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The Role of Mediator Kinases CDK8/19 in the Acquired Resistance to CDK4/6 Targeting Drugs in Breast Cancer

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Abstract

Breast cancer remains the highest cause of worldwide cancer-related mortality. Unfortunately, acquired therapeutic resistance occurs in the majority of cases of estrogen receptor-positive breast cancer. An effort to discover drugs which act synergistically with Palbociclib and prevent the development of resistance and tumor growth is essential to improving patient outcomes. Cyclin dependent kinases (CDK) 8 and 19 are Mediator complex proteins. The Mediator complex is an enzymatic module regulating transcription. The goal of the research is to elucidate the mechanism by which CDK8/19 inhibitor, SNX631, prevents acquired resistance to CDK4/6 inhibitor, Palbociclib. The present study aims to elucidate the role of the senescence-associated secretory phenotype (SASP) in Palbociclib resistance, and furthermore, to investigate if SASP is mitigated by a novel CDK8/19 inhibitor, SNX631. It was determined that SASP was suppressed by Palbociclib and therefore not a contributor to resistance. TGF-B, a member of SASP, was identified as a key modulator of both tumorigenesis and cell cycle arrest in breast cancer. The possibility that SNX631 suppresses TGF-B signaling to prevent Palbociclib resistance was explored. Overall, it was determined that SNX631 suppresses TGF-B/Smad3 signaling preventing changes to TGF-B signaling that occur throughout Palbociclib treatment. Lastly, alternative mechanisms of Palbociclib resistance are present, and they should be further explored in the context of SNX631.

Introduction

The Insufficiency of Breast Cancer Therapy

Worldwide, breast cancer remains the highest cause of cancer-related mortality for women. Estrogen receptor-positive (ER+)/ human epidermal growth factor receptor 2-negative (HER2-) breast cancer makes up 40% of breast cancers (13). This breast cancer type is commonly treated with endocrine therapy (ET), along with targeted chemotherapy. One such target of chemotherapies are proteins involved in the progression of the cell cycle, such as cyclin-dependent kinases (CDK), because it is through the cell cycle that all proliferation events occur. Palbociclib (IBRANCE™) is a highly selective CDK4/6 inhibitor which is currently used to treat ER+/HER2- breast cancers. However, 15% of patients exhibit *de novo* or early resistance to Palbociclib, and 50% develop resistance within 25 months (8). Acquired resistance to Palbociclib leads to poor patient outcomes, however, a better alternative has not yet been developed. A potential remedy for acquired resistance to Palbociclib is combined targeted therapy, where multiple drugs act synergistically to sustain sensitivity of the cancer to Palbociclib. However, most chemotherapy drugs require cell cycling to be toxic, where Palbociclib directly inhibits cell cycling, so synergistic drug combinations are hard to identify (1).

Palbociclib-induced Senescence

CDK4 and its paralog, CDK6, form a complex with cyclin D protein which phosphorylates retinoblastoma protein (Rb). The phosphorylation of Rb destabilizes the

association with E2 transcription factor-1 (E2F1), and E2F1 is subsequently released. The release of E2F1 facilitates the expression of genes that assist in the synthesis of DNA in order for the cell to progress from the G1 phase to the S phase of the cell cycle (1). The endogenous inhibitor of CDK4/6 is p15-INK^{4B}, which is therefore a critical tumor suppressor. Much like p15-INK^{4B}, CDK4/6 inhibition by Palbociclib causes the cell cycle to be halted in the G1 phase, preventing cell division, and slowing the growth of cancer (1).

Senescence is defined as the irreversible arrest of the cell cycle, so senescent cells do not divide, however they are still metabolically active and secrete a variety of factors (13). Senescent cells exhibit other phenotypic alterations such as increases of cell size, vacuolization, cell spreading, cellular complexity, and senescence-associated β-galactosidase activity (3). The senescence-associated secretory phenotype (SASP) is a label for the cytokine secretions that occur by the senescent cancer cells. Secreted proteins such as interleukins (IL), tumor necrosis factors (TNF), and transforming growth factors (TGF) exhibit paracrine activity and are paradoxically able to modify the tissue environment in a way that promotes proliferation and enhances tumorigenesis (13). Tumor-supporting behaviors of cytokines such as angiogenesis, drug resistance, proliferation, and apoptosis resistance can occur in response to stimuli such as oncogenic activity or cellular senescence (3). Reviewers found that in preclinical and clinical breast cancer models, exposure to the IL-17 family of cytokines had pro-cancer effects for all except IL-17E, which had mixed results (4).

Alternatively, cytokines can play a role in the suppression of cancer. Researchers found that Th1 therapy in combination with dual tyrosine kinase inhibitor, lapatinib, sensitizes HER+ breast cancer cells to lapatinib (12). Another study showed that TGF-B engages RAS-induced senescence to prevent malignant transformation in human mammary epithelial cells (3).

Consistently, cells treated with Palbociclib exhibit phenotypes of senescence within days (see Figure S1). Since senescent cells can change the tumor microenvironment to support growth via SASP, and breast cancer cells exhibit phenotypes of senescence in response to treatment with Palbociclib, it was hypothesized that SASP is a tool that ER+ breast cancer uses to confer resistance to Palbociclib.

CDK8/19 Inhibition to Prevent Resistance

Another cyclin-dependent kinase, CDK8 and its paralog, CDK19, acts as a mediator of gene expression by regulating transcription. CDK8/19 is a member of the submodule of the Mediator Complex, which associates numerous activator, repressor, and chromatin remodeling proteins, with RNA polymerase II in preparation for transcription (6). CDK8/19 is involved in the expression of genes aiding in glucose uptake, stemness, and tumor invasiveness (9). High levels of CDK8 promote expression of c-Myc and cyclin D, proteins involved in cell division. Additionally, CDK8 is affected by estrogen receptor signaling for transcription regulation (9). On the other hand, CDK8/19 inhibition lowers the levels of glucose transporters and glucose uptake to reduce cellular proliferation (8). Inhibition of CDK8/19 to treat ER+/HER2- breast cancer is promising, as it has been shown to reduce invasiveness, metastasis, and cell proliferation. In other cancer types, CDK8/19 inhibition can increase stem cell differentiation (9). In organismal systems, targeting CDK8 can increase differentiation of cancerous cells and reduce stem cell character, as well as improve NK cell cytotoxicity (9). CDK8/19 inhibition fails to induce apoptosis in ER+ breast cancer, but it suppresses growth through synergy with other anticancer compounds (9).

Previous data shows that a novel CDK8/19 inhibitor, SNX631, when used in combination with Palbociclib, prevents acquired resistance to Palbociclib by ER+ breast cancer cells *in vitro* (see Figure S2). Microscopy also shows a reduction in senescent phenotypes in MCF7 treated with the combination (see Figure S1). It is not yet understood how SNX631 works to prevent resistance to Palbociclib in breast cancer. Since CDK8/19 is closely related to the expression of cytokines and cell proliferation, is regulated by estrogen receptor, and has been shown to prevent acquired resistance to Palbociclib, it was hypothesized that CDK8/19 inhibition reduces the tumor-promoting activity of SASP to prevent the acquisition of Palbociclib resistance in ER+ breast cancer. A proposed mechanism is shown in Figure 1. Therefore, this research aims to

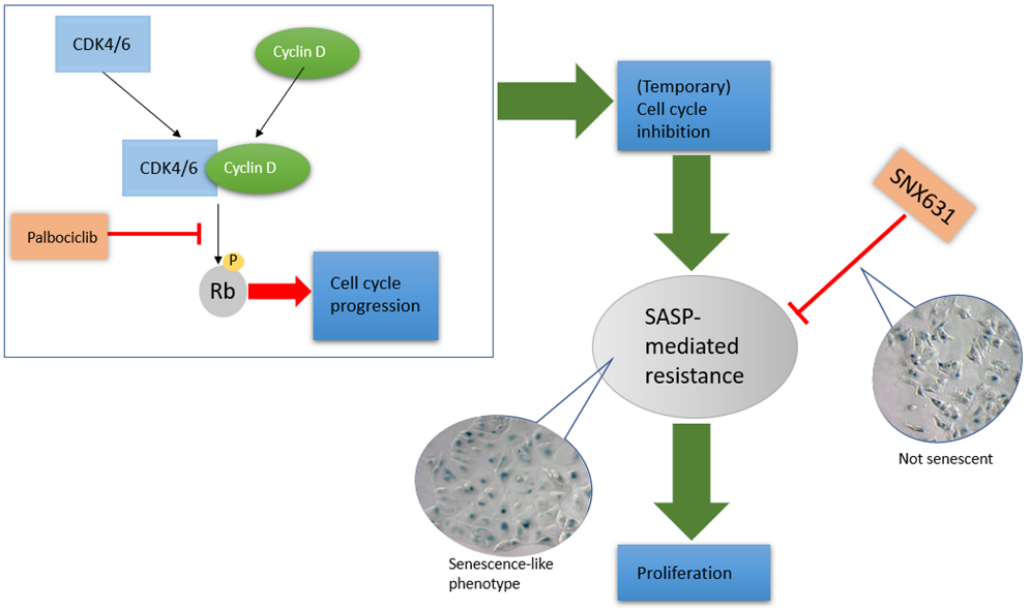

Figure 1. The proposed mechanism of how resistance to Palbociclib occurs. The blue-outlined box shows the proteins involved in the progression of the G1 phase of the cell cycle. The callout bubbles are microscopic images of MCF7 cells to demonstrate senescent morphology.

answer the question, "Does mediation of SASP by SNX631 prevent the acquisition of Palbociclib resistance in ER+ breast cancer?"

The Role of TGF-B in Cancer

Transforming growth factor-beta (TGF-B) is a super-family of crucial signaling molecules with numerous functions, some of which stimulate tumorigenesis, some which suppress it (2). The effect of TGF-B changes throughout tumor progression and depends on the

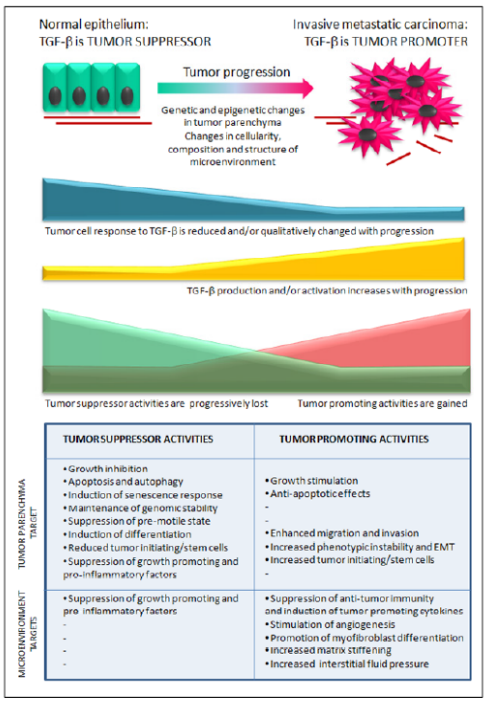

Figure 2. A general timeline of changes that occur in carcinomas regarding TGF-B activity and responses. Source: Ben-Baruch, A. (2012). The Inflammatory Milieu of Tumors: Cytokines and Chemokines That Affect Tumor Growth and Metastasis. Bentham Science Publishers.

type of tumor and its microenvironment. TGF-B production and activation tends to increase with tumor progression. Tumor-promoting activities such as growth stimulation, enhanced migration, and increased phenotypic instability occur (2). This trend is summarized in Figure 2.

TGF-B molecules bind to TGF-B receptor II (TBRII) and recruits TBRI. TBRI and TBRII are serine kinases which phosphorylate Smad 2/3. A subsequent signal cascade involving the Smad super-family regulates transcription (2). There are multiple signaling pathways that TGF-B is involved in, but the canonical Smad-signaling has primarily tumor-promoting outputs (2) (See Figure S4 for TGF-B signaling pathway).

In breast carcinomas, there is a high correlation between TGF-B1 expression and disease progression regardless of other variables, however compromised TGF-B pathways do not lead to tumorigenesis alone, rather they require oncogenesis to have occurred (2). And in some cases, overexpression of TGF-B signaling is associated with drug resistance, and chemotherapy has been shown to increase circulating TGF-B (2).

In normal human mammary epithelial cells (HMEC), TGF-B inhibits growth by suppressing C-MYC, cyclin D, cdc25, and upregulating CDK inhibitors such has p15-INK^{4B} (3). TGF-B leads to G1 arrest and inactive Rb. In response to oncogene activation in HMECs, TGF-B induces senescence as a protective anti-tumor mechanism (3). Additionally, loss of TBRII in HMECs resulted in tumor progression through an overexpression of chemokines (2). This information indicates that TGF-B has tumor-suppressing activity in noncancerous cells but exhibits predominantly tumor-promoting activity in cancerous cells. TGF-B expression and response requires cellular context and has complex chemotherapy dose responses (2).

CDK8 is a coregulator of several transcription factors such as TGF-B/Smad and NF-κB. CDK8 was shown to contribute to invasiveness of colon cancer through downregulation of matrix metalloproteinase (MMP inhibitor) TIMP3 via TGFB/Smad driven expression of a TIMP3-targeting microRNA, miR181b (6). CDK8 can interact independently with TGF-B signaling pathways. For example, CDK8 can phosphorylate the linker region of Smad proteins, enhancing transcriptional activity (2).

Since TGF-B has been shown to have tumor-supporting activity in breast cancer models, and is affected by CDK8/19 action, I hypothesized that an increase of TGF-B paracrine activity contributes to Palbociclib resistance in ER+ breast cancer cells, and that CDK8/19 inhibition by SNX631 contributes to the prevention of resistance by attenuating TGF-B activity. This study

aims to answer the question, “What is the role of TGF-B in acquired resistance to Palbociclib by ER+ breast cancer, and how does SNX631 affect it?”

The overall goal of the study was to elucidate the mechanism by which SNX631 can prevent acquired therapeutic resistance to Palbociclib. A better understanding of the mechanism of action of SNX631 can facilitate the use of the more effective drug combinations to treat cancer in clinical studies, improving outcomes for breast cancer patients.

Materials and Methods

Breast cancer cell culture.

A line of MCF7 estrogen receptor-positive breast cancer cells was maintained at the University of South Carolina for the following experiments. Cultured MCF7 was incubated at 37 °C with 5% CO_2 and cultured in DMEM (Dulbecco's Modification of Eagle's Medium with 4.5 g/L glucose & L-glutamine; Corning, Corning, NY) supplemented with 10% FBS (Fetal bovine growth serum (HyClone BGS: GE Healthcare life Sciences, HyClone Laboratories, Logan, UT)), 1% sodium pyruvate (Sigma Aldrich, St. Louis, MO), 1% penicillin/streptomycin/L-glutamate (HyClone Laboratories, Logan, UT), and 10µg/mL insulin (Sigma Aldrich, St. Louis, MO). For experiments, cells were seeded at a concentration of $1.5x10^3$ cells per well on a 96-well tissue culture dish. Outside wells contained 200 µL phosphate-buffered saline (PBS). Cells were seeded at a concentration of $3.85x10^5$ cells per well on a 12-well tissue culture dish. Seeded cells were incubated for 24 h before being treated. Drugged media was replaced every 5 days unless mentioned otherwise.

Conditioned media production and collection.

Conditioned media was produced by culturing MCF7 in growth medium with 500 nM Palbociclib. Four 96-well plates of cells were cultured for 3 days under 500 nM Palbociclib and 4-96 well plates of cells were cultured for 7 days, producing '3-day conditioned media' and '7-day conditioned media', respectively. One hundred µL of 3- and 7- day conditioned media was carefully removed from each well and dispensed directly into the corresponding well of a plate with freshly seeded cells.

Conditioned media concentration pilot.

MCF7 cells were cultured in a p150 cell culture dish with 500 nM Palbociclib for 3 days to produce '3-day conditioned media'. 13.5 mL of 3-day conditioned-media was collected and centrifuged for 5 mins at 200 rpm. The supernatant was collected, and the pellet was discarded. 13 mL of conditioned media was diluted with fresh growth media at 10% intervals to produce solutions of 0%-90% conditioned media. Two mL solutions of 500 nM Palbociclib in 0%-90% conditioned media were also prepared. 200 µL of conditioned media solution was placed onto MCF7 cells in 96-well plates as shown in Figure S4.

The plates were stained with Sulforhodamine-B (SRB) after 7 days according to the manufacturer's instructions (see detailed methods). Absorbance of SRB solution represents cell viability which was displayed in a line graph vs. % conditioned media. Error was represented by the standard deviation of 6 replicates. The trend was used to determine the highest percentage of conditioned media which did not significantly affect cell viability to use in further experiments.

Conditioned media with drug combination.

Twelve 96-well plates of freshly seeded MCF7 cells were prepared, four with 50% 3-day conditioned media, four with 50% 7-day conditioned media, and four with 100% fresh media. All the 96-well plates were treated with Palbociclib or a combination of Palbociclib and SNX631 as shown in Figure S4 and described in the Detailed Methods section. One plate from each group of media types (3-day, 7-day, fresh) was stained with SRB every 5 days, and the remaining plates were replaced with fresh drugged media. Absorbance data for each treatment group was displayed in bar graphs. The error was determined by the standard deviation of replicates.

TGF-B inhibitor pilot.

MCF7 cells were seeded in two 96- well plates and treated with a range of 312.5 nM-40 μM TGF-B receptor I (TBRI) inhibitor (sb431542, Tocris Bioscience, Bristol, UK) (1:2dilution) against a 0.1% DMSO vehicle. One plate was stained with SRB after 3.5 days of treatment and the other was stained with SRB after 7 days. Average absorbance was plotted versus TBRI inhibitor concentration to determine the highest concentration of TBRI inhibitor which did not significantly affect cell viability.

TGF-B inhibitor and Palbociclib combination.

MCF7 cells in a 96-well plate were treated with a range of 125-1000 nM Palbociclib. One half of the replicates (3 wells in each row) were supplemented with 0.1% DMSO vehicle, the other half were treated with 2.5 μM TGF-B inhibitor. Every 5 days, one plate was stained with SRB, and the rest received fresh drugged media. The absorbance of the wells was read and plotted vs. Palbociclib concentration on a logarithmic scale. Error was represented by the standard deviation of the replicates.

Microscopy.

A representative picture of control (50% conditioned media), 500 nM Palbociclib + 50% conditioned media, 125 nM Palbociclib + 50% conditioned media, and 500 nM Palbociclib + 500 nM SNX631 + 50% conditioned media for each media type was taken at day 5. Images were taken using the Zeiss Axio-Imager M1 Upright Phase Contract Motorized Trinocular Microscope in Phase 0 with an Olympus 4x objective and a Zeiss 10x objective. Images were taken again on day 12.

Conditioned media with drug release.

MCF7 cells were seeded onto four 12-well cell culture plates 24 h prior to treatment. Two plates received only fresh media, and two received 50% 3-day conditioned media. One well contained 100% fresh media (negative control); 2 wells contained only 50% 3-day conditioned media. Wells in triplicate were treated with 125 nM Palbociclib, 500 nM SNX631, or 125 nM Palbociclib + 500 nM SNX631 (see Figure S6). Drugged media was replaced on day 5 for all the plates. On day 10, 2 of the 4 plates had drugged media replaced with fresh drugged media and 2 of the 4 plates had drugged media replaced with fresh undrugged media. On Day 16, the cells were stained with crystal violet according to the manufacturer's instructions. Images of the stained 12-well plates were taken with the BIO-RAD Chemi-Doc Imager (Hercules, CA) fitted with the White Sample Tray, set to Coomassie Blue with automatic exposure time.

RNA sequencing.

MCF7 cells were seeded in p100 tissue culture dishes at $1x10^6$ cells per well. In replicates of 3, the cells were treated with 0.1% DMSO, 500 nM SNX631, 500 nM Palbociclib, or 500 nM Palbociclib + 500 nM SNX631. Ten additional dishes were treated with 500 nM Palbociclib for

21 days, and 5 dishes were not treated (control). After 3 and 21 days of treatment, the cells were trypsinized and collected. Total miRNA was purified with the miRNAeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. One µg of total RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quantabio, Beverly, MA). RNA quality was evaluated on RNA-1000 chip using Bioanalyzer (Agilent, Santa Clara, CA, USA). Illumina sequencing was performed by Medgenome (Foster City, CA). Library preparation, post-processing of the raw data and data analysis were performed by the USC CTT COBRE Functional genomics Core.

Gene Set Enrichment Analysis was conducted using the phenotypes DMSO, Palbociclib, SNX631, and COMBO. The gene sets used in the analysis were "Reactome Senescence Associated Secretory Phenotype (SASP), Wikipathways TGF-B Receptor Signaling" and "Hallmark TGFB Signaling". Significance was determined using False Discovery Rate <25% and p-value <5%.

Results

Conditioned media replicates the effect of a higher Palbociclib dosage.

A range of conditioned media solutions were tested to find an appropriate percentage of conditioned media that would not significantly affect cell viability. A treatment group with 500 nM Palbociclib and conditioned media was also tested preliminarily. Absorbance data shows that conditioned media does not exhibit a clear trend of increasing or decreasing cell viability (Figure 3A). Additionally, the standard deviation of absorbance between the 6 replicates was unusually large. The large variability between replicates and lack of a clear trend shows that conditioned

media has an unpredictable effect on MCF7 cells. For cells treated with 500 nM Palbociclib and conditioned media, an increased percentage of conditioned media was negatively correlated with cell viability after 7 days. The standard deviation of absorbance was also slightly smaller, indicating less variability among Palbociclib-treated MCF7. This preliminary data also suggests that a combination of conditioned media and Palbociclib does significantly decrease cell viability after 7 days, which is not consistent with the expectation that tumor-supporting cytokines in conditioned media would improve cell viability. For future experiments, 50% conditioned media was chosen because it did not exhibit a marked decrease in cell viability compared to the fresh media control group but was also high enough to cause an effect when combined with drug.

An experiment that combined varying concentrations of Palbociclib, SNX631, and 3- and 7- day conditioned media was conducted to observe the effect of the conditioned media on Palbociclib resistance development. The decrease in cell viability from the 3-day conditioned media + 500 nm Palbociclib treatment group is roughly double that of the 3-day conditioned media control after 9 days (Figure 3B). It has been shown that cells maintain an equilibrium concentration of Palbociclib between the intracellular and extracellular space (7). This data suggests that the conditioned media contains residual Palbociclib, and that adding 500 nM Palbociclib to conditioned media simply increases the dosage of Palbociclib, further inhibiting cell growth. The decrease in cell viability was not due to nutrient depletion since the 3- and 7-day old 'control' media reached the same absorbance as fresh media. Among cells with conditioned media, cell viability still increased throughout 15 days, indicating resistance to

Palbociclib. However, it cannot be said that the conditioned media provided a 'boost' to resistance, because viability was lower compared to cells with fresh media.

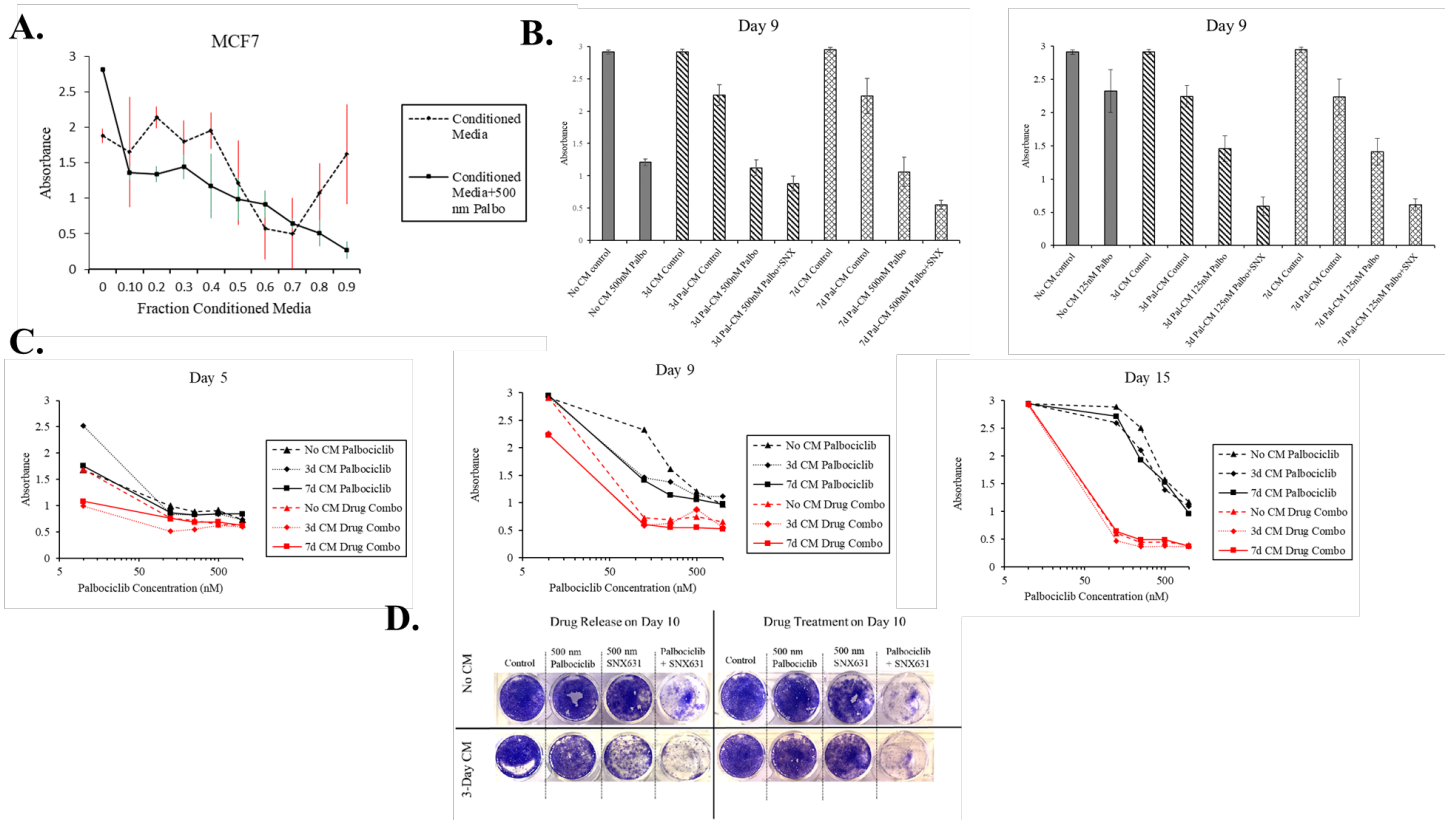

Figure 3. (A) A graph of absorbance vs. fraction conditioned media of MCF7 as the result of a 7-day cell viability assay. (B) Absorbance for treatment groups combining conditioned media, 125 nM Palbociclib, 500 nM Palbociclib, and 500 nM SNX631 after 9 days of treatment. (C) A graph of absorbance vs. Palbociclib concentration with varying media types, with or without SNX631 after 5, 10, and 15 days of treatment. (D) Images of MCF7 after 16 days treatment with or without conditioned media and with or without drug release at day 10, after crystal violet stain.

Additionally, there is no significant difference in cell viability between cells treated with Palbociclib or the combination after 15 days. This suggests that conditioned media affects untreated cells differently than treated cells. There may be a compensatory effect occurring between the additional Palbociclib dosage and tumor-promoting cytokines in the conditioned media, however an experiment to prove this is necessary.

The combination of SNX631 and Palbociclib was able to prevent Palbociclib resistance for all media types.

In both 3-day and 7-day conditioned media treatment groups, the absorbance of cells treated with both 500 nM Palbociclib and 500 nM SNX631 remained low after 15 days, indicating that the combinatory inhibition of CDK4/6 and CDK8/19 prevented the development of resistance to Palbociclib (Figure 3). This has been shown to occur in cells with fresh media as well (See Figure S1). Since this trend is observed regardless of media type, the ability of SNX631 to prevent resistance is not dependent on cytokine secretions by senescent cells.

RNA sequencing shows that the expression of genes involved in SASP is low in Palbociclib vs. Control MCF7 after 3 and 21 days of treatment.

Sequencing of mRNA transcripts from MCF7 cells treated with Palbociclib or DMSO for 3 and 21 days was conducted to compare how gene expression changes over time in response to Palbociclib. Gene Set Enrichment Analysis (GSEA) was used to analyze a gene set for the senescence-associated secretory phenotype. GSEA shows that the genes were significantly under-expressed in Palbociclib vs. DMSO after 3 and 21 days of treatment (Figure 4). In fact, the enrichment score (ES) of Palbociclib vs. Control after 21 days (ES=-0.55) was lower than Palbociclib vs. DMSO (ES=-0.47) after 3 days. The combination of SNX631 and Palbociclib was shown to slightly further decrease expression of SASP genes after 3 days vs. Palbociclib alone (ES=-0.28), however this result was not significant.

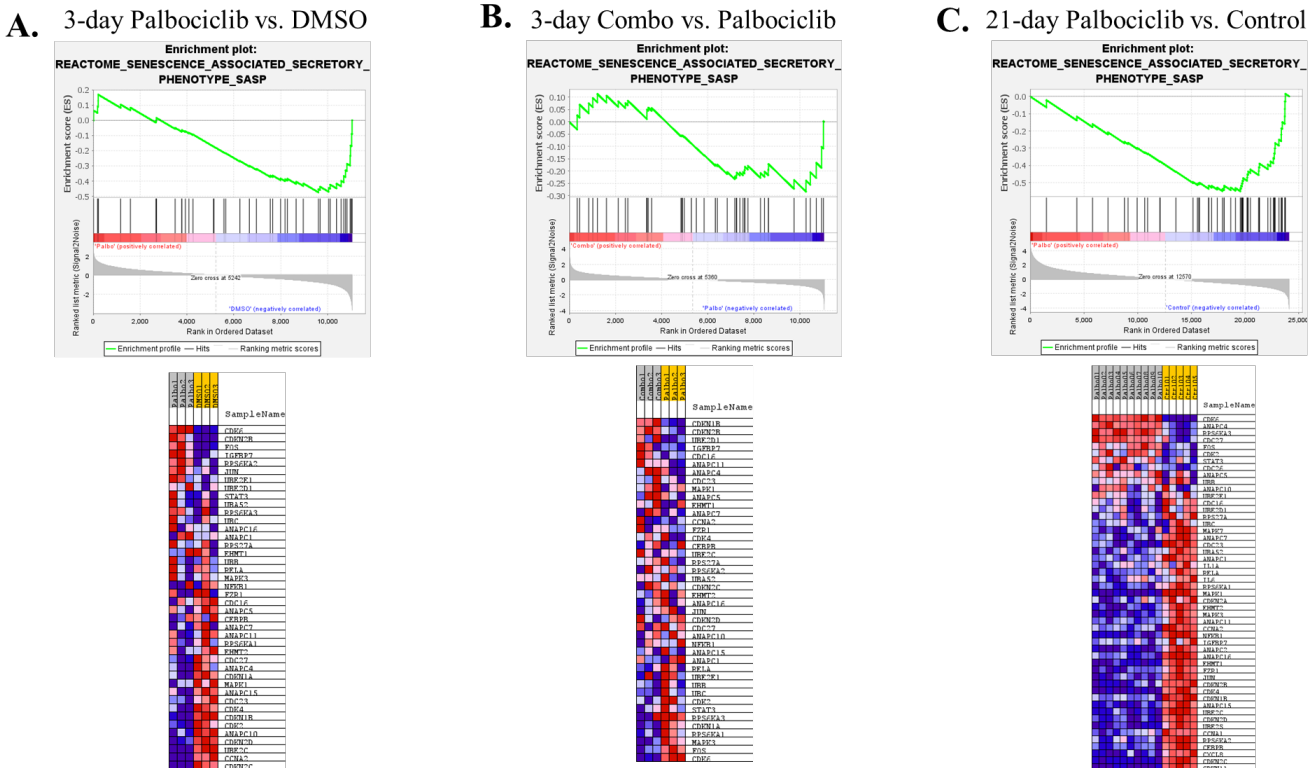

Figure 4.GSEA Enrichment Score Plots and Heat maps of the 25 most affected genes in the SASP gene set for MCF7 comparing (A) 3 days Palbociclib vs. control. (B) 3-day combination vs 3-day Palbociclib and (C) 21-day Palbociclib vs. Control.

A combination of CDK4/6 inhibition and TBRI inhibition produced a slight increase in MCF7 cell viability.

A TBRI inhibitor was combined with Palbociclib to determine if TBRI inhibition by SNX631 was an avenue for prevention of Palbociclib resistance. Alone, TBRI inhibition did not have a significant effect on MCF7 cell viability below 5 µM (Figure 5A). 2.5 µM concentration of TBRI inhibitor was chosen for future experiments. TBRI inhibition in combination with Palbociclib improved viability of MCF7 cells over 20 days compared to those treated with Palbociclib alone (Figure 5B), which conflicts with the expectation that the combination would decrease viability vs. Palbociclib alone. There is also a lack of sensitivity to Palbociclib demonstrated by continued cell growth over 10 days, which suggests that resistance occurs with or without TBRI activity. This does not replicate the synergistic negative effect exhibited by SNX631 and is not consistent with the hypothesis that TBRI was the avenue through which SNX631 prevents resistance.

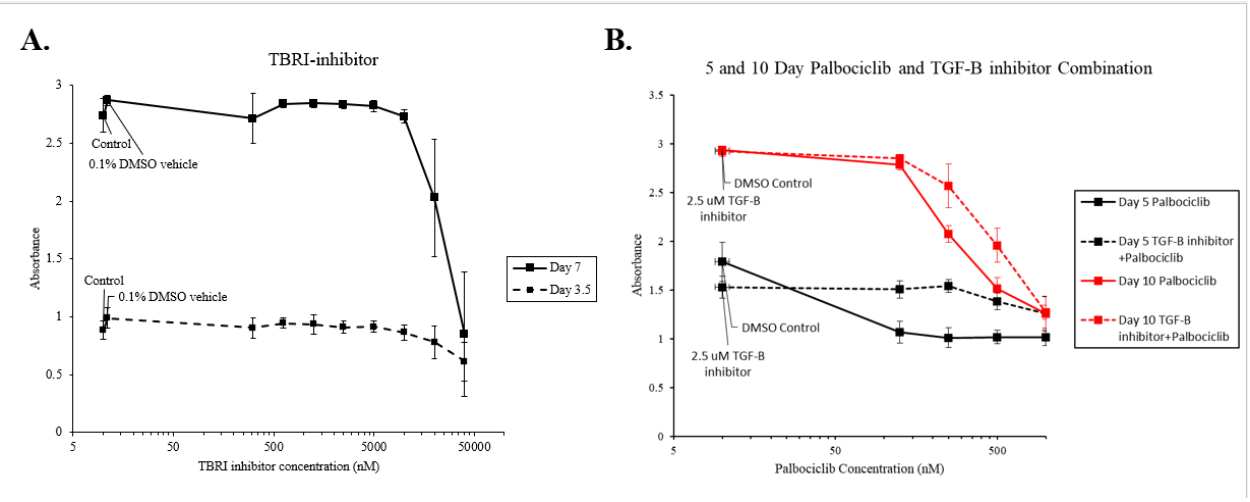

Figure 5. (A) Absorbance vs TBRI inhibitor concentration after 3.5 and 7 days of treatment. Alone, TBRI inhibitor does not have a significant effect on cell viability below 5 µM. (B) Absorbance vs Palbociclib concentration with and without TBRI inhibitor after 5 and 10 days of treatment.

Among genes that are upregulated in response to TGF-B1, Palbociclib-treated MCF7 saw an alteration of expression over time.

GSEA of a set of genes that are expected to be upregulated in response to TGF-B1 stimulation shows that those genes are significantly upregulated in Palbociclib-treated MCF7 vs. DMSO after 3 days (ES=0.5). However, after 21 days of Palbociclib treatment, the same genes are significantly downregulated (ES=-0.29) (Figure 6). This is not consistent with the fact that TGFB1 transcripts are upregulated vs control cells at both 3 (fc= 1.36) and 21 days (fc=1.14) of Palbociclib treatment (Figure 7), suggesting that sensitivity to TGF-B1 is reduced as resistance develops.

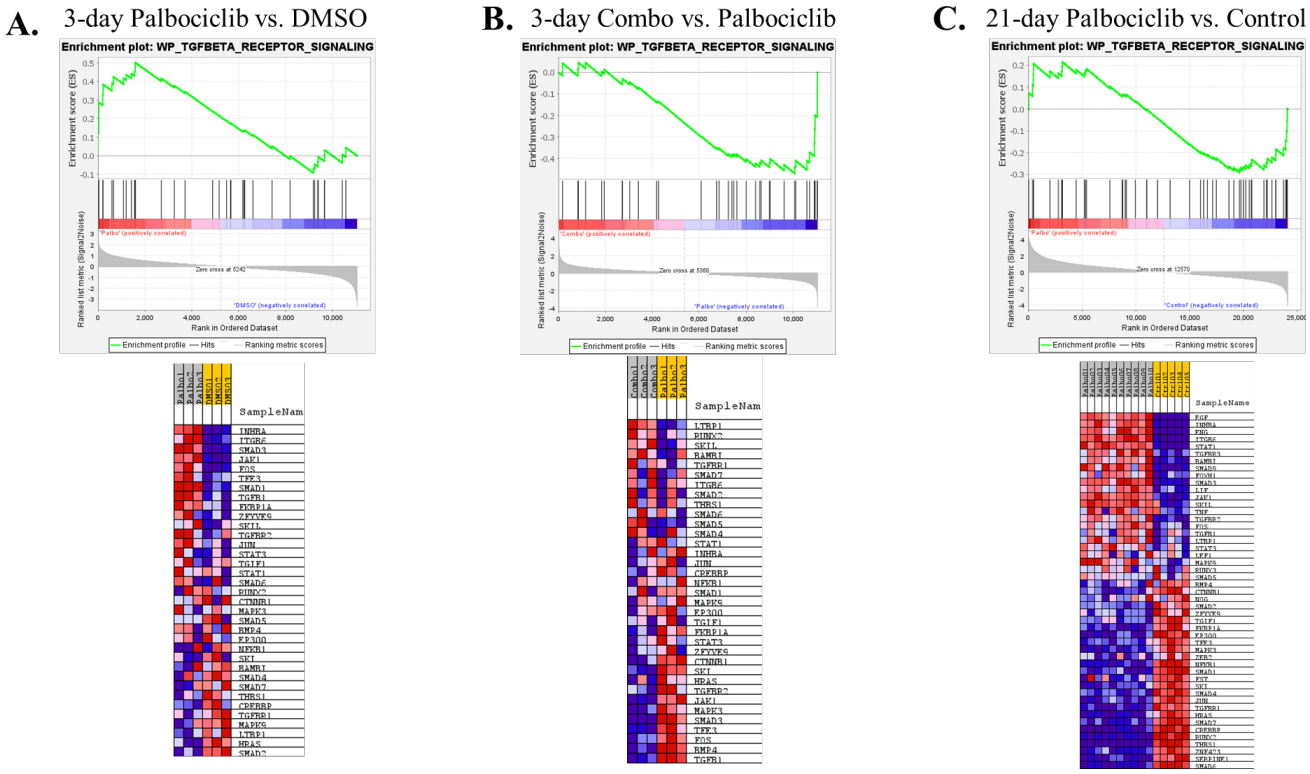

Figure 6. GSEA enrichment plots and heat maps comparing expression of a set of genes involved in TGF-B1 signaling between treatment groups (A) 3-day Palbociclib vs. DMSO. (B) 3-day Palbociclib + SNX631 vs. Palbociclib (C) 21-day Palbociclib vs. Control.

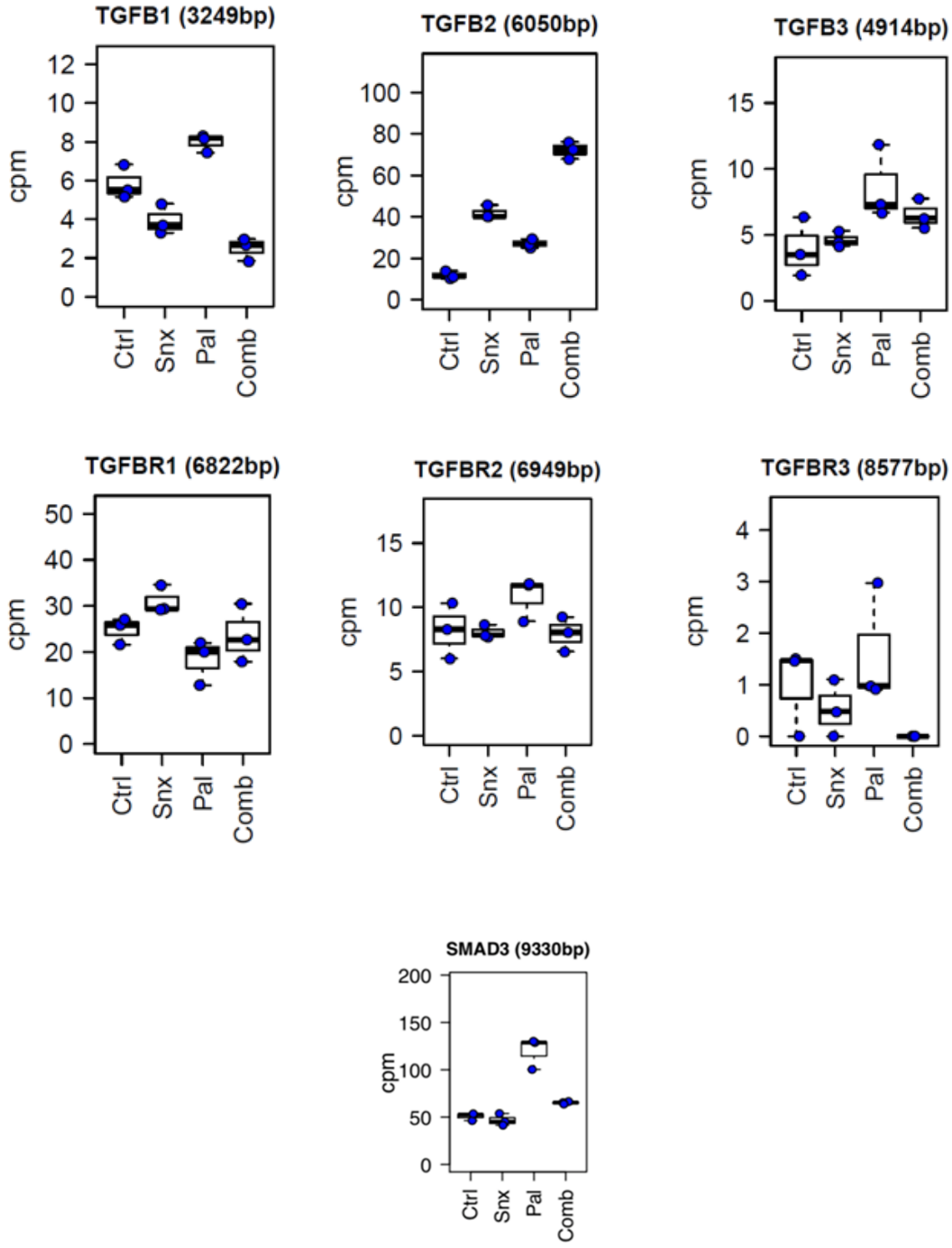

Figure 7. Box and Whisker plots showing counts per million of mRNA transcripts of TGFB, TGF-B receptor genes, and Smad3 for each treatment group after 3 days of treatment.

The combination of Palbociclib and SNX631 causes significant downregulation of TGF-B receptor signaling vs Palbociclib alone.

GSEA shows that the combination treatment significantly downregulates TGF-B1 associated genes compared to Palbociclib alone after 3 days (ES=-0.47). The 3-day combination treatment showed the lowest number of TGFB1 transcripts (2.5 cpm), compared to 3-day Palbociclib (7.9 cpm) 21-day Palbociclib (15.1 cpm) and DMSO (5.3 cpm). This result suggests that SNX631 suppresses TGF-B1 to prevent the development of resistance to Palbociclib.

Among genes associated with TBRI signaling, the combination significantly downregulated these genes vs. Palbociclib alone after 3 days. Those genes were not significantly enriched vs. DMSO. This suggests that the combination treatment suppresses the changes in TBRI signaling that occur with Palbociclib treatment.

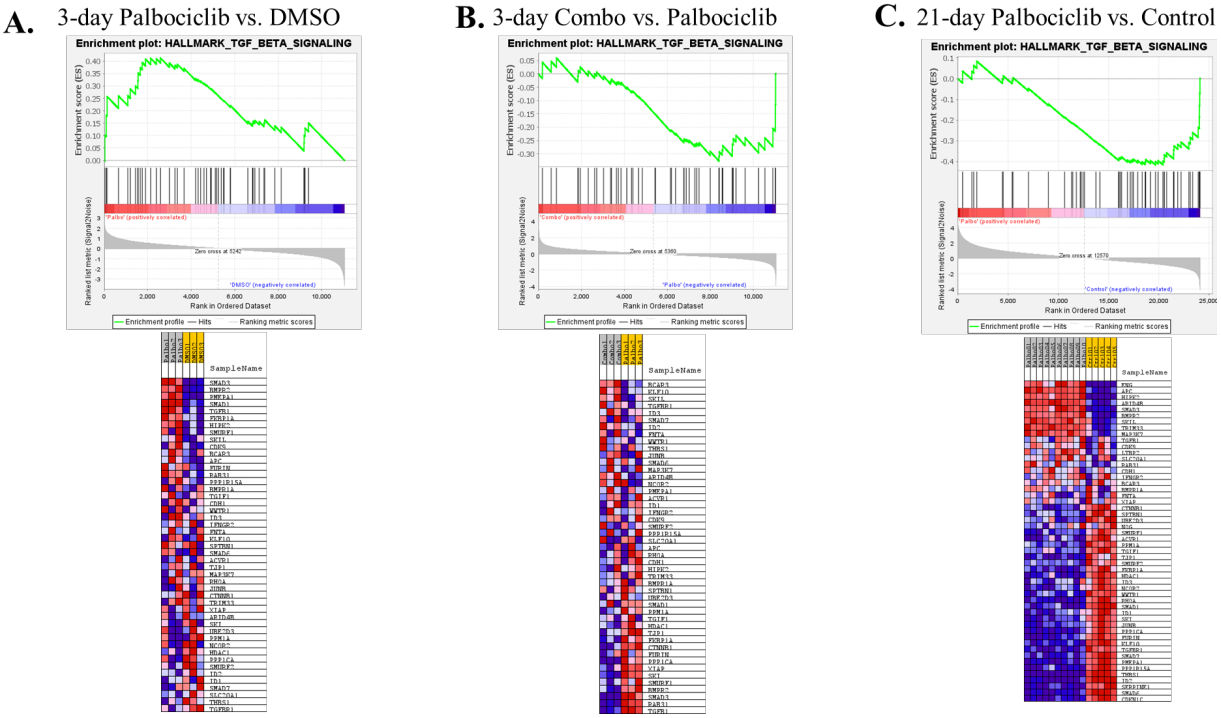

Figure 8. GSEA enrichment plots and heat maps for a set of genes that is involved in TGF-B receptor signaling. (A) 3-day Palbociclib vs. DMSO. (B) 3-day Palbociclib + SNX631 vs. Palbociclib (C) 21-day Palbociclib vs. Control.

Other Palbociclib resistance mechanisms are prevalent according to RNA sequencing.

There are various mechanisms of resistance to Palbociclib established by the literature. For example, the activity of CDK6 increases and facilitates phosphorylation of Rb in absence of CDK4 activity. RNA sequencing shows that transcripts of CDK6 are significantly upregulated in Palbociclib treated cells and less so in combination-treated cells. Additionally, CDK2/cyclin E phosphorylates Rb after the initial phosphorylation by CDK4/cyclin D. Palbociclib resistance can be conferred by an increase in CDK2/cyclin E activity, where Rb can be phosphorylated without CDK4/6. RNA sequencing shows that CDK2 expression increases after 21 days of Palbociclib treatment but remains low for combination-treated cells (Table I). Current research shows that combined CDK2 and CDK4/6 inhibition prevents resistance to Palbociclib in breast cancer cells by enhancing C-MYC modulated senescence (10). C-MYC expression is high in combination-treated cells, suggesting that C-MYC modulated senescence is maintained (Table I).

Smad3 suppression has been cited as a possible mechanism for Palbociclib resistance (11). Inhibition of Smad3 activity leads to loss of cell cycle arrest in cancer by overexpression of cyclin D (11). Consistent with this hypothesis, the gene encoding Smad3 is significantly downregulated in the combination vs. Palbociclib after 3 days. Also, CCNDI expression (encoding cyclin D) is high in all treatment groups (Table I). Together, CDK8/19 inhibition may prevent resistance by suppressing the TGF-B/Smad signaling cascade.

P53 is tumor suppressor protein involved in the regulation of the cell cycle. P53 loss-of-function mutations have been associated with the resistance of many chemotherapies by various cancers. RNA sequencing shows a significant increase in expression of TP53 (p53 encoding-gene) in combination-treated MCF7 cells, which may contribute to sustained cell cycle arrest (Table I).

Fold-change vs. Control					
GENE	**PROTEIN PRODUCT**	**Treatment Group**			
		3-day Palbociclib	**21-day Palbociclib**	**3-day SNX631**	**3-day Pal +SNX**
CDK2	CDK2	0.53	1.14	0.78	0.45
CCNA2	cyclin A	0.29	0.72	0.86	0.31
CDK6	CDK6	2.64	2.09	0.72	1.12
CCND1	cyclin D1	1.63	1.36	1.07	1.36
TP53	p53	0.72	0.37	1.12	0.86
CMYC	c-myc	0.89	0.57	1.31	1.81
SMAD2	Smad2	0.60	N/A	0.67	0.69
SMAD3	Smad3	2.35	1.45	0.92	1.28

Table I. Shows the fold-change relative to DMSO control in counts per million of gene transcripts associated with drug resistance or cell cycle arrest for each treatment group analyzed with mRNA sequencing.

Discussion

The eventual progression of ER+ breast cancer, despite treatment with endocrine therapy and CDK4/6 inhibitors, prompts the investigation of the mechanisms of acquired drug resistance. There are many mechanisms of Palbociclib resistance that have been established by the literature. ER+ breast cancer exhibits phenotypes of senescence after exposure to Palbociclib, and senescence-associated secretory phenotypes that result from senescence can become tumor-supporting and promote drug resistance. However, combination therapy with CDK8/19 inhibitor SNX631 reduces senescence phenotypes, so it was hypothesized that SNX631 was also preventing SASP-induced resistance to Palbociclib.

This idea inspired experimentation with media conditioned with secretions from Palbociclib-treated cells. Cell viability assays show that conditioned media had a negative effect on cell growth over 20 days compared to fresh growth media. Because literature demonstrates that residual Palbociclib remains in the media, it cannot be effectively discerned whether the decrease in cell viability was due to tumor-suppressing cytokine secretions or due to an

unintentional higher dosage of Palbociclib. A further study with conditioned media where Palbociclib was first removed would be beneficial to separating the effects of these confounding variables. Additionally, the pilot experiment where varying concentrations of conditioned media were used, and the crystal violet assay provide evidence that suggests that conditioned media has a variable effect on cell growth that cannot be accurately predicted, especially in the presence of Palbociclib.

GSEA analysis showed that genes associated with SASP were significantly downregulated in Palbociclib-treated MCF7 after 3-days and 21-days. This suggests that SASP is not a driver of resistance in MCF7, as previously hypothesized. This could be possibly explained by the reversal of senescence in the cells as they reenter the cell cycle. A further study could investigate the SASP on a protein level, with a proteome analysis of conditioned media, to determine which cytokines are being secreted from the cells.

GSEA regarding the combination treatment was not significant, making it difficult to discern the effect of SNX631 on cytokine secretions. However, cell viability assays show that the combination treatment prohibits cell growth regardless of the addition of conditioned media, suggesting that the success of SNX631 should not be fully contributed to SASP attenuation.

Since TGF-B is a key signaling molecule, has been associated with tumorigenesis, and has been shown to be affected by CDK8/19, it was hypothesized that TGF-B signaling was an avenue through which SNX631 inhibition might prevent acquired Palbociclib resistance.

However, cell viability assays showed that a combination of Palbociclib and TBRI inhibitor did not inhibit cell growth over 20 days, but rather improved it. This does not replicate the effect of SNX631 in combination with Palbociclib, and therefore does not support the hypothesis. GSEA analysis shows that TGF-B signaling is heavily upregulated in Palbociclib-

treated cells, coinciding with an increase in genes encoding TGF-B1. The combination decreases the signaling closer to levels of untreated cells. The data may indicate a lower reliance upon TGF-B signaling after 21 days of Palbociclib treatment, where the combination treatment maintains TGF-B sensitivity and activity. Alternatively, combination treatment could cause a switch of predominant TGF- B isoform from type 1 to type 2, which serve different functions. Overall, the CDK8/19 inhibition leads to abrogation of TGF-B-induced Palbociclib resistance. TGF-B gene transcripts experience significant translational regulation, so conclusions made based on RNA sequencing must be confirmed at a protein level.

According to RNA sequencing, Palbociclib-treated cells exhibit published mechanisms of resistance such as suppression of Smad3, overexpression of cyclin D, CDK6, CDK2, and suppression of p53. There is some evidence that SNX631 counteracts these mechanisms, giving cause to further examine these resistance mechanisms in the context of SNX631.

Conclusions

This study intended to explore SASP as a mechanism of Palbociclib resistance and examine the ability of SNX631 to attenuate SASP. The conditioned media experiment was not a successful determinant of the presence of tumor-supporting cytokine secretions. The study found that the combination treatment suspended proliferation of the breast cancer cells even in the presence of conditioned media, which suggests that SNX631 works regardless of cytokine secretions. GSEA also suggests that SASP is suppressed in Palbociclib-treated cells.

Research shows that the role of TGF-B in tumorigenesis and cell cycle arrest is complex. It was hypothesized that TGF-B is suppressed by CDK8/19 inhibition. The study found that in this context, an increase of TGF-B signaling occurs with Palbociclib treatment, but

paradoxically, inhibition of TBRI is associated with an increase in cell growth. Based on RNA sequencing data, SNX631 was shown to suppress TGF-B/Smad signaling in combination with Palbociclib, which supports the hypothesis that TGF-B inhibition is key to the prevention of Palbociclib resistance. Due to these contradictory results, the role of TGF-B signaling on Palbociclib resistance is complex, and the effect of SNX631 on TGF-B has yet to be fully understood.

Overall, this study indicates that further scientific investigation should be directed towards understanding how SNX631 affects the TGF-B/Smad axis, along with previously suggested mechanisms of resistance of Palbociclib. Knowledge regarding the effects of combinatory CDK4/6 and CDK8/19 inhibition is essential for improving treatment of estrogen receptor-positive breast cancer.

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Supplementary Materials

Supplementary Figures

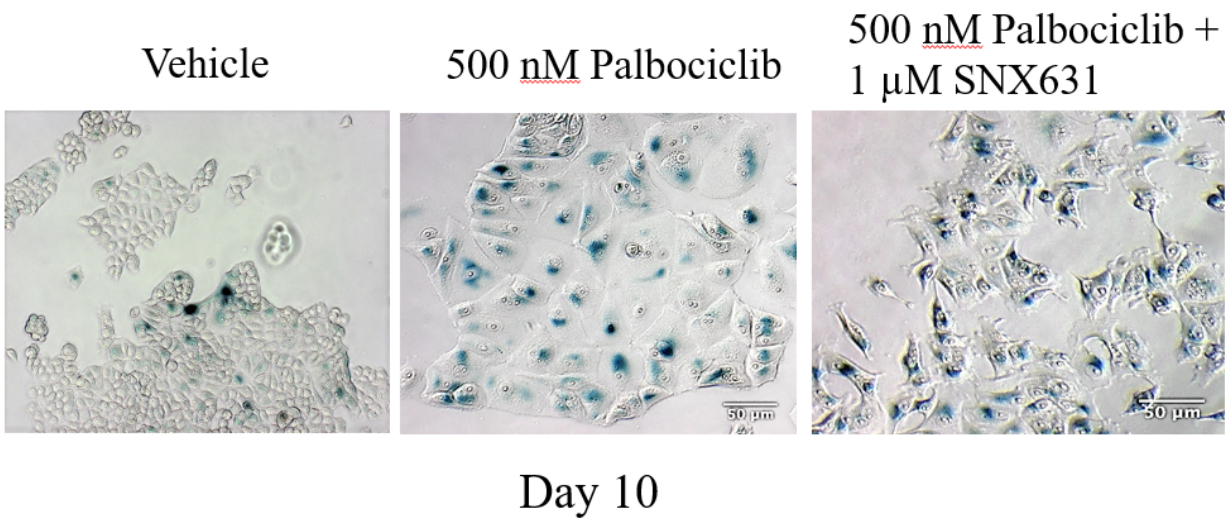

Figure S1. Microscopic images of MCF7 cells after 10 days of treatment with either 500 nM Palbociclib or 500 nM Palbociclib + 1 µM SNX631 or equivalent DMSO vehicle control show that senescent morphology occurs in Palbociclib-treated cells but occurs less strongly in combination-treated cells.

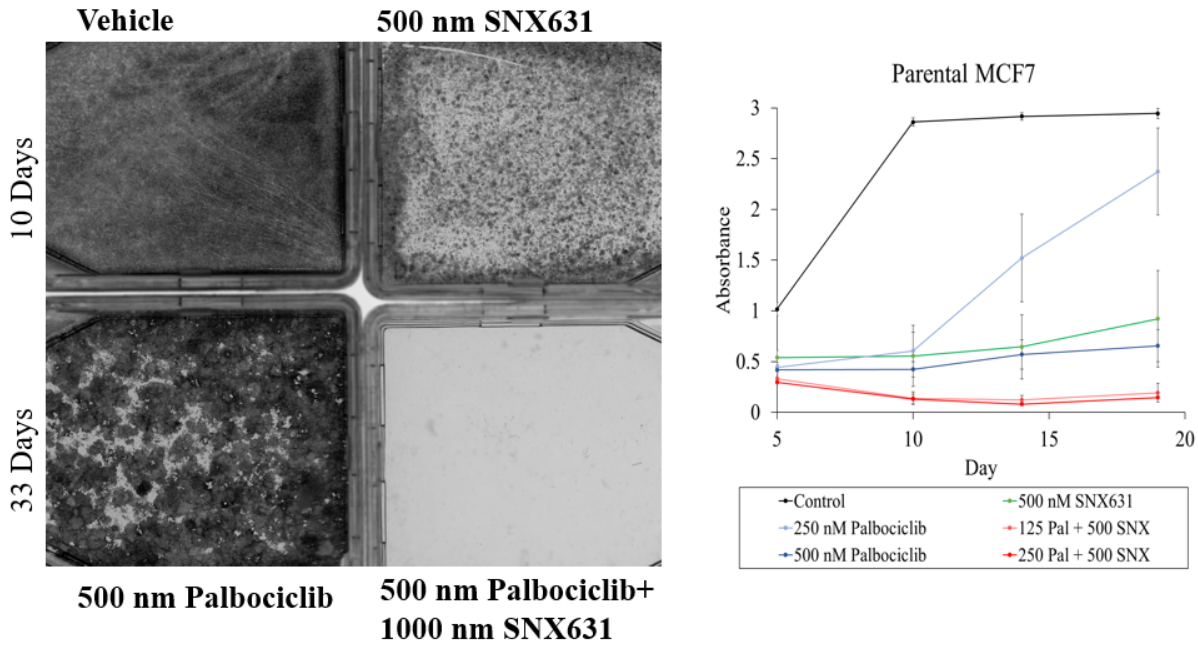

Figure S2. **(A)**Crystal violet staining of flasks with MCF7 cells treated with Palbociclib for 33 days, SNX631 for 10 days, or a combination for 33 days. **(B)** A preliminary cell viability assay shows the effects of a combination of Palbociclib and SNX631 on MCF7 cell viability after 20 days. Palbociclib and SNX631 combination treatment conditions (red lines) show no cell growth over 20 days.

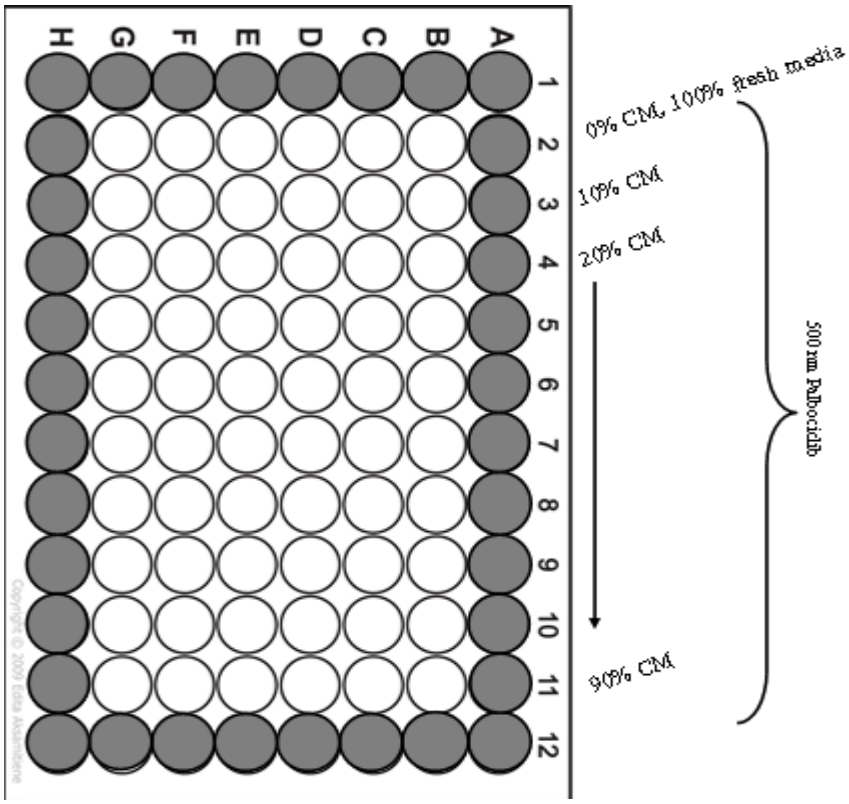

Figure S3. A diagram of the experimental setup of the 96-well plates for the Conditioned media pilot experiment. One plate included 500 nM Palbociclib with a dilution of conditioned media.

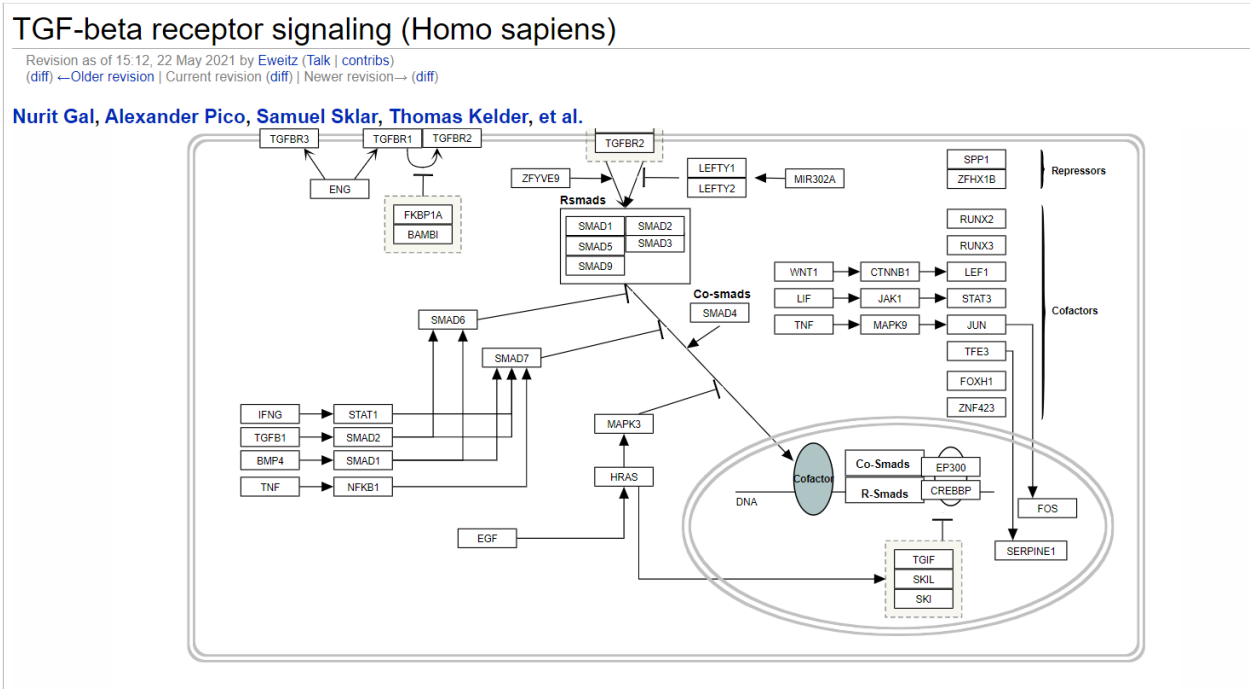

Figure S2. A simplified pathway of TGF-B/Smad signaling leading to transcription regulation. Source: wikipathways

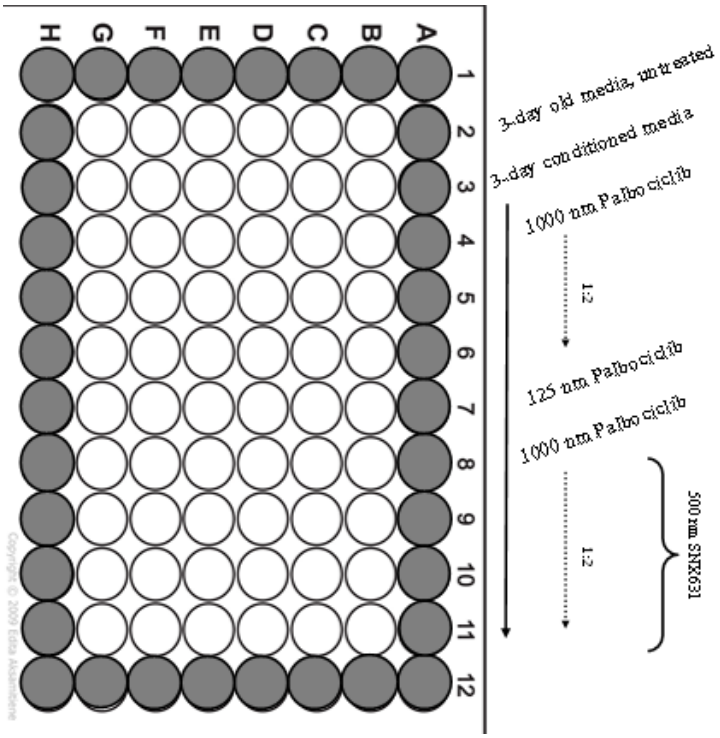

Figure S5. A diagram of the experimental setup of the 96-well plates for the conditioned media and drug combination experiment.

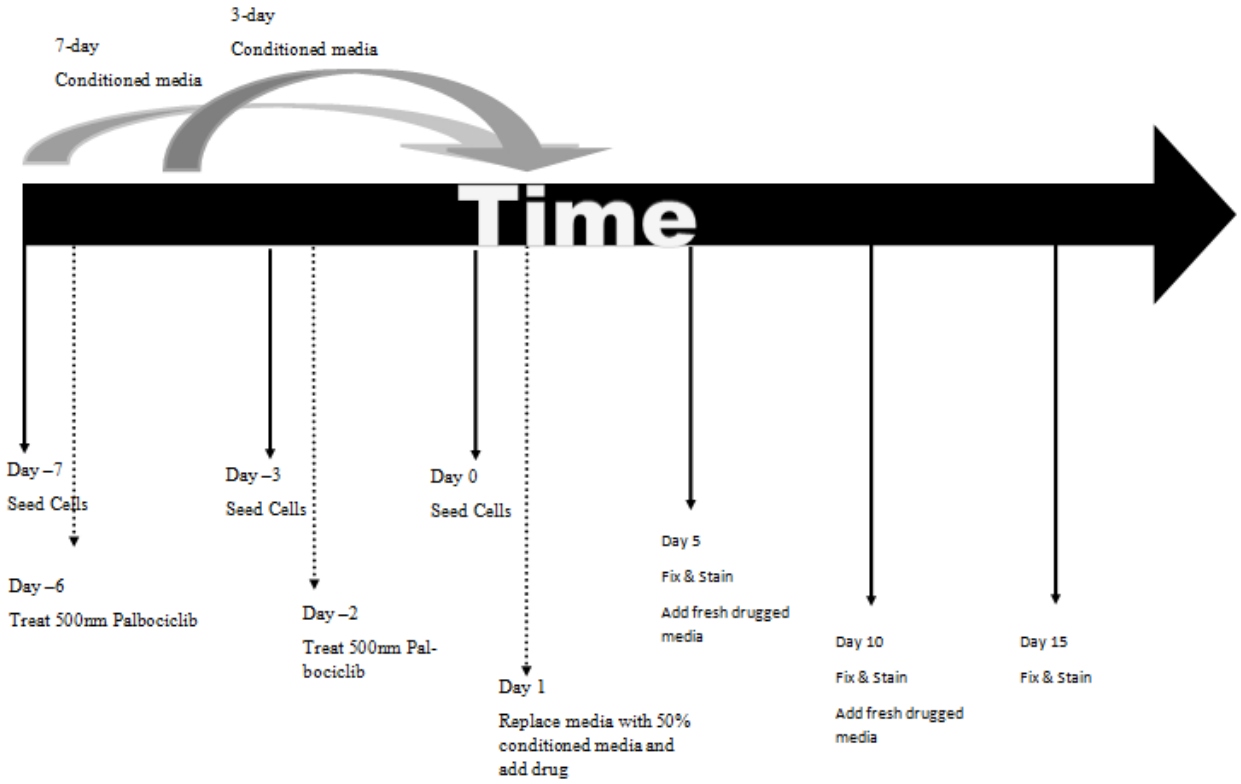

Figure S6. A timeline of plating, treating, and staining to produce conditioned media and conduct the conditioned media experiment.

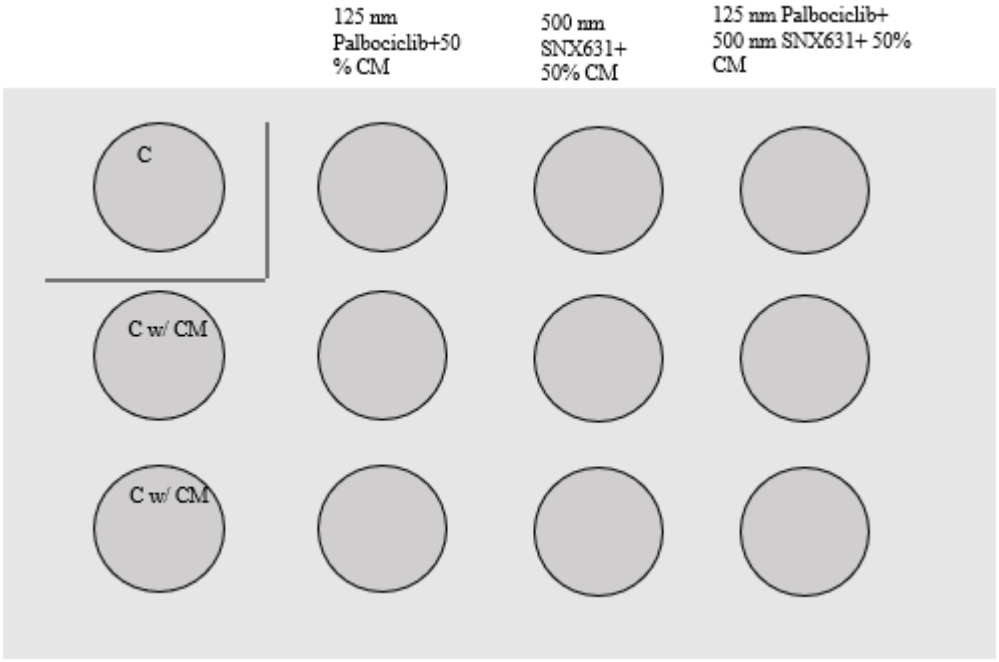

Figure S7. A diagram of the experimental setup of the conditioned media with drug release experiment. C is 'control', a treatment group with 100% fresh media, C w/ CM is a treatment group with 50% conditioned media but without drugs.

Detailed Methods

Serial Dilution of Palbociclib and SNX631.

24 h after seeding, media was removed from the wells of the 96-well culture dish. 100 µL fresh media was loaded into each well. Solutions of 8.5 mL 8 µM Palbociclib and 13 mL 4 µM Palbociclib were prepared. Thirty-two mL of 2 µM SNX631 was prepared. Four concentrations of Palbociclib were tested: 1000 nM, 500 nM, 250 nM, and 125 nM. To prepare the serial dilution, 100 µL of 4 µM Palbociclib stock was placed into the third row of the plate with a multichannel pipettor. The 4 µM stock was carried to each subsequent row, diluting the concentration by 0.5. This was repeated with the 8 µM Palbociclib stock for rows 8-11. One hundred µL of 2 µM SNX631 stock was added to rows 8-11 for the 'combination' treatment. 100 µL media was removed from the 'combination' wells, and 100 µL 100% conditioned media was added to all the wells, producing a 50% conditioned media solution for the entire plate.

Sulforhodamine-B staining protocol.

MCF7 cells were fixed with 10% ice-cold trichloroacetic acid in dH_2O and incubated at 4 °C for 10 minutes. The plates were rinsed with water. The cells were then stained with 0.4% sulforhodamine-B in 1% acetic acid and incubated at room temperature for 10 minutes. After rinsing with 1% acetic acid in dH_2O, the plates were incubated at 37 °C (no CO_2) overnight. The SRB crystals were solubilized in a 20 µM TRIS base and rocked for 1-2 h. Absorbance of the crystals in each well was measured with a BIO-RAD iMark™ Microplate Reader (Hercules, CA) at 570 nm excitation wavelength.

Crystal Violet staining protocol.

Media was aspirated off the cells. They were washed gently with non-sterile PBS. The cells were fixed with 1-2 mL 80% methanol, 20% acetic acid solution and incubated for 10 minutes at room temperature. The cells were gently washed with room temperature non-sterile (PBS). The cells were then stained with 1-2 mL 0.5% Crystal Violet in 10% methanol and incubated at room temperature for 5 minutes. The stain was washed out with dH_2O and dried on the bench overnight.

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