15.6nM, 31.3nM, 62.5nM, 125.0nM, 250.0nM, and 500.0nM and combined with vemurafenib concentrations of 0.25μM, 0.5μM, and 1μM. It was predicted that there would be synergy between vemurafenib in the WIDR (S) cell line because they have a mutational BRAF V600E status and are sensitive to SVC112, in contrast it was predicted that there would be an additive or slightly antagonistic interaction between vemurafenib and SVC112 in the MIP101 (R) because they have a wild type BRAF V600E mutational status.

Based on the results of the combined drug growth assay between SVC112 and vemurafenib, there is moderate synergy between SVC112 and vemurafenib at all doses of vemurafenib. Figure 6a illustrates the results of the 3-day combined drug growth assay between vemurafenib and the combination index (CI) values are shown to the right of the MIP101 (R) graph. CI values were only calculated for the WIDR (S) cells because the equation to produce a CI value requires a response curve for both drugs. Because MIP101 cells are so resistant to SVC112 and are BRAF V600E WT there was no response curve for either SVC112 or vemurafenib and so no CI value could be calculated. Synergy has various levels determined based on the numerical CI values and these levels are defined in Table 1. The response to SVC112 combined with vemurafenib ranged from moderate synergy to additive interactions at vemurafenib concentrations ranging from 1.25 μM to 5 μM (Figure 6a). The relationship between BRAF V600E mutations and sensitivity to SVC112 should be further elucidated and vemurafenib should be further investigated as a combination partner for SVC112.

A second drug that SVC112 was put in combination with was MLNO128, an mTORC1 and mTORC2 inhibitor. Translation is commonly dysregulated in cancer and mTORC1 and mTORC2 both play a role in cap-dependent translation regulation. It was hypothesized combining MLNO128 and SVC112 would be a way to further deplete CRC cell lines of proteins necessary for proliferation like Myc and would be an efficacious way to inhibit growth. The response of WIDR cells to a combination of SVC112 and MLNO128 ranged from moderate synergy at low doses of MLNO128 (0.25 μM) to antagonism at high doses of MLNO128 (1 μM) (Figure 6a). Based on the results of 3-day combined drug growth assays MLNO128 and SVC112 fluctuate between synergistic and antagonistic responses in CRC WIDR (S) cells. This response is
potentially because at some point the inhibition of the cMyc pathway is saturated, so at high doses of MLNO128 antagonism is observed and at low doses synergy is observed. This response will have to be further investigated in other CRC cell lines to verify that it is not unique to WIDR (S) cells.

Interactions between vemurafenib and MLNO128 were confirmed through clonogenic assay as shown in Figure 6b. The two drugs interacted similarly in a clonogenic experiment as they did in the 3-day combined drug growth assay demonstrating that the effects of the drug combinations is not a transient response.

Interestingly, when SVC112 and vemurafenib were combined and then immunoblotted for reduction in short half-life proteins there was a significant reduction in levels of cMyc in the vemurafenib treated WIDR (S) sample that was not observed in the SVC112 treated sample (Figure 6c). The decrease in cMyc with treatment of vemurafenib alone can be explained because V600E BRAF mutant WIDR are sensitive to vemurafenib and so proliferative proteins like cMyc will be expressed at lower rates. The reason that there may not have been a combinatorial effect seen is because of the relatively low levels of SVC112 used, 0.05μM, and the high levels of vemurafenib used, 1.25μM. What this could mean is that any combined effects between SVC112 and vemurafenib are overwhelmed by the excess levels of vemurafenib used when treating the cells. Levels of p53 are reduced in the combination only at 4hr and 24hr in WIDR (S) cells. This observation may be artificial and a result of inconsistent loading or transfer of the western blot and so should be repeated to verify the phenomenon. The same reduction in p53 levels after treatment with a combination of SVC112 and vemurafenib was not observed in MIP101 (R) cells. Actin was used as the loading standard to verify that there are similar protein levels in each well and so any reduction in specific protein levels is a results of drug treatment and not inconsistent protein loading.

