Triple Negative Breast Cancer

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Abstract

Cancer is the second leading cause of death worldwide and has an impact on everyone. Approximately every two minutes, a woman in the United States is diagnosed with breast cancer and recently there has been an increase in the incidences of breast cancer. Breast cancer is the leading cause of cancer related deaths in women worldwide with a fatality rate of about 2.6%. Triple negative breast cancer (TNBC), a subtype of breast cancer, makes up approximately 10-15% of all breast cancers. It is a subset of ER-negative breast cancer and does not have estrogen receptor alpha, progesterone receptor, and HER2 markers. It is usually a more aggressive form of breast cancer, has more metastasis, and the recurrence free survival is short. As of now, there only exists targeted therapies to treat ER-positive breast cancer. For TNBC, the identifying factors, the markers, that aid in treatment success are lacking and therefore no previous targeted therapies are effective. The only option that exists is chemotherapy, however it is usually not effective and comes with many adverse side effects. There exists a great need to continue exploring treatment options for TNBC and in order to do so, the pathways and mechanisms that are unique to it must be explored. It has been shown that ERRα provides the metabolic needs and energy of the rapidly dividing tumor cells and a high level of ERRα expression has been linked to worse outcomes for breast cancer patients. Additionally, the mitogen-activated protein kinase (MAPK) pathway is involved in cell proliferation and in cancer cells the receptors responsible for activating this pathway are overexpressed. The PI3K-Akt pathway is also involved in cell proliferation and a mutation in the Akt protein, or an upregulation of it, is associated with the formation of tumors.

MDA-MB-231 cells were studied to see how tamoxifen, XCT-790, and U0126 effected its cell proliferation. Tamoxifen is a selective estrogen receptor modulator, meaning an

antagonist for ERs in breast tissue, XCT-790 is a specific antagonist of ERRα, and U0126 is an inhibitor of MEK/ERK or the MAPK pathway. It was discovered that the inhibition of MAPK signaling, U0126, and the use of an antagonist of ERRα, XCT-790, lead to a significant decrease in cell proliferation in the TNBC cells. For the first time, there has been seen that a link exists between ERRα expression and the MAPK signaling pathway. This lays down the foundation for a possible therapeutic use of the MAPK inhibitors in treating TNBC that expresses ERRα.

Introduction

As the second leading cause of death worldwide, cancer is not something to take lightly, and treatment options need to be developed to slow its effects. Cancer is second to heart disease, but in some high-income countries, deaths from cancer are more common (Hulvat, 2020). In 2022, the three most common types of cancer among women were breast, uterine corpus, and thyroid cancer, while among men the most common types included colon, prostate, and melanoma of the skin (Miller et al., 2022). The existence of many forms of cancer, each with its own unique characteristics, and the deadly effects of it, necessitate many different treatment options and research in developing effective treatments for each type.

Significant progress has been made at curbing the deadly effects of cancer, however, there is a lot more that needs to be achieved. Between the years of 1991 and 2015, the overall death rate from cancer in the United States has fallen by 26%. This is partly due to the increase in screening, prevention, and successful treatments (Hulvat, 2020). As of January 1, 2022, more than 18 million Americans who had a history of cancer were alive but many more did not make it (Miller et al., 2022). Additionally, treatment options don't only need to be more effective at treating cancer but need to be made more accessible to all no matter the family income. In high-income countries, there are higher incidence rates for all cancers, specifically lung, colorectal, breast, and prostate, however mortality rates are declining. In low-income and middle-income countries, there are higher rates of stomach, liver, cervical, and esophageal cancers but mortality rates remain high (Hulvat, 2020). The statistics show that income effects mortality rates, so it is important to continue to develop treatment methods that are both effective at treating the cancer and accessible to all.

Breast cancer is a very important type of cancer to develop treatments for as it has a very high incidence rate in women and a rate that slowly keeps on increasing. Approximately every two minutes, a woman is diagnosed with breast cancer in the United States. According to the National Breast Cancer Foundation, that correlates to 1 in 8 women in the United States being diagnosed with breast cancer in their lifetime (*Breast Cancer Facts & Stats*, 2020)**.** The highest rates for breast cancer incidence are found in Western Europe, the United States, and Israel while the lowest rates are seen in Africa and Asia (Hulvat, 2020). In North America, Europe, and Oceania, breast cancer is the most common cancer found specifically in women (Torre et al., 2016). Figure 1 shows the estimated age-standardized incidence rates (ASR) of cancer in both men and women in the world (Hulvat, 2020). It shows that per 100,000 people in the world, the ASR for breast cancer is slightly greater than 45 and is followed by prostate which is slightly less than 30, and then lung, colorectum, cervix uteri, stomach, liver, corpus uteri, thyroid, and ending with ovary which is about 7. Breast cancer is leading all other types of cancers worldwide and is substantially higher than the second most form of cancer, prostate, by about 15 ASR per 100,000 people. Due to its high rate worldwide, breast cancer is an important cancer to research.

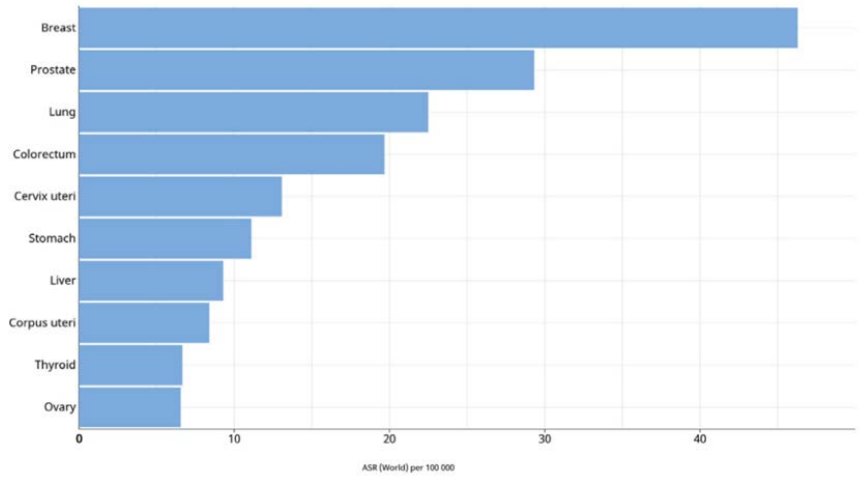


Figure 1. Estimated Age-Standardized Incidence Rates (ASR) of Cancer per 100,000 People Worldwide in 2018 (Hulvat, 2020).

Not only are there high incidence rates for breast cancer, recently there has been an increase in the incidences of breast cancer (Hulvat, 2020) showing that over the years there has been an even greater need to develop treatments for it. This partially can be due to advances made in early detection, awareness, and changes in reproductive patterns (Torre et al., 2016). Between 1973-2012, the incidence rates of breast cancer were collected in selected countries. Figure 2 depicts the trends of breast cancer incidences during this time frame, and it is seen that generally there has been an increase over time (Torre et al., 2016). The figure shows the breast cancer incidence rate per 100,000 in selected countries between 1973 and 2012. The countries studied were organized into regions in Africa, Latin America, Asia, North America and Oceania, Northern and Eastern Europe, and Western and Southern Europe. In predominately all the countries, there is an increase in the breast cancer rate over time. Some exceptions include Israel and the United States which have both begin to show a slight decrease in rate in the more recent years however there still exists a large increase compared to 1973. Since the rate for breast cancer is so large and keeps on increasing over the years, it is important to research and develop new methods to treat it before it gets to the point of death.

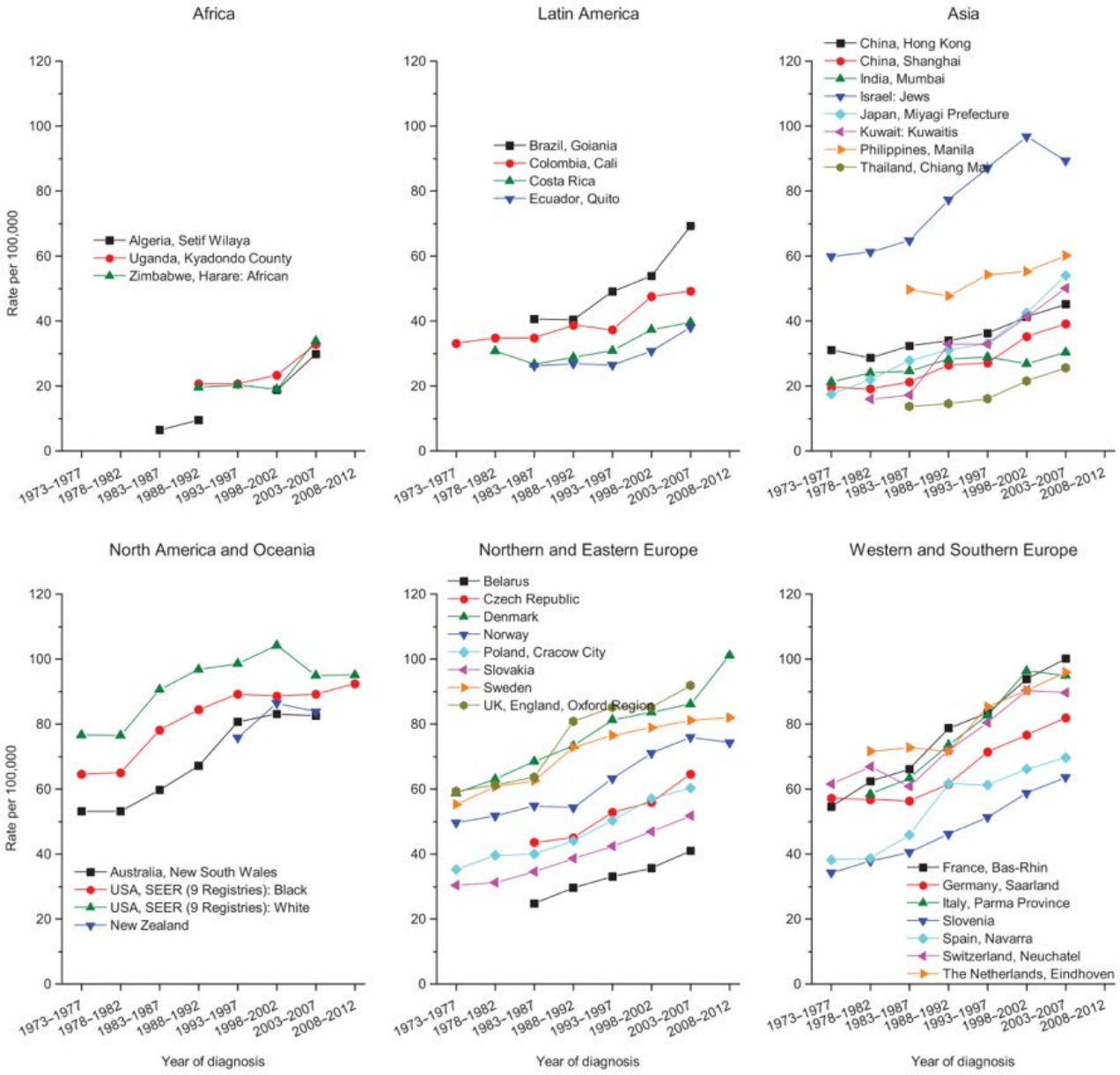


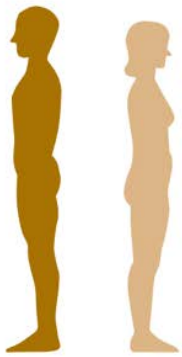
Figure 2. Breast Cancer Incidence Rate per 100,000 in Selected Countries Between 1973 and 2012 (Torre et al., 2016).

Besides the fact that there are so many women impacted by breast cancer, breast cancer is also the leading cause of cancer related deaths in women worldwide (Torre et al., 2016) so this shows that treating it is of even more importance. The American Cancer Society notes that about 1 in every 39 women diagnosed with breast cancer will die from it as the fatality rate is about

2.6% (*Triple-Negative Breast Cancer*, 2023). The mortality rates differ depending on many factors including ethnicity, race, and income. In the United States, the lowest mortality rates for breast cancer are among Korean women while the highest is seem among black women (Hulvat, 2020). In many high-income countries, breast cancer mortality rates have been decreasing since around 1990. This is despite the increase in incidences, which follows the patterns of many other forms of cancer (Torre et al., 2016).

Tremendous progress has been made at slowing down the fatality rates of breast cancer however there is still a long road ahead. Figure 3 shows the estimated number of cancer survivors by site in the United States as of January 1, 2022 (Miller et al., 2022). Among women, there are 4,055,770 breast cancer survivors followed by uterine corpus and thyroid that are both in the 800,000s. Breast cancer is the leading form of cancer survivors in the United States among women meaning there are approximately 4 million women who live in the United States with a history of breast cancer. It should be noted that this information is partially due to the fact that breast cancer is the most common form of cancer found in women in the United States (Torre et al., 2016).

Male	
Prostate	3,523,230
Melanoma of the skin	760,640
Colon & rectum	726,450
Urinary bladder	597,880
Non-Hodgkin lymphoma	451,370
Kidney & renal pelvis	376,280
Oral cavity & pharynx	311,200
Testis	303,040
Leukemia	300,250
Lung & bronchus	287,050
All sites	**8,321,200**



Female	
Breast	4,055,770
Uterine corpus	891,560
Thyroid	823,800
Melanoma of the skin	713,790
Colon & rectum	710,670
Non-Hodgkin lymphoma	394,180
Lung & bronchus	367,570
Uterine cervix	300,240
Ovary	246,940
Kidney & renal pelvis	230,960
All sites	**9,738,900**

Figure 3. Estimated Number of Cancer Survivors by Site in the United States as of January 1, 2022 (Miller et al., 2022).

Many different women are susceptible to breast cancer, and it is disease that impacts all walks of life and therefore everyone will benefit from treatment. Along with many other diseases, breast cancer has both a genetic predisposition and is also affected, but not caused, by environmental factors. About a quarter of the hereditary breast cancer cases are from a mutation in either the BRCA1, BRCA2, PTEN, TP53, CDH1, or STK11 gene. Those with a mutation in this gene have an 80% risk of getting breast cancer in their lifetime (Shiovitz & Korde, 2015). In addition to the genetic level, there are environmental risk factors that cause an increase in the occurrence of breast cancer. According to the US Centers for Disease Control and Prevention, alcohol is a risk factor for developing breast cancer among esophagus, colon, liver, oropharynx, and larynx cancers. In 2014, it was determined that for the women in the United States diagnosed with breast cancer, alcohol as a factor was seen in 16.4% of the cases. Another risk factor is the level of physical activity one engages in as it has also been seen that inactivity causes an increased risk in breast cancer incidences. Furthermore, there is a higher mortality rate for those inactive compared to those active. A study was done on women between the ages of 15 and 39 and it was seen that physical activity, along with high intake of plants and low red meat intake, decreased the risk of developing breast cancer (Hulvat, 2020). In addition to alcohol consumption and inactivity, other risks include long menstrual history, recent use of oral contraceptives, later age at first birth, use of menopausal hormone therapy, excess body weight (specifically seen by postmenopausal cancer), and weight gain after age 18. Breastfeeding, unlike most other factors in life, decreases the risk of developing breast cancer (Torre et al., 2016). These are all among the many contributing factors in developing breast cancer and can affect all different types of women, so it is important for the entire human population to continue looking for more effective treatments.

Types of Breast Cancer

There are many different subtypes within breast cancer and to find the most effective treatment each one must be looked at individually. The different subtypes arise from expression of the different markers in the tumor cells including estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The cells that express the ERα marker are called estrogen receptor positive (ER-positive) and those that do not express the marker are called estrogen receptor negative (ER-negative) (Musheyev et al., 2023). Figure 5 shows a summary of different subtypes within breast cancer and their receptor profiles, prevalence rates, and subcategories. The subtypes include hormone positive which is ER+ and PR+, HER2 positive which is HER2+, and triple negative breast cancer (TNBC) which is ER-, PR-, and HER2-. ER-positive accounts for roughly 70% of the breast cancer cases which is why it has previously been heavily studied (Barzaman et al., 2020). For breast cancer that expresses HER2, existing treatment includes monoclonal antibodies and chemotherapy, usually used in combination (Barzaman et al., 2020). For breast cancer that is ER-positive, existing treatments inhibit ERα function by using endocrine therapies to either block the synthesis of estrogen, promote the degradation of ERα, and to antagonize the binding of estrogen to ERα. Antagonizing the binding of estrogen to ERα is done through the usage of selective estrogen receptor modulators (SERM) and a drug that has been seen to be successful is tamoxifen which will be discussed in more detail below (Musheyev et al., 2023). However, resistance develops, and sometimes ER-positive patients cannot be treated with these targeted therapies. This leads to a need to explore other treatment options for ER-positive, and at the same time a need to explore treatments for ER-negative which these existing drugs do not treat.

Table 1
Breast cancer subtypes category.

Breast cancer subtype	*Receptor profile*	*Subtype prevalence*	*Subcategories*
Hormone positive	ER+ or PR+	60%	Luminal A & B
HER2 positive	HER2+	20%	–
Triple negative breast cancer	ER−, PR− and HER2−	10–20%	Basal-like 1 (BL-1), basal-like 2 (BL-2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem cell-like (MSL), and luminal androgen receptor (LAR)

Figure 5. Subtypes of Breast Cancer and Prevalence (Barzaman et al., 2020).

TNBC is a subset of ER-negative breast cancer which does not have targeted therapies to treat it. As its name explains, it is negative for the three markers: estrogen receptor alpha, progesterone receptor, and HER2. Within TNBC there are six categories; basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem cell-like, and luminal androgen receptor (Barzaman et al., 2020). TNBC makes up approximately 10-15% of all breast cancers and it is found to be more common in women who have the BRCA1 mutation, women who are below the age of 40, and in African American women (Manna et al., 2016). Compared to other forms of breast cancer, TNBC is normally found in younger women when women don't necessarily go for routine mammograms. Therefore, its early stages normally go undetected while for other forms of cancer it would normally be detected early on since older women generally go for routine mammograms. TNBC can affect anyone thus there is a strong need to be able to treat it. Additionally, TNBC tumors are usually more aggressive and there is more metastasis compared to other subtypes of breast cancer. The recurrence free survival is very short, so it likely comes back faster and is more aggressive so therefore harder to treat (Manna et al., 2016). Compared to other forms of breast cancer, the five-year survival rates are not as promising. Based on data collected from 2011 to 2017, patients with localized TNBC have a five-year survival rate of 91% compared to the 95% seen by other forms of breast cancer. For patients with distant TNBC, the five-year survival rate drops down to 12% which means that those with TNBC are twice more likely to die from it within 5 years compared to other forms of

breast cancer (*Cancer Stat Facts: Female Breast Cancer Subtypes*, n.d.). All of this points to a greater need to explore treatment options for those with TNBC as it effects everyone and has poorer prognoses.

There are targeted therapies, such as monoclonal antibodies and traditional chemotherapeutic drugs, that exist for the ER, PR markers, and HER2 protein. However, these therapies are only effective in treating ER-positive breast cancer and not ER-negative since the identifying factors, the markers, that aid in treatment success are lacking. Therefore, right now there are no targeted therapies against TNBC. The only option that exists is chemotherapy, but it is usually not effective and comes with many adverse side effects. It is quite important to find treatments that specifically target TNBC, and tremendous progress has been made with treating it, but it is not completely controlled.

In contrast to familiar forms of treatment such as chemotherapy and radiotherapy, target therapies address the root cause of the disease. This minimizes damage to healthy cells that chemotherapy and radiotherapy are known to cause. Target therapies work in a multitude of ways. They can target the proteins that are found in the tumor cells which allow them to grow, stop the growth of the tumor cells by starving them of the necessary hormones, mark the tumor cells for degradation by the immune system, boost the immune system so it can be more successful at killing the cancer cells, and also stop the signals that form the blood vessels which are necessary for the tumor growth (*Targeted Therapy to Treat Cancer*, 2022). However, just like in non-targeted therapies, the tumor cells eventually will become resistant to the treatment and the treatment will no longer be effective. Therefore, it is helpful to have multiple types of targeted therapies available so that when one type is no longer effective, another type can be used. To be able to create such a therapy for TNBC, it is important to understand the markers

found in TNBC and how it works on a cellular level to know what the target therapies need to target.

Long-Term Effects

With every disease out there, there are long term health effects associated with treatment and even lasting through survival, so it is important to look for treatments that are the least harmful. One of the categories of effects specifically for breast cancer is associated with sexual concerns. Firstly, some of the chemotherapeutic agents that are used can lead to premature menopause since they are gonadotoxic. Some of the hormonal treatments used can create menopausal symptoms including night sweats, hot flashes, and atrophic vaginitis (Miller et al., 2022). Specifically by surgeries such as mastectomies, there are many musculoskeletal changes that can affect daily life. A study was performed by Rangel et al on 94 Portuguese women between the ages of 23 and 72 who had survived breast cancer. They reported that there were many musculoskeletal changes that occurred after recovery. Figure 4 shows the number of women who reported changes following a mastectomy (Rangel et al., 2019). Disorders included shoulder, neck, and arm pain, lymphedema, difficulties in everyday activities, difficulty in raising the arm, and overall decreased quality of life. Outside of sexual and musculoskeletal concerns, there are also concerns regarding other organs of the body. Some forms of treatments can cause cognitive impairment, others cause fatigue, and some can even lead to congestive heart failure (Miller et al., 2022). The study done by Rangel et al showed that women who engaged in physical activity, such as through physiotherapy, post-surgery were less likely to develop many of the possible complications (Rangel et al., 2019). Since there are a lot of negative side effects of previously existing treatments, it is important to continue to look for a more successful treatment that is less invasive and comes with less side effects.

Table 2 Musculoskeletal disorders and perceived quality of life (QOL) reported by participants ($n = 94$)

Musculoskeletal changes and quality of life perception (QOL)	Answers	*n* (%)
Lymphedema	87	29 (33)
Arm pain ipsilateral to surgery	89	43 (48)
Shoulder pain	87	41 (47)
Difficulty in raising the arm	90	31 (34)
Neck pain	90	53 (59)
Difficulties in the activities of daily living	91	33 (36)
Decreased quality of life	91	72 (79)

Figure 4. Musculoskeletal Disorders Associated with Mastectomies (Rangel et al., 2019).

ERRα, MAPK and PI3K-Akt Pathways

TNBC does indeed lack the important markers that are usually targeted, however there are other unique targets that exist in TNBC. This is important as it gives the option of a place to be able to control the cancer. Breast tissue, in addition to the kidneys, skeletal muscle, intestinal tract, and heart, express estrogen-related receptor alpha (ERRα) (Fagerberg et al., 2014). ERRα is an orphan nuclear receptor and takes care of the energy demands of the cell (Manna et al., 2016). Therefore, ERRα provides the metabolic needs and energy of the rapidly dividing tumor cells as they spread. This was proven in a study in mice where ESRRA, the gene that encodes ERRα, was removed. The removal of ESRRA caused a delay in the growth of a tumor showing the importance of ERRα in providing the needs of proliferating cancer cells (Manna et al., 2016). ERRα has been used as a prognostic marker for ovarian, colon, prostate, and breast cancer and therefore is a possible place to consider targeting (F. Wu et al., 2009). Since it is found in cancer cells and is responsible for the rapid growth of the tumors, ERRα is thus a possible place to investigate targeting and treating TNBC.

ERRα is like estrogen receptor alpha (ER$\boldsymbol{\alpha}$) and this connection can aid in determining possible treatments for cancers that expresses ERRα including TNBC. They are both

transcription factors, however, ERR**α** regulates transcription of enzymes involved in glycolysis, citric acid cycle, and lipid, amino- and nucleic acid metabolism and ER**α** regulates expression of genes involved in proliferation. There exists an inverse relationship between the expression of ERR**α** and ER**α**. In cases where there are high levels of ER**α**, such as in ER-positive breast cancer cells, there are low levels of ERR**α**. In cases where there is low expression of ER**α**, such as in TNBC and other ER-negative cancer cells, ERR**α** levels are high (Manna et al., 2016). A very high level of ERR*α* expression has been linked to worse outcomes for breast cancer patients (F. Wu et al., 2009). Previous findings have shown that the downregulation of ERR**α** slows down cell proliferation and tumorigenicity in ER-positive and ER-negative breast cancers (Manna et al., 2016). Since ERR**α** is partly responsible for cell growth and proliferation in ER-negative breast cancers, including TNBC, it is a good place to try targeting when developing treatments for TNBC.

ERR**α** does not act alone. It has been seen that a link exists between ERR**α** and the mitogen-activated protein kinase (MAPK) pathway. This pathway regulates cell differentiation, proliferation, and death in eukaryotic cells including human cells. Since it is involved in cell proliferation and death, it is therefore involved in the continuous proliferation of cancer cells. Thus, the MAPK pathway, along with ERR**α**, are possible locations to investigate in treating cancer, including TNBC. The MAPK pathway consists of three sequentially activated protein kinases, extracellular-signal-regulated kinases (ERKs), Jun amino-terminal kinases (JNKs), and stress-activated protein kinases (p38/SAPKs) (Morrison, 2012). A schema of the complexity of the pathway is seen in Figure 6 (Fang & Richardson, 2005). Extracellular signal proteins such as growth factors bind to the tyrosine receptors on the surface of the cell and through phosphorylation of the kinases the signal reaches the nucleus. Once it has entered the nucleus,

this will affect gene expression and protein function. In cancer cells, the receptors responsible for activating the MAPK pathway are often overexpressed. An overexpression of the receptors leads to more binding of the extracellular signal proteins and hyperactivation of the pathway which leads to transcription factor activation or histone/chromatin modification and causes an increase in cell proliferation. Specifically, in TNBC, there is an overexpression of epidermal growth factor receptor (EGFR) which is seen in higher rates compared to other forms of breast cancer (Dent et al., 2007; Masuda et al., 2012). Targeting the MAPK pathway is therefore a good place to try treating TNBC cells as it is partly responsible for cell proliferation and is seen to be overexpressed in TNBC cells.

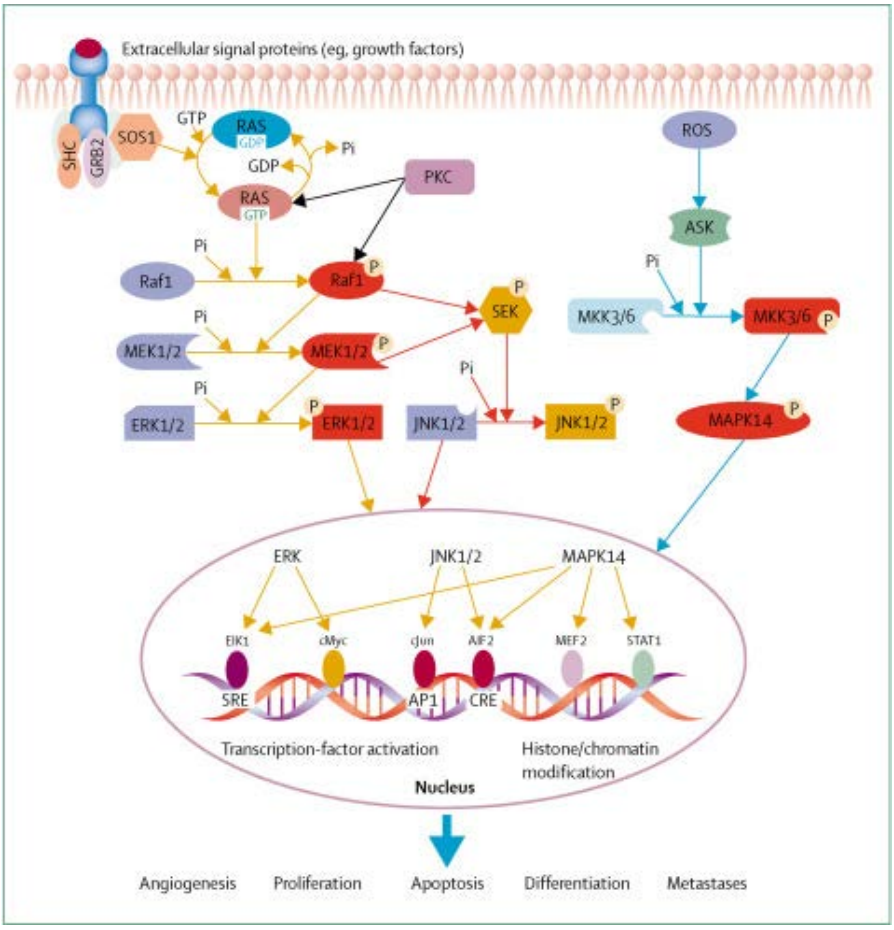


Figure 6. MAPK Pathway (Fang & Richardson, 2005).

Many parts of the MAPK pathway effect cell proliferation and tumor growth and finding the specific proteins involved in the cell growth in TNBC is important in treating it. The specific protein kinase that is important for treating TNBC is the ERKs, subdivided into ERK1/2 and ERK 5, where ERK1/2 is specifically responsive to growth factors and induces cell growth and differentiation (Morrison, 2012). Therefore, high levels of phosphorylated ERK (pERK) indicate that there is cell growth and proliferation. Another way the MAPK pathway is involved in TNBC is through the Bim protein. The MAPK pathway phosphorylates Bim and thus prevents the accumulation of the proapoptotic protein. If left unphosphorylated, the Bim protein would eventually cause apoptosis but in TNBC it does not cause apoptosis and therefore the cells will continuously grow (Mehnert et al., 2011). Both ERK and Bim, parts of the MAPK pathway, are involved in the continuous growth of cancer cells and are good markers to look into when developing treatments for TNBC.

Another pathway that also plays an important role in cancer formation is the PI3K-Akt pathway and is also another marker to consider when treating cancers including TNBC. It is a signaling cascade that is activated by nutrients, hormones, or growth factors and is involved in cell proliferation, survival, and metabolism. A mutation in the Akt protein, or an upregulation of it, is associated with the formation of tumors as it inhibits pathways that are involved in apoptosis and induces the pathways that are involved in cell growth (Madhunapantula et al., 2011). Knowing this information about the role of the PI3K-Akt pathway in tumor cells is another place to consider when trying to develop a treatment to target cancer including TNBC.

Breast Cancer Treatments

Tamoxifen is a drug that in 1977 was approved by the Food and Drug Administration to use in the treatment of women with breast cancer (Ent & Sborne, 1998). It is a selective estrogen receptor modulator meaning an antagonist for ERs in breast tissue, but agonist for ERs in other parts of the body (Manna et al., 2016). It inhibits the binding of estrogen to the estrogen receptors and competitive inhibition thereby makes tamoxifen effective at inhibiting tumor growth in the breast cells. Specifically, the trans isomer of tamoxifen is used (as citrate salt), since trans has a higher affinity for the estrogen receptors compared to the cis isomer. As seen in Figure 7, tamoxifen has a dimethylaminoethoxy side chain which, in addition to being trans, makes it more effective at its antiestrogenic activity. Due to its antiestrogenic activity, it inhibits the expression of estrogen-regulated genes and blocks the G1 phase of the cell cycle. This includes growth factors and angiogenic factors which have been seen to be secreted by tumor cells to stimulate growth. Therefore, there is an overall decrease in cell proliferation so tumors regress (Ent & Sborne, 1998).

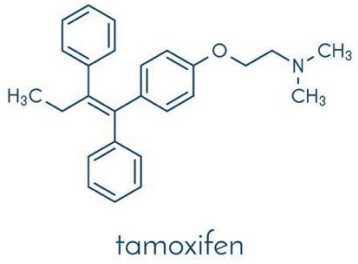


Figure 7. Structure of Tamoxifen.

Tamoxifen has been found to be effective at treating ER-positive breast cancer but generally ineffective at treating ER-negative breast cancer as it is an antagonist for ERs in breast tissue which is found in ER-positive and not ER-negative. It is possible that ERβ may influence

the tamoxifen response in ER-negative breast cancer and cotargeting the PI3K-Akt pathway may also cause ER-negative cells to more sensitive to the effects of tamoxifen (Manna et al., 2016). Interestingly, a study was done in Sweden that has shown that there is a relationship between the effects of tamoxifen on ER-negative breast cancers and ERRα expression. Breast cancer patients treated with tamoxifen who had a higher expression of ERRα in their tumor cells had better results compared to those with low expression of ERRα. Also, those with lower expression of ERRα, the recurrence-free survival time was also decreased meaning low expression of ERRα is not good because the cancer came back faster compared to those with high expression of ERRα. Figure 8 shows the recurrence-free survival in a study of ER-negative cancer cells with high and low expressions of ERRα and treated with tamoxifen. As seen in the left portion of the figure, a higher ERRα expression is associated with a higher recurrence-free survival rate when treated with tamoxifen (Manna et al., 2016). Tamoxifen has been shown to be effective at treating those with high expression of ERRα, so it is an important drug to continue exploring and use as a stepping board to try and treat those with TNBC since patients with TNBC tend to have high expression of ERRα.

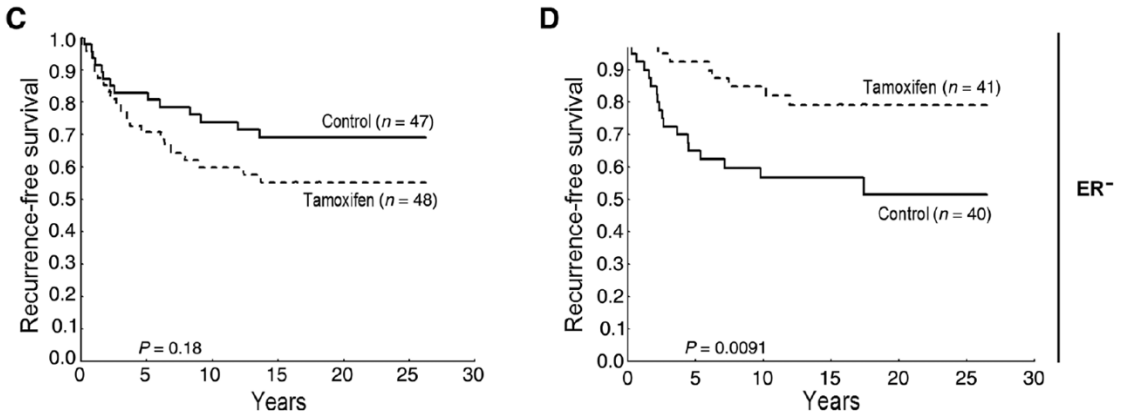


Figure 8. Recurrence-Free Survival in ER-negative Cancer Cells Treated with Tamoxifen (Manna et al., 2016).

XCT-790 is a synthetic chemical that was designed to act as a specific antagonist of ERRα (F. Wu et al., 2009). It is very selective so only targets ERR$\boldsymbol{\alpha}$ and not similar receptors like ERRγ or ER$\boldsymbol{\alpha}$. Previous studies have shown that XCT-790 indeed effects TNBC cells. Specifically, it decreases the G2/M phases of the cell cycle and therefore inhibits cell growth and proliferation and at the same time induces mitochondrial-related apoptosis in the cells. XCT-790 suppresses SOD1/2 expression and thereby increases reactive oxygen species (ROS) generation. ROS generation causes the death of cells further inhibiting the proliferation of the tumor cells. XCT-790 also effects cell growth by effecting signal pathways including AKT/ROS and ERK/ROS positive feedback loops, NF-κB/ROS, and ROS/p38-MAPK (Y.-M. Wu et al., n.d.). All this data combined shows that XCT-790 has potential in treating and slowing down TNBC cell growth.

U0126 is an inhibitor of MEK/ERK (*GPR30-Mediated HMGB1 Upregulation in CAFs Induces Autophagy and Tamoxifen Resistance in ERα-Positive Breast Cancer Cells*, n.d.). U0126 does not have a substantial effect on the activity of other kinases including protein kinase C, Abl, Raf, MEKK, ERK, JNK, MKK-3, MKK-4/SEK, MKK-6, Cdk2, or Cdk4. Since it is very specific, this drug is an important tool for effecting mitogen-activated protein kinase- mediated signal transduction. U0126 inhibits the MAPK cascade, specifically MEK1 and MEK2, which leads to ERK1 and ERK2 activation (Favata et al., 1998). Since U0126 effects the MAPK pathway, there is a possibility that this drug is useful in inhibiting cell growth and differentiation.

These three drugs, tamoxifen, XCT-790, and U0126, should all be experimented with individually and in combination to see their effects on TNBC cells. A link is seen between ERRα expression and the MAPK pathway and each of these drugs have the potential to affect the cell proliferation. Together these drugs have the potential to inhibit tumor growth and have the

possibility of achieving a positive result on TNBC cells. Further research on the effects of tamoxifen, XCT-790, and U0126 on TNBC is required to determine whether these drugs are effective and whether they can be used in vivo in patients diagnosed with TNBC.

Materials and Methods

Cells used in the experiments included MDA-MB-231 cells. All of the cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were grown in a 37°C incubator with humidified 5% CO_2 atmosphere. The cells were treated with 100 nM tamoxifen in ethanol, 10 μM U0126 in DMSO, and 10 μM XCT-790 in DMSO, alone or in combination (Musheyev et al., 2023).

Neutral Red Assay

MDA-MB-231 cells were used for the neutral red assay. Cells were seeded in replicates of 6 in a 96 well plate at a density of 2,500 cells/well and were allowed to attach overnight. The next day, media was changed to the assay media and was supplemented with drugs alone or in combination. Treatment options included treatment with XCT-790, tamoxifen, U0126, XCT-790 with tamoxifen, U0126 with XCT-790, and U0126 with tamoxifen. Cell proliferation was examined 6 days later using the supravital neutral red dye. The media was removed from the well plate and 0.2 ml of 0.04 mg/ml neutral red was added to each well and the plate was incubated at 37°C for 30 minutes. 0.2 ml of solution of 0.5% formalin, 1% CaCl2 (v/v) was added to each well to wash, kill, and fix the cells. 0.2 ml solution of 1% acetic acid, 50% ethanol was added to each well so the neutral red incorporated into the viable cells could be released into the supernatant. The absorbance was recorded at 540 nm using a microtiter plate spectrophotometer (Musheyev et al., 2023).

Scratch Assay

MDA-MB-231 cells were used for the scratch assay. Cells were seeded in 6-well plates in DMEM media and 10% FBS and grown to confluency in monolayer overnight. A wound/scratch was created along the diameter of each well using a 200 µl pipette tip. The cell debris was removed by washing the cells with PBS and then fresh DMEM media supplemented with 1% FBS was added to each cell with the drugs alone or in combination. The Keyence BZX-800 microscope was used to image the cells and their migration, and the data was analyzed and plotted using Excel (Musheyev et al., 2023).

Results

The MDA-MB-231 cells were serum starved for 24 hours and treated with 10 µM XCT-790, 100 nM tamoxifen, or 10 µM U0126, alone or in combination for 48 hours. The treatments supplemented were either XCT-790, tamoxifen, U0126, XCT-790 with tamoxifen, U0126 with XCT-790, or U0126 with tamoxifen. Figure 10 shows the cells under 20x magnification using a BZX-800 microscope from Keyence. By just looking at the image of the cells alone, it seems that some of the drugs do have an effect on the cell density of the TNBC cells. The control did not include any drugs and shows that the cells survived and are pretty confluent. Treatment with tamoxifen alone, the cells seem pretty similar to the control cells indicating that tamoxifen did not have an effect on cell growth and density. As compared to the control, treatment with XCT-790 alone showed a significant decrease in the density of cells. Treatment with U0126 alone, there is also a decrease in the growth of cells compared to the control however not as much as XCT-790. Treatment with XCT-790 with tamoxifen, there is a drastic decrease in cell density as it appears that very few cells are attached. The cells appear unhealthy and the majority of the

cells seen are dead cells. In comparison to the control, treatment with U0126 with tamoxifen has similar amounts of attached cells. Treatment with U0126 with XCT-790, there is also a drastic reduction in attached cells compared to the control and the cells do not appear healthy. Overall, it seems that XCT-790 alone, XCT-790 with tamoxifen, and U0126 with XCT-790 showed the best results in stopping cell growth as it appears that the cells are less confluent.

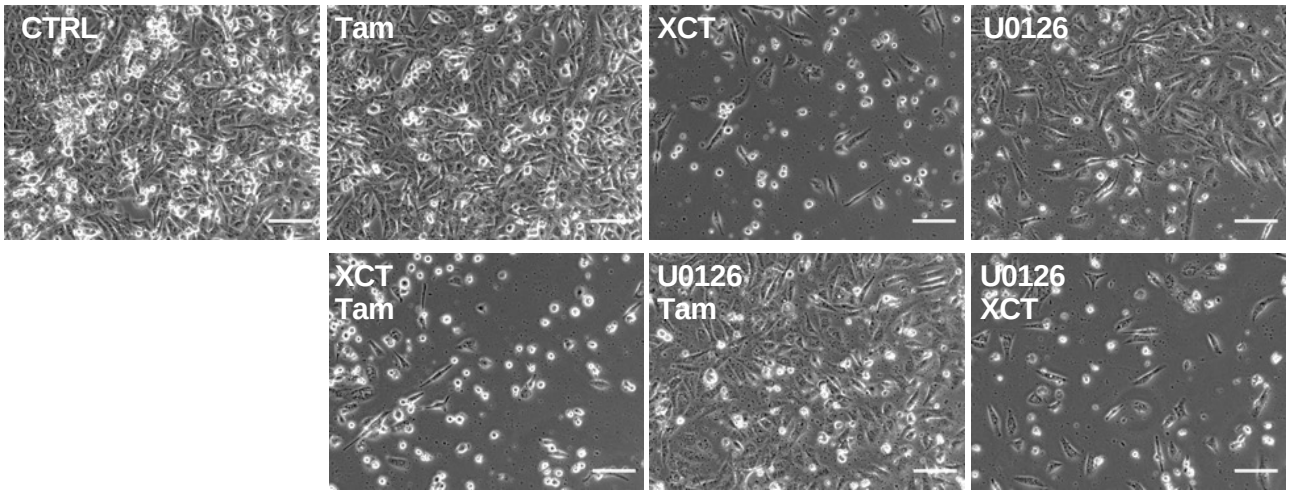


Figure 10. MDA-MB-231 cells were serum starved for 24 hours and treated with 10 μM XCT-790, 100 nM tamoxifen, or 10 μM U0126, alone or in combination for 48 hours. Cells were imaged under 20x magnification using a BZX-800 microscope from Keyence. Scale bar represents 100 μm.

Neutral Red Assay

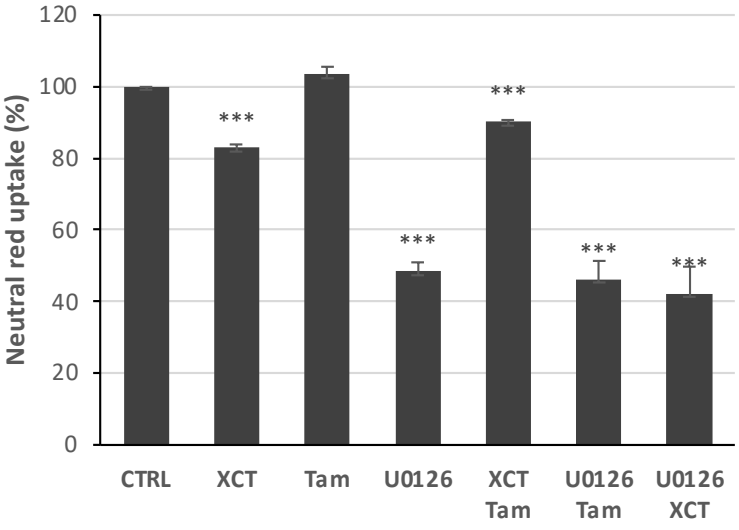
The neutral red assay, as its name sounds, is based on the ability of living cells to bind to the neutral red dye. The dye pentrates the cells through nonionic passive diffusion and once in the cell, the neutral red is collected in the lysosomes (Gilbert & Friedrich, n.d.). By measuring the absorbance spectromotometrically using a plate reader, as seen in Figure 11, the number of alive and dead cells can be determined since the dead cells cannot retian the dye. Therefore, the absorbance of dye is directly proportional to the number of viable cells and the neutral red assay

succesfully quanitifies cell proliferation (Neutral Red Uptake Assay for the Estimation of Cell Viability: Cytotoxicity, n.d.). This assay is useful in determing the effects of drugs on the viability of cells. So, compared to the controls, the lower the neutral red absorbtion indicates that less cell proliferation has occurred and therefore those drugs in that particular combination were effective at stopping the cell growth.

Figure 11. Plate Reader Used to Measure Absorbance.

To quantify cell viabilty, the neutral red assay was performed to see the amount of uptake in the viable cells. The MDA-MB-231 cells were seeded in a 96-well plate in full serum media and then the next day supplemented with either XCT-790, tamoxifen, U0126, XCT-790 with tamoxifen, U0126 with XCT-790, or U0126 with tamoxifen. The neutral red assay was performed 6 days after treatment to see if the change in confluency was due to changes in the cell proliferation. After the neutral red dye was added, the absorbance values obtained from the plate reader were taken and the averages were determined for each drug and combination of drugs. Figure 12 shows a graph and corresponding table of all of the average values of absorbances in comparison to the control, the cells without any treatment. The results further prove that some of the drugs did have an effect on the cell proliferation of the TNBC cells. As compared to the control, untreated cells whose neutral red uptake was 100%, cells treated with XCT-790 had about 80% neutral red uptake which is a 20% reduction in absorbance or cell viability. Treatment

with tamoxifen alone did not show any significant change compared to the control as the uptake was about 103% indicating that it did not effect cell viabilty in this cell line. Related to the control, treatment with U0126 alone had about a 50% uptake of neutral red dye which is about a 50% decrease indicating that U0126 did have an effect on cell viability. Treatment with XCT-790 with tamoxifen had about a 90% uptake showing that there was an effect on cell viabilty however not significantly. As compared to the control, treatment with U0126 with tamoxifen had about a 50% uptake which is a 50% decrease in cell viability. Treatment with U0126 with XCT-790 had about a 40% uptake which is about a 60% decrease compared to the control.



Day 6	AVG
CTRL	100
XCT	82.85063
Tam	103.3686
U0126	48.45085
XCT Tam	90.09522
U0126 Tam	46.20755
U0126 XCT	42.21676

Figure 12. Average Absorbance Values from Neutral Red Assay. MDA-MB-231 cells were seeded in 96-well plate in complete DMEM media, supplemented with 10% FBS and treated with 10 µM XCT-790, 100 nM tamoxifen, or 10 µM U0126, alone or in combination for 6 days and cell viability was measured using Neutral Red Assay as described in "Materials and Methods". *** represents $P<0.001$.

It is seen that treatment by tamoxifen alone did not effect cell proliferation in this cell line. However, XCT-790 and U0126 both showed a decrease in absorbance with about a 20% decrease and a 50% decrease respectively. When used in combination, tamoxifen with XCT-790

caused the absorbance to actually increase when compared to XCT-790 alone. This shows that XCT-790 alone had a bigger effect on cell proliferation then in combination with tamoxifen and therefore tamoxifen hinders the effects of XCT-790, the two ER antagonists do not work well when used together. Tamoxifen with U0126 had a slight decrease in absorbance but results were pretty similar to U0126 without tamoxifen showing that tamoxifen does not effect U0126's effect on cell proliferation for this cell line. The biggest change in percent of neutral red uptake is seen by the use of XCT-790 and U0126 in combination. This shows that the use of U0126 in combination with XCT-790 had the biggest impact on the MDA-MB-231 cells. This is consistent with the image of the cells in Figure 10 where the cells treated with XCT-790 and U0126 in combination have the fewest cells depicted. Altogether, the best results from the neutral red assay were obtained by using a combination of XCT-790 and U0126, or more importantly, targetting ERRα and the MAPK pathway simultaneously are the most effective at stopping cell proliferation in MDA-MB-231 cells.

Scratch Assay

The scratch assay, or wound healing assay, is a useful tool in measuring cell motility and whether the treartments are effective at inhibiting the migration of TNBC cells. Cell migration is determined based on the cells ability to grow in a place where the cells were destroyed. A confluent cell monolayer is destroyed and a cell-free region is generated. The cells can then prolifereate and migrate into the now empty space, or the wound bed. After the cells are allowed to grow, images are taken and using computer software the distance from one side of the scratch to the other side can be measured (Martinotti & Ranzato, 2020). If the distance between the two sides of the wound is very small or nonexistant, that indicates that the cells have had no problem continuing to grow, compared to a distance that is quite large which indicates that the cells were

unable to grow and fill the empty space. So, compared to the controls, the larger the wound indicates that less cell migration has occurred and therefore those drugs in that particular combination were effective at stopping the cell motility.

MDA-MB-231 cells were grown in a 6-well plate and then the next day a scratch was made and the cells were treated with XCT-790, tamoxifen, U0126, XCT-790 with tamoxifen, U0126 with XCT-790, or U0126 with tamoxifen. Figure 13 shows the images of the cells 0 hours and 17 hours after the scratch was made and Figure 14 shows the wound closure percentage of each sample 17 hours after the scratch was made. In Figure 13, the images are for the control and for each drug alone or in combination and the top left of each set is an image taken immediately after the scratch was made. As seen by the control, there is about a 90% wound closure and there no longer is a visible scratch compared to 0 hours indicating that the cells were able to migrate despite the inflicted wound. Treatment with XCT-790 had about a 50% statistically significant wound closure as the wound partially began closing. Treatment with tamoxifen alone showed significant cell migration as there is about a 90% wound closure and the scratch is no longer visible indicating that the cells did not have a problem migrating just like the control. Treatment with U0126 shows that the cells did migrate over the inflicted wound with had about a 60% statistically significant wound closure. Treatment with XCT-790 and tamoxifen also showed that the cells were able to migrate with about a 50% statistically significant wound closure. Treatment with U0126 and tamoxifen showed that the cells were able to slightly grow across the wound with about a 70% wound closure. Treatment with XCT-790 and U0126 had about a 50% statistically significant wound closure.

Based on the quantification of the scratch assay in Figure 14, cells with tamoxifen alone had nearly the same amount of wound closure as the control which means that tamoxifen was

unable to stop cell migration in the TNBC cells and this was also seen in image of the cells Figure 13. Treatment with XCT-790 alone or U0126 alone both had a statistically significant effect on inhibiting cell migration. This is also the case with treatment of XCT-790 in combination with either tamoxifen or U0126. Overall, based on the scratch assay results, treatment with XCT-790 and U0126 had statistically significant inhibition on cell migration whether used alone or in combination with another drug. Again, targetting ERRα, through XCT-790, and the MAPK pathway, through U0126, simultaneously, is the most effective at inhibiting cell motility and migration in MDA-MB-231 cells.

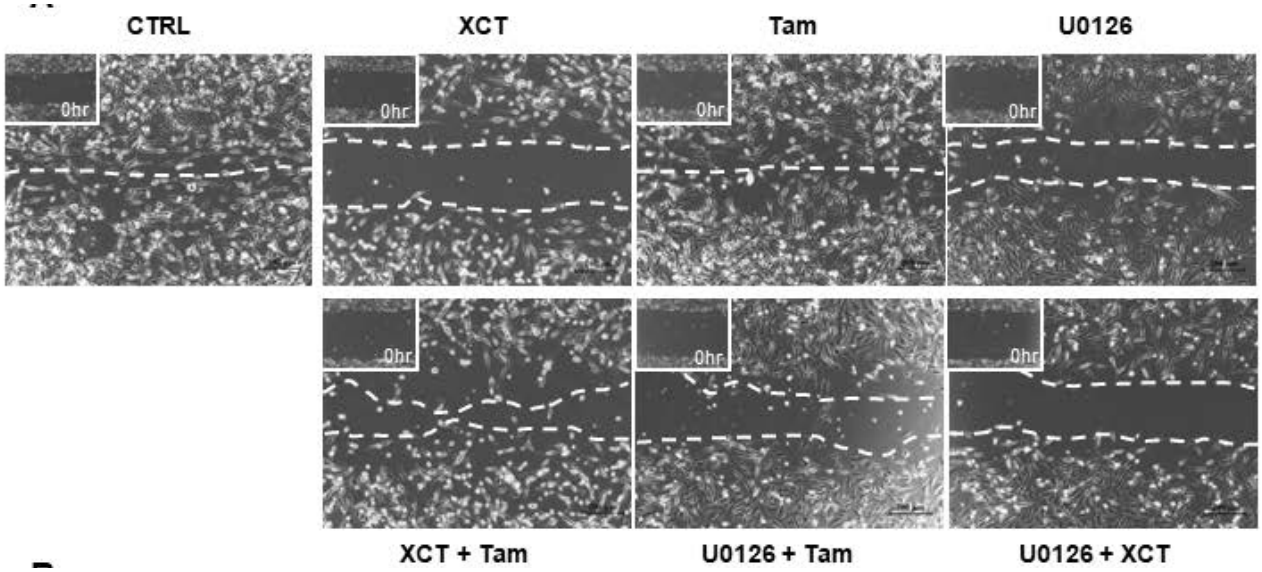


Figure 13. MDA-MB-231 wound closure measured 17 hours post generation. Cells were seeded in a 6-well plate in complete DMEM media and allowed to attach overnight. Next day, cells were placed in reduced (1%) serum media and following 8-10 hours of pretreatment with 10μM XCT-790, 100nM tamoxifen, or 10μM U0126, alone or in combination and wound/scratch was generated.

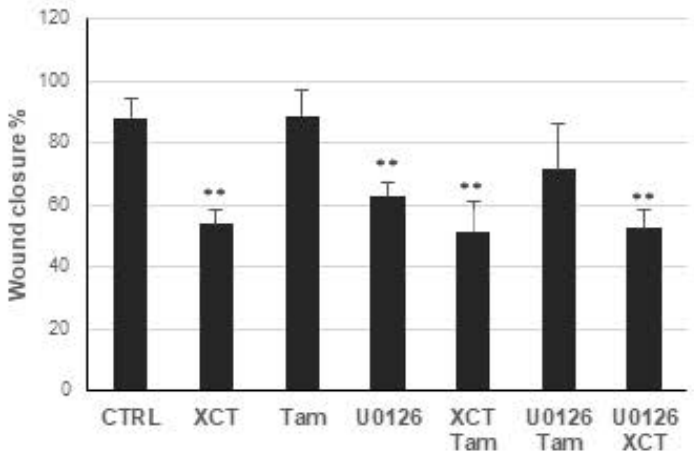


Figure 14. Wound closure perecentage of MDA-MB-231 cells 17 hours after scratch based on Figure 13. Graphed using paired student's t-test with ** representing P<0.01.

Discussion

TNBC is an important form of cancer to study due to it being highly aggressive and having a poorer prognosis. It is the most dangerous subtype of common cancers and makes up approximately 10-15% of all breast cancers. The recurrence free survival is very short, so it likely comes back faster and is more aggressive. Much progress has been made in developing treatments for ER-positive breast cancers however, ER-negative breast cancer is far behind and there are less treatment options available for those suffering with TNBC. There is a great need to develop an effective drug for TNBC as the only current option available is nonspecific chemotherapy, which is notorious for only prolonging the disease and not getting rid of what it is causing it. However, it is more difficult to develop a successful treatment option as the biomarkers normally targeted in other forms of breast cancer are lacking. Therefore, it is important to first investigate other pathways that are involved to possibly be able to target those.

Previous studies have shown that tamoxifen has been shown to be helpful in treating patients with TNBC who have a high ERRα expression (Manna et al., 2016), so these

experiments were done to help investigate that and to investigate signaling pathways that are responsible for ERRα induced tamoxifen sensitivity. The aim of all of it is to identify treatments to target TNBC. Although both the neutral red assay and scratch assay did not show these anticipated successful results regarding tamoxifen, the experiments helped shed light on other ways to help slow down the cell proliferation in TNBC cells.

It was discovered in these experiments that the MAPK signaling pathway is regulated by tamoxifen and by ERRα, and that weakening this signaling pathway is possibly a therapeutic strategy to treat TNBC. Although the mechanism of tamoxifen is not clear, it seems that tamoxifen might behave similarly to ERRα as it does to ERα. Both assays showed that XCT-790 and U0126 slowed down cell proliferation when used alone and even more so when used in combination. XCT-790 is a specific antagonist of ERRα and U0126 is an inhibitor of MEK/ERK or the MAPK pathway. ERRα is known to provide the metabolic needs and energy of the rapidly dividing tumor cells allowing them to spread throughout the body and the MAPK pathway is known to regulate cell proliferation. Altogether, the assays showed that targeting ERRα (by XCT-790) and the MAPK pathway (by U0126) are effective ways to slow down the growth of TNBC cells.

The connection that is seen between ERRα and ERK signaling pathway are important findings as we know that ERRα is expressed in TNBC, and higher expression is usually indicative of a more aggressive form of cancer. Also, the receptors responsible for activating the MAPK pathway are often overexpressed in cancer cells leading to an increase in cell proliferation. Therefore, inhibitors of the MEK/ERK pathway, such as U0126, are good drugs to continue exploring for treating TNBC. This was seen in both assays where XCT-790 and U0126

both indeed slowed the cell proliferation of TNBC cells by slowing down cell growth or inhibiting the migration across the wound.

At this point, there are no clear ways to tackle the cancer progression in TNBC patients as the exact molecular pathways are unknown. This data helped show for the first time that there indeed exists a link between ERRα and ERK signaling pathway. ERRα directly binds to ERK so usage of inhibitors of the MEK/ERK signaling pathway are possibly a worthy treatment for TNBC. This was proven in both assays where it was seen that usage of the MEK1/2 inhibitor, U0126, alone or in combination with XCT-790 induced apoptosis and inhibited the migration of the TNBC cells. These studies helped show that ERRα plays a significant role in the mechanisms of TNBC growth, and that cotargeting ERRα and the MAPK pathway is a practical approach to treating TNBC. These are exciting findings for TNBC patients and there is hope that treating TNBC will no longer be a dream but be a reality.

Looking ahead, more research needs to be done to explore these options and to look into the role of ERRα in both TNBC and healthy cells and the effects on their respective metabolic pathways. It is important to note that there are not yet any ERRα inhibitors that have been approved for use in humans. Additionally, it is crucial to investigate the effects that these treatment options have on non-cancerous cells in TNBC patients. In developing a proper treatment, it is important that it be as specific as possible and only effect the TNBC cells and not the rest of the cells in the patient's body. This can be achieved by doing the same experiments on healthy non-cancerous breast tissue cells in addition to the TNBC cells. The findings in this study are just the beginning in what is hopefully an end to unnecessary TNBC deaths.

Attached to the end please find the paper where my original work was published.

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Inhibition of ERK signaling for treatment of ERRα positive TNBC

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Keywords

TNBC (triple-negative breast cancer), ERRα, MAPK, ERK, Tamoxifen, XCT-790

Abstract

Breast cancer is the second leading cause of cancer-related deaths in women and triple-negative breast cancer (TNBC), in particular, is an aggressive and highly metastatic type of breast cancer that does not respond to established targeted therapies and is associated with poor prognosis and worse survival. Previous studies identified a subgroup of triple-negative breast cancer patients with high

expression of estrogen related receptor alpha (ERRα) that has better prognosis when treated with tamoxifen. We therefore set out to identify common targets of tamoxifen and ERRα in the context of TNBC using phosphoproteomic analysis. In this study, we discovered that phosphorylation of mitogen-activated protein kinase 1 (MAPK1) is regulated by tamoxifen as well as ERRα. Additionally, we showed that inhibition of MAPK signaling together with the use of a selective ERRα inverse agonist, XCT-790, leads to a significant upregulation of apoptosis and paves way for the therapeutic use of MAPK inhibitors for treatment of ERRα expressing TNBC.

Introduction

Breast cancer is one of the most common cancers in women and is the second leading cause of cancer-related deaths in women [1,2]. The rate at which breast cancer is diagnosed is astonishing, with about a quarter of a million women diagnosed with invasive breast cancer in the United States annually and 1 in 8 women diagnosed within their lifetime [3]. Risk factors such as age, body-mass-index, and ethnicity are confounding factors for developing breast cancer [3–6]. Certain ethnic groups are disproportionately affected by breast cancer with a higher mortality despite a lower incidence, indicating that there remains a lot about this disease that we still do not know [3–5].

Further subclassification of breast cancer based on molecular status of the tumor reveals a correlation between prognosis and receptor expression. Tumors are classified based on expression of the following known molecular markers: estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [5,7]. ERα expressing tumors account for 70% of breast cancers [8] and carry a better prognosis than triple negative breast cancer (TNBC), classified as tumors that do not express ERα, PR and no amplification of HER2, which account for 15-20% of breast cancers [5,9–12]. Unlike ERα expressing tumors, TNBCs tend to affect younger, premenopausal women, have a higher mortality rates of 40% within the first 5 years after diagnosis,

and disproportionately affect African Americans [3,5]. A combination of the poor prognosis in TNBC and predominance in African Americans might be driving the racial disparity of breast cancer survival. Therefore, the difference in prognosis and receptor expression between ERα expressing tumors and TNBCs requires diverging therapeutic approaches in the treatment of the two cancers.

Approaches to treating ERα expressing tumors focus on inhibiting ERα function with endocrine therapies by either antagonizing the binding of estrogen to ERα (selective estrogen receptor modulators), promoting ERα degradation (selective estrogen receptor degraders), or by blocking estrogen synthesis (aromatase inhibitors) [13,14]. An example of one of the earlier used selective estrogen receptor modulators (SERM) is tamoxifen. Approved by the FDA in 1977, it was found to be most beneficial in tumors with high expression of ERα and continues to be used therapeutically today [14,15]. Patients respond very well to tamoxifen treatment with 40-50% reduction in both distant and local recurrence with 5 years of treatment [8]. However, new or acquired resistance develops in approximately 30% of cases and tumors can spontaneously convert to hormone-independent proliferation or can lose ERα expression altogether, creating a greater need for understanding molecular mechanisms of hormone receptor negative breast cancer [15–18]. Therefore, further research has focused on identifying alternative molecular pathways responsible for tumor progression as druggable targets, [9,10,12,19] and such research overlaps with the search for targeted treatments for TNBCs.

Since TNBC tumors do not express any known markers, treatment options for TNBC patients are extremely limited and currently no targeted therapies exist for such patients [12]. This leaves chemotherapy as the only treatment option for TNBC [11,12,20–22] with a three-year overall survival of 74% compared to 89% in non-TNBC tumors, indicating that chemotherapy is not very effective [20]. Besides for leading to a significantly lower survival in ERα negative tumors as

compared to ERα expressing tumors, this treatment option burdens patients with the adverse effects of chemo-toxicity such as alopecia, myelosuppression, gastrointestinal disturbances, nephrotoxicity, neurotoxicity, cardiotoxicity, and infertility, calling into question any benefit from chemotherapy at the cost of a reduced quality of life [23]. Current scientific work aims to offer insights into molecular drivers that may be leveraged in the treatment of TNBC by further classifying tumor microenvironment, identifying tumor cellular signatures and mRNA expression profiles [9,10,12,19]. One such molecular driver that is currently explored is estrogen related receptor α (ERRα) [24,25].

ERRα is an orphan nuclear receptor that is part of the superfamily of transcription factors, which include ERRα, ERRβ, ERRγ [26]. ERRα is structurally most similar to ERα and there is an overlap in ERα and ERRα binding to response elements in promoters of genes whose expression they regulate [27]. Despite their homology, ligands, such as estrogen, that bind to ERα do not bind to ERRα, therefore signaling between the two molecules is quite divergent [27]. Though its function in metabolic processes in the muscle heart and liver has been described, its role in tumorigenesis is not fully understood [28–32]. Metabolic functions that are regulated or that are thought to influence ERRα include glycolysis, cholesterol metabolism, fatty acid oxidation, and oxidative metabolism [28,31]. In breast cancer, ERRα expression is mutually exclusive with ERα expression and is corelated with a more aggressive and metastatic disease [27]. Previous studies have shown that although ERRα was a negative predictive marker for progression free survival and disease recurrence, in TNBC-basal-like tumors, when treated with tamoxifen, ERRα expression was associated with a slightly prolonged distal metastasis-free survival, while those treated with chemotherapy alone, had significantly shorter interval to metastasis. Additionally, patients with elevated nuclear expression of ERRα who were treated with tamoxifen had a better prognosis than patients with elevated nuclear ERRα who were not treated with tamoxifen, indicating that there is a therapeutic benefit to treating ERRα-expressing TNBC patients with tamoxifen and that the action of

tamoxifen in TNBCs is ERRα dependent. [25]. These findings indicated that a subgroup of ERα-negative patients respond to tamoxifen and this effect is dependent on ERRα expression. This evidence prompted our current study, whose aim was to investigate the relationship between ERRα and tamoxifen in the context of TNBC. To investigate this relationship, phosphoproteomic analysis was performed in TNBC cells to identify common pathways that are regulated by both tamoxifen as well as XCT-790, an inverse agonist of ERRα [33] and to identify therapeutic targets. Our findings identify MAPK1 (also known as ERK2) as a common target for tamoxifen and ERRα and show that treatment of TNBC cells with a MEK1/2 inhibitor together with XCT-790 leads to activation of apoptosis and inhibition of cell migration and invasion, suggesting that ERK is a potential novel therapeutic target that should be considered for TNBC treatment.

Materials and Methods

Cell culture and treatments

MDA-MB-231, MDA-MB-436, MDA-MB-157, Hs 578T, BT-549 sh-control and sh-ERRα cells were grown in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (R&D, a Bio-Techne Brand) and 1% penicillin–streptomycin (Gibco). Cells were cultured in a 37°C incubator with a humidified 5% CO2 atmosphere. MDA-MB-231, MDA-MB-436, MDA-MB-157, Hs 578T, BT-549 cells were purchased from ATCC. sh-ERRα and sh-control cells were a kind gift from Dr. Marina Holz.

Cells were treated with either 100 nM tamoxifen in ethanol (Millipore), 10 μM U0126 in DMSO (Tocris, a Bio-Techne Brand), or 10 μM XCT-790 in DMSO (Tocris, a Bio-Techne Brand), alone or in combination.

Immunoblotting

Following treatment, cells were lysed in ice cold lysis buffer (RIPA buffer with Triton®X-100, Halt™ Protease & Phosphatase Single-Use Inhibitor Cocktail (100X), ThermoScientific). Insoluble materials were centrifuged out at 14,000 rpm and 4°C for 10 min. Using the Bradford assay (Coomassie Protein Assay Reagent, ThermoScientific) and the Eppendorf BioPhotometer, cell protein concentrations were measured and normalized. 4X LDS Sample Buffer (Invitrogen B0008) and Bolt™ 10x Sample Reducing Agent (Invitrogen) were added to the samples followed by denaturation for 10 minutes at 70°C. Samples were resolved through electrophoresis using NuPage™ 4-12% Bis-Tris Gels (Invitrogen) and then transferred onto a nitrocellulose membrane (ThermoScientific) for staining. Immunoblots were detected using the following primary antibodies: ERRα #13826, Phospho-p44/42 MAPK (ERK1) (Tyr204)/(ERK2) (Tyr187) #5726, Phospho-p90RSK1 (Ser380) #12032, RSK1 #8408, p44/42 MAPK (ERK1/2) #4695, Phospho-MAPK Substrates Motif [PXpTP] MultiMab™ #14378, Phospho-mTOR (Ser2448) #5536, PARP #9532, and β-Actin #4970. All primary antibodies were ordered from Cell Signaling Technology. After staining with primary antibodies, nitrocellulose membranes were treated with IRDye conjugated secondary antibodies (IRDye® 680RD Donkey anti-Mouse IgG Secondary Antibody, IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody, Li-COR), and then imaged using the Odyssey-Clx Li-COR infrared detection instrument. Quantification of immunoblots was performed using Image Studio 5.2 (Li-COR).

Immunoprecipitation assay

Cells were lysed in ice cold IP lysis buffer (Pierce™ IP Lysis Buffer, Halt™ Protease & Phosphatase Single-Use Inhibitor Cocktail (100X), Thermo Scientific). Lysates were subjected to immunoprecipitation with either Sepharose®Bead Conjugated p44/42 MAPK (Erk1/2) antibody

#5736 (Cell Signaling Technology) or Protein A Agarose beads, 50% slurry (EMD Millipore) overnight at 4^0C. Beads were pelleted from solution, washed twice with IP buffer and once with PBS and boiled using Invitrogen's LDS Sample Buffer and Reducing Agent according to manufacturer's instructions.

Phosphoproteomics sample processing and data analysis

Cells were lysed in RIPA lysis buffer, the supernatant was collected following centrifugation at 21000xg for 10 min, and an acetone precipitation was performed overnight at -20°C. The samples were re-suspended in 7 M urea, reduced with 5 mM DTT (dithiothreitol) and alkylated with 15 mM CAA (chloroacetamide). A standard tryptic digest was performed overnight at 37°C. Solid Phase Extraction (SPE) was then performed using C18 Prep Sep™ cartridges (Waters, WAT054960), followed by reconstitution in 0.5% TFA (trifluoroacetic acid). The SPE cartridge was washed with conditioning solution (90% methanol with 0.1% TFA), and equilibrated with 0.1% TFA. The sample was passed (1 drop/sec) through the equilibrated cartridge, then desalted. The sample was then eluted (1 drop/sec) with an elution solution (50% ACN (acetonitrile) with 0.1% TFA. The sample was then TMT labeled according to kit specifications (ThermoFisher Scientific, 90110), with the exception that labeling was performed for 6hrs instead of 1 hr. Following labeling, another SPE was performed, as stated above. Phosphopeptide enrichment using Titansphere Phos-TiO Kit (GL Sciences, 5010-21312) was then performed. Briefly, samples were reconstituted in 100 µL of Buffer B (75% ACN, 1% TFA, 20% lactic acid – solution B in the kit). The tip was conditioned by centrifugation with 100 µL of Buffer A (80% ACN, 1% TFA), followed by conditioning with Buffer B (3000xg, 2min). The samples were then loaded onto the tip and centrifuged twice (1000xg, 5min). The tip was then washed with 50 µL of Buffer B, followed by 2 washes with 50 µL of Buffer A (1000xg, 2min). The samples were eluted with 100 µL of elution 1 (20% ACN, 5% NH4OH) then 100 µL of elution 2

(20% ACN, 10% NH4OH) (1000xg, 5min). A final clean-up step was performed using C18 Spin Columns (Pierce, 89870).

Mass spectrometry, data filtering, and bioinformatics

Mass spectrometry analysis was carried out as follows: to separate peptides, reverse-phase nano-HPLC was performed by a nanoACQUITY UPLC system (Waters Corporation). Peptides were trapped on a 2 cm column (Pepmap 100, 3 μM particle size, 100 Å pore size), and separated on a 25cm EASYspray analytical column (75 μM ID, 2.0 μm C18 particle size, 100 Å pore size) at 45ºC. The mobile phases were 0.1% formic acid in water (Buffer A) and 0.1% formic acid in acetonitrile (Buffer B). A 180-minute gradient of 2-30% buffer B was used with a flow rate of 300 nl/min. Mass spectral analysis was performed by an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific). The ion source was operated at 2.4kV and the ion transfer tube was set to 275oC. Full MS scans (350-2000 m/z) were analyzed in the Orbitrap at a resolution of 120,000 and 4e5 AGC target. The MS2 spectra were collected using a 0.7 m/z isolation width and analyzed by the linear ion trap using 1e4 AGC target after HCD fragmentation at 30% collision energy with 50ms maximum injection time. The MS3 scans (100-500 m/z) were acquired in the Orbitrap at 50,000 resolution, with a 1e5 AGC, 2 m/z MS2 isolation window, at 105ms maximum injection time after HCD fragmentation with a normalized energy of 65%. Precursor ions were selected in 400-2000 m/z mass range with mass exclusion width of 5 – 18 m/z. Polysiloxane 371.10124 m/z was used as the lock mass.

The raw mass spectrometry data was searched with MaxQuant (1.6.6.0). Search parameters were as follows: specific tryptic digestion, up to 2 missed cleavages, a static carbamidomethyl cysteine modification, variable protein N-term acetylation, and variable phospho(STY) as well as methionine

oxidation using the human UniProtKB/Swiss-Prot sequence database (Downloaded Feb 1, 2017). MaxQuant data was deposited to PRIDE/Proteome Xchange

Fractionation

Following treatment, MDA-MB-231 cells were harvested, and using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoScientific), nuclear fractionation was performed according to manufacturer's instructions.

Cell proliferation assays

Cells were seeded in replicates of 6, at a density of 2,500 cells/well in 96 well plates and allowed to attach overnight. Next day, media were changed to assay media, supplemented with or without agents as indicated. Cell proliferation was assayed after 6 days using the supravital dye neutral red (NR) incorporation. The medium was removed, 0.2 ml of medium containing 0.04 mg/ml NR was added per well, and incubation was continued for 30 min at 37 °C. Cells were then rapidly washed and fixed with a 0.2-ml solution of 0.5% formalin, 1% $CaCl_2$ (v/v), and the NR incorporated into the viable cells was released into the supernatant with a 0.2-ml solution of 1% acetic acid, 50% ethanol. Absorbance was recorded at 540 nm with a microtiter plate spectrophotometer. Experiments were performed a minimum of three times. Cytotoxicity graphic data were presented as the mean percentages of control ± standard deviation (STDEV).

Wound healing assay

Cells were seeded in 6-well plates in complete DMEM media, supplemented with 10% FBS and grown to confluency in monolayer overnight. Wound/scratch was created along the diameter of each well using a 200µl pipette tip. Cell debris were removed by washing once with PBS, followed by

addition of fresh DMEM media supplemented with 1% FBS, with or without agents as indicated. Cell imaging and migration were measured using Keyence BZX-800 microscope. Data was analyzed and plotted using Excel. Experiments were performed a minimum of three times.

Boyden chamber assay

Cells were cultured in media without serum and treated with either XCT-790, tamoxifen and/or U0126, as indicated. Nineteen hours later, 1×10^5 cells per well were plated on tissue culture inserts with 8.0-μm pores. The inserts were incubated with serum-free media containing 10 μM XCT-790, 100 nM tamoxifen or 10 μM U0126, alone or in combination. Complete media were added to the lower chamber and cells allowed to migrate for 15 hours. After 15 hours, cells remaining on the upper side of the membrane were scraped off, and the cells that had migrated to the lower side of the membrane were fixed in 4% paraformaldehyde. The insert membranes were removed, stained, and mounted on coverslips using DAPI Fluoromount. Images were collected at 10× magnification using Keyence BZX-800 microscope. Nuclei were counted manually, and data were analyzed using two-tailed Student's *t* test and plotted using Excel. Experiments were performed a minimum of three times.

Microscopy

Cells were imaged under phase contrast lens using the Keyence BZX-800 microscope with a 20x objective.

Statistical analysis

All experiments were repeated at least thrice. Data was analyzed using Excel and significance of data was determined using t-tests, where * represents P values <0.05, ** represents P values <0.01, and *** represents P values <0.001.

Results

To identify common targets that are regulated by both tamoxifen as well as ERRα inverse agonist XCT-790 in TNBC, phosphoproteomic analysis was performed on MDA-MB-231 cells (Figure 1A, Supplementary Figure 1 and Supplemental Table 1). Bioinformatic analysis identified 307 unique targets with statistically significant phosphorylation (P<0.05) changes (Figure 1B and Supplemental Table 2). Further analysis of the data identified 19 unique phosphosites that were changed in both tamoxifen and XCT-790 treated cells (Table 1). A specific direct target which was of particular interest to us was MAPK1, whose phosphorylation was upregulated on tyrosine 187 (also known as p-ERK Y204/187) upon treatment with tamoxifen as well as with XCT-790. MAPK1 is a member of the Ras/Raf/MEK/ERK signaling pathway that regulates cell cycle progression as well as apoptosis and warrants further study as an important player in TNBC progression.

To validate phosphoproteomic findings, MDA-MB-231 cells were grown in serum-free media for 24 hrs and treated with 100 nM tamoxifen and 10 μM XCT-790, alone or in combination as indicated and probed with an antibody that recognizes proteins that are phosphorylated at the threonine within the PXpTP motif, also known as the phospho-MAPK substrate motif (Figure 2). As compared to the untreated control, phosphorylation changes in phospho-MAPK substrate motif [PXpTP] are readily seen and are indicated by asterisks. In tamoxifen or XCT-790 treated samples, phospho-MAPK substrate changes are seen by 15-30 min post treatment (Figure 2A) and in samples treated with

combination of tamoxifen and XCT-790, phospho-MAPK substrate changes are pronounced as early as 5 min post treatment (Figure 2B). Counterstaining of the membrane with an antibody specific for the phosphorylation of MAPK1 on tyrosine 187 (p-ERK Y204/187) showed upregulation in phosphorylation upon treatment with XCT-790 and tamoxifen, alone or in combination. Therefore, western blot analysis validated the phosphoproteomic findings and further showed that XCT-790 and tamoxifen treatments regulate MAPK signaling pathway globally in TNBC. To further validate phosphoproteomic findings and explore the relationship between ERRα, tamoxifen, and ERK in the context of TNBC, MDA-MB-231 cells were grown in serum-free media for 24 hrs and treated with tamoxifen and XCT-790, alone or in combination over a time course of 5, 15, 30 and 60 min (Figure 3). Immunoblotting verified that ERRα inhibition by XCT-790 caused statistically significant upregulation of p-ERK Y204/187 as early as 5, 15 and 30 min post treatment (Figure 3A and B). Likewise, tamoxifen treatment caused statistically significant upregulation of p-ERK Y204/187 as early as 5, 15 and 30 min post treatment and similar results were observed in the combination of XCT-790 and tamoxifen treated samples (Figure 3D and E). Importantly, statistically significant upregulation of p-RSK1 S380, a direct downstream target of ERK, was observed in XCT-790 and tamoxifen treated samples, alone or in combination (Figure 3A and C). This finding validates phosphoproteomic's findings and indicates that MEK/ERK/RSK signaling pathway is regulated by ERRα and tamoxifen in TNBC. Additionally, this effect is specific to the MAPK signaling pathway, as XCT-790 or tamoxifen treatment had no impact on activation of mTOR on S2448 (Figure 3A and D). Mammalian target of rapamycin (mTOR) is a parallel signaling pathway that is activated by growth factors and is often hyperactivated in cancer, including breast cancer [34]. These results indicate that ERRα and tamoxifen regulate p-ERK and that this effect is direct and specific to the MAPK signaling pathway.

In an effort to define the relationship between ERRα levels and tamoxifen response, as well as to explore the role of ERRα in the phosphorylation of ERK, MDA-MB-231 cells with stable knockdown of ERRα (shERRα), were grown in serum-free media for 24 hrs and treated with tamoxifen for 5, 15, 30 and 60 min (Figure 4). As compared to control, MDA-MB-231 cells with reduced ERRα expression showed 2-fold upregulation of phosphorylation of p-ERK on Y204/187 (Figure 4A and B). This validates our previous findings and indicates that the effect of XCT-790 on ERK phosphorylation is due to its inhibition of ERRα. Furthermore, treatment of either MDA-MB-231 cells or cells with reduced expression of ERRα, showed that there is an inverse relationship between ERRα expression and phosphorylation of ERK on Y204/187, such that high ERRα expression in MDA-MB-231 cells is correlated with low p-ERK Y204/187 expression and reduced ERRα expression in sh-ERRα cells is correlated with increased phosphorylation of p-ERK on Y204/187 (Figure 4 A and C). This finding further validates phosphoproteomic data and shows that inhibition of ERRα by XCT-790 as well as use of tamoxifen activate ERK signaling, and this has tremendously important implications for TNBC patients as we have identified a signaling pathway and a specific molecular target whose inhibition can be used therapeutically.

Since ERK is known to have both cytoplasmic as well nuclear functions, it is important to determine subcellular localization of p-ERK and its function. MDA-MB-231 cells were grown in serum-free media for 24 hrs, treated with tamoxifen and XCT-790, alone or in combination for 30 min, followed by preparation of nuclear and cytoplasmic fractions. As seen in Figure 4D, tamoxifen and XCT-790 treatment, alone or in combination, upregulated phosphorylation of ERK on Y204/187 specifically in the cytoplasm, and no p-ERK expression was detected in the nuclear fraction at this time point. Consistent with ERK's cytoplasmic function, its downstream target RSK1 was also phosphorylated on S380 upon tamoxifen as well as XCT-790 treatment, alone or in combination, and this was observed only in the cytoplasmic fraction and not in the nuclear fraction. The presence of histone H3

mainly in the nuclear fraction and presence of actin mainly in the cytoplasmic fraction served as technical control (Figure 4D).

Since XCT-790 and tamoxifen treatments upregulate phosphorylation of ERK, this paves the way for investigating the use of MAPK inhibitors as a therapeutic strategy for TNBC patients. To that end, we investigated the effectiveness of U0126, a MEK1/2 inhibitor, in the context of TNBC cells. MDA-MB 231 cells were grown in serum free media and treated with 10 μM XCT-790, 100 nM tamoxifen and 10 μM U0126, alone or in combination (Figure 5). Following 48 hrs of treatment, cells were imaged with Keyence BZX-800 microscope under phase contrast (Figure 5A). Tamoxifen treatment alone did not have an effect on cell growth and density, however U0126 treatment and to a greater extent XCT-790 treatment showed a significant reduction in cell growth and density. Additionally, combination of tamoxifen together with XCT-790 as well as U0126 together with XCT-790 had a drastic and pronounced effect on cell density with very few cells attached. The attached cells were not healthy and appeared spindle-like or rounded up and majority of cells were floating dead cells. To quantify cell viability, relative uptake of neutral red dye by the lysosomes of live cells was measured. MDA-MB-231 cells were seeded in a 96-well plate in full serum media, treated with 10 μM XCT-790, 100 nM tamoxifen and 10 μM U0126, alone or in combination and neutral red assay was performed 6 days post treatment (Figure 5B). Consistent with previous observation, cells treated with XCT-790 had approximately 20% reduction in cell viability, while treatment of cells with U0126 alone or in combination with tamoxifen or XCT-790 had greater than 50% reduction in cell viability.

To further investigate whether the effect on cell viability is due to cell death, rather than inhibition of cell growth, expression level of cleaved-PARP fragment, a marker of apoptotic cell death was investigated (Figure 5C and D). MDA-MB-231 cells were grown in serum free media for 24 hrs and

treated with 10 μM XCT-790, 100 nM tamoxifen and 10 μM U0126, alone or in combination for 24 hrs. As expected, U0126 treatment blocked activation of ERK signaling as seen via reduction of p-ERK Y204/187 and XCT-790 treatment reduced ERRα protein levels. Additionally, p-ERK Y204/187 and p-RSK1 S380 levels were upregulated upon treatment with XCT-790 and tamoxifen, validating previous data (Figure 5C). Most importantly, we observed statistically significant upregulation in apoptosis as measured by quantification of cleaved-PARP fragment (Figure 5D), indicated by the arrow (Figure 5C). The most notable increase in cleaved PARP levels was observed in samples treated with a combination of U0126 and XCT-790, which exhibited the greatest level of cleaved PARP when compared to cells treated with either U0126 or XCT-790, alone.This finding is very exciting as it indicates that the use of MEK inhibitors together with inhibition of ERRα is effective at inducing apoptosis in TNBC cells and is a first indication of designing a targeted therapy for treatment of TNBC.

We subsequently wanted to examine whether such therapy is effective at inhibition migration and invasion of TNBC cells. To test the effect on cell motility, wound-healing assay was performed and quantified using MDA-MB-231 cells (Figure 6A and C). Though tamoxifen treatment alone did not have an effect on cell migration, treatment with either XCT-790 or U0126 had a statistically significant effect on inhibiting cell migration and this effect was also seen in combination therapy of XCT-790 with either tamoxifen or U0126. To test the effect on cell invasion, trans-well migration assay was performed and quantified using MDA-MB-231 cells (Figure 6B and D). Similar trend was observed in the trans-well migration assay as in the wound-healing assay. In particular, both XCT-790 and U0126 treatments had statistically significant inhibition on cell migration and this effect was maintained in all three combinations of treatments.

To verify that such therapy is beneficial to treatment of TNBC and is not unique to MDA-MB-231 cells, expression levels of ERRα and active signaling of MEK/ERK/RSK pathway were investigated in a panel of TNBC cells (Figure 7A). As expected, all of the tested TNBC cells express ERRα, but they also show active MEK/ERK/RSK signaling as indicated by phosphorylation of ERK on Y204/187 and RSK on S380. To identify the mechanism of XCT-790 action on ERK signaling, we wanted to see whether ERRα and ERK interact using co-immunoprecipitation assay using whole cell lysates. We confirmed that ERK and ERRα interact using MDA-MB 231 (Figure 7B) as well as high-ERRα expressing MDA-MB 436 (Figure 7C) cell lines.

Discussion

Despite significant strides that have been made in the development of treatments for hormone receptor positive breast cancer, progress in the treatment of TNBC has lagged behind, leaving patients with few treatment options. Results from previous studies identified a subset of patients with TNBC who have high ERRα expression and respond to tamoxifen treatment [25]. This finding prompted us to further investigate the role of ERRα in conferring tamoxifen sensitivity in TNBC tumors and to discover signaling pathways responsible for ERRα induced tamoxifen sensitivity, with the aim of identifying specific druggable target for the treatment of TNBC. In our study, we discovered that the MAPK signaling pathway is regulated by tamoxifen as well as ERRα and that attenuation of this signaling pathway may be a promising therapeutic strategy for the treatment of TNBC. Though the mechanism of tamoxifen action in TNBC cells is not clear, since ERRα and ERα can regulate a subset of common target genes, specifically ones with high relevance to breast tumor biology [35], it is possible that tamoxifen might have a similar mechanism of action on ERRα as it does on ERα. Of the targets we identified through phosphoproteomic analysis, MAPK1

phosphorylation at Y204/187 was particularly interesting as the MAPK pathway has been implicated in many processes associated with cancer progression including, tumor proliferation, invasion, metastasis, migration, and apoptosis [36,37]. MAPK1 is part of a kinase signaling cascade that begins extracellularly with the EGFR receptor and cascades through a series of kinases including Ras, Raf, MEK, and ERK 1/2 consecutively [38]. ERK 2 is also known as MAPK1 and is a serine threonine kinase [39]. The identification of ERK as a target of ERRα and tamoxifen identifies an important target whose modulation should be further explored clinically as a therapy in TNBC.

Further validation of the phosphoproteomic result with immunoblotting confirmed that XCT-790 as well as tamoxifen treatment led to an increase in ERK phosphorylation on Y204/187 as well as phosphorylation of the downstream kinase cascade. Our results show that ERRa directly binds to MAPK1, and through this interaction leads to modulation of MAPK1 phosphorylation on Y204/187 as well as downstream signaling. Since MAPK activation is known to support cellular survival, we hypothesized that this activation might be a compensatory cellular signal in response to treatment. Such compensatory responses have been previously described and have been implicated in the development of drug resistance [40]. Crosstalk from other pathways such as PI3K/Akt/mTOR or activation feedback loops within the Raf/MEK/ERK signaling pathway have been previously implicated in pro-survival compensatory mechanisms and drug resistance, thus encouraging further research into the use of drug combinations that act on multiple targets and disrupt the molecular compensatory mechanisms [37,41,42]. Though activation of Raf/MEK/ERK signaling is associated with tumor progression, it also identified an ERRα dependent target whose inhibition can be explored clinically for treatment of TNBC tumors.

Strategies to curb tumor progression through the inhibition of the MAPK pathway have been previously described and are currently used in the treatment of colon cancer and melanoma [43–53].

In breast cancer, preclinical studies described reduced tumor volume in xenograft models, induced cell cycle arrest, and increased apoptosis with the attenuation of the MAPK pathway [54,55]. Though these results have yet to be validated in clinical trials, inhibition of EGFR in breast cancer was tested clinically due to evidence of EGFR overexpression in nearly half of TNBCs [43,44]. Results from those trials were limited showing benefit only in certain subgroups of patients and clinical trials exploring the use of MEK inhibitors in breast cancers are currently underway. In our work, we showed that a combination of ERRα inverse agonist and MEK inhibition leads to statistically significant reduction in cell proliferation, upregulation of apoptosis as well as inhibition of cell migration and invasion.

TNBC is a highly aggressive and metastatic form of breast cancer with a poor prognosis and poor patient outcome. To date, we do not have a clear understanding of molecular pathways that modulate cancer progression and therefore no targeted treatment therapies exist for TNBC patients. What is exciting and important is that we, for the first time, present data that show a direct link between ERRα and ERK signaling pathway. ERRα is a transcription factor that is highly expressed in TNBC and is associated with a more aggressive cancer and a poor outcome. We have shown that ERRα directly binds to ERK and modulates its phosphorylation on Y204/187 as well as downstream pathway activation, indicating that inhibitors of the MEK/ERK signaling pathway should be considered therapeutically for treatment of TNBC. As a proof of this concept, we showed that the use of MEK1/2 inhibitor alone or in combination with XCT-790 induces apoptosis as well as inhibits migration and invasion in TNBC cells. This is a highly exciting finding for TNBC patients and indicates that further investigation into clinical use of MEK inhibitors should be done for treatment of TNBC.

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Figure legends

Figure 1: Phosphoproteomic analysis of XCT-790 and tamoxifen treated MDA-MB-231 cells.

(A) Steps of the work flow chart of samples treated for the phosphroproteomic analysis. (B) Outcome of number of identified unique phosphorylated proteins in each group.

Table 1: Significant phosphoproteomic changes inXCT-790 as well as tamoxifen treated samples. A table of statistically significant hits from phosphoproteomic analysis of cells treated with XCT-790 as well as tamoxifen.

Figure 2: XCT-790 and tamoxifen treatments regulate signaling of MAPK pathway. (A) MDA-MB-231 cells were grown in starvation media for 24 hrs and treated with either 10 μM XCT-790 or 100 nM tamoxifen for 5, 15, 30 or 60 minutes. Cells were lysed as described in "*Materials and Methods"* and indicated proteins were detected by immunoblot. (B) MDA-MB-231 cells were grown in starvation media for 24 hrs and treated with 10 μM XCT-790 and 100 nM tamoxifen for 5, 15, 30 or 60 minutes. Cells were lysed as described in "*Materials and Methods"* and indicated proteins were detected by immunoblot. * indicates phosphorylation changes in [PXpTP] motif as compared to control. 'C' is untreated control.

Figure 3: XCT-790 and tamoxifen treatments upregulate phosphorylation of p-ERK on Y204/187. (A) MDA-MB-231 cells were grown in starvation media for 24 hrs and treated with either 10 μM XCT-790 or 100 nM tamoxifen for 5, 15, 30 or 60 minutes. Cells were lysed as described in "*Materials and Methods"* and indicated proteins were detected by immunoblot. (B) Quantification of p-ERK Y204/187 protein levels normalized to total ERK signal from *'A'*. (C) Quantification of p-RSK1 S380 protein levels normalized to actin signal from *'A'*. (D) MDA-MB-231 cells were grown in starvation media for 24 hrs and treated with 10 μM XCT-790 and 100nM tamoxifen for 5, 15, 30 or 60 minutes. Cells were lysed as described in "*Materials and Methods"* and indicated proteins were detected by immunoblot. (E) Quantification of p-ERK Y204/187 protein levels normalized to actin signal from *'D'*. (F) Quantification of p-RSK1 S380 protein levels normalized to actin signal from *'D'*. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$.

Figure 4: Tamoxifen potentiates phosphorylation of ERK on Y204/187 in the absence of ERRα. (A) sh Control and sh ERRα cells were treated with 100 nM of tamoxifen for 5, 15, 30, or 60

591 minutes. Cells were lysed as described in *"Materials and Methods"* and the indicated proteins were
592 detected by immunoblot. (B) Relative levels of p-ERK Y204/187 normalized to actin, ** represents
593 $P<0.01$. (C) Relative levels of p-ERK Y204/187 and ERRα, normalized to actin from *'A'*. (D) MDA-
594 MB 231 cells were treated with 10 μM XCT-790 and/or 100 nM tamoxifen for 30 minutes followed
595 by lysis with NE-PER Nuclear Cytoplasmic extraction kit as described in *'Materials and Methods'*
596 and lysates from nuclear and cytoplasmic extractions were immunoblotted with the indicated
597 antibodies.

598

599 **Figure 5: Inhibition of ERRα potentiates apoptotic effects of U0126 in TNBC cells.** (A) MDA-
600 MB-231 cells were serum starved for 24 hrs and treated with 10 μM XCT-790, 100 nM tamoxifen, or
601 10 μM U0126, alone or in combination for 48 hrs. Cells were imaged under 20x magnification using
602 BZX-800 microscope from Keyence. Scale bar represents 100 μm. (B) MDA-MB-231 cells were
603 seeded in 96-well plate in complete DMEM media, supplemented with 10% FBS and treated with 10
604 μM XCT-790, 100 nM tamoxifen, or 10 μM U0126, alone or in combination for 6 days and cell
605 viability was measured using Neutral Red cytotoxicity assay as described in *"Materials and*
606 *Methods*". (C) MDA-MB-231 cells were serum starved for 24 hrs and treated with 10 μM XCT-790,
607 100 nM tamoxifen, or 10 μM U0126, alone or in combination for 24 hrs as indicated. Cells were
608 lysed as described in *"Materials and Methods"* and immunoblotted for indicated proteins. Arrow
609 indicates cleaved PARP fragment. (D) Quantification of cleaved PARP fragment from "*B"*
610 normalized to actin. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$.

611

612 **Figure 6: Inhibition of ERRα together with U0126 prevents cell migration and invasion in**
613 **TNBC cells.** (A) MDA-MB-231 cells were seeded in a 6-well plate in complete DMEM media and

allowed to attach overnight. The following day cells were placed in reduced (1%) serum media and following 8-10 hrs of pretreatment with 10rμM XCT-790, 100nM tamoxifen, or 10rμM U0126, alone or in combination and wound/scratch was generated. Wound closure was measured 17 hrs post generation. (B) Cell migration/Boyden chamber assay was performed as described in *"Materials and Methods".* Representative images of MDA-MB-231 cells stained with 4′,6-diamidino-2-phenylindole (DAPI) following 15 hrs migration assay are shown. (C) Quantification of the Wound Healing assay from *(A)* was performed and graphed using paired Student's t-test. * represents P<0.05. ** represents P<0.01. (D) Histogram representing the number of cells migrated relative to untreated control. * represents *P*<0.05, ** represents *P*<0.01 and *** represents *P*<0.001.

Figure 7: ERRα directly binds to ERK. (A) The represented TNBC cell lines were lysed as described in *"Materials and Methods"* and immunoblotted for the indicated proteins. (B) MDA-MB 231 cells were lysed and immunoprecipitated as described in *"Materials and Methods"* and proteins were detected by immunoblot. (C) MDA-MB 436 cells were lysed and immunoprecipitated as described in *"Materials and Methods"* and proteins were detected by immunoblot.

Supporting information

Supplemental figure 1: Principal component analysis (PCA). Principal component analysis of protein abundance of MDA-MB 231 cells treated with tamoxifen, XCT-790 or tamoxifen plus XCT-790.

Conflict of Interest

Authors declare no conflict of interest.

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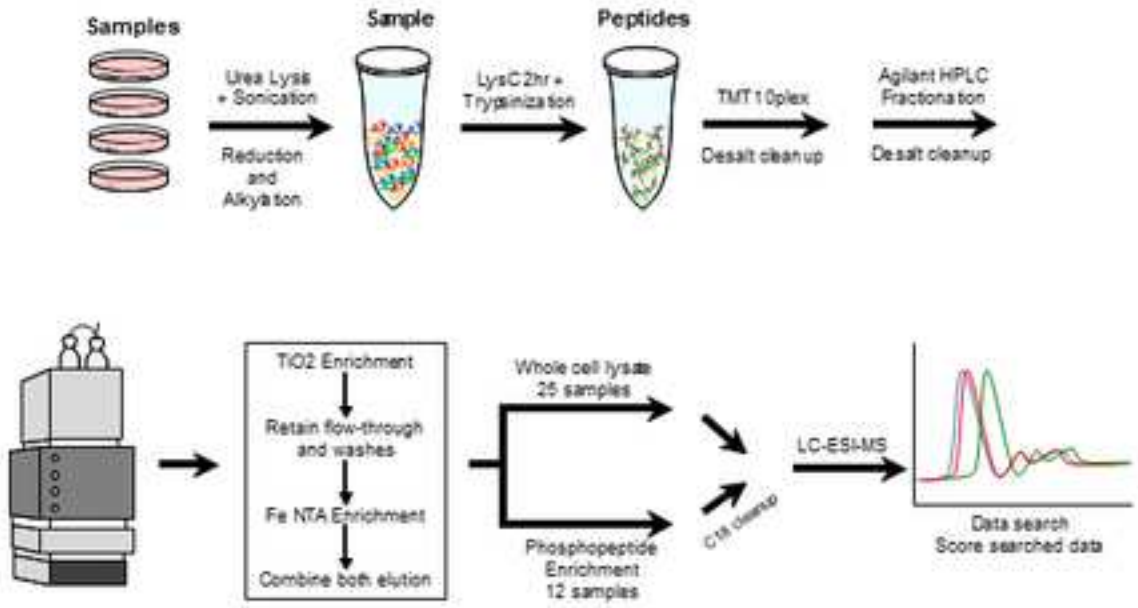
Figure 1

A



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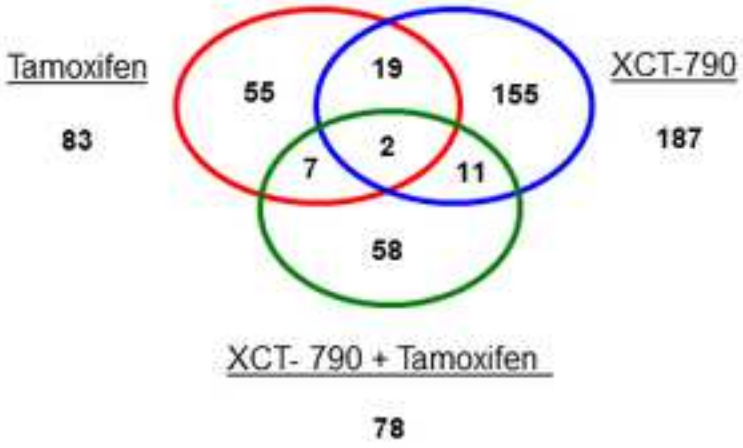


Figure 2

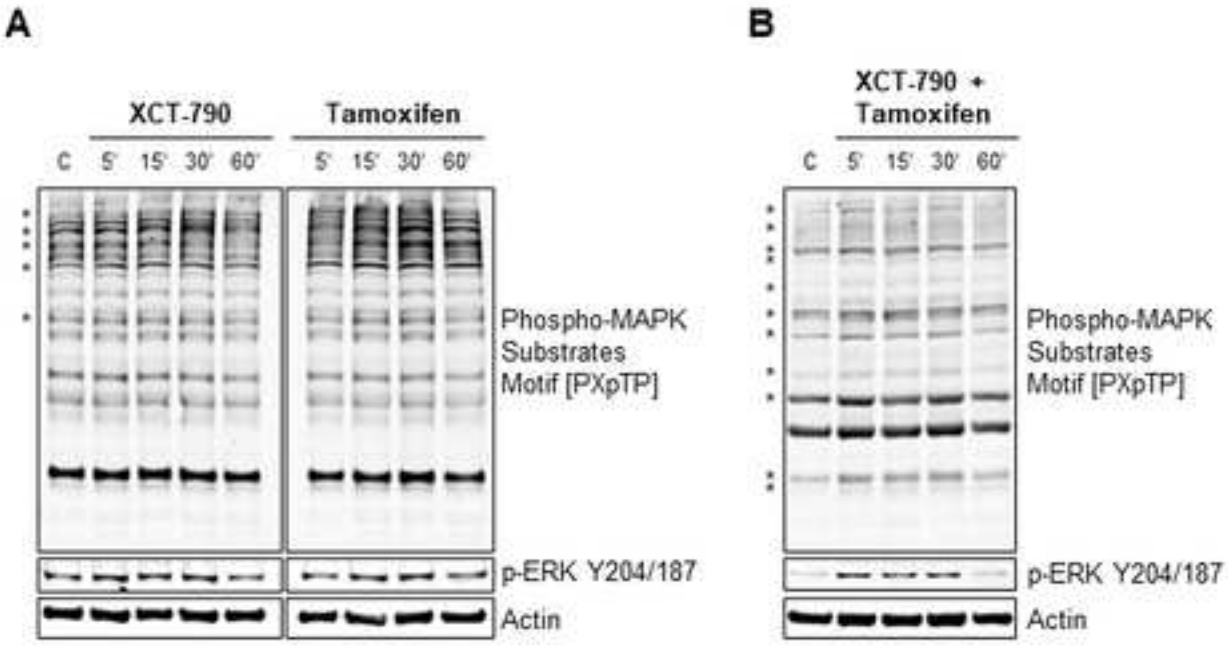


Figure 3

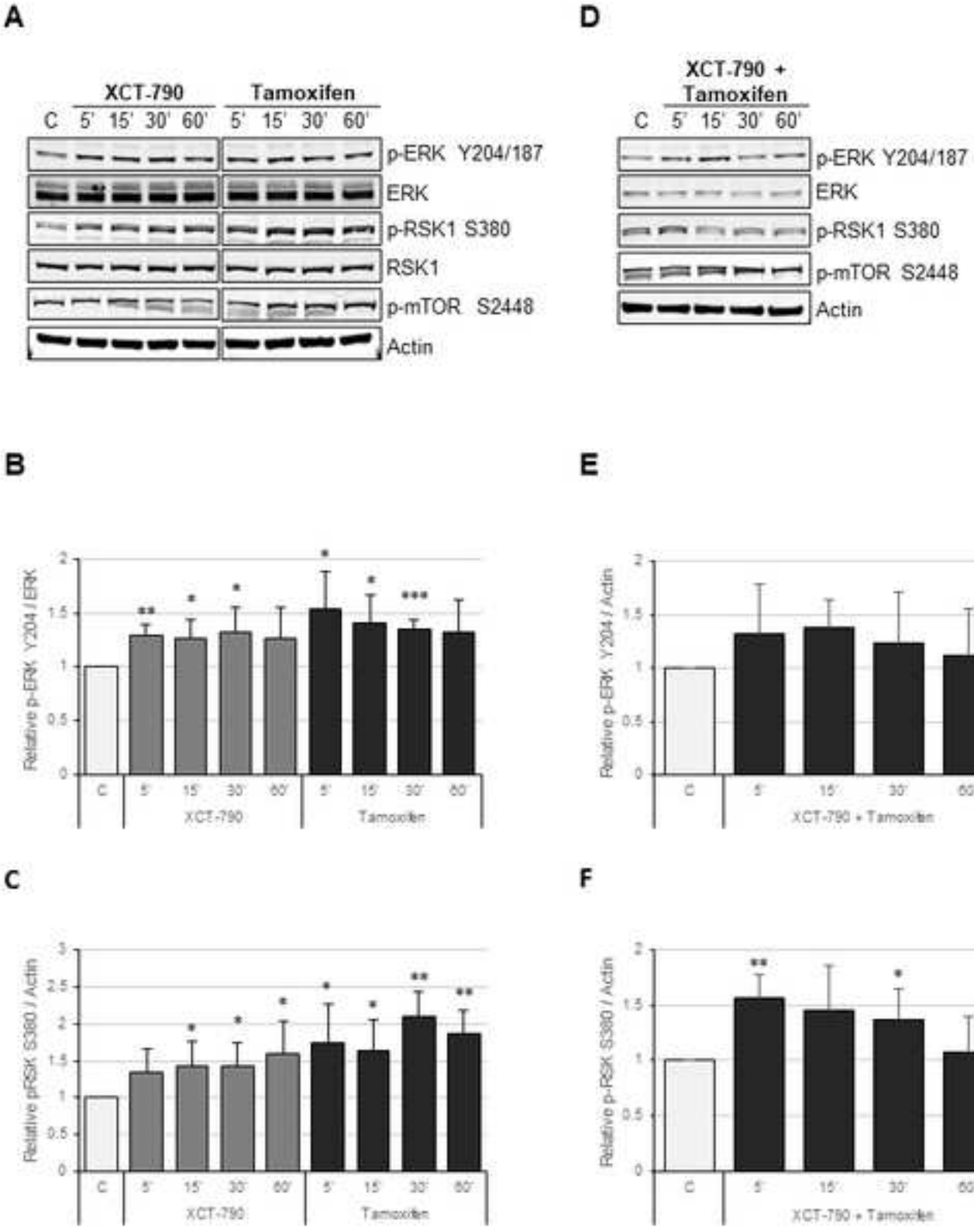
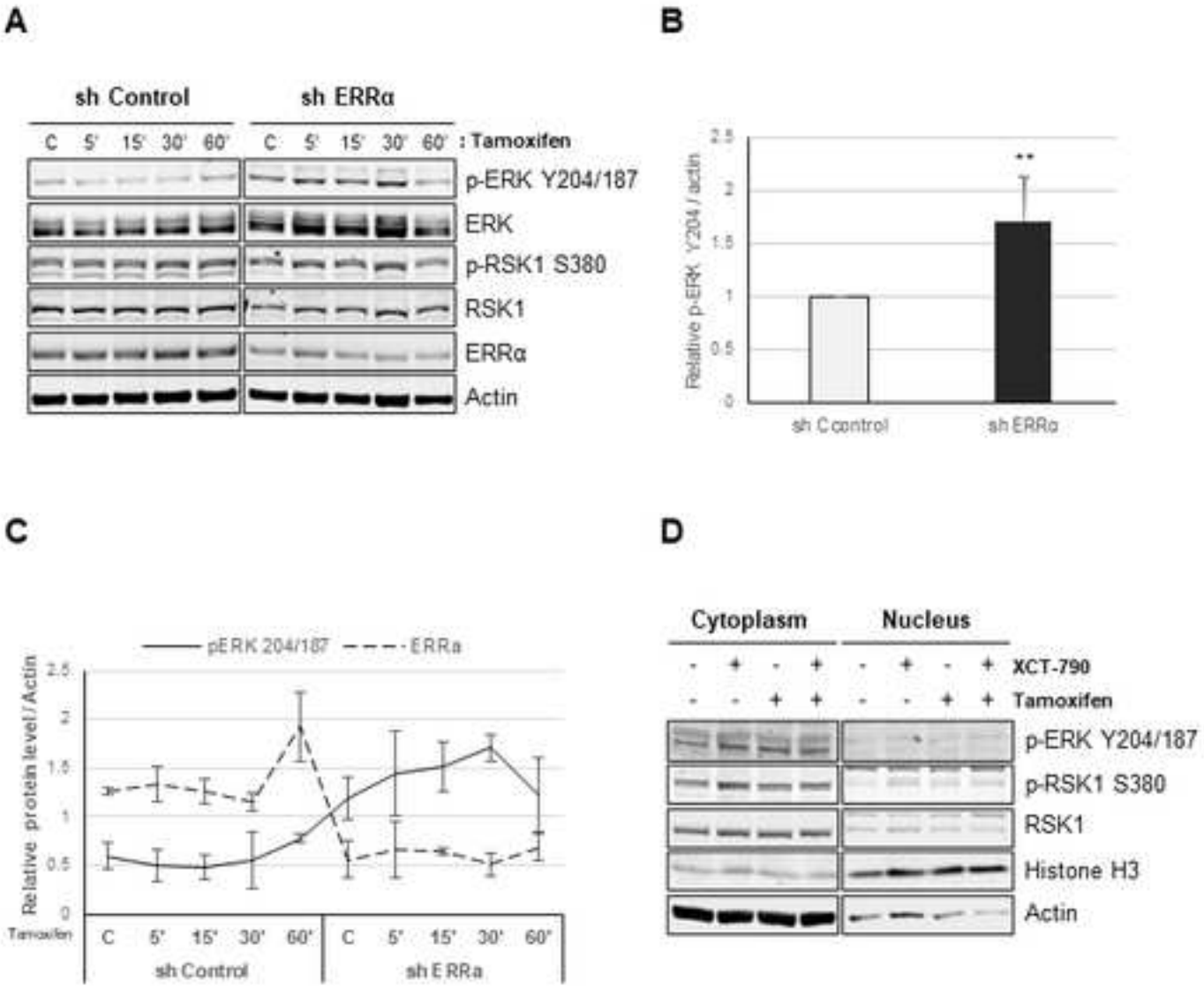


Figure 4



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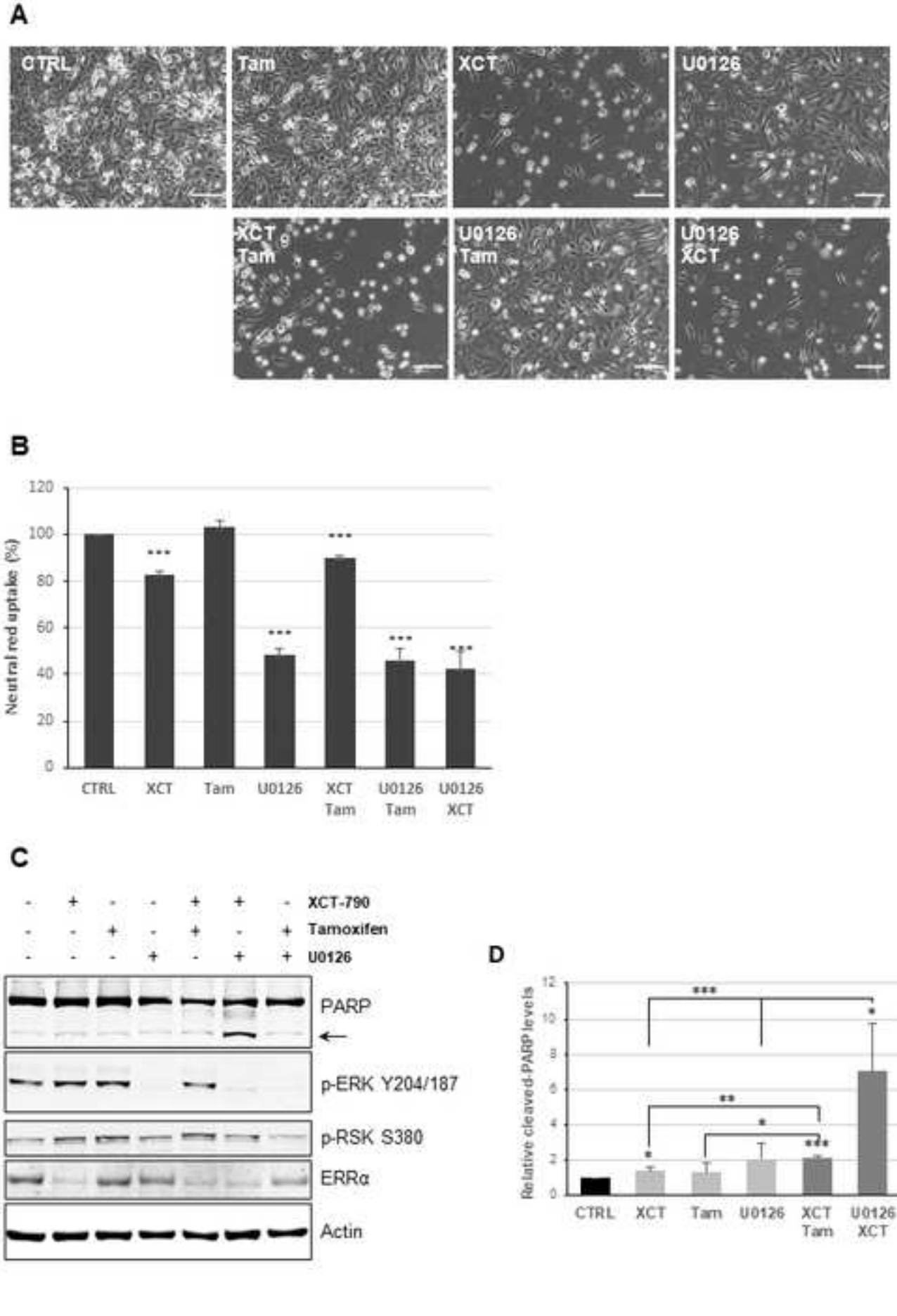

Figure 6

Figure 6

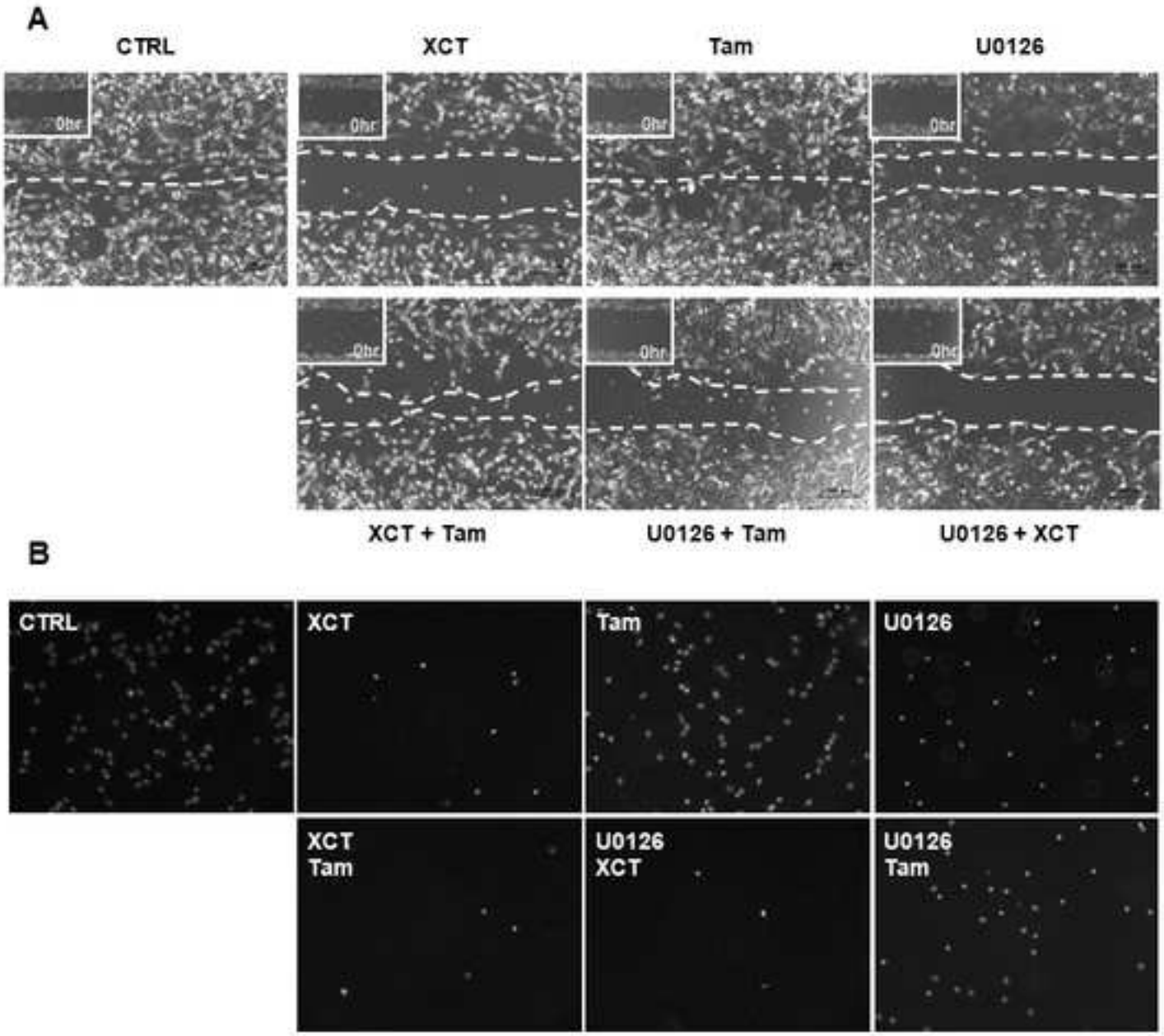

Figure 7

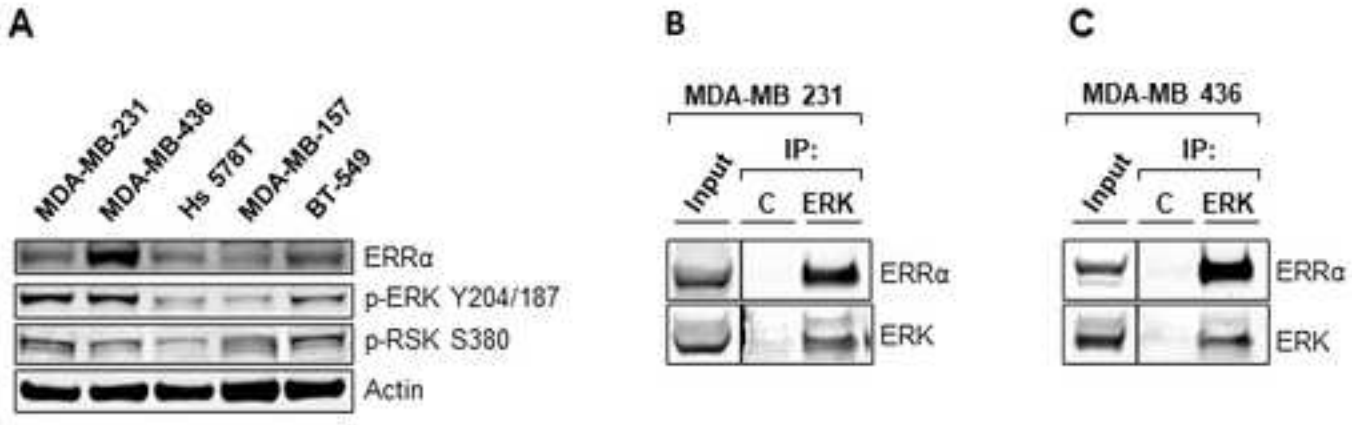

Table 1

Protein	Protein names	Gene names	Position
Q03252	Lamin-B2	LMNB2	420
Q9Y2U5	Mitogen-activated protein kinase kinase kinase 2	MAP3K2	239
Q08170	Serine/arginine-rich splicing factor 4	SRSF4	431
Q9Y2D5	A-kinase anchor protein 2	AKAP2	720
Q3KQU3-2	MAP7 domain-containing protein 1	MAP7D1	796
Q9BWF3-3	RNA-binding protein 4	RBM4	86
Q9UKV3-5	Apoptotic chromatin condensation inducer in the nucleus	ACIN1	408
Q8WZ73-3	E3 ubiquitin-protein ligase rififylin	RFFL	212
Q5JSH3-2	WD repeat-containing protein 44	WDR44	163
Q13547	Histone deacetylase 1	HDAC1	421
Q96RT1-7	Protein LAP2	ERBB2IP	1015
P42858	Huntingtin	HTT	432
Q13158	FAS-associated death domain protein	FADD	194
Q9NXH8	Torsin-4A	TOR4A	63
Q8IYB3-2	Serine/arginine repetitive matrix protein 1	SRRM1	595
Q9UQ35	Serine/arginine repetitive matrix protein 2	SRRM2	1014
Q8NCF5	NFATC2-interacting protein	NFATC2IP	204
Q15366-7	Poly(rC)-binding protein 2	PCBP2	317
P28482-2	Mitogen-activated protein kinase 1	MAPK1	187