

COMBINATION TREATMENT WITH CDK4/6 AND CDK8/19 INHIBITORS IN
ER-POSITIVE BREAST CANCER

By

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Thesis Summary

Cyclin-dependent kinases (CDK) 4 and 6 are the main drivers of proliferation in estrogen receptor (ER)-positive breast cancer cells. CDK4/6 inhibitors, such as the FDA-approved cytostatic drug, Palbociclib, induce G1 arrest and prevent cell cycle progression. Prolonged treatment with CDK4/6 inhibitors can lead to drug resistance in the clinic as well as in the lab. The mechanisms behind this resistance are varied, with preliminary evidence suggesting that it may be influenced by the senescence-associated secretory phenotype (SASP), CDK6 hyperactivation, or degradation of the retinoblastoma protein (Rb).

In contrast to the cell cycle mediating CDK4/6, CDK8 and 19 are involved in regulating transcription. CDK8/19 phosphorylate the C-terminal domain (CTD) of RNA polymerase II (Pol II), inducing the elongation of transcription. Studies have shown that high CDK8/19 expression is associated with shorter relapse-free survival and failure of systemic therapy in breast cancer. Preliminary data suggests the inhibition of CDK8/19 successfully prevents resistance to CDK4/6 inhibitors, resulting in complete elimination of cancer cells.

This project will help establish why combination therapy with CDK8/19 inhibitors prevents drug resistance to CDK4/6 inhibitors via the suppression of ER-regulated transcription of mitogenic factors. This drug combination therapy may have clinical relevance for preventing drug resistance in ER-positive breast cancer.

Introduction

ER-positive breast cancer makes up approximately 70% of human breast cancers.¹ The ER is the primary transcription factor driving oncogenesis in ER-positive breast cancer.¹ Selective estrogen receptor modulators, aromatase inhibitors, and selective estrogen receptor degraders in combination with each other or with cytostatic drugs, comprise the current standard

of care for women with ER-positive breast cancer.² However, many ER-positive breast cancer patients do not respond to these therapies or ultimately develop resistance despite an initial response.²

Cancer is characterized by uncontrolled proliferation due to dysregulation of various cell cycle proteins. CDK 4 and CDK 6 belong to the family of serine-threonine kinases and are the main drivers of proliferation in ER-positive breast cancer.³ As seen in Figure 1, Rb regulates cell cycle entry by restricting progression from the G1 phase into the S phase of the cell cycle. CDK4/6, when bound to Cyclin D1 (CCND1), overcomes this restriction by phosphorylation of the Rb protein, resulting in the release of E2F transcription factors and progression into S phase.³

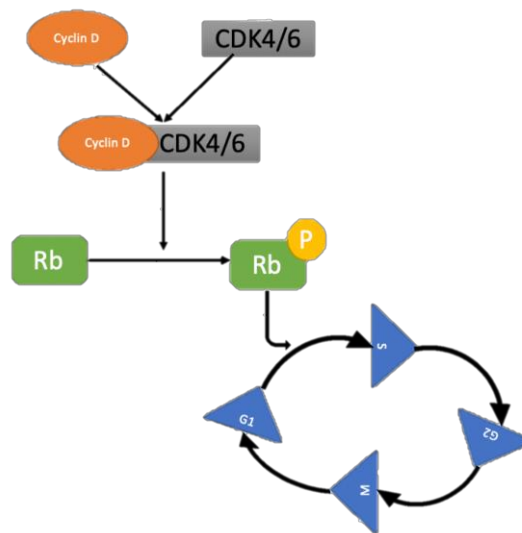


Figure 1. Pathway of CDK4/6 in cell cycle progression.

