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Therapeutic Resistance in Breast Cancer Cells Can Result from Deregulated EGFR Signaling.

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Abstract

The epidermal growth factor receptor (EGFR) interacts with various downstream molecules including phospholipase C (PLC)/protein kinase C (PKC), Ras/Raf/MEK/ERK, PI3K/ PTEN/Akt/GSK-3, Jak/STAT and others. Often these pathways are deregulated in human malignancies such as breast cancer. Various therapeutic approaches to inhibit the activity of EGFR family members including small molecule inhibitors and monoclonal antibodies (MoAb) have been developed. A common problem with cancer treatments is the development of drug-resistance. We examined the effects of a conditionally-activated EGFR (v-Erb-B:ER) on the resistance of breast cancer cells to commonly used chemotherapeutic drugs such as doxorubicin, daunorubicin, paclitaxel, cisplatin and 5-flurouracil as well as ionizing radiation (IR). v-Erb-B is similar to the EGFR-variant EGFRvIII, which is expressed in various cancers including breast, brain, prostate. Both v-Erb-B and EGFRvIII encode the EGFR kinase domain but lack key components present in the extracellular domain of EGFR which normally regulate its activity and ligand-dependence. The v-Erb-B oncogene was ligated to the hormone binding domain of the estrogen receptor (ER) which results in regulation of the activity of the v-Erb-ER construct by addition of either estrogen (E2) or 4-hydroxytamoxifen (4HT) to the culture media. Introduction of the v-Erb-B:ER construct into the MCF-7 breast cancer cell line increased the resistance to the cells to various chemotherapeutic drugs, hormonal-based therapeutics and IR. These results point to the important effects that aberrant expression of EGFR kinase domain can have on therapeutic resistance.

1. Introduction

Multiple signaling pathways are induced by EGFR and EGFR-related molecules including Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/GSK-3β/mTORC1, Jak/STAT and PLC/PKC. These pathways play critical roles in growth regulation and are abnormally regulated in numerous human cancers (McCubrey et al., 2012a; McCubrey et al., 2012b; Davis et al., 2014, McCubrey et al., 2014a, McCubrey et al., 2014b). An overview of the interactions between these pathways and how they are involved in drug-resistance is presented in Figure 1. Also included in this figure is an overview of how chemotherapeutic drugs and 4HT can induce reactive oxygen species (ROS) which can stimulate many pathways and regulatory molecules including: ataxia–telangiectasia serine/threonine kinase (ATM), TP53, microRNAs (miRs), cell cycle ($p21^{Cip-1}$) and apoptotic regulators (PUMA, NOXA). EGFR, PLC/PKC and ROS can also induce the calcium calmodulin kinases (CaMKs) which can in turn regulate multiple signaling pathways (Franklin et al., 2006; Wu et al., 2006; You et al., 2010; Dai et al., 2017).

1.1. Involvement of EGFR Family Members in Breast Cancer.

Mutations and genetic alterations occur at EGFR family member genes in breast cancer which leads to the abnormal expression of these and other downstream pathways (Davis et al., 2014; Sokolosky et al., 2014; McCubrey et al., 2014a; McCubrey et al., 2014b). Due to the critical involvement of EGFR family members in breast cancer, multiple effective approaches to target various EGFR family members have been developed including small molecule kinase inhibitors and MoAb. A schematic overview of EGFR family members is presented in Figure 2.

1.2 EGFR1 and the EGFR variant III (EGFRvIII) in Breast Cancer.

EGFR1 is often called EGFR. The truncated EGFR gene (EGFRvIII) has been proposed to be involved in the malignant transformation of many types of cancers, (e.g., brain, breast, ovarian prostate and others) (Gan et al., 2013). It is constitutively-active and lacks part of the ligand

binding domain. The v-Erb-B is an oncogene contained in the avian erythroblastosis virus. It also lacks part of the ligand binding domain.

Introduction of the *EGFRvIII* into breast cancer cells resulted in HER2 phosphorylation, potentially through heterodimerization and cross-talk (Tang et al., 2000). MCF-7 is an estrogen receptor positive (ER+) breast cancer cell line with a low tumorigenic potential. MCF-7/EGFRvIII cells were more tumorigenic than parental MCF-7 cells in athymic nude xenograft experiments.

The EGFRvIII protein has been detected in human cancers, but not in normal tissues. EGFRvIII and EGFR mRNAs were observed in primary invasive breast cancers upon laser capture microdissection (LCM)/RT-PCR. EGFRvIII mRNA transcripts were detected in 67.8% of pure breast cancer cells (Ge et al., 2002). 57.1% of the infiltrating breast carcinomas expressed both EGFR1 wild type (WT) and EGFRvIII mRNAs in the same tumor. EGFRvIII mRNAs were not detected in normal breast tissues. Immunohistochemical analysis confirmed these results. Co-expression of EGFRvIII and EGFR WT proteins was observed in some human invasive breast cancer tissues, but co-expression was not detected in normal breast samples.

In a different study consisting of 55 breast cancer cell lines and 170 primary breast cancers similar results were not observed. The authors concluded that expression of EGFRvIII was extremely rare in the breast cancer cells examined in their study (Rae et al., 2004).

The expression of EGFRvIII mRNA in women with breast cancer was examined in another study by RT-nested PCR. EGFRvIII mRNAs were detected in peripheral blood from 30% of 33 low risk, early stage patients. EGFRvIII mRNAs were detected in 56% of eighteen patients selected for neoadjuvant chemotherapy. EGFRvIII mRNAs were observed in 63.6% of eleven patients with disseminated disease but not in any of forty control women (Silva et al., 2006). EGFRvIII expression was associated with ER- or HER2+ expression in early stage patients.

The expression of EGFR and its phosphorylation status as well as the presence of EGFRvIII, were examined by immunohistochemistry in a study of 225 breast cancer patients.

Patient outcomes were also followed (Nieto et al., 2007). 48% of the patients expressed EGFR and over half (54%) of the patients expressed activated (phosphorylated)-EGFR. 4% of the patients were positive for EGFRvIII. EGFR expression was correlated with negative hormone receptor (HR) status, worse relapse-free survival and overall survival than patients that did not express EGFR This study concluded that EGFR expression was an important prognostic indicator in the HER2+ and the ER-/progesterone receptor negative (PR-) subgroups.

EGFRvIII expression is also associated with the Wnt/β-catenin pathway and downstream target gene expression. The expression of many these genes is associated with self-renewal and cancer stem cells (CSCs). EGFRvIII expression has linked with increased *in vitro* mammosphere formation and tumor formation (Del Vecchio et al., 2012).

EGFRvIII has also been associated with estrogen-independence and tamoxifen-resistance in breast cancer such as the MDA-MB-361 breast cancer cell line which is an ER+/PR+, luminal mammary carcinoma. Tamoxifen is an ER agonist/antagonist. 4-hydroxytamoxifen (4HT, Afimoxifene) is a selective estrogen receptor modulator (SERM) and is the major active metabolite of tamoxifen. Higher levels of activated Akt and ERK were detected in MDA-MB-361 cells transfected with EGFRvIII than non-transfected cells. EGFRvIII had pronounced stimulatory effects on tumorigenicity of MDA-MB-361 cells. In addition, EGFRvIII expression has been correlated with PR expression. EGFRvIII expression was associated with an absence of PR protein in invasive human breast cancer specimens (Zhang et al., 2009).

1.3 HER2 and Breast Cancer.

HER2 is the EGFR family member the most frequently implicated in breast cancer. Many drugs have been developed to target HER2. HER2 (a.k.a. ErbB2, EGFR2, Neu) is overexpressed in approximately 15-30% of women with breast cancer. Overexpression can be due to gene amplification or increased transcription of the HER2 gene. The humanized MoAb herceptin targets HER2 and is used in therapy. It is one of the first MoAb-based therapeutics that was approved by

the FDA in 1998. A problem with herceptin therapy is the development of resistance. The cyclin dependent kinase (CDK) inhibitor p57 ($p57^{Kip2}$) is a downstream target of Akt and is important in HER2-mediated tumorigenicity. Akt is a negative regulator of $p57^{Kip2}$ as overexpressing Akt decreases $p57^{Kip2}$ expression while inhibition of Akt results in $p57^{Kip2}$ stabilization. Akt phosphorylates $p57^{Kip2}$ on S282 and T310 and Akt promotes cytoplasmic localization of $p57^{Kip2}$. HER2/Akt activation results in increased turnover of $p57^{Kip2}$ by ubiquitination. This results in HER2-mediated cell proliferation (Zhao et al., 2013). In contrast, HER2+ breast cancer patients with high levels of $p57^{Kip2}$ have a better overall survival.

HER2 can interact with EGFRvIII to enhance tumorigenesis (Yu et al., 2008). EGFRvIII can also interact with chemokine receptor 4 (CXCR4) (Rahimi et al., 2010; Rahimi et al., 2011). These interactions activate signaling pathways important in migration, invasion and tumorigenesis. It is more difficult to down-regulate the constitutive, ligand-independent nature of EGFRvIII. Thus, the signaling complexes induced between EGFRvIII and HER2 and CXCR4 may be prolonged in comparison to interactions between EGFR and HER2 and CXCR4. CXCR4 is highly expressed in breast cancers. CXCR4 is implicated in metastasis, CSCs and resistance to targeted therapy such as the dual EGFR/HER2 inhibitor lapatinib (Müller et al., 2001; Mukherjee and Zhao, 2013). Lapatinib-resistant breast cancer cells were derived. Src and CXCR4 were involved in the resistance to lapatinib as well as the invasiveness of the cells (De Luca et al., 2014).

1.4. EGFR3 and Breast Cancer.

The expression and role of the EGFR3 receptor in breast and other cancers is complex. EGFR3 lacks a functional kinase domain. EGFR3 binds the EGFR family ligands neuregulin 1 (NRG1) and neuregulin 2 (NRG2). Binding of these ligands results in the dimerization with another EGFR family member and a conformation change which leads to activation of downstream signaling. EGFR3 can heterodimerize with any of the other three EGFR family members (Holbro et al., 2003).

1.5. EGFR4 and Breast Cancer.

The role of EGFR4 in breast cancer is different than either EGFR or HER2 complex (Pitfield et al., 2006; Mill et al., 2011). EGFR4 expression has been associated with improved outcomes in certain breast cancer patients undergoing various therapies (Portier et al., 2013). HER2+ patients which also expressed EGFR4 exhibited a significant delay in the development of metastasis after herceptin treatment. Furthermore, these patients exhibited a significant improvement in progression free survival after herceptin treatment. In contrast, nuclear EGFR4 was associated with resistance to herceptin and associated with a poor outcome in HER2-positive breast cancer. It is possible to prevent EGFR4 cleavage with a secretase inhibitor and inhibit EGFR tyrosine kinase activity by nerbtinib treatment. This combination resulted in decreased EGFR4 nuclear translocation and enhanced the response to herceptin. Preventing nuclear translocation of EGFR4 is important in herceptin-sensitivity (Mohd Nafi et al., 2014). Nerbtinib is approved by the USA FDA to treat HER2 positive breast cancer patients that are resistant to herceptin.

Upon analysis of 238 primary invasive breast cancer patients, the localization of the intracellular domain of EGFR4 and the presence of certain alternatively-spliced exons of EGFR4 were determined to have prognostic significance in ER+/HER2- breast cancers (Fujiwara et al., 2014). Patients with HER2/EGFR4 expression displayed a delay in the development of metastasis after neo-adjuvant herceptin therapy as well as an improvement in progression free survival (Portier et al., 2013).

1.6. Interactions between the EGFR and PLC/PKC Pathways.

Activated EGFR and EGFRvIII can induce the PLC/PKC pathway (Kadamur and Ross., 2013; An et al., 2018). This can result in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG). PKC is a multi-gene family of serine (S)/threonine (T) kinases. Depending on the cell type and target molecules PKCs can have either tumor suppressor or tumor promoter functions (Aeder et al., 2004; Uht et al., 2007;

Fan et al., 2009; Ma et al., 2013; Antal et al., 2015). PKCs phosphorylate and activate many effector molecules including GSK-3 (Goode et al., 1992). These events can influence many processes important in normal cells growth as well as cancer. These phosphorylation events influence tumor angiogenesis, invasion, proliferation, and survival. Substrates phosphorylated by PKC include cell cycle regulators such as TP53 and $p21^{Cip-1}$, cell growth and proliferation regulators Ras/Raf and glycogen synthase kinase 3 (GSK-3), cell motility regulators including integrins, apoptotic regulators Bcl-2 and Bad, and the transcription factor NF-κB.

Various PKC isoforms have different functions in terms of tumor growth. In glioblastoma (GBM), PKCα, PKCη and PKCδ elicit pro-tumorigenic functions. In contrast, high levels of PLCγ are correlated inversely with survival of GBM (Mawrin et al., 2003). EGFR can interact with PKCs to transmit the growth promoting signaling to mTORC1 which results in proliferation of GBM cells. This was shown to be independent of Akt (Fan et al., 2009). Suppression of PKC decreased the viability of GBM cells. PKCη can stimulate proliferation of GBM cells by the Akt/mTORC1 pathway (Aeder et al., 2004). PKCs such as PKCη can interact with the Raf/MEK/ERK pathway to stimulate proliferation (Uht et al., 2007).

1.7. Downstream of the EGFR and PKC Pathways-The PI3K/PTEN/Akt/mTORC1 Ras/Raf/MEK/ERK and TP53 Pathways.

Mutations in downstream components of the EGFR pathway can contribute to cancer in many cell types. Examples include loss of functional *PTEN* and mutations at *PIK3CA*. These mutations may confer sensitivity to drugs which target mTORC1 such as rapamycin, rapalogs, PI3K/mTOR dual inhibitors and metformin (Iijima et al., 2002, Wheler et al., 2014a; Wheler et al., 2014b; Steelman et al., 2016).

The PI3K/PTEN/Akt/mTORC1 pathway is frequently altered in breast cancer, in part due to activating mutations in *PIK3CA* and inhibition of PTEN activity by multiple mechanisms. In studies by Wheler et al, the mutational status of key genes in this pathway were examined in 57

women with metastatic breast cancer. 216 mutations were observed in seventy genes (Wheler et al., 2014a; Wheler et al., 2014b).

The most common gene alterations observed in this study included: *TP53, PIK3CA, CCND1 (Cyclin D), MYC, HER2, MCL1, PTEN, FGFR1, GATA3, NF1, PIK3R1, BRCA2, EGFR, IRS2, CDH1, CDKN2A, FGF19, FGF3* and *FGF4*. Many different types of genetic alterations were observed which included point mutations, amplifications, deletions, splice mutations, truncations, fusions and rearrangements. In some cases, there were multiple alterations in the same gene. This study documents the important roles of gene mutations in breast cancer and also indicates the possibility of therapeutic intervention with precision medicine as many of the genes encode proteins which are druggable targets (Wheler et al., 2014a; Wheler et al., 2014b).

In a study with 104 formalin-fixed paraffin-embedded triple negative breast cancer (TNBC) patient samples, the mutational status of key genes in the PI3K/PTEN/Akt/mTORC1, Raf/MEK/ERK and TP53 pathways were determined in 44 genes. *TP53* was determined to be mutated in greater than 80% of the TNBC patient samples. *PIK3CA* mutations were detected in 29.8 of the patient samples. Amplification or deletion of PI3K-associated genes were detected in 7.7% of the patient samples. Mutations at the Raf/MEK/ERK pathway members were detected in 8.7% of the TNBC patient samples. Finally, mutations in cell cycle regulators were detected in 14.4% of the TNBC patient samples examined in this group (Kriegsmann et al 2014).

1.8. PKC, Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR Pathways Involvement of Breast Cancer Drug Resistance.

PKC has been implicated in multi drug-resistance which occurs in many cancers including breast cancer due to deregulated expression or activity of MDR-1 (Pgp) (Fine et al., 1996; Rumsby et al., 1998; Garraway et al., 2002). A brief depiction of the roles of these pathways in the phosphorylation of drug transporters is present in the right side of Figure 1. PKC can also activate MDR-1 through the Raf/MEK/ERK pathway. PLC can also induce MDR-1 expression which

occurs via the Raf/MEK/ERK pathway (Yang et al., 2001). Introduction of activated v-Ha-Ras or v-Raf-1 induced MDR-1 expression and transformation of rat epithelial cells as well as resistance to chemotherapeutic drugs (Burt et al., 1988). Overexpression of PKCα can increase the tumorigenicity of MCF-7 cells (Ways et al., 1995).

Both the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways lies downstream of EGFR and EGFRvIII. These pathways are frequently implicated in the resistance of breast and other cancers to chemotherapeutic drugs as well as hormonal-based therapies (Weinstein-Oppenheimer et al., 2001; Davis et al., 2003; Lee et al., 2004; Steelman et al., 2010; Abrams et al., 2010; Taylor et al., 2011; McCubrey et al., 2011; Steelman et al., 2011; Sokolosky et al 2011, Davis et al., 2014; McCubrey et al., 2014b, Wheler et al., 2014b).

The PI3KAkt pathway has effects on both MDR-1 and the breast cancer resistance protein (BCRP) and drug resistance (Lee et al., 2005; Steelman et al., 2008; Abrams et al., 2010; Steelman et al., 2010; Zhang et al., 2020).

Kinases and phosphatases can regulate Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways and influence sensitivity to targeted therapy, hormonal-based and chemotherapeutic drugs. Thus, these pathways have many sites of regulation and disrupting one molecule can have consequences on the activity of many downstream molecules which regulate cellular growth and malignant transformation (Lee et al., 2004; McCubrey et al., 2008; Steelman et al., 2008; Steelman et al., 2011; Solokosky et al., 2011; McCubrey et al., 2012a; McCubrey et al., 2012b; McCubrey et al., 2014a). PKC can also regulate MAPK kinase phosphatase-1 (MKP-1) activity which regulates the activity of the Raf/MEK/ERK pathway and drug resistance in some cells (Valledor et al., 1999). Thus, there are many sites of interaction of these pathways which can result in drug resistance.

2. Materials & Methods.

2.1. Cell Lines and Growth Factors.

MCF-7 cells (Soule et al., 1973) were purchased from the ATCC (Rockville, MD, USA) and used at low passage numbers. MCF-7 cells were maintained in a humidified 5% CO_2 incubator with (RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA).

MCF-7 cells (Soule et al., 1973) were chosen for these studies as they are ER+ and wild type (WT) at TP53. The MCF-7 cell line represents a well characterized model system to investigate drug resistance in ER+, TP53 WT breast cancer. In addition, MCF-7 cells contain a mutation in *PIK3CA* which is frequently altered in breast cancer [Turturro et al., 2016]. Piqray (Alpelisib) is a PI3K-p110α inhibitor that was approved by the FDA in 2019 for treatment of HR+, HER2-, *PIK3CA*-mutated, advanced or metastatic breast cancer patients following progression on or after an endocrine-based regimen (Andre et al., 2019). The MCF-7 cell line was used in pre-clinical studies to evaluate the effects of Piqray on ER+ breast cancer (Chen et al, 2017). Other ER+ breast cancer cell lines lack WT TP53 (e.g., T47D). Triple negative breast cancer cell lines (e.g., MDA-MBA-231) are HR- and often have mutant TP53 and other oncogenes.

2.2. Analysis of Cell Sensitivity to Anticancer Agents.

Chemotherapeutic and hormonal based drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sensitivities of the cells to doxorubicin, paclitaxel, daunorubicin, cisplatin or 5-fluorouracil were investigated by characterizing the effects of these agents on proliferation as described (Steelman et al., 2008; Abrams et al., 2010; Steelman et al., 2011). The four different cell types (MCF-7, MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR) and MCF-7 + v-Erb-B:ER (4HT + DoxR) were seeded in 96-well cell culture plates (BD Biosciences, San Jose, CA) at a density of 5,000 cells/well in 100 µl/well of phenol red free RPMI-1640 containing 5% charcoal stripped (CS) FBS as described in reference (Steelmen et al., 2011). Cells were plated in 6 wells for every culture condition. Cells were incubated for days to permit cells to adhere to the bottom of each well. Cells were subsequently treated with serial two-fold dilutions of the different

chemotherapeutic drugs, some in the presence of 100 nM 4HT. The four different cell lines were then incubated at 37°C for four days until the extent of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, St. Louis, MO) reduction in each well was quantified at 530 nm. Data were plotted with GraphPad software. Inhibitory concentration 50% (IC_{50}) is defined in this context as the drug dose that causes the cells to proliferate to a level that is half as much as when the cells were incubated in the absence of drugs as determined by MTT assays as described (Steelman et al., 2008).

2.3. Infection of MCF-7 Cells with Retroviral Vector Encoding v-Erb-B:ER and Selection of Drug Resistant Cells.

MCF-7 breast cancer cells were infected with v-Erb-B:ER viral stocks as described (McCubrey et al., 2004) in six well Corning tissue culture plates (Corning, Elmira, NY). Neo^r MCF-7 cells were isolated by selection in medium containing 2 mg/ml G418 as described (McCubrey et al., 2004). The nomenclature of the v-Erb-B:ER-infected cells is MCF-7 + v-Erb-B:ER. 4 hydroxytamoxifen-resistant (4HTR) MCF-7 + v-Erb-B:ER(4HTR) were isolated by culturing MCF-7 + v-Erb-B:ER cells in the presence of 100 nM 4HT for two months. 4HTR and doxorubicin-resistant MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells were isolated by culturing MCF-7 + v-Erb-B:ER in the presence of 100 nM 4HT and 25 nM doxorubicin for 2 months.

2.4. Colony Formation in the Absence and Presence of Radiation.

Approximately 2,000 cells were seeded into each well of Corning six well plates in RPMI 1640 containing phenol red dye as a pH indicator and 10% FBS and allowed to adhere to the bottom of the well for 24 h before ionizing radiation (IR). Each treatment condition was performed in triplicate with each cell line and each IR dose. The next day the plates were irradiated with Gammacell 40 (Atomic Energy of Canada Limited, Cs137 source) with different doses of ionizing IR). Cells were seeded in triplicate for each treatment condition. IR, ranging from 0 to 8 grays was administered to the cells, and the media was removed, and the different selective media were

provided. The media in the wells were changed every three days and then after approximately three weeks of culture, the number of colonies determined after fixing the cells for 10 min with 3:1 mixture of methanol:acetic acid and stained for 10 min with 0.5% crystal violet (Sigma, St. Louis, MO, USA) in methanol.

3. Results.

To determine the effects of v-Erb-B:ER expression on the sensitivity of cells to chemotherapeutic drugs, we infected MCF-7 cell line with a retrovirus encoding v-Erb-B:ER which is similar in structure to the naturally occurring EGFRvIII truncated EGFR oncoprotein observed in breast other cancers, but not normal tissues. The activity of the v-Erb-B:ER can be increased by treatment of the cells with either E2 or 4HT which induces the dimerization of the v-Erb-B:ER protein as they contain the hormone binding domain of the ER linked to the v-Erb-B oncogene in the chimeric construct (McCubrey et al., 2004). We chose to activate the v-Erb-B:ER with 4HT in these studies with MCF-7 cells as they are ER+ and E2 would increase proliferation.

3.1 Effects of v-Erb-B on Proliferation in the Absence of Chemotherapeutic Drugs.

We examined the effects of v-Erb on proliferation of MCF-7, MCF-7 + v-Erb-B:ER and the two drug-resistant MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER(4HTR+ DoxR) lines in the presence and absence of 25 nM 4HT and five different chemotherapeutic drugs (Figures 3-5). MCF-7 cells proliferated to the highest level in the absence of chemotherapeutic drugs and 4HT (Figure 3, Panel A). In the absence of 4HT, the MCF-7 + v-Erb-B:ER cells proliferated, but to not as high levels as observed in MCF-7 cells. In contrast, the 4HTR MCF-7 + v-Erb-B:ER(4HTR) proliferated slower and the 4HTR + DoxR MCF-7 + v-Erb-B:ER(4HTR+DoxR) proliferated the slowest. Thus, cells which were drug-resistant proliferated slower than either MCF-7 or MCF-7+ v-Erb-B:ER cells.

3.2 Effects of v-Erb-B on Proliferation in the Presence of Chemotherapeutic Drugs.

When the same experiment was performed in medium containing 4HT, a different result was observed. The ER agonist/antagonist 4HT suppressed the proliferation of MCF-7 and MCF-7+ v-Erb-B:ER cells (Compare Panels B vs A). However, the MCF-7 + v-Erb-B:ER and MCF-7 cells proliferated to similar levels in the presence of 4HT. Thus, the growth of the MCF-7 + v-Erb-B:ER was not as inhibited as MCF-7 cells (compare Panels B vs Panel A) as the MCF-7 cells proliferated to higher levels in the absence of 4HT (Panel A). The drug-resistant MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-ERB-B:ER (4HTR+ DoxR) cells proliferated at lower levels in the presence of 4HT. We have observed that continuous culture of MCF-7 cells in medium containing 4HT results in downregulation of the ER (Steelman et al., 2011).

When MCF-7 cells were cultured in 25 nM doxorubicin in the absence of 4HT, they proliferated to a higher extent than any of v-Erb-B:ER-transduced cells (Figure 3, Panel C). In contrast when the cells were cultured in the presence of 25 nM doxorubicin and 4HT (Figure 3, Panel D), the MCF-7 + v-Erb-B:ER cells proliferated to a higher level, demonstrating the effects of the activated v-Erb-B:ER on the ability to cells to grow in the presence of the chemotherapeutic drug doxorubicin and the ER antagonist 4HT. The amount of growth of MCF-7 in the presence of 4HT+ Dox were lower than the levels of growth when MCF-7 cells were cultured in just doxorubicin (Compare Panels D vs C) or just 4HT (compare Panels D and B). In contrast, the levels of growth of the MCF-7 + v-Erb-B:ER in 4HT and doxorubicin were higher than MCF-7 cells.

When MCF-7 and MCF-7 + v-Erb-B:ER cells were cultured in 25 nM daunorubicin, they proliferated to similar levels (Figure 4, Panel A). The 4HTR and 4HTR + DoxR MCF-7 + v-Erb:ER cells proliferated to lower levels in medium containing daunorubicin. When MCF-7 and MCF-7 + v-Erb-B:ER cells were cultured in 25 nM daunorubicin in the presence of 4HT, MCF-7 + v-Erb-B:ER proliferated to higher levels than MCF-7 cells (Figure 4, Panel B). The levels of proliferation of MCF-7 and MCF-7 + v-Erb-B:ER cells were lower in the presence of

4HT+daunorubicin than in just daunorubicin (Compare Panels A and B). The 4HTR and 4HTR + DoxR MCF-7 + v-Erb:ER cells proliferated to lower levels in medium containing 4HT+ daunorubicin.

MCF-7 cells proliferated to higher levels in the presence of 5 nM paclitaxel than the various MCF-7 cells containing v-Erb-B:ER (Figure 4, Panel C). In contrast, when the cells were plated in the presence of 4HT and 5nM paclitaxel, MCF-7 + v-Erb-B:ER cells proliferated to higher levels (Figure 4, Panel D) than MCF-7 cells. The levels of proliferation of MCF-7 + v-Erb-B:ER in 5nM paclitaxel in the presence and absence of 4HT were similar (Compare Panels D and C). In contrast, the level of proliferation of MCF-7 cells decreased when they were cultured in 4HT + paclitaxel in comparison to when they were cultured in just paclitaxel (Compare panels D and C). The 4HTR and 4HTR + DoxR MCF-7 + v-Erb:ER cells proliferated to lower levels in medium containing 4HT and paclitaxel.

MCF-7 proliferated to higher levels in the presence of 250 nM 5-fluoruoracil than the various MCF-7+ v-Erb-B:ER cells (Figure 5, Panel A). In contrast when the cells were plated in the presence of 4HT and 250 nM 5-fluorouracil, MCF-7 + v-Erb-B:ER cells proliferated to higher levels than the other cells (Panel B). The levels of proliferation of MCF-7 and MCF-7 + v-Erb-B:ER were reduced when they were cultured in 4HT and 5-fluorouracil than when they were cultured in 5-fluoruoracil by itself. In summary, in the absence of 4HT, MCF-7 cells proliferated to higher levels than the v-Erb-B:ER transformed cells. However, in the presence of 4HT, the MCF-7 + v-Erb-B:ER proliferated to higher levels than MCF-7 cells demonstrating that activation of the v-Erb-B:ER enhanced growth in the cells in the presence of 5-fluoruoracil.

MCF-7 cells proliferated to higher levels in the presence of 1 µM cisplatin than the various MCF-7 + v-Erb-B:ER cells (Figure 5, Panel C). In contrast, when the cells were plated in the presence of 4HT and 1 µM cisplatin, MCF-7 + v-Erb-E:ER cells proliferated to higher levels than MCF-7 cells (Figure 5, Panel D). The level of proliferation of MCF-7 and MCF-7 + v-Erb-B:ER

cells decreased when they were cultured in 4HT + 1 μM cisplatin in comparison to when they were cultured in just cisplatin (Compare panels D and C).

Thus, in the absence of 4HT, MCF-7 proliferated to higher levels than cells containing v-Erb-B:ER. However, in the presence of 4HT, MCF + v-Erb-B:ER proliferated to higher levels than MCF-7 cells demonstrating that activation of v-Erb-B:ER could increase the resistance of the cells to 4HT. The drug-resistant cells that were selected in either 4HT or 4HT + DoxR proliferated to lower levels than either MCF-7 or MCF-7 + v-Erb-B:ER.

3.3 Effects of v-Erb-B:ER on Chemoresistance of MCF-7 Breast Cancer Cells.

Figures 6-10 present the IC_{50} curves for the MCF-7 and drug-sensitive and -resistant MCF-7/v-Erb-B:ER cells. Table 1 presents the IC_{50} values for the MCF-7 and drug-sensitive and -resistant MCF-7/v-Erb-B:ER cells.

The IC_{50} for MCF-7 cells cultured in doxorubicin in the absence of 4HT was 55 nM. In contrast, when the MCF-7 cells were cultured with 25 nM 4HT, the doxorubicin IC_{50} decreased slightly to 45 nM (Figure 6, Panel A). The IC_{50} for MCF-7 + v-Erb-B:ER cells cultured in doxorubicin in the absence of 4HT was 70 nM. In contrast, when the MCF-7 + v-Erb-B:ER cells were cultured with 25 nM 4HT, the doxorubicin IC_{50} increased 1.7-fold to 120 nM (Figure 6, Panel B). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR) cells cultured in doxorubicin in the absence of 4HT was 120 nM, in contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the doxorubicin IC_{50} increased 9.2-fold to 1,100 nM (Figure 6, Panel C). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells cultured in doxorubicin in the absence of 4HT was 280 nM. In contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the doxorubicin IC_{50} increased 2.9-fold to 800 nM (Figure 6, Panel D).

The IC_{50} for MCF-7 cells cultured in daunorubicin in the absence of 4HT was 30 nM. In contrast, when the MCF-7 cells were cultured with 25 nM 4HT, the daunorubicin IC_{50} decreased 1.7-fold to 18 nM (Figure 7, Panel A). The IC_{50} for MCF-7 + v-Erb-B:ER cells cultured in

daunorubicin in the absence of 4HT was 30 nM, in contrast, when the MCF-7 + v-Erb-B:ER cells were cultured with 25 nM 4HT, the daunorubicin IC_{50} increased 2.1-fold to 63 nM (Figure 7, Panel B). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR) cells cultured in daunorubicin in the absence of 4HT was 60 nM, in contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the daunorubicin IC_{50} increased 4.2-fold to 250 nM (Figure 7, Panel C). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells cultured in daunorubicin in the absence of 4HT was 130 nM. In contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the daunorubicin IC_{50} increased 2.3-fold to 300 nM (Figure 6, Panel D). The MCF-7 + v-Erb-B:ER (4HT + Dox) resistant cells were more resistant to daunorubicin than the 4HTR MCF-7 + v-Erb-B:ER(4HTR) cells.

The IC_{50} for MCF-7 cells cultured in cisplatin in the absence of 4HT was 120 µM. In contrast, when the ER+ MCF-7 cells were cultured with 25 nM 4HT, the cisplatin IC_{50} decreased 1.8-fold to 65 µM (Figure 8, Panel A). The IC_{50} for MCF-7 + v-Erb-B:ER cells cultured in cisplatin in the absence of 4HT was 200 µM. In contrast, when the MCF-7 + v-Erb-B:ER cells were cultured with 25 nM 4HT, the cisplatin IC_{50} increased 1.5-fold to 300 µM (Figure 8, Panel B). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR) cells cultured in cisplatin in the absence of 4HT was 200 µM. In contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the cisplatin IC_{50} increased 1.5-fold to 300 µM (Figure 8, Panel C). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells cultured in cisplatin in the absence of 4HT was 250 µM. In contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 µM 4HT, the cisplatin IC_{50} increased 2.5-fold to 400 µM (Figure 8, Panel D). The MCF-7 + v-Erb-B:ER (4HT + Dox) resistant cells were more resistant to paclitaxel than the 4HTR MCF-7 + v-Erb-B:ER(4HTR) cells.

The IC_{50} for MCF-7 cells cultured in paclitaxel in the absence of 4HT was 9.5 nM. In contrast, when the ER+ MCF-7 cells were cultured with 25 nM 4HT, the paclitaxel IC_{50} decreased 2.4-fold to 4 nM (Figure 9, Panel A). The IC_{50} for MCF-7 + v-Erb-B:ER cells cultured in

paclitaxel in the absence of 4HT was 10 nM. In contrast, when the MCF-7 + v-Erb-B:ER cells were cultured with 25 nM 4HT, the paclitaxel IC_{50} increased 15-fold to 100 nM (Figure 9, Panel B). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR) cells cultured in paclitaxel in the absence of 4HT was 28 nM. In contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the paclitaxel IC_{50} increased 3.6-fold to 100 nM (Figure 9, Panel C). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells cultured in paclitaxel in the absence or absence of 4HT was 200 nM.

The IC_{50} for MCF-7 cells cultured in 5-fluorouracil in the absence of 4HT was 2.5 µM. In contrast, when the ER+ MCF-7 cells were cultured with 25 nM 4HT, the 5-fluorouracil IC_{50} increased slightly to 3.2 µM (Figure 10, Panel A). The IC_{50} for MCF-7 + v-Erb-B:ER cells cultured in 5-fluorouracil in the absence of 4HT was 3 µM. In contrast, when the ER+ MCF-7 + v-Erb-B:ER cells were cultured with 25 nM 4HT, the 5-fluorouracil IC_{50} increased 2-fold to 6 µM (Figure 10, Panel B). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR) cells cultured in 5-fluorouracil in the absence of 4HT was 6 µM. In contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the 5-fluorouracil IC_{50} increased 1.5-fold to 9 µM (Figure 10, Panel C). The IC_{50} for MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells cultured in 5-fluorouracil in the absence of 4HT was 5 µM. In contrast, when the MCF-7 + v-Erb-B:ER (4HTR) cells were cultured with 25 nM 4HT, the cisplatin IC_{50} increased 1.8-fold to 9 µM (Figure 10, Panel D).

3.5 Effects of v-Erb-B:ER on Colony Formation in the Absence and Presence of Radiation.

In the absence of radiation and either 4HT or doxorubicin treatment, uninfected MCF-7 cells had an approximately 1.5-fold higher plating efficiency than MCF-7 + v-Erb-B:ER and 2.3- and 3.6-fold higher plating efficiency than MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER(4HTR+ DoxR) cells respectively. Thus, in the absence of drugs and radiation, the v-Erb-B:ER oncoprotein suppressed colony formation. Furthermore, the drug-resistance cells had even lower colony formation that unselected MCF-7 + v-Erb-B:ER cells.

The colony numbers were normalized to untreated cells to determine the effects of the v-Erb-B:ER on various treatments in more detail (Figure 11). When the number of colonies was examined after two grays of radiation (Figure 11, Panel A), in the absence of any drug treatment, 1.6-fold more colonies were observed in MCF-7 + v-Erb-B:ER than in MCF-7 + v-Erb-B:ER(4HTR) cells. After 4 grays of radiation 1.4-fold more colonies were observed in MCF-7 than in MCF-7 + v-Erb-B:ER cells and 3.6- and 3-fold more colonies than in MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER(4HTR + DoxR) cells respectively. Thus, in the absence of 4HT and doxorubicin, the drug- resistant cells had lower levels of colony formation after 2 or 4 grays of radiation than either MCF-7 or MCF-7 + v-Erb-B:ER cells. After 6 and 8 grays of radiation, few colonies were observed in all cell types. In the absence of 4HT (Panel A),

The MCF-7 cells, which lacked the v-Erb:B:ER construct displayed similar colony formation as the MCF-7/v-Erb-B:ER cells at 2 gray and even slightly more at 4 gray (MCF-7 vs MCF-7 + v-Erb-B:ER cells). If phenol red was significantly activating the v-Erb-B:ER significantly, then the MCF-7 + v-Erb-B:ER cells would have been predicted to be more resistant to radiation and exhibited higher cloning ability.

3.6. Effects of v-Erb-B:ER on Colony Formation in 4HT, Doxorubicin, 4HT+Doxorubicin in Absence and Presence of Radiation.

The abilities of the parental MCF-7, MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER (4HTR+ DoxR) to form colonies in the presence of 4HT, doxorubicin and 4HT+doxorubicin were examined to determine the effects of the activated v-Erb-B:ER oncogene on the colony formation in the presence of radiation (Figure 11).

When the cells were plated in medium containing 100 nM 4HT in the absence of radiation (Panel B), the highest colony formation was observed in MCF-7 + v-Erb-B:ER cells which was 2-fold greater than observed in MCF-7 cells. The MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER (4HTR+ DoxR) cells had 1.7- and 1.3-fold more colonies than MCF-7 cells.

After 2 grays of radiation treatment and culture in the presence of 100 nM 4HT, 2.5, 2.1, and 2.8 fold more colonies were observed in MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER (4HTR+ DoxR) cells respectively than in MCF-7 cells demonstrating the ability of the activated v-Erb-B:ER to allow colony formation after 2 grays of radiation in the presence of 4HT. With higher doses of radiation, few colonies were observed in any of the MCF-7 and various MCF-7 + v-Erb-B:ER cells.

When the cells were plated in medium containing 25 nM doxorubicin in the absence of radiation (Panel C), the highest colony formation was observed in MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells which was 1.3-fold greater than observed in MCF-7 cells. The MCF-7 + v-Erb-B:ER had 1.2-fold more colonies than MCF-7 cells. The MCF-7 + v-Erb-B:ER (4HTR) had 0.8-fold less colonies than MCF-7 cells.

After 2 grays of radiation treatment and culture in the presence of 25 nM doxorubicin, 1.8-, 1.7-, and 2-fold more colonies were observed in MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER (4HTR+ DoxR) cells respectively than in MCF-7 cells demonstrating the ability of the v-Erb-B:ER to allow colony formation after 2 grays of radiation in the presence of doxorubicin. Less colonies were observed after culture with doxorubicin than with 4HT. With higher doses of radiation, few colonies were observed in any of the MCF-7 and MCF-7 + v-Erb-B:ER cells.

When the cells were plated in medium containing 100 nM 4HT + 25 nM doxorubicin in the absence of radiation (Panel D), the highest colony formation was observed in MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells which was 4.6-fold greater than observed in MCF-7 cells. The MCF-7 + v-Erb-B:ER (4HTR) had 3-fold more colonies than MCF-7 cells. The MCF-7 + v-Erb-B:ER had 1.9 -fold more colonies than MCF-7 cells.

After 2 grays of radiation treatment and culture in the presence of 100 nM 4HT and 25 nM doxorubicin, 3.4-, 3.1-, and 4-fold more colonies were observed in MCF-7 + v-Erb-B:ER, MCF-7

+ v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER (4HTR+ DoxR) cells respectively than in MCF-7 cells demonstrating the ability of the v-Erb-B:ER to allow colony formation after 2 grays of radiation in the presence of 4HT and doxorubicin.

After 4 grays of radiation treatment and culture in the presence of 100 nM 4HT and 25 nM doxorubicin, the differences in colony formation in the MCF-7 and the various MCF-7 + v-Erb-B:ER cells were modest. Namely, 1.3-, 1.2-, and 1.5-fold more colonies were observed in MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER (4HTR+ DoxR) cells respectively than in MCF-7 cells. In summary, the v-Erb-B:ER oncoprotein could stimulate some resistance to 2 grays of radiation.

4. Discussion.

Many studies have indicated interactions between EGFR, PKC and breast cancer. They have been summarized recently in a review [Hsu and Hung, 2016]. Angiotensin (Ang II) was demonstrated to activate the Raf/MEK/ERK pathway via EGFR and PKC signaling in breast cancer [Greco et al., 2003]. Nine PKC isozymes were expressed in primary breast cancer cells, but Ang II only induces a translocation from the cytosol to the plasma membrane with four of them. Only PKC- α and - β1/2 were involved in stimulation of cell proliferation [Greco et al., 2003]. In 2005, PKC-zeta was shown to be required for EGF/EGFR-induced chemotaxis of breast cancer cells [Sun et al., 2005]. Other studies have shown that inhibition of PKCα preferentially targets breast cancer CSCs. PKCα inhibition specifically targets CSCs but has minor effects on non-CSCs. Generation of CSCs from non-stem cells involves a shift from EGFR to PDGFR/PKCα signaling and PKCα-dependent activation Fra1, an important component of the AP-1 transcription factor complex. Activation of Fra1 is important in induction of EMT which is associated with drug resistance Thus, PKCα is associated with EGFR/PDGFR signaling, EMT, and drug resistance in breast cancer [Tam et al., 2013]. Recently, the hormonally up-regulated neu-associated kinase

[HUNK] tyrosine kinase has been shown to phosphorylate EGFR potentially by a PKC-dependent mechanism [Williams et al., 2020].

Our studies were undertaken to examine some of the effects of the aberrant expression of v-Erb-B oncogene, related to both the EGFR and the mutant EGFRvIII gene, on the growth of breast cancer cells. EGFR, EGFRvIII and the related HER2 gene have been implicated as contributing oncogenic factors in many cancer types. Identification of mechanisms by which they can cause drug resistance could further our treatment of these diseases. Introduction of the v-Erb-B:ER oncogene conferred resistance of MCF-7 breast cancer cells to certain chemotherapeutic and hormonal based drugs as well as two grays of radiation.

We realize that the presence of the estrogen receptor (ER) hormone binding (HB) domain of the ER in the v-Erb-B construct does complicate interpretation of results, however, it is an inducible and not constitutive construct. Introduction of v-Erb-B or EGFRvIII by themselves would result in constitutive expression. In our scenario with v-Erb-B:ER, we can observe that addition of tamoxifen (4HT) results in both resistance to chemotherapeutic drugs and 4HT. In contrast, with constitutively active oncogenes, the effects of the oncogenes are not necessarily so clear as different clones or pools may express different levels of the activated oncogene and some may lose expression of the activated oncogene, especially if it is not necessary for the cells to grow under the given culture conditions. We observed this when we introduced activated Raf, Akt, or PTEN oncogenes into various cell types (Weinstein et al., 2001; Davis et al., 2003; Steelman et al., 2008; Steelman et al., 2011). We have demonstrated that the v-Erb-B:ER construct can be induced by estrogen or 4HT (McCubrey et al., 2004; McCubrey et al., 2015) and results in activation of the Raf/MEK/ERK and PI3K/PTEN/Akt and other signaling pathways.

We do not believe that the differences in responses to 4HT and chemotherapy between MCF-7 + v-Erb-B:ER and MCF-7 + v-Erb-B:ER (4HTR) were due to down-regulation of the v-Erb-B:ER construct in the 4HT resistant cells. Previously, we have shown that growth of MCF-

7/ΔAkt-1:ER cells in the presence of 4HT results in increased expression of ΔAkt-1:ER compared to unselected cells (Steelman et al., 2011).

Increased expression of the Raf/MEK/ERK pathway has been observed in tamoxifen-resistant breast cancer (Haagenson et al., 2014). Treatment of MCF-7 cells with 4HT increased the levels of MAPK kinase phosphatase-2 (MKP-2) but not MKP-1 protein levels. Enhanced expression of MKP-1 or MKP-2 suppressed E2-induced proliferation. MKP-2 was determined to increase sensitivity to tamoxifen while MKP-1 overexpression did not. ERK1/2 phosphorylation was lost in MKP-1 and MKP-2 overexpressing cells. Interestingly, in tamoxifen-resistant MCF7-TAMR cells, higher levels of MKP-2 mRNA and proteins were detected as well as activated ERK1 and ERK2 proteins. Thus, MKP-2 may play a role in the sensitivity of cells to tamoxifen. 4HT may disrupt the normal feedback loop between MKP-2 and ERK1,2. PKC also can regulate the activity of MKP-1 and MKP-2 (Zhang et al., 2001; Trappanese et al., 2016:). 4HT can also regulate PKC activity in some cell types by ROS (Gundimeda et al., 1996). We have previously observed that 4HT can activate ERK1,2 in MCF-7 cells [Steelman et al., 2011].

One of the problems of radiation therapy (RT) in the treatment of breast cancer is that RT may stimulate the development of radio-resistant CSCs. RT may induce the expression of genes associated with stemness. RT may stimulate the reprogramming of differentiated breast cancer cells into CSCs by inducing the re-expression of stemness genes. Interestingly, the RT-induced stemness can be blocked by targeting the NF-κB pathway with disulfiram (DSF) and copper. DSF inhibits aldehyde dehydrogenase (ALDH) which is also a marker for CSCs. DSF binds copper which inhibits the proteosome and NF-κB activation. This was shown to inhibit primary mammary tumors in mice (Wang et al., 2014).

NF-κB expression is associated with doxorubicin-resistance in breast cancer. Defects in TP53 are necessary for the transcriptional activation of NF-κB target genes that doxorubicin can induce. A TP53-deficient background was determined to correlate with the NF-κB-dependent gene

expression signature. The presence of a TP53-deficient background in breast cancer patients was associated with a reduced disease-free survival (Dalmases et al., 2014).

Drug- and radiation-resistance remain key problems in cancer therapy. We are elucidating the involvement of various signaling pathways and how they can influence the development of drug-resistance. The EGFR is a key player in cell growth. Deregulated EGFR expression has been implicated in cancer development as well as drug-resistance. Our studies have indicated that deregulated EGFR expression, in the form of an inducible v-Erb-B:ER gene can confer drug-resistance as well as radio-resistance.

Author Contributions:

Conceptualization and Data Acquisition: L.S.S., W.H.C., S.M.A., S.L.A., L.C., L.M., S.R., A.M.M., G.M., M.C., M.L., S.C. and J.A.M. performed the experiments and researched the various topic areas and wrote multiple sections. **Funding acquisition;** J.A.M., L.C. and S.R. were involved with funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Figure Legends

Figure 1. Interactions between EGFR, ROS/CaMK, TP53, PLC/PKC, Ras/Raf/MEK/ERK/p90Rsk, PI3K/PTEN/PDK1/Akt/mTORC1/GSK-3 Signaling Pathways and their Effects on Cell Growth and Drug Resistance. Green arrows indicate stimulation of pathway. Red blocked arrows indicate inhibition of pathway. In top right of this Figure is a panel depicting EGFR, EGFRvIII and v-Erb-B:ER and how they can transmit signals to multiple

downstream pathways involved in growth, gene expression and drug resistance. Addition of the hormone binding domain of estrogen receptor (ER) to the v-Erb-B oncogene makes the activity of v-Erb-B:ER dependent upon E2 or 4HT for activity.

Figure 2. Schematic Diagram of EGFR Family Members and EGFRvIII and v-Erb-B. The four different EGFR family members as well as additional names that the particular EGFR family member are known as are listed underneath the family member. The regions deleted in EGFRvIII and v-Erb-B as well as the lack of a ligand binding domain in HER2 and the defective kinase domain present in EGFR3 are indicated. Abbreviations: L1 and L2 are ligand binding domains, CR1 and CR2 are cysteine-rich domains. TM are transmembrane domains. CT are C-terminal domain which contains the phosphorylation sites. JM are juxta membrane domains. Conserved domains of the four different EGFR family members are indicated by similar shading.

Figure 3. Growth Curves of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR), MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells in the absence and presence of 4HT and either no drugs or doxorubicin. Panel A) growth of the cells in the absence of 4HT and chemotherapeutic drugs, Panel B) growth of the cells in the presence of 25 nM 4HT, Panel C) growth of cells in the absence of 4HT and 25nM doxorubicin (Dox), Panel D) growth of the cells in the presence of 25 nM 4HT and 25 nM Dox. Symbols: solid red squares = MCF-7 cells, solid blue upward triangles = MCF + v-Erb-B:ER cells, solid orange downward triangles = MCF-7 + v-Erb-B:ER (4HTR) cells, solid green diamonds = MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells. Each data point was performed in sextuplicate.

Figure 4. Growth Curves of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR), MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells in the absence and presence of 4HT and either daunorubicin or paclitaxel. Panel A) growth of the cells in the presence of 25 nM daunorubicin (Dau), Panel B) growth of the cells in the presence of 25 nM 4HT and 25 nM Dau, Panel C) growth of cells in the presence of 5 nM paclitaxel (Pac), Panel D) growth of the cells in the presence of 25

nM 4HT and 5 nM Pac. Symbols: solid red squares = MCF-7 cells, solid blue upward triangles = MCF + v-Erb-B:ER cells, solid orange downward triangles = MCF-7 + v-Erb-B:ER (4HTR) cells, solid green diamonds = MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells. Each data point was performed in sextuplicate.

Figure 5. Growth Curves of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR), MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells in the absence and presence of 4HT and either 5-fluorouracil or cisplatin. Panel A) growth of the cells in 250 nM fluorouracil (5FU), Panel B) growth of the cells in the presence of 25 nM 4HT and 250 nM 5FU, Panel C) growth of cells in the presence of 1 µM cisplatin (Cis). Panel D) growth of the cells in the presence of 25 nM 4HT and 1 µM Cis. Symbols: solid red squares = MCF-7 cells, solid blue upward triangles = MCF + v-Erb-B:ER cells, solid orange downward triangles = MCF-7 + v-Erb-B:ER (4HTR) cells, solid green diamonds = MCF-7 + v-Erb-B:ER (4HTR + DoxR) cells. Each data point was performed in sextuplicate.

Figure 6. IC_{50} Analysis of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR) and MCF-7 + v-Erb-B:ER (4HTR + Dox) cells in the absence and presence of 4HT and doxorubicin. Panel A) MCF-7 cells, Panel B) MCF + v-Erb-B:ER cells, Panel C) MCF-7 + v-Erb-B:ER (4HTR) cells and Panel D) MCF-7 + v-Erb-B:ER (4HTR + Dox) cells. Black squares equal growth in the absence of 4HT, green triangles equals growth in the presence of 25 nM 4HT. Downward arrows pointing to X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated three times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with or without 4HT where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P<0.0001$, ** $P < 0.005$. Each data point was performed in sextuplicate.

Figure 7. IC_{50} Analysis of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR) and MCF-7 + v-Erb-B:ER (4HTR + Dox) cells in the absence and presence of 4HT and

daunorubicin. Panel A) MCF-7 cells, Panel B) MCF + v-Erb-B:ER cells, Panel C) MCF-7 + v-Erb-B:ER (4HTR) cells and Panel D) MCF-7 + v-Erb-B:ER (4HTR + Dox) cells. Black squares equal growth in the absence of 4HT, green triangles equals growth in the presence of 25 nM 4HT. Downward arrows pointing to X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated three times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with or without 4HT where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P<0.0001$. Each data point was performed in sextuplicate.

Figure 8. IC_{50} Analysis of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR) and MCF-7 + v-Erb-B:ER (4HTR + Dox) cells in the absence and presence of 4HT and Cisplatin. Panel A) MCF-7 cells, Panel B) MCF + v-Erb-B:ER cells, Panel C) MCF-7 + v-Erb-B:ER (4HTR) cells and Panel D) MCF-7 + v-Erb-B:ER (4HTR + Dox) cells. Black squares equal growth in the absence of 4HT, green triangles equals growth in the presence of 25 nM 4HT. Downward arrows pointing to X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated three times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with or without 4HT where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P<0.0001$, ** $P < 0.005$. Each data point was performed in sextuplicate.

Figure 9. IC_{50} Analysis of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR) and MCF-7 + v-Erb-B:ER (4HTR + Dox) cells in the absence and presence of 4HT and Paclitaxel. Panel A) MCF-7 cells, Panel B) MCF + v-Erb-B:ER cells, Panel C) MCF-7 + v-Erb-B:ER (4HTR) cells and Panel D) MCF-7 + v-Erb-B:ER (4HTR + Dox) cells. Black squares equal growth in the absence of 4HT, green triangles equals growth in the presence of 25 nM 4HT. Downward arrows pointing to X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated three times and similar results were obtained. Statistical analyses were performed in relationship to

cells not treated with or without 4HT where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P<0.0001$, NS = not statistically significant. Each data point was performed in sextuplicate.

Figure 10. IC_{50} Analysis of MCF-7, MCF + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR) and MCF-7 + v-Erb-B:ER (4HTR + Dox) cells in the absence and presence of 4HT and 5-Fluorouracil. Panel A) MCF-7 cells, Panel B) MCF + v-Erb-B:ER cells, Panel C) MCF-7 + v-Erb-B:ER (4HTR) cells and Panel D) MCF-7 + v-Erb-B:ER (4HTR + Dox) cells. Black squares equal growth in the absence of 4HT, green triangles equals growth in the presence of 25 nM 4HT. Downward arrows pointing to X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated three times and similar results were obtained. Statistical analyses were performed in relationship to cells not treated with or without 4HT where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P<0.0001$, ** $P < 0.005$, * $P < 0.05$. Each data point was performed in sextuplicate.

Figure 11. Colony Formation of MCF-7, MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER (4HTR) and MCF-7 + v-Erb-B:ER (4HTR +DoxR) cells in Absence and Presence of Radiation. Panel A) MCF-7, MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER(4HTR + DoxR) were plated in six well plates and treated with different amounts of radiation in the absence of drugs and the number of colonies determined approximately 3 weeks of culture. The number of colonies in the untreated cultures (no radiation, no chemotherapeutic drugs was normalized to 100% for each cell type). Panel B) MCF-7, MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER(4HTR + DoxR) were plated in six well plates and treated with different amounts of radiation and then cultured with 100 nM 4HT. The number of colonies was determined after 3 weeks of culture. Panel C) MCF-7, MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER(4HTR + DoxR) were plated in six well plates and treated with different amounts of radiation and then cultured with 25 nM doxorubicin. The number

of colonies was determined after 3 weeks of culture. Panel D) MCF-7, MCF-7 + v-Erb-B:ER, MCF-7 + v-Erb-B:ER(4HTR) and MCF-7 + v-Erb-B:ER(4HTR + DoxR) were plated in six well plates and treated with different amounts of radiation and then cultured with 100 nM 4HT + 25 nM doxorubicin. The number of colonies was determined after 3 weeks of culture. MCF-7 cells red squares, MCF-7 + v-ErbB:ER dark blue triangles, light blue circles, MCF-7 + v-Erb-B:ER (4HTR), purple downward triangles, MCF-7 + v-Erb-B:ER (4HTR + DoxR). Each data point was performed in triplicate.

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Figure 1

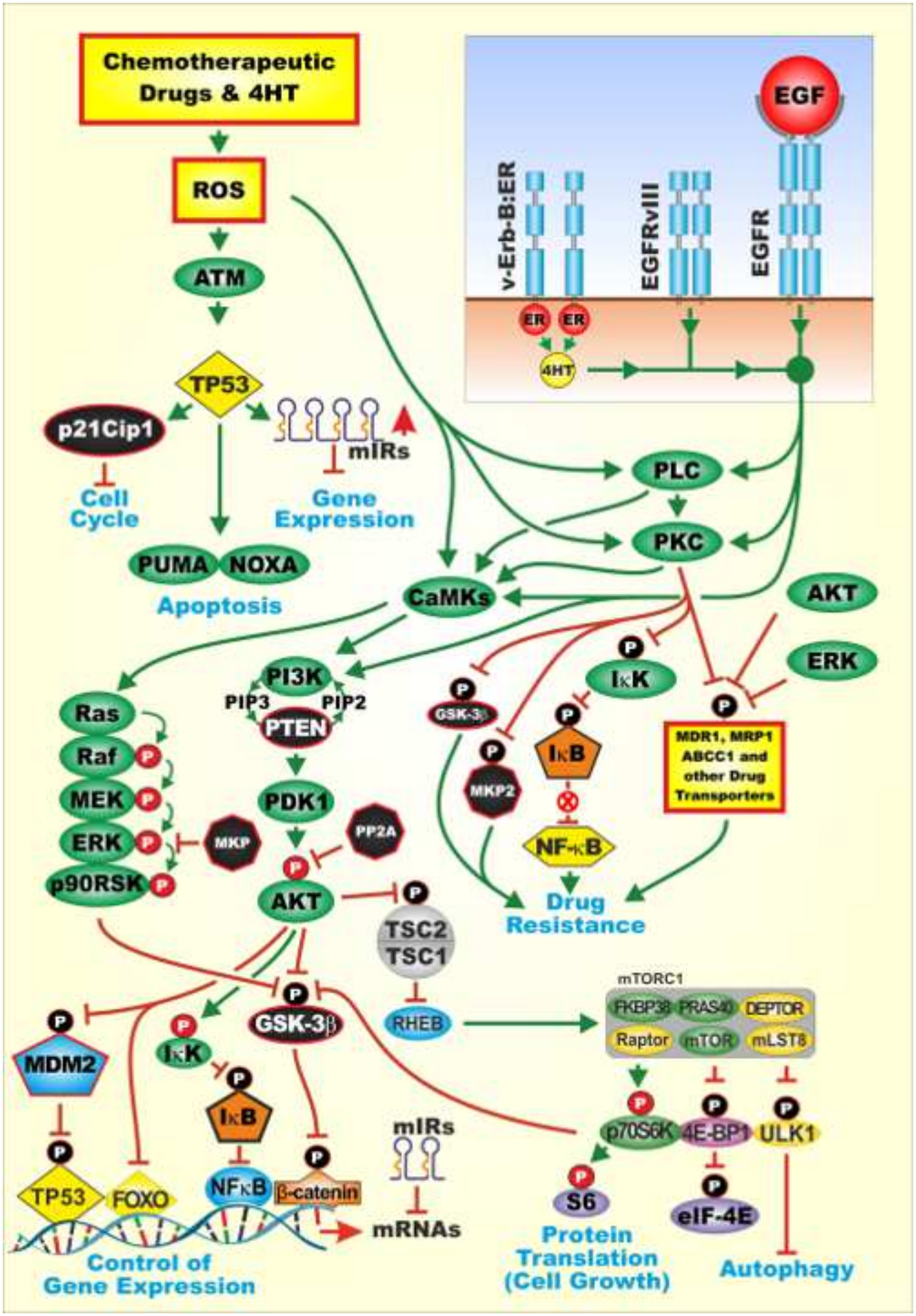

Chemotherapeutic Drugs & 4HT
ROS
ATM
TP53
p21Cip1
Cell Cycle
miRs
Gene Expression
PUMA
NOXA
Apoptosis
EGF
v-Erb-B:ER
EGFRvIII
EGFR
ER
ER
4HT
PLC
PKC
CaMKs
AKT
ERK
PI3K
PIP3
PIP2
PTEN
Ras
Raf
MEK
ERK
p90RSK
MKP
PDK1
PP2A
AKT
GSK-3β
MKP2
IκK
IκB
NF-κB
MDR1, MRP1 ABCC1 and other Drug Transporters
Drug Resistance
TSC2
TSC1
RHEB
mTORC1
FKBP38
PRAS40
DEPTOR
Raptor
mTOR
mLST8
p70S6K
4E-BP1
ULK1
S6
eIF-4E
Protein Translation (Cell Growth)
Autophagy
MDM2
TP53
FOXO
NFκB
β-catenin
mRNAs
Control of Gene Expression

Figure 2

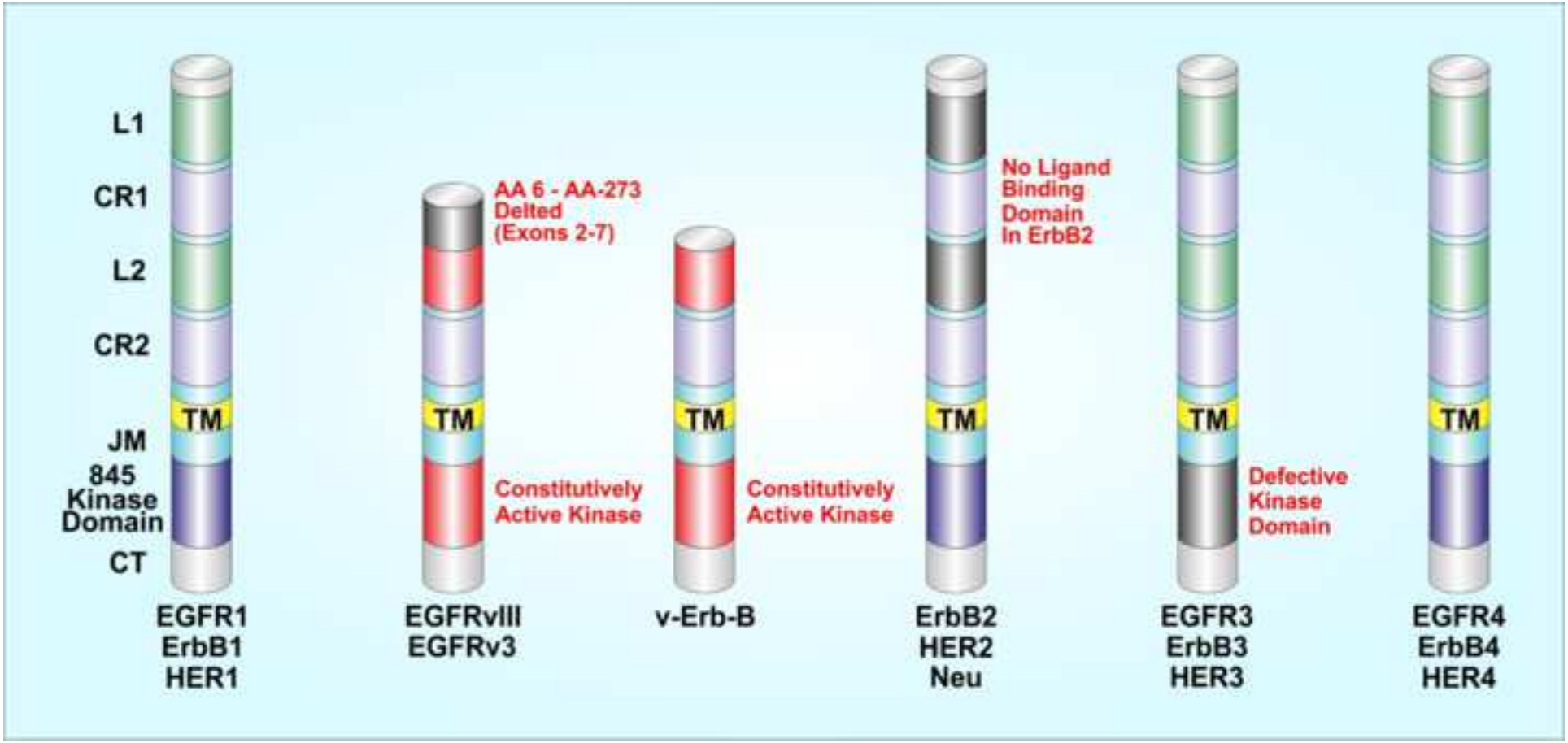

L1
CR1
L2
CR2
TM
JM
845
Kinase
Domain
CT
EGFR1
ErbB1
HER1
AA 6 - AA-273
Delted
(Exons 2-7)
Constitutively
Active Kinase
EGFRvIII
EGFRv3
TM
Constitutively
Active Kinase
v-Erb-B
TM
No Ligand
Binding
Domain
In ErbB2
ErbB2
HER2
Neu
TM
Defective
Kinase
Domain
EGFR3
ErbB3
HER3
TM
EGFR4
ErbB4
HER4

Figure 3

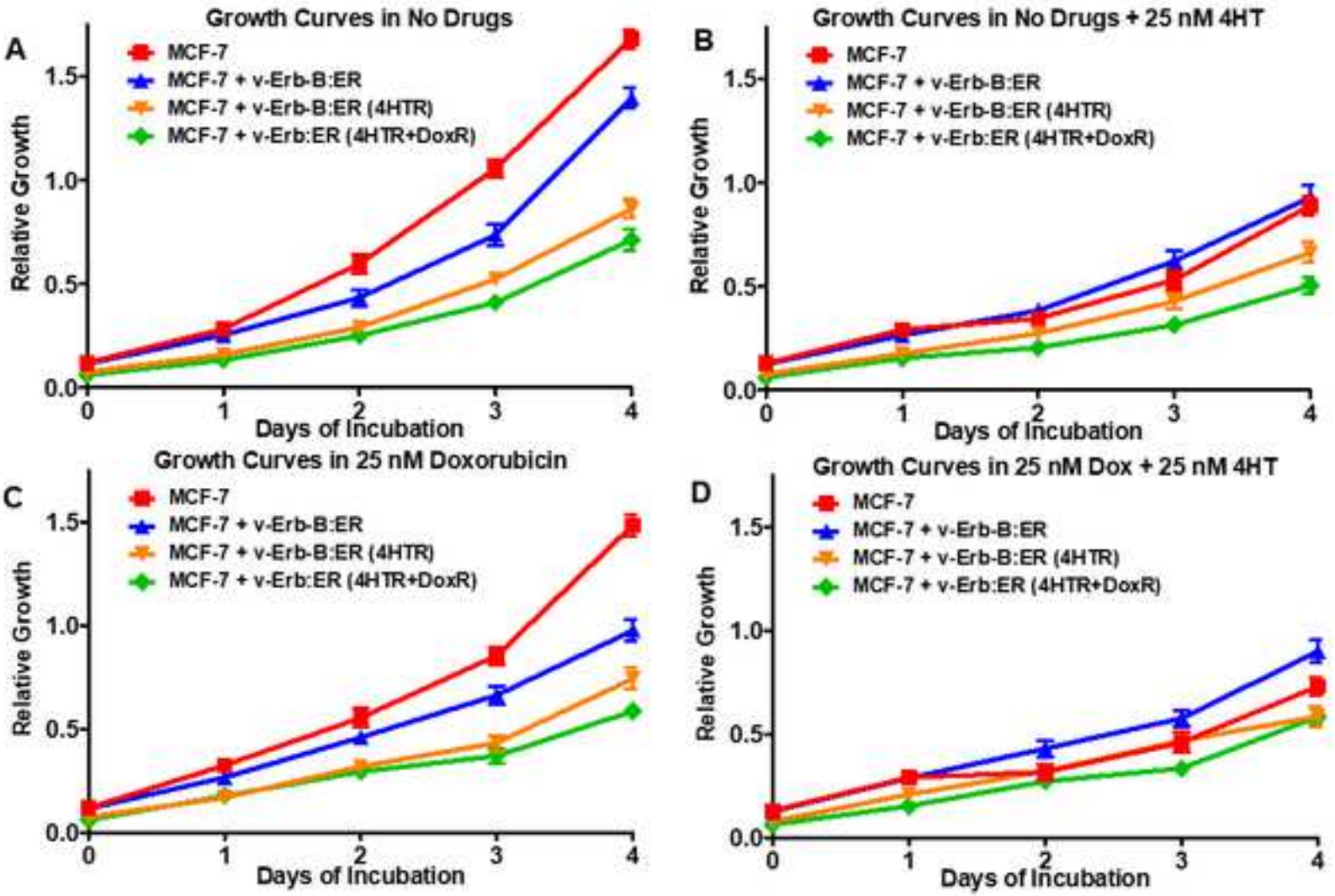

A
Growth Curves in No Drugs
MCF-7
MCF-7 + v-Erb-B:ER
MCF-7 + v-Erb-B:ER (4HTR)
MCF-7 + v-Erb:ER (4HTR+DoxR)
Relative Growth
Days of Incubation
B
Growth Curves in No Drugs + 25 nM 4HT
C
Growth Curves in 25 nM Doxorubicin
D
Growth Curves in 25 nM Dox + 25 nM 4HT

Figure 4

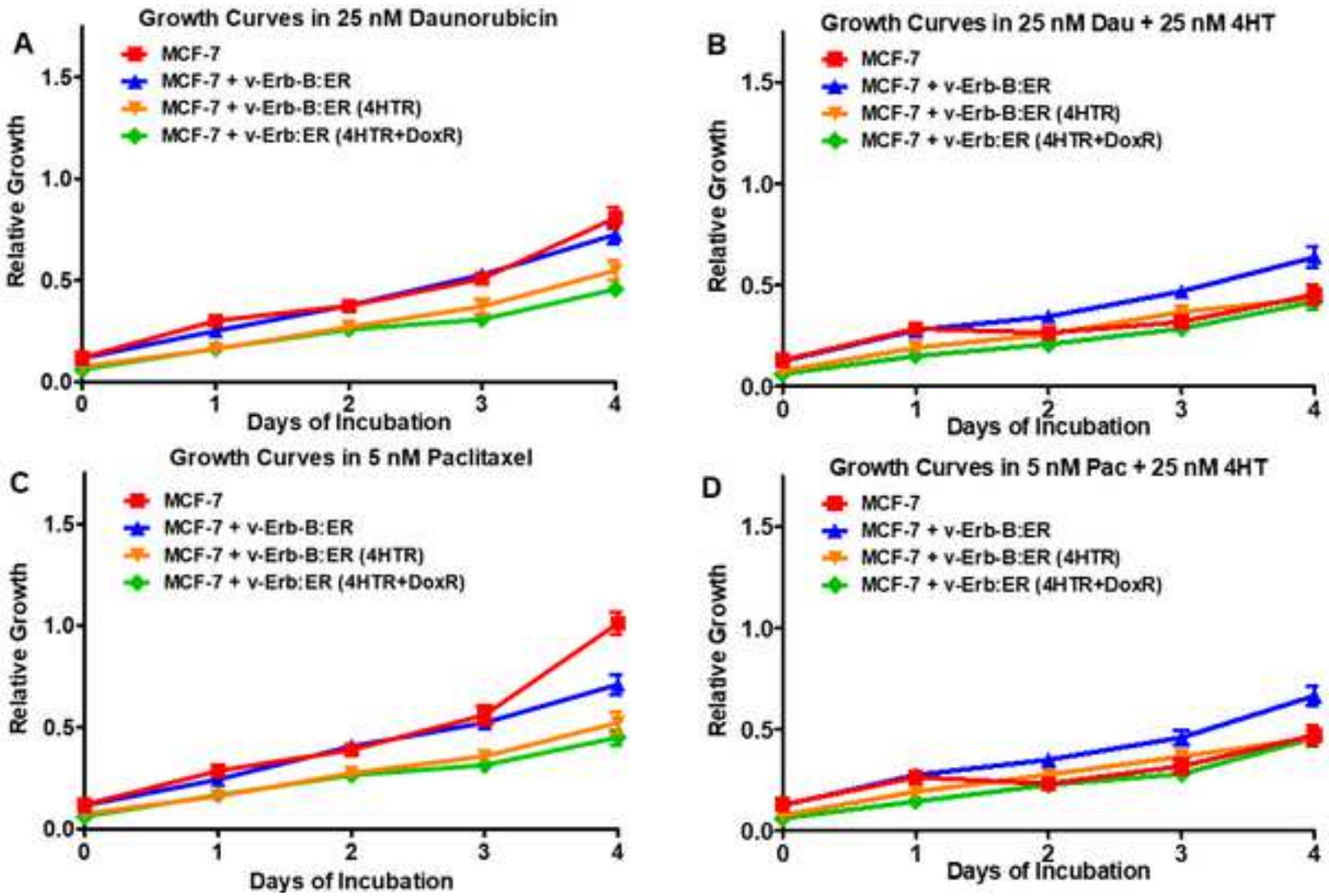

A
Growth Curves in 25 nM Daunorubicin
MCF-7
MCF-7 + v-Erb-B:ER
MCF-7 + v-Erb-B:ER (4HTR)
MCF-7 + v-Erb:ER (4HTR+DoxR)
Relative Growth
Days of Incubation
B
Growth Curves in 25 nM Dau + 25 nM 4HT
C
Growth Curves in 5 nM Paclitaxel
D
Growth Curves in 5 nM Pac + 25 nM 4HT

Figure 5

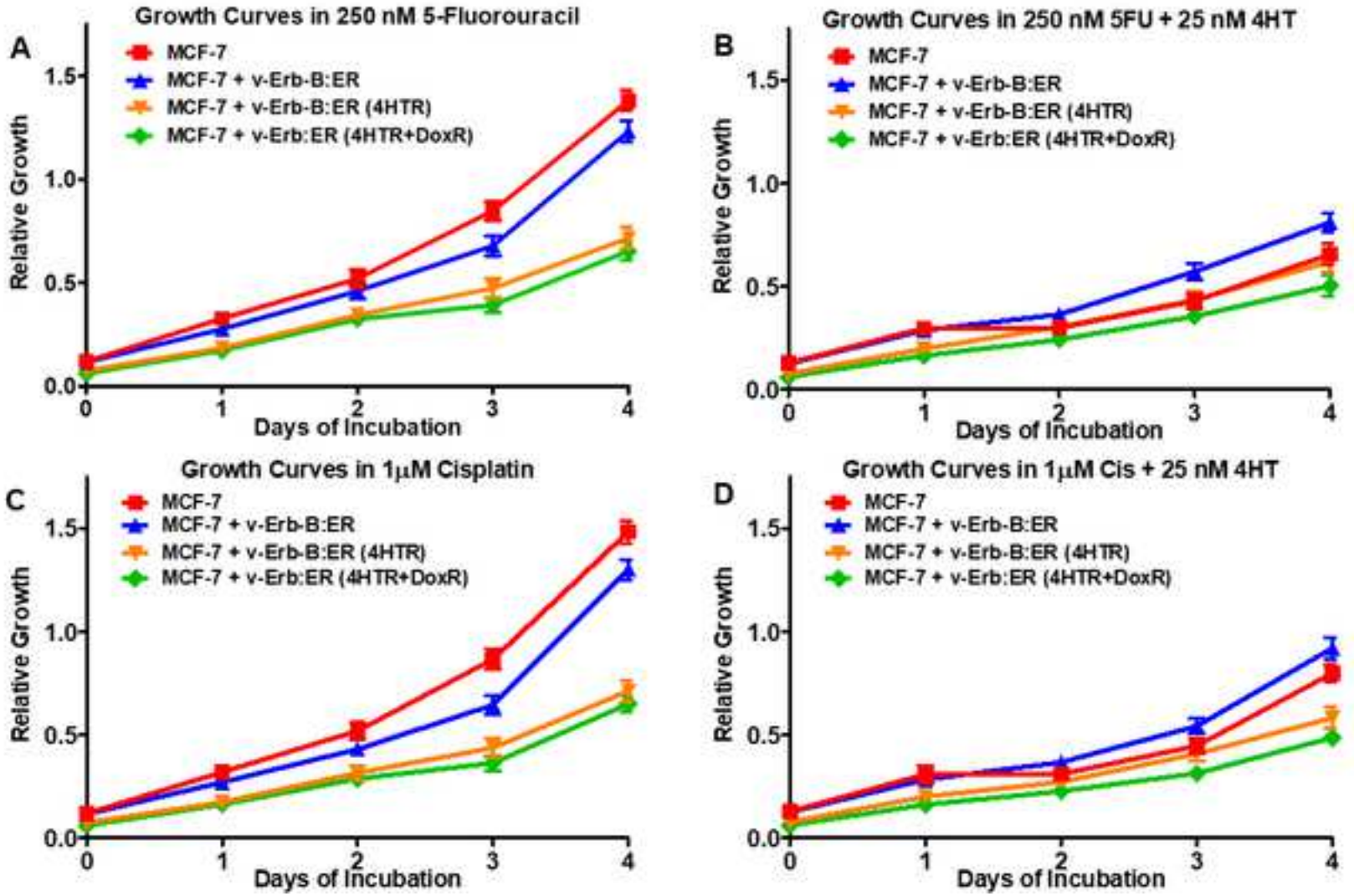

A
Growth Curves in 250 nM 5-Fluorouracil
MCF-7
MCF-7 + v-Erb-B:ER
MCF-7 + v-Erb-B:ER (4HTR)
MCF-7 + v-Erb:ER (4HTR+DoxR)
Relative Growth
Days of Incubation
B
Growth Curves in 250 nM 5FU + 25 nM 4HT
C
Growth Curves in 1μM Cisplatin
D
Growth Curves in 1μM Cis + 25 nM 4HT

Figure 6

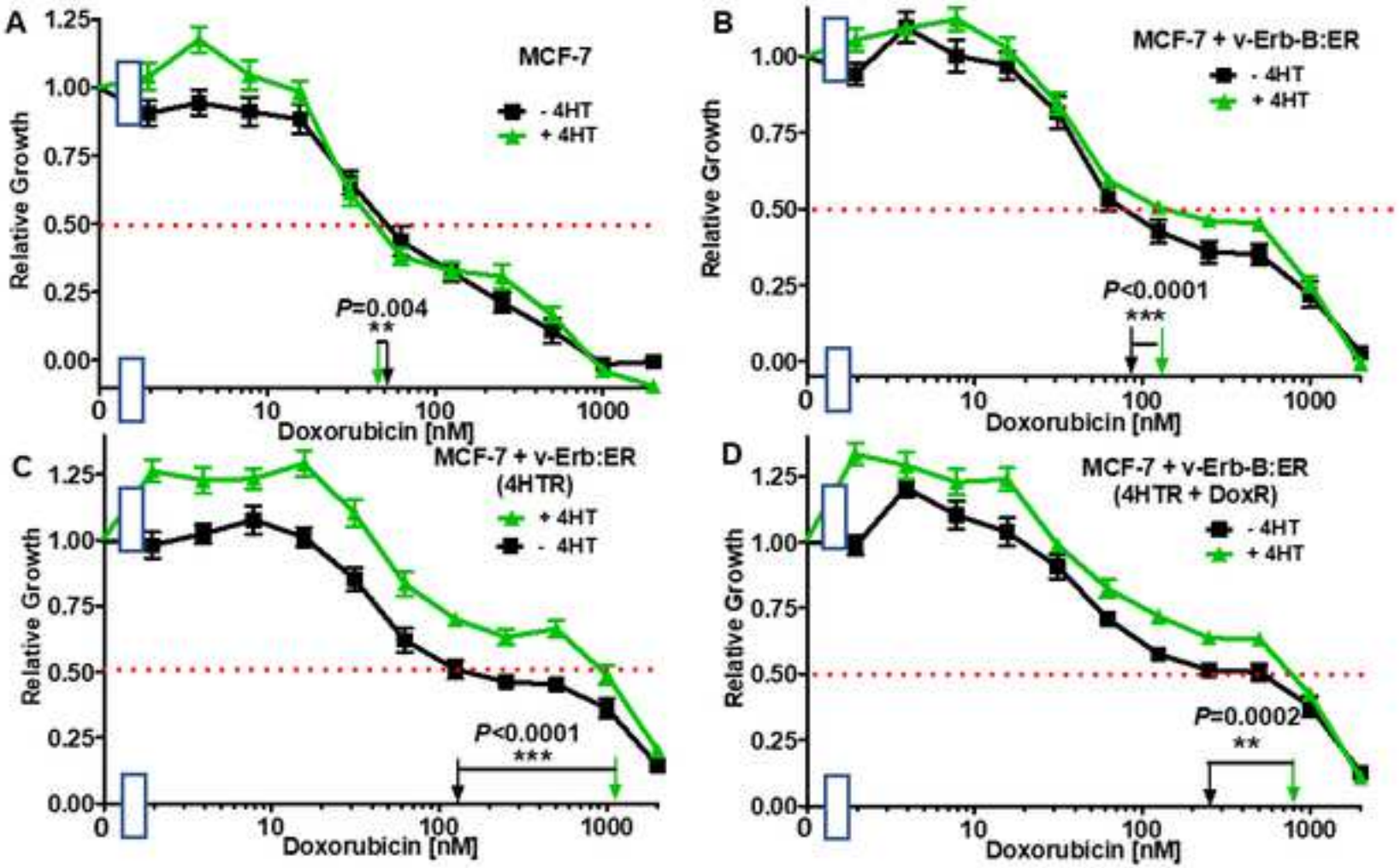

A
MCF-7
- 4HT
+ 4HT
Relative Growth
P=0.004
**
Doxorubicin [nM]
B
MCF-7 + v-Erb-B:ER
- 4HT
+ 4HT
Relative Growth
P<0.0001
***
Doxorubicin [nM]
C
MCF-7 + v-Erb:ER
(4HTR)
+ 4HT
- 4HT
Relative Growth
P<0.0001
***
Doxorubicin [nM]
D
MCF-7 + v-Erb-B:ER
(4HTR + DoxR)
- 4HT
+ 4HT
Relative Growth
P=0.0002
**
Doxorubicin [nM]

Figure 7

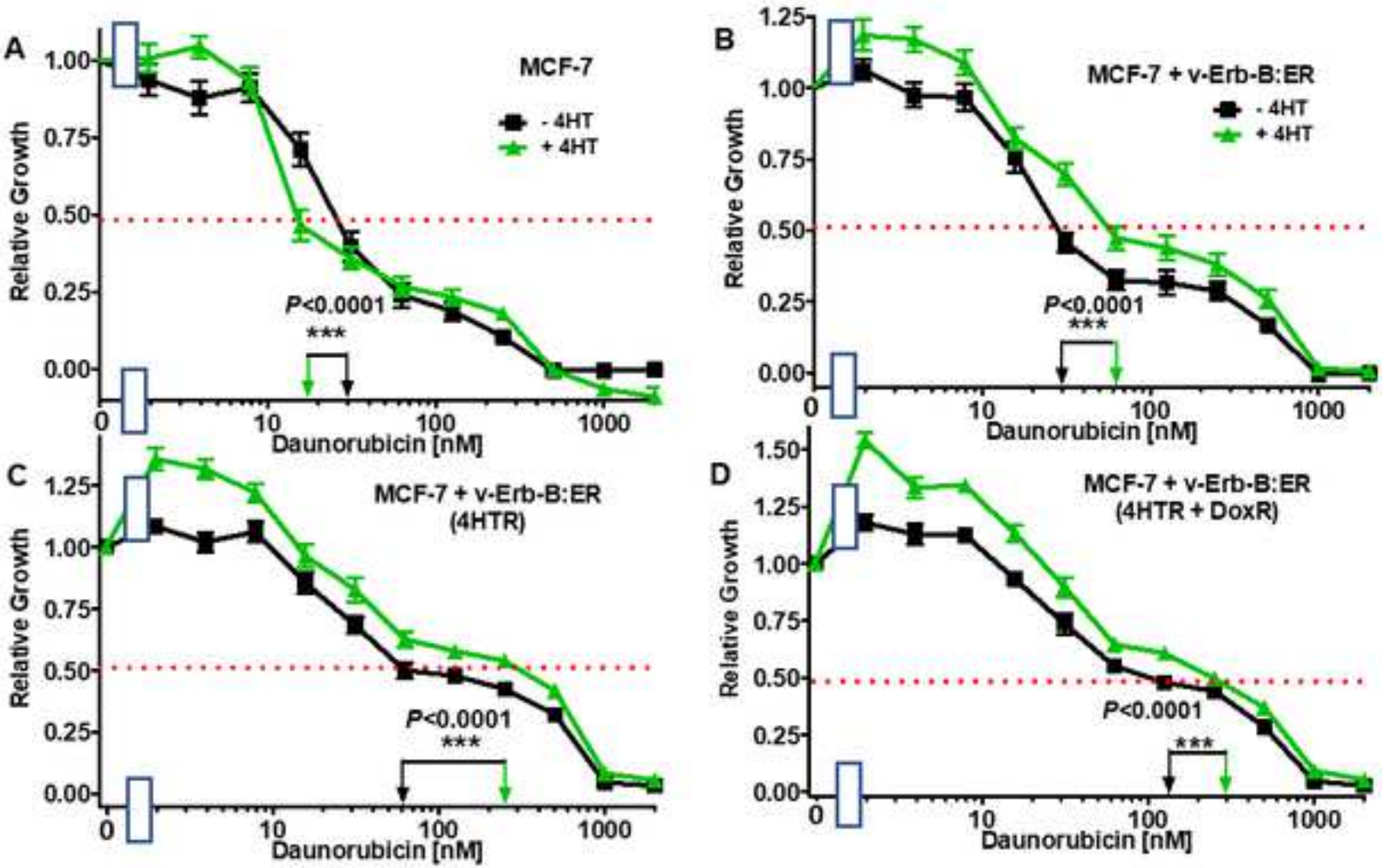

A
MCF-7
- 4HT
+ 4HT
Relative Growth
1.00
0.75
0.50
0.25
0.00
P<0.0001
***
0
10
100
1000
Daunorubicin [nM]
B
MCF-7 + v-Erb-B:ER
- 4HT
+ 4HT
Relative Growth
1.25
1.00
0.75
0.50
0.25
0.00
P<0.0001
***
0
10
100
1000
Daunorubicin [nM]
C
MCF-7 + v-Erb-B:ER
(4HTR)
Relative Growth
1.25
1.00
0.75
0.50
0.25
0.00
P<0.0001
***
0
10
100
1000
Daunorubicin [nM]
D
MCF-7 + v-Erb-B:ER
(4HTR + DoxR)
Relative Growth
1.50
1.25
1.00
0.75
0.50
0.25
0.00
P<0.0001
***
0
10
100
1000
Daunorubicin [nM]

Figure 8

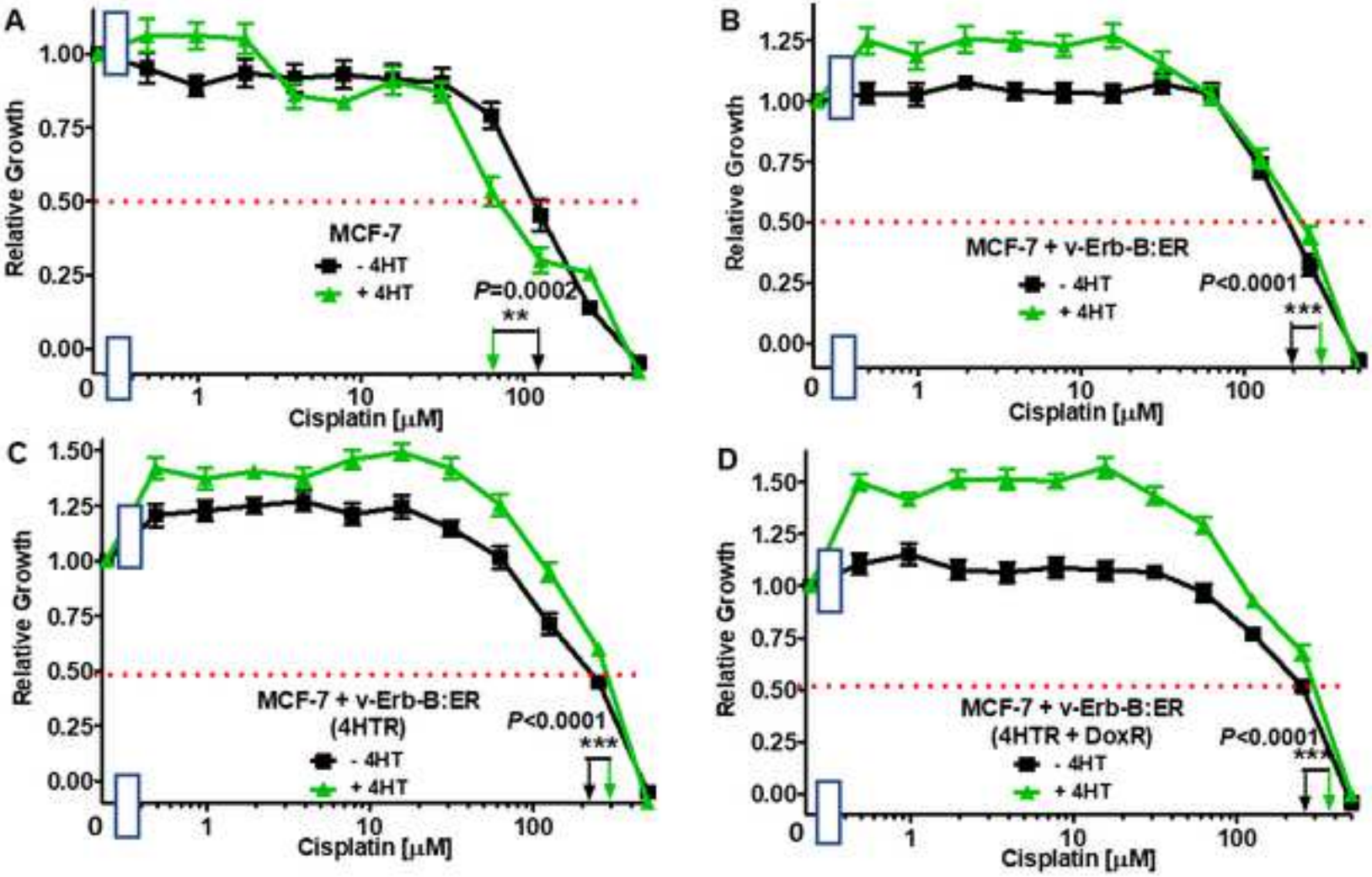

A
Relative Growth
1.00
0.75
0.50
0.25
0.00
0
1
10
100
Cisplatin [μM]
MCF-7
- 4HT
+ 4HT
P=0.0002
**
B
Relative Growth
1.25
1.00
0.75
0.50
0.25
0.00
0
1
10
100
Cisplatin [μM]
MCF-7 + v-Erb-B:ER
- 4HT
+ 4HT
P<0.0001
***
C
Relative Growth
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
1
10
100
Cisplatin [μM]
MCF-7 + v-Erb-B:ER
(4HTR)
- 4HT
+ 4HT
P<0.0001
***
D
Relative Growth
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
1
10
100
Cisplatin [μM]
MCF-7 + v-Erb-B:ER
(4HTR + DoxR)
- 4HT
+ 4HT
P<0.0001
***

Figure 9

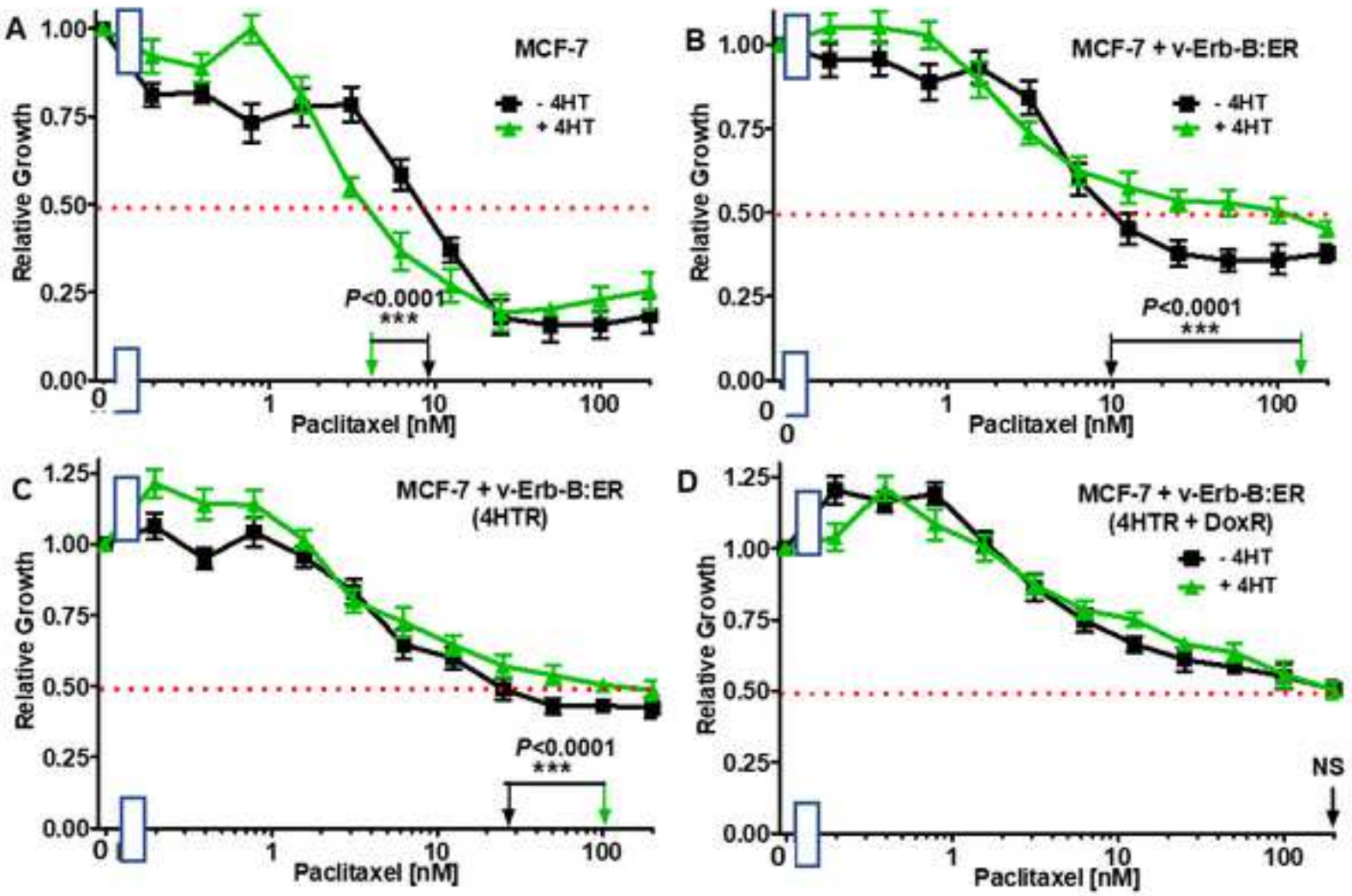

A
MCF-7
- 4HT
+ 4HT
Relative Growth
1.00
0.75
0.50
0.25
0.00
P<0.0001
***
0
1
10
100
Paclitaxel [nM]
B
MCF-7 + v-Erb-B:ER
- 4HT
+ 4HT
Relative Growth
P<0.0001
***
Paclitaxel [nM]
C
MCF-7 + v-Erb-B:ER
(4HTR)
1.25
Relative Growth
P<0.0001
***
Paclitaxel [nM]
D
MCF-7 + v-Erb-B:ER
(4HTR + DoxR)
- 4HT
+ 4HT
Relative Growth
NS
Paclitaxel [nM]

Figure 10

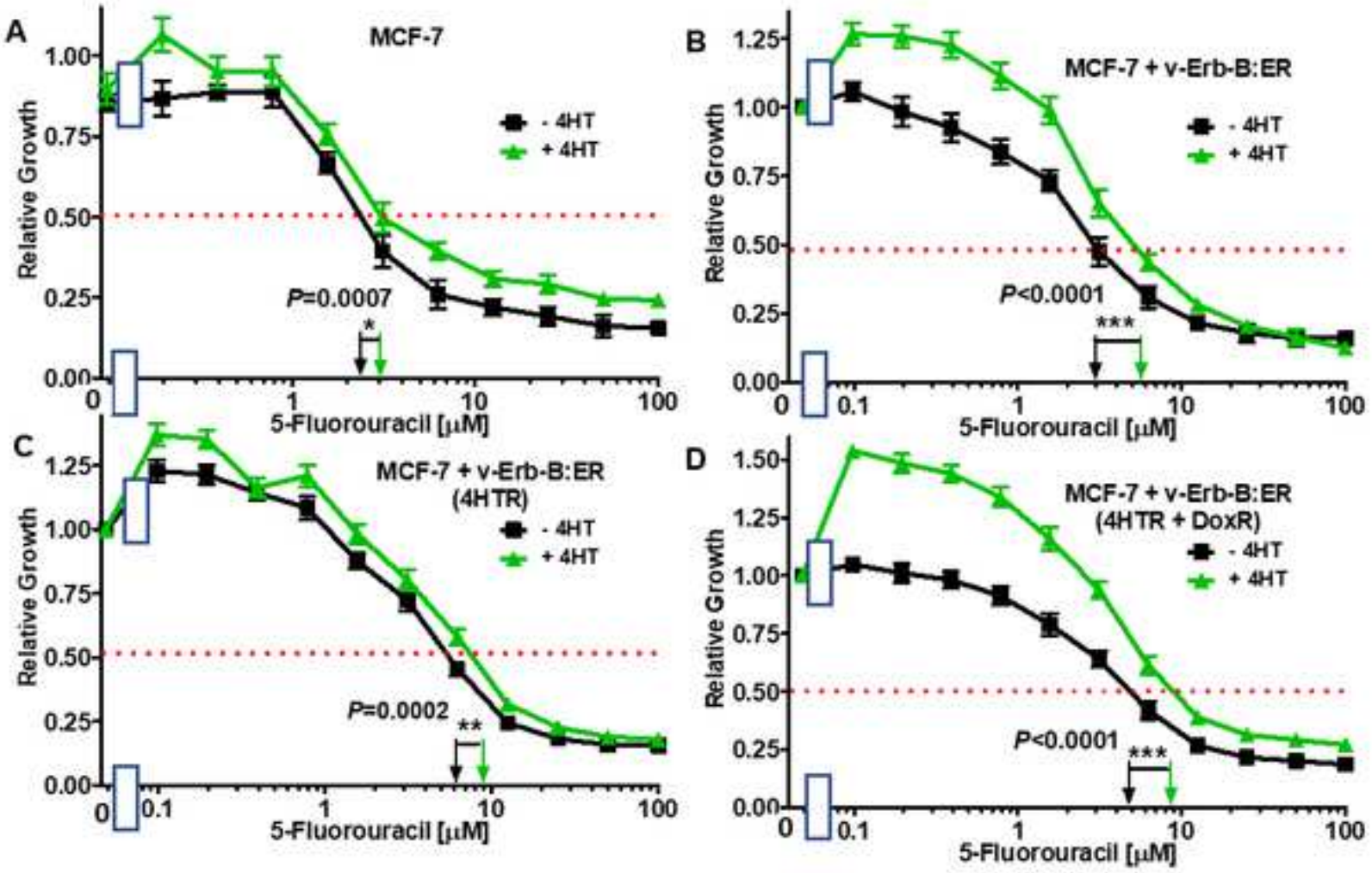
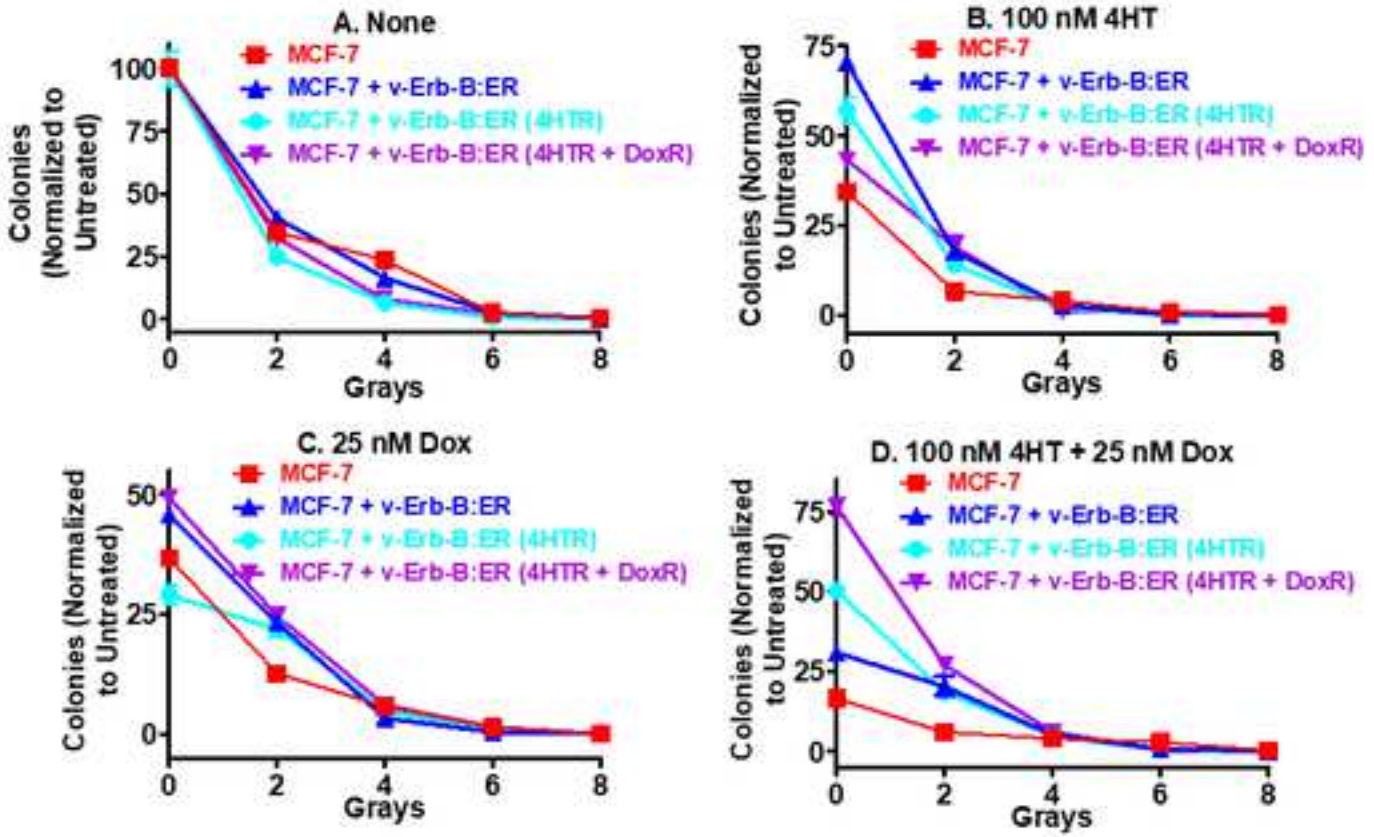

A
MCF-7
- 4HT
+ 4HT
Relative Growth
P=0.0007
*
5-Fluorouracil [μM]
B
MCF-7 + v-Erb-B:ER
P<0.0001
***
C
MCF-7 + v-Erb-B:ER
(4HTR)
P=0.0002
**
D
MCF-7 + v-Erb-B:ER
(4HTR + DoxR)
P<0.0001
***

Figure 11


A. None
MCF-7
MCF-7 + v-Erb-B:ER
MCF-7 + v-Erb-B:ER (4HTR)
MCF-7 + v-Erb-B:ER (4HTR + DoxR)
Colonies (Normalized to Untreated)
Grays
B. 100 nM 4HT
C. 25 nM Dox
D. 100 nM 4HT + 25 nM Dox

Author Contributions:

Conceptualization and Data Acquisition: L.S.S., W.H.C., S.M.A., S.L.A., L.C., L.M., S.R., A.M.M., G.M., M.C., M.L., S.C. and J.A.M. performed the experiments and researched the various topic areas and wrote multiple sections.