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SCHOOL OF MEDICINE

Thesis

EXPLORATORY ROLE OF PROTEIN KINASE CK2 SYNERGY IN TREATMENT OF BREAST CANCER

by

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B.S, George Washington University, 2012

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EXPLORATORY ROLE OF PROTEIN KINASE CK2 SYNERGY IN TREATMENT OF BREAST CANCER

ANIL AJIT SHAH

ABSTRACT

Breast cancer is the second most common type of cancer among women. The serine-threonine protein kinase CK2 is overexpressed in many cancers, including lung, prostate, hematologic cancers, and breast (Pinna, 2013). Here, we examined the potential of CK2 inhibition alone and in combination with chemotherapy to treat breast cancer. We performed cell viability assays on five breast cancer cell lines treated with CK2 chemical inhibitors or small interfering RNAs, and chemotherapeutic drugs, to test if a synergistic effect could be attained. We also tested if CK2 inhibition would change the stem-like phenotype, epithelial-to-mesenchymal (EMT) marker expression, and CK2 subunit gene expression in the HS578T cell line. We concluded that in the five cell lines utilized, CK2 inhibition had no synergistic effect with chemotherapeutic drugs. CK2 inhibition had no effect on the stem-like phenotype of HS578T cells. However, CK2 inhibition did show a pattern of inhibition of EMT marker expression. Finally, we found that CK2 inhibition appears to activate a compensatory feedback loop for the transcription of the alpha subunit. This may explain the lack of synergy, and bears further investigation in future studies.

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ABBREVIATIONS

CSC	Cancer Stem Cells
TBCA	Tetrabromocinnamic acid
DMSO	Dimethyl sulfoxide
CTB	Cell titer blue
EMT	Epithelial-mesenchymal transition
LCIS	Lobular Carcinoma *in situ*
DCIS	Ductal Carcinoma *in situ*

INTRODUCTION

Breast Cancer

Statistics

Cancer is a disease that has plagued the entire world. The American Cancer Society states that breast cancer is the second leading cause of cancer deaths in females in the United States, second only to lung cancer. Breast cancer is also the second most common cancer among women, with the first being skin cancers. According to the American Cancer Society (www.cancer.org), 232,340 new cases of invasive breast cancer will be diagnosed in women in 2014 and about 39,620 women will die of breast cancer. Though there have been many advances in the treatment of breast cancer ranging from new surgical techniques to new drug development, further advances are urgently needed. As our understanding of the different molecular bases of breast cancer progresses, there will undoubtedly be better and more specific treatment options available in the future.

Breast Cancer Subtypes

Breast cancer is a heterogeneous disease that is difficult to characterize molecularly. Breast cancer lacks the step-wise molecular progression that has been characterized in colon cancer (Malhotra et al. 2010). Histologically, breast

cancer can be identified into two broad categories: *in situ* carcinomas and invasive (infiltrating) carcinomas. Within *in situ* carcinomas there are ductal and lobular subclasses. Within invasive carcinomas there are tubular, ductal lobular, invasive lobular, infiltrating ductal, colloid, medullary, and infiltrating ductal. Despite the lack of molecular markers there have been attempts to molecularly classify breast cancer. Subtypes that have been identified by gene expression include: claudin low, basal like, Her2 enriched, normal breast-like, luminal A, and luminal B (Malhotra et al. 2010). While not fully validated or universally available, molecular subtyping is being used more and more to guide treatment decisions. One promising step towards this more personalized medicine approach is the OncotypeDX assay, which is already being widely used at Boston Medical Center to help with treatment decisions in patients with breast cancer. OncotypeDX is a RT-PCR based assay for expression of 21 genes that can be performed on fixed paraffin-embedded tissue. It is used to predict the risk of local recurrence of ductal carcinoma *in situ*, and the risk of distant recurrence in patients with node-negative, estrogen-receptor-positive breast cancer (Paik et al. 2004; Paik et al. 2006). These are clinical situations in which decision-making is difficult for doctors and for patients, as some patients will not recur and would be over-treated with chemotherapy, but in others, treatment will prevent recurrence. OncotypeDx has been validated and adopted by the American Society for Clinical Oncology and National Comprehensive Cancer Network into treatment guidelines. As more predictive tests like this are utilized, better outcomes for

patients will be achieved. OncotypeDx testing is approved by most insurance companies, and can be used to make decisions about adjuvant chemotherapy.

Breast Cancer Staging

The 7th edition of the American Joint Committee on Cancer gives a very comprehensive staging guideline for breast cancer. Breast cancer is staged based on the TNM system. This system stages cancer based on T (primary tumor), N (regional lymph nodes), and M (distant metastases). Stage 0 consists of carcinomas *in situ* with no regional lymph nodes and no metastases. These also encompass the categories of ductal or lobular carcinomas *in situ*, with the former having cancer cells in ducts and the latter having the cancer cells in the milk-producing lobules. Stage I is divided into IA and IB. IA consists of a tumor that is less than 20mm at its greatest aspect. IB consists of either no evidence of primary tumor or a tumor the size of that found in IA but could include axillary lymph nodes. Stage II is divided into IIA and IIB. In IIA the tumor is not greater than 50mm at its greatest aspect and could have axillar lymph node involvement. IIB could have a tumor that is bigger than 20mm and greater than 50mm as its greatest aspect and also axillar lymph node involvement. Stage III is divided into IIIA, IIIB, and IIIC. IIIA can have tumor of sizes anywhere ranging from 20mm or greater than 50mm but also involve clinically fixed axillary lymph nodes. IIIB has tumors of any measurement but have extended as far as the chest wall and/or skin and can include clinically fixed lymph node involvement. Stage IIIC can have

any size tumor but has metastases in ipsilateral infraclavicular lymph node, supraclavicular lymph nodes, or internal mammary lymph nodes. Stage IV can have any size tumor with any extent of lymph node involvement, but with detectable distant metastases as detected by radiology or histology (Edge and American Joint Committee on Cancer, 2010).

Breast Cancer Treatments

Throughout the years, breast cancer treatments have evolved through clinical trials that guide optimal treatment, based upon tumor stage. Surgery, radiation, and chemotherapy are the most common types of treatment given to patients. Surgery is usually the first line of defense if the tumor is operable. Radiation therapy usually follows breast-conserving surgery (lumpectomy or partial mastectomy) because of the decrease in mortality and recurrence (Maughan et al. 2010).

Mainly, treatment of breast cancer depends on the current TNM stage of the cancer, however molecular marker status, hormone receptor status, and other non-staging factors are sometimes considered. At Stage 0 breast cancer, it depends on the histology of the cancer. If it is a LCIS, usually there are no recommended treatments. Women are recommended to undergo regular breast examinations. The American Cancer Society recommends a clinical breast exam every three years for women who are in the 20-30 year old range and yearly mammograms for women once they reach 40 years of age. DCIS are much more

prone to progress to invasive types of breast cancer. In this case, breast-conserving surgery followed by radiation therapy is usually done (Maughan et al. 2010). At Stages I and II, the most frequent treatment is breast conserving surgery or mastectomy. In these cases, there could also be lymph node involvement, which will influence the use of radiation or systemic adjuvant therapies (Maughan et al. 2010). At Stage III, the cancer is considered locally advanced and often inoperable, in these cases, chemotherapy combined with local therapies is carried out (Maughan et al. 2010). Often, preoperative (neoadjuvant) chemotherapy decreases the size of the tumor, sometimes making it operable. At Stage IV breast cancer (metastatic), treatment goals are usually palliative. Hormone receptor status is strongly considered in this case and endocrine therapy along with chemotherapy is often used (Maughan et al. 2010). A major problem lies in the recurrence of breast cancer. This could be due to cancer cells becoming resistant to treatments, in part due to the presence of cancer stem cells within the tumor.

Cancer Stem Cells and Drug Resistance

There are several different theories as to why some cancers stop responding to radiation and chemotherapy. One such theory is related to molecular subclasses of cancers. It is thought that these varying molecular mechanisms are implicated in providing resistance to chemotherapy in different molecular classes of cancers (Andre and Pusztai, 2006). Another recurring

theme is the hypothesis of cancer stem cells (CSC). The idea behind this is that not all cancerous cells have the ability to form tumors and that there is a subclass of cells within cancers that essentially have the ability to proliferate and form metastases (Riley and Desai, 2009). It is important to note that these CSC are not stem cells arising from specific tissues, but are cells that have gained stem-like properties during their neoplastic progression (Hermann et al. 2007).

CSC have been identified in many types of cancers including breast cancer. There has been much research into CSC because of the idea that if specific therapies could be found to kill CSC, that would overcome their resistance to chemo- and radiotherapy and improve outcomes for patients (Liu and Wicha, 2010).

Protein Kinase CK2

The Holoenzyme of CK2

Protein Kinase CK2 (formerly casein kinase II) was first described in 1954 (Burnett and Kennedy, 1954) and since, the kinase has been implicated in a wide array of cellular processes. CK2 is a highly ubiquitous and conserved tetrameric serine-threonine kinase and is one of about 518 kinases predicted in the human genome (Manning et al. 2002). The holoenzyme is composed of two catalytic subunits, α and α’ and two regulatory subunits, β. The α, α’, and β are about 42, 38, and 26 kDa respectively. CK2 plays a myriad of roles in biological processes,

however for the scope of this work, only certain aspects will be highlighted (for a more complete review see: Pinna, 1997; Pinna, 2002; Trembley et al. 2009). CK2 is found in both the nucleus and cytoplasm and can shuttle between different compartments of the cell (Ahmad et al. 2008). CK2 is also unusual in that it has the ability to use GTP as well as ATP to donate phosphate groups (Pinna, 1990; Niefind et al. 1999).

CK2 in Gene Expression and Cell Cycle Regulation

CK2 has been implicated in signaling pathways that are known to affect gene expression. Gene expression regulation relies on different signaling pathways, involving kinases, transcription factors, and other proteins. One such pathway is regulated by the Akt kinase. Akt plays major roles in metabolism, proliferation, cell survival, and angiogenesis. It also plays major roles in cancer, diabetes, cardiovascular diseases, and neurologic diseases (Hers et al. 2011). CK2 was established as an effector of Akt. Interaction of CK2α or CK2β with Akt was shown to increase Akt kinase activity *in vitro* and *in vivo* (Di Maira et al. 2005; Guerra, 2006). Aside from directly modulating Akt activity, CK2 also phosphorylates PTEN, a phosphatase that negatively regulates Akt activity and inactivates it (Arevalo, 2006). In collaboration with the Neel lab, our group also successfully identified the phosphorylated residues of PTEN by CK2 (Miller et al. 2002). CK2 was also shown to be associated with the transcription factor NFκB. It was shown that PKCζ, bound to CK2, phosphorylates IκBα, thus degrading the

amount of IκB, furthermore activating NFκB (Bren et al. 2000; Dominguez et al. 2009). The Sonenshein lab, in collaboration with our lab also identified that aberrant expression of CK2 in breast cancer cells promotes increased levels of NFκB/Rel, a result suggesting that increased levels of CK2 in primary tumors could result in NFκB activation (Romieu-Mourez et al. 2002; Romieu-Mourez et al. 2001). CK2 was also found to be a novel binding activator of the JAK-STAT pathway, which is a key signaling cascade involved in differentiation, as well as cell survival and proliferation (Zheng et al. 2011).

CK2 has been found to be essential in cell cycle progression and required at the G0/G1, G1/S, and G2/M checkpoints (St-Denis and Litchfield, 2009). The G1/S phase of the cell cycle is the committed step in which the cell is preparing to divide and duplicate its DNA. There are many proteins and kinases that play a role in this checkpoint and some examples are p53 and the FACT complex. In 1992, it was found that cells containing p53 arrested in G1 whereas when p53 was lost, cells underwent uncontrolled gene amplification (Yin et al. 1992). In G1, CK2 phosphorylates murine double minute 2 (MDM2), disrupting the MDM2-p53 interaction, and leading to the stabilization of p53 (Allende-Vega et al. 2005). Additionally, CDKs are important kinases that control the cell cycle by controlling negative regulators of the cell cycle (Harper et al. 1993). CK2 has been reported to interact with the FACT complex, SSRP1, and regulate CDK activity (Keller and Lu, 2002; Li et al. 2005; Tapia et al. 2004). Another transition checkpoint that CK2 is involved in is the G2/M phase. At this phase, in mitosis, the spindle

apparatus has formed and equal separation of chromosomes into two identical daughter cells occurs. A study showed that when cells were treated with nocodazole (spindle formation disruptor) and CK2 inhibitors, a novel role of CK2 and p53 was found showing the importance of CK2 in cdc2 activity and mitotic checkpoint arrest (Sayed et al. 2001). Albeit cell cycle regulation involves a host of other proteins in many signaling cascades, the role of CK2 in cell cycle regulation and cellular proliferation proves to be central.