When MIP101 (R) cells were treated with MLNO128, there was a significant reduction in cMyc at 24 hours in the MLNO128 only treated sample and in the combined MLNO128 and SVC112 sample. MLNO128 inhibits the mTORC1 and mTORC2 pathways preventing mTORC1 from signaling for increased expression of cMyc. A similar depletion of cMyc in SVC112 only treated WIDR (S) cells may not be observed because WIDR (S) are treated with 0.05μM SVC112 and so are treated close to their
IC50 (0.047μM) while both WIDR (S) and MIP101 (R) are being treated with MLNO128 well past the IC50 for the drug. Levels of p53 may be reduced in MLNO128 only treated MIP101 (R) cells and in the SVC112 + MLNO128 treated MIP101 (R) cells at 24 hours. It is likely that any observed reduction in p53 in the combination sample of MIP101 (R) is entirely due to addition of MLNO128 if the reduction in p53 in this sample is to be believed. Immunoblotting for p53 levels in MIP101 cells should be repeated in order to verify that the reduction in p53 at 24 hours is a real phenomenon. The combination of SVC112 and vemurafenib or MLNO128 had no clear combined effects on short half-life proteins in WIDR (S) and MIP101 (R) samples.
Basal gene expression data shows correlations with sensitivity/resistance to SVC112

Based on the single drug 3-day growth assay data for the CRC panel compiled in Figure 4a, a heat map was generated by the University of Colorado Anschutz Bioinformatics core based on RNA Seq data compiled by the Eckhardt lab (University of Colorado, Aurora, CO) for six sensitive CRC cell lines and 12 resistant CRC cell lines (Pitts et al., 2014). The RNA Seq data analysis performed by the bioinformatics core identified 100 genes, 50 of which have decreased (blue) RNA expression in sensitive cell lines and increased (red) RNA expression in resistant cell lines. The other 50 genes identified had increased (red) RNA expression in sensitive cell lines and decreased (blue) RNA expression in resistant cell lines (Figure 7a). A Fisher exact test was performed on this data to identify genes that are statistically significant in the impact their RNA expression levels have on sensitivity to SVC112 (Table 2).

Based on the genes identified through RNA Seq data analysis, various small molecule inhibitors were selected to combine with SVC112 and with the goal of identifying a specific pathway that can modulate sensitivity to SVC112. Two genes of interest ABCB1 (p-value = 0.043) and ABCB4 (p-value =0.035) have RNA expressed at higher levels in resistant cell lines (Table 1). ABCB1 and ABCB4 are both members of the ATP binding cassette (ABC) transporter protein family, which are associated with multi-drug resistance (Hammond et al., 2016). These proteins have been shown to play...
Verapamil is a calcium channel blocker that has been demonstrated to bind to P-glycoproteins like ABCB1 or ABCB4 and inhibit the drug-pump activity of these proteins (Yusa and Tsuruo, 1989). Verapamil sensitizes tumor cells with up regulated ABC proteins to chemotherapeutics through competitive binding of P-glycoprotein transporters, thus increasing intracellular concentrations of chemotherapeutics (Yusa and Tsuruo, 1989). Verapamil is so effective at blocking the activity of ABCB1 and ABCB4 P-glycoprotein drug pumps that it has been used to verify if cell lines do or don’t express ABCB1 or ABCB4.

Table 2: Genes identified by the Chan lab as being potentially indicative of sensitivity or resistance to SVC112. Half of the genes are expressed at higher levels in sensitive cell lines and half are expressed at higher levels in resistant cell lines. A Fisher exact test was performed based on the results of the heat map generated by the Chan laboratory. The Fisher exact test results are listed above along with the subsequent statistical relevance of the results. Genes that were targeted with small molecule inhibitors are highlighted and were identified as ABCB1, ABCB4, and APCDD1.
have active ABCB1 or ABCB4 mutations (Duan et al., 2004). Verapamil in combination with SVC112 was used to determine if multi-drug resistance through ABCB1 and ABCB4 mutations is a factor in resistance to SVC112 in some resistant CRC cell lines.

