The Search for a Targeted Treatment Against Triple-Negative Breast Cancer

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Introduction

Breast cancer is the most common type of cancer worldwide, having surpassed lung cancer in 2020. It is the most common type of cancer to affect women in almost every country, and can and does occur in males, but more rarely. It is also the most common cause of death by cancer among women worldwide, and in the United States, it is second only to lung cancer. (1) Incidence rates rose sharply in the 1980s and 1990s, most likely due to more widespread mammographic screening, but dropped in the early 2000s, due at least in part to reduction in hormone replacement therapy in menopausal women. (2) According to the data from the 2018 SEER cancer survey, there is a 1 in 8 chance that a woman will be diagnosed with breast cancer in her lifetime, and once diagnosed, a 1 in 39 chance that she will die from it. (3) The American Cancer Society estimated that in 2019, over a quarter million new cases of breast cancer were diagnosed in the US alone. (4)

The risk of disease for each individual is different, and is affected by many factors. According to the American Cancer Society, the factors that increase breast cancer risk the most are the presence of a mutation in the BRCA genes, being older than 65, pre-existing hyperplasias or carcinomas in the breast, and high levels of estrogen post-menopause. (5) The prognosis for individuals diagnosed with breast cancer is also affected by these factors, as well as how advanced the disease is when it is diagnosed. If breast cancer is diagnosed before it metastasizes outside the immediately surrounding tissue, the patient has a much better chance of surviving longer than if the cancer had already spread throughout the body.

The types and arrangements of cells in the tumor also play a large role in prognosis. Breast cancers are often categorized by molecular subtype, based on what receptors and enzymes

are expressed in the cells. The presence of these distinct molecular markers affects how the cells behave, including how and where they metastasize, and what treatments can be used against them.

History of breast cancer treatment

For thousands of years, doctors and scientists have puzzled over the causes and treatment of cancerous tumors. Breast cancer in particular is often at the forefront of cancer research, because of its prevalence. Many advancements in cancer treatments, including combination chemotherapy and hormone therapy, were made in the context of breast cancer. Over the course of history, understanding of what causes the development of such tumors has improved, and with it, the ability to treat patients suffering from them. Until the end of the 19th century, cancer treatments focused on diet, pain relief, and radical surgery and cautery to remove superficial tumors. These treatments often proved ineffective, and frequently led to the death of the patient due to the progression of the disease or complications from surgery. Effective treatments for cancer only began to arrive at the end of the 19th century, with the discovery of X-rays and their tissue-killing properties. For decades, radiotherapy was the only way to slow the spread of cancer. Chemotherapy came half a century later, based on the discovery of the long-term effects of chemical weapons used in World War 2. (6)

Before the 1960s, using combinations of drugs in chemotherapy was still anathema in the medical world and was met with great resistance and skepticism, but by the 1970s, the standard treatments for cancer consisted of cocktails of nonspecific cytotoxic chemotherapeutic agents. One such program, called CMFVP (Cyclophosphamide, Methotrexate, Fluorouracil, Vincristine, and Prednisone), brought about a dramatic decrease in mortality from breast cancer. (7) The

introduction of these combination treatments coincided with a decrease in the rates of radical mastectomy in favor of less invasive procedures that preserved the breast tissue, in part because these treatments were as effective as they were against cancerous tumors.

In general, combination chemotherapy guarantees a wider range of interaction, and causes more tumor cells to be killed without risking a toxic dose of any single drug. In addition, it is less likely to decrease in effectiveness over time due to cells developing resistance. These combination regimens aimed to aggressively curb the proliferation of cells in advance of tumor removal surgery, in order to both reduce the size of the tumor, thereby making the surgery easier and more likely to be successful, and prevent micrometastases from causing the cancer to recur. The prevailing belief of the medical community for decades was that if the right combination of cytotoxic compounds was used, chemotherapy could eventually conquer the most stubborn tumor. However, nonspecific cytotoxic chemotherapy has significant drawbacks. It kills cells regardless of whether they are cancerous or not. Often, chemotherapeutic agents target cells that are actively dividing, which does include cancer cells, but also healthy cells in tissues that constantly replenish their cells, such as the digestive tract, bone marrow, and skin. In addition, no matter how strong the regimen, chemotherapy is by no means guaranteed to clean up all or even most micrometastases in the body.

It is now understood that many cellular diseases, including cancer, can be attributed to dysfunction on the part of individual genes and molecules, resulting from a myriad of complex and interrelated processes. Modern medical research often follows the strategies of rational drug design, which is the process of developing a pharmaceutical solution to a problem by aiming to address it at the source. In the case of modern targeted cancer therapy, the goal was to find a more reliable and less harmful way of killing cancer cells by using the factors that distinguish

them from healthy cells and cause them to become cancerous. The development of targeted therapies began with the discovery of hormone therapy in the 60s and the invention of monoclonal antibodies in the 1970s and 80s, and has been continually advancing in the decades since then.