CK2 in Apoptosis

Apoptosis is programmed cell death that relies on an intricate network of proteins to regulate cell survival and is a process in normal cellular function. In a study in 2001 by Wang, it was shown that CK2 has a key anti-apoptotic role in cancer cells. The authors showed that decreasing levels of nuclear CK2 had an effect of induction of apoptosis (Wang et al. 2001). Furthermore it was shown that overexpression of the CK2 catalytic subunit α, but not the β subunit, also blocks and protects against apoptosis (Guo et al. 2001). Additionally, considering that phosphorylation plays essential roles in almost all cellular processes, CK2 has been found to phosphorylate a number of proteins involved in apoptotic signaling pathways. PTEN is another protein that controls cell cycle by regulating levels of phosphatidylinositol 3,4,5-triphosphate (Sun et al. 1999). CK2 phosphorylates PTEN, a tumor suppressor protein; it phosphorylates Bid, a protein involved in apoptosis and regulated by Fas; and also p53 (Desagher et al.

2001; Torres et al. 2003; Shen and White, 2001). Thus, CK2 plays a central role in apoptosis, and interacts with proteins involved in several apoptotic pathways.

CK2 in Embryonic Development

CK2 has been implicated in regulation of development in plants, invertebrates, and vertebrates. In 1986, Schneider et al. showed that CK2 was most highly active in 12 and 14 day-old mouse embryos as compared to other embryonic time points (Schneider et al. 1986). In 1994 Mestres showed the varying levels of CK2 in different organ systems during mouse embryogenesis. They found that on day 10.5, CK2 was most elevated in the neuroepithelia. On day 11.5 and on CK2 was found in all epithelial layers. On day 16.5 and onwards, CK2 was much more highly expressed in the tissues involved in organogenesis than those tissues associated with the secondary mesenchyme (Mestres et al. 1994). Our lab has engineered mice with disruptions of either the CK2α or CK2α' subunits. Mice heterozygous for CK2α+/- are outwardly normal and display a regular life span, whereas mice homozygous for CK2α-/- are embryonic lethal, most likely due to defects in heart formation (Lou et al. 2007). It was further elucidated that CK2α-/- homozygous embryos had defects in neural tube formation. The CK2α-/- embryos had no compensatory mechanism for upregulating CK2α', however CK2β was reduced in the homozygous embryos compared to the heterozygotes (Seldin et al. 2008). Mice heterozygous for CK2α'+/- are phenotypically similar to the wild types, whereas the males

homozygous for CK2α'-/- exhibit defects in spermatogenesis consistent with globozoospermia (Xu et al. 1999). Mouse embryos heterozygous for CK2 β+/- are for the most part phenotypically viable, however a fraction are embryonic lethal, and homozygous CK2β-/- embryos are only able to undergo the implantation process, but do not survive post-implantation (Buchou et all 2003). Though the role of CK2 in embryogenesis is very complex, these works show that different subunits of the protein kinase are essential at different aspects of embryogenesis. The efforts spent on creating knockout mouse models for the different subunits were essential in elucidating some of the properties of each subunit.

CK2 in Signaling Pathways: Normal and Cancer

A multitude of different signaling pathways contribute to embryonic development, differentiation, and growth. Of these, the Wnt, Notch, and the Tgfβ superfamily signaling pathways are all critically important and CK2 is known to play a role in each. The role of CK2 role in these pathways will be briefly discussed.

Wnt signaling is important in processes involving in cell fate, cell polarity, and induction of embryogenesis (Cadigan and Nusse, 1997). Wnt is a signaling cascade that regulates the intracellular levels of β-catenin. Wnt proteins are effectively glycoproteins that are rich in the amino acid cysteine and can act through either the 'canonical' or 'non-canonical' pathways, with the former

regulating cell proliferation and survival and the later regulating tissue polarity (Dominguez et al. 2009). The Wnt pathway has been seen to become aberrant during cancer progression. In 2000, Song showed that CK2 is actually a positive regulator of Wnt signaling because of its role in phosphorylating β-catenin and other Wnt signaling intermediates. A Wnt-1 transfected murine breast cell line (C57MG) that was treated with CK2 inhibitors had lower levels of β-catenin and slower proliferation rates, showing the positive role CK2 has in the Wnt pathway (Song, 2000).

Notch signaling is another pathway that is tightly regulated by a series of posttranslational modifications. This signaling cascade plays central roles in normal animal development, binary and inductive cell fate, and has even been implicated in B and T cell development in the immune system (Fortini, 2009). The Notch signaling cascade is also dysregulated in cancer. It was shown that CK2 does in fact phosphorylate Notch at two specific amino acid sites, which results in a decrease of its transcriptional activity (Ranganathan et al. 2011). These data show that CK2 is a negative regulator of Notch signaling. Furthermore, CK2 was also shown to be a positive regulator of the Notch pathways in lung cancer. In 2013, Zhang and his group found that when CK2's alpha subunit was inhibited by CX4945, Notch1 transcriptional activity was downregulated in the A549 and H1299 lung cancer cell lines (Zhang et al. 2013).

The Tgfβ signaling pathway is another cascade that is involved in cell differentiation, proliferation, and development. This pathway also includes

several important growth factors and among these is bone morphogenic protein. In 2010, it was shown that CK2 negatively regulates the differentiation of osteoblasts by associating with BMP receptor type Ia. Normally BMP2 is allowed to release this interaction and allow differentiation of the osteoblasts, but when this interaction was inhibited, a negative regulation followed (Bragdon et al. 2010). However, another study in 2006 showed that when CK2 was inhibited, Tgfβ was not able to induce expression of ApoE and it was shown that Tgfβ actually activates CK2 (Singh, 2006). Though broad aspects of these signaling pathways were shown, these sources show that CK2 can play a negative and positive role in the Tgfβ superfamily signaling cascade. Though this signaling pathway is crucial in normal development, it also has a central role in cancer as it has been implicated in malignant progression, invasiveness, dissemination and metastasis via a host of processes involved in cell mobilization, evasion of immune surveillance, and microenvironmental dysregulation (Massagué, 2008).

As shown through the studies referenced above, these signaling pathways play a role in normal development. However, in cancers, these pathways do become dysfunctional and CK2 plays a role in regulating these pathways

CK2 and Cancer

The hallmarks of cancer include the ability of cells to elude regulated cellular proliferation, differentiation, and apoptosis. Because CK2 is important in embryonic development, cell proliferation and cell death, it is perhaps not

surprising that it plays roles in cancer as well. Overexpression of CK2 provides a survival advantage for cells by being able to suppress apoptosis and stimulate cellular proliferation (Ahmad et al. 2008). As cancer cells overexpress CK2, it becomes an attractive option for cancer therapy (Ahmad et al. 2008). Also interesting is the fact that CK2 is in the nuclear and cytoplasmic compartments of the normal cell, but in cancer cells its distribution is markedly higher in the nucleus (Ahmad et al. 2008; Laramas et al. 2007). Increased levels of CK2 have been found in cancers of the lung, colon, prostate, hematologic malignancies, and breast cancer (Munstermann et al. 1990; Daya-Makin et al. 1994; Landesman-Bollag et al. 2001; Ahmed, 1994; Piazza et al. 2012). Additionally, overexpression in many different cancers, including prostate and colorectal, has been associated with disparaging survival and prognosis (Laramas et al. 2007; Lin et al. 2011). Though CK2 has been implicated in many cancers, no mutations have yet been found in CK2. In the 2013 edition of Protein Kinase CK2, edited by Lorenzo Pinna, it is noted that no mutations could have been found in CK2 for different reasons such as: upregulation of activity, protein, or mRNA may progress cancers, there could be a loss rather than a gain of function, or even that CK2 is not an underlying cause of cancer but is a colluding kinase with other proteins (Pinna, 2013) Even if CK2 is not a prototypical oncogene, it most certainly is a driver of neoplasia. It is thus more urgent now than ever to uncover more of the molecular mechanisms associated with this protein kinase and cancers.

CK2 and Breast Cancer

Though CK2 has an established role in many different cancers, the growing number of breast cancer-related deaths makes it especially important to examine its role and therapeutic potential in this type of cancer. In studies done in 2001, it was shown that CK2 is more highly expressed and has higher activity in breast cancer samples as compared to normal tissues, both in carcinogen-induced mammary tumors in rats and in human breast tumor samples (Landesman-Bollag et al. 2001). This study also showed a causative role for CK2 in mammary tumorigenesis: transgenic mice that overexpress the α subunit in the mammary gland developed mammary adenocarcinomas (Landesman-Bollag et al. 2001). This study not only showed that CK2 is overexpressed in breast cancer, but that it also contributes to the development of the disease. More recently, in an immunohistochemical study of 1000 breast carcinomas, a strong correlation was identified between poor prognosis and CK2α (Giusiano et al. 2011). Additionally, CK2 was later identified as a gene that was significantly increased in breast cancer samples as compared to normal samples (Liu et al. 2007).

Thus, CK2 is an attractive target for cancer therapy. Its role in cell proliferation, apoptosis, cell cycle checkpoints, as well as other functions give tumors with high levels of CK2 a favorable environment for tumorigenesis, even if

the molecular pathways involved in the pathogenesis of the disease do not depend on CK2 alone (Sarno and Pinna, 2008).

OBJECTIVES

As shown by the preceding literature, CK2 plays crucial roles in normal cellular functions, embryogenesis, cell-cycle checkpoints, apoptosis, and developmental signaling pathways. CK2 has also been implicated in the progression of breast cancer. Therefore, through the specific aims listed below, a primary goal of this study is to further elucidate the role of CK2 in breast cancer.

The specific aims of this study are to:

1. Test the ability of chemical or siRNA inhibitors of CK2 to affect the viability of breast cancer cell lines *in vitro*.
2. Test the ability of CK2 inhibition to synergize with chemotherapy to kill breast cancer cell lines.
3. Test the ability of CK2 inhibition to modulate the expression of stem cell markers and EMT markers in breast cancer cell lines.

With this study, we hope to identify additional roles that CK2 plays in cancer cell survival. Because of the ever-growing need for information in cancer therapeutics, a major breakthrough could be achieved if CK2 is identified as a valid target and if CK2 inhibition could synergize with chemotherapeutic agents in breast cancer.

METHODS

Materials

CX-4945 [5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid)] was kindly obtained from Cylene Pharmaceuticals. Doxorubicin and paclitaxel were chemotherapeutic drugs used for breast cancer cell lines. TBCA was purchased from EMD Millipore via Calbiochem.

Cell Culture

The HS578T, MCF7, 6604, 59, 7367 breast cancer cell lines were monitored bi-weekly for growth rates and morphology consistency. Cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin (Cellgro Manassas, VA) in a 5% CO_2 incubator.

Cell Lines

HS578T is a human mammary breast cell line derived from the carcinosarcoma of a 74-year-old Caucasian female, which is epithelial in origin (Hackett et al. 1977).

MCF7 is a human mammary breast adenocarcinoma cell line derived from a pleural effusion from a 69-year-old Caucasian female (Soule et al. 1973).

The 7367, 59, and 6604 cell lines were derived from murine breast cancer cell lines that originated in the MMTV-CK2α transgenic mice, in our laboratory.

The 6604 cell line was derived from an adenocarcinoma and exhibits epithelial features. The 7367 cell line was derived from a spindle cell carcinoma and the 59 cell line was derived from a spindle cell sarcoma of mammary gland origin. Both 7367 and 59 cell lines were produced from tumors of epithelial origin but which exhibited histological features that resemble mesenchymal cells, that is have undergone epithelial to mesenchymal transition (EMT).

Cell Viability Assays

HS578T, MCF7, 6604, 59, 7367 were seeded at densities of 10,000 cells per well in Corning 96 well solid black flat bottom TC-treated polystyrene microplates. Cells were allowed to adhere for 24 hours and then treated according to the treatment layout planned beforehand. Treatment was allowed to continue for 24 hours and cell viability was analyzed by Cell Titer Blue assay, according to manufacturer's instructions (Promega).

CK2 Peptide Kinase Assays

The 7367 cell line was seeded at a density of about 50% confluence in a 6 well plate. Cells were allowed to adhere for 24 hours and then treated with the CK2 inhibitors TBCA and CX4945, to obtain a dose response and time course response. After 24-hour treatment, cells were harvested using RIPA buffer. Protein was then quantified using BCA Protein Assay Reagent (Pierce). A range of 2-5ug (masses were consistent in each assay) of protein lysate were

incubated in CK2 kinase buffer (100mM Tris pH 8.0, 20mM $MgCl_2$, 100mM NaCl, 50mM KCl, 0.1μg/μl BSA and 100μM Na_3VO_4) with or without 1mM solution of the specific protein kinase CK2 substrate peptide RRREEETEEE (Sigma-Genosys, The Woodlands, TX) and 5μCi of [γ-^{32}P]-GTP (6000Ci/mmol) at 30°C for 10 min. The reaction was stopped by adding 25 μl of 100mM ATP in 0.4N HCl. Samples were spotted onto a P81 Whatman filter and washed in 150mM H_3PO_4, 3 times, 5 minutes each, to remove unincorporated [γ-^{32}P]-GTP. Two ml scintillation fluid was added to each sample and phosphorylation of peptide substrates was quantified in an automatic scintillation counter. The samples were assayed in duplicate and background kinase activity in the absence of the specific peptide substrate was subtracted (Adapted from Kuenzel *et al.* 1987).

Western Blot Analysis

Protein extracts were prepared by aspirating all media in cell culture and then washing 3 times with cold 1x PBS solution. Collected cells were then lysed in RIPA buffer. Lysate protein content was quantified by BCA protein assay (Pierce, Rockford, Ill.). Protein extracts (40-100mg) were diluted in 2X sample loading buffer (100mM Tris-Cl pH 6.8, 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), heated at 95° C for 5 minutes, and separated by electrophoresis on a 7-9% SDS polyacrylamide gels (Laemmli 1970) and transferred onto Immobilon-P PVDF membranes by semi-dry electroblotting at

40mA (for membrane of about 40cm^2) for 90 minutes using an electroblotter (Owl Scientific, Woburn, MA). Membranes were blocked in 5% fat-free milk, 1xPBS, 0.05% Tween, incubated with primary antibody, washed in 1x PBS and 0.05% Tween, and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), washed again and visualized by ECL (Pierce, Rockford, Ill.) according to the manufacturer's instructions. Primary antibodies were rabbit a-human-CK2α (1:1000) (Abcam) and mouse monoclonal anti-β-actin, (1:5000) (Sigma, St. Louis, MO).

RNA Isolation and RT-qPCR

RNA was isolated from HS578T cells using Qiagen RNeasy Plus Mini Kit. cDNA was then made using Promega's GoScript Reverse Transcription system from 1.3μg of harvested RNA. cDNA was then diluted 1:20 and qPCR analysis was done using Promega's goTaq qPCR system, using the manufacturer's instructions.

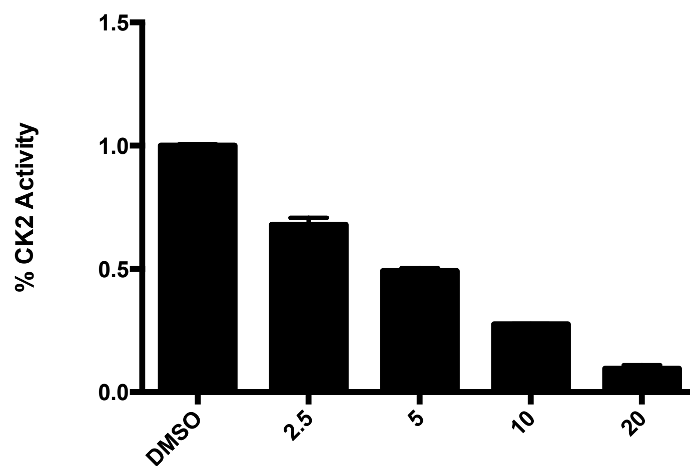
CK2α siRNA Transfection

RNA knockdown of CK2α was accomplished using a specific CK2α siRNA sequence developed in the lab, and siRNA control. siRNA was introduced into cells using Lipofectamine 2000 transfection agent according to the manufacturer's instructions. Transfections were allowed to proceed for 24 hours.

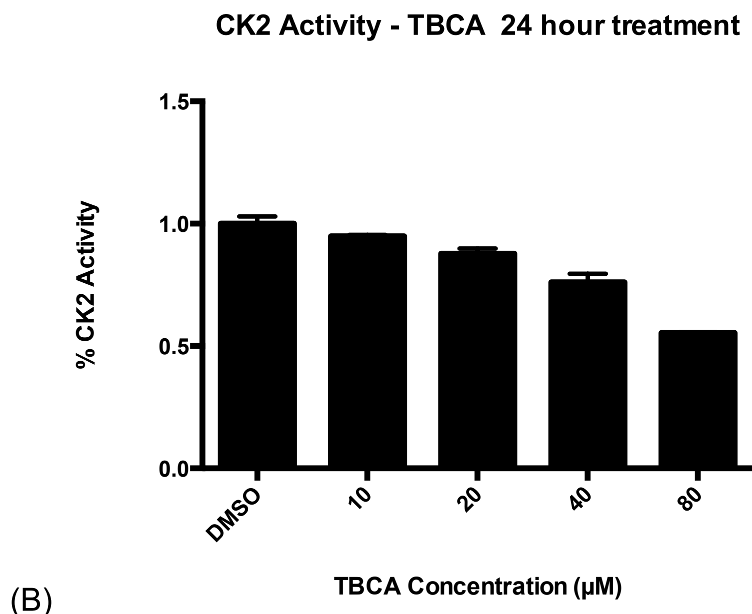
RESULTS

CK2 Peptide Kinase Assay:

To measure the potency of the CK2 inhibitors TBCA and CX4945, on 7367 cell line, 70% confluent cells were treated with the indicated doses for 24 hours (Figure 1). Cells were seeded in a 6 well plate and allowed to adhere for 24 hours before being treated with inhibitors for another 24 hours. A dose dependent pattern of inhibition of CK2 activity was achieved with CX4945 with an IC50 of about 5 µM. A dose dependent pattern of inhibition of CK2 activity was also achieved with TBCA; however, an IC50 was not achieved, at 80 µM, the highest dose used.



(A)



(B)

Figure 1: CK2 Activity With Inhibitors CX4945 and TBCA. Data was obtained by performing a specific CK2 kinase peptide assay on 7367 cells. Cells were treated with either DMSO or 2.5-25 μM CX4945 (A) or 10-80 μM TBCA (B). Results are shown as percent inhibition of the DMSO-treated cells.

Single Dose Cell Viability Assays:

To test the cytotoxicity of CK2 inhibitors, TBCA and CX4945, as well as the chemotherapeutic agents, doxorubicin and paclitaxel, cell viability assays were performed on the mouse breast cancer cell lines 7367, 6604, 59 and the human breast cancer cell lines HS578T and MCF7. The results were obtained using cell titer blue (CTB) as described in the methods. Firstly, single dose response experiments were performed on 10,000 cells seeded in a 96 well cell culture microplate, to obtain a dose dependent pattern for each drug (Figures 2, 4, 6, 8, and 10). CX4945 was most toxic to 7367 cells, while having decreased killing efficacy in 6604, 59, HS578T, and MCF7. A dose dependent pattern of

cytotoxicity was achieved with doxorubicin in each cell line—with a range of 0.1-3.2 μM for 7367 and 0.2-1.6 μM for the remaining cell lines. A dose dependent pattern was not achieved with paclitaxel, instead in each cell line a plateau effect was seen with the indicated doses. The range of paclitaxel used was between 0.3-2.4 μM. TBCA was most toxic to the human breast cancer cell line MCF7, while having almost no effect on the other cell lines with doses of 10 and 20 μM.

Combination Dose Cell Viability Assays:

To examine whether inhibition of CK2 might potentiate chemotherapeutic treatment of the cell lines, combination drug experiments were conducted with the CK2 inhibitor, CX4945 and chemotherapeutic agents to test for synergy between the two (Figures 3, 5, 7, 9, and 11). To calculate an additive, antagonistic, or synergistic effect, single doses of the drugs used in combination were also tested in parallel. The combination dose experiments showed a similar reproduction of the minimal cytotoxicity of CX4945 to each cell line. Also, a dose dependent pattern of cell death was again achieved by doxorubicin. In the combination experiments, CK2 inhibitor with chemotherapeutic agent did not result in synergistic cytotoxicity (Figures 3, 5, 7, 9, and 11). In addition, in cell lines 6604 and MCF7, the combination of CK2 inhibitor with doxorubicin resulted in an antagonistic effect (Figures 5 and 11); the combined treatment has a protective effect over that of doxorubicin alone.

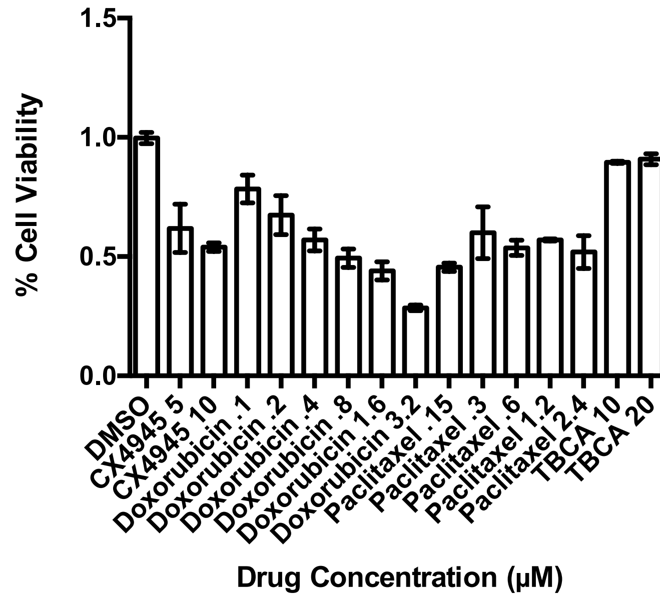


Figure 2: Single Dose Responses on 7367 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on 7367 cells. The graph shows single dose response for CX4945, doxorubicin, paclitaxel, and TBCA for the indicated range of dose concentrations.

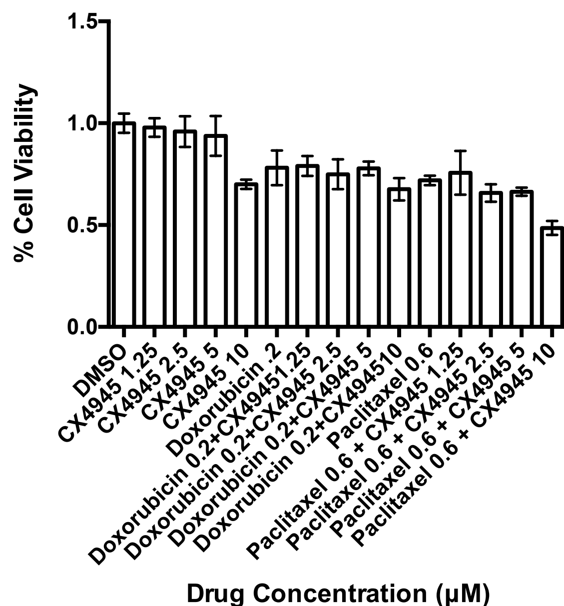


Figure 3: Combination Dose Responses on 7367 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on 7367 cells. The graph shows single cell viability results for a range of CX4945 doses and single doses of Doxorubicin and paclitaxel as well as results from CX4945 combined with doxorubicin and paclitaxel. (Experiment done by Yajuan Shao)

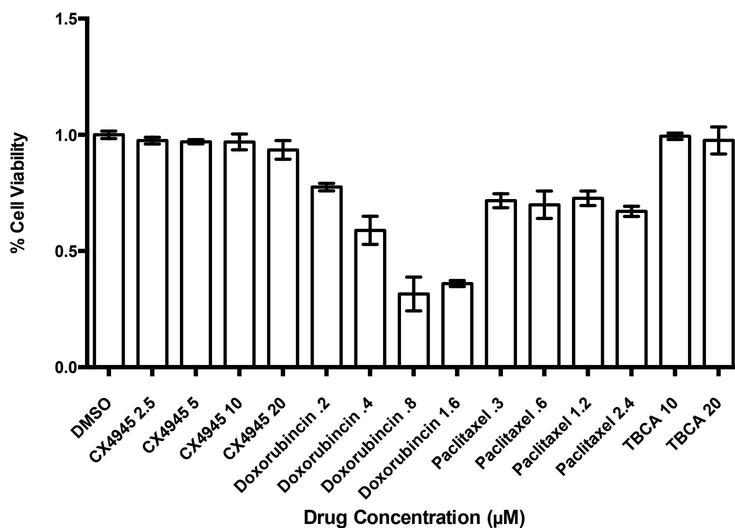


Figure 4: Single Dose Responses on 6604 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on 6604 cells. The graph shows

single dose response for CX4945, doxorubicin, paclitaxel, and TBCA for the indicated range of dose concentrations.

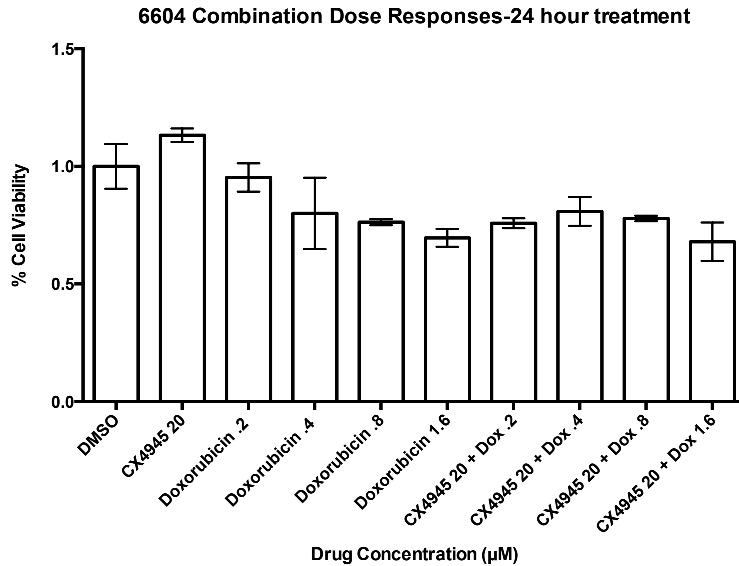


Figure 5: Combination Dose Responses on 6604 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on 6604 cells. The graph shows single cell viability results for a range of CX4945 doses and single doses of doxorubicin as well as results from CX4945 combined with doxorubicin.

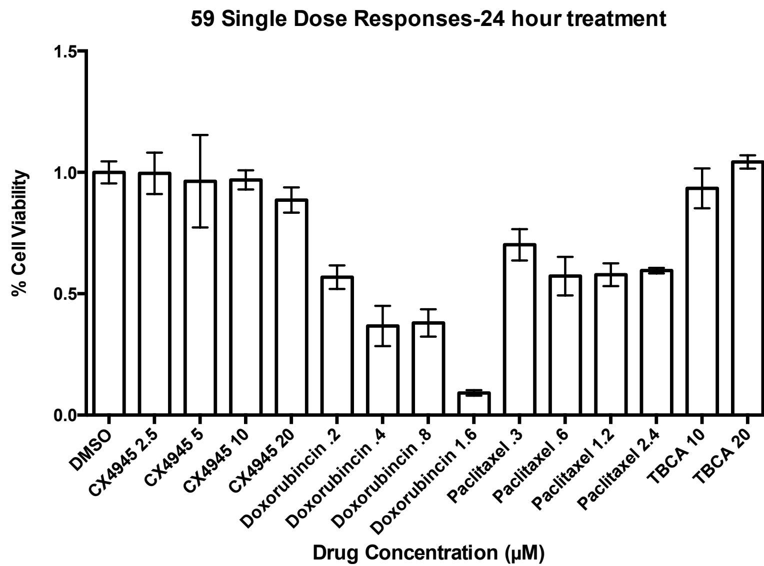


Figure 6: Single Dose Responses on 59 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on 59 cells. The graph shows single

dose response for CX4945, doxorubicin, paclitaxel, and TBCA for the indicated range of dose concentrations.

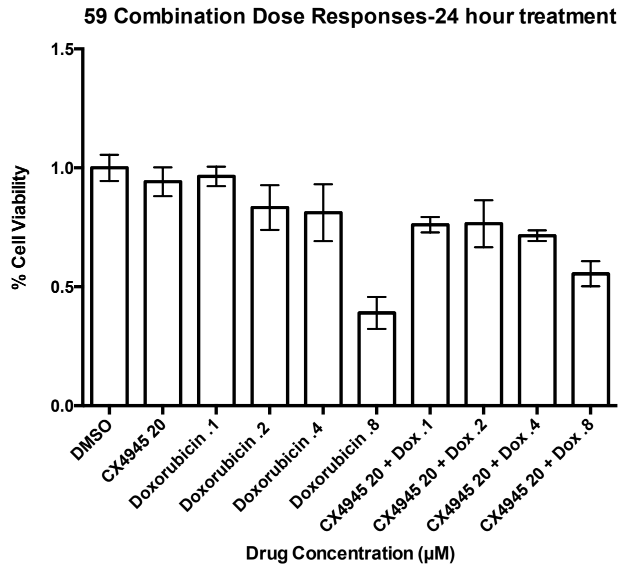


Figure 7: Combination Dose Responses on 59 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on 59 cells. The graph shows single cell viability results for a range of CX4945 doses and single doses of doxorubicin as well as results from CX4945 combined with doxorubicin.

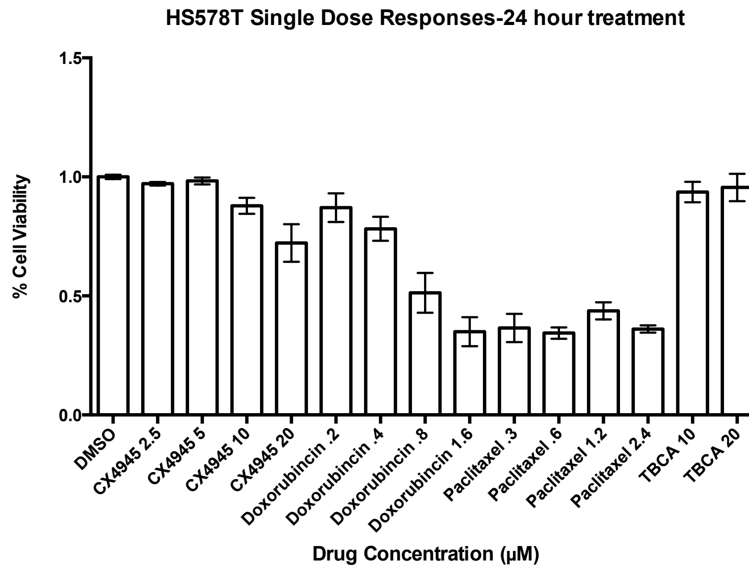


Figure 8: Single Dose Responses on HS578T Cells. Data was obtained by performing Cell Titer Blue cell viability assay on HS578T cells. The graph shows

single dose response for CX4945, doxorubicin, paclitaxel, and TBCA for the indicated range of dose concentrations.

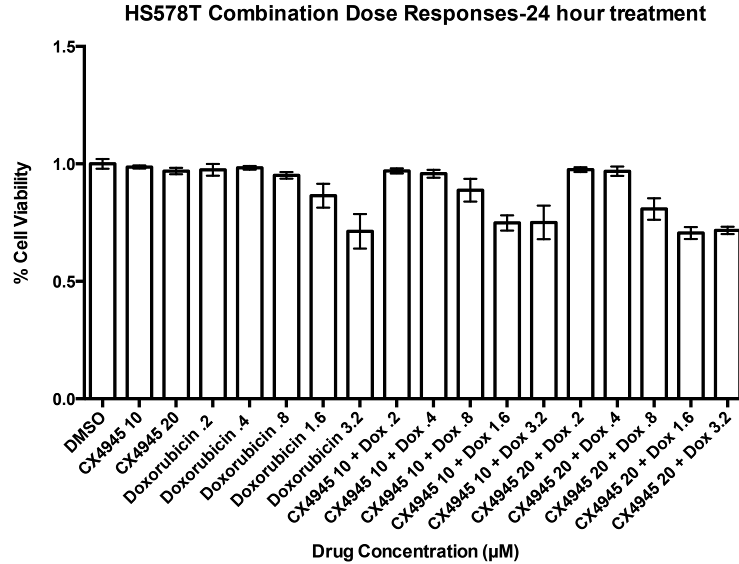


Figure 9: Combination Dose Responses on HS578T Cells. Data was obtained by performing Cell Titer Blue cell viability assay on HS578T cells. The graph shows single cell viability results for a range of CX4945 and doxorubicin as well as results from CX4945 combined with doxorubicin.

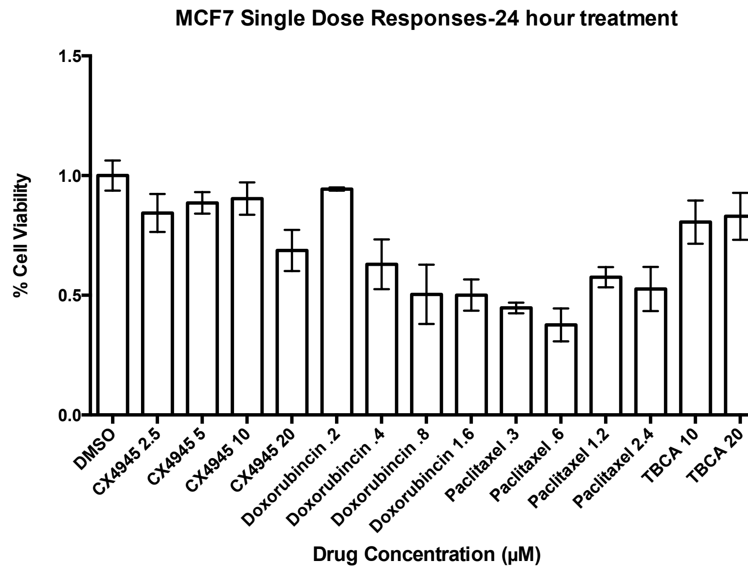


Figure 10: Single Dose Responses on MCF7 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on MCF7 cells. The graph shows single dose response for CX4945, doxorubicin, paclitaxel, and TBCA for the indicated range of dose concentrations.

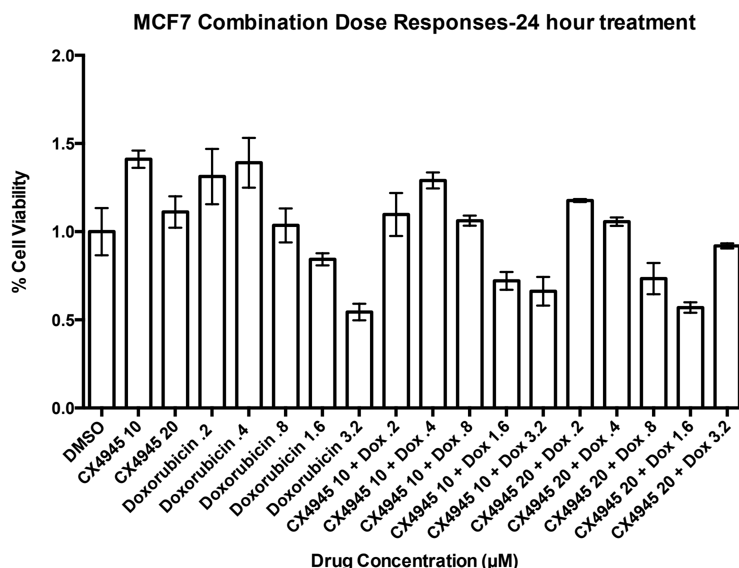


Figure 11: Combination Dose Responses on MCF7 Cells. Data was obtained by performing Cell Titer Blue cell viability assay on MCF7 cells. The graph shows single cell viability results for a range of CX4945 and doxorubicin as well as results from CX4945 combined with doxorubicin.

Quantification of Synergy: CK2 Inhibition with Chemotherapeutic Agent

With the results of the combination dose experiments, synergy was calculated using the Chou-Talalay method between CK2 inhibition and doxorubicin (Chou, 2010).

7367 Cell Line**:**

In the 7367 cell line, we found that a CX4945 dose of between 5 and10 μM killed about 50% of the cell lines as compared to the DMSO control. We showed that this particular cell line was fairly sensitive to the CK2 inhibitor, CX4945. However, 7367 is shown to be much more resistant to TBCA, with doses of 10 and 20 μM not having much cytotoxicity. In investigation of the

chemotherapeutic agents, we found that doxorubicin was very effective in killing this cell line. Paclitaxel on the other hand showed plateau effect through a range of dose concentrations. The single dose experiments were done to obtain a dose dependent manner of killing, which could then be applied to a combination dose experiment. To investigate the role of synergy, we then picked varying doses of chemotherapeutic agent and CK2 inhibitor. Figure 16 shows the synergy values of each triplicate of each combination dose. As explained earlier, a synergy value of 1 is an additive effect, greater than 1 is an antagonistic effect, and less than 1 is a synergistic effect. As shown, the combination dose values on 7367 cells display a pattern of additive effect when treated with a combination of chemotherapeutic drug and CK2 inhibitor.

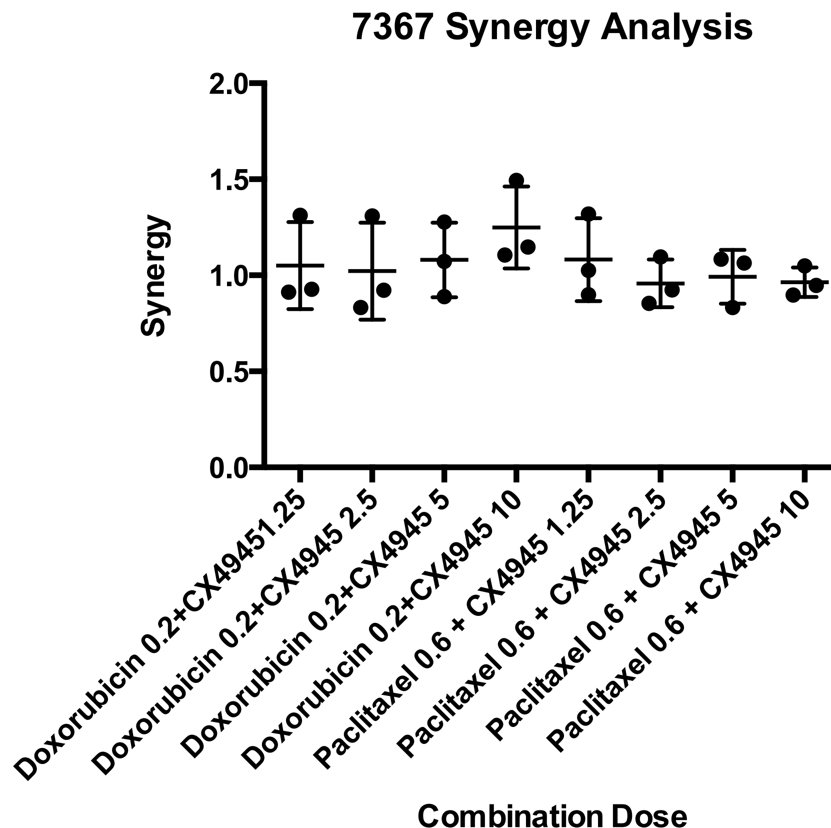


Figure 12: Synergy Values For Combination Doses – 7367 Cell Line. These data are calculated values of synergy for the combination dose results on 7367 cell line.

6604 Cells:

In the 6604 cell line, we found that a CX4945 dose range of between 2.5-20 µM did display a slight pattern of dose dependent killing, however, even in this range we were not able to obtain an IC50 for CX4945. Similarly, TBCA did not have much effect either on this cell line, suggesting that 6604 cells were fairly resistant to CK2 inhibitor's cytotoxicity. In investigation of the chemotherapeutic agents, we found that doxorubicin was very effective in killing this cell line.

Paclitaxel, again, showed plateau effect through a range of dose concentrations. Figure 17 shows the synergy values of each triplicate of each combination dose. Because of the repeat ineffectiveness of Paclitaxel in obtaining a dose dependent killing, it was abandoned in following combination experiments. As shown, there does not seem to be any unifying theme of synergy in this cell line. It could be argued, despite the variability, that at lower concentration of doxorubicin at the given dose of CX4945 there is some synergy. This cell line could potentially be more sensitive to lower doses of chemo agent coupled with a CK2 inhibitor.

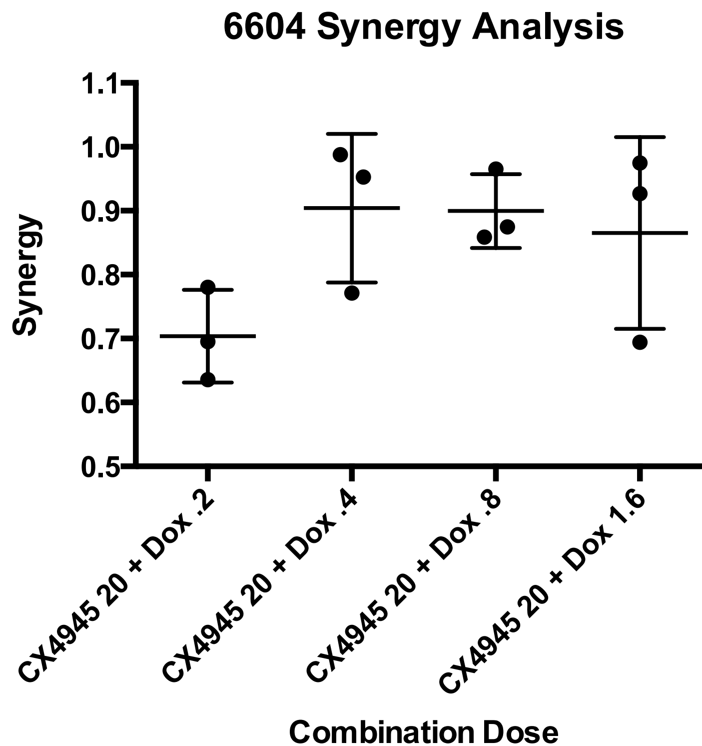


Figure 13: Synergy Values For Combination Doses – 6604 Cell Line. These data are calculated values of synergy for the combination dose results on 6604 cell line.

59 Cells:

In the 59 cell line, we found a similar occurrence as in 6604, in that, CX4945 dose range of between 2.5-20 μM did display a slight pattern of dose dependent killing, however, even in this range we were not able to obtain an IC50 for CX4945. Similarly, TBCA did not have much effect either on this cell line, suggesting that 59 cells were fairly resistant to CK2 inhibitor's cytotoxicity. In investigation of the chemotherapeutic agents, we found that doxorubicin was very effective in killing this cell line. Paclitaxel, again, showed a plateau effect through a range of dose concentrations. Figure 18 shows the synergy values of each triplicate of each combination dose. Because of the repeat ineffectiveness of Paclitaxel in obtaining a dose dependent killing, it was abandoned during the combination experiment. Because of the reproduction of results with paclitaxel in this cell line as well as 6604, its use was no longer warranted. In the case of the 59 cell line, there does seem to be a unifying pattern of synergy. At higher doses of doxorubicin, at a given concentration of CX4945, there seems to be less synergy. This is similar to 6604, in that the lower concentration of chemo agent used did show some level of synergy.

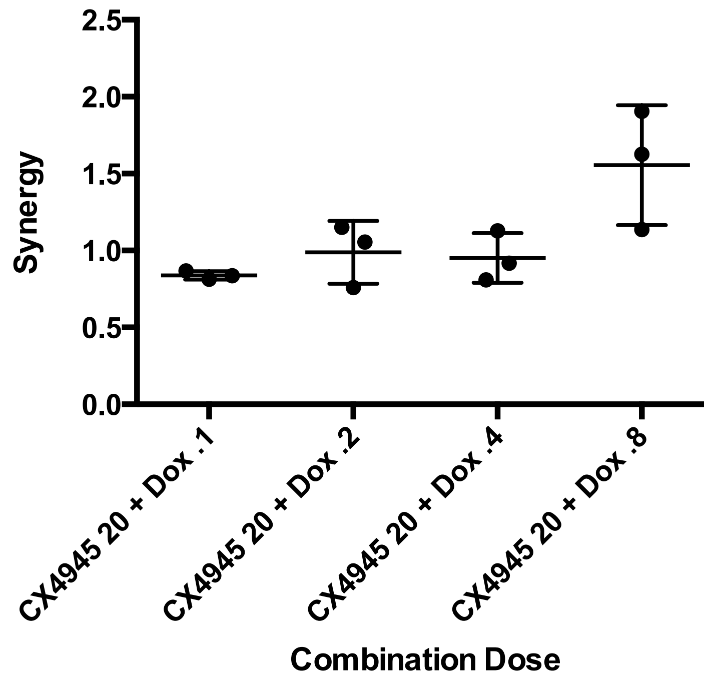


Figure 14: Synergy Values For Combination Doses – 59 Cell Line. These data are calculated values of synergy for the combination dose results on 59 cell line.

HS578T Cells:

In the HS578T cell line, CX4945 was a much more potent killer than the three mouse breast cancer cell lines used. With the same range of concentrations used as in the mouse breast cancer cell lines, 2.5-20 μM, we were closer at finding an IC50 for CX4945. However, similar to the mouse cell lines, TBCA did not have much effect, suggesting that this cell line was also partially resistant to TBCA. In investigating the chemotherapeutic agents, we found that doxorubicin was very effective in killing this cell line. Paclitaxel, again,

showed a plateau effect through a range of dose concentrations. Figure 19 shows the synergy values of each triplicate of each combination dose. As before, because of the repeat ineffectiveness of Paclitaxel in obtaining a dose dependent killing, it was abandoned during the combination experiment. In examination of the synergy in this human cell line, an interesting observation can be made in the contrast in synergy when compared to the mouse cell line. In this case, at a given concentration of CX4945, there seems to be more synergy at increasing concentration of doxorubicin. Despite the variability at the 3.2 μM concentration of doxorubicin, this is the trend that seems to be happening here.

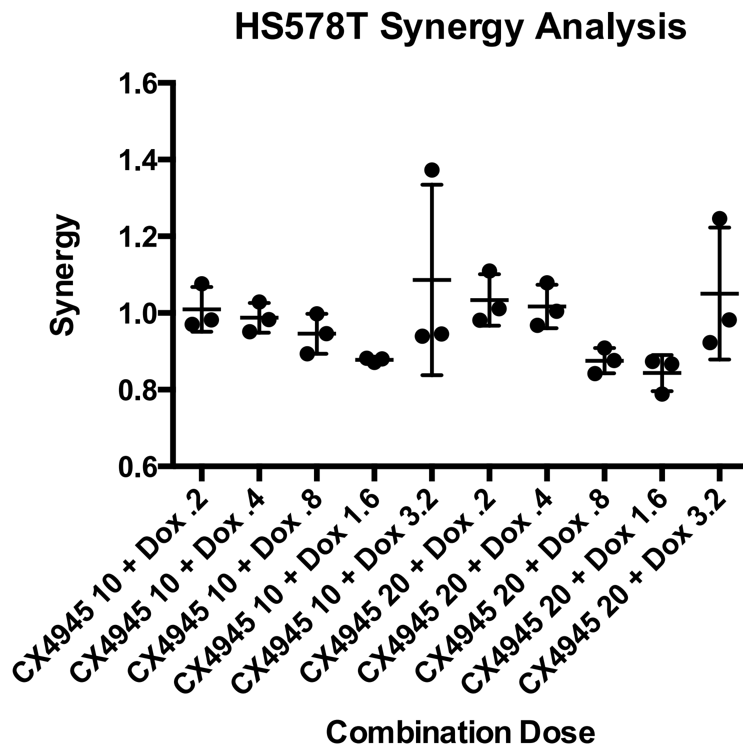


Figure 15: Synergy Values For Combination Doses – HS578T Cell Line. These data are calculated values of synergy for the combination dose results on HS578T cell line.

MCF7 Cells:

In the MCF7 cell line, CX4945 showed variable results in killing between the 2.5-20 μM ranges. What is interesting about this cell line, is that TBCA was much more effective in cell killing. Though, there were variable results at the 10 and 20 μM concentrations used, there was more killing here than seen in the HS578T human cell line and the mouse cell lines. In investigation of the chemotherapeutic agents, we found that doxorubicin was very effective in killing this cell line. Paclitaxel, again, showed plateau effect through a range of dose concentrations. Figure 20 shows the synergy values of each triplicate of each combination dose. Because of the repeat ineffectiveness of Paclitaxel in obtaining a dose dependent killing, it was abandoned during the combination experiment. Investigating the synergy values for this cell line, a confirmation can be made in the potency of combination therapeutics in comparison with human and mouse cell lines. Like HS578T, there is more synergy in the combination doses with higher concentrations of doxorubicin, despite the outlier at doxorubicin 3.2 μM coupled with CX4945 20 μM.

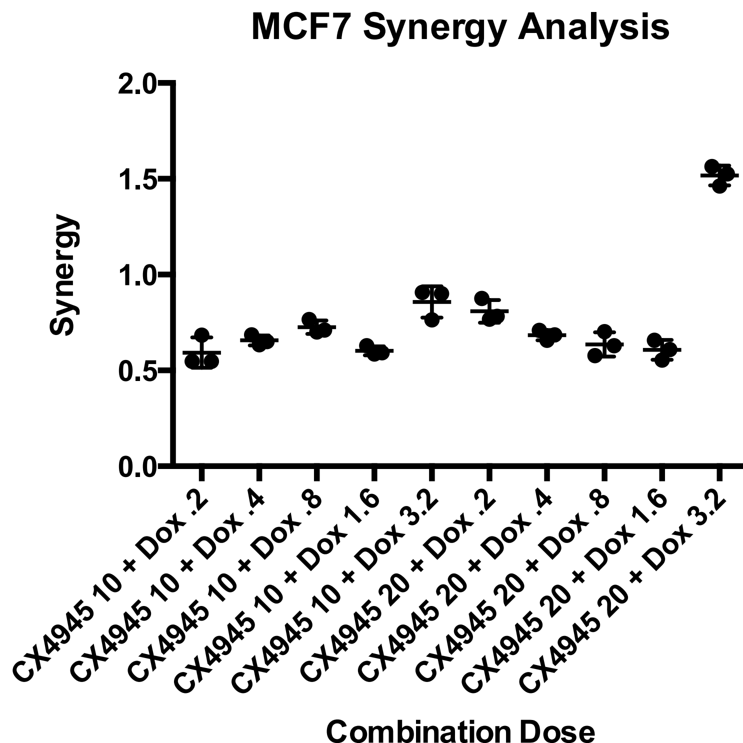


Figure 16: Synergy Values For Combination Doses – MCF7 Cell Line. These data are calculated values of synergy for the combination dose results on MCF7 cell line.

CK2 Activity by Peptide Kinase Assay Testing the Potency of TBCA:

To reassess the potency of the CK2 inhibitor TBCA, a specific CK2 peptide kinase assay was performed using recombinant CK2 protein (Figure 12). This was done to rule out the possibility of the drug either degrading in culture or there being a cell uptake issue. CX4945 at a concentration of 20 μM was used as a positive inhibitor control. The results show that TBCA does indeed inhibit CK2 compared to the DMSO control, just as effectively as CX4945 at 10 and 20 μM.

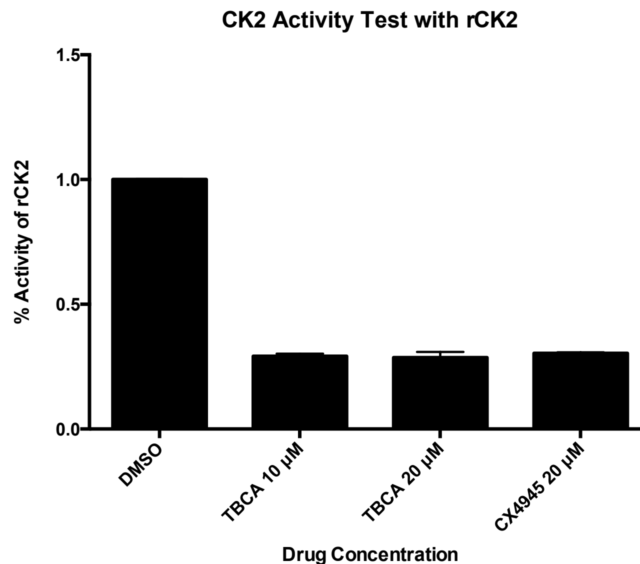


Figure 17: Recombinant CK2 (rCK2) Activity with TBCA and CX4945 Treatment. The following data was obtained by performing a CK2 kinase peptide assay on recombinant CK2 protein. These data shows CK2 activity reduction with inhibitor TBCA at two concentrations as well as CK2 activity reduction with CX4945 used as a positive control.

CK2α siRNA Transfection and Doxorubicin Dosage:

To confirm the validity of the single and combination dosages in the cell viability assays performed on the given cells lines we took an alternative approach to CK2 inhibition. A CK2α siRNA transfection was performed on 7367 cells simultaneously with doxorubicin treatment (Figure 12). 7367 cells were transfected with a siRNA control (Luciferase) as well as siRNA CK2α. Each was also dosed with increasing concentrations of doxorubicin. A dose dependent pattern of cytotoxicity was achieved on the blank, luciferase control, as well as the CK2α transfected cells. Through these results we observed that the cells transfected with CK2α siRNA had decreased killing in combination with

doxorubicin compared to the control. The combination of the CK2 inhibition coupled with doxorubicin also showed to be protective against the cells and from this we were able to conclude that a synergistic effect could not be attained.

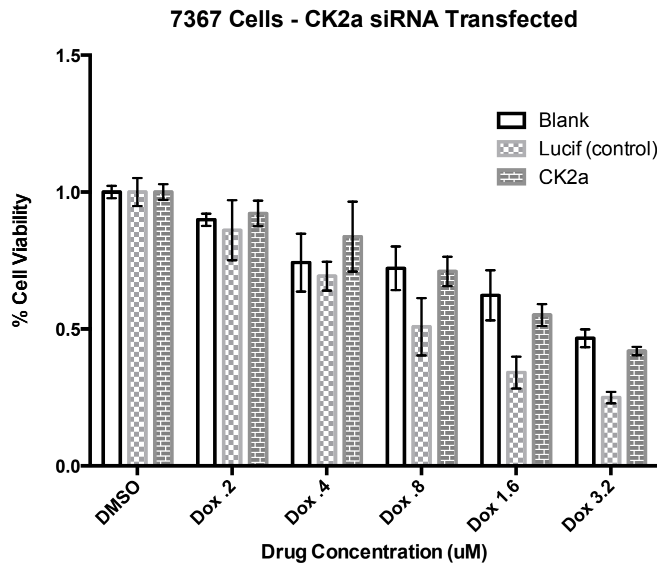


Figure 18: Doxorubicin Treatment on CK2α siRNA Transfected 7367 Cells. The following data was obtained by performing Cell Titer Blue cell viability assay on 7367 cells transfected with siRNA to knockdown the CK2α protein. The graph shows the dose response with a range of concentrations of doxorubicin on the transfected cells as well as Luciferase as a control.

RT-qPCR analysis:

From the previous experiments, we showed that a synergistic effect was not achieved in using CK2 inhibition and doxorubicin. However to start to elucidate a role of CK2 in CSC's, epithelial to mesenchymal transition, CK2 regulation, and to validate the potency of CK2 inhibition by siRNA, we measured gene expression levels of selected genes in HS578T cells inhibited by CK2. To examine the mRNA levels of stem cell marker genes, EMT marker genes, and the CK2α, α', and β genes, RT-qPCR was performed on cDNA from HS578T

cells, which were transfected for (1) CK2α siRNA, (2) CX4945 5 μM and TBCA 20 μM for 48 hours, and (3) CX4945 10 μM for 24 hours.

To examine a potential link between CK2 and CSCs, several known stem cell marker genes were examined in the human breast cancer cell line HS578T. We wanted to examine whether CK2 inhibition, either achieved with a chemical inhibitor or by interference at the transcriptional level, would impair the ability of this cell line to maintain its stem-like cell population. This stem-like population typically comprises 0.3-4% of the total cell number as measured by ALDH positivity with the Aldefluor assay (Lohberger et al. 2012). If this were the case, we would preliminarily conclude that CK2 plays a role in the stem cell like phenotype of this cell line.

Cyp1b1 is a marker that shows activation of the Ahr signaling pathways and has recently been shown to be important in stem-like cell phenotypes in breast cancer (Yang et al. 2008; Han et al. 2010). When inhibited by CK2 we see inconsistent activation of Cyp1b1. The control siRNA induced Cyp1b1 about 5 fold; there was no change in its expression upon siRNA for CK2α. Furthermore, inhibition with 5 μM CX4945 for 24hours was more potent than 10 μM for 48 hours, while TBCA had no effect on the expression of Cyp1b1. With no clear pattern of change, we concluded that the inhibition of CK2 had no clear effect on the expression of Cyp1b1 in this model system.

SOX2 is a transcription factor that is known to interact with other proteins to regulate several stem cell differentiation factors and is a key regulator of

pluripotency (Boyer et al. 2005; Zhang et al. 2012). A recent study showed that SOX2 expression is significant in the early stages of breast tumor development and that SOX2 is a promoter of mammosphere development (Leis et al. 2011). When inhibited by siRNA for CK2α, TBCA 20 μM for 48 hours, and CX4945 10 μm for 24 hours, only minor effect were seen in the expression of SOX2. Similarly to Cyp1b1 however, upon inhibition with CX4945 5 μM for 48 hours there was a 5-fold change decrease in the expression of SOX2. Again, no clear pattern of change in expression was established for SOX2 in this given model system.

STELLA is a protein that is critical for pluripotency, oocyte development, and development through implantation (Saitou et al. 2002). Another study found elevated levels of STELLA in breast cancer tissues as well as the MCF7 cell line, which further promotes the idea that there are cells that exhibit stem-like characteristics (Ezeh et al. 2005). The control siRNA inducted STELLA slightly less than 5-fold while knockdown of CK2α was able to induce STELLA slightly more than 5 fold. CK2 inhibition with TBCA 20 μM for 48 hours and CX4945 10 μM for 24 hours had no effect on the induction of STELLA. Again, CX4945 5 μM for 48 hours had the greatest effect at inducing the gene with over 5-fold change decrease in expression. No clear pattern of change in expression of STELLA was seen in this model, however.

NANOG is another protein that is involved in embryonic development and stem cells. This protein was also highly expressed in breast cancer tissue and

breast cancer cell lines (Ezeh et al. 2005). The control siRNA, siRNA for CK2α, TBCA 20 μM for 48 hours, and CX4945 10 μM for 24 hours had only minor effects in the induction of NANOG. Again, CX4945 5 μM for 48 hours was able to induce NANOG expression about 4-fold, more than any of the other model conditions. There was however no clear pattern of change in the expression of NANOG in this model.

OCT4 is also shown to be elevated in breast cancer tissue and breast cancer cell lines (Ezeh et al. 2005). The control siRNA, siRNA for CK2α, CX4945 5 μM for 48 hours, and TBCA 20 μM for 48 hours had no effect on the expression of OCT4. In this case, CX4945 10 μM for 24 hours showed only slightly greater induction of OCT4 than the other scenarios of CK2 inhibition. No clear pattern of change in the expression of OCT4 was established in this model.

RUNX1 is crucial in hematopoiesis and has been implicated as a tumor suppressor gene in breast cancer (Janes, 2011). In this case, the control siRNA, siRNA for CK2α, CX4945 5 μM for 48 hours and 10 μM for 24 hours, and TBCA 20 μM for 24 hours had only minor induction effects as compared to the DMSO. No clear pattern of change in expression of RUNX1 was established with the given conditions.

MSI is highly expressed in stem cells of nervous tissue and the CNS progenitor cells and more recently has been implicated in the growth of multiple types of cancers, including breast tumors (Good et al. 2008; Glazer et al. 2012). The control siRNA inducted MSI about 4 fold; there was no change in its

expression upon siRNA for CK2α. Furthermore, inhibition with 5 μM CX4945 for 48 hours was more potent than either 10 μM for 24 hours or TBCA 20 μM for 24 hours on the expression of MSI. No clear pattern of change in expression of MSI was established with the given conditions.

Our results show that stem cell marker genes had a fold increase or decrease from control of no greater than 5-fold with no unifying pattern among all the markers (Figure 13). We were able to conclude that in the given model system, no clear pattern of gene expression change of stem cell markers was achieved with CK2 inhibition and no clear conclusion can be made on the maintenance of HS578T's stem-like population.

CK2 has been shown to play an important role in EMT in breast cancer (Deshiere et al. 2013, and Seldin lab, unpublished). Several different EMT marker genes were examined in HS578T cells (Figure 14). The same cDNA from HS578T cells, treated for CK2 inhibition as outlined above, was then tested for its expression for the EMT markers TWIST1, TWIST2, SNAIL1, SLUG, TGF-β, FN1, and VIM. A general trend of inhibition of EMT markers was noticeable with CK2 inhibition.

With markers, TWIST1 and SNAIL1 there was a general pattern of decrease in expression with CK2 inhibition with chemical inhibitor or inhibition at the transcriptional level. CX4945 10 μM for 24 hours had the greatest effect in decreasing expression of TWIST1, while TBCA 20 μM for 48 hours had the greatest effect in decreasing expression of SNAIL1.

With TWIST2, there was also a general pattern of decrease in expression with CK2 inhibition. In this case however, the control siRNA decreased expression almost 10 fold, greater than any of the other conditions. The siRNA for CK2α, CX4945 5 μM and 10 μM, and TBCA 20 μM for 48 hours decreased expression of TWIST2 almost 5 fold.

SLUG and FN1 showed similar patterns of expression with CK2 inhibition and showed a decrease in overall expression. The control siRNA decreased expression almost 6-fold for SLUG and 4-fold for FN1. The siRNA for CK2α decreased expression slightly higher than 2 fold for each gene. CX4945 5 μM for 48 hours and TBCA 20 μM for 48 hours decreased expression of both genes almost 2 fold, however, CX4945 10 μM for 24 hours decreased expression slightly higher than 6-fold for SLUG and 4-fold for FN1.

TGF-β and VIM showed similar patterns of expression. The siRNA control showed the greatest decrease in expression at almost 3 fold. The siRNA for CK2α had no effect on either gene. The TBCA 20 μM treatment for 48 hours and CX4945 10 μM treatment for 24 hours were able to decrease expression between up to 2 fold. CX4945 5 μM for 48 hours however had no effect on either gene.

To validate the effectiveness of the siRNA inhibition and to explore possible effects of CK2 inhibition on the regulation of CK2, we examined the effect of CK2 chemical inhibition and interference of the alpha subunit on its transcriptional regulation. RT-qPCR was performed on all three CK2 subunit

genes with the reverse transcribed RNA obtained as outlined above (Figure 15). We found that for the α' and β subunits, there is a variable effect when inhibiting with siRNAs or CK2 inhibitors. For the alpha prime subunit, the control siRNA, siRNA for CK2α, and CX4945 5 μM and 10 μM, and TBCA 20 μM had no effect on the expression. The control siRNA, siRNA for CK2α, and CX4945 and TBCA treatments, had no effect on the beta subunit. The α subunit, however, showed drastic changes in its expression with CK2 inhibition. While the control siRNA had very little effect on the expression of the alpha subunit, as expected the treatment with siRNA for CK2α showed an almost 10-fold reduction in expression, indicating that the CK2 siRNA worked as intended. Surprisingly, chemical inhibition with CX4945 5 μM for 48 hours increased expression by about 10-fold, TBCA 20 μM for 48 hours increased expression almost 30-fold, and CX4945 10 μM for 24 hours increased expression almost 40-fold. This suggests that there might be a compensatory feedback loop for the transcription of the alpha subunit. When the alpha subunit's transcription is impaired, in our case by specific knockdown of the alpha subunit, it may signal to ramp up its own transcription, perhaps in order to maintain a constant cellular level of the mRNA.

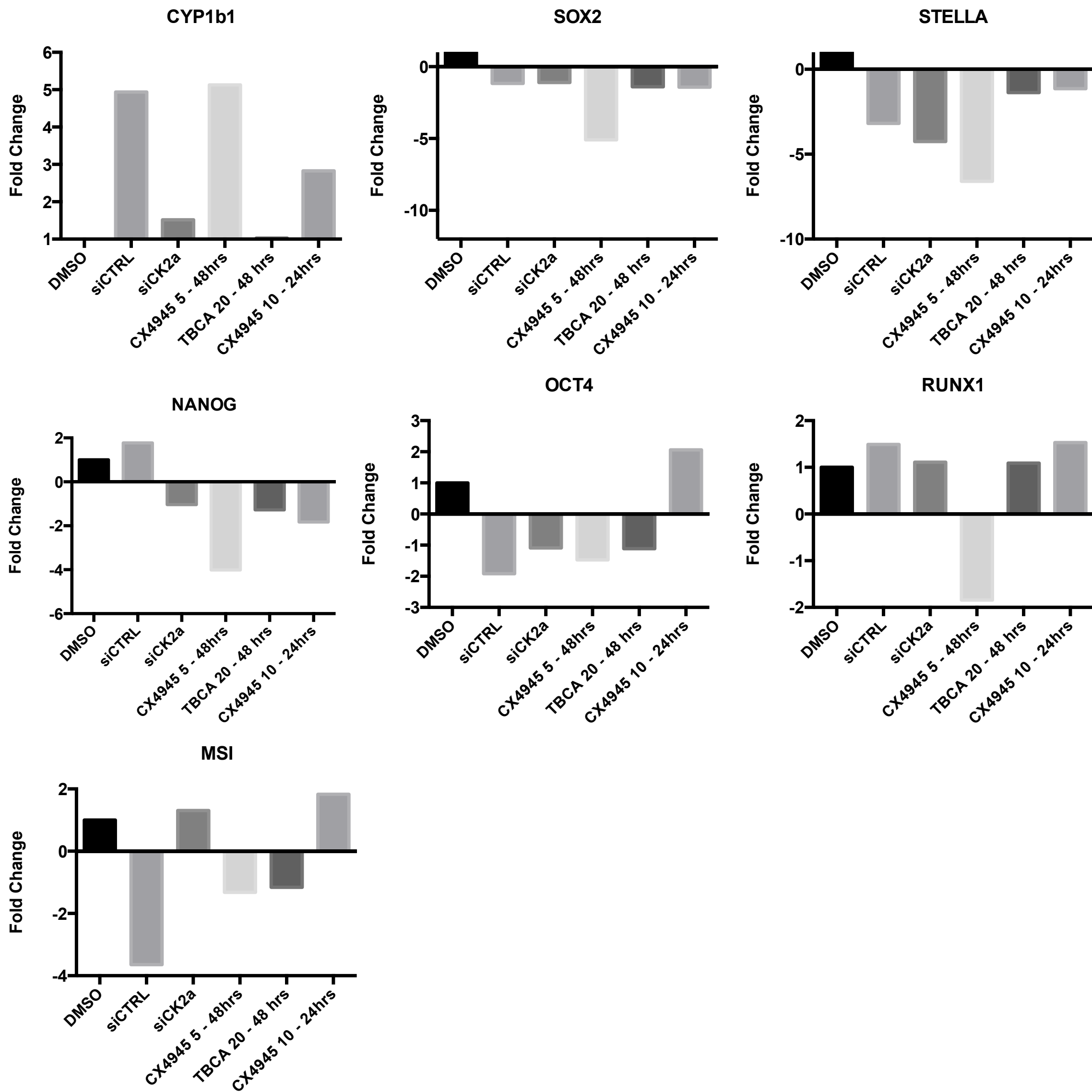


Figure 19: RT-qPCR Stem Cell Marker Fold Change on HS578T Cells. The following data shows fold change from control (DMSO) on HS578T with siRNA treatments as well as CX4945 and TBCA treatments for both 24 and 48 hours on known stem cell markers. Results were normalized against GAPDH as a positive/loading control.

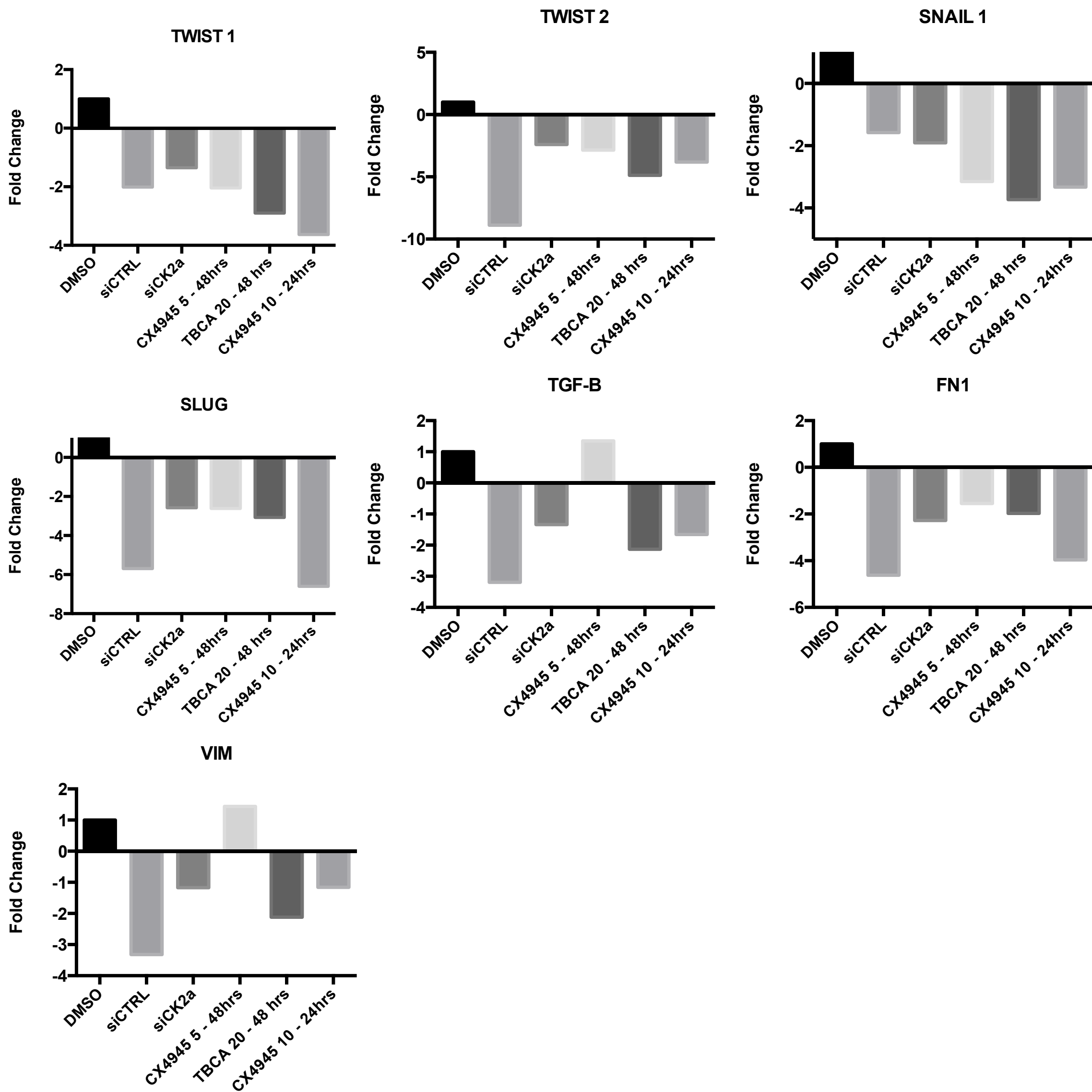


Figure 20: RT-qPCR EMT Marker Fold Change on HS578T Cells. Data show fold change from control (DMSO) on HS578T with siRNA treatments as well as CX4945 and TBCA treatments for both 24 and 48 hours on various known EMT markers as indicated. Results were normalized against GAPDH as a positive/loading control.