Small-molecule inhibitors of CDK4/6 prevent phosphorylation of the Rb protein, inducing G1 arrest and preventing cell cycle progression. Preclinical trials have demonstrated the ability of CDK4/6 inhibitors to selectively inhibit the growth of ER-positive breast cancer cells, act synergistically with endocrine therapy, and reverse endocrine resistance.⁴ Additionally, phase III trials have shown that CDK4/6 inhibitors in combination with hormonal therapy significantly

improve progression-free survival in ER-positive breast cancer compared to hormonal therapy alone.⁵ Palbociclib, in particular, is a highly selective CDK4/6 inhibitor and was the first agent within the drug class to be synthesized and studied.⁶ Following the results of the clinical trials PALOMA-1, PALOMA-2 and PALOMA-3, Palbociclib has been approved by the US Food and Drug Administration for the treatment of hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative advanced or metastatic breast cancer in combination with an aromatase inhibitors or fulvestrant.⁷⁻¹⁰ Similar to hormone therapies, prolonged treatment with CDK4/6 inhibitors can lead to drug resistance in the clinic as well as in the lab. The mechanisms behind this resistance are varied; however, preliminary evidence suggests that it may be due to SASP, CDK6 hyperactivation, or the loss of Rb.¹¹

In contrast to the cell cycle mediating CDK4/6, CDK8 and its paralog, CDK19, are serine-threonine kinases that are involved in regulating transcription.¹² CDK8/19 along with Cyclin C (CCNC), MED12, and MED 13 comprise the transcriptional Mediator complex.¹² As part of the Mediator complex, CDK8/19 functions by phosphorylating the C-terminal domain, CTD of Pol II, inducing the elongation of transcription. CDK8/19 displays selective activity and phosphorylates the CTD of Pol II only once silent genes become activated by transcription-initiating signals, demonstrating the role of CDK8/19 in transcriptional reprogramming and the development of drug resistance.¹³ CDK8/19 has previously been identified as an amplified oncogene in colorectal cancers.¹⁴ Previous studies have also shown that high CDK8/19 expression is associated with shorter relapse-free survival and failure of systemic therapy in breast cancer.¹³ As a result, CDK8/19 has become a target for drug discovery in oncology.

Small molecule CDK8/19 inhibitors have been developed and have shown *in vitro* and *in vivo* efficacy in various cancers. These inhibitors include Senexin B, 15W, and its analog

SNX631, all of which display high selectivity for CDK8/19.¹⁵ Treatment with CDK8/19 inhibitors has been shown to suppress tumor growth and augment the effects of hormonal therapy in ER-positive breast cancer.¹⁶ Current data also lends to the theory that combination therapy with CDK8/19 inhibitors prevents drug resistance to CDK4/6 inhibitors via the suppression of ER-regulated transcription of mitogenic factors. Based on our preliminary data and supporting literature, we hypothesize that SNX631's prevention of Palbociclib resistance is driven by SASP.

Materials and Methods

Cell Culture and Reagents

All cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained at 37 °C with 5% CO₂. T47D cells were maintained in RPMI-1640 medium with 10% FBS, 1% penicillin-streptomycin, and 8 µg/mL insulin. PC3 cells were maintained in DMEM-high glucose medium with 10% FBS and 1% penicillin-streptomycin. MCF7 cells were maintained in DMEM-high glucose medium with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1 mM sodium pyruvate and 10 µg/mL insulin. Cells were subcultured 1-2 days per week.

Senescence-associated β -galactosidase (SA- β -gal) Assay

MCF7 cells were treated with 500 nM Palbociclib alone and in combination with 500 nM SNX631. Growth medium was removed from MCF7 cells. Cells were washed 3 times in phosphate-buffered saline (PBS) then covered in fix solution (PBS, 2% formaldehyde, 0.2% glutaraldehyde) for 10 minutes at room temperature. Cells were washed again 3 times in PBS. Staining solution [dH₂O, citric acid/Na₂PO₄, K₃Fe(CN)₆ (50mM), K₄Fe(CN)₆ (50mM), NaCl (5M), MgCl₂-6H₂O (1M), X-gal] was added to cells and incubated at 37°C for 48 hours. For

long-term storage, cells were washed in PBS, overlaid with 50% glycerol in H₂O, and stored at 4°C. Approximate percentage of SA-β-gal⁺ cells was assessed by brightfield light microscopy.