RKO (S) and DLD1 (R) cells were treated with a combination of SVC112 and Verapamil. Synergy was observed between the two drugs when growth inhibition data was analyzed using Calcusyn. The RKO cell line has an IC50 of 29 nM and the DLD1 cell line has an IC50 of 515 nM. In the RKO (S) cells there was moderate synergy, CI values ranged from 0.923 in the low dose of verapamil to 0.314 in the high dose of verapamil (Figure 8c). When SVC112 and Verapamil were combined in DLD1 (R) cells there was strong synergy at high doses of SVC112 and verapamil and antagonism at low doses of SVC112 and verapamil (Figure 8c). This is indicative of the fact that ABCB1 and ABCB4 P-glycoprotein are expressed at higher levels in resistant cell lines and a way to sensitize DLD1 (R) to SVC112 may be to block the P-glycoprotein pumps through verapamil, thus demonstrating the potential reliance of DLD1 (R) cells on P-glycoprotein pumps to survive treatment with SVC112. A combination of verapamil and SVC112 should be tested in a greater number of CRC cell lines to verify synergy between the two drugs and further illuminate the role of ABCB1 and ABCB4 P-glycoproteins in resistance to SVC112.

A second gene identified through Fisher exact test of the RNA Seq data as having a high probability of modulating sensitivity or resistance to SVC112 is APCDD1. APCDD1 is a membrane bound glycoprotein that inhibits Wnt and is expressed at higher levels in resistant CRC cell lines and expressed at lower rates in sensitive cell lines (Clevers and Nusse, 2012). Nuclear and cytosolic accumulation of β-catenin, the downstream target of

![Figure 7: The signaling pathway for APCDD1, Wnt and β-catenin with the known effects of ICG-001 and hypothesized effects of SVC112.](image)
Wnt, has been shown to play a role in CRC carcinogenesis (Pignatelli, 2002). Furthermore, a target protein for β-catenin is c-Myc, with the correlation between nuclear β-catenin levels and c-Myc protein expression being high (Pignatelli, 2002). Cytosolic β-catenin has been shown to have a short half-life, enabling SVC112 to potentially deplete cells dependent on the Wnt signaling pathway of a necessary secondary messaging molecule (Bommer et al., 2012). Based on these known interactions it was hypothesized that blocking Wnt signaling through the Wnt inhibitor ICG-001 would replicate the effects of APCDD1 and increase response to SVC112 in RKO (S) cells while not effecting response in SW480 (R) cells. This is the expected result because if RKO cells rely on the activation of the Wnt pathway for proliferative signaling, then depleting this pathway doubly through the Wnt inhibitor ICG-001 and SVC112 blocking production of cMyc should result in a robust anti-proliferative response. Because SW480 (R) cells already produce APCDD1 at higher levels, the Wnt pathway is constitutively repressed, thus further repression of Wnt through ICG-001 and depletion of β-catenin through SVC112 should result in a weak anti-proliferative response.

RKO (S) and SW480 (R) were treated with ICG-001 at concentrations of 1μM, 5μM, and 20μM and with concentrations of SVC112 ranging from 7.1nM to 500.0nM. The SW480 cell line has an IC50 of 477 nM and the RKO cell line has an IC50 of 29 nM. The results of this experiment indicate that sensitivity of RKO to SVC112 is modulated through the addition of ICG-001 and thus the blocking of the Wnt signaling pathway (Figure 8c). There was noticeable synergy in RKO (S) cells at low concentrations of SVC112 and high concentrations of ICG-001 and this synergy reduced until at high concentrations of SVC112 there was only an additive interaction between the two drugs. In SW480 (R) there was no noticeable synergy (Figure 8c), which is expected if Wnt signaling is already inhibited through increased expression of APCDD1 in these cells. Sensitivity to SVC112 can be modulated through combination of ICG-001 and SVC112 because these two drugs can target the Wnt signaling pathway by inhibiting the pathway at two important points. ICG-001 can inhibit Wnt, restricting downstream signaling for β-catenin, and SVC112 can block transcription of short half-life proteins like c-Myc.
Single Drug Treatment of Acute Myeloid Leukemia cells with SVC112