The compound now known as tamoxifen was the first selective estrogen receptor modulator (SERM) to be discovered. It was found in a 1960s search for an effective contraceptive, but animal tests yielded perplexing results - it functioned as an estrogen analogue in mice, but an estrogen receptor antagonist and effective contraceptive in rats. Eventually, it was found to induce ovulation in humans. Naturally, this meant that tamoxifen could not be used as a contraceptive, but in the early 1970s, its antitumor properties began to be investigated in earnest. (8) In the human uterus and liver, tamoxifen acts as an ER agonist, which is why it did not work as a contraceptive, but it has the opposite effect on ER in breast tissue, slowing the growth of breast cancer. Tamoxifen has since been used with great success to treat ER-positive breast cancer, and sparked the interest of the scientific community in the therapeutic possibilities of SERMs.

The following decade saw the creation of the first monoclonal antibodies, which were first developed in the 1970s and 80s and reached clinical trials in the early 1990s. (9) Monoclonal antibodies are produced from clones of a single white blood cell, and can theoretically be made to bind to just about any epitope, which makes them extremely versatile in the medical field and other biotechnological applications. Monoclonal antibodies used to treat cancer are made to bind to specific receptors on the surface of cancer cells, attacking only cells that express those factors and leaving neighboring cells that do not express them. The first monoclonal antibodies originated from mouse cells, but chimeric, humanized, and human

antibodies were developed soon after, in order to prevent the adverse reactions the human immune system had to the foreign proteins. The development of monoclonal antibodies was a revolutionary step in cancer treatment, because now it was within the realm of possibility to intentionally target specific molecular factors within cancer cells and manufacture customized therapies.

Types of breast cancer

The markers commonly used to classify breast cancers include the estrogen receptors (ER), progesterone receptors (PR), and the growth factor HER2. The estrogen and progesterone receptors are referred to collectively as hormone receptors (HR). For each of these factors, there exists targeted therapies that selectively attack the cells that express them.

The most common and most easily treatable molecular subtypes of breast cancer are the luminal types, which are HR-positive. They are generally responsive to targeted hormone therapies, such as tamoxifen. The HR-negative breast cancers are not as easy to treat, in the absence of hormone receptors that can be selectively blocked. HR-negative, HER2-positive breast cancer used to have a much worse prognosis than it does now, before the discovery of treatments that targeted HER2, such as trastuzumab. Trastuzumab, a humanized version of a murine anti-HER2 antibody, was the first monoclonal antibody to reach clinical trials in 1992, and soon brought about a dramatic reduction in the mortality rate of HER2-positive breast cancer.

Triple-negative breast cancer (TNBC) is the most difficult type of breast cancer to treat. It makes up approximately 15% of total breast cancer cases, and is characterized as lacking expression of ER, PR, and HER2. (10) It occurs almost twice as often in African-American

women as in women of other races in the US, and primarily affects younger women under the age of 40, (11) before the usual age when they start routine mammograms. Early stage breast cancers are often detected in mammograms, but because TNBC develops in women at an age that is considered a relatively low risk for breast cancer, the likelihood that it will be caught in an early stage at a routine mammogram is lower than other breast cancers that tend to develop at an older age. Even if it is caught early, patients who are diagnosed with TNBC have a significantly higher risk of recurrence and death than other early-stage breast cancers. SEER data collected from 2011 to 2017 puts the five-year survival rate for patients diagnosed with localized TNBC at 91%, as opposed to other types of breast cancer, all of which have a survival rate of over 95%. This gap only grows as the disease progresses, with distant TNBC having a five-year survival rate of 12%, less than half of the average five-year survival rate of distant tumors of all types of breast cancer. (12) If first-line treatment with chemotherapy fails, TNBC also has a much higher risk of relapse than other breast cancers within the first three years of follow-up. (13) While successful treatment of TNBC with chemotherapy is correlated with a decreased risk of recurrence, that decrease is outweighed by chemotherapy's low rate of success against TNBC. (14) The disparity in success rates of treatment between different types of breast cancer can be attributed to the use of targeted treatments in addition to traditional chemotherapy against other breast cancers, as opposed to TNBC, which is only treated with chemotherapy. This is because there is currently no targeted treatment available for TNBC, due to its lacking expression of all the biological markers that are used as targets. Because chemotherapy is currently the only treatment available for TNBC, patients are more likely to have residual disease after treatment, which contributes to recurrence rates. In addition, TNBC is associated with more aggressive tumors than other breast cancers. Because of this, there is a much shorter window of time in

which it can be treated, since the tumors grow and metastasize so quickly. All of these factors taken together contribute to a poorer prognosis for those with TNBC than other types of breast cancer.

The development of treatment options against TNBC has lagged behind other breast cancers, in part because the mechanisms by which TNBC spreads and progresses are complex and not well understood. In other types of breast cancer, the pathways that contribute to tumorigenesis are known to be related to the activity of HER2 and HR, depending on which of the factors are expressed by the cell. Cells that express those factors are therefore vulnerable to substances that inhibit them, and that fact is taken advantage of by targeted therapies. Lacking these factors, these pathways are regulated by different factors in TNBC, the identities and relative effects of which are still being investigated. The discovery of new treatments for TNBC will depend on new research into these alternative regulation mechanisms, particularly those that rely on a distinct molecular target present in the cells. The low rate of success in treating TNBC with traditional chemotherapy alone highlights the need for a targeted therapy against it, and thus a better understanding of the mechanisms that drive it.

Targeted therapies tend to fight cancer effectively by addressing the root cause of the disease while minimizing damage to healthy cells, in contrast to chemotherapy and radiotherapy, which attack all proliferating cells in a particular part of the body, healthy or not. Targeted therapies are often used in combination for the same reasons that nonspecific therapies are. However, while using multiple drugs in treatment decreases the rate at which tumor cells acquire resistance, eventually the treatment will cease to be effective and the harm to healthy cells will outweigh the therapeutic effects. This occurs in both targeted and non-targeted therapies, leading to the disease becoming more and more difficult to treat over a long period of time. Therefore, in

addition to treating TNBC itself, targeted treatments against TNBC would also be useful in treating other types of breast cancer that have become resistant to the therapies that have been used up until that point. In order to develop such a therapy, it is crucial to understand how TNBC works on a cellular level.

ERRα

While TNBC lacks expression of the proteins usually used as targets for treatment, it does express a distinctive marker of its own, namely the estrogen-related receptor alpha (ERRα). In addition to breast tissue, ERRα is also expressed in the kidneys, heart, intestinal tract, and skeletal muscle. (15) ERRα is an orphan nuclear receptor, which means that it is homologous to a different nuclear receptor, but has no known ligand. In the case of ERRα, it is most similar to estrogen receptor alpha (ERα). Despite their similarity, the two receptors play different roles in the cell. While ERα regulates expression of genes involved in proliferation, ERRα regulates transcription of enzymes involved in metabolism, particularly relating to mitochondrial function, oxidative phosphorylation, and lipid metabolism. (16, 17) Given that it can function as a transcription factor without regulation by a ligand, it is a constitutively active receptor. (18) Studies have shown that inhibiting ERRα slows proliferation of cells that have been subject to stress. (19) A study in mice showed that tumorigenesis was significantly delayed when ESRRA, the gene that codes for ERRα, was removed from their genome entirely, demonstrating the role of ESRRA as an oncogene and the importance of ERRα in fulfilling the metabolic needs of cancer cells. (20) Proliferating cancer cells necessarily consume nutrients and energy at an accelerated rate, requiring more and more from their environment as the tumor grows. In order to maintain their growth, they must adapt to their increasing energy demands by altering their metabolic pathways. This causes them to use metabolic pathways ordinarily only used by cells in

a state of stress. Through its function as a regulator of metabolism and stress response, ERRα provides cancer cells with the energy to proliferate and adapt to unfavorable conditions they will encounter as they spread throughout the body.

Recent discoveries have shown that in addition to regulating metabolism, ERRα also functions as a modulator of ERα activity. There are certain genetic elements in the genome, called estrogen response elements, that can be regulated through the binding of ERα, and because of the homologies between ERRα and ERα, there are some response elements that can be bound by both receptors. (19) In some cases, ERRα and ERα produce the opposite effects when bound to the same response element. For example, the genes ERBB2, which codes for HER2, and GRB7, which codes for a small protein that interacts with HER2 and similar proteins, are both genes that increase a cell's sensitivity to tamoxifen treatment when bound to ERα, and decrease it when bound to ERRα. (21) Competitive inhibition of ERα by ERRα at these genes is thus theorized to be partially responsible for higher ERRα expression being associated with poor response to hormone therapy in ER-positive breast cancer. (18)

Even in ER-negative breast cancer, ERRα shows an association with the ER signalling pathway. Tamoxifen, while effective as a treatment against ER-positive breast cancer, is generally ineffective at treating TNBC and other ER-negative breast cancers. However, studies have shown that some ER-negative breast cancer cells can be sensitized to tamoxifen by inhibiting the Akt pathway. (22-24) The Akt pathway is a metabolic pathway that plays many important roles in cell proliferation, survival, and metabolism, and mutation or abnormal upregulation of the Akt protein is associated with the formation of melanomas and other malignancies. (25) This implies that tamoxifen, as well as being an estrogen receptor modulator, has some ER-independent mechanism of action that may make it useful to shed light on the inner

workings of ER-negative breast cancers. In addition, recent analysis of microarray data from 2000 breast cancer cases worldwide and tumor samples derived from a Swedish study done from 1976 to 1990 showed a correlation between ERRα expression and tamoxifen sensitivity in ER-negative breast cancers. Higher expression of ERRα in the tumor cells was associated with improved prognosis for TNBC patients treated with tamoxifen, but in tumors with low expression of ERRα, tamoxifen was not only ineffective at slowing cancer, it even decreased recurrence-free survival times. (20)

ERRα expression is inversely correlated with ERα expression in breast tissue, which means that ERRα levels are often low in ER-positive breast cancer cells and elevated in TNBC and other ER-negative breast cancers. (21) High ERRα expression in TNBC cells is also correlated with more aggressive tumors and worse prognosis. (26) This relationship makes ERRα a potential candidate for a biomarker of TNBC, indicating that concepts for targeted treatments against TNBC that specifically target ERRα merit further investigation.

