

# Breast Cancer Therapeutics Based on Fusarochromanone and EGFR Inhibitors

**Natalie Carroll**

Jackson Laboratory

**Alena Smith**

Southern Arkansas University

**Brian A. Salvatore**

Louisiana State University in Shreveport

**Elahe Mahdavian** (✉ [Elahe.Mahdavian@lsus.edu](mailto:Elahe.Mahdavian@lsus.edu))

Louisiana State University in Shreveport

---

## Research Article

**Keywords:** TNBC, fusarochromanone, EGFR, mTOR, everolimus, erlotinib, lapatinib, combination cancer therapies, synergy

**Posted Date:** March 13th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-289662/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background:** Fusarochromanone (FC101) is a small molecule with potent anti-cancer activity. It was originally derived from the fungal plant pathogen, *Fusarium equiseti*, and it has also been synthesized in non-racemic form in our lab. Numerous studies reveal the promising biological activity of FC101, including potent anti-angiogenic and anti-cancer activity. While FC101 is potent as a single drug treatment across many cancer cell lines, current cancer therapies often incorporate a combination of drugs in order to increase efficacy and decrease the development of drug resistance. In this study, we leverage drug combinations and cellular phenotypic screens to address important questions about FC101's mode of action and its potential synergies as an anti-cancer therapeutic agent in triple negative breast cancer (TNBC).

**Method:** We hypothesized that FC101's activity against TNBC is similar to the known mTOR inhibitor, everolimus, because FC101 reduces the phosphorylation of two key mTOR substrates, S6K and S6. Since everolimus synergistically enhances the anti-cancer activities of known EGFR inhibitors (erlotinib or lapatinib) in TNBC, we performed analogous studies with FC101. Phenotypic cellular assays helped assess whether FC101 (in both single and combination treatments) acts similarly to everolimus.

**Results:** FC101 outperformed all other single treatments in both cell proliferation and viability assays. Unlike everolimus, however, FC101 brought about a sustained decrease in cell viability in drug washout studies. None of the other drugs were able to maintain comparable effects upon removal of the treatment agents. Although we observed slightly additive effects when the TNBC cells were treated with FC101 and either EGFR inhibitor, those effects were not truly synergistic in the manner displayed with everolimus.

**Conclusion:** Our results rule out direct inhibition of mTOR by FC101 and suggest that FC101 acts through a different mechanism than everolimus. This lays the foundation for the refinement of our hypothesis in order to better understand FC101's mode of action as a novel anti-cancer agent.

## Background

### 1.1. Cancer statistics and treatment modalities

Cancer claims many lives, and it continues to be a significant human health challenge [1]. In 2021, there will be an estimated 1.9 million new cancer cases diagnosed and 608,570 cancer deaths in the United States [2]. Although surgery, radiation, and therapeutics are often effective when the disease is detected early, many late-stage cancers are only diagnosed after the primary tumor cells have metastasized. [3]. Systemic chemotherapies are nonselective drugs that affect both cancer cells and any rapidly growing normal cells, causing severe side effects, preventing effective long-term use [4]. Therefore, these drugs are often administered at sub-optimal levels, contributing to even poorer clinical outcomes [4].

In an effort to improve efficacy and safety of indiscriminate chemotherapy, molecularly targeted cancer therapies have emerged representing a paradigm shift in the treatment of cancer [5]. Targeted therapies

are tailored to disrupt a single and specific molecular entity that drives cancer development and progression [5, 6]. These targets are typically part of a signaling cascade that run awry and their modulation can reverse the phenotypic cancerous progression.

The clinical outcomes of targeted drugs used as single treatments for advanced cancer have unfortunately been disappointing [7]. Genomic sequencing has revealed that advanced cancer acquires complex genomic and proteomic heterogeneity to support its abnormal phenotypes [8]. This heterogeneity is fueled by genome instability that leads to changes in the expression levels (copy number), the functions of many genes and their network of protein interactions [8]. Furthermore, advanced cancer evolves to gain a multitude of escape mechanisms to evade inhibition of the primary drug target. This reprogramming activates cancer cell survival mechanisms, leading to drug resistance and poor clinical outcomes [9].

Drug resistance represents a significant clinical challenge that requires targeting any compensatory pathways that develop to support the resistance phenotype [10]. Advances in cancer genome sequencing provide a reliable experimental framework to identify molecular drivers of drug resistance [11]. Dual cancer therapeutics targeting both the primary oncogenic and the secondary adaptive survival pathways have the potential to significantly improve clinical outcomes [10,11]. Thus, rational combination therapies have emerged to overcome drug resistance, representing a second paradigm shift in cancer treatment. Preclinical investigation of synergistic activities of combination therapies for advanced cancer is an area of increasing interest in oncology research [11, 12].

## **1.2. Triple Negative Breast Cancer (TNBC)**

Breast cancer is the most common malignancy in women and the second most common cancer, worldwide [13]. Breast cancer is highly heterogeneous with subtypes having distinct genomic, proteomic, morphologic, and clinical behavior [14]. Triple positive breast cancer (TPBC) is characterized by overexpression of three types of membrane protein receptors, HER<sup>+</sup>, ER<sup>+</sup>, PR<sup>+</sup> [14]. There are effective targeted therapeutics against TPBC, including endocrine therapy [tamoxifen] and anti-HER therapies [herceptin, lapatinib] [14]. However, there are no effective targeted therapeutics for triple negative breast cancer (TNBC) since it lacks the prominent expression of HER2, ER, and PR receptors.

A large body of experimental evidence establishes the importance of EGFR (HER1) receptor overexpression and hence its significance as a promising therapeutic target in TNBC [15,16]. However, targeting EGFR in TNBC using single-action kinase inhibitors (lapatinib and erlotinib) leads to drug resistance and poor preclinical and clinical outcomes. Cancer's compensatory response is known to arise from unleashing the inhibitory feedback on EGFR signaling [17]. This results in activation of mTOR and PI3K-AKT, two major downstream signaling pathways from EGFR [17]. Thus, the disruption of cooperation and crosstalk between EGFR and either mTOR or PI3K-AKT signaling has received significant attention towards establishing effective therapeutic modalities for TNBC [17].

Targeting mTOR activation to overcome EGFR-directed drug resistant phenotype in TNBC is well documented in recent research [17,18]. Preclinical data indicates significant improvement and synergy in anti-cancer drug response in TNBC when EGFR and mTOR inhibitors are combined vs. single therapeutic approaches [19,20]. Notably, the combination treatment consisting of lapatinib (EGFRi) and everolimus (mTORi) has advanced to clinical trials for the treatment of TNBC [20]. There is currently an increasing demand for other novel mTOR inhibitors to establish and validate new drug combinations that can effectively block the oncogenic networks responsible for drug resistance in TNBC.

### 1.3. Drug Panel

Our drug panel of kinase inhibitors for this project included: FC101, erlotinib (EGFRi), lapatinib (EGFRi), and everolimus (mTORi). The selection criteria for this group of kinase inhibitors incorporates experimental data on selectivity, potency, and clinical relevance. We chose kinase inhibitors that were the most selective and potent for their respective targets and were FDA-approved drugs thus had the best safety profiles.

Both erlotinib and lapatinib are small molecule synthetic drugs that are FDA-approved for the treatment of human metastatic cancer [25]. Both drugs also belong to a class of reversible EGFR kinase inhibitors, but they differ in structure, target-specificity, and mechanism of action. Erlotinib binds to EGFR's ATP-binding site and thus is a type-I competitive inhibitor, whereas lapatinib binds to EGFR's allosteric site and thus is a type-II allosteric inhibitor [26]. Structurally, erlotinib has a smaller head group, consisting of a substituted quinazolinamine ring, compared to lapatinib's larger quinazoline head group [27]. In 2004, erlotinib as a single drug, was FDA approved for the treatment of patients with metastatic non-small cell lung cancer and pancreatic cancer [28]. Lapatinib was approved by the FDA in 2007 for the treatment of advanced metastatic breast cancer in conjunction with the chemotherapy drug, capecitabine [29]. While both lapatinib and erlotinib inhibit heterodimerization of EGFR and HER2, their signal transduction effects, induced on downstream kinases differ slightly [30]. Lapatinib prevents activation (and thus phosphorylation) of both Erk1/2 and AKT [29], whereas erlotinib blocks the activation and thus phosphorylation of only ERK1/2 [26].

Everolimus, a selective mTOR inhibitor, is a structural analog of rapamycin but has a slightly different drug-like properties [31]. Like rapamycin, everolimus does not directly inhibit the catalytic activity of mTOR. Everolimus binds to the intracellular protein FKBP12 to promote its interaction with mTOR complex at a non-catalytic domain (adjacent to the kinase domain). This bimolecular association causes Raptor's release from this newly formed mTOR complex, without raptor, mTOR1 is rendered catalytically inactive towards phosphorylation of its downstream proteins, 4E-BP1/S6k. Phosphorylation of 4E-BP1/S6k is required for their interactions with the eukaryotic initiation factors (eIF4B, eIF4E), which regulates activation of protein synthesis. Everolimus effectively downregulates protein synthesis that is in great demand for cellular proliferation and survival of cancer [31]. Thus, everolimus acts as an anti-cancer drug by inhibiting mTOR signaling, reducing S6K phosphorylation, and protein synthesis.

### 1.4. Fusarochromanone's potential as an mTOR inhibitor

In this context, we believe that the small molecule, amino flavonoid, fusarochromanone (FC101) possesses remarkable potential as an mTOR pathway modulating agent for cancer drug discovery. Lee *et al.* first isolated and determined the structure of FC101 via NMR and mass spectrometry (Fig. 1) [21]. The unique therapeutic potential of FC01 merited the issue of three patents covering its total synthesis and its use as a treatment for solid tumors and angiogenic diseases [22]. In 2014, Mahdavian *et al.*, employed a novel Sonogashira coupling methodology to synthesize FC101 in enantiomerically pure form [Supplementary Material]. This allowed for further investigation of its therapeutic potential and provided new opportunities to modify the molecule as a lead compound for the development of new anti-cancer agents.

FC101 is a cancer-specific cytotoxic agent that exhibits 10–100 times the effects against cancer cells *vs.* normal cells of the same type. This is the result of differential uptake/permeability by cancer cells and selective toxicity against them [22]. Furthermore, the most invasive cancer cells, particularly the oncogenic BRAF mutant- MAPK driven cancer types are most sensitive to FC101's anti-cancer activity [23]. This includes significant *in-vitro* inhibition of proliferation (sub- $\mu$ M IC<sub>50</sub>), migration, and induction of apoptosis in *in-vitro* models of melanoma, bladder, and TNBC [Figure 1][23,24]. Additionally, FC101 affects multiple molecular targets and cellular processes, leading to modulation of multiple cancer signaling pathways simultaneously [Williams-Hart unpublished]. This is an important drug attribute with the potential to mitigate the development of drug resistance, offering a unique therapeutic advantage. Notably, drug-resistant MCF-7-DOX cells are 8-fold more sensitive to FC101's cytotoxicity than MCF-7 cells [Supplementary Materials].

While FC101's exact mechanism of action is currently unknown, it might have a similar mode of action to inhibitors of the mTOR and MAPK signaling pathways. FC101 modulates both the MAPK and mTOR pathways downstream of EGFR, which are vital to cancer cell proliferation, survival, and development of drug resistance in advanced cancer (e.g. TNBC). Prior Western Blotting experiments have confirmed that FC101 simultaneously inhibits the activity of the MAPK pathway (corresponding to p-ERK reduction) and the mTOR pathway (corresponding to p-S6K, and p-S6 reduction) in MAPK-driven *TNBC* cancer cells [Supplementary Materials]. Thus, FC101's multifaceted effects on mTOR/MAPK in TNBC warrant further investigations of its pre-clinical potential based on a synergistic anti-cancer drug response. Herein, we focus on FC101's potential as a promising mTOR inhibitor (mTORi) to overcome single-therapy-driven drug resistance produced by known EGFR inhibitors (EGFRi). Specifically, the purpose of this project was to evaluate FC101's synergistic activity when used in drug combinations with two known EGFR inhibitors (erlotinib and lapatinib) through cellular phenotypic screens in TNBC. We used the known mTOR inhibitor, everolimus, as a control drug to benchmark FC101's anticancer activity in single and combination treatments. This study will lay the foundation for elucidation of FC101's exact mechanism of action. Here, we report FC101's ability to produce inhibition of cell proliferation, viability, and the survival of TNBC compared to everolimus in single and combination treatments with erlotinib and lapatinib.

## Materials And Methods

Our drug panel of kinase inhibitors for this project included: FC101, erlotinib (ChEML553), lapatinib (ChEML554), and everolimus (ChEMBL1908360). FC101 is not commercially available, it was synthesized in our medicinal chemistry laboratory at the LSUS Chemistry Department. Lapatinib (Tykerb), erlotinib (Tarceva), and everolimus (Afinitor) were purchased from Selleckchem, Inc (Houston, TX, USA). Three cellular phenotypic screens (Crystal Violet, MTT, and Drug Wash Out) were used to determine the overall drug response of each compound in single and combination treatment modalities in TNBC (MB-MDA-231).

## 2.1. Cell lines and Mammalian Cell Culture

The triple negative breast cancer (TNBC) cell line, MDA-MB-231 was kindly Dr. Maria Dragoi at the LSUHSC-INLET lab (Shreveport, LA, USA). The cells were grown in a culture medium of high glucose Dulbecco's modified Eagle's medium (DMEM, 02-0111-0500). All media was supplemented with 10% Fetal Bovine Serum (FBS, S11550, Sigma Aldrich) and 1% penicillin/streptomycin (P4333, Sigma Aldrich). Trypsin was purchased from Sigma Aldrich (Cat# 25-0510). Cells were incubated in appropriate culture plates or microwell plates and incubated in a humidified incubator at 37° C and 5% CO<sub>2</sub>.

## 2.2. Crystal Violet Staining Assay

Cells were plated at a density of 10,000 cells per 100 µL media in a 96-well tissue culture plate. A dose-response for each of the drugs was performed with treatments of 0.05 µM, 0.1 µM, 0.5 µM, 1.0 µM, 1.5 µM, 2 µM, and 3 µM. Cells were placed in humidified incubator at 37°C and 5% CO<sub>2</sub>. After 48 hours of treatment, the growth medium was aspirated from the wells. The cells were gently washed twice with 150 µL of tap water. Cells were fixed with 100 µL of 100% methanol for 20 minutes. The methanol was aspirated and 40 µL of crystal violet staining solution was added to each well and allowed to incubate at room temperature for 1 hour. The staining solution was removed by aspiration and the wells were rinsed 3 times with 250 µL of tap water. The plates were inverted and gently tapped on a paper towel to remove excess moisture. Plates were dried overnight. 50 µL of 10% acetic acid was added to each well and allowed to incubate for 2 hours. Absorbance at 595 nm was measured.

A second set of crystal violet experiments was performed after determining a set of optimal doses for each drug based on the dose response curves and the MTT viability assay. The test samples consisted of single treatments as well as combination treatments. Single treatments included 0.5 µM FC101, erlotinib, and everolimus, while lapatinib was tested at 1 µM. Combination treatments included 0.5 µM FC101 with 0.5 µM erlotinib or 1 µM lapatinib as well as 0.5 µM everolimus with 0.5 µM erlotinib or 1 µM lapatinib. In addition, DMSO-treated negative control wells were included. Cells were placed in a humidified incubator at 37°C and 5% CO<sub>2</sub> for 24, 48, or 72 hours. Crystal violet staining color intensity was quantified by scanning plates using an Odyssey Infrared Imaging System (LI-COR Biosciences), followed by data analysis using manufacturer's software.

## 2.3 MTT Viability Assay

A Trevigen TACS MTT Cell Assay (Cat # 4890-25-K) was used to measure cell viability. Cells were plated at a density of 10,000 cells per 100  $\mu\text{L}$  medium in a 96-well tissue culture plate. The next day the growth medium was aspirated and fresh growth medium with respective treatment conditions were added. The test samples consisted of single treatments as well as combination treatments. Single treatments included 0.5  $\mu\text{M}$  FC101, erlotinib, and everolimus, while lapatinib was tested at 1  $\mu\text{M}$ . Combination treatments included 0.5  $\mu\text{M}$  FC101 with 0.5  $\mu\text{M}$  erlotinib or 1  $\mu\text{M}$  lapatinib as well as 0.5  $\mu\text{M}$  everolimus with 0.5  $\mu\text{M}$  erlotinib or 1  $\mu\text{M}$  lapatinib. In addition, DMSO-treated negative control wells were included. Cells were placed in a humidified incubator at 37°C and 5%  $\text{CO}_2$  for 24, 48, or 72 hours. At the appropriate time point, 10  $\mu\text{L}$  per well of the MTT reagent (5 mg/mL) was added, and plates were returned to the incubator for 2 hours until a purple dye was visible. 100  $\mu\text{L}$  of the detergent reagent was added to all wells, and plates were immediately covered with foil. Plates were placed in a dark drawer to incubate overnight at room temperature. The absorbance in each well was read at 570 nm in a microplate reader, SpectroMax M2/ M2e spectrometer (Molecular Devices, San Jose, CA, USA).

## 2.4 Drug-Wash out Assay

To determine whether the drug treatments could sustain an impact after their removal, we performed a drug washout assay, in which the drugs were incubated with the cells for a total of 72 hours. Then the growth medium was removed, and fresh growth medium without inhibitors was added for cells to recover for another 72 hours. The cells were transferred to a 96-microtiter plate (10,000 cells per 100  $\mu\text{L}$ , six replicates). Cell viability was measured at 72-hour drug treatment and the 72-hour period after drug washout using Trevigen TACS MTT Cell Assay. Treatment concentrations included FC101 0.5  $\mu\text{M}$ , erlotinib 0.5  $\mu\text{M}$ , lapatinib 1  $\mu\text{M}$ , everolimus 0.5  $\mu\text{M}$ , and combinations of FC101 or everolimus with erlotinib and lapatinib.

## 2.5 Statistical Analysis

All cell culture experiments including crystal violet, MTT, and drug washout assays were performed in triplicate three times. All graphing and statistical analyses were performed in GraphPad Prism. Statistical significance was determined using an ANOVA. P-values less than 0.01 were considered statistically significant. Results are reported as mean  $\pm$  SE (n = 3), P < 0.01.

## Results

Our previous experiments indicated FC101's anti-cancer activity might be directly related to its inhibition of mTOR signaling pathway. Here, we explored this hypothesis and evaluated FC101's potential to produce synergistic anti-cancer effects in combination treatment modality in TNBC using three cellular phenotypic screens. We screened drug response in TNBC that resulted from either single and combination treatments of FC101, two EGFR inhibitors (erlotinib and lapatinib), and a known mTOR inhibitor (everolimus) as a control. After establishing the optimal doses from single-treatment experiments, we paired FC101 with either EGFR inhibitors and compared drug response to Everolimus. We used three well-

documented phenotypic assays, growth (crystal violet assay), viability (MTT assay), and survival (drug wash out assay).

### 3.1 Crystal Violet Proliferation Assay

The crystal violet dye binds and stains the protein and DNA in adhering live cells, thus the assay can be used to evaluate the drug-induced reduction in cellular growth/viability. The non-adhering cells are first removed and then the crystal violet dye bound to the adhering viable cells are solubilized. The color intensity resulted from the crystal violet dye is then measured at its  $\lambda_{\text{max}}$  of 570nm, thus  $A_{570}$  is directly proportional to the number of adhering live cells. For all single drug treatments, a dose range of 0.05  $\mu\text{M}$ -3.0  $\mu\text{M}$  was used to treat MDA-MB-231 breast cancer cells in a 96-well cell culture plate for 48 hours. The dose response curve shown in Fig. 2 represents the normalized color intensity (%control) versus drug concentration for each experiment. For dual drug treatment experiments, a concentration of 0.5  $\mu\text{M}$  was chosen for FC101, erlotinib, and everolimus, and a concentration of 1.0  $\mu\text{M}$  was chosen for lapatinib.

A second round of crystal violet assays were then conducted with each chosen dose combination at time points of 24, 48, and 72 hours (Fig. 3). At 48 hours, all single and combination treatments had significantly lower proliferation levels than the untreated control cells ( $p < 0.01$ ) (Fig. 3). In single drug treatment experiments, FC101 (0.5  $\mu\text{M}$ ) induced the greatest inhibition of cell proliferation as compared to lapatinib (1.0  $\mu\text{M}$ ), erlotinib (0.5  $\mu\text{M}$ ), or everolimus (0.5  $\mu\text{M}$ ). The combination treatment of FC101 (0.5  $\mu\text{M}$ ) and lapatinib (1.0  $\mu\text{M}$ ) had a slightly lower average  $A_{570}$  value, but there was no significant difference between the two when compared with FC101 (0.5  $\mu\text{M}$ ) treatment alone. Similarly, the combination treatment of FC101 (0.5  $\mu\text{M}$ ) and erlotinib (0.5  $\mu\text{M}$ ) had a slightly lower average  $A_{570}$  value, but there was also no significant difference between the two when compared with FC101 (0.5  $\mu\text{M}$ ) treatment alone. The combination of everolimus (0.5  $\mu\text{M}$ ) and lapatinib (1.0  $\mu\text{M}$ ) had a significantly lower level of proliferation than the everolimus (0.5  $\mu\text{M}$ ) treatment alone. However, in the combination of everolimus (0.5  $\mu\text{M}$ ) and erlotinib (0.5  $\mu\text{M}$ ), proliferation was not significantly different than the everolimus (0.5  $\mu\text{M}$ ) treatment alone.

### 3.2. MTT Viability Assay

A hallmark of aggressive TNBC is its self-sufficiency for unchecked growth and survival. Therefore, inhibition of cellular viability is a crucial feature of any anti-cancer agent against TNBC. Cell viability can be conveniently screened using a well-documented MTT assay. A 96-well microscale MTT assay was used to determine if single and combination treatments with FC101 and control drugs inhibited the viability of MDA-MB231 cells. The MTT assay measures cellular viability by evaluating the mitochondrial integrity and activity of its most important class of enzymes, dehydrogenases. Metabolically active and viable cells have intact mitochondria and therefore have high dehydrogenase enzyme activity. Most anti-cancer drugs induce cytotoxicity by compromising the mitochondrial integrity, thus the reduction in dehydrogenase enzyme activity can be used as an end point for drug response on viability. The MTT assay relies on the reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), a

yellow water-soluble tetrazolium dye, primarily by the mitochondrial dehydrogenases, to purple formazan crystals. The formazan product is released by solubilizing the cells and measured spectrophotometrically at  $\lambda_{\text{max}}$  570 nm. The amount of color produced determined as  $A_{570}$  is directly proportional to the number of metabolically viable cells.

Single and combination treatments of FC101 and lapatinib at various concentrations were first tested in a 48-hour MTT assay as shown in Fig. 4. In single treatment experiments, FC101 (0.05  $\mu\text{M}$  and 0.1  $\mu\text{M}$ ) did not induce significant reduction in TNBC cellular viability as compared to control DMSO ( $p < 0.001$ ). However, the viability of FC101-treated cells at 0.5  $\mu\text{M}$  was significantly lower than the DMSO-treated control cells ( $p < 0.001$ ). All the single treatments of lapatinib (0.1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , and 1.0  $\mu\text{M}$ ) displayed significantly lower cell viability levels than the control cells. When analyzing the combination of FC101 and lapatinib against the respective equivalent concentration of FC101 alone, the only combination treatment that displayed significantly lower viability was the combination of FC101 0.05  $\mu\text{M}$  and Lapatinib 1.0  $\mu\text{M}$  ( $p < 0.01$ ). However, when the same combination was analyzed against the equivalent single lapatinib dose of 1.0  $\mu\text{M}$ , the significance was lost, indicating the effect was due to lapatinib rather than a synergistic effect. Except for the combination of FC101 0.05  $\mu\text{M}$  and Lapatinib 0.1  $\mu\text{M}$ , which did not display significantly lower viability than control cells, all of the other FC101 and lapatinib combination treatments were significantly lower than the control cells. From these results, the combination of 0.5  $\mu\text{M}$  FC101 and 1.0  $\mu\text{M}$  Lapatinib was selected for use in future time response experiments because it was the most effective drug dose and had significantly ( $p < 0.001$ ) lower viability when compared to control cells(Fig. 4).

Figure 5 represents the MTT time response curve results for the combination treatments of FC101 and everolimus with each of the EGFR inhibitors (lapatinib and erlotinib) over the time points of 24, 48, and 72 hours.

At 48 hours, cell viability in all single and combination treatments was significantly lower than the control experiments ( $p < 0.001$ )( Fig. 6). Viability for the combination of FC101 (0.5  $\mu\text{M}$ ) and lapatinib (1.0  $\mu\text{M}$ ) was significantly lower than FC101 (0.5  $\mu\text{M}$ ) alone or lapatinib alone (1.0  $\mu\text{M}$ )( $p < 0.001$ ). Viability for the combination of FC101 (0.5  $\mu\text{M}$ ) and erlotinib (0.5  $\mu\text{M}$ ) was also significantly lower than FC101 (0.5  $\mu\text{M}$ ) alone and Erlotinib alone (0.5  $\mu\text{M}$ )( $p < 0.001$ ). Viability for the combination of everolimus (0.5  $\mu\text{M}$ ) and lapatinib (1.0  $\mu\text{M}$ ) was significantly lower than everolimus (0.5  $\mu\text{M}$ ) alone and lapatinib (1.0  $\mu\text{M}$ ) alone ( $p < 0.001$ ). Viability for the combination of everolimus (0.5  $\mu\text{M}$ ) and erlotinib (0.5  $\mu\text{M}$ ) was also significantly lower than everolimus (0.5  $\mu\text{M}$ ) alone and erlotinib (0.5  $\mu\text{M}$ ) alone ( $p < 0.001$ ). When comparing the FC101 based combinations with the everolimus based combinations, there was no significant difference of viability between the combination of FC101 with erlotinib versus the combination of everolimus and erlotinib. The combination of FC101 and lapatinib compared to the combination of everolimus and lapatinib showed a differing result with everolimus and lapatinib having significantly lower viability than FC101 and lapatinib combination treatment. Viability for FC101 (0.5  $\mu\text{M}$ ) treatment was significantly lower than all single treatments of lapatinib, erlotinib, and everolimus. FC101 alone did not show any significant difference when compared to the combination treatment of everolimus and erlotinib. The only

condition in which FC101 was not equal or superior in reducing cell viability was when compared to everolimus and lapatinib combination treatment. In this case, everolimus and lapatinib combination treatment displayed significantly lower viability than FC101 alone (Fig. 6).

### 3.3. Drug Washout Survival Assay

To determine TNBC (MDA-MB-231) cellular growth recovery after drug treatments, cells were treated for 72 hours followed by the removal of the growth medium containing the drug, and then replaced with drug-free fresh growth medium. The cells were placed back in the incubator and allowed to recover for another 72 hours. An MTT viability assay was performed on the drug-treated cells at 72 h and recovered cells. Fold changes in viability levels at 72 hours of treatment were graphed against the fold changes in viability levels at 72 hours after treatment removal (Figs. 7 and 8). When drugs were removed, both lapatinib (1.0  $\mu\text{M}$ ) and erlotinib (0.5  $\mu\text{M}$ ) single treated cells were able to recover to the viability level of untreated control cells with no significant difference in viability among the three. All other treatments including FC101 alone and in combination with lapatinib or erlotinib as well as everolimus alone and in combination with lapatinib or erlotinib maintained a significant decrease in viability when compared to untreated cells ( $p < 0.001$ ). However, when comparing the fold change in viability for cells treated for 72 hours against the viability fold change of drug washout cells, more notable results were observed. There was no significant difference in viability when cells were treated with FC101 (0.5  $\mu\text{M}$ ) for 72 hours when compared to the drug washout cells. Therefore, even when the FC101 treatment was removed, the cells were not able to recover indicating a robust and durable drug response. This inability for cells to recover was also seen in the FC101 (0.5  $\mu\text{M}$ ) and lapatinib (1.0  $\mu\text{M}$ ) combination treatment [Figure 7]. For all other treatment conditions, the cells recovered their viability levels in a significant ( $p < 0.001$ ) manner after the treatments were removed. Cell viability recovered by 66% after lapatinib (1.0  $\mu\text{M}$ ) treatment and subsequent drug washout. However, when FC101(0.5  $\mu\text{M}$ ) was added for a combination treatment with lapatinib (1.0  $\mu\text{M}$ ), the cell viability recovered only 11% [Figure 7].

When erlotinib (0.5  $\mu\text{M}$ ) was tested after cell treatment and subsequent drug washout, viability recovered 74%. In contrast, when FC101 (0.5  $\mu\text{M}$ ) was added for a combination treatment with erlotinib (0.5  $\mu\text{M}$ ), the cell viability was only recovered by 23%. FC101(0.5  $\mu\text{M}$ ) treatment alone with drug washout actually provides an additional 8% decrease in viability after the drug was removed. FC101 (0.5  $\mu\text{M}$ ) was significantly more effective in sustaining a decrease in viability when compared to everolimus (0.5  $\mu\text{M}$ ) because when Everolimus was removed, cells recovered by 40%. When everolimus (0.5  $\mu\text{M}$ ) was used in combination with lapatinib (1.0  $\mu\text{M}$ ) then washed out, a 54% recovery of viability was observed. When everolimus (0.5  $\mu\text{M}$ ) was used in combination with erlotinib (0.5  $\mu\text{M}$ ) then washed out, a 20% recovery in viability was observed. These results indicated that FC101 was superior in its ability to provide a sustained decrease in viability both when used by itself and in combination with lapatinib or erlotinib. Interestingly, when FC101 was used alone as a treatment, its viability levels continued to decrease even after its removal, whereas when FC101 was combined with the other drugs, cells were able to regain some viability.

## Discussion

Cancer drug resistance is a significant clinical challenge that can be effectively addressed by the development of combination therapies [11]. Using both genomic and proteomic information, new combination therapies can be developed that target compensatory oncogenic signaling pathways and achieve better clinical outcomes in treating cancer. Combination treatments are generally synergistic, blocking both the primary oncogenic target as well as its secondary (drug-induced) targets, which evolve as the cancer cells adapt to survive [11].

TNBC is among the most aggressive types of cancer, and it has the least favorable prognosis [13]. TNBC lacks the overexpression of ER, PR, and HER2/Neu, and thus it offers the fewest available therapeutic options. TNBC's aggressiveness and poor prognosis are both correlated to the overexpression of EGFR [18]. Yet, in clinical trials, patients often do not respond favorably to EGFR inhibitors alone, due to the development of drug resistance [20]. A variety of pre-clinical and clinical evidence indicates that combining an EGFR inhibitor with an mTOR inhibitor produces synergistic drug response and effectively circumvent drug resistance in TNBC [17,18, 19, 20]. There is currently an increasing demand for other novel mTOR inhibitors to establish and validate new drug combinations that can effectively block the oncogenic networks responsible for drug resistance in TNBC. In this context, FC101 also serves as a promising anti-cancer agent for TNBC, because it powerfully modulates the mTOR pathway.

We hypothesized that FC101's activity against TNBC is similar to that of the known mTOR inhibitor, everolimus, since FC101 reduces the phosphorylation of two key mTOR substrates, S6K and S6 [supplementary materials]. Since the activity of everolimus is enhanced when it is combined with known EGFR inhibitors (erlotinib or lapatinib), we performed analogous studies with FC101. Phenotypic cellular assays helped assess whether FC101 (in both single and combination treatments) acts similarly to everolimus.

The treatment of cultured TNBC cells with FC101 or everolimus (a known mTOR inhibitor), along with EGFR inhibitors (erlotinib or lapatinib) was used to measure synergistic effects. A slight additive effect was seen in the crystal violet and MTT assays for the combination of FC101 (0.5  $\mu$ M) and erlotinib (0.5  $\mu$ M). However, FC101 combinations were not as robust as everolimus combinations in enhancing the synergistic drug response. For example, at 48 hours, the combination of FC101 and erlotinib had an additional 14% decrease in viability (*vs.* FC101 alone). However, everolimus combined with erlotinib had an additional 25% decrease in viability (*vs.* everolimus alone) [Figure 6].

For combinations involving lapatinib, however, there was a greater difference between FC101 and everolimus. In the MTT assay at 48 hours, FC101 (0.5  $\mu$ M), combined with lapatinib (1.0  $\mu$ M) had an additional 13% decrease in viability from FC101(0.5  $\mu$ M) alone, while everolimus (0.5  $\mu$ M) combined with lapatinib (1.0  $\mu$ M) showed an additional 52% decrease *vs.* everolimus alone. Interestingly, while everolimus/lapatinib (0.5  $\mu$ M/1.0  $\mu$ M) combination treatment was more effective at reducing viability than everolimus/erlotinib (0.5  $\mu$ M/0.5 $\mu$ M) in the MTT (52% *vs.* 25%), this difference was not seen in the

FC101 combination treatments. The FC101/lapatinib (0.5  $\mu$ M/1.0  $\mu$ M) and FC101/erlotinib (0.5  $\mu$ M/0.5 $\mu$ M) combination treatments performed similarly (13% vs 14%).

These findings negate our original hypothesis that FC101 combined with EGFR inhibitors would produce a similar synergistic effect as everolimus in similar combinations. That hypothesis was based upon FC101's ability to reduce pS6K and p-S6 downstream of mTOR, but it also assumed that FC101 inhibited mTOR directly. This surprising outcome can be explained in-part by considering the significant genomic heterogeneity and molecular complexity of cancer. Natural selection ultimately leads to redundancy and crosstalk between cancer cell signaling pathways. For example, similar cancer cell phenotypes can be achieved through a variety of different mechanisms, such as the reduction in p-S6K and p-S6. Notably, our results confirm that FC101's reduction in p-S6K and p-S6 may not represent direct mTOR inhibition. Thus, FC101 may exploit a unique mechanism in effecting the mTOR signaling and reducing p-S6K and p-S6. This may include the inhibition of proteins involved in the regulatory crosstalk involving feedback loops between the two canonical EGFR downstream pathways: RAF/MEK/ERK (MAPK) and AKT/mTOR. Extensive crosstalk between these two key pathways is well-documented and may be modulated by treatment with FC101. This suggests that a currently unidentified and novel protein interaction may facilitate FC101's mode of action.

Notably, FC101 as a single treatment outperformed all other single treatments in the crystal violet, MTT, and drug wash-out assays. For example, in the MTT viability assay (48h post treatment), the control-normalized drug-induced % decrease for FC101, everolimus, lapatinib, erlotinib treatments was 45%, 30%, 15%, 10%, respectively. Furthermore, in the drug wash-out assay, FC101 produced the most robust reductions of viability between the 72-hour treatment and the 72-hour period after drug washout. There were no significant differences for FC101's effects on cell viability between the two time points, indicating that FC101 was able to affect cells in a sustained manner.

FC101 (0.5  $\mu$ M), alone as well as in combine with lapatinib (1.0  $\mu$ M) and erlotinib (0.5  $\mu$ M) all produced a sustained decrease in cell viability with no significant difference between the two time points. Moreover, three treatment conditions were the only ones that maintained the decrease in cell viability. All other single and combination treatments showed a significant recovery of cell viability between the two time points. FC101 clearly produces a sustained treatment effect, blocking the ability of cancer cells to recover once treatments are removed. This supports the possibility for successful long-term treatment outcomes and reaffirms FC101's unique and multi-targeting pharmacology.

## Conclusion

Addressing drug resistance using rationally designed combination therapies is a very important approach in cancer drug discovery today. As a novel anti-cancer agent with unique structure and function, FC101 has significant promise not only as a research tool but also clinical application. In this study, we examined TNBC-directed FC101's mode of action and possible synergy with EGFR inhibitors. We ruled out our original hypothesis that FC101 combined with EGFR inhibitors would produce a similar synergistic

effect as everolimus in similar combinations. The results demonstrate that as an mTOR signaling modulator, FC101 acts through a different mechanism as everolimus. This lays the foundation for hypothesis refinement in order to better understand FC101's mode of action. Future studies will include either confirming or ruling out other possible mechanisms for FC101's ability to reduce the expression of p-S6K and p-S6, including: protein phosphatase-dependent mechanisms, disruption of protein-protein interactions involved in mTOR signal transduction, and effects on scaffolding proteins.

## Abbreviations

Fusarochromanone – FC101

TNBC – Triple Negative Breast Cancer

mTORi – mTOR inhibitor

EGFRi – EGFR inhibitor

μM – micromolar

## Declarations

### Ethics approval and consent to participate

'not applicable'

### Consent for publication

'not applicable'

### Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available because Natalie Carroll has taken a new job and currently does not have access to the computer that contains the data, but are available from the corresponding author on reasonable request.

### Competing interests

'not applicable'

### Funding

Research reported here was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 5 P20 GM103424-18 and 3 P20 GM103424-15S1.

## Authors' contributions

“NC” performed all experiments; analyzed and interpreted all data, and created all graphs. This project was her thesis research and part of the requirement for her MS degree in Biological Sciences at LSUS.

“AS” performed the preliminary experiments and analyzed the data that formed the basis for the hypothesis of this project.

“BAS” helped with the synthesis of FC101 and edited the manuscript.

“EM” was the primary research supervisor for NB, helped with the synthesis of FC101, designed all experimental methodologies, was a major contributor in writing the manuscript.

## Acknowledgements

'not applicable'

## References

1. Cancer Facts & Figures 2021. <https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2021/cancer-facts-and-figures-2021.pdf>. Accessed on 18 Feb.2021.
2. Cancer facts and statistics. <https://www.cancer.org/research/cancer-facts-statistics.html>. Accessed 18 Feb.2021.
3. Metastatic Cancer: When Cancer Spreads. Available online. <https://www.cancer.gov/types/metastatic-cancer>. Accessed 18 Feb.2021.
4. Huang CY, Ju DT, Chang CF, Muralidhar Reddy P, Velmurugan, BK. A review on the effects of current chemotherapy drugs and natural agents in treating non-small cell lung cancer. *Biomedicine (Taipei)*. 2017; 7: 23. doi:10.1051/bmdcn/2017070423.
5. Targeted Cancer Therapies. <https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet>. Accessed on 18 Feb.2021.
6. Hassanpour, SH, Dehghani, M. Review of cancer from perspective of molecular. *J Cancer Res Pract*. 2017; 4:127-129. doi:10.1016/j.jcrpr/201707001.
7. Sams-Dodd F. Target-based drug discovery: is something wrong?. *Drug discovery*. 2005; 10:139-147. doi:10.1016/S1359-6446(04)03316-1.
8. Zhang J, Spath SS, Marjani SL, Zhang W, Pan W. Characterization of cancer genomic heterogeneity by next-generation sequencing advances precision medicine in cancer treatment. *Precision clinical medicine*. 2018; 1:29-48. doi:10.1093/pcmedi/pby007.
9. Viswanathan VS, Ryan MJ, Dhruv HD Gill, S Eichhoff, O M Seashore-Ludlow, et al. Dependency of a therapy-resistant state of cancer cells on a lipid peroxidase pathway. *Nature*. 2017; 547:453-457. doi:10.1038/nature23007.

10. Raghavendra NM, Pingili D, Kadasi S, Mettu A, Prasad S. Dual or multi-targeting inhibitors: The next generation anticancer agents. *European Journal of Medicinal Chemistry*. 2018; 143: 1277-1300. doi:10.1016/j.ejmech.2017.10.021.
11. Tosi D, Perez-Gracia E, Atis S, Vie N, Combes E, Gabanou M, Larbouret C, Jarlier M, Mollevi C, Torro A, Del Rio M, Martineau P, Gongora C. Rational development of synergistic combinations of chemotherapy and molecular targeted agents for colorectal cancer treatment. *BMC Cancer*. 2018; 18. doi: 10.1186/s12885-018-4712-z.
12. Malone ER, Oliva M, Sabatini PJ, Stockley TL, Siu LL. Molecular profiling for precision cancer therapies. *Genome Med*. 2020; 12. doi:1186/s13073-019-0703-1.
13. Breast Cancer. <https://www.cancer.org/cancer/breast-cancer.html>. Accessed on 18 Feb.2021.
14. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J, Cardoso F. Breast cancer. *Nat Rev Dis Primers*. 2019; 5. doi: 10.1038/s41572-019-0111-2.
15. Lawrence RT, Perez EM, Hernández D, Miller CP, Haas KM, Irie HY, Lee SI, Blau CA, Villén The Proteomic Landscape of Triple-Negative Breast Cancer. *Cell Report*. 2015; 11: 630-644. doi: 10.1016/j.celrep.2015.03.050.
16. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi, GN, Ueno NT. Role of Epidermal Growth Factor Receptor in Breast Cancer. *Breast Cancer Research and Treatment*. 2012; 136:331-345. doi: 1007/s10549-012-2289-9.
17. Madden JM, Mueller KL, Bollig-Fischer A, Stemmer P, Mattingly RR, Boerner Abrogating phosphorylation of eIF4B is required for EGFR and mTOR inhibitor synergy in triple-negative breast cancer. *Breast Cancer Research and Treatment*. 2014; 147:283-293. doi: 10.1007/s10549-014-3102-8.
18. Zhou X, Tan M, Stone Hawthorne V, Klos KS, Lan KH, Yang Y, Yang W, Smith TL, Shi D, Yu D. Activation of the Akt/Mammalian Target of Rapamycin/4E-BP1 Pathway by ErbB2 Overexpression Predicts Tumor Progression in Breast Cancers. *Clin Cancer Res*. 2004; 10:6779-6788. doi: 10.1158/1078-0432.CCR-04-0112.
19. Buck E, Eyzaguirre A, Brown E, Petti, F, McCormack S, Haley JD, Iwata KK, Gibson NW, Griffin G. Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors. *Mol Cancer Ther*. 2006; 5: 2676-2684. doi:10.1158/1535-7163.MCT-06-0166.
20. Gadgeel SM, Lew DL, Synold TW, LoRusso P, Chung V, Christensen SD, Smith DC, Kingsbury L, Hoering A, Kurzrock R. Phase I study evaluating the combination of lapatinib (a Her2/Neu and EGFR inhibitor) and everolimus (an mTOR inhibitor) in patients with advanced cancers: South West Oncology Group (SWOG) Study S0528. *Cancer Chemother Pharmacol*. 2013; 72: 1089-1096. doi:10.1007/s00280-013-2297-4.
21. Lee YW, Mirocha CJ, Shroeder DJ, Walser MM. TDP-1, A Toxic Component Causing Tibial Dyschondroplasia in Broiler Chickens, and Trichothecenes from *Fusarium roseum* Graminearum. *Applied and Environmental Microbiology*. 1985; 50, 102-107. doi:10.1128/AEM.50.1.102-107.1985.

22. Dreau D, Foster M, Hogg M, Culberson C, Nunes P, Wuthier RE. Inhibitory Effects of Fusarochromanone on Melanoma Growth. *Anti-Cancer Drugs*. 2007; 18:897-904. doi:10.1097/CAD.0b013e3280d94298.
23. Mahdavian E, Williams-Hart T, Furmanski B, Kevil C, Gopi K, Yoon-Jee K, Salvatore BA, Huang S, Clifford J. Biological Activities of Fusarochromanone: A Potent Anti-Cancer Agent. *BMC Research Notes*. 2014; 7. doi:10.1186/1756-0500-7-601.
24. Mahdavian E, Marshall M, Martin PM, Cagle P, Salvatore BA, Quick QA. Caspase dependent signaling underlies glioblastoma cell death in response to the fungal metabolite, fusarochromanone. *Int J Mol Med*. 2014; 34: 880-885. doi:10.3892/ijmm.2014.1842.
25. Kuang YH, Shen T, Chen X, Sodani K, Hopper-Borge E, Tiwari AK, Lee JW, Fu LW, Chen ZS. Lapatinib and erlotinib are potent reversal agents for MRP7 (ABCC10)-mediated multidrug resistance. *Biochem Pharmacol*. 2010; 79: 154-161. doi: 10.1016/j.bcp.2009.08.021.
26. Iyer R, Bharthuar A. A review of erlotinib—an oral, selective epidermal growth factor receptor tyrosine kinase inhibitor. *Expert Opin Pharmacother*. 2010; 11:311-320. doi: 10.1517/14656560903551283.
27. Wainberg ZA, Anghel A, Desai AJ, Ayala R, Luo T, Safran B, Fejzo MS, Hecht JR, Slamon DJ, Finn RS. Lapatinib, a dual EGFR and HER2 kinase inhibitor, selectively inhibits HER2-amplified human gastric cancer cells and is synergistic with trastuzumab in vitro and in vivo. *Clin Cancer Res*. 2010; 16: 1509-1519. doi: 10.1158/1078-0432.CCR-09-1112.
28. Cohen MH, Johnson JR, Chen YF, Sridhara R, Pazdur R. FDA drug approval summary: erlotinib (Tarceva) tablets. *Oncologist*. 2005. 10: 461-466. doi: 10.1634/theoncologist.10-7-461.
29. Voigtlaender M, Schneider-Merck T, Trepel M. Lapatinib. *Recent Results Cancer Res*. 2018; 211:19-44, doi:10.1007/978-3-319-91442-8\_2.
30. Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, Owens, G, Alligood KJ, Spector NL. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene*. 2002; 21: 6255-6263. doi: 10.1038/sj.onc.1205794.
31. Hasskarl J. Everolimus. *Recent Results Cancer Res*. 2018; 211:101-123, doi:10.1007/978-3-319-91442-8\_8.

## Figures

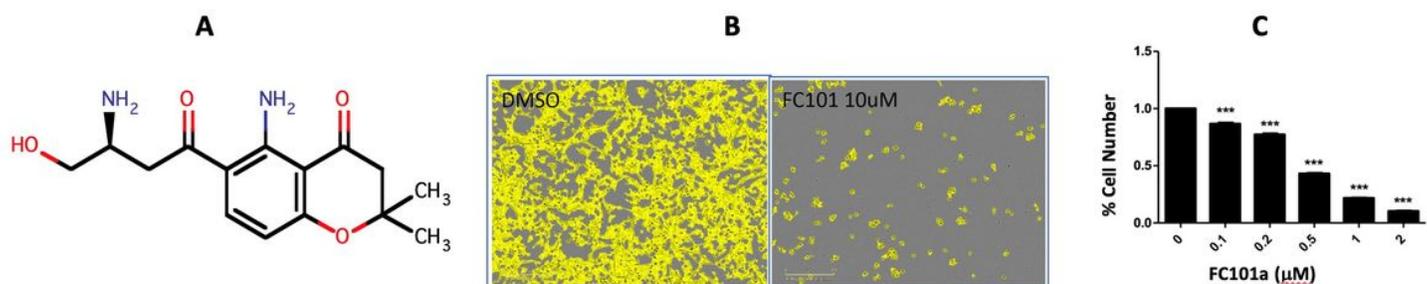


Figure 1

A. The 2D Chemical Structure of Fusarochromanone. B. Phase contrast Images of TNBC cells (MDA-MB231) treated with FC101 (10uM and DMSO), 72h post treatment. C. The effect of FC101 on TNBC cell number using Incucyte Zoom Confluence.

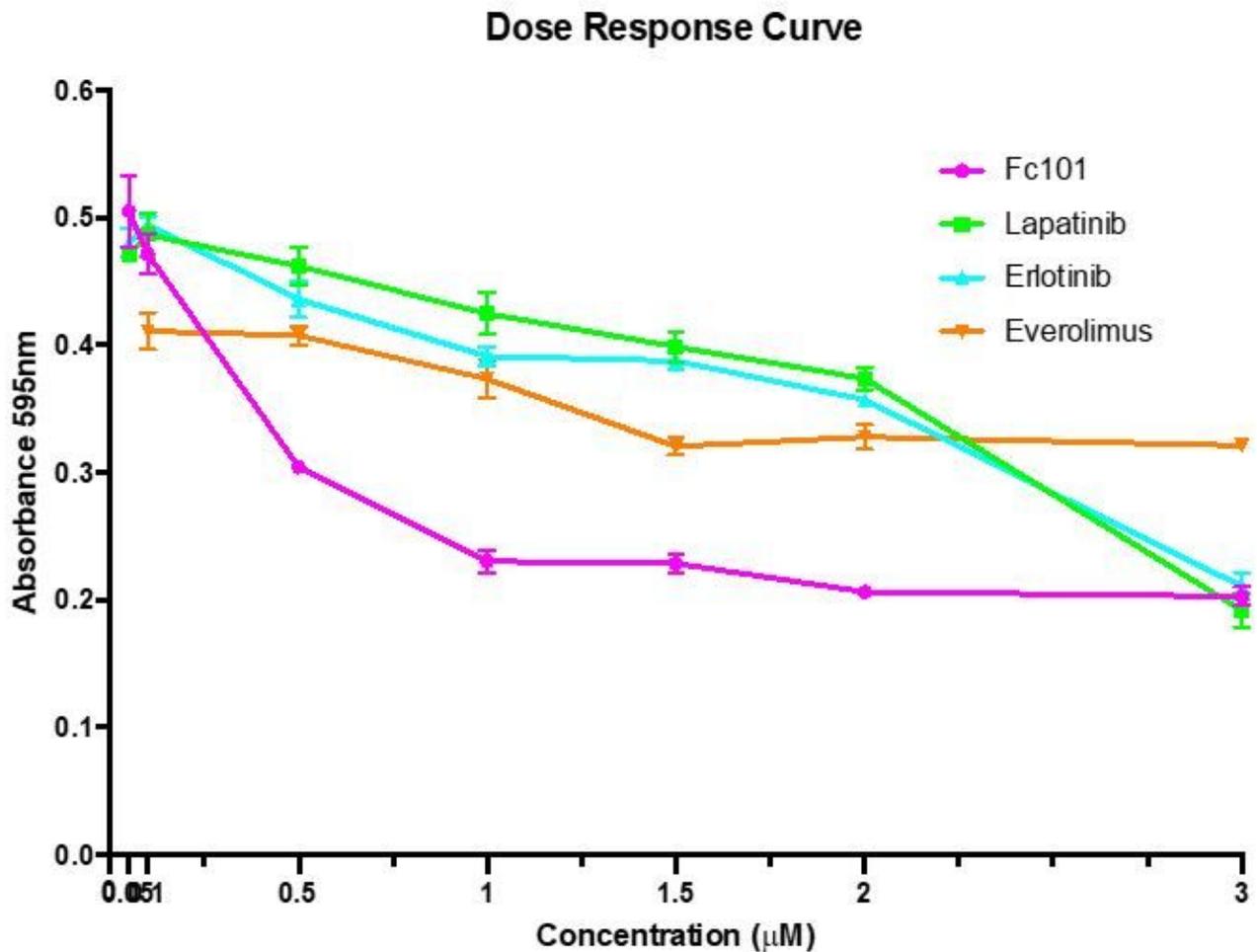
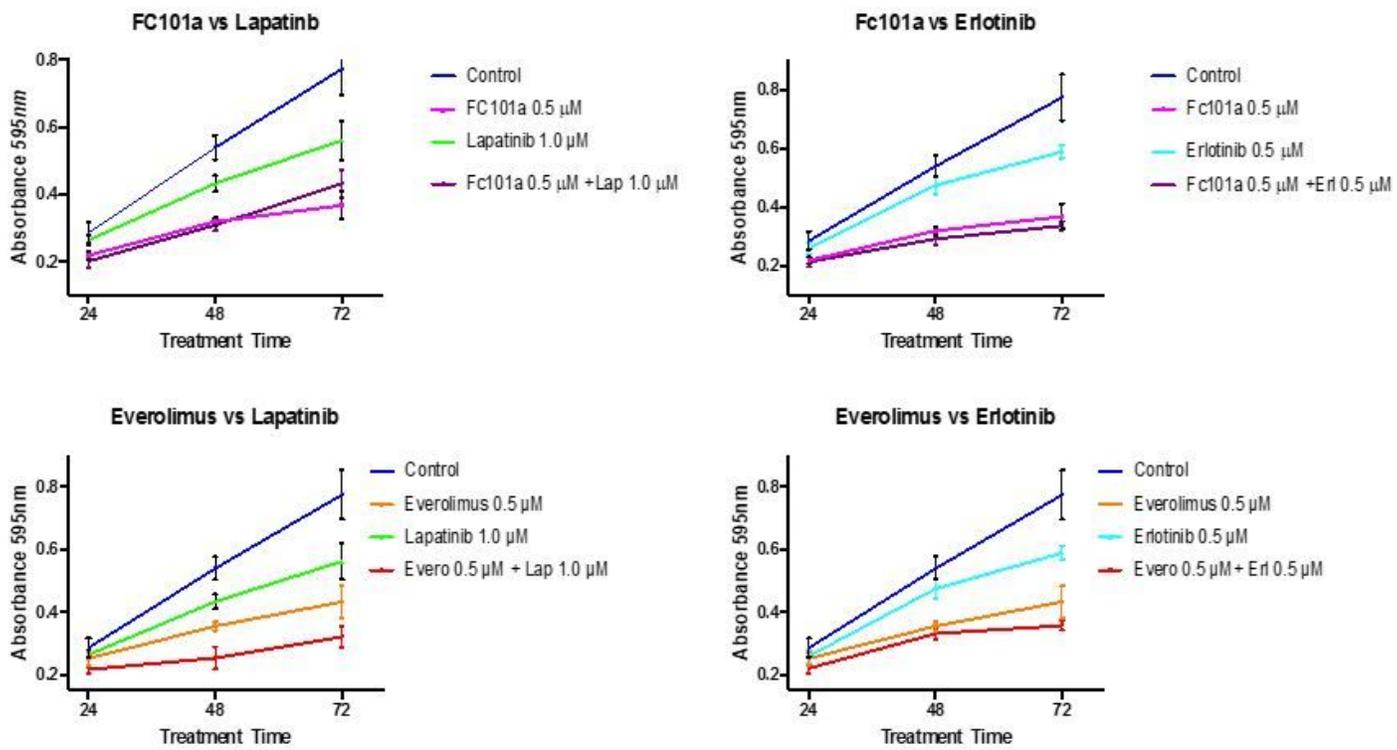


Figure 2

Crystal Violet Assay- Dose Response Curve, 48h-Post Drug Treatment Drug panel included FC101, lapatinib, erlotinib, and everolimus at concentrations 0 µM, 0.05 µM, 0.1 µM, 0.5 µM, 1.0 µM, 1.5 µM, 2 µM, and 3 µM. Graph represents normalized color intensity A595 (% control) vs. drug concentration.

# Crystal Violet Assay



**Figure 3**

Crystal Violet Staining Assay- Time Response Curve. Treatment concentrations included FC101 0.5  $\mu$ M, Lapatinib 1.0  $\mu$ M, Erlotinib 0.5  $\mu$ M, Everolimus 0.5  $\mu$ M, and combinations of FC101 or everolimus with lapatinib or erlotinib. Graph represents normalized A595 (% control) vs. time course treatments at 24, 48, and 72 hours.

## MTT 48 hour Assay

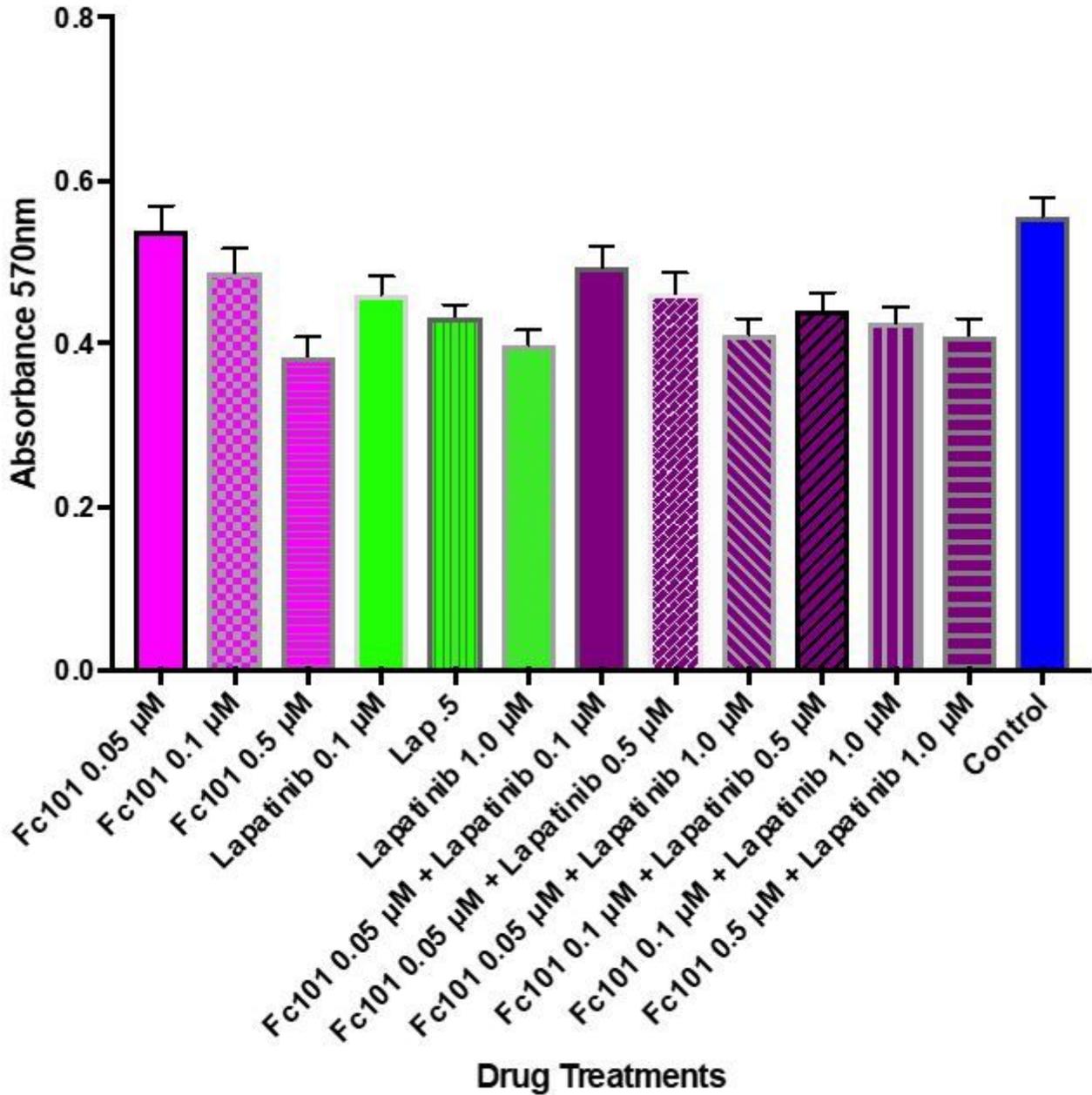
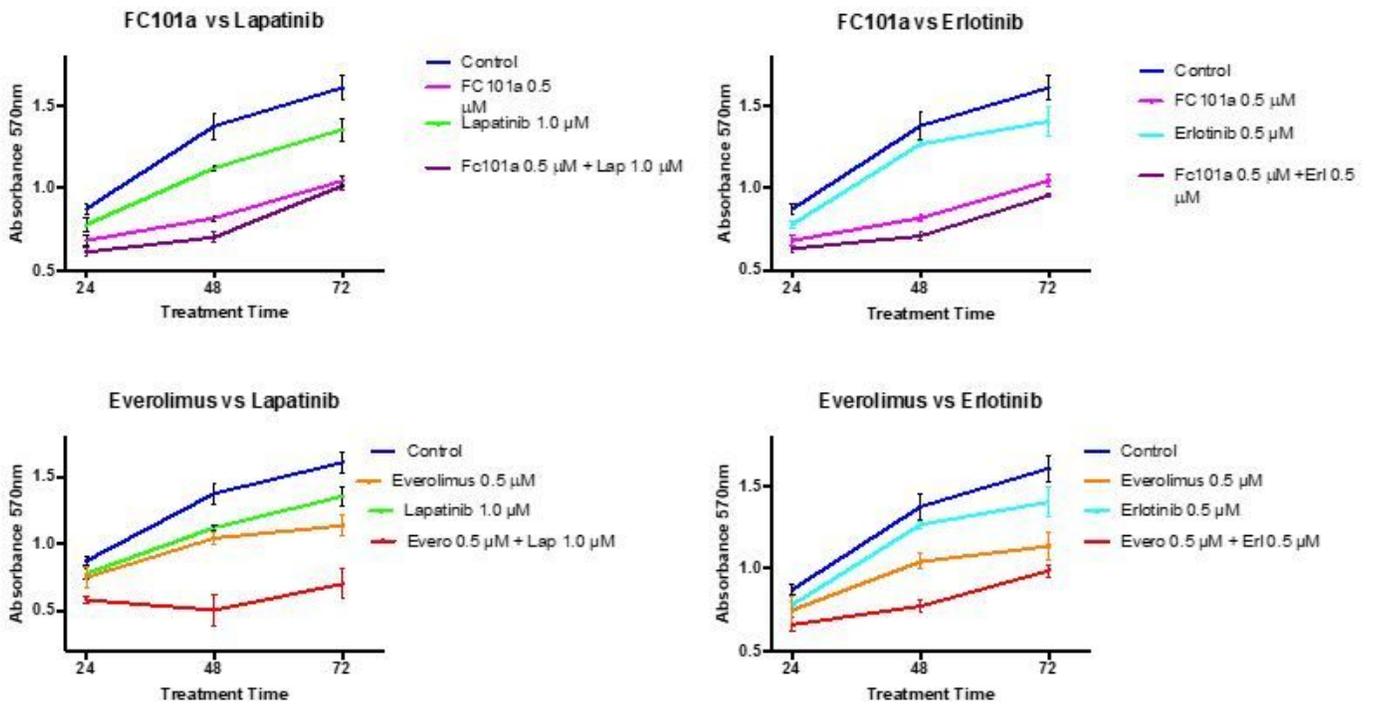


Figure 4

MTT Viability Assay- Dose Response Curve. FC101 0.05 μM, 0.1 μM, 0.5 μM and Lapatinib 0.1 μM, 0.5 μM, 1.0 μM. Each concentration of FC101 was paired with an equal or higher concentration of Lapatinib. Graph represents normalized color intensity A570 (% control) vs. each single and combination treatment at 48h.

# MTT Assay



**Figure 5**

MTT Viability Assay- Time Response Curve. Treatment concentrations included FC101 (0.5  $\mu$ M), lapatinib (1.0  $\mu$ M), erlotinib (0.5  $\mu$ M), everolimus (0.5  $\mu$ M), and combinations of either FC101 (0.5  $\mu$ M) everolimus (0.5  $\mu$ M) with lapatinib (1  $\mu$ M), or erlotinib (0.5  $\mu$ M). Graph represents normalized color intensity A570 (% control) vs. time course treatments at 24, 48, and 72 hours.

## MTT 48 hour Assay

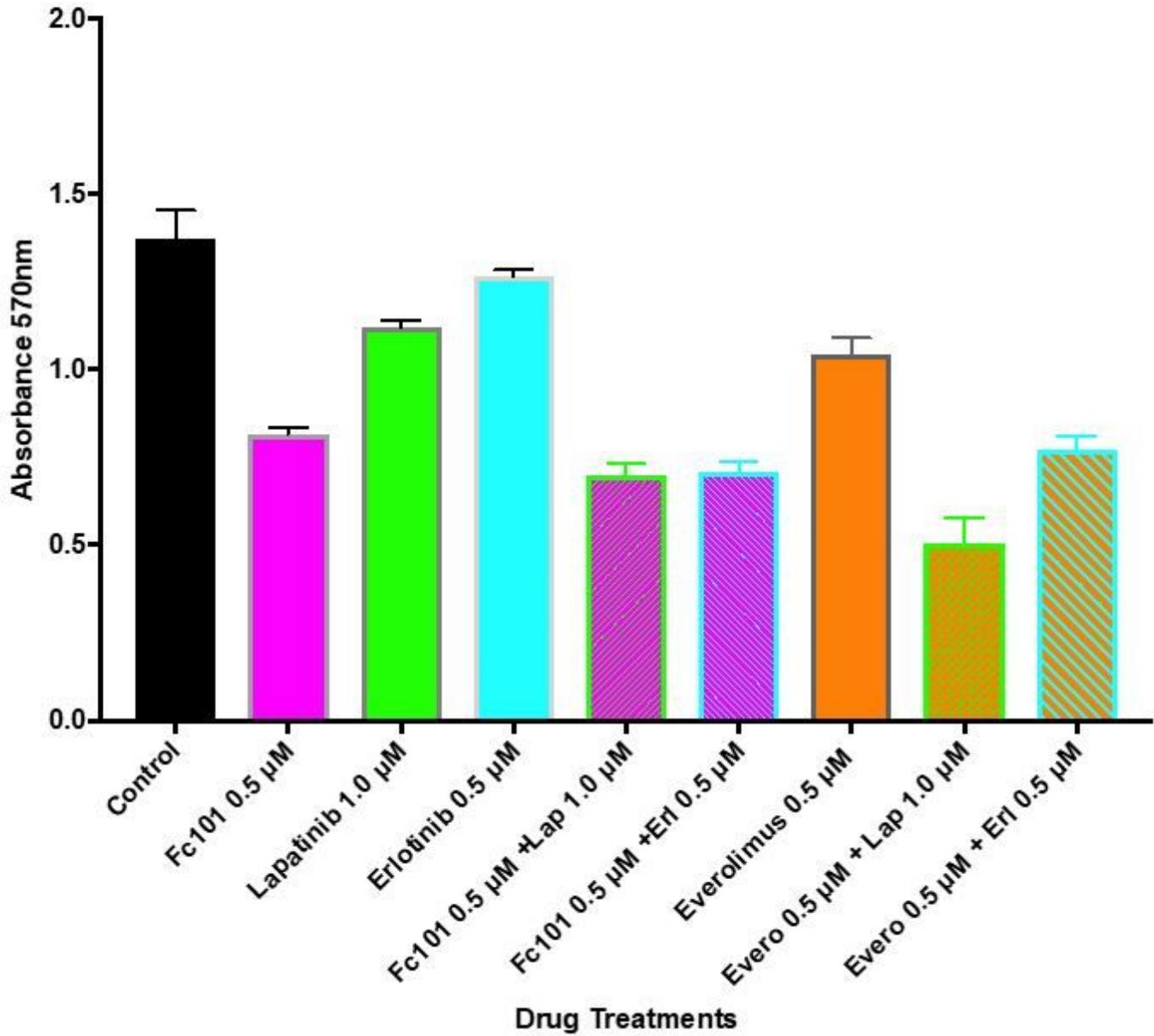
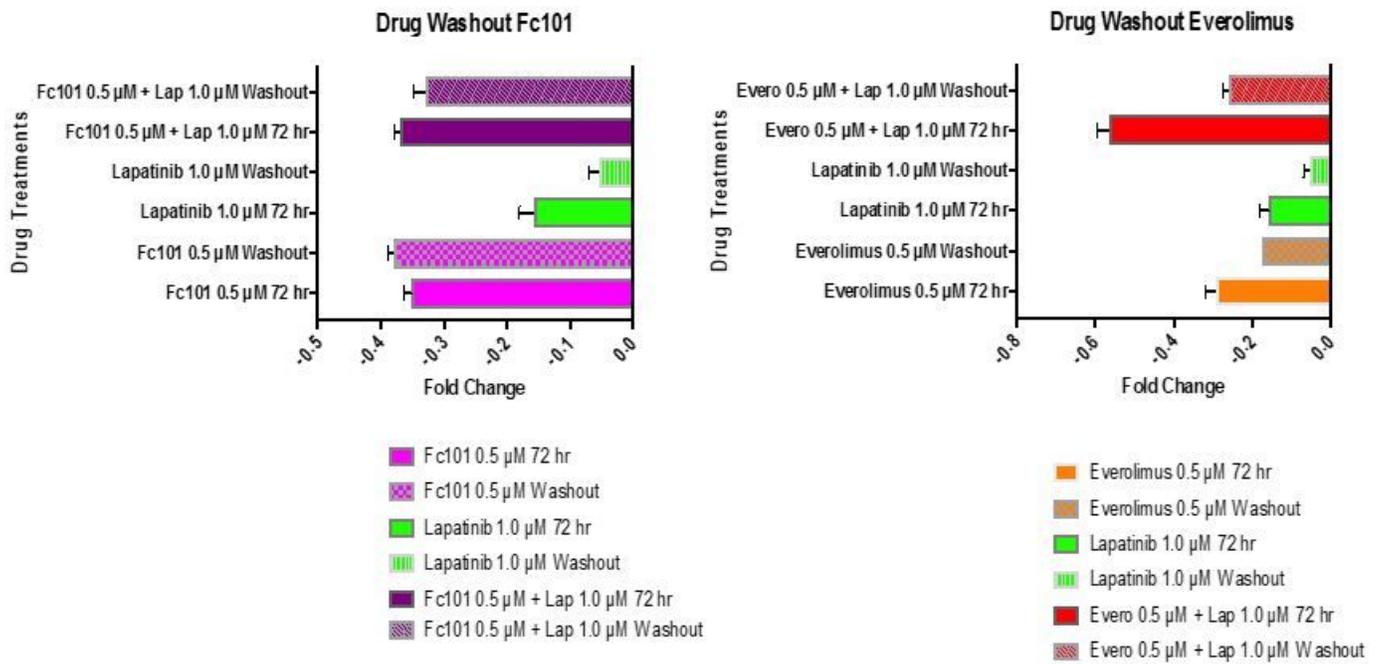


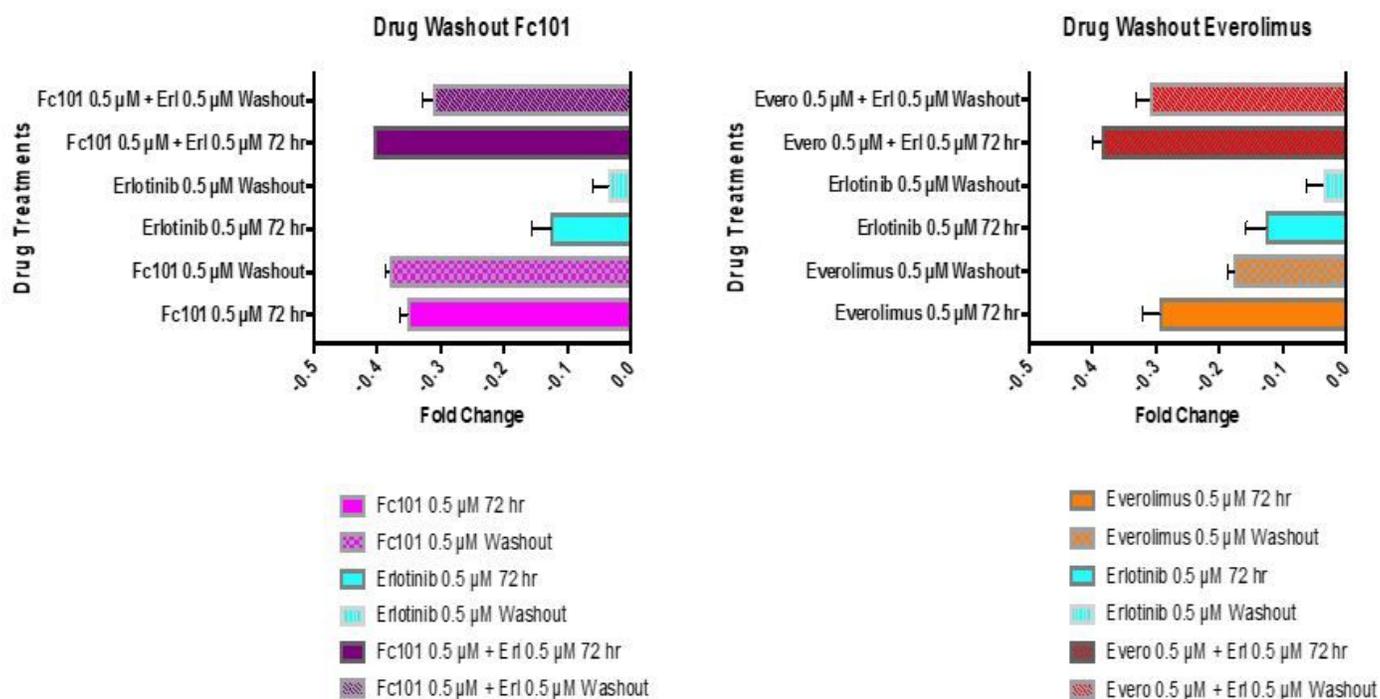
Figure 6

MTT Viability Assay- 48h Post Drug Treatment. MTT viability assay depicting 48-hour time point. Treatment concentrations included FC101 0.5 µM, lapatinib 1.0 µM, erlotinib 0.5 µM, everolimus 0.5 µM, and combinations of FC101 or everolimus with lapatinib or erlotinib.



**Figure 7**

Drug washout assay. Fold changes in viability for the 72-hour treatment are graphed alongside viability fold changes of the 72-hour recovery after drug washout. Solid bars represent 72-hour treatment condition, patterned bars represent drug washout conditions. Treatment concentrations included FC101 0.5  $\mu$ M, lapatinib 1.0  $\mu$ M, everolimus 0.5  $\mu$ M, and combinations of FC101 or everolimus with lapatinib.



**Figure 8**

Drug washout assay. Fold changes in viability for 72-hour treatment are graphed alongside viability fold changes of 72-hour recovery after drug washout. Solid bars represent 72-hour treatment condition, patterned bars represent drug washout conditions. Treatment concentrations included FC101 0.5  $\mu$ M, erlotinib 0.5  $\mu$ M, everolimus 0.5  $\mu$ M, and combinations of FC101 or everolimus with erlotinib.