Acute myeloid leukemia (AML) cell lines were obtained from the DeGregori lab and were evaluated for sensitivity to SVC112. Ten cell lines were tested for IC50 values and were then evaluated for their response to SVC112 through immunoblotting. Sensitivity to SVC112 ranged between IC50s of 3 nM for AML-EOL-1 to 7,774nM for Kasumi-3. Of the cell lines evaluated a sensitivity of less than 200 nM was determined to be sensitive, and above 200 nM was determined to be resistant (Figure 9a). Six AML cell lines were determined to be sensitive to SVC112 and four were resistant (Figure 9a). Nathan Gomes of the Su lab performed 3-day growth assays to determine IC50 in 4 AML cell lines, Kasumi-3, THP-1, MV-4-11, and AML-EOL-1.

Based on the determined IC50s to SVC112, cell lines were evaluated for a potential biomarker that could predict sensitivity to SVC112. RNA Seq data was not available for AML cell lines and so potential biomarkers predictive of sensitivity had to be selected based the known ability of SVC112 to deplete AML cells of Mcl-1. The Mcl-1 inhibitor S63845 showed that while Mcl-1 is not an efficacious biomarker for efficacy of S63845, the Bcl-2 family protein Bcl-x<sub>L</sub> does show potential as a predictive biomarker with higher expression in resistant MM cell lines and lower expression in sensitive MM cell lines (Kotschy et al., 2016). Because both S63845 and SVC112 deplete dependent cells of Mcl-1, it was thought that Bcl-x<sub>L</sub> could also work as a biomarker to predict for
sensitivity to SVC112. Immunoblotting was performed on cell lines basal levels of Mcl-1, Bcl-2, and Bcl-xL. Based on these basal levels it was observed that neither Mcl-1 nor Bcl-2 were predictive for sensitivity to SVC112, however there is an observed gradient in Bcl-xL expression levels and sensitivity to SVC112 mimicking the pattern observed in S63845 sensitivity (Figure 9c).

SVC112’s effect on AML cell lines was then evaluated through immunoblotting. Four sensitive and four resistant cell lines were selected, treated with 200 nM SVC112 and harvested after 2 hours. Previous experiments have demonstrated that there is a response to SVC112 after only two hours (Figure 5d). The results of this experiment demonstrate that SVC112 depletes sensitive and resistant AML cell lines of Mcl-1 (Figure 9b). Depletion of Mcl-1 is correlated with an increase in apoptosis as observed through the increase in cleaved PARP in sensitive AML cell lines after treatment with SVC112 (Figure 9b). If over expression of Mcl-1 is a molecular mechanism for evading apoptosis in cells then SVC112 is a viable candidate for treatment of AML especially because of SVC112s unique ability to inhibit both cap-dependent and cap-independent translation. However, Mcl-1 overexpression is not a universal survival mechanism and so molecular characterization of cell lines may be necessary for effective treatment of patients.
Figure 9: Figure 9a shows the panel of ten AML cell lines tested for sensitivity to SVC112 and their corresponding IC50 values. Kasumi-3, THP-1, MV-4-11, and AML-EOL-1 cell lines were tested for sensitivity to SVC112 by Nathan Gomes from the Su laboratory at University of Colorado, Boulder, CO. Six AML cell lines show IC50 values within the sensitive range, while four AML cell lines showed IC50s in the resistant range (technical N=3). Figure 9b shows the levels of Mcl-1 before and after treatment with SVC112 in four sensitive cell lines and four resistant cell lines. Also blotted is the parp cleavage associated with these blots. The upper band of parp is uncleaved, representing little apoptotic activity and is at 116 kDa. Cleaved parp is at 89 kDa and represents apoptotic activity. The result of these blots imply that SVC112 may reduce Mcl-1 levels in some resistant and some sensitive cell lines. Potentially elevated parp cleavage in cell lines where Mcl-1 is depleted may indicate induction of apoptosis (N=1). Figure 9c shows a potential negative correlation between Bcl-x\textsubscript{L} expression and sensitivity to SVC112. B-actin blotting did not work in the cell lines MonoMac6 and U937, however because there is strong banding in Bcl-x\textsubscript{L} for these cell lines there is still reason to believe the negative correlation between sensitivity to SVC112 and Bcl-x\textsubscript{L} expression (technical N=3). All error bars are representative of standard error (SEM).
**Discussion:**

SVC112 is a novel anti-cancer agent that has been shown to inhibit translation elongation. SVC112 was evaluated for its anti-cancer effects in a variety of cancer cell lines. Based on the results of these experiments, SVC112 shows promise as a treatment for cancers that overexpress short half-life proteins that are known to be translated through cap-dependent and cap-independent translation. Disparities in response to SVC112 were investigated through the combination of SVC112 with other targeted inhibitors. Genes that play a role in sensitivity or resistance to SVC112 were investigated through combined drug 3-day growth assays.

A panel of forty-four colorectal carcinoma cell lines was evaluated for sensitivity to SVC112, which was defined as an IC50 below 200 nM in a 3-day growth assay in 96-well format. A minority of CRC cell lines were sensitive to SVC112, with only thirteen of the forty-four lines defined as sensitive to SVC112 (Figure 4). SVC112s ability to prevent protein synthesis in sensitive and resistant CRC cell lines was investigated through AHA amino-acid incorporation, there was an observed reduction in protein synthesis in the sensitive HCT116 cell lines, however there was no corresponding reduction in protein synthesis in the resistant HCT15 cell line (Figure 5). SVC112 may reduce protein synthesis in sensitive cell lines and may not reduce protein synthesis in resistant cell lines. Confounding this result though is the fact that cell lines were treated with SVC112 for 24 hours and because of this prolonged exposure there could be off-target effects of SVC112 that distorts the results of this experiment. In future experiments SVC112 should only be put on the cells for 2-4 hours to more accurately measure the effects of the drug. The AHA amino-acid incorporation experiment should be repeated to determine if the results are reproducible and should be performed on more resistant and sensitive CRC cell lines to see if the trend in disparities in protein production holds for multiple cell lines.

Based on the results presented in Figure 8, ABCB1 and ABCB4 may play a role in resistance to SVC112 in some CRC cell lines and some instances of CRC resistance to SVC112 may be due to the development of multi-drug resistant P-glycoproteins that actively remove chemotherapeutics from the intracellular fluid. The high expression of ABCB1 and ABCB4 is not uniform across resistant cell lines and so it is unlikely to be
the exclusive reason for resistance to SVC112. Additionally, up-regulation of ABCB1 and ABCB4 P-glycoprotein pumps is an adaptation made by numerous malignant colorectal carcinomas as a method to develop resistance to chemotherapeutics, and these mutations are unlikely to already be present in patients whose first line of treatment were to be SVC112 (Hammond et al., 2016). The role of ABCB1 and ABCB4 P-glycoproteins in resistance to SVC112 will have to be further investigated through repeating the experiment conducted in Figure 8c as well as by using shRNA to knock out ABCB1 and ABCB4 P-glycoproteins in resistant cell lines.

More promising for why SVC112 is effective in some cell lines as opposed to others is SVC112s’ demonstrated ability to deprive cell lines of short half-life proteins that have cancer driving effects and are known to switch between cap-dependent and cap-independent translation. In Figure 5 it was demonstrated that SVC112 effectively depletes sensitive WIDR (S) cells of cMyc, p53, and survivin. SVC112 did not deplete MIP101 (R) cells of short half-life proteins like cMyc, p53, or survivin indicating that these proteins expression and response to SVC112 should be investigated further.