Sulforhodamine B (SRB) Assay

MCF7 cells were plated into 96-well plates at a density of 1.5×10^3 cells/well. PC3 cells were plated into 96-well plates at a density of 2.25×10^3 cells/well. T47D cells were plated into 96-well plates at a density of 750 cells/well. Cells were plated 24 hours prior to the addition of treatment. Cells were treated with Palbociclib (ranging from 15.625 nM to 4000 nM) alone and in combination with SNX631 (500 nM or 1000 nM). Growth medium and drug were replenished every 3-4 days. At 7 days, 13 days, and 20 days of treatment, medium was removed from one plate of each cell line. 200 μL of cold 10% trichloroacetic acid was added to each well and plates were incubated at 4°C for 10 minutes. 96-well plates were washed by submerging in water 5 times. Washed plates were then dried. 100 μL of 0.4% SRB in 1% acetic acid was added to each well and plates were incubated at room temperature for 10 minutes. Plates were submerged in 1% acetic acid 5 times, dried, and incubated overnight. 200 μL of 20 mM Tris, pH 10 (unbuffered) was added to each well. Plates were placed on a rotary shaker for 2 hours. Absorbance was measured at 570 nm on a microplate reader.

Results

To further evaluate the role of senescence in CDK4/6 inhibitor resistance, the ratio of senescent MCF7 cells to normal MCF7 cells treated with Palbociclib alone compared to Palbociclib in combination with SNX631 was quantified using SA-β-gal staining. Figure 2 shows that there was little difference in SA-β-gal staining between treatment groups; however, cells treated with Palbociclib alone were significantly larger than cells under combination treatment. This increase in size is a phenotype of senescence which was attenuated with the

addition of SNX631 to Palbociclib. SNX631's lack of effect on the Palbociclib-induced ratio of SA- β -gal⁺ cells is obfuscated by its significant effect on cell morphology. These results suggest a nuanced role for SASP in the development of resistance and the ability of CDK8/19 inhibitors to suppress resistance.