The MAPK pathway

In addition to its role in metabolism and ERα-related signalling, evidence also exists for crosstalk between ERRα and the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is an important signalling pathway present in all eukaryotic organisms that regulates cell survival, proliferation, and apoptosis. (27) This pathway is activated by growth factors at receptor tyrosine kinases on the cell surface, which then propagate a signal through phosphorylation of successive kinases that eventually reaches transcription factors in the nucleus as well as targets in the cytoplasm. The receptors that activate the MAPK pathway are often overexpressed in cancer, causing hyperactivation of the pathway and promoting cell

proliferation. One of the most common such receptors includes the epidermal growth factor receptor (EGFR), which is related to HER2, and overexpressed in TNBC at a higher rate than other breast cancers. (28, 29) Considering that the MAPK pathway is highly active in TNBC cells, this implicates the MAPK pathway as one of the major drivers of tumorigenesis and proliferation in TNBC. (26)

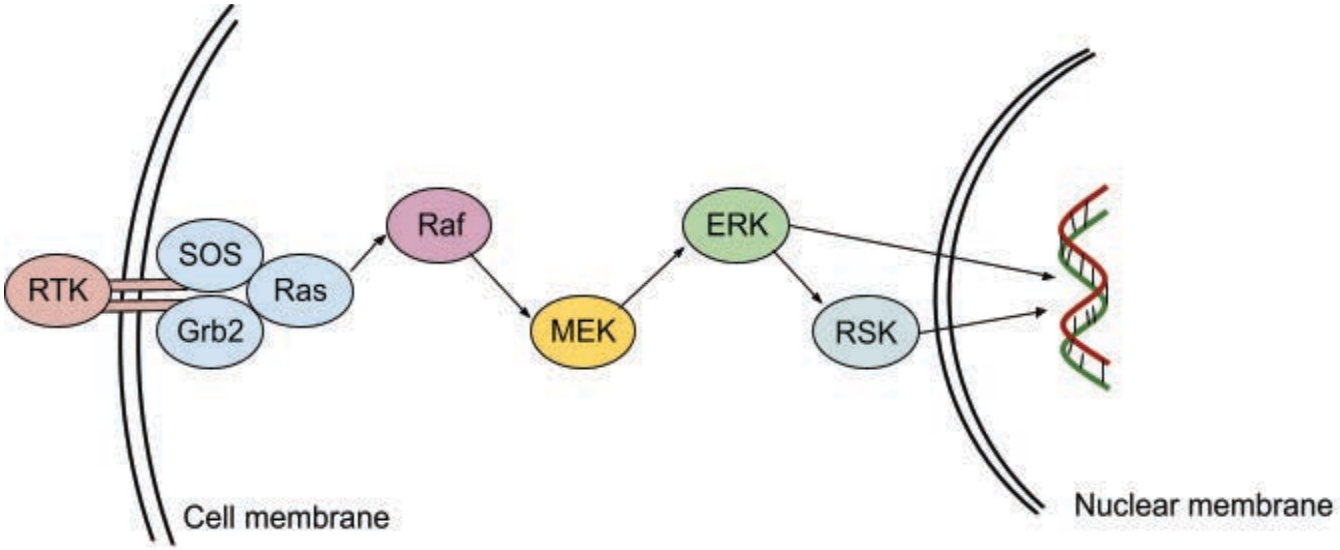

Figure 1: a simplified diagram of the MAPK pathway. From left to right: a receptor tyrosine kinase (RTK) forms a complex with the proteins SOS, Grb2, and Ras, which propagates a signal down the pathway to Raf, MEK, ERK, and RSK. Finally, the signal crosses the nuclear membrane, where it can reach and regulate transcription factors.

One of the kinases that transfers the signal across the nuclear membrane is called ERK. The level and duration of ERK signalling influences cell fate and behavior through various mechanisms, including stabilization of mRNA and recruitment of transcription factors. (27) ERK is activated by MEK, the kinase directly upstream. High levels of pERK (phosphorylated ERK) indicate that the pathway is active and causing cell growth and proliferation. ERK also activates downstream kinases such as RSK, which also travels into the nucleus when phosphorylated. (30, 31) In addition to affecting proliferation through regulating transcription, the MAPK pathway is

responsible for preventing the accumulation of the proapoptotic protein Bim by phosphorylating it. Phosphorylation of Bim marks it for proteasomal degradation, while accumulation of unphosphorylated Bim eventually causes apoptosis. (32) In breast cancer and other cancers of epithelial tissues, the MAPK pathway also plays a role in epithelial-mesenchymal transition (EMT), which is the reprogramming of tumor cells to change their behavior to become more invasive. (26)

As-yet unpublished data shows that TNBC cells that have been treated with tamoxifen have elevated levels of pERK. The same upregulation of pERK has been observed in TNBC cells that have been treated with the ERRα antagonist XCT-790. XCT-790 was the first ERRα antagonist discovered, identified in 2004 through high-throughput screening. (33) It is used in research to study the functions of ERRα in various contexts, due to its potency and selectivity. While activation of the MAPK signalling pathway is the opposite of the desired effect of a treatment for cancer, it also implies a link between ERRα, tamoxifen, and the MAPK pathway in TNBC cells. Given this link, the combined effects of MAPK inhibitors, ERRα inhibitors, and tamoxifen on breast cancer cells should be studied in order to determine the role of ERRα in TNBC cells, investigate the pathways that affect tumorigenesis and the progression of TNBC in the absence of HR and HER2, and assess the effects of a treatment targeting ERRα on these pathways.

Methods

Neutral Red assay

The NR assay was used to measure the effect of XCT-790, tamoxifen, and U0126, both individually and in combination, on the proliferation of cultures of TNBC cells over the course

of several days. The assay was performed on MBA-MD-231 cells as well as MBA-MD-436 cells.

The cells were serum starved overnight and then plated in 96-well plates at a density of 2500 cells per 100 µl in DMEM with 10% FBS. They were then treated with tamoxifen, XCT-790, U0126, and each combination of two drugs, and incubated at 37°C. The tamoxifen treatment was at a concentration of 100 nmol, and the XCT-790 and U0126 treatments were each at a concentration of 10 µmol. The NR assay was then performed according to standard protocol on each plate of cells on the sixth day after plating and drug treatment.. A 1:100 dilution of NR dye in DMEM with 10% FBS was prepared. The media was aspirated, 200 µl of the dye dilution was added to each well, and the plate was incubated for 45 minutes. The excess dye was discarded, and the plate was washed with $CaCl_2$-formaldehyde solution to fix the cells. 200 µL of ethanol was added in each well, and the plate was shaken for 45 min. Finally, the absorbance was measured in a plate reader.

Western blots

In preparation for each western blot, the cells were serum starved and incubated overnight at 37°C, treated and incubated, lysed, centrifuged to isolate the proteins, and normalized by volume. The normalized lysates were run on gels at 165 volts for 60 minutes. The resulting membranes were blocked with antibodies and scanned.

A western blot was performed on MDA-MB-231 cells that were given the same treatments as the ones tested with the NR assay - XCT-790, tamoxifen, U0126, and all combinations of two drugs. The cells were incubated with the treatments for 24 hours, and levels of ERRα, pERK, cleaved PARP, and survivin were measured. Another western blot was

performed on MBA-MD-231 cells in order to further investigate how some of the treatments take effect over time, specifically XCT-790 and tamoxifen. The cells were treated with XCT-790, tamoxifen, and a combination of both; one group of cells was treated for 15 minutes, and another treated for 30 minutes.

Results

In this study, TNBC cell cultures were treated *in vitro* with XCT-790, tamoxifen, and the MEK1/2 inhibitor U0126. The Neutral Red (NR) assay was used to assess how each drug and combination of drugs affects proliferation over time. The NR assay is a cytotoxicity and proliferation assay that measures the relative amount of living cells in a culture. It is based on a red dye that living cells can absorb through their lysosomes, but dead cells cannot. Breast cancer is a highly heterogeneous disease, and different tumors may have very different properties. (34) Even a single tumor may comprise many different kinds of cells. In order to ascertain that the effects of the treatments being investigated are generalizable across TNBC and not specific to a genetic quirk of one lineage of cells, two different cell lines, MDA-MB-231 and MDA-MB-436, were tested.

In order to see the effects of each drug treatment on the various pathways in more detail, western blots were also performed to qualitatively analyze the relative levels of proteins expressed in the cells. Variations were measured in the levels of pERK and pRSK, activated versions of proteins in the MAPK pathway, as well as pmTOR (mammalian target of rapamycin), a protein that is not involved in the MAPK pathway, as a negative control. In addition, the levels of ERRα itself and the general apoptotic markers Poly-[ADP-ribose] polymerase (PARP) and survivin were measured. PARP is a small protein involved with DNA repair, especially with

single-strand breaks. Higher expression of PARP promotes cell survival, and during the process of apoptosis, PARP is deactivated by being cleaved into smaller polypeptides by caspase proteins. High levels of cleaved PARP, as opposed to active uncleaved PARP, is thus a marker of apoptosis. BRCA mutations are associated with overexpression of PARP in breast cancer, as are dysfunctional HR pathways, but the primary cause of this overexpression is unknown. (35, 36) Survivin is an inhibitor of apoptosis that is often abnormally upregulated in tumor cells, particularly in breast and lung cancers. It contributes to tumorigenesis by inhibiting apoptotic pathways involving caspase proteins, such as those that cleave and inactivate PARP. (37) It is regulated by many different factors and signalling pathways, including the MAPK pathway as well as other unrelated pathways such as Wnt and Akt. (38)

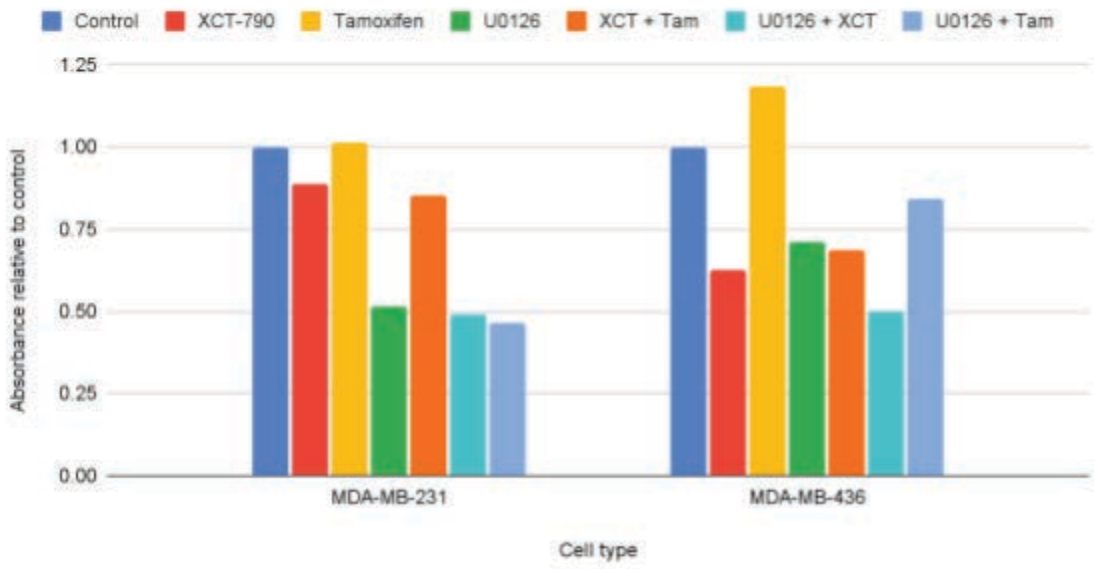

Figure 2: the absorbances of the MDA-MB-231 cells and MDA-MB-436 cells relative to their respective controls, as measured on the sixth day after plating and drug treatment.

The results of the NR assay can be seen in Figure 2. Over the course of the six days of incubation, the absorbance of the cells rose at different rates, indicating that the cells proliferated in that span of time at different rates according to the drug treatments. Higher absorbance

indicates greater proliferation. Treatment with only tamoxifen did not produce any change in cell proliferation rate compared to control in the MDA-MB-231 cell line, and increased it by approximately 20% in the MDA-MB-436 cell line. In contrast, XCT-790 and U0126 both successfully slowed the rate of cell proliferation. In the MDA-MB-231 cells, U0126 was consistently much more effective than XCT-790 in slowing cell proliferation, reducing absorbance by 50% while XCT-790 only reduced absorbance slightly. In contrast, in the MDA-MB-436 cells, U0126 had approximately the same level of effect as XCT-790, both reducing absorbance by between 60 and 75 percent. In the MDA-MB-231 cells, the addition of tamoxifen to the XCT-790 treatment had minimal effect, the cells having approximately the same absorbance as those treated with only XCT-790. The same lack of effect on the part of tamoxifen was observed in the combination with U0126, which also showed little change from the cells treated with only U0126. On the other hand, in the MDA-MB-436 cells, the addition of tamoxifen caused a dramatic reduction in the effectiveness of the U0126 treatment, even more than the difference between the tamoxifen-only treatment and the control group. However, the cells treated with the combination of tamoxifen and XCT-790 showed little change from the cells treated with only XCT-790.

The reasons for the disparity between the effects the treatments had on the two cell lines is unclear, probably due to slight genetic differences in the ways the metabolic pathways are regulated. The treatment that was the most consistently effective at slowing proliferation was the combination of XCT-790 and U0126, which showed an approximate 50% reduction in absorbance from control in both cell lines. In the cells given the combination treatments, the drugs had additive effects, indicating that the drugs in the treatments operated independently of one another and their effects did not cancel out.

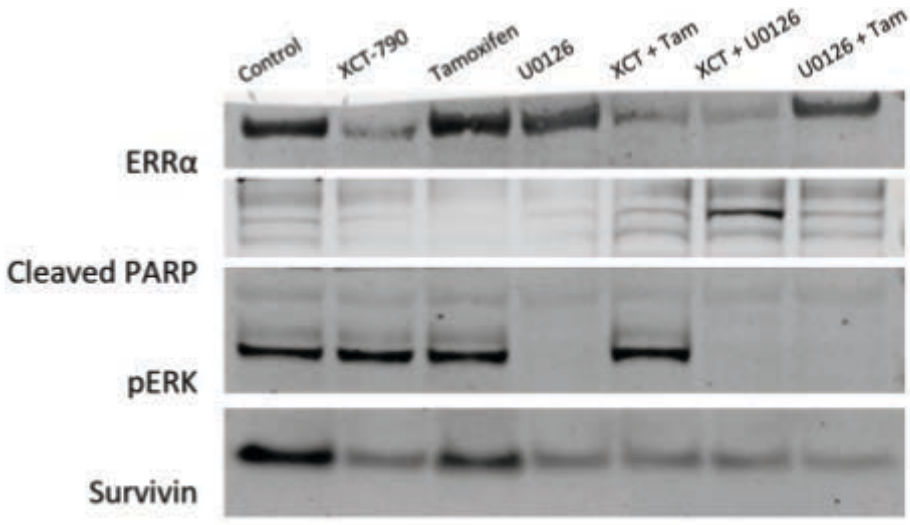

Figure 3: western blot of MDA-MB-231 cells after incubation for 24 hours.

The results of the western blot in Figure 3 show that ERRα is reduced in all the cells that were treated with XCT-790 or a combination treatment that included XCT-790, indicating that XCT-790 is successfully inhibiting ERRα. Similarly, pERK is reduced in the cells that were treated with U0126 or a combination that included U0126, indicating that U0126 is successfully inhibiting the MAPK pathway. The presence of cleaved PARP in the cells treated with the combination of U0126 and XCT-790 indicates that PARP is involved in the apoptosis induced by the treatment. However, cleaved PARP is present in much lower amounts in all the other cells, including the control, in roughly equal amounts. The control cells show a high level of survivin expression. In all the other cells, survivin is reduced in relation to control. The cells treated with only tamoxifen show a smaller reduction in survivin compared to control than the other treatments, but a reduction nevertheless. All the other treatments show a much more pronounced reduction in the level of survivin.

The results of the western blot in Figure 4 indicate that pERK levels rose in the cells treated with tamoxifen. This is most noticeable in the cells treated for 15 minutes, where the

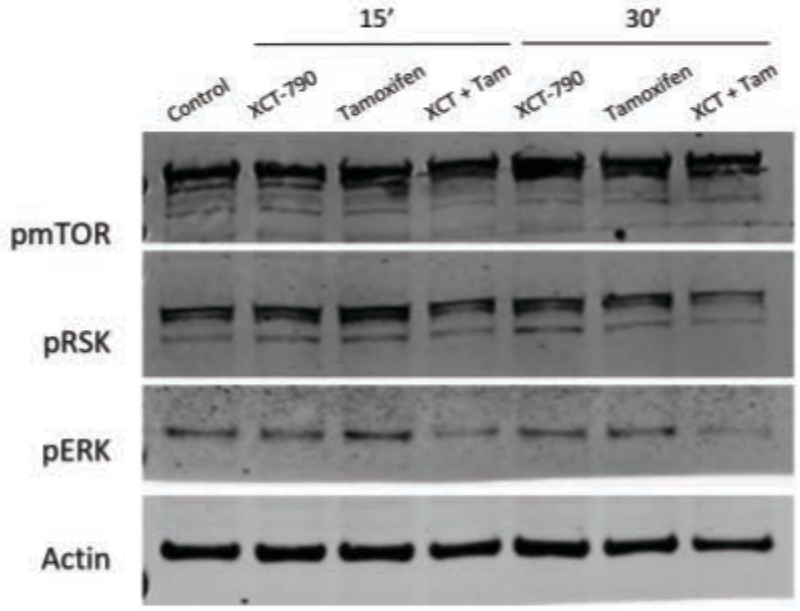

Figure 4: western blot of MDA-MB-231 cells after incubation for 15 and 30 minutes.

level of pRSK rose as well. The effect became less pronounced in the 30 minute treatment. The cells treated with XCT-790 also showed an elevation in the levels of pERK and pRSK, but to a slightly lesser extent than in the cells treated with tamoxifen, and the effect only manifests in the 30 minute treatment. However, in the cells treated with the combination of tamoxifen and XCT-790, the levels of pERK and pRSK actually went down to a level even lower than that of control. Similarly to the XCT-790-only treatment, this effect was most noticeable in the 30 minute treatment. The level of pmTOR remained unchanged from control in all the treated cells, which indicates that the tamoxifen and XCT-790 treatments selectively upregulate the MAPK pathway and have minimal effect on other pathways within the cell that involve pmTOR.

Discussion

The results of the NR assays show that tamoxifen does not decrease the proliferation of TNBC cells in vitro, as expected in a cell culture lacking ER expression. In fact, it even increased the proliferation by a considerable amount in the MDA-MB-436 cell line. XCT-790

and U0126, however, do slow the proliferation of the cells, and that effect is compounded when they are used in combination. This shows that targeting both ERRα and the MAPK pathway is effective at preventing the growth of TNBC, and therefore potentially a suitable approach for a targeted therapy against it.

The western blots showed that XCT-790 and U0126 did successfully inhibit ERRα and the MAPK pathway respectively. The combination of XCT-790 and U0126, found to be the most consistent inducer of apoptosis in the cells in the NR assay, was the only treatment to show an increase in the level of cleaved PARP in the cells. However, the lack of a difference between the amount of cleaved PARP in all the other cells, including the control group, indicates that the apoptosis caused by the other treatments cannot be solely attributed to the presence of cleaved PARP. In addition, all the treated cells, including those only treated with tamoxifen, expressed lower levels of survivin compared to control. The fact that the tamoxifen treatment decreased survivin levels indicates that despite its failure to induce apoptosis in ER-negative cells, it does demonstrate a certain level of anti-tumor capability that is independent of its inhibition of estrogen receptors.

The western blots also showed that XCT-790 and tamoxifen treatment selectively upregulate the MAPK pathway, shown by the increase in the levels of pERK and pRSK in the treated cells, and the unchanged levels of pmTOR, which is not involved in the MAPK pathway. The difference between the time it took for tamoxifen and XCT-790 to show their effect indicates that the mechanisms of action by which XCT-790 and tamoxifen affect the expression of proteins in the MAPK pathway may be different. This upregulation was not as visible in the cells treated overnight as it was in the cells treated for 15 and 30 minutes, which indicates that it may be a temporary or short-term effect. However, when treated with a combination of XCT-790 and

tamoxifen, the cells actually showed a downregulation of the MAPK pathway rather than an upregulation. This raises questions about the relationship between ERRα and tamoxifen in TNBC cells, whether the two signalling cascades interfere with one another, and how they affect the MAPK pathway. Future research into this area could potentially prove useful not only for TNBC, but for other ER-negative breast cancers and other cancers that express high levels of ERRα.

Conclusion

Triple negative breast cancer poses a serious threat to women's health. It is the most dangerous subtype of the most common cancer, known for its aggressive tumors and poor prognosis. There is an unfulfilled and urgent need for an effective targeted treatment against TNBC, as all targeted treatments against breast cancer rely on the presence of estrogen and progesterone receptors (HR) or the growth factor HER2, none of which are expressed in TNBC cells. Without the option to use targeted treatment, the current standard for treatment of TNBC is nonspecific chemotherapy, which often fails to eliminate cancerous tumors, thereby prolonging the disease. Targeted treatments against cancer are more effective than nonspecific chemotherapy, shown by lower mortality rates and higher recurrence-free survival rates. As a historical example of targeted treatments improving the prognosis for cancer patients, HER2+/HR- breast cancer used to have the worst prognosis of all breast cancers before the discovery of trastuzumab and other anti-HER2 therapies dramatically improved survival rates. The development of a targeted therapy for TNBC would potentially enable a similar improvement in prognosis. In addition, non-TNBC breast cancers can become resistant to anti-HR and anti-HER2 therapies over time, due to the selective pressures imposed by treatment. The discovery of a targeted treatment that works independently of HR and HER2 would

therefore not only help improve the prognosis of TNBC itself, but also would be invaluable in the fight against other types of breast cancer.

The main issues to address in the search for such a treatment are that the metabolic mechanisms that drive TNBC are not as well understood as they are in other types of breast cancer, as well as the lack of a known reliable biomarker in TNBC to use as a target for treatment. A promising candidate for such a biomarker is the estrogen-related receptor alpha (ERRα), which regulates cell metabolism, specifically oxidative phosphorylation within mitochondria. Inhibiting ERRα in TNBC cells has previously been shown to affect the MAPK pathway, possibly due to its role in the cellular stress response. The same effect on the MAPK pathway has been observed in TNBC cells treated with tamoxifen, which implies a link between ERRα, tamoxifen, and the MAPK pathway. Future research in this area should take into account the fact that there are currently no ERRα inhibitors approved for use in humans. It is possible that investigation into ERRα as a target for treatment of TNBC would be the impetus to accelerate research into ERRα inhibitors.

This study investigates the effects of tamoxifen, the ERRα inhibitor XCT-790, and the MEK1/2 inhibitor U0126 on TNBC cells *in vitro*. The study compares the effects of each drug individually and in combination, in terms of how it affects both the proliferation of the cells and the relative levels of proteins expressed within them. The results of the study demonstrate that targeting both ERRα and the MAPK pathway through the use of XCT-790 and U0126 is quite effective at slowing the proliferation rate of TNBC cells, reducing the proliferation rate to about half that of untreated cells. Therefore, ERRα can be considered a potential target for future development of targeted treatments against TNBC. In addition, this study has shown that while XCT-790 and tamoxifen both cause a short-term upregulation of the MAPK pathway in TNBC

cells on their own, treating TNBC cells with both drugs at the same time actually downregulates the pathway, which raises questions about how interrelated the ERRα and ERα pathways are and how they are regulated in the absence of estrogen receptors. The data produced by this study support the hypothesis that ERRα plays a significant role in the mechanisms by which TNBC grows and progresses, and that dual-targeting ERRα and the MAPK pathway is a viable approach to treating TNBC.

Moving forward, this research can be refined in order to assess the specificity of such treatments and investigate how they affect non-TNBC cells. A successful targeted treatment against TNBC would not only attack TNBC cells, but also leave non-cancerous cells relatively unaffected. A future direction for this line of investigation would be to test the treatments used in this study on both non-TNBC breast cancer cells and on healthy non-cancerous breast tissue cells, in order to determine the effects on those cells in relation to TNBC cells. There is still much research to be done into the role of ERRα both in and out of TNBC, and how the metabolic pathways ordinarily regulated by estrogen receptors in TNBC cells are affected by ERRα. Studies that aim to determine how the MAPK pathway and ERRα are linked and how their effect on TNBC differs from that of tamoxifen will be invaluable in increasing understanding of the mechanisms by which TNBC grows and progresses, so that understanding could be used to find a promising avenue toward a targeted treatment of TNBC.

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