Depletion of pro-survival proteins did not induce apoptosis in CRC cell lines as was initially hypothesized. Apoptosis levels were measured in Figure 5 through annexin-5 staining and subsequent flow-cytometry, the results of this experiment indicate that there is no induction of apoptosis in response to treatment with SVC112 in WIDR (S) or MIP101 (R) cell lines.

Combination partners for SVC112 in CRC were investigated. Because SVC112 has already shown potential as a radiation sensitizer and it was hypothesized that SVC112 could sensitize CRC cell lines to other chemotherapeutics through protein deprivation. There was limited evidence that there was a more than additive effect between SVC112 and vemurafenib or MLNO128. The CI values calculated through calcsyn demonstrate what could be a moderate synergy effect between SVC112 and MLNO128 or vemurafenib. Common CRC chemotherapeutics were investigated in combination with SVC112 and demonstrated moderate synergy and additive effects with SVC112.

There is evidence that cells that repress the Wnt signaling pathway through increased expression of APCDD1 are more resistant to SVC112. This is based on the
fact that the Wnt inhibitor protein APCDD1 is enriched in resistant cell lines, blocking the Wnt signaling pathway (Figure 8a). A reason for this disparity in RNA seq data is that cell lines that have developed constitutive activation of the Wnt signaling pathway as a path to tumorogenesis may be more likely to be sensitive to SVC112, because both cytosolic β-catenin and c-Myc (a downstream target of β-catenin) are short half-life proteins easily depleted through the introduction of SVC112. This argument is supported through the observed synergy between ICG-001 and SVC112 at low doses of SVC112 and high doses of ICG-001. This is possibly happening because ICG-001 inhibits Wnt, blocking production of β-catenin and therefor only limited levels of SVC112 were needed to block translation of more β-catenin, modulating RKO (S) sensitivity to SVC112. This same effect was not observed in SW480 (R) perhaps because APCDD1 is already expressed at higher levels, blocking the Wnt signaling pathway. Because of this, adding a second Wnt inhibitor, ICG-001 did very little to modulate sensitivity to SVC112 in DLD1 (R) cells. The possible role of the Wnt signaling pathway in sensitivity or resistance to SVC112 should be further investigated through repeating the combination of ICG-001 and SVC112 in RKO (S) and SW480 (R) cell lines. Cytosolic β-catenin levels should also be measure before and after treatment with SVC112 in each cell line to see if as predicted, RKO have higher cytosolic β-catenin levels initially that are depleted with treatment of SVC112 and if SW480 have lower basal cytosolic β-catenin levels that are impacted by treatment with SVC112.

SVC112 may be a viable treatment option for acute myeloid leukemia based on its' ability to induce apoptosis and block production of Mcl-1. Figure 9a results, demonstrate that five out of ten AML cell lines were determined to be sensitive to SVC112 with an IC50 of 200 nM used as a cut-off. When cells were treated with SVC112 PARP cleavage increased (Figure 9c) demonstrating that in contrast to CRC cell lines, apoptosis is induced in AML cells with the introduction of SVC112 at 200 nM. The ability of SVC112 to effectively deplete AML cell lines of Mcl-1 protein, which has been indicated as a protein they become dependent on, was also demonstrated, showing a method through which apoptosis may be induced in AML cell lines (Figure 9c). Lastly, a protein that negatively correlates with sensitivity to SVC112 was identified as Bcl-x_L, showing a path to determining if a patient could effectively respond to
SVC112 in the clinic. The reason for the correlation between Bcl-xL expression and response to SVC112 may be because Bcl-xL is the Bcl-2 family protein that has the most weight when concerned with meeting the threshold of mitochondrial outer membrane permeabilization (MOMP). In order for MOMP to occur a basal level of BAX, BAK, and BOK pro-apoptotic proteins have to be unbound by bcl-2 anti-apoptosis proteins like Mcl-1, Bcl-2, and Bcl-xL (Kalkavan and Green, 2017). It is possible that there is are more Bcl-xL proteins binding BAX, BAK, and BOK and therefore the relative levels of Bcl-xL are higher when compared to Bcl-2 or Mcl-1, so Bcl-xL determines the ability of these cells to enter apoptosis more than Mcl-1 or Bcl-2. In order to determine if Bcl-xL is expressed at higher levels and so plays more of a role in the meeting of a threshold for apoptosis mass spectrometry should be performed to determine relative protein expression levels between Bcl-2 family proteins. Additionally, Bcl-xL should be knocked out in cell lines using shRNA to determine its' importance in providing resistance to SVC112.
**Future Aims:**

In order to further elucidate mechanisms of resistance to SVC112, the exact mechanism of action for SVC112 should be elucidated. This would enable more detailed and exact investigation into modes of resistance to SVC112 in the resistant cell lines. The heat map in Figure 8a should be repeated with different cell lines selected. The cell lines that should be selected when this is repeated should be the most resistant cell lines, with approximately ten times the IC-50 of the sensitive cell lines. This will allow for genes that may play a larger role in sensitivity or resistance to be identified as opposed to general multi-drug resistance genes. To determine efficacy of SVC112 in CRC, mouse experiments should be done using patient derived xenografts. This will allow for determination of efficacy of SVC112 with the inclusion of challenges like drug delivery and drug toxicity. The inclusion of all of this data will mean a more accurate and realistic determination of SVC112’s efficacy in treating colorectal carcinoma.

For evaluation of SVC112’s efficacy in treating AML cell lines, SVC112 should be tested in a wider panel of AML cell lines which could be obtained through a variety of cell line banks. This may provide a more representative sample of leukemias that respond to SVC112. An siRNA knockdown of Mcl-1 should also be performed in a sample of these AML cell lines to determine if Mcl-1 is actually a protein that these AML cell lines are dependent on. Through these experiments SVC112’s ability to deprive AML cell lines of Mcl-1 and that deprivations impact on AML cell lines would be better understood and characterized.
Supplementary Figures:

Supplementary Figure 1: Supplementary Figure 1a shows the dose response curves of CRC cell lines COLO678, GP5D, MIP101, CL34, KM12C, MDST8, WIDR, and SW48. The result demonstrated by these CellTiter Glo dose response curves confirms that sensitivity and resistance is reproducible (technical N=3). Supplementary figure 1b shows the clonogenics performed on more CRC cell lines with SVC112 confirming that the effects of SVC112 are long lasting and consistent between sensitive and resistant cell lines (technical N=3). Clonogenics were performed by Annika Gustafson (Su Laboratory), Anna Capasso (Eckhardt Laboratory), and Kelli Robertson (Eckhardt Laboratory). All error bars are representative of standard error (SEM).
Supplementary Figure 2: Supplementary Figure 2a and 2b show the 3-day combined drug growth assays for PF-502 and Trametenib. They were also evaluated for efficacy as combination partners for SVC112 but showed no synergy at any drug concentrations (technical N=3). Supplementary Figure 2c shows the CI values for all four drugs evaluated graphed in clusters based on combination partner concentration. WIDR (S) cells treated with PF502, a PI3 kinase/ mTOR combined inhibitor, showed additive interactions with SVC112. Trametinib, a MEK inhibitor showed antagonistic interactions when combined with SVC112. All error bars are representative of standard error (SEM).
Supplementary Figure 3: Supplementary Figure 3a MG-132 was also evaluated in combination with SVC112 based on RNA seq data however the CL34 cells did not produce a sufficient growth curve to calculate CI values from or draw any meaningful conclusions from (biological N=3). MG-132 is a proteasome inhibitor that was thought to be efficacious in combination with SVC112 based on enriched pathways identified by Aik Choon Tan (University of Colorado, Aurora, CO). Error bars are representative of standard error (SEM).
References:


