SHORT CHAIN FATTY ACIDS EXHIBIT SELECTIVE ESTROGEN RECEPTOR DOWNREGULATOR (SERD) ACTIVITY IN BREAST CANCER

A Thesis

by

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ABSTRACT

Breast cancer is one of the most common cancers affecting women in the United States. Hormone receptor positive (HR+) breast tumors are the most prevalent, accounting for 70% of breast cancer cases. Therapies that target hormone receptors such as the estrogen receptor (ER) or that target estradiol synthesis are most commonly used to treat these patients; however, some patients may develop resistance to these treatments. One mechanism of resistance is the development of a constitutively active estrogen receptor alpha (ERα, ESR1) due to somatic mutations in the ligand binding domain (LBD). The most common of these mutations are ESR1-Y537S and ESR1-D538G. Fulvestrant, a hormone therapy, is used to treat patients with these mutations. Previous studies have shown that histone deacetylase (HDAC) inhibitors downregulate ESR1. Some dietary derived short chain fatty acids: butyrate, propionate, and acetate are reported to exhibit HDAC inhibitory activity. We investigated their effects as SERDs in MCF-7 and T47D cells expressing both wild-type and mutant ESR1. Propionate and butyrate exhibited similar induction of histone acetylation. Although acetate induced ESR1 degradation, acetate may not function independently of HDAC inhibition to downregulate ESR1. HDAC inhibitors: Panobinostat, Vorinostat, and Entinostat are currently being investigated as breast cancer treatment in clinical trials. We observed that Panobinostat, Vorinostat, and Entinostat downregulated wild-type and mutant ESR1and induced histone acetylation. These results suggest that HDAC inhibitors act as SERDs and may be clinically efficacious for treating ER-positive endocrine resistant breast cancer patients and this is currently being investigated.

DEDICATION

To my grandmother Harriet Greenleaf Schoeller, you have provided so much for me. You have stressed the importance of education to me. Stressed that it would never be all textbooks and tests and that sometimes the greatest education is the one you don't find in school, but the one that you can find around you. You listened to me ramble on about whatever career path I had decided upon, you let me use all the printer paper when I was going to become a city planner, listen to me be a meteorologist while the real one gave an accurate forecast on TV, attended my off key clarinet concerts in the living room and told me I sounded wonderful. Without you I wouldn't have had the chance to explore anything I wanted or be where I am today.

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NOMENCLATURE

4-OHA	4-hydroxyandrostenedione
ADCC	Antibody-dependent cellular cytotoxicity
AhR	Aryl hydrocarbon receptor
AI	Aromatase inhibitor
AR	Androgen receptor
Bcl-2	B-cell lymphoma 2
CDK	Cyclin dependent kinase
DCIS	Ductal carcinoma in situ
DFS	Disease free survival
EGFR	Human epidermal growth factor receptor 1
ER	Estrogen receptor
ERα, ESR1	Estrogen receptor alpha
GEP	Gene expression profiling
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor

HSP90	Heat shock protein 90
IDC	Invasive ductal carcinoma
IGF, IGF-1	Insulin-like growth factor 1
IGFBP3	Insulin-like growth factor binding protein 3
IGF1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
IBC	Inflammatory breast cancer
ILC	Invasive lobular carcinoma
IM	Intramuscular injection
H12	Helix 12
LBD	Ligand binding domain
LBP	Ligand binding pocket
LCIS	Lobular carcinoma in situ
MAPK	Mitogen-activated protein kinase
mTOR	Molecular target of rapamycin
OS	Overall survival
PARP	POLY (ADP-RIBOSE) POLYMERASE
pCR	Pathological complete response

PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
PPAR	Peroxisome proliferator activated receptor
PR	Progesterone Receptor
RTK	Receptor tyrosine kinase
SARM	Selective androgen receptor modulator
SCFA	Short chain fatty acid(s)
SERCA	Selective estrogen receptor covalent antagonist
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
SHBG	Sex hormone binding globulin
SRC	Steroid receptor coactivator
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TNBC	Triple negative breast cancer
Treg	Regulatory T cells
TTP	Time to progression
VEGF	Vascular endothelial growth factor

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1. BACKGROUND AND INTRODUCTION

1.1 Prevalence and Risk Factors for Breast Cancer

Breast cancer is the most commonly diagnosed cancer in women with exception of melanomas and other skin cancers, but breast cancer accounts for the highest incidence of new cancer cases each year (1). One in 8 women, or 13% of all women will be diagnosed with breast cancer in their lifetime in the United States (1,2). Since the early 2000s, rates of new breast cancer cases have increased by about 0.5% each and has been attributed to declines in fertility and increases in body weight; however, the incidence of deaths due to breast cancer have decreased (2,3). These improvements are due to greater understanding of menopausal hormone use, improved screening and education of the disease, and increased understanding of how to treat breast cancer. Detection of the disease early, has been important for improving prognosis of the disease (4).

New breast cancer cases diagnosed at a rate of 126.0 persons per 100,000 persons, across all races and ethnicities in women, and an estimated 281,550 new cases are expected in 2021 in the United States (2). Breast cancer is the second most common cause of cancer-related death in the United States, behind lung and bronchial cancer, with 20.1 deaths per 100,000 persons, and a total of 43,600 deaths in women expected in 2021 (1,2). The majority of breast cancer cases are diagnosed in women over 50 years of age; 82% of women are 50 years or older when diagnosed, and they represent 90% of all women who die of breast cancer (5,6). The probability of being diagnosed with breast cancer is highest for women in their seventies, death due to cancer is most likely to occur when women are in their eighties (6). The median age of diagnosis is 62 years and the median age of death due to breast cancer is 68 years. However, there are discrepancies, the median age of diagnosis for White women is 63 years, whereas for Black women it is 60 years.

The median age of death due to breast cancer for White women is 70 years, but for Black women it is 63 years (6).

There are disparities in breast cancer occurrence and outcomes between different racial and ethnic groups (1,2,6). Non-Hispanic White women have the highest incidence of breast cancer diagnosis and Asian/Pacific Islander and non-Hispanic white women are more likely to be diagnosed with local-stage breast cancers than non-Hispanic Black women, Hispanic women or American Indian and Alaska Natives (2). While non-Hispanic White women and non-Hispanic Black women have similar incidences of breast cancer, Black women are much more likely to die of breast cancer (1,2). Black women are more likely to be diagnosed with metastatic breast cancer, about 8%, than other races and ethnicities which average around 5% to 6% (6). Black women are also the only group that are more likely to be diagnosed with high grade rather than low or intermediate grade tumors and are the most likely to be diagnosed with triple negative breast cancer (TNBC) compared to other racial or ethnic groups (2,6,7). Incidences of breast cancer and mortality rates are lower in Asian/Pacific Islander, American Indian/Alaska Native, and Hispanic women compared to White or Black women (2).

A variety of risk factors affect a women's likelihood of developing breast cancer, and these factors range from ones that we can influence to those that we cannot, such as genetic risk factors (5). Age is one of the best understood risk factors, age is directly proportional with risk of developing breast cancer (5,8). Family history of breast cancer is a well known risk factor for developing breast cancer, those with a first degree relative who have had breast cancer are two to three times more likely to develop breast cancer than people who do not have a first degree relative with breast cancer (5,8). Family history of ovarian cancer, previous personal history of breast cancer or non-cancerous diseases, as well as previous history of radiation therapy can all

contribute to increased risk of developing breast cancer (5,8). Additionally, genetic factors can increase risk of developing breast cancer. Germline mutations to *BRCA1* and *BRCA2* are some of the most well known are important genes associated with increased risk of developing breast cancer (5,8). Some risk factors that cannot be changed may be less intuitive, like menarche before 12 or starting menopause after 55, but longer duration of hormone exposure can potentially increase risk. Some risk factors associated with breast cancer are ones that women may have greater control over such as exogenous hormone use during menopause and certain birth controls typically used in premenopausal women (5,8). Certain lifestyle factors such as increased alcohol consumption and decreased physical activity and smoking are all associated with an increased risk for developing breast cancer and are factors that women have influence over (8). Some lifestyle factors that women can influence are not necessarily controllable though, such as increased weight after menopause or women not having their first pregnancy until after 30, not breastfeeding, or not having a full-term pregnancy (5,8).

Men account for 1% of breast cancer cases (9). In 2021 it is expected that 2,650 new cases of breast cancer will occur in men and 530 deaths are expected (2). Incidence of male breast cancer is higher for non-Hispanic Black men than any other group, at a rate of 1.9 men per 100,000. This group also faces the highest mortality rate, at 0.5 men per 100,000. Non-Hispanic White men have the next highest incidence of breast cancer with 1.3 in 100,000 men being diagnosed and a mortality rate of 0.3 in 100,000 men (10). Age also plays a determining factor in male breast cancer, with incidence increasing as age increases (9,11).

Men diagnosed with breast cancer tend to be diagnosed at later stages, than women. The overall rarity of breast cancer in men and therefore lack of awareness and testing likely leads to the disease being detected at later stages (9). Known risk factors for men are similar to those seen

in women. Age is a contributing factor, though average age of diagnosis in men (71) is higher than observed in women (62). Family history of breast cancer corresponds with an increased likelihood of men being diagnosed with breast cancer, with the relative risk increasing 2.5-fold (11). Genetic mutations in *BRAC1 and BRCA2* can increase the likelihood of male breast cancer. The *BRCA2* gene is more likely to contribute to the development of male breast cancer whereas *BRCA1* seems to play a more limited role in the development of male breast cancer (9,11). Additionally, Klinefelter syndrome, a genetic disorder, is a strong risk factor for developing breast cancer (9,11). Men with *BRCA* mutations or Klinefelter syndrome that develop breast cancer are more likely to do so at a younger age (9). Other conditions that may lead to the development of breast cancer in males include: lack of physical activity and obesity as well as conditions that impact estrogen and androgen ratios such as administration of exogenous estrogen or testosterone, prostate cancer treated with estrogen, orchitis and epididymitis (9,11).

1.2 Molecular Subtyping of Breast Cancer

There are different ways to classify breast cancer. Molecular subtyping is prominently used since it may also identify targets of therapy for breast cancer (12-14). Global gene expression profiling (GEP) is used to identify molecular subtype, for example, PAM50 identifies 50 genes that classifies breast cancer into molecular intrinsic subtypes (12-16). Frequently though, immunohistochemistry (IHC) is useful in clinical settings to identify a select group of proteins used for molecular subtyping since GEP applications are largely confined to research settings (12,13,16). Between the two methods of molecular subtyping there are overlaps in their different definitions, although each subtype is not inherently synonymous (12,13,15,16).

1.2.1 Immunohistochemical Subtyping of Breast Cancer

IHC molecular subtyping of breast cancer determines whether the cell is hormone receptor (HR) positive or negative, and this primarily refers to the estrogen receptor (ER) and the

progesterone receptor (PR) (12,13,15). HER2 status is also assessed positive or negative, and highly invasive TNBC, which does not exhibit expression of ER, PR, or HER2 (12,13,15). Classification by IHC markers is recommended by St. Gallen Expert Consensus due to cost and practicality for clinical settings (13,16).

1.2.2 Intrinsic Molecular Subtyping of Breast Cancer

Luminal A is the most common, accounting for 50%-60% of all breast cancer cases and this subtype has the best prognosis since these cancers tend to be low-grade (15). Luminal A breast cancers are HR+ and HER2- and express low levels of the proliferation marker Ki-67 (12,13,15,16). They typically have low histological grade, a low degree of nuclear pleomorphisms, and low mitotic activity conferring slow tumor growth (15). The relapse rate of luminal A tumors is 27.8%; recurrence in bone is most common, with lower occurrence of metastasis in the liver, lung, and central nervous system (15). Hormone therapies and third-generation aromatase inhibitors are the main basis of treatment used to treat people with luminal A cancers.

Luminal B breast cancers account for approximately 15%-20% of breast cancer cases (15). Luminal B is HR+, be HER2 positive or negative, and expresses high levels of Ki-67 (12,13,16). HER2 is expressed in around 30% of luminal B cases (15). This subtype has a poorer prognosis than luminal A cancers and these tumors a higher histological grade and a higher proliferative index (12,15). Patients with luminal B tumors have a higher incidence of recurrence and lower overall survival rates compared to luminal A tumors (15). Bone is the most common area of recurrence, it is observed in 30% of patients with this subtypes, 13.8% of patients exhibit cancer recurrence in the liver and survival rate after relapse is approximately 1.6 years (17). Higher expression of genes associated with all proliferation result in a more aggressive

phenotype and luminal B has a greater expression of Ki-67 than luminal A and also increased expression of cyclin B1 and can express epidermal growth factor receptor (EGFR) (15,17). Approximately 6% of patients diagnosed with luminal B tumors may be ER negative and HER2 negative (17). For luminal-B cancers patients chemotherapies are considered the most effective course for treatment, therapies such as are paclitaxel and doxorubicin are used (12,15,17).

HER2-enriched tumors are approximately 15%-20% of breast cancer cases (15,17). The IHC and intrinsic subtype do not match perfectly since only 70% of HER2+ tumors will overexpress HER2 (15,17). It is not uncommon too for HER2+ breast cancers to express ER, but at low levels and these tumors are classified as luminal B and are generally resistant to hormonal therapies (15). HER2-enriched breast cancer cases are considered to be more aggressive tumors which are highly proliferative, have a high histological grade, and p53 mutations are common (12,15,17). The overall 10 year survival rate is 50%-55% for patients with this tumor subtype (15,17). Increased PI3K signaling, increased *IGF1R* levels, and lower lymphocytic infiltration can lead to decreased survival for patients with HER2-enriched breast cancer, and patients with this phenotype of HER2-enriched breast cancer have an overall 10-year survival rate of 12% (15,17,18). Monoclonal antibodies, such as trastuzumab have greatly increased efficacy of treatment of HER2-enriched tumors in both early stage and metastatic cases (12,15,17). These tumors are also more sensitive to chemotherapeutics, such as doxorubicin than their luminal A or luminal B counterparts (17).

Basal-like breast cancers are 10%-20% of all breast cancers (17). Basal-like, which generally corresponds to TNBC, is HR- and HER2-, and is particularly common among women with the *BRCA1* gene mutations (15,17). The term basal-like and TNBC has been used interchangeably in literature, even though they are not exactly the same, they have some

differences (15,17). These tumors commonly express genes found in breast myoepithelial cells such as CK5 and CK17, P-cadherin, caveolin 1 and caveolin 2, nestin, CD44, and EGFR (15,17). These tumors have the poorest prognosis among the molecular subtypes. They normally occur in women at a younger age than other molecular subtypes and have a higher incidence in Black women (12,17). These tumors tend to be invasive ductal carcinomas and are associated with a high histological grade and nuclear grade and have high mitotic and proliferative indices, as well as frequently spread to the lymph node (15,17). They may also have necrotic or fibrotic zones and have high rates of metastasis to the lung and brain and lymph nodes (15). Relapse in the first three years is not uncommon, even if the patient is responsive to chemotherapy (17). It has been hypothesized that the high rate of p53 mutations in these tumors may lead to their aggressiveness and the poor prognosis for patients (17). Currently chemotherapeutic drugs are used to treat these cancers, platinum salts carboplatin are a commonly employed for their treatment (14,17).

Normal-like breast tumors account for 5%-10% of all breast carcinomas (15,17). This tumor subtype is poorly understood and not well characterized (15,17). These tumors express genes more characteristic of adipose tissue genes, and their prognosis is considered slightly worse than luminal A breast cancers (12,17). These tumors do not express either hormone receptor or HER2 and are frequently designated as TNBC. They differ from basal-like tumors in that they are negative for CK5 and EGFR (15,17). Due to the paucity of cases some researchers doubt the existence of this subtype and believe it may be due to contamination of tumor tissue with normal tissue during testing (15,17).

Claudin-low breast cancer is a relatively new characterization, having been identified within the past 20 years and this subtype is identifiable by its low expression of genes involved in tight junctions and intercellular adhesion (12,15,17). This subtype is found in around 12-14%

of tumors and is normally found as a high grade invasive ductal carcinoma, that may also present as a metaplastic or medullary differentiation (17). Claudin low tumors have a poor prognosis despite having low expression of genes related to cell proliferation (15,17). However, they overexpress genes related to mesenchymal differentiation and epithelial-mesenchymal transition. These tumors also highly express certain immune related genes, indicating a high infiltration of immune cells in the tumors. These tumors are similar to TNBC, however about 20% of tumors are hormone receptor positive (17).

1.3 Histological Subtypes of Breast Cancer

Histological subtypes may also be used to identify breast cancer. Most breast cancers are carcinomas, specifically adenocarcinomas and this accounts for approximately 90% of all breast cancer cases (19). Ductal carcinomas account for around 80% of breast cancer cases, with invasive ductal carcinoma (IDC) being more common than ductal carcinoma in situ (20,21). Lobular carcinomas also occur, with invasive lobular carcinoma accounting for 5-15% of cases (21,22). Lobular carcinoma in situ (LCIS), can be difficult to identify due to lack of symptoms and problems in detecting these tumors during mammography (22). LCIS are most frequently identified in women undergoing biopsies for other causes (22). LCIS is extremely rare in men, is predominately observed in premenopausal women (22).

Invasive ductal carcinomas (IDC) are a diverse group of tumors that display a plethora of morphological features (22). IDC no specific type (NST) is the most common form of IDC constituting 40%-75% of cases. Tumor size and grade can vary widely, and necrosis and calcification are not uncommon in these tumors (22). IDC can be further subdivided based on differentiation: well-differentiated or grade 1, moderately differentiated or grade 2, and poorly differentiated or grade 3 (20). The determination of differentiation is based on nuclear

pleomorphism, gland and tubule formation, and mitotic index. IDCs express a wide range of molecular phenotypes, with no molecular classification being singularly associated with this histological subtype (22).

Ductal carcinoma in situ (DCIS) is noninvasive, having not invaded the stroma, it is limited to the ducts and lobules (22). Unlike IDC, DCIS has a maintained myoepithelial layer of cells, however these cells may have diminished in number (22). DCIS is potentially malignant and can be viewed as a precursor for IDC, and the higher the grade of tumor, the more likely it is to develop to IDC (21,22). Detection of these tumors has improved greatly with routine mammogram screenings (22). Prognosis after initial diagnosis is also generally good with DCIS tumors and death is rare. Death after initial diagnosis is usually attributable to undetected invasiveness of the tumor or recurrence of an invasive tumor after treatment (22). DCIS has 5 subtypes: comedo, cribiform, micropapillary, papillary, and solid; however, the majority of DCIS tumors exhibit a mix of these subtypes (20).

Invasive lobular carcinoma (ILC) is common in older women diagnosed with breast cancer and its incidence appears to be increasing in postmenopausal women (22). These tumors are generally uniform and are round and small. Various subtypes for ILC exist, classic type ILC tumors are made up of small, uniform cells lacking cohesion or in single-file linear cords that surround the lobules in a concentric pattern (22,23). Pleomorphic lobular carcinoma is similar to classic ILC, but displays signet ring cells and apocrine differentiation, this subtype is also likely to be HR negative and may express HER2 (22).

Lobular carcinoma in situ (LCIS), like DCIS is considered to be a potential risk factor for invasive lobular carcinoma, and approximately 25%-35% of patients diagnosed with LCIS will develop an invasive carcinoma which can be lobular carcinomas or ductal carcinomas (22,23). It

is not uncommon for LCIS to be found from a breast specimen or biopsy carried out for different reasons (22). LCIS typically does not have a high impact on the normal architecture of the breast, and lobules are typically recognizable. LCIS cells rarely present changes such as pleomorphism, changes in mitosis, and necrosis, that are commonly seen in ductal carcinomas (22).

Tubular carcinoma is typically a low-grade tumor that makes up 1%-2% of invasive breast carcinoma cases (22,24). These tumors are made up of well-differentiated tubular structures with an open lumina and are surrounded by abundant stroma and they are unlikely to recur locally, spread to lymph nodes, or metastasize to distant sites (22,24). These tumors primarily occur in women in their 60s or 70s and are more commonly found in White compared to Black women and are very rale in male patients (24). These tumors almost always ER+ and PR+ and are typically HER2- and EGFR- (24). Because these tumors have a good prognosis, less aggressive surgical treatment and adjuvant therapies are used and their ten year disease-free survival rates have been reported between 93.1%-99.1% and overall survival rates have been reported between 99%-100% (21,22,24).

Medullary carcinoma is a rare subgroup of invasive breast carcinomas that have highly malignant characteristics and a favorable prognosis when compared to invasive ductal carcinoma (21,22,25). Medullary breast carcinomas are 5% of all invasive breast carcinomas (21,22,25). The subtype has distinct features used for diagnosis that include: complete circumscription, syncytial growth pattern compromising 75% of the tumor, at least intermediate nuclear grade, diffuse lymphocytic infiltrate, and lack of intraductal components or glandular differentiation (22,25). These tumors are commonly associated with mutations in *BRCA1* gene, with 7.8%-19% of medullary carcinomas coming from patients with *BRCA1* mutation carriers and 35%-60% of *BRCA1* carriers with breast cancer experiencing medullary features (22,25). TP53 mutations are

also common in these tumors (25). Medullary carcinomas are generally associated with the basal-like molecular subtype (21,25). Like basal-like cancers, medullary carcinomas are also associated with onset at a young age, ranging from 45-54 years old (25). These tumors typically have an active immune response and show an increased number of activated cytotoxic lymphocytes, which some have attributed to the favorable prognosis of medullary carcinoma (21,22,25). These tumors are normally sensitive to radiation and chemotherapy and breast conserving surgery followed by radiation is prescribed for patients with tumors 3 cm or smaller. The overall 10 year survival rate is 74% for patients with medullary breast cancer (25).

Mucinous carcinoma is rare and accounts for around 2% of all invasive breast carcinomas (21,22,26). Mucinous carcinomas are divided into pure type mucinous carcinoma which is made up of tumor tissue with extracellular mucin production in over 90% of the tumor and mixed type mucinous carcