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Anticancer Effects and Mechanisms of CFI-400945 and Radiation in Breast Cancer

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Abstract

Breast cancer is a leading cause of death in women and development of new treatments is essential. Polo-like Kinase 4 (PLK4) controls centriole duplication and its inhibition by CFI-400945 induces genomic instability and aneuploidy. Radiation therapy (RT) also induces aneuploidy leading to cell death, although development of radioresistance is common. We hypothesized that CFI-400945 and RT act synergistically in breast cancer. Colony formation assays identified synergistic anticancer effects of CFI-400945 and RT, with combinatorial effects also observed for RT with either siRNA inhibition of PLK4 or with the PLK4 inhibitor Centrinone B. This suggests that the antiproliferative effect of these combinations are, at least partly, mediated through PLK4. Immunocytochemistry for Centrin showed significant overamplification of centrioles in combination compared to single agent treatment, suggesting a possible combined mechanism of action. These results support further translational studies of CFI-400945 and RT as a combination treatment to improve breast cancer outcomes.

Keywords

Breast Cancer, Radiation, CFI-400945, Combination Therapy, Patient-Derived Organoids, Centriole Duplication, Genomic Instability, Aneuploidy.

Summary for Lay Audience

Despite recent advances in breast cancer diagnostics and treatment, poor outcomes are still frequently observed once cancer has spread, or metastasized, to distant organs. Developing new therapeutic strategies to treat advanced or metastatic disease is therefore of significant importance. A promising anti-breast cancer drug called CFI-400945 has been developed to target a protein called Polo-Like Kinase 4 (PLK4) that is involved in the regulation of cancer growth. This project investigated CFI-400945 as a combination therapy with radiation therapy in an effort to develop novel and more effective treatment strategies. We used both breast cancer cell lines and patient-derived organoids, the latter of which are clinically relevant models developed from individual breast cancer patients. We found that CFI-400945 and radiation had a much greater anticancer effect when used as a combined treatment versus individually, regardless of the various sequencing of drug and radiation treatments. CFI-400945 predominantly acts by inhibition of PLK4, but can also inhibit other proteins, such as Aurora Kinase B. By comparing results of combining CFI-400945 with radiation to combinations with other inhibitors of PLK4 or other proteins, we identified that the combination effect of CFI-400945 and radiation is due, at least partially, to the PLK4 inhibition effect, though other inhibitory effects could also be involved, including targeting of Aurora Kinase B. To examine the anticancer mechanisms of the combination of CFI-400945 and radiation, we investigated the cell machinery needed for division and tumour growth. We found that the cells treated with CFI-400945 or radiation individually had amplified centrioles (needed for proper cell division), and that this was further increased when the treatments were used in combination. By enhancing this overamplification, cell division occurred abnormally in cancer cells leading to cell death. Future studies are aimed at delving into these findings further. The results of these studies will also support the development of clinical trials and eventual clinical use of this combination to improve outcomes in breast cancer patients.

Co-Authorship Statement

This thesis was written by Sierra Pellizzari and edited by Dr. Armen Parsyan and Dr. Alison Allan. The experiments in results section 3.5 were performed by postdoctoral fellow Dr. Vasudeva Bhat. All other experiments and data analysis from this thesis were performed by Sierra Pellizzari.

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Abbreviations

AURKB – Aurora Kinase B
BME – Basement Membrane Extract
BRCA1/2 – Breast Cancer Gene 1/2
CFA – Colony Formation Assay
CFI – Campbell Family Institute
CK5/6 – Cytokeratin 5/6
Ct – Cycle Threshold
DMSO – Dimethyl Sulfoxide
EGFR – Epidermal Growth Factor Receptor
EHS – Engelbreth-Holm-Swarm Sarcoma
ER – Estrogen Receptor
FBS – Fetal Bovine Serum
HER2 - Human Epidermal Growth Factor Receptor 2
IC50 – Half Maximal Inhibitory Concentration
IFN – Interferon
K_i – Inhibition Constant
PARP – Poly ADP Ribose Polymerase
PD-1 – Programmed-death Protein-1
PD-L1 – Programmed-death Ligand 1
PDO – Patient-Derived Organoid
PDX – Patient-Derived Xenograft
PLK – Polo-like Kinase
PLK4 – Polo-like Kinase 4
PR – Progesterone Receptor
ROS – Reactive Oxygen Species
RT – Radiation Therapy
SASS6 – Spindle Assembly Abnormal Protein 6 Homolog
SF – Surviving Fraction
TIL – Tumour-infiltrating Lymphocyte
TNBC – Triple Negative Breast Cancer
TRK – Tyrosine Receptor Kinase

1 Introduction

Breast cancer, particularly its triple negative subtype, represents a significant burden on the healthcare system in Canada and worldwide[1]. Current therapeutic options for triple negative breast cancer (TNBC), especially in the metastatic setting, are limited based on the lack of known therapeutic targets expressed by these tumours, leaving general cytotoxic chemotherapy as the most commonly used treatment[2]. New therapeutic options and precision medicine approaches in which treatment plans are personalized to the individual patient, including combination treatment regimens, are of great interest. The novel drug CFI-400945, developed within the University Health Network, Toronto, Canada, inhibits Polo-like Kinase 4 (PLK4) that is often aberrantly expressed in breast cancer and is involved in centriole duplication and mitotic fidelity[3]. Inhibition of PLK4 is known to induce genomic instability and aneuploidy, leading to cell death[3]. CFI-400945 was generally well tolerated in a Phase I clinical study and has now entered Phase II studies in breast cancer[4]. Another breast cancer treatment modality, radiation therapy (RT), acts via induction of DNA damage, improper repair of which can also induce genomic instability and aneuploidy[5]. It is hypothesized that combination treatment with an aneuploidy-inducing drug such as CFI-400945 could improve the efficacy of anticancer effects of RT. In support of this, previous preliminary studies from our laboratory demonstrated a synergistic effect of CFI-400945 and RT in limited TNBC models[6]. In the current study, we confirmed these findings and expanded the work to clinically-relevant TNBC models and investigated the mechanistic implications of combination treatment.

1.1 Breast Cancer

Breast cancer is the most common cancer diagnosed in women worldwide, with one in eight Canadian women likely to be diagnosed in their lifetime[1]. This represents 25% of all cancer incidences in women and is a significant burden on the Canadian healthcare system and population[1]. In addition, breast cancer is a leading cause of cancer death, with one in thirty women in Canada predicted to die from the disease[1]. These statistics have remained relatively consistent over the past several years with only a slight decrease in

mortality, attributed to earlier detection and treatment[7]. Detection and treatment are of major focus in breast cancer research. A key obstacle in treating breast cancer is its heterogeneity, as there are several differentiating molecular characteristics which affect treatment feasibility, as well as individual patient heterogeneity which leads to inconsistencies in response to current therapeutics[8]. Studies have used next-generation sequencing to identify more than 1600 probable driver mutations in 93 genes in breast cancer, clearly demonstrating the heterogeneity of this disease[9]. Despite this increased understanding of breast cancer molecular complexity, treatment decisions and options are still primarily based on broad molecular subtypes divided by expression of hormone receptors and human epidermal growth factor receptor 2 (HER2)[8]. Luminal A and Luminal B subtypes express the estrogen receptor (ER) and in some cases also the progesterone receptor (PR)[10]. The Luminal subtypes have relatively better prognosis compared to other subtypes of breast cancer, as they grow at a slower rate and can be treated using endocrine-based therapies[10]. The HER2-enriched subtype expresses high levels of the HER2 receptor, which represents a key therapeutic target leading to improved prognosis over TNBC[10]. TNBC lacks expression of these three therapeutic targets (ER, PR, HER2) and is the subtype with the highest mortality due to its aggressive nature, propensity to metastasize, heterogeneity within the subtype, and limited treatment options[11].

1.2 Triple Negative Breast Cancer

Triple negative breast cancer accounts for approximately 15-20% of all breast cancers and has poor survival outcomes compared to other subtypes[12]. There is currently no standard treatment for TNBC as it represents a heterogenous group of tumours which lack expression of the most common therapeutic targets; including hormone receptors or HER2 receptor[2]. Overall survival is also decreased in the TNBC subtype compared to other breast cancer subtypes, highlighted in a study by Dent *et al.* which demonstrated that the percentage of patients who succumbed to TNBC was 42.2% compared to 28% of patients with Luminal or HER2+ breast cancer and a median survival time of 4.2 years in TNBC patients and 6 years for patients of the other breast cancer subtypes[12]. In addition, a greater proportion of TNBC patients experience distant metastasis than other breast cancer subtypes[12]. Location, as well as prevalence, of metastasis varies between breast

cancer subtypes, with TNBC having higher rates of metastasis to the lung and brain or distant nodes, while ER/PR+ or HER2+ breast cancers more frequently metastasize to the bone or liver[13–15].

1.3 Current Therapeutic Options

The cornerstone of curative treatment for non-metastatic (Stages I-III) breast cancer is surgical removal of the primary breast tumour[16] and sampling (sentinel node biopsy) or removal of the associated axillary lymph nodes (axillary lymph node dissection)[17]. Surgical treatment is often accompanied by radiation treatment, chemo-, hormonal or targeted therapies. In metastatic disease (Stage IV) systemic control of cancer through chemotherapy, targeted, hormonal and/or immunotherapy is the primary treatment, although radiotherapy and sometimes surgical removal of the tumour or metastatic deposits can be utilized.

Radiation therapy (RT) is an integral treatment for breast cancer in the curative and palliative setting[18]. RT can be used after surgery at the primary tumour site or regional nodes, and this has been demonstrated to reduce both local and distant recurrence as well as decreasing mortality[19]. RT use prior to surgery is currently being investigated in clinical trials (NCT03978663). In some situations, RT is also used at metastatic sites and targeted stereotactic ablative RT in oligometastatic breast cancer seems to improve disease control and survival[20].

In patients with Luminal subtypes of breast cancer, endocrine therapy is a commonly used treatment approach, and has been shown to reduce recurrence and mortality rates[21]. One of the most common forms of endocrine therapy that has been used for many years is Tamoxifen, an effective anti-estrogen targeted therapy with a low incidence of serious negative side effects[22]. In postmenopausal patients, aromatase inhibitors are often used as a monotherapy or added to Tamoxifen treatment regimens to improve outcomes[23].

For HER2+ breast cancer patients, anti-HER2 receptor antibodies (such as trastuzumab or pertuzumab) or tyrosine kinase inhibitors of HER2 or epidermal growth factor receptor (EGFR) such as lapatinib can be utilized[24]. Since HER2 receptors are expressed at higher levels in cancer cells than normal tissue and signalling is involved in cell proliferation

and differentiation, targeting this receptor has proven to be an effective treatment strategy[25].

General systemic chemotherapy remains the standard therapeutic option in TNBC which lacks expression of hormone receptors or HER2 receptors[11]. Taxane, anthracycline, and platinum-based therapies can be used as monotherapy or combination therapy in metastatic cancer, adjuvant or neoadjuvant to surgery, or in combination with other systemic therapies or radiation therapy[26]. Certain molecular profiles indicate greater likelihood of improved response, such as Breast Cancer Gene 1/2 (BRCA1/2) mutations providing benefit to those receiving platinum-based compound treatments[27]. However, only about 20% of TNBC patients experience pathologic complete response[28], and resistance is prevalent due to a variety of factors including overexpression of drug efflux pumps, cancer stem cells, hypoxia, ineffective or altered apoptotic pathways, and/or alterations in other signalling pathways[11]. With limited therapeutic options, there is a clear need for development and investigation of new targeted treatments for TNBC.

A recently advanced and improved therapeutic option for breast cancer including TNBC is immunotherapy. Current immunotherapy consists of immune checkpoint inhibitors that target CTLA-4, programmed death protein-1 (PD-1) and programmed death ligand-1 (PD-L1)[29]. These therapies function by blocking immunosuppressive receptors or improving the anti-tumour function of tumour-infiltrating lymphocytes (TILs)[30]. Certain populations of patients, including those with a high proportion of TILs or high expression of PD-L1, typically respond better to immunotherapy[31]. Currently the PD-L1 inhibitor Atezolimumab is approved for use in metastatic breast cancer[32], and other immunotherapies are being investigated[29]. Drugs targeting PD-1/PD-L1 signalling, including avelumab and pembrolizumab, have been evaluated in multiple cancer types including TNBC. These studies demonstrate promising results, and improved outcomes may be possible by utilizing combination approaches with chemotherapy or targeted therapies, or through personalized approaches based on a patient's specific cancer biomarkers[29,33].

Several therapeutic targets have been evaluated in TNBC or are currently in preclinical or clinical studies. A significant proportion of TNBC tumours have mutations in the *BRCA1* or *BRCA2* genes, which can alter or decrease homologous recombination repair of

DNA[34]. Poly-ADP ribose polymerase (PARP) inhibitors are of interest in TNBC due to their ability to induce synthetic lethality in BRCA-deficient tumour cells by blocking non-homologous end-joining repair of DNA damage[35]. Olaparib, veliparib, niraparib, rucaparib and talazoparib are some of the PARP inhibitors being investigated or approved[36]. Olaparib and talazoparib were both determined to be more effective than conventional chemotherapy approaches in BRCA-mutant breast cancer and are FDA approved, while veliparib is undergoing a phase 3 clinical trial to confirm efficacy and lack of toxicity[36]. A number of other targets are of interest including the PI3K/AKT/mTOR pathway which is often altered in TNBC and inhibitors of which are in clinical trials and approved in ER/PR+ and HER2+ breast cancer subtypes[37]. Other tyrosine kinase receptors such as EGFR, FGFR and VEGFR often mediate hallmarks of cancer such as proliferation, angiogenesis and metastasis and inhibitors for these targets are in clinical trials, although consistent efficacy in TNBC has not yet been proven[38]. Overall, further work in investigating and developing targeted treatment options for TNBC is required.

1.4 Precision Medicine

Recently, treatment for many cancers including breast cancer has shifted to a more personalized approach, known as precision medicine. As previously mentioned, the heterogeneity of breast cancer has significant clinical implications as there is both inter-tumour heterogeneity, meaning differences between patients, and intra-tumour heterogeneity, indicating molecular differences between different areas of a single patient's tumour or changes over time in a single patient[39]. The goal of a precision medicine approach is to understand and consider the unique molecular profile of a patient's tumour when determining a treatment plan in order to identify the therapeutic options with the greatest likelihood of efficacy[40]. Other factors, including a patient's environment and lifestyle impacts, should also be considered in this approach[41]. The typical method for precision medicine is integrating OMICs information about the tumour (such as genomics, transcriptomics, proteomics, etc.) with drug sensitivity data which can be obtained through large scale drug screens[41]. Data is available on the results of such large-scale drug screens in cell line panels such as the Cancer Cell Line Encyclopedia, which stores information about compound sensitivity and gene expression

and sequencing for 479 cancer cell lines and allows identification of predictors to drug response[42]. However, most patient tumours are not well-represented by cell lines and as a result, preclinical testing of compounds often does not translate well into clinical practice[43].

To attempt to mitigate this problem, biobanks of breast cancer patient-derived organoid (PDO) lines and/or patient-derived xenografts (PDX) derived from both primary and metastatic tumour sites have been developed[8]. Precision medicine requires the ability to correlate identified genomic alterations (from data collected through next-generation sequencing or other genetic screening procedures) to clinical outcomes from clinical trials or general clinical practice to match a patient with an effective therapy[44]. These PDO/PDX biobanks allow characterization of a large number of heterogenous patient tumours, including genomic sequencing and *in vitro* drug screens, providing further resources for precision medicine approaches[8]. Precision medicine holds promise as "the future of cancer treatment" as it acknowledges that cancer is heterogenous and thus its treatments must differ as well for each patient. This approach can be used to treat primary and metastatic tumours as well as to combat resistance, and will continue to gain strength as more genomic and drug sensitivity data is collected and correlated. In order for a precision medicine approach to be feasible and effective, development of novel targeted therapies is crucial. One such targeted therapy of interest in this study is the Polo-like Kinase 4 (PLK4) inhibitor, CFI-400945.

1.5 Polo-like Kinase 4

The cell cycle is a highly regulated process whereby cells replicate and divide their genetic material evenly between two daughter cells[45]. One of the hallmarks of cancer is uncontrolled proliferation[46], often a result of abnormal expression of cell cycle proteins[47]. The Polo-like Kinase (PLK) family has 5 members which independently act in the processes of centriole and centrosome duplication, mitotic entry, spindle fiber assembly and attachment, and cytokinesis[48]. PLK4 stands out from the other PLK family members in structure, having only a single Polo-box motif at the C-terminal rather than two, likely indicating differences in how it functions[49]. PLK4 expression gradually increases from S to M phase of the cell cycle, then is reduced by APC/C dependent proteolysis[50]. PLK4 is highly expressed in embryonic tissues during development and in actively dividing

tissues in adults[50]. During the G2 phase, PLK4 is localized to the nucleolus, then to the centrosomes during M phase, followed by localization to the cleavage furrow during cytokinesis[51]. PLK4 colocalizes with Centrin at the centrioles throughout the cell cycle, and it has been observed that excess PLK4 activity results in centriole amplification[52]. PLK4 is a crucial player in the process of duplication of centrioles, key components of the centrosomes which are microtubule organizing centres in cells[53]. PLK4 undergoes autophosphorylation, followed by recruitment of STIL centriolar assembly protein and spindle assembly abnormal protein 6 homolog (SASS6) which allow duplication of the centriole to occur properly[53].

The role of PLK4 in cancer is complex and not well-understood. Since PLK4 is known to have a key role in mitotic fidelity through centriole duplication[48], aberrant expression of PLK4 is a contributor to the development and progression of cancer[54]. This is supported by the finding that PLK4 is often aberrantly expressed in several cancers, including breast cancer[3,55,56]. PLK4 expression was also identified to be associated with poor survival outcomes in breast cancer patients[57]. Inhibition of PLK4 results in abnormal cell division, aneuploidy and genomic instability leading to cell death[58]. A study by Mason *et al.* demonstrated that shRNA knockdown of PLK4 in MDA-MB-468 breast cancer cells resulted in significantly reduced tumour growth *in vivo* compared to MDA-MB-468 cells expressing endogenous levels of PLK4[3]. However, other studies indicate that PLK4 may be a tumour suppressor, as *Plk4* heterozygous mice developed liver and lung tumours due to the resulting multiple mitotic errors[59]. Despite this conflicting and unclear understanding of the function of PLK4 in cancer development, the results of several studies indicate that in human breast cancer, PLK4 is a promising therapeutic target[3,4].

1.5.1 CFI-400945

A novel, small-molecule inhibitor of PLK4 called CFI-400945 was developed at the Campbell Family Institute (CFI) for Breast Cancer Research, at the University Health Network in Toronto, Canada after identification through a drug discovery program. CFI-400945 was derived from indolinone after an ELISA assay on a focused kinase library identified it as a PLK4 inhibitor[60]. This drug binds to the ATP site of PLK4 and its chemical structure was optimized to improve oral bioavailability by inclusion of a cyclopropane ring and morpholine-containing compound[61]. CFI-400945 is a potent

inhibitor of PLK4 with a half maximal inhibitory concentration (IC50) value of 2.8 nM and inhibition constant (K_i) value of 0.26 nM[60]. It also demonstrates specificity for PLK4 without significant inhibition of other PLK family members up to a 50 μM dose[60]. It is known that CFI-400945 has a bimodal effect on centriole duplication: at low doses PLK4 activity is partially inhibited resulting in lower levels of autophosphorylation and thus less degradation, causing increased PLK4 levels and increased centriole amplification[3]. At high concentrations when PLK4 is fully inhibited, it lacks activity and centrioles do not duplicate[3]. In both scenarios, the aberrant centriole duplication leads to mitotic infidelity, cell cycle arrest and cell death (**Fig. 1**)[3].

Despite the specificity of CFI-400945 for PLK4 over other PLK family members, it still demonstrates inhibitory activity of other kinases with effective concentrations under 100 nM[3]. These include Aurora Kinase B (AURKB), Tyrosine Receptor Kinase (TRK) A/B, and Tie-2/TEK[3]. AURKB is involved in the attachment of the spindle fibre to the kinetochore, and its inhibition could result in chromosomal missegregation and aneuploidy as well[62]. It has been suggested that AURKB inhibition from CFI-400945 could contribute to its effects in cancer cells[63]. In cancer cells treated with CFI-400945, excess centriole amplification was observed, which can be attributed to the effects of inhibiting PLK4, however there was also multinucleation and excess centrosomes, the effects of which are likely related to other effects such as AURKB inhibition[3,63]. CFI-400945 was well tolerated in a TNBC MDA-MB-468 xenograft model at both low and high doses (3.0 g/kg and 9.4 g/kg, respectively) given once per day for 21 days[60].

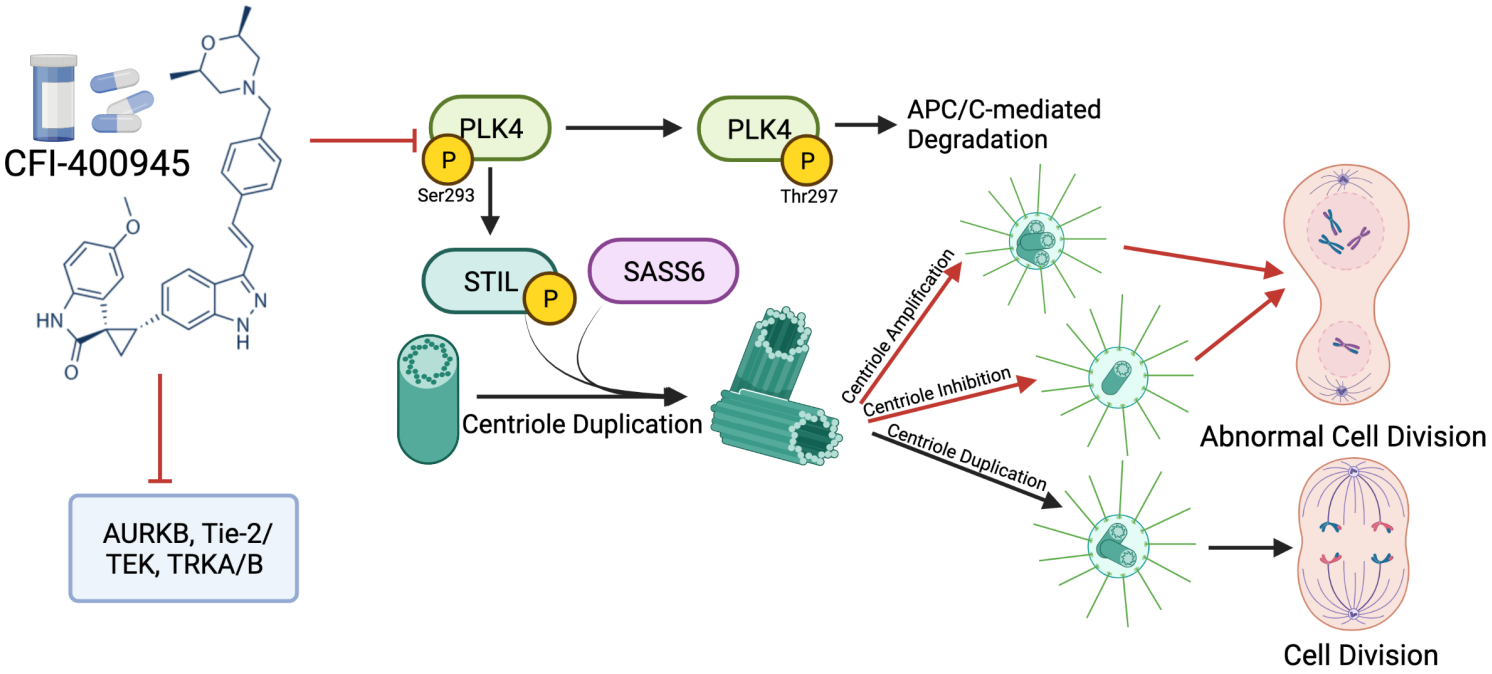

Figure 1. Cellular action of CFI-400945. CFI-400945 functions primarily through inhibition of PLK4, involved in the process of centriole duplication by recruitment and phosphorylation of STIL and SASS6. Autophosphorylation of Ser293 is crucial for function, while Thr297 marks PLK4 for APC/C-mediated degradation. Inhibition of PLK4 can result in centriole amplification or inhibition, resulting in abnormal cell division. Off-target effects of CFI-400945 include inhibition of AURKB, Tie-2/TEK, and TRKA/B. *Figure created with BioRender.com.*

For clinical use, orally available CFI-400945 exists in tablet form. A Phase I clinical trial evaluated the safety and tolerability of CFI-400945 in advanced solid tumours of multiple types, including 8% breast cancer, and found that the drug was generally well-tolerated[4]. The most common higher-grade adverse effect was neutropenia, occurring in 21% of patients[4]. This common side effect was observed at higher doses, and a dosage of 64 mg was recommended for phase II trials[4]. All other side effects observed were low grade including fatigue, nausea, diarrhoea, anorexia, vomiting, dyspepsia, hypomagnesaemia, and dehydration[4]. These side effects were manageable with dose interruptions, reductions or intervention with full recovery[4]. Observed efficacy in this study was low, though the patient population had a large number of previous treatments and efforts are being made to improve the efficacy[4]. CFI-400945 is currently undergoing Phase II clinical trials in breast cancer to optimize dosage[3], as well as in additional Phase II clinical trials in prostate and breast cancer patients to evaluate efficacy and biomarkers for treatment response (NCT03385655, NCT03624543). One such potential biomarker that will be investigated is PTEN loss, which was identified as a positive biomarker for PLK4 inhibition and response in preclinical studies[3]. Altered dosing schedules and combination treatments with immune checkpoint inhibitors are currently being investigated (NCT04176848). Additional studies on combination therapy regimens of CFI-400945 with other treatment options such as RT will be beneficial to improving patient response.

1.6 Radiation Therapy

Radiation therapy functions to control cancer cell proliferation or cause cell death, accomplished through several mechanisms of action[64]. A primary mode through which this occurs is via DNA damage in cancer cells to prevent proper transmission of genetic information during proliferation or to directly cause cell death[64]. Energy from the radiation creates single-strand breaks, double-strand breaks, base or sugar damage and crosslinks within DNA[64]. DNA double-strand breaks are a major mechanism of RT as they are the most harmful to the cell and can cause chromosomal rearrangements or loss of genetic information through repair[64,65]. Simple double-strand breaks are rapidly repaired, while complex double-strand breaks which have instances of other forms of DNA damage clustered around the break result in genomic instability and lethality[64]. Radiation creates double-strand breaks through high energy damage to the DNA sugar

back bone[66]. In addition, RT causes oxidation of bases which are removed through base excision repair, but when this repair system is overwhelmed further double-strand breaks are formed[65]. When double-strand breaks are formed, they are typically repaired by non-homologous end joining or homologous repair, resulting in cells regaining normal function, however DNA damage accumulated from radiation induces apoptosis, necrosis and senescence which prevent cancer cells from proliferating[65].

An additional mechanism of RT is through the generation of reactive oxygen species (ROS)[66]. ROS from RT are formed primarily through radiolysis of extracellular water and partially due to endogenous production in the mitochondria[66]. The produced ROS can disrupt the electron transport chain and interact with biological molecules causing cellular stress and damage to DNA and other organelles[66]. The plasma membrane is damaged by ROS through lipid peroxidation which can lead to cell death[65]. As well, the mitochondria can be damaged by ROS, causing release of cytochrome c and activation of apoptotic pathways[65]. Cellular stress from ROS and RT itself can also disrupt endoplasmic reticulum function which results in accumulation of misfolded proteins and induction of apoptosis or autophagy[65]. There is also evidence that RT can induce endothelial cell death and vascular damage to cause cancer cell death and delay of tumour growth[67,68]. Finally, high levels of ROS from RT stabilize p53 expression which initiates signalling pathways promoting apoptosis[66].

RT also indirectly functions through inducing anti-tumour immune responses and immunogenic cell death[69]. Activation of the NFkB transcription factor or interferon (IFN) response pathway by RT results in release of pro-inflammatory cytokines including tumour necrosis factor alpha, IFN-α, IFN-β, and IFN-γ[69]. The inflammatory response can cause immune cell maturation, and recognition and destruction of the cancer cells[69]. As well, apoptosis and necrosis of cancer cells directly due to radiation results in increased antigen release and the priming of immune cells for anti-tumour immunity (**Fig. 2**)[69].

Despite being a commonly used therapy for many types of cancer including TNBC, patients may not respond to RT or the response may not last due to development of

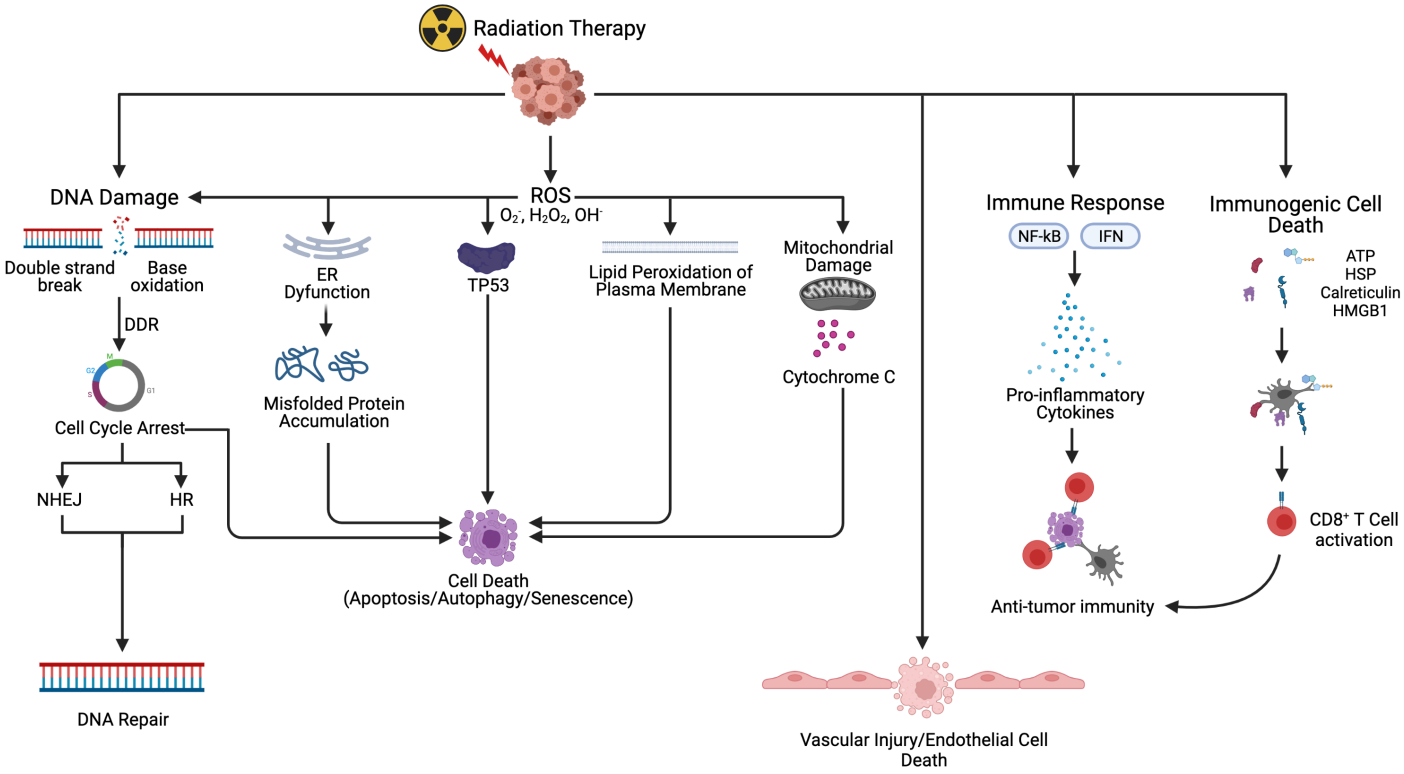

Figure 2. Mechanisms of action of RT in cancer cells. RT causes DNA damage in cells directly or via ROS generation, repair of which may occur abnormally in cancer cells resulting in cell death. ROS from RT also may induce cell death through mitochondrial damage, ER dysfunction, lipid peroxidation of plasma membrane, or stabilization of TP53. High dose of radiation can also activate vascular endothelial cell death. RT can induce activation of NFκB and IFN pathways to initiate anti-tumour immunity or activate immunogenic cell death. ATP – adenosine triphosphate, DDR - DNA damage repair, HMGB1 – high mobility group box 1, HR - homologous repair, HSP – heat shock protein, NHEJ - non-homologous end joining, ROS – reactive oxygen species, RT – radiation therapy. *Figure created with BioRender.com. From Bhat et al., 2022.*

radioresistance[70]. TNBC is particularly prone to radioresistance compared to other BC subtypes, limiting the use and efficacy of RT as a treatment option[71]. Many mechanisms of radioresistance have been theorized, including non-coding RNAs affecting signalling pathways involved in cellular processes[72], cell cycle regulation, or hypoxia[73]. While radioresistance mechanisms may be diverse among patients and are not fully understood, several approaches have been developed to combat the development and persistence of this resistance. One such approach which has been demonstrated to hold promise is the use of combination therapy regimens with RT in which chemotherapy, immunotherapy, or targeted agents act to radiosensitize cancer or act synergistically with RT to improve outcomes[5].

1.6.1 Combination Therapies with Radiation

Multimodality treatment approaches are used in various cancers, including breast cancer and hold significant promise in breast cancer treatment. Several types of therapeutic agents have been investigated in combination with RT for the treatment of breast cancer. PARP inhibitors, which block the base-excision repair pathway in cells, have been investigated in combination with RT in TNBC in a Phase I clinical trial which found no dose limiting toxicities, and recommended future study of efficacy[74]. Of great interest is the combination of RT with immunotherapies as it is known that the immune response is involved in RT-mediated tumour responses[75]. A Phase I clinical study of RT with immune checkpoint inhibitor pembrolizumab showed overall response rate of 13.2% in a mix of metastatic cancers including 6 patients with metastatic breast cancer, and recommended further study on this combination and biomarkers of response[76]. Clearly, combinations of RT with these and other targeted treatments need further investigation in breast cancer as current information about efficacy and response is very limited.

Due to the aneuploidy and mitotic abnormality-inducing features of CFI-400945, a combination therapy with RT, which has similar effects on deregulating genetic stability, is of interest. Preliminary studies from our lab indicate that the combination of CFI-400945 and RT is promising for the treatment of breast cancer[6]. Colony formation assays in human TNBC cell lines MDA-MB-231, MDA-MB-468 and MDA- MB-436 demonstrated that the combination of RT and CFI-400945 was more effective at reducing colony formation than the individual monotherapies. As well, the synergy score was

calculated for the combination in MDA-MB-231 cells using SynergyFinder and maximal synergy was detected around the IC50 and ID50 of CFI-400945 and radiation respectively. While this provides some promising evidence about the potential efficacy of combination therapy with CFI-400945 and RT, further studies in more advanced models of breast cancer are needed in order to fully elucidate the potential impact and mechanism of this combination.

1.7 Models of Breast Cancer

Research on understanding the biology, prognosis, imaging techniques, biomarkers, and potential therapeutic options and treatment response for breast cancer is limited by the preclinical models of breast cancer available for study. The current models of breast cancer that are most commonly used and widely available include *in vitro* immortalized BC cell lines, *in vivo* murine syngeneic models and *in vivo* xenograft human models of breast cancer cell lines or human tissue (patient-derived xenografts, PDX). Each of these models have distinct strengths and limitations, making them best suited for specific stages and types of research.

The first immortalized breast cancer cell line was developed in 1958 with the BT-58 cell line[77], though the development of other lines was not widespread until the 1970s, when the MCF-7 line was developed[78] as was the MD Anderson series (MDA-MB)[79] which remain some of the most commonly used breast cancer cell lines in the present day. Immortalized breast cancer cell lines are advantageous in that they are relatively inexpensive, grow quickly and self-replicate almost infinitely, require fewer resources and are less laborious than other models[80]. Many cell lines are thoroughly tested and validated, making them reliable models, and they can be, and often already have been, analyzed for gene expression that provides valuable insight in studies using these models[81]. Immortalized cell lines can be co-cultured with other cell types such as fibroblasts for studies on how these cell types influence each other[82], which is particularly valuable in understanding the process of cancer cell invasion and metastasis. However, immortalized breast cancer cell lines have drawbacks in that they may experience genotypic and phenotypic drift over time, causing changes in the cell population from the original cancer from which they were derived[80]. Genetic drift in cell lines has been shown to greatly alter responses to drugs compared to original tumours, limiting usefulness in

drug testing[80]. As well, they are typically grown in two-dimensions and may not reflect the heterogeneity found in patient breast cancer samples[83]. As a result, these cell lines often do not accurately portray treatment response of the majority of patients unless a panel of several cell lines is used[84]. Neve et al. described a model system of 51 cell lines that they found could be used to identify predictive molecular biomarkers of treatment response to Trastuzumab[84], though use of a model system like this is far more complex than a single cell line. Finally, immortalized breast cancer cell culture also lacks the tumour microenvironment as they are grown on plastic and do not include the complex cell-cell and cell-matrix interactions and conditions found in a patient tumour[83]. These interactions can alter cell phenotype, morphology, polarization and differentiation, making them crucial elements for understanding breast cancer[85,86].

There is a low success rate of translating anticancer drugs from development to clinical use, and one of the main contributing factors for this is this lack of translational preclinical models[87]. Therefore, models that more accurately capture patient sample heterogeneity and microenvironment are of benefit for translational research. Xenograft models are often seen as the solution to these drawbacks of immortalized breast cancer cell lines. A xenograft is defined as the transplant of tissue or cells from one species to another, and in this context, often human breast cancer cells or tumours are implanted into mice for study. A PDX model involves implantation of a patient tumour sample into an immunocompromised/immunodeficient mouse followed by passaging into additional mice for expansion and maintenance[88]. Implantation can occur heterotopically/ subcutaneously, allowing for easy observation and measurement, or orthotopically into the mammary fat pad which allows more interaction with the microenvironment[89]. PDX models are able to recapitulate the original patient tumour and more accurately predict drug response than cell lines, as well as allow for the metastatic process to occur, making them a powerful model in translational breast cancer research[90]. PDX models are also useful in development and testing of preclinical imaging techniques[91]. However, PDX models have a low success rate of development, are expensive, labour-intensive and time-consuming, and are limited in their ability to reflect the tumour microenvironment as the mice lack a competent immune system[92].

To overcome the lack of immune system in these models, murine syngeneic mouse models as well as humanized PDX models have been developed. Murine syngeneic models are those where the cancer is of the same genetic origin as the mouse and these can be spontaneous, arising either naturally in the mouse or through specific genetic alterations purposefully induced in the mouse, or carcinogen-induced tumours[93]. These models better represent the stromal environment around the tumour including immunosurveillance and immunoediting mechanisms, making them particularly useful for studies on immunotherapies, a budding area of research[93]. However, since these are not human cancers, they may not accurately reflect the biology of patient tumours, thus limiting their use[94]. To address this, humanized mice are immunodeficient mice that have human hematopoietic stem cells implanted in them which eventually act to create a functioning immune system[95]. These humanized models can be time-consuming and challenging to develop with engraftment failure or early mouse death, and some versions of humanized mice pose ethical questions as they require human fetal tissue, thus these models also have several limitations[96]. Due to the clear limitations of each of these breast cancer models, other translational models that do not use mice are of great interest, and organoid and patient-derived organoid (PDO) models can bridge the gap between cell lines and murine models.

1.7.1 Patient-Derived Organoids

Organoid models are considered to be an *in vitro* or *ex vivo* model in which tissues are grown in three dimensions and form structures that replicate the origin organ or tissue[97]. The first work on organoids began in 1907 after Wilson found that sponge cells could be recombined after dissociation to form a whole organism again[98]. Initial development of modern organoids was performed using healthy intestinal stem cells which could be propagated and form the crypt-villus-like structures reflecting origin morphology, as well as give rise to all cell lineages of the gut[99]. These organoid structures are grown in Matrigel or Basement Membrane Extract (BME) which are gel solutions that form a scaffold of extracellular matrix which allow cells to come together in three dimensions rather than adhered to a plate[100]. Matrigel is the most commonly used commercial matrix gel, and it is formed from collagen IV which is isolated from Engelbreth-Holm-Swarm (EHS) sarcoma and *in vivo* it is a main component of tumour stroma and laminin[101]. BME

and BME-2 are also derived from EHS sarcoma with major components laminin, collagen IV, entactin, and heparan sulfate proteoglycans, but have a lower protein concentration than Matrigel[102]. However, the composition and stability of Matrigel and BME/BME-2 can be variable due to its biological origin, and as a result, synthetic matrices are becoming more commonly used[103]. These scaffold solutions can contain various growth factors and nutrients to nurture the organoids, and these factors can be altered to include alternative differentiation of stem cells in some cases[104]. Since the first organoid types developed were derived from normal or healthy tissue, it was necessary for the cells used to be stem cells which could generate the other cell types needed to self-organize into the organ-like structure. This organoid technology progressed into development of organoid structures from malignant tissue which could self-renew, the first of which was colorectal cancer cells[104]. Soon after, protocols were developed from healthy and malignant pancreas, stomach, prostate and liver tissues, which formed the foundation for organoid use in cancer studies and development of other organoid types including ovarian cancer and breast cancer[105].

Breast organoid development began in 1977 when Emerman and Pitelka observed that mouse breast cells could form mammary acinus structure in a three dimensional matrix surface with the addition of insulin, hydrocortisone and prolactin to induce differentiation and isolation[106]. A similar strategy was then used to develop breast cancer organoids, adding growth factors and apoptotic inhibitors to the medium used with breast tumour tissue to induce growth and differentiation of the breast cancer stem cells present, which are the foundation of breast cancer organoid growth[8,99,107,108]. Sachs and colleagues went on to develop a breast cancer organoid biobank, consisting of 95 organoids which cover a wide range of genotypes, phenotypes and pathologies for use in drug screening and precision medicine approaches[8]. This group and others in recent years have also optimized the protocols involved in digestion and development of organoid cultures, resulting in an improved success rate of breast cancer organoid development[109].

Organoids have a number of benefits as a model for studying cancer. It has been demonstrated in many different cancer types including breast cancer that organoids maintain the heterogeneity of the original tumour from which they are derived, and are able to capture and maintain the genetic and morphological features of the original

patient tumour[8,41]. Organoids also better reflect tumour biology as they maintain cell-cell interactions, cell-matrix interactions, and cell polarization[110]. These models can recapitulate other features of tumours which can be important in biology and treatment response, such as the hypoxic core of cells found in patient tumours[111]. Organoids can be used for high-throughput drug screening over PDX models, which makes them a very useful tool in drug development and testing and precision medicine[97]. Since these three-dimensional models more accurately capture the heterogeneity and biology of patient tumours, they also better reflect patient response to drugs and other treatments such as RT[41]. PDO cultures can be developed using less patient tissue than PDX models, have a higher success rate of establishment, and they pose less ethical issues with respect to the use of animals in research[112]. Organoids are also more cost-effective than PDX models and can be genetically manipulated with relative ease using CRISPR-Cas9 gene editing. This allows investigation into the effects and treatment responses of knockouts of specific genes, either genes known to be involved in cancer development, such as BRCA1/2 in breast cancer, or novel genes that may be implicated[97]. These genetically manipulated organoids can be a crucial player in drug resistance studies[113]. Organoid models can also be easily used in additional experiments for analysis including immunocytochemistry and immunohistochemistry, flow cytometric analysis, RTqPCR and RNA sequencing which can provide further insight into understanding mechanisms of tumour growth, drug response and resistance[105].

Despite the many advantages of organoid models in cancer studies, there does exist some drawbacks and limitations to using these in certain experimental situations. Since these models are relatively new to widespread use in breast cancer, the protocols for development and the composition of the medium used for growth is not standardized and can vary between groups and institutions[110]. This can cause variability in results due to these factors not directly related to the tumour itself. The materials used for organoid development and propagation, particularly the Matrigel or BME/BME-2 and supplements for medium can be expensive, and these models are far more costly and time consuming than cell lines, though still less so than PDX models[105]. An additional limitation can be a lack of patient samples available for development of organoids, particularly in institutions without a pre-existing protocol and system for easily obtaining patient samples. This lack of available samples can be caused by low patient consent rates, difficulty in obtaining

ethics board approvals for study, or delays and reductions in the number of patient surgeries and biopsies being performed. A major limitation in the use of organoids is that they lack innervation, blood vessels and blood supply, and do not have a functioning immune system or immune cells including TILs[97,114]. As a result, immunotherapy, which is a budding area of research, cannot easily be investigated using PDO models. To overcome this issue, several groups are attempting strategies such as coculture of organoids with immune cells, although further studies are required[115]. These limitations, though significant, do not undermine the great advantages of organoid models, hence their use is becoming more widespread in studies on cancer biology and therapeutics.

1.8 Rationale for Study

As previously discussed, CFI-400945 was developed and found to be a potent inhibitor of PLK4 that resulted in aberrant centriole duplication leading to abnormal mitotic division, genomic instability and cell death[3]. CFI-400945 was shown to be generally well-tolerated in a Phase I dose-escalation trial in patients with advanced solid tumours[4]. However, this Phase I trial found low response rates, which could be attributed to the advanced stages of cancers, many prior treatments, and lack of biomarkers to indicate response[4]. Due to the aneuploidy and mitotic abnormality-inducing features of this drug, a combination therapy with RT which has similar effects on deregulating genetic stability is of great interest. Previous work by Dr. Parsyan using immortalized TNBC cells *in vitro* and *in vivo* supports the combinatorial anticancer effects of CFI-400945 and RT[6]. However, the mechanism of action of this combination requires further study, and use of a broader panel of patient-derived models such as PDOs will augment the ability of this therapeutic approach to be translated into clinical trials and use in patient management in the future.

1.9 Overall Hypothesis and Objectives

In my thesis, we hypothesized that the PLK4 inhibitor CFI-400945 and radiation therapy have a synergistic anticancer effect by increasing genomic instability in breast cancer.

To investigate this hypothesis, my project had three major objectives:

Objective 1:

To further examine anticancer effect of CFI-400945 and RT in combination in immortalized TNBC cell lines and PDOs.

Objective 2:

To investigate whether the combined anticancer effects of RT and CFI-400945 in TNBC are PLK4-specific.

Objective 3:

To investigate the mechanism(s) of the synergistic effect of CFI-400945 and RT in TNBC.

2 Materials & Methods

2.1 Cell Culture and Treatment Conditions

The MDA-MB-468 human TNBC cell line was obtained from Dr. Ann Chambers (London Health Science Centre, London, Canada) and was cultured in α-MEM + 10% fetal bovine serum (FBS). The MDA-MB-231 human TNBC cell line was obtained from Dr. Ann Chambers (London Health Science Centre) and was cultured in DMEM:F12 + 10% FBS. The SUM-159 human TNBC cell line was obtained from Asterand Inc. (Detroit, MI, USA) and was cultured in HAMS:F12 + 5% FBS, 0.5% insulin, 0.1% hydrocortisone and 1% HEPES. The 4T1 mouse breast cancer cell line was obtained from Dr. Ann Chambers (London Health Science Centre) and was cultured in DMEM +10% FBS, 1% NEAA, and 1% L-Glutamine. All cell lines were tested for mycoplasma and authenticated by RADIL (Maine, USA) via polymerase chain reaction comparison to ATCC. CFI-400945 (Cat. No. S7552) and AZD-1152 (Cat. No. S1147) were obtained from SelleckChem (Pennsylvania, USA) and Centrinone B (Cat. No. 5690) was obtained from Tocris Bioscience (Bristol, UK). Paclitaxel was obtained from London Health Science Centre. RT was performed using the Cobalt-16 radiation unit at the London Regional Cancer Program. Doses of RT and drug were selected by determining the ID50 or IC50 respectively for each cell line using dose response curves.

2.2 Colony Formation Assays

Colony formation assays (CFA) were performed as previously described[6]. Briefly, cells were seeded in 6 well plates at colony forming density (52 cells/cm^2 for MDA-MB-231, SUM159 and 4T1, and 104 cells/cm^2 MDA-MB-468). Adhered cells were treated with RT 16-20 hours later followed immediately by drug or vehicle control-supplemented media at the indicated doses, then grown for 7-14 days prior to fixation with acetone:methanol (1:1 vol/vol) and staining with 0.5% crystal violet in ddH_2O. Plates were imaged, colonies consisting of >50 cells were counted, and numbers were compared to vehicle control. Fold-decrease was calculated by dividing the control by the treatment % survival values. In pre-treatment studies, cells were treated with RT or drug 16-20

hours after seeding, then 4 days later the drug or RT, respectively, was added. For siRNA studies, cells were transfected with either scrambled control or PLK4 targeting siRNA. Cells were detached 16 hours after transfection using cell dissociation solution and re-plated in CFA conditions.

2.3 Organoid Culture and Formation Assays

All work with PDOs was carried out under protocols approved by the Western University's Health Sciences Research Ethics Board (HSREB; protocol #118019, 118685 **[Appendix A,B]**). The BPDXO58 and PDO66 lines were developed and provided by Princess Margaret Cancer Centre (Toronto, Canada). Both PDO lines were generated from TNBC tumour tissue surgical samples. BPDXO58 was grown first as a PDX in mice, then excised and propagated as PDO, while PDO66 was grown directly as a PDO. Organoids were propagated in 24-well cell culture plates in 50 µl of growth factor reduced basement membrane-like extract, type 2 (BME-2) (Bio-Techne, Minnesota, USA), Cat. No. 3533-010-02), with 500 µl Breast Cancer Organoid Media[8]. Media was changed 2 times per week and organoids were passaged approximately every 7-14 days. For CFI-400945 and RT testing, organoids were dissociated and 2000 cells/well were plated in 48 well plates in 25 µl BME-2 with 250µl Breast Cancer Organoid Media. The next day, organoids were treated with RT, followed immediately by replacement of media containing CFI-400945 at the indicated doses or vehicle control (dimethyl sulfoxide, DMSO). Media and drug were replenished 2 times per week. Organoids were counted by two independent observers in at least 2 random fields in each well and number was compared across treatment conditions. Organoids were defined by threshold size and morphology as agreed upon by observers. Synergy was calculated using SynergyFinder software[116].

2.4 siRNA Knockdowns

2.4.1 PLK4 silencing in TNBC cells

SUM-159, MDA-MB-231 and MDA-MB-468 TNBC cells were plated at a density of 5000 cells/cm^2 and maintained at 37º C in an incubator with 5% CO_2 overnight. Cells were then transfected with 100 nM of PLK4 targeting (si1-GGACUUGGUCUUACA ACUA; si2-GAAGAUAGCAAUUAUGUGU) or non-targeting siRNAs (scrambled

control; Cat. No. D-001810-10-05) purchased from Dharmacon (Colorado, USA). Transfection was performed using Lipofectamine RNAiMAX transfection reagent from Invitrogen (Massachusetts, USA) according to the manufacturer's protocol. Cells were collected 48 hours after transfection for RNA extraction and RT-qPCR analysis. Western blots to confirm PLK4 knockdown were not performed due to a lack of effective antibodies available for this target, despite multiple commercially available antibodies being tested.

2.4.2 RT-qPCR analysis

Total RNA was isolated 48 hours post transfection using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed into complementary DNA using the Superscript IV VILO master mix (Invitrogen) according to manufacturer's protocol. *PLK4* knockdown was quantified using Brilliant III SYBR green qPCR master mix (Agilent Technologies, Inc, California, USA) on the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Massachusetts, USA). All reactions were carried out in triplicates for each biological replicate. The cycle threshold (Ct) values of *PLK4* were normalized to *GAPDH* internal control to calculate ΔC_t values. The difference in *PLK4* expression between scrambled control and *PLK4* knockdown samples were determined by calculating fold change with $2^{-\Delta\Delta C_t}$ method as previously described[117]. The gene expression was determined using the following primers: *PLK4*, forward 5'-CCT TCT GCA AAT CTG GAT GG-3', reverse 5'-ACA GTG GTT TGG GAA TCT GC-3' and *GAPDH*, forward 5'-AAG GTG AAG GTC GGA GTC AAC-3', reverse 5'-GGG GTC ATT GAT GGC AAC AAT A-3'.

2.5 Immunocytochemistry

Cells were seeded on glass coverslips at 10,000 cells/well in 24 well plates, treated with RT 16-20 hours after seeding followed immediately by CFI-400945 or vehicle control. After 24 hours of treatment, cells were washed with PBS, fixed with 100% methanol at -20ºC for 20 minutes, and washed 3 times with PBS. Cells were then incubated with blocking buffer (5% BSA, 0.5% Triton X-100 in PBS) for 1 hour at room temperature, then incubated with primary Centrin antibody (Sigma-Aldrich, Missouri, USA), Cat. No. 04-1624, 1:1000) for 2 hours at room temperature in blocking buffer, then with 1:500

Alexafluor 488 Anti-Mouse secondary antibody (Invitrogen, Cat. No. A11029) in PBS for 1 hour. Coverslips were mounted using Vectashield with DAPI. Images were taken using Nikon Eclipse Ti2 Confocal microscope.

2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (Dotmatics, San Diego, USA). IC50/ID50 doses of compounds and RT were calculated from single-agent dose response curves using a nonlinear regression model. Comparison of control, single agent and combination effects for CFA and immunocytochemistry was performed using Two-Way ANOVA. Statistical significance was defined as $p \leq 0.05$.

3 Results

3.1 CFI-400945 and RT demonstrate a combinatorial anticancer effect in TNBC cell lines

Our previous study suggested a synergistic effect of CFI-400945 and RT in TNBC cell lines[6]. To further investigate and confirm this finding, an expanded series of TNBC cell lines (human and mouse) were exposed to CFI-400945 and RT as single agents or in combination. Initially, dose-response curves were created from single-agent CFA for RT (**Fig. 3A**) and CFI-400945 (**Fig. 3B**) to determine ID50 and IC50 values (**Table 1**). Near-IC50/ID50 doses of CFI-400945 and RT, respectively, were selected for combination treatment studies (10 nM CFI-400945 + 2 Gy RT [MDA-MB-468], 4 nM CFI-400945 + 2 Gy RT [MDA-MB-231], 7 nM CFI-400945 + 5 Gy RT [SUM159], and 6.25 nM CFI-400945 + 2.5 Gy RT [4T1]). Using these doses, further CFA were performed to compare single agents to combination treatments and identify combinatorial effects. In the MDA-MB-468 TNBC cell line, a combinatorial effect was observed with 10nM of CFI-400945 and 2 Gy RT (**Fig. 4A**). Single agent treatment with 10nM CFI-400945 resulted in a 2.3-fold decrease in colony formation compared to vehicle control, 2 Gy RT reduced colony formation by 1.5-fold compared to no radiation, while combination treatment resulted in a 11.1-fold decrease in colony formation (**Table 2**). Similar combinatorial effects were observed in three additional TNBC cell lines; MDA-MB-231, SUM159 and 4T1, although extent of response varied (**Fig. 4B-D**). In the 4T1 cell line, a range of doses for both CFI-400945 and RT were compared in a combination matrix [**Appendix C**], and a Bliss synergy score of 16.63 was determined using SynergyFinder software[116] with maximal synergy observed near 2.5 Gy and 6.25 nM CFI-400945 (**Fig. 4E).**

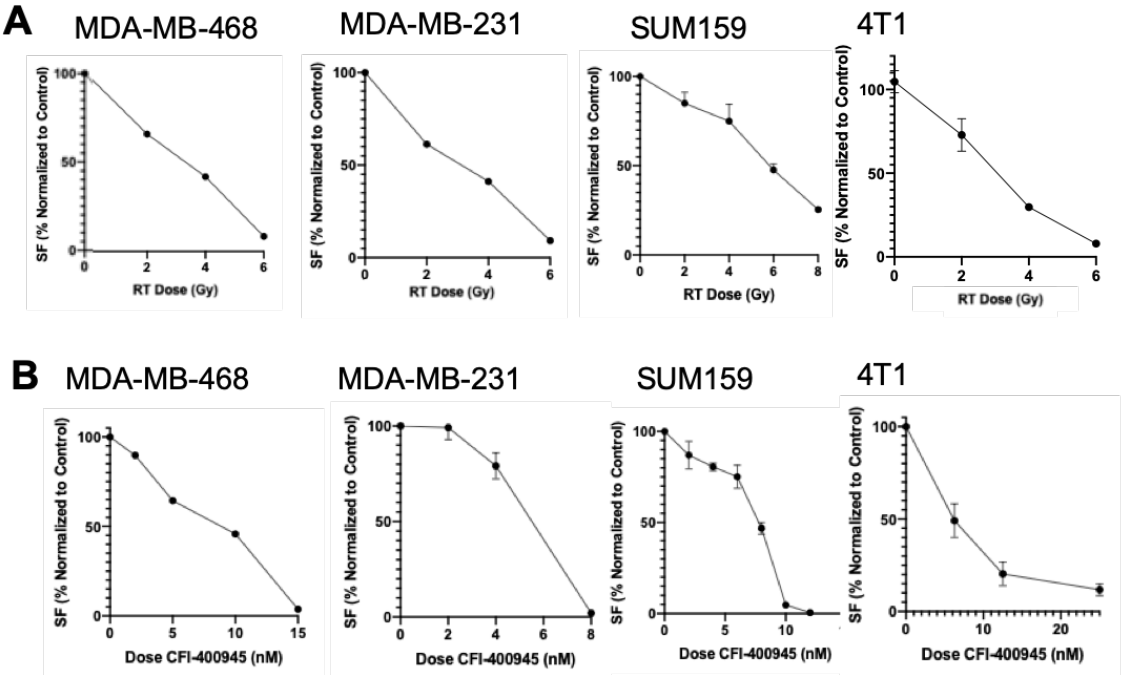

Figure 3. Dose response curves of single agent treatments in TNBC cell lines. Colony formation assays were performed on cell lines (MDA-MB-468 (n=1 [RT, CFI-400945]), MDA-MB-231 (n=1 [RT], n=3 [CFI-400945]), SUM159 (n=3 [RT, CFI-400945], and 4T1 (n=3 [RT, CFI-400945])) by treating with a range of doses of (A) RT and (B) CFI-400945 to identify ID50 or IC50 values using non-linear regression analysis. The number of colonies of 50+ cells were counted after 7-14 days using crystal violet staining and treatment arms were normalized to control (no RT, or DMSO). SF – Surviving Fraction.

Table 1. ID50 and IC50 values of RT and CFI-400945 in TNBC lines, calculated by non-linear regression analysis of data presented in Figure 3.

Treatment	MDA-MB-468	MDA-MB-231	SUM159	4T1
RT	2.96 Gy	2.72 Gy	5.64 Gy	2.86 Gy
CFI-400945	7.10 nM	4.78 nM	7.34 nM	6.28 nM

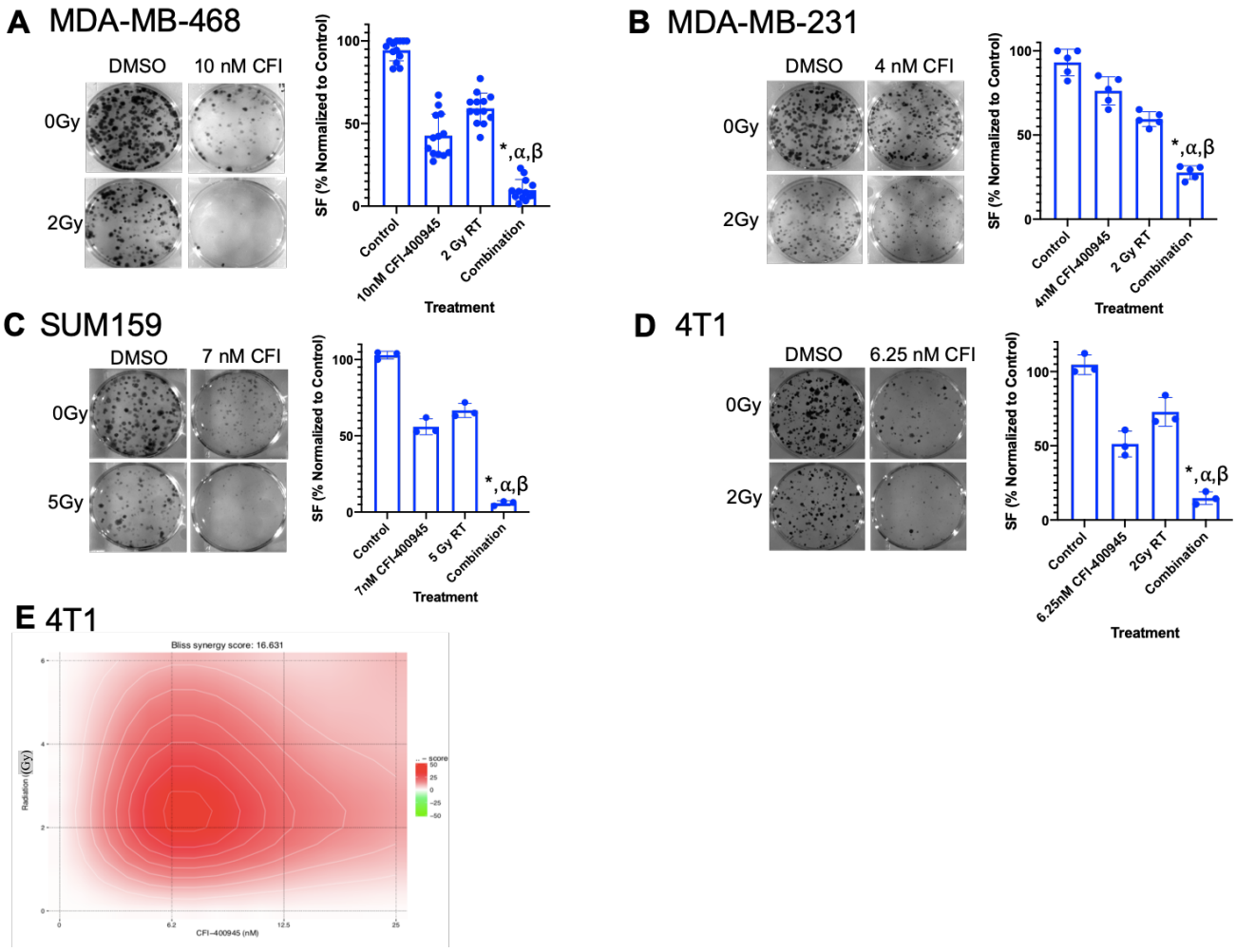

Figure 4. Anticancer effects of CFI-400945 and RT in TNBC *in vitro*. Combination of CFI-400945 and RT indicates improved efficacy in decreasing colony formation in (A) MDA-MB-468 (n=13), (B) MDA-MB-231 (n=5), (C) SUM159 (n=3) and (D) 4T1 (n=3) immortalized breast cancer cell lines compared to single agent treatment with CFI-400945 or RT. CFA were performed by treating cells with RT and CFI-400945 at pre-determined ID50/IC50 doses. After 7-14 days, colonies of 50+ cells were counted after crystal violet staining and the number of colonies in treatment arms were normalized to control (no radiation, DMSO). (E) In 4T1, synergy of combined CFI-400945 and RT was observed across several dose levels. Bliss synergy scores were calculated with SynergyFinder and are displayed in the heatmap, where intensity of red indicates higher degree of synergy. *$p \leq 0.05$ compared to control, $^{\alpha}p \leq 0.05$ compared to RT only, $^{\beta}p \leq 0.05$ compared to CFI-400945 only. SF – Surviving Fraction.

Table 2. Fold decrease in colony formation after simultaneous treatment, CFI-400945 4-day pre-treatment, or RT 4-day pre-treatment combinations of CFI-400945 and RT compared to control (set to 1 indicating no change).

Treatment	MDA-MB-468	SUM159
RT Only	(2 Gy) 1.5*	(5 Gy) 1.5*
CFI-400945 Only	(10nM) 2.3*	(7 nM) 1.4*
Simultaneous Combination	$11.1^{*,\alpha,\beta}$	$4.4^{*,\alpha,\beta}$
Drug Pre-treatment Combination	$13.1^{*,\alpha,\beta}$	$2.3^{*,\alpha,\beta}$
RT Pre-treatment Combination	$8.9^{*,\alpha}$	$1.9^{*,\beta}$

*$p \leq 0.05$ compared to control, $^{\alpha}p \leq 0.05$ compared to RT only, $^{\beta}p \leq 0.05$ compared to CFI-400945 only. Data from rows 2-5 also presented in Table 4.

3.2 Sequential treatment of breast cancer cells with CFI-400945 or RT does not enhance combination anticancer effects compared to simultaneous treatment

We next investigated whether pre-treating TNBC cells with CFI-400945 or RT for 4 days prior to the other would improve efficacy of this combination or whether the combination acted similarly to when administered simultaneously. In MDA-MB-468 cells, there was no significant difference in anticancer effects of the RT and CFI-400945 combination treated simultaneously or with pre-treatment of either drug or RT (**Fig. 5A**). All combinations, whether simultaneous or with pre-treatment of one agent, resulted in significant combinatorial effects compared to single agent treatments or control. In SUM159 cells, there was no significant difference between simultaneous or RT pre-treatment, but CFI-400945 pre-treatment resulted in a significantly reduced anticancer effect compared to simultaneous ($p \leq 0.05$) (**Fig. 5B, Table 2**). However, even with pre-treatment the combination had much greater effect on reducing colony formation than the single agent RT or CFI-400945 treatments or control. Based on these findings, a simultaneous administration was selected for the future experiments.

3.3 CFI-400945 and RT demonstrate synergistic effect in PDO models

To further confirm the synergistic effects of CFI-400945 and RT in a more translationally relevant experimental model, organoid formation assays were performed using BPDXO58 and PDO66 models treated with CFI-400945, RT or combination for 14 days at a range of doses to examine effect of combination treatment on ability of PDOs to form and grow. Synergy in reduction of organoid formation was observed across several dosages in both PDO lines (Bliss synergy score 17.88 in BPDXO58, 11.93 in PDO66) [**Appendix D**]. Maximal synergy was observed between 1-2.5 Gy RT and 2-5 nM CFI-400945 in the BPDXO58 organoid line (**Fig. 6A**), while in PDO66 synergy was greatest between 2.5-5 Gy with the same 2-5 nM CFI (**Fig. 6B**). At 12.5 nM CFI-400945 alone, organoid formation was almost completely abolished in both lines. More than 50% inhibition of organoid formation was observed in all combination treatments, regardless of doses, with dose-dependent increases in inhibition.

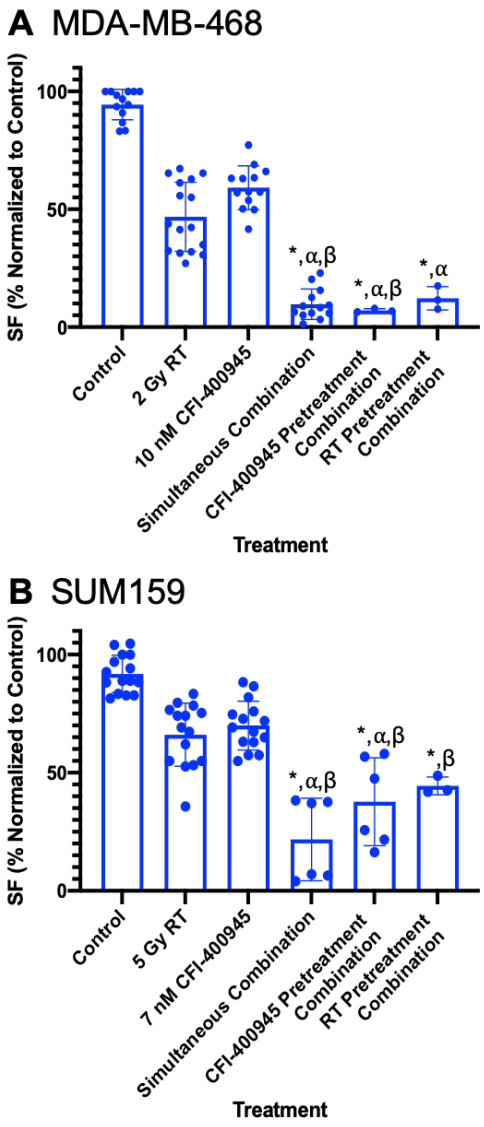

Figure 5. Effects of pre-treatment with CFI-400945 or RT on combination efficacy in TNBC cell lines. Colony formation is not further decreased in combination treatment by pre-treatment of cells with CFI-400945 for 4 days prior to RT or RT 4 days prior to initiation of CFI-400945 treatment in (A) MDA-MB-468 cells or (B) SUM159 cells (n=3). CFA were performed by treating cells with RT and CFI-400945 at pre-determined ID50/IC50 doses. After 7-14 days, colonies of 50+ cells were counted after crystal violet staining and the number of colonies in each treatment arm were normalized to control (no RT, DMSO) and compared to the simultaneous dosing conditions in MDA-MB-468 (n=13), SUM159 (n=6). *$p \leq 0.05$ compared to control, $^{\alpha}p \leq 0.05$ compared to RT only, $^{\beta}p \leq 0.05$ compared to CFI-400945 only. SF – Surviving Fraction.

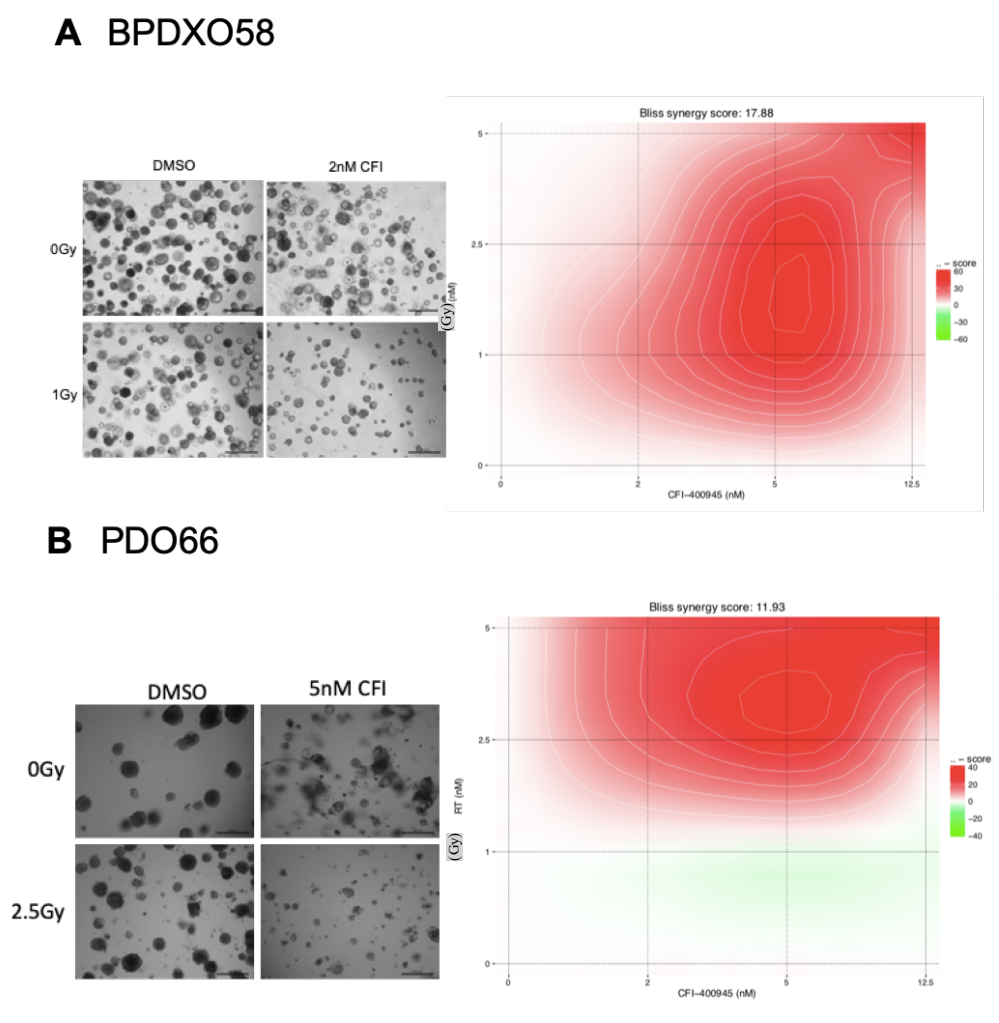

Figure 6. Effects of CFI-400945 and RT in TNBC PDO models. Organoid formation assay using various concentrations of CFI-400945 alone or in combination with various doses of RT in (A) BPDXO58 and (B) PDO66 PDO models. Dissociated PDOs plated in BME were treated with 0-5 Gy RT followed by media supplemented with 0-12.5 nM CFI-400945. Media and drug were replenished twice per 7 days. Bright-field microscopy images (4× magnification) were taken 14 days following treatment. The number of organoids were counted by 2 independent observers in at least 3 random fields per each well. The counts were normalized to respective unirradiated controls in each group. Scale bars = 500 μm. Average number of organoids was normalized to that of control (no-RT, no-drug). Synergy of combined CFI-400945 and RT was observed across several dose levels. Bliss synergy scores were calculated with SynergyFinder and are displayed in the heatmap, where intensity of red indicates higher degree of synergy (n=2).

3.4 Combination of RT with Centrinone B, AZD1152, or Paclitaxel demonstrate combinatorial anticancer effect in TNBC cell lines

CFI-400945 is known to be a potent inhibitor of PLK4, though it has other known inhibitory effects on kinases including AURKB[3]. To investigate specificity of the combination effect observed with CFI-400945 and RT to PLK4 inhibition, the most prominent target of CFI-400945, combination treatments of RT with the specific PLK4 inhibitor Centrinone B, the AURKB inhibitor AZD1152, or the cytotoxic chemotherapeutic agent Paclitaxel were tested in three TNBC cell lines. Again, the IC50 dose of Centrinone B, AZD1152, or Paclitaxel were determined for each cell line (**Fig. 7A-C, Table 3**), then used in combination with the respective ID50 dose of RT. The results of this analysis are presented in **Table 4** and **Fig. 8**. Significant decreases in colony formation in combination Centrinone B and RT treatment compared to single agents were observed in MDA-MB-468 (**Fig. 8A**) and SUM159 (**Fig. 8C**), while the decrease in MDA-MB-231 cells did not reach statistical significance (**Fig. 8B**). For the combination of AZD1152 with RT, colony formation was significantly reduced compared to single agent AZD1152 or RT in each cell line (**Fig. 8A-C**). Interestingly, the combination of Paclitaxel with RT also demonstrated a combinatorial anticancer effect. In MDA-MB-468 and MDA-MB-231 cell lines, the combination showed significantly decreased colony formation relative to single agent Paclitaxel or RT (**Fig. 8A, B**). In SUM159 cells, a similar trend was observed though the decrease in colony formation only reached significance in contrast to Paclitaxel as a single agent, not RT (**Fig. 8C**).

3.5 PLK4 inhibition enhances RT response in TNBC

To further confirm that the specificity of the observed combinatorial interaction between RT and CFI-400945 is at least partly dependent on PLK4 inhibition, we employed loss of gene function studies. TNBC cells were knocked down using two independent siRNAs targeting *PLK4*. We observed more than 75% knockdown in *PLK4* expression in all three TNBC cell lines transfected with si*PLK4*_si2 (**Fig. 9A-C**). Therefore, si*PLK4*_si2 was used in downstream experiments (subsequently referred to as si*PLK4*). To examine the

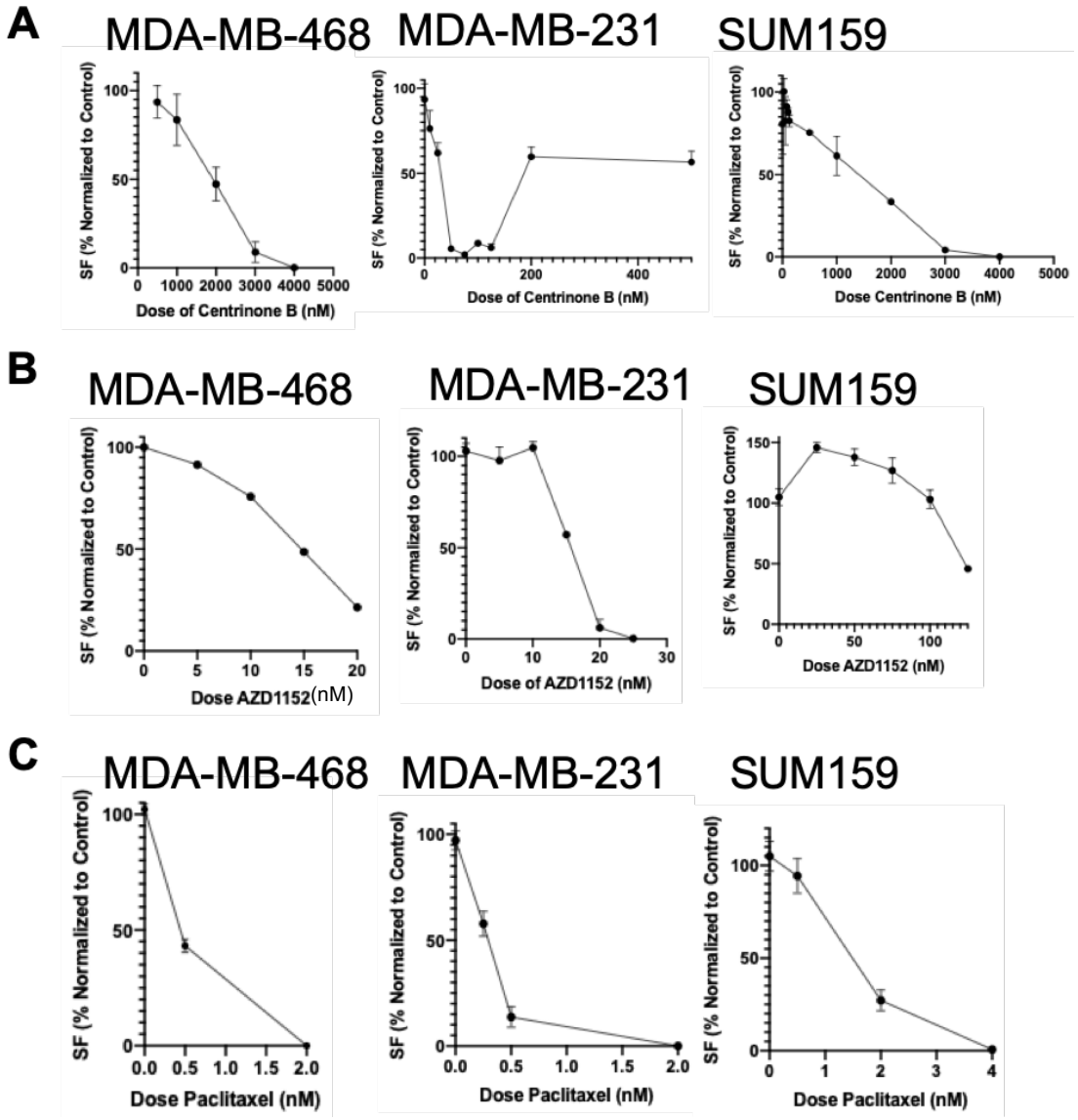

Figure 7. Dose response curves of single agent treatments in TNBC cell lines. CFA in cell lines (MDA-MB-468 (n=3 [Centrinone B, Paclitaxel], n=1 [AZD1152]), MDA-MB-231 (n=3) and SUM159 (n=3)) were performed by treating cells with a range of doses of (A) Centrinone B, (B) AZD1152 and (C) Paclitaxel to identify IC50 values via non-linear regression analysis. The number of colonies of 50+ cells were counted after 7-14 days using crystal violet staining and treatment arms were normalized to vehicle control. SF – Surviving Fraction.

Table 3. IC50 values of Centrinone B, AZD1152 and Paclitaxel in TNBC lines, calculated by non-linear regression analysis of data presented in Figure 7.

Treatment	MDA-MB-468	MDA-MB-231	SUM159
Centrinone B	1805.00 nM	25.07 nM	1050.00 nM
AZD1152	14.18 nM	15.40 nM	124.70 nM
Paclitaxel	0.49 nM	0.28 nM	1.10 nM

Table 4. Fold decrease in colony formation of TNBC lines after single agent and combination treatments of RT with PLK4 inhibitors CFI-400945 or Centrinone B, AURKB inhibitor AZD1152, or chemotherapeutic agent Paclitaxel compared to control (set to 1 indicating no change).

Treatment	MDA-MB-468	MDA-MB-231	SUM159
RT Only	1.5*	1.4*	1.5*
CFI-400945 Only	2.3*	1.2	1.4*
CFI-400945 +RT	11*,α,β	3.5*,α,β	4.4*,α,β
Centrinone B Only	1.2*	4.3*	1.2*
Centrinone B +RT	2.3*,α,β	17*,α	3.3*,β
AZD1152 Only	1.6*	2.2*	1.4*
AZD1152 +RT	3.1*,α,β	4.5*,α,β	3.9*,α,β
Paclitaxel Only	2.4*	1.7*	1.1
Paclitaxel +RT	4.3*,α,β	4.5*,α,β	2.4*,β

*$p \leq 0.05$ compared to control, $^{\alpha}p \leq 0.05$ compared to RT only, $^{\beta}p \leq 0.05$ compared to matched compound only. Data presented in rows 2-5 also presented in Table 2.

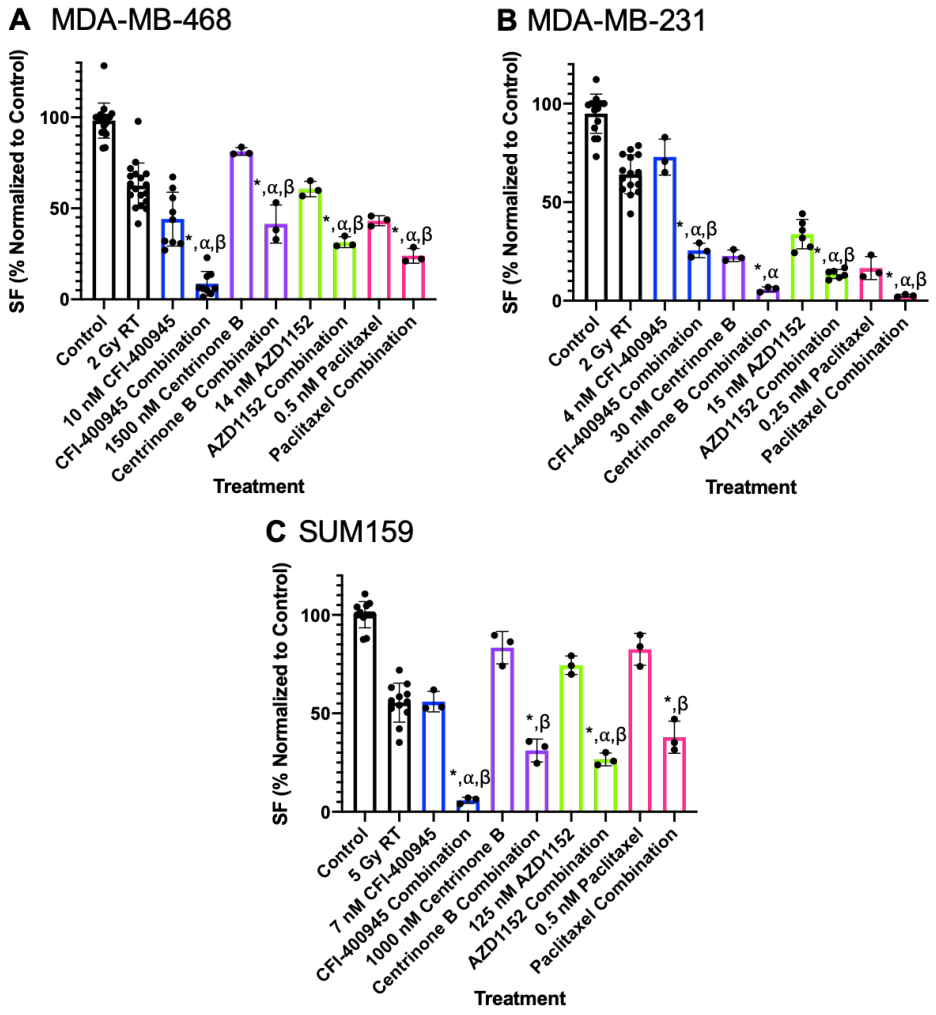

Figure 8. Effects of Centrinone B, AZD1152 or Paclitaxel in combination with RT in TNBC cell lines. Combination treatment of (A) MDA-MB-468 (n=18 [CFI-400945, RT], n=3 [Centrinone B, AZD1152, Paclitaxel]), (B) MDA-MB-231 (n=15 [CFI-400945, RT], n=3 [Centrinone B, AZD1152, Paclitaxel]) and (C) SUM159 (n=12 [CFI-400945, RT], n=3 [Centrinone B, AZD1152, Paclitaxel]) cells with RT with the specific PLK4 inhibitor Centrinone B, the AKB Inhibitor AZD1152, and the non-specific chemotherapeutic agent Paclitaxel results in reduced colony formation relative to single agents similar to CFI-400945 and RT, and effects varied between cell lines. CFA were performed by treating cells with RT and drugs at pre-determined ID50/IC50 doses. After 7-14 days, colonies of 50+ cells were counted after crystal violet staining and the number of colonies in each treatment arm were normalized to control (no RT, DMSO). *$p \leq 0.05$ compared to control, $^{\alpha}p \leq 0.05$ compared to RT only, $^{\beta}p \leq 0.05$ compared to matched compound only. SF – Surviving Fraction.

role of PLK4 in the combination effect observed between CFI-400945 and RT, TNBC cells transfected with either non-targeting (scrambled control; scsi) or *PLK4* targeting (si*PLK4*) siRNA were plated in CFA conditions. Cells were then exposed to the pre-determined ID50 doses, 5 Gy (SUM-159), 2 Gy (MDA-MB-231), and 2 Gy (MDA-MB-468) of RT. Following RT treatment, cells were cultured for 7 days (SUM-159 and MDA-MB-231) or 14 days (MDA-MB-468). Knockdown efficiency of *PLK4* was confirmed in these experiments (**Fig. 9D-F**). TNBC cells exposed to RT or deficient in *PLK4* showed a significant reduction in their colony forming ability as compared to scrambled control (**Fig 10A-F**). This decrease was further exacerbated in irradiated cells that were deficient in *PLK4* expression (**Fig 10A-F**). These observations suggest that that the synergistic action of RT and CFI-400945 is at least partly mediated by PLK4.

3.6 Combination of RT and CFI-400945 increases centriole amplification

CFI-400945 is known to induce centriole duplication[3] while RT is known to induce centriole splitting and centrosome amplification[118]. In order to investigate the combination effects of RT and CFI-400945 on centriole number, immunocytochemistry for Centrin was performed. Centrin is a centriole-associated protein used to identify centriole number per spindle pole in cells. An increased proportion of cells with overamplification of centrioles (≥3) at spindle poles was observed in all treatments, and the proportion of cells with centriole overamplification was significantly increased in combination treatment of 50 nM CFI-400945 and 8 Gy RT over single agent 8 Gy RT or 50 nM CFI-400945 ($n=4$, $p \leq 0.05$, **Fig. 11A-B**). Single agent 100 nM CFI-400945 resulted in a drastic increase in centriole overamplification over control, however no significant difference was observed between this single treatment and the combination of 100 nM CFI-400945 and 8 Gy RT. The latter observation is likely due to the saturation of the CFI-400945 effect on centriole overamplification at a dose as high as 100 nM.

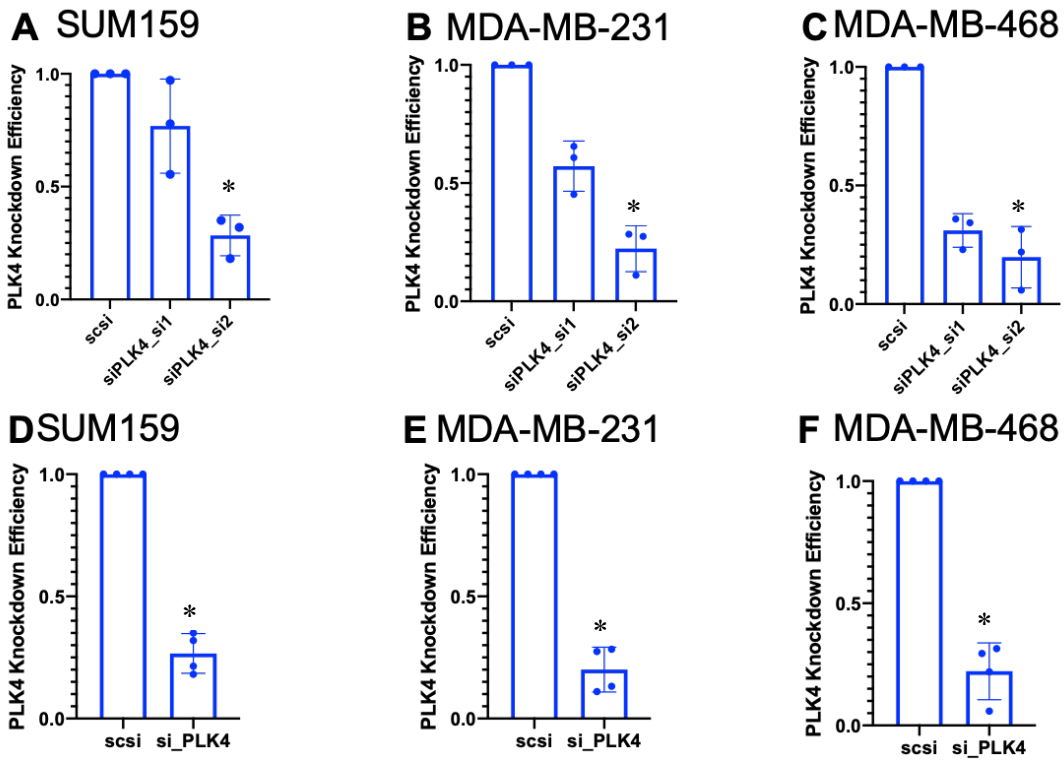

Figure 9. PLK4 knockdown efficiency in TNBC cells. (A) SUM-159, (B) MDA-MB-231 and (C) MDA-MB-468 cells were depleted of PLK4 using two independent siRNAs, and the knockdown efficiency was determined by RT-qPCR analysis (*p≤0.05, n=3). siPLK4_si2 siRNA treatment resulted in >75% knockdown in PLK4 transcript level in all cell lines. PLK4 knockdown was also confirmed in (D) SUM-159, (E) MDA-MB-231 and (F) MDA-MB-468 cells that were used for the colony formation assays presented in Figure 10 (n=4, *p≤0.05).

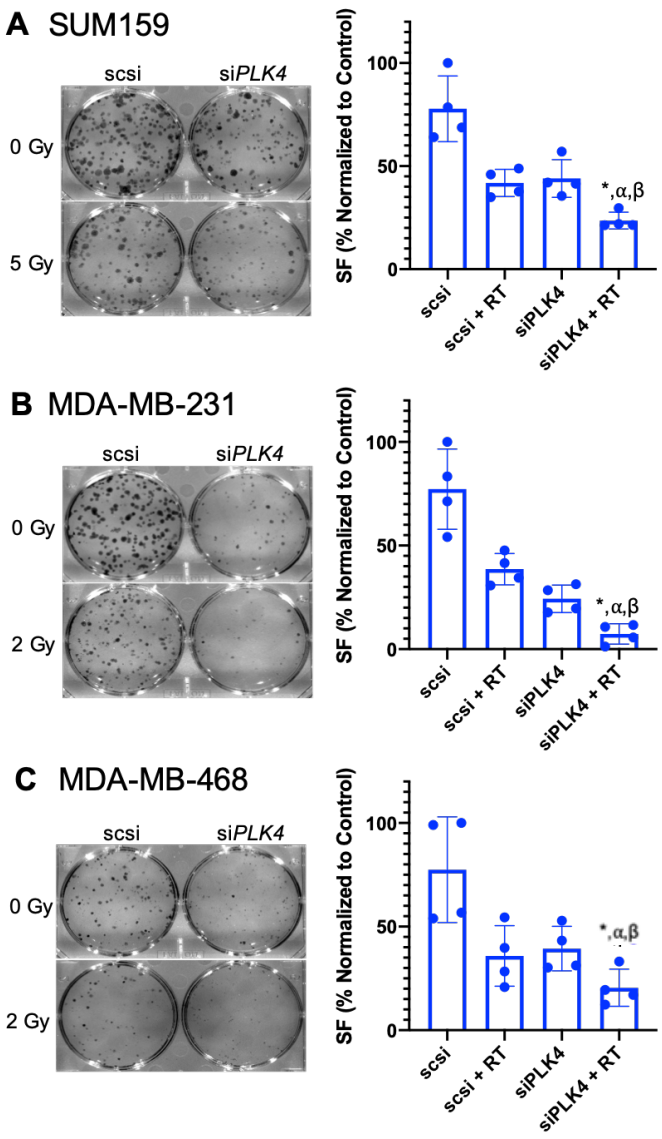

Figure 10. Effect of PLK4 knockdown in combination with RT in TNBC cell lines. PLK4 knockdown and RT demonstrate increased anticancer effects over single agent treatments in (A) SUM-159, (B) MDA-MB-231 and (C) MDA-MB-468 cells transfected with either nontargeting control (scsi) or PLK4 targeting siRNA (siPLK4) exposed to either pre-determined ID50 doses of radiation or no radiation. Average number of colonies generated in scsi, radiation alone (scsi+RT), siPLK4 and the combination (siPLK4+RT) are shown as bar graph. Representative images of colony formation of (A) SUM-159, (B) MDA-MB-231 and (C) MDA-MB-468 TNBC cells under different treatment conditions are shown (n=4, *$p \leq 0.05$ compared to control, $^{\alpha}p \leq 0.05$ compared to RT Only, $^{\beta}p \leq 0.05$ compared to siPLK4 only). SF – Surviving Fraction.

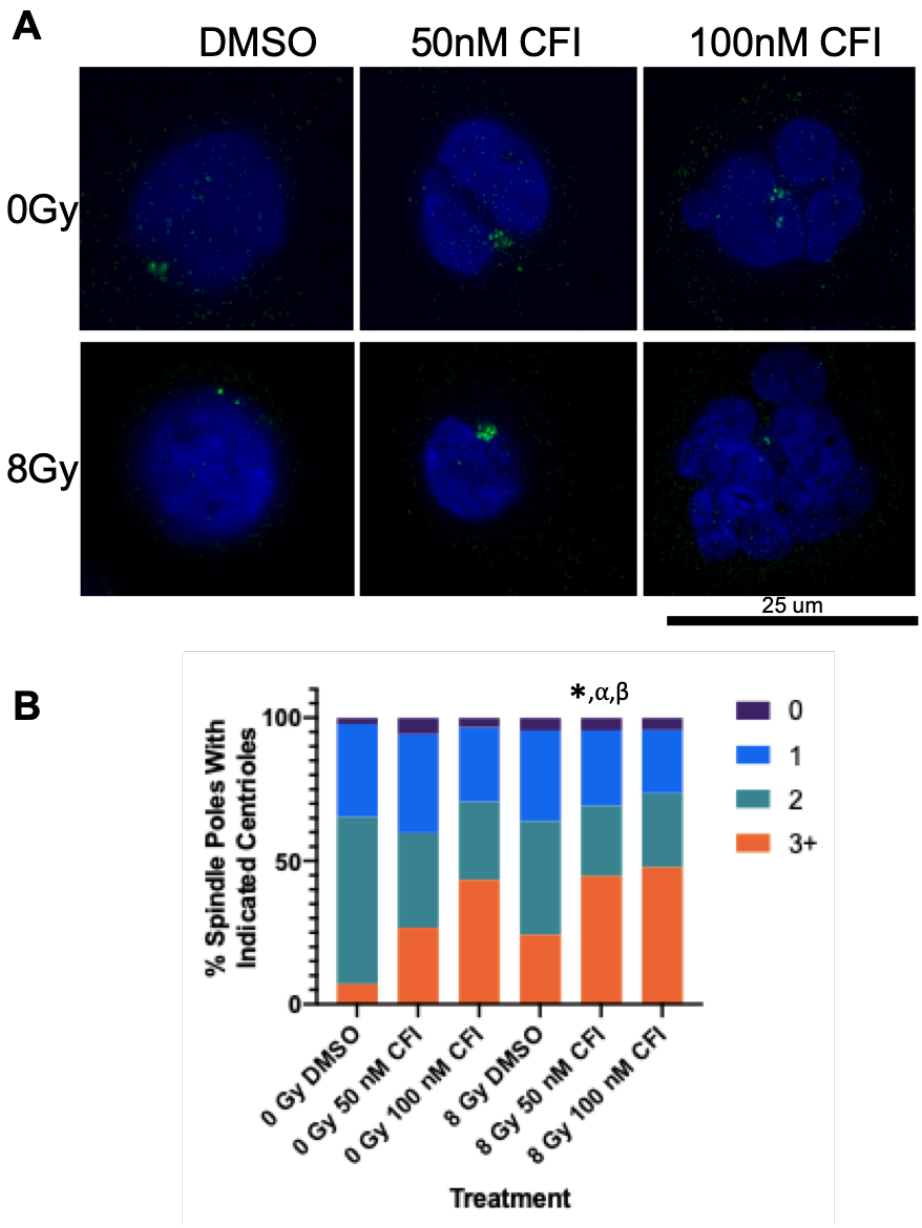

Figure 11. Effects on centriole duplication of CFI-400945 and RT combination treatment in MDA-MB-468 cell line. (A) Representative images depicting nuclear staining by DAPI (blue) and centriole staining by Centrin (green). (B) Immunocytochemistry for Centrin indicated increased centriole amplification (≥3 centrioles) after 24 hours at spindle poles in single agent treatments and this was significantly increased relative to single agent RT or CFI-400945 after combination treatment of 50 nM CFI-400945 and 8 Gy RT (n=4, *$p \leq 0.05$ compared to control, $^{\alpha}p \leq 0.05$ compared to RT Only, $^{\beta}p \leq 0.05$ compared to CFI-400945 only).

4 Discussion

TNBC often has poor outcomes due to a lack of effective treatment options, particularly in advanced or metastatic disease[2,11,26]. Development of combination therapy regimens, multimodality approaches (such as chemotherapy combined with RT) and targeted therapies in TNBC constitute a heightened focus in breast cancer research. Combination treatments with chemotherapeutic or targeted agents and/or RT that could act synergistically can improve anti-tumour activity of the treatment and improve patient outcomes[26]. New strategies based on the aforementioned approach are under investigation in several clinical trials looking at the oncologic benefits of combining RT with various targeted agents[74,76,119]. Combination therapies not only can provide synergistic anticancer action and increase efficacy and improve outcome of treatment, but also reduce toxicities of each individual agent through dose reduction in combination treatments. PLK4 inhibition is a promising targeted therapeutic strategy which has not yet been explored thoroughly in combination with other drugs or RT. The focus of the current study was to investigate anticancer potential and mechanisms of action of PLK4 inhibition by CFI-400945 in combination with RT. CFI-400945 is an orally-available drug, an advantage over other treatment options, and appears to be well-tolerated as a monotherapy in breast cancer[4]. However, response of TNBC is low compared to ER+ subtypes of breast cancer in preliminary results of ongoing clinical trial (NCT03624543), therefore investigating CFI-400945 as a combination therapy with RT could be of benefit for future clinical studies[4].

4.1 Summary and Implications of Findings

In this study, we evaluated the anticancer effects and mechanisms of action of PLK4 inhibitor CFI-400945 in combination with RT in TNBC immortalized cell lines and PDO models. We observed that CFI-400945 and RT act in synergistic fashion in both immortalized TNBC lines and PDOs to reduce colony or organoid formation, respectively. Maximal synergy was found at doses of RT and CFI-400945 near the IC50 for both in each particular model. These results are in line with previous preclinical data indicating that CFI-400945 and RT can act synergistically in TNBC cell lines MDA-MB-231, MDA-MB-468 and MDA-MB-436, PDO lines BPTO19, BXTO64 and BXTO81, as well as a MDA-MB-231 xenograft mouse model[6]. These results are also supported by

previous findings in glioblastoma where PLK4 knockdown led to radiosensitivity, identifying that PLK4 could be a promising therapeutic target in combination with RT[120]. Thus, the combination of CFI-400945 and RT is a promising combination which we delved into further. Overall, no significant differences in combinatorial anticancer effects were observed between various sequencing of treatment regiments, such as application of the drug and RT in a simultaneous fashion versus using one of those modalities shortly before the other. In MDA-MB-468, no significant differences were observed between simultaneous treatment or pre-treatment with CFI-400945 or RT. In SUM159 cells, the simultaneous treatment was actually significantly more effective at reducing colony formation than the CFI-400945 pre-treatment condition before applying radiation. This observation could be due to the variation in length of culturing time for the CFA in MDA-MB-468 (14 days) and SUM159 cells (7 days). Since MDA-MB-468 cells take longer to form detectable colonies, the CFA was performed for 14 days without cells/colonies overtaking the culture well, while in SUM159 cells that time was limited to 7 days due to their faster growth rate. As a result, the shorter, 7-day treatment length of SUM159 cells for CFA significantly reduced the length of combination treatment, since the 4-day pre-treatment condition meant that RT was administered only 3 days prior to the experiment endpoint. This may not be enough time for a large proportion of the cells to experience the effects of the combination that result in cell arrest or death. Based on these results, a simultaneous treatment of drug and RT was used in the experiments going forward.

Next, we aimed to investigate if combinatorial effects of RT with CFI-400945 are mediated through specific inhibition of PLK4. CFI-400945 has potent inhibitory activity against PLK4, but is also known to inhibit other kinases including AURKB[3]. To specifically examine the PLK4 inhibition mechanism in combination with RT, an additional PLK4 inhibitor, Centrinone B, was tested in combination with RT, as was an siRNA knockdown approach. Studies have demonstrated Centrinone B as a specific inhibitor of PLK4 which does not have the same inhibitory activity against AURKB and other kinases as CFI-400945, and is considered highly specific to PLK4 inhibition[121]. In our study, we observed that PLK4 inhibition from Centrinone B also demonstrated a combinatorial anticancer effect with RT in TNBC cell lines, but only achieved statistical significance in anticancer effect compared to both single agent RT or Centrinone B in the

MDA-MB-468 line. However, we experienced difficulties in selecting the correct IC50 dose of Centrinone B for use in these studies in MDA-MB-231 cells due to a biphasic effect observed on cell survival. These results indicating that a combinatorial anticancer effect could be achieved with Centrinone B and RT suggest that PLK4 inhibition is a major contributing factor to the combination effects of CFI-400945 with RT. It has been previously observed that in cervical tumour HeLa cells, Centrinone B enhanced mitotic catastrophe in cells exposed to 2.5 Gy RT. In contrast, Centrinone B treatment of EMT6 murine mammary cells reduced cell growth and mitotic catastrophe after ionizing radiation[122]. The conflicting results in previous studies between HeLa and EMT6 cells, along with our findings that the extent of the combinatorial anticancer effects of Centrinone B and RT vary by cell line, may indicate the influence of heterogeneity between cancer types and individual cancers of the same type on treatment response. This reinforces the benefit of precision medicine approaches which could identify biomarkers of positive treatment response. Furthermore, loss of function of *PLK4* also demonstrated combinatorial effect in the TNBC cell lines similar to drug-induced PLK4 inhibition. This confirms that the combinatorial effect of CFI-400945 and RT is likely at least partially mediated through PLK4 inhibition mechanisms.

It has been suggested in previous studies that the inhibition of AURKB is a key contributor in the failure of mitotic division and amplification of centrioles and centrosomes in cells treated with CFI-400945[63]. As a result, the AURKB inhibitory effect could be contributing to the mitotic abnormalities in TNBC cells after CFI-400945 treatment that acts with RT to enhance the anticancer effect of the treatments. Our findings in this study that showed that AZD1152 and RT act in a combinatorial manner to decrease colony formation in TNBC[5]. This could indicate that inhibition of AURKB by CFI-400945 could also play a role in the synergistic effects on cell proliferation/colony formation observed with RT and CFI-400945. AURKB inhibition is known to exert cellular effects similar to PLK4 inhibition since it targets the microtubules or microtubule-organizing centres which maintain genomic integrity and mitotic fidelity[121,123,124]. AURKB is localized to the centromeres and microtubules and is involved in the processes of chromosome alignment, kinetochore orientation and cytokinesis, and this kinase is often aberrantly expressed in breast cancer[125]. Similar to our results, in the colon cancer cell line HCT116, AZD1152 has been previously found to

enhance radiosensitivity by increasing mitotic catastrophe[126]. Since our results demonstrated that the anticancer effects of AURKB with RT varied by cell line, it is possible that different inhibitory mechanisms are involved depending on the cell line profiles, such as the kinome and proteome. This further emphasizes the value of a precision medicine approach in clinical decision making and treatment of patients with breast cancer.

We also tested the combination of chemotherapy agent Paclitaxel with RT and found a combinatorial anticancer effect. Paclitaxel is a taxane-based chemotherapeutic agent which binds to the tubulin component of microtubules, stabilizing them and rendering them non-functional, inducing cell cycle arrest[127]. We selected Paclitaxel to test in combination with RT since it is a commonly used therapeutic option, that also acts in the same area of microtubules and microtubule-organizing centres in cells to CFI-400945, AZD1152 and Centrinone B[127]. Paclitaxel has been previously identified as a radiosensitizing agent, in line with our findings that there is a combination anticancer effect between Paclitaxel and RT. This combination has been investigated in clinical trials in non-small cell lung cancer and breast cancer with promising results[127–129]. Since Paclitaxel, AURKB inhibitors, and PLK4 inhibitors all demonstrate combinatorial anticancer effects with RT, further studies on combination therapy regimens with RT and drugs targeting microtubules and microtubule organizing centres are warranted. Based on our results, the action of CFI-400945 and RT is likely multifactorial, involving PLK4, AURKB, and possibly other known or unknown off-target effects of this drug. It appears that regulation of centrioles, centrosomes, microtubules and kinetochores may play an essential role in enhancing radiosensitivity which can be exploited therapeutically.

Next, we aimed to examine potential mechanisms of action of CFI-400945 and RT in combination. In this study, we observed an increase in the percentage of cells with overamplified centrioles after single agent CFI-400945 at doses of 50 nM and 100 nM, and single agent RT at 8 Gy. In combination treatment conditions of 50 nM CFI-400945 with 8 Gy RT, there was a further increase over single agents in the portion of cells with 3 or greater centrioles, a characteristic indicative of a likelihood for genomic instability, aneuploidy, mitotic catastrophe, and mitotic arrest[130,131]. However, we did not observe an increase in centriole overamplification between the 100 nM CFI-400945 treated cells and

those treated with combination of 100 nM CFI-400945 and 8 Gy, likely due to a saturating CFI-400945 effect on centriole overamplification. Taken together, our findings suggest that CFI-400945 and RT assert their synergistic anticancer effects at least in part through overamplification of centrioles. Our combination therapy studies had suggested that that the mechanisms of CFI-400945 and RT could be multifactorial, involving the DNA damage response and centriole duplication leading to aneuploidy. Aneuploidy can result in mitotic arrest, mitotic catastrophe and cell death in cancer cells, and targeted therapies which exacerbate aneuploidy could work in combination with RT which is known to induce mitotic abnormalities[132]. Abnormal centriole amplification leads to asymmetrical centrosomes causing chromosome missegregation and aneuploidy[133]. CFI-400945 is known to have a biphasic effect on centriole duplication at spindle poles: at doses from 10 to100 nM it induces overamplification of centrioles, with a large proportion of cells having 3 or greater centrioles per spindle pole, while at doses over 200 nM it prevents centriole duplication resulting in 1 centriole per spindle pole[3], and our results reflected the overamplification effect. Radiation has also been shown to induce centrosome amplification in cancer cells, the main cause of which is often centriole splitting or overamplification[122,130,134]. Future studies investigating mechanisms of the observed effects on centriole overamplification upon combination treatment might identify new targets and outline new avenues for enhancing radiosensitivity.

Based on our studies, the combination of CFI-400945 and RT shows clear promise in TNBC as a therapeutic option that warrants further investigation and future consideration in translational and clinical studies. The results of this study provide insight into possible mechanisms of this combination including inhibition of PLK4, AURKB, and increased centriole overamplification, which can be delved into further to create a comprehensive understanding in future studies.

4.2 Potential Limitations of the Study

This project had some limitations which could affect the ability of the results to be translationally relevant. Only two PDO models were used to test for synergy between CFI-400945 and RT due to a lack of patient samples available, slow growth and development, and financial restrictions. As a result, these findings may not necessarily reflect the responses of patients as the heterogeneity of all patients is not represented, and

these experiments should be expanded to further PDO models to assist in identifying markers of sensitivity and resistance to this combination treatment. However, our results were consistent across three human TNBC cell lines, one mouse cell line, and two PDO models, and were in line with previous findings that included an additional TNBC cell line and 3 other PDO models[6], demonstrating strong promise for this combination treatment of CFI-400945 and RT. The quantification method for organoid formation assays relied on human observation and counting of all organoids present across several horizontal planes under a microscope, which can be imprecise due to missed or duplicate counts as well as requiring the observer to decipher if organoid is large enough to be counted. This is a known limitation to many organoid studies currently, though accuracy was improved in our study by utilizing two independent observers, and experiments were performed in duplicate for each PDO line, demonstrating consistency. Colony formation and organoid formation assays are the current gold standard experimental procedure when investigating the effects of RT[135], therefore they were used consistently in this study. Computational and artificial intelligence strategies for counting organoid formation are in development and could mitigate this limitation in the future.

In this study, we confirmed synergy of CFI-400945 and RT in the 4T1 cell line and two PDO lines, and previous findings in MDA-MB-468, MDA-MB-231 and MDA-MB-436 cell lines demonstrated synergy as well[6]. However, time and resource availability limited the number of doses of the drugs (CFI-400945, Centrinone B, AZD1152, Paclitaxel) and RT combinations which were used to look at whether there was a combinatorial anticancer effect in the other TNBC cell lines. As a result, it is difficult to confirm whether the combinatorial effects observed in MDA-MB-468, MDA-MB-231 and SUM159 cells in this study are actually synergistic since a matrix of doses is needed for the statistical calculations. In addition, the extent of off-target effects of CFI-400945 inhibiting kinases other than PLK4 made pinpointing a mechanism of action of the combination of CFI-400945 and RT difficult. We looked at several possible mechanisms and were able to identify the centriole overamplification effect, though the future studies discussed in the previous paragraph could be used to further identify mechanisms.

Due to time constraints we limited the mechanistic studies on centrioles to the MDA-MB-468 model. However, we plan in the future to expand this to more models and these are underway in the other cell lines (MDA-MB-231 and SUM159) used in this thesis.

Finally, animal studies would further improve the ability of this work to be translated to clinical research, including understanding the role of immune system. The lack of immune system in PDO and PDX models is a limitation, therefore expanding the work testing the combination of CFI-400945 and RT into 4T1 syngeneic mouse models where the immune system is intact would provide interesting insight into the efficacy of this combination. Our promising results *in vitro* using 4T1 cell culture have provided the foundation for this combination to be tested in this syngeneic *in vivo* model.

4.3 Future Directions

To improve understanding of the cellular response to the combination of CFI-400945 and RT, studies directly investigating aneuploidy and mitotic catastrophe would be beneficial. This could be done using chromosome missegregation or lagging chromosome immuno-fluorescent assays. Examining whether the extent of chromosome missegregation and aneuploidy is further enhanced under combination treatment with CFI-400945 and RT would indicate a clear intracellular mechanism of this anticancer effect. In addition, studies on apoptosis, senescence and cell cycle arrest would provide valuable information on the fate of cells after combination treatments. CFI-400945 has been demonstrated to increase apoptosis in MDA-MB-468 cells after 24-72 hours, but to a lesser extent in MDA-MB-231 and HMEC cell lines, where instead cells continued to undergo endoreduplication (replication of nuclear gene content in cells arrested in S phase) without progressing to cell division[3]. RT is also a known inducer of senescence in both normal and cancerous human cells[136], therefore a combination treatment of CFI-400945 and RT may be causing cell cycle arrest and senescence in TNBC cells and this should be studied. In this study we examined the effect of the combination of CFI-400945 and RT on centriole duplication, though in the future, expansion into examining centrosome amplification, an additional key aspect of mitotic catastrophe and aneuploidy, is also important. RT is known to induce centrosome amplification in cancer cells, and CFI-400945 can also cause amplified centrosomes resulting from overduplication of

centrioles[3,122]. Identifying whether centrosomes are amplified in this combination would provide further proof to the effect it is having in enhancing abnormal mitotic division.

An overarching strategy to investigate the cellular responses to this combination treatment would be to use RNA sequencing to compare the transcriptome of control and single agent-treated cells or organoids to those treated with the combination of CFI-400945. By analyzing differences in pathway activation, the mechanisms by which this combination has a synergistic anticancer effect can be pinpointed. Finally, in order for this promising combination treatment to move from bench to bedside, further translational studies are required. While xenografts of MDA-MB-231 cells in mice responded well to this combination[6], these mouse studies can be expanded into further cell line xenografts, PDX models and 4T1 syngeneic mouse models which will confirm efficacy in a more representative set of models. Following positive results in mouse studies, and dependent on outcomes of the current Phase II trial of CFI-400945 as a monotherapy, clinical trials using the combination of CFI-400945 and RT should be considered.

4.4 Final Conclusions

In summary, this project identified that CFI-400945 and RT are a promising combination for a multimodality treatment approach in TNBC, demonstrating efficacy in immortalized TNBC cell lines and translational PDO models. Our understanding of how these therapies act in combination against TNBC has also been improved, as we established that the observed anticancer effects were at least partially mediated by PLK4 inhibition by CFI-400945 with other possible contributing mechanisms including inhibition of AURKB. These inhibitory effects increase centriole amplification in TNBC cells, likely leading to aneuploidy, mitotic catastrophe, cell cycle arrest and cell death. These findings provide a foundation for future studies investigating the mechanism of this combination further. The promising results of this study also indicate that further translational studies using this combination are warranted. This combination could eventually be tested in clinical studies pending results of the Phase II trial on CFI-400945 as a monotherapy in breast cancer, and this combination could be used to improve outcomes in TNBC patients.

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Appendices

Appendix A: HSREB Approval Letter for Study #118019: Establishing a Biobank of Breast Surgical Specimens for Research.

Western Research

Date: 25 January 2021

To: Dr. Armen Parsyan

Project ID: 118019

Study Title: Establishing a Biobank of Breast Surgical Specimens for Research

Application Type: HSREB Initial Application

Review Type: Delegated

Full Board Reporting Date: 09/Feb/2021

Date Approval Issued: 25/Jan/2021

REB Approval Expiry Date: 25/Jan/2022

Dear Dr. Armen Parsyan

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
Biobank of Breast Surgical Specimens - Telephone Script - 2020.11.19 Version 1.0	Telephone Script	19/Nov/2020	Version 1.0
Biobank of Breast Surgical Specimens - Research Coordinator Telephone Script - 2021.01.07 Version 1.0	Telephone Script	07/Jan/2021	Version 1.0
Biobank of Breast Surgical Specimens - Protocol - 2021.01.07 Version 1.1	Protocol	07/Jan/2021	Version 1.1
Biobank of Breast Surgical Specimens - LOI - 2021.01.25 Version 1.2	Written Consent/Assent	25/Jan/2021	Version 1.2 -

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB , except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Karen Gopaul , Ethics Officer on behalf of Dr. Joseph Gilbert, HSREB Vice-Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Appendix B: HSREB Approval Letter for Study #118685: Anticancer Effects of Radiation Therapy Combined with PLK4 Inhibitor CFI-400945 in Breast Cancer.

Western Research

Date: 30 March 2021

To: Dr. Armen Parsyan

Project ID: 118685

Study Title: Anticancer Effects of Radiation Therapy Combined with PLK4 Inhibitor CFI-400945 in Breast Cancer

Application Type: HSREB Initial Application

Review Type: Delegated

Full Board Reporting Date: 20/April/2021

Date Approval Issued: 30/Mar/2021 14:55

REB Approval Expiry Date: 30/Mar/2022

Dear Dr. Armen Parsyan

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. **All other required institutional approvals and mandated training must also be obtained prior to the conduct of the study**.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
There are no data collection instruments being used	Other Data Collection Instruments	08/Mar/2021	
Effects of Radiation with PLK4 Inhibitor in Breast Cancer - Study Protocol - 08Mar2021	Protocol	08/Mar/2021	Version 08 Mar 2021

Documents Acknowledged:

Document Name	Document Type	Document Date	Document Version
Effects of Radiation with PLK4 Inhibitor in Breast Cancer - Budget - 08Mar2021	Study budget	08/Mar/2021	08 Mar 2021

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB , except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Ms. Nicola Geoghegan-Morphet , Ethics Officer on behalf of Dr. Joseph Gilbert, HSREB Vice-Chair

Appendix C: 4T1 Colony Formation Assay Data. Data is presented as % survival normalized to control. Values are shown in triplicate and were input into SynergyFinder software to determine Bliss synergy score data displayed in Figure 3E.

Dose CFI-400945 (nM)	Dose RT (Gy) 0	2	4	6
0	100.00	84.043	27.66	10.11
	112.23	68.09	31.38	5.85
	101.59	66.49	30.32	7.98
6.25	43.62	19.15	4.23	1.06
	49.47	10.64	1.60	0.53
	60.64	14.36	2.13	0.00
12.5	27.66	6.91	1.60	0.00
	19.68	4.26	0.53	0.00
	15.96	2.66	1.06	0.00
25	15.43	2.66	0.00	0.00
	10.64	2.13	0.00	0.00
	10.64	0.53	0.53	0.00

Appendix D: PDO Organoid Formation Assay Data. Data is presented as % survival normalized to control for **(A)** BPDXO58 and **(B)** PDO66. Values are shown in duplicate and were input into SynergyFinder software to determine Bliss synergy score data displayed in Figure 6A-B.

A. BPDXO58

Dose CFI-400945 (nM)	Dose RT (Gy) 0	1	2.5	5
0	103.60	80.23	56.30	18.75
	100.00	211.90	178.57	-
2	69.59	49.19	41.70	9.59
	161.90	61.90	90.48	-
5	56.67	38.75	27.97	4.90
	280.95	42.86	4.76	-
12.5	1.26	0.64	0.31	0.32

B. PDO66

Dose CFI-400945 (nM)	Dose RT (Gy) 0	1	2.5	5
0	100.00	87.73	125.15	100.61
	100.00	22.14	87.47	17.02
2	102.45	96.93	102.45	82.21
	105.42	29.08	90.79	6.47
5	93.25	86.50	69.33	47.24
	52.80	8.57	21.79	2.16
12.5	14.11	9.82	19.02	30.06

Curriculum Vitae

Name: Sierra Pellizzari

Post-secondary Education and Degrees:
University of Western Ontario
London, Ontario, Canada
2016-2020 BMSc

Honours and Awards:
Ontario Graduate Scholarship
2021-2022

Department of Surgery Research Day Poster Award
2022

London Oncology Research and Education Day Poster Award
2022

Anatomy and Cell Biology Research Day Oral Presentation Award
2021

Translational Breast Cancer Research Scholarship
2020-2021

MCAHN Scholarship
2016-2022

Western Scholarship of Excellence
2016-2017

Ontario Principals Council Scholarship
2016-2017

Publications:

Anticancer Effects of Radiation Therapy Combined with Polo-Like Kinase 4 (PLK4) Inhibitor CFI-400945 in Triple Negative Breast Cancer. Armen Parsyan, Jennifer Cruickshank, Kelsey Hodgson, Drew Wakeham, Sierra Pellizzari, Vasudeva Bhat and David W.Cescon. *The Breast*, April 2021 (IF: 4.38).

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