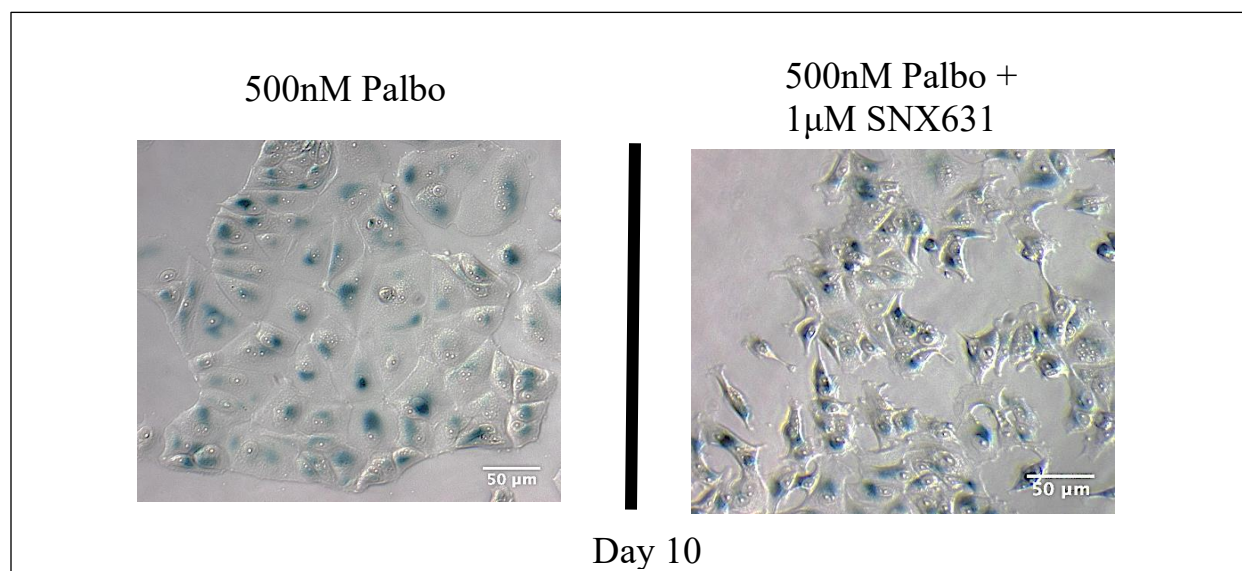


Figure 2. Representative SA- β -gal stains of 10 days of treatment with 500 nM Palbociclib (left) and 500 nM Palbociclib with 1000 nM SNX631 (right).

Cell proliferation under drug was assessed using SRB assays with 3 different cell lines. Figure 3 shows that the addition of SNX631 to T47D cells treated with Palbociclib displayed little effect on cell proliferation. Combinatorial treatment had slightly better effects in decreasing cell survival in PC3 cells (Figure 4). The most significant effects of treatment with Palbociclib and SNX631 were seen in MCF7 cells (Figure 5 and 6). As seen with the MCF7 cells, prolonged treatment with Palbociclib resulted in cells overcoming CDK4/6 inhibition and re-entering the cell cycle at approximately days 7-11. Of note, SNX631 did not display efficacy in cell death when used as monotherapy, which is consistent with its mechanism of action. However, the addition of SNX631 to cells treated with Palbociclib resulted in sustained growth arrest and the prevention of Palbociclib resistance.

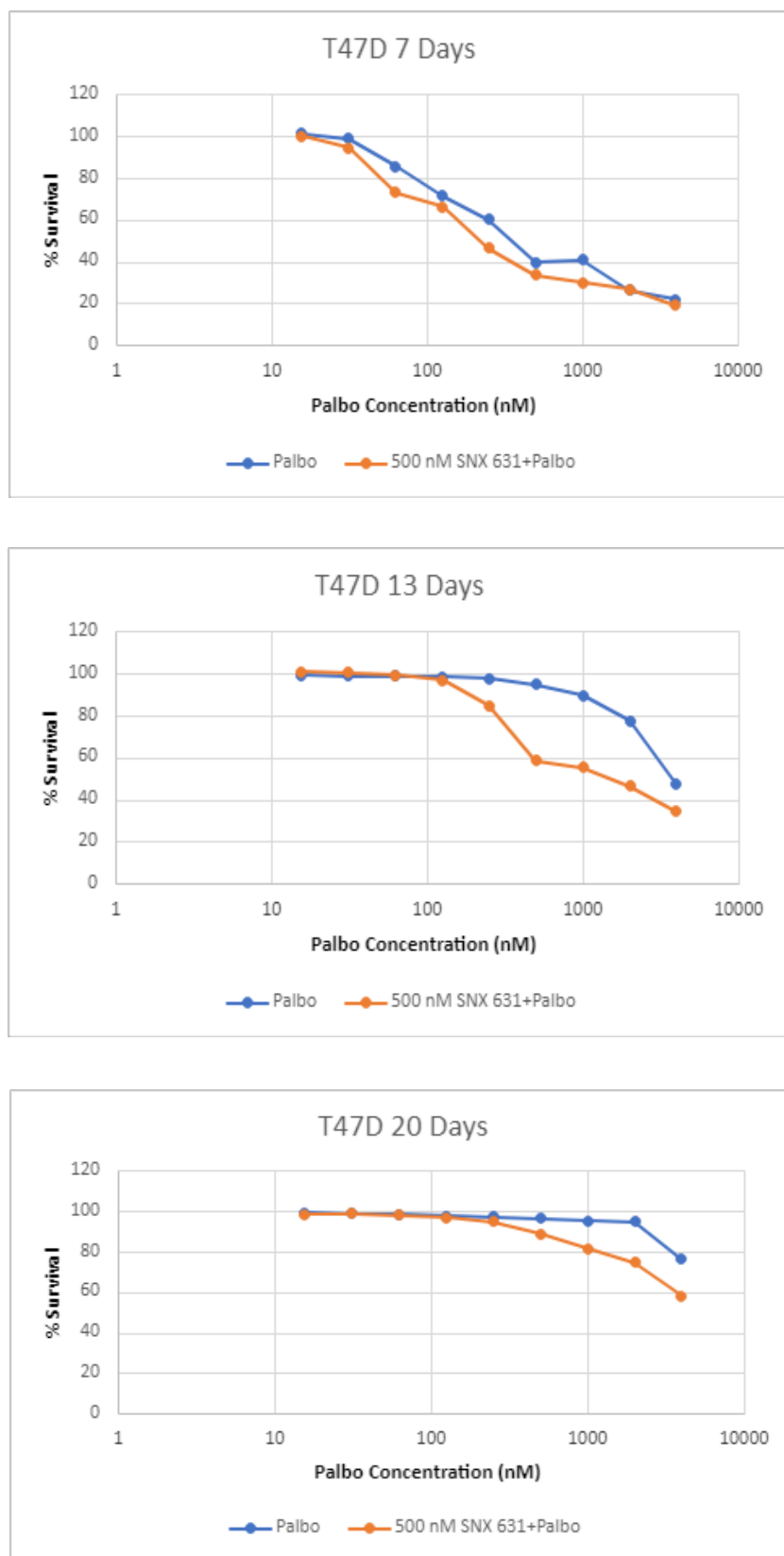


Figure 3. SRB viability analysis of T47D cells treated with 15.625 nM-4000 nM Palbociclib (Palbo) alone (blue line) or Palbociclib in combination with a constant concentration of 500 nM SNX631 (orange line) at days 7, 13, and 20.

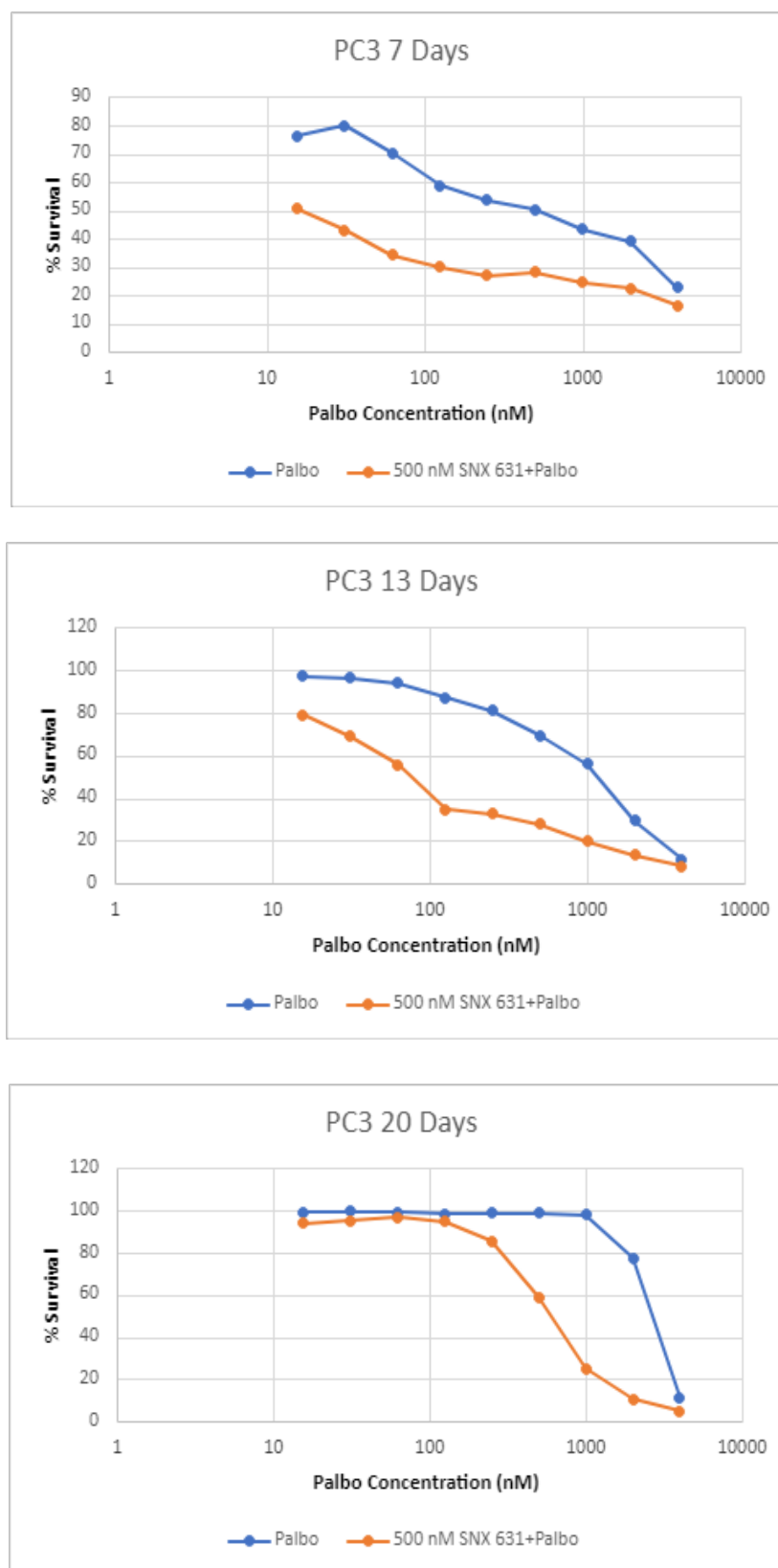


Figure 4. SRB viability analysis of PC3 cells treated with 15.625 nM-4000 nM Palbociclib (Palbo) alone (blue line) or Palbociclib in combination with a constant concentration of 500 nM SNX631 (orange line) at days 7, 13, and 20.

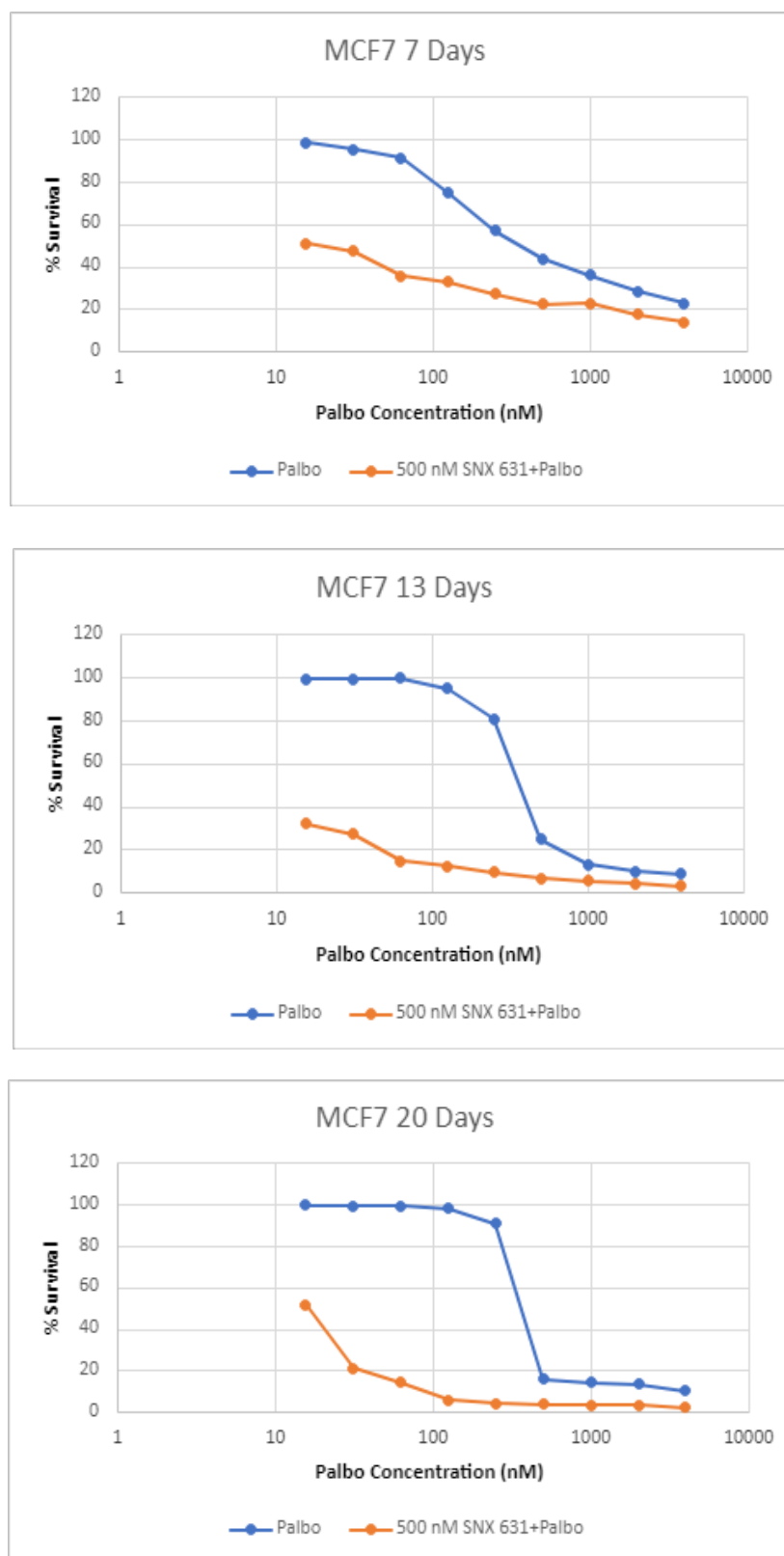


Figure 5. SRB viability analysis of MCF7 cells treated with 15.625 nM-4000 nM Palbociclib (Palbo) alone (blue line) or Palbociclib in combination with a constant concentration of 500 nM SNX631 (orange line) at days 7, 13, and 20.

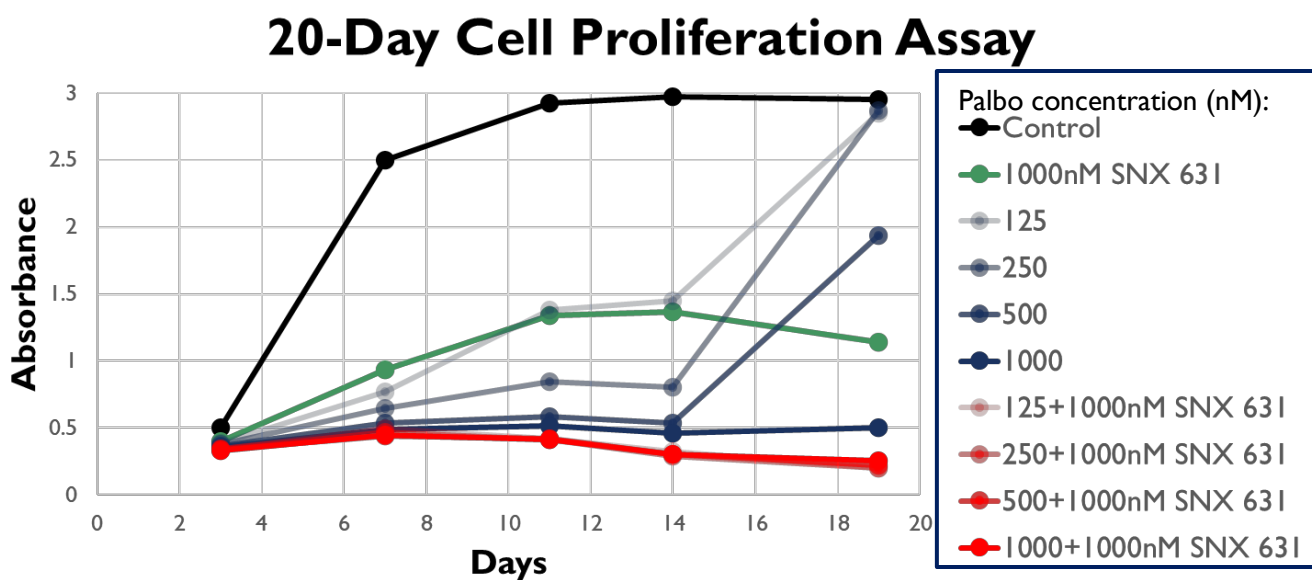


Figure 6. SRB viability analysis of MCF7 cells treated with 125nM-1000nM Palbociclib alone (blue lines), 1 μ M SNX631 alone (green line), or Palbociclib in combination with 1 μ M SNX631 (red lines) over 19 days. Media was changed at days 7, 11, and 14.

Discussion

Further studies are required to determine the exact mechanisms of the observed resistance with CDK4/6 inhibitors. Some of the observed results supported our hypothesis of SNX631's attenuation of SASP, such as the change in cell morphology, while others were contradictory, such as the lack of difference in SA- β -gal⁺ cells between treatment groups. The results of our senescent quantification experiments implicate that SASP and its associated transcription-regulating factors is a promising area of research to elucidate the mechanisms behind CDK4/6 and CDK8/19 inhibitor combinatorial treatment efficacy. Due to the indistinguishability in SA- β -gal staining between treatment groups, a different method of senescent quantification will need to be utilized in future experiments.

Further experiments are also needed to evaluate the lack of efficacy of Palbociclib and SNX631 in T47D cells despite their similarities to the MCF7 cells in which combination treatment was effective. PC3 cells were chosen to conduct *in vitro* experiments due to their

ability to grow well in immunocompromised mice and to be used in future *in vivo* experiments. Additionally, PC3 cells have previously demonstrated sensitivity to SNX631 alone. Because CDK4/6 and CDK8/19 inhibition showed success in PC3 cells *in vitro*, our lab has since conducted experiments in mice to evaluate the efficacy of Palbociclib and SNX631 *in vivo*. In these *in vivo* experiments, SNX631 monotherapy was shown to be effective in tumor eradication whereas the addition of Palbociclib to SNX631 showed minimal effects and displayed an additive rather than synergistic effect. MCF7 cells have consistently been the best model for demonstrating CDK4/6 and CDK8/19 inhibitor combination treatment efficacy. The results of our MCF7 cell viability experiments have laid the groundwork for further *in vivo* investigation of CDK4/6 and CDK8/19 inhibition. Given the eradication of tumor cells *in vitro* caused by Palbociclib and SNX631, examining this combination's effect on tumors *in vivo* will provide additional evidence of clinical benefit for ER-positive breast cancer patients. The cause behind Palbociclib resistance is multifaceted; therefore, as well as further investigation of SASP, ongoing Western Blotting experiments are evaluating the combination's effect on CDK6, Rb, and other cell cycle proteins. Future studies are also warranted to examine the effects of combinatorial treatment in additional cell lines and with other CDK4/6 inhibitors such as Ribociclib and Abemaciclib. In conclusion, the complementary effects of CDK4/6 and CDK8/19 inhibitors found in these *in vitro* experiments suggest that this combination may be a promising therapy for preventing drug resistance in ER-positive breast cancers.

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