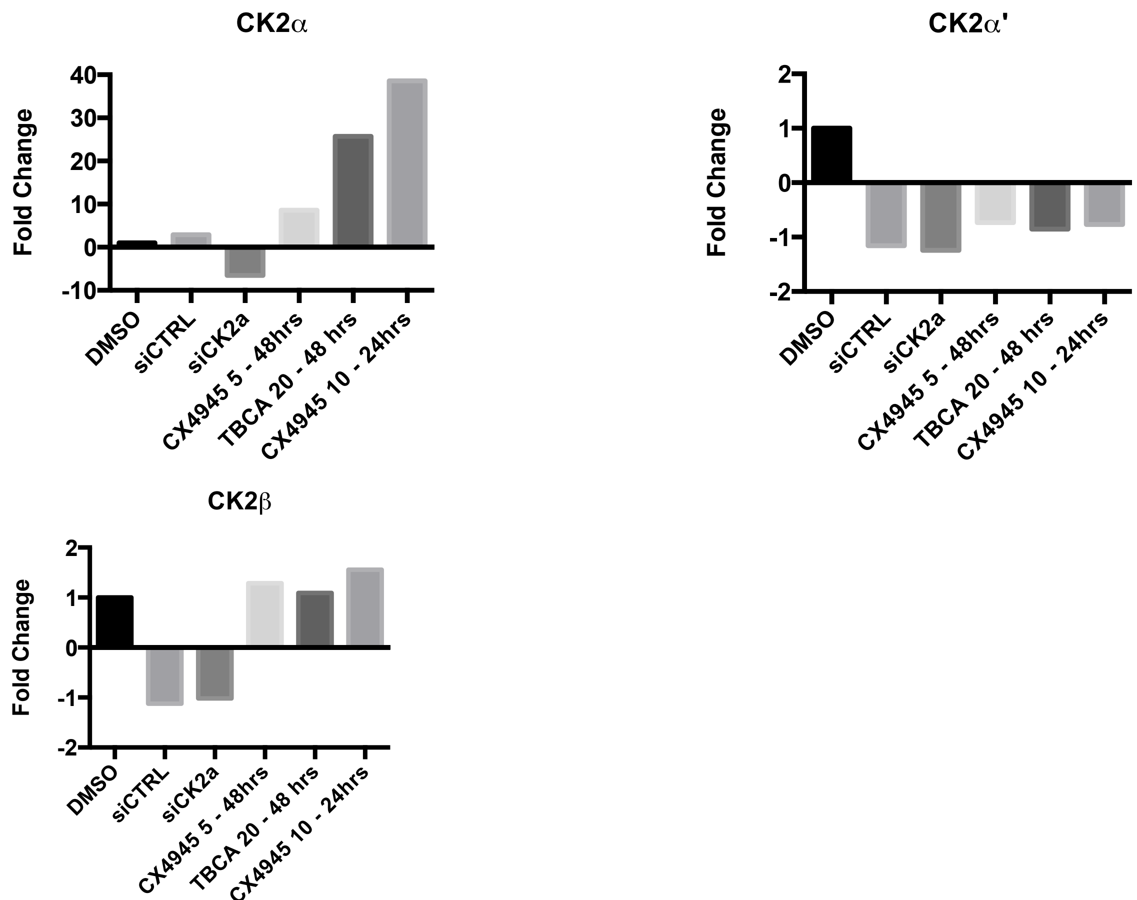


Figure 21: RT-qPCR CK2 Genes Fold Change on HS578T Cells. Data show fold change from control (DMSO) on HS578T with siRNA treatments as well as CX4945 and TBCA treatments for both 24 and 48 hours on the CK2α, α', and β genes. Results were normalized against GAPDH as a positive/loading control.

Western Blot:

With the unexpected result suggesting a feedback loop on the CK2α subunit gene expression upon chemical inhibition, we wanted to further investigate this effect at the total protein level of CK2. We performed a western blot on the HS578T human breast cancer cell line with the given treatments: siRNA control, siRNA for CK2α, and a range of concentrations of CX4945, from 2.5-20 μM (Figure 16). Actin was used as a loading control. We found that when

knocking down CK2α using siRNA, there is no visible band, as expected; the control siRNA does show a band for CK2α. Interestingly, there seems to be a trend to an increase in CK2α protein levels with higher doses of CX4945. This does correlate with our results on the CK2 subunit gene expression using siRNA and chemical inhibitors of CK2. This further suggests that there is a compensatory feedback loop for the regulation of CK2.

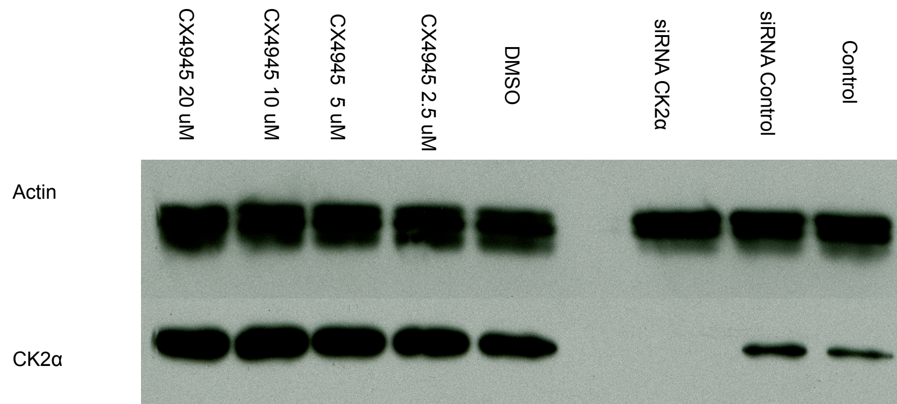


Figure 22: Western Blot Analysis HS578T Cells. The following shows western blot on HS578T human breast cancer cell line with siRNA and CX4945 treatments. A range of 2.5-20 μM for CX4945 was used. Actin was used as a loading control.

DISCUSSION

The purpose of this study was to investigate whether CK2 inhibition could be utilized in breast cancer therapy. The major findings in this study were (i) CK2 inhibition does not synergize with chemotherapeutics for enhanced killing of breast cancer cells, (ii) CK2 inhibition with siRNA results in an antagonistic effect when coupled with chemotherapeutics, (iii) CK2 inhibition, obtained either by CK2 inhibitors or CK2α siRNA, does not result in reduced "stemness" in the HS578T human breast cancer cell line, (iv) CK2 inhibition results in a slight reduction of EMT marker expression in HS578T, and (v) CK2 inhibition with the chemical inhibitors results in upregulation of CK2α mRNA. Levels of the mRNA for the CK2α' and CK2β subunits were unchanged. This could explain why the chemical inhibitors are ineffective, and in fact there was some antagonism of killing with CK2 inhibitors and chemotherapy. Future studies would best be focused on optimizing siRNA inhibition. Despite the limited scope of this study and the negative results on synergy of CK2 inhibitors with cancer drugs, some interesting conclusions can be drawn.

In investigating the potency of the CK2 inhibitors, CX4945 and TBCA, it was found that CX4945 was a much more effective inhibitor of CK2 at lower concentrations than TBCA, on cells. However, when the potency of TBCA was tested on recombinant CK2, TBCA and CX4945 were able to inhibit the protein equally. Since both drugs are ATP-competitive inhibitors, the difference in potency on cells is puzzling. Experiments to test the inhibition potential of

CX4945 and TBCA were repeated several times and consistent results were obtained, with CX4945 being much more potent, at lower concentrations. Possible explanations of this could be that TBCA is not stable in tissue culture medium. Another possibility is that TBCA is not able to enter cells as efficiently as CX4945, a permeability issue. Lastly, drugs can be pumped out of cells differentially after they get in.

Consistent with these observations, we found that CX4945 was more effective in killing breast cancer cells than TBCA. Therefore, CX4945 was chosen in the combination experiments. The doxorubicin and paclitaxel were both able to kill breast cancer cells, however it was only with the doxorubicin that a dose dependent manner of killing was reproduced consistently. It could be hypothesized that this could be due to the different mechanisms of action of the two drugs. Doxorubicin is an intercalating agent where as paclitaxel is a microtubule poison—the breast cancer cells could be more sensitive to killing by the former mechanism.

In this study, we were not able to obtain synergistic killing with CK2 chemical inhibitors and doxorubicin. In most cases, there was only an additive effect between the two drugs. Furthermore, when utilizing CK2 inhibition at the transcriptional level with siRNA, we obtained a drastic antagonistic effect between CK2 inhibition and doxorubicin.

Others have reported that CK2 inhibition, either in combination with chemotherapeutic drugs or alone, can kill cancer cells. Recently, CX4945 was

shown to exhibit antitumor, antiproliferative activity, and to inhibit angiogenesis in tumor and endothelial cells; in addition CX4945 was accepted into clinical trials to treat a range of different cancers (Siddiqui-Jain et al. 2010). Additionally, another study demonstrated the ability of CX4945 ability to overcome drug resistant cells and synergize with vinblastine, a microtubule drug used to treat different types of cancers (Zanin et al. 2012). Since CK2 has also been implicated in other types of cancers, there has been research into therapeutic responses using CK2 inhibition in lung cancers, glioblastoma, and hematologic malignancies. One of the main motivations for conducting our study were the synergy experiments with CK2 inhibition on chronic lymphocytic leukemia and hematologic malignancies, (Prins et al. 2013). Additionally, CK2 inhibition was seen to synergize with the drug bortezomib in treating multiple myeloma and mantle cell lymphoma (Manni et al. 2013). Another cancer that CK2 inhibition could have promising therapeutic effects in is glioblastoma. CK2 is overexpressed and contributes to the tumorigenesis of glioblastoma and could potentially be used in the treatment of the cancer (Ji and Lu, 2013; Zheng et al. 2013). The most likely explanation for our differing results is the different cancer model we used.

In this study we also examined the role of CK2 in cancer stem cells and EMT. We were not able to see a reduction of the "stemness" of the human breast cancer cell line, HS578T, by measuring it with stem cell markers mRNA expression. Recently, Zhang and group published a study showing the effects of CK2 inhibition on the stem-like population in human lung cancer cells. They

showed that CK2 is a positive regulator of Hh/Gli signaling, and when CK2 is inhibited at the transcriptional level, it down regulates the pathway (Zhang et al. 2012). This is especially important because of the role the Hh pathway places in the preservation of CSCs. CK2 could thus play a role in the upkeep of the stem-like population, and though we were not able to produce results that correlated with its involvement, there could be ways to further investigate it. For example, preliminary results from our lab show reduced ALDH positivity on several breast cancer cell lines upon CK2 inhibition. These results indicate an effect of CK2 on the maintenance of the stem cell population. Our results need to be repeated on the HS578T cell line as well as others breast cancer cell lines. Additionally, more stem cell markers could be utilized.

The promising aspect of our study came in the investigation of CK2 and EMT in HS578T cells. We were able to produce results that showed the effect of CK2 inhibition, with chemical inhibitors and at the transcriptional level, on the pattern of reduction of EMT marker expression. Recently, CK2 inhibition, using CX4945, was shown to inhibit several key pathways that regulate EMT, such as: Wnt, Smad and non-Smad TGFbeta pathways, and focal adhesion pathways (Kim and Hwan Kim, 2013). Though our study was not as comprehensive, we were able to show similar effects on other EMT markers with CK2 inhibition. Certainly, these experiments would need to be repeated to show reproducibility, and expanded to include other EMT assays, but CK2 appears to play a role in EMT progression at least of one breast cancer cell line. Given the importance of

EMT in invasive cancers, it would be very promising if CK2 could be utilized to block this property of cells.

Despite the shortcomings of this study, many future directions were established to investigate the role of CK2 as a clinically relevant target for breast cancer. Firstly, in the scope of this experiment, synergy between CK2 inhibition and chemotherapeutics could be further investigated. One limitation of this study was the use of a 24-hour time point in the treatment of cells. In the future, different time points could be studied and in different combinations of drugs. For instance: CK2 inhibition could be allowed to go on for 24 hours while subsequently treating with chemotherapeutic agents for 48 hours. Different combinations of time points could be utilized for a more comprehensive study. However, ultimately given our results that CK2 inhibition with chemical drugs results in more CK2 being made, future studies should be directed at utilizing CK2 siRNA with varying doses and schedules of chemotherapy, and varying cell lines.

The feedback upregulation of CK2 mRNA and protein occurring in the presence of chemical inhibitors of CK2 is a novel and particularly interesting observation made in this study. This opens the door to the development of a new line of investigation studying the self-regulation of CK2. If the regulation of CK2 could be further elucidated, it might allow better use of the therapeutic potential of CK2 inhibitors in disease. It might also shed light on the role of CK2 in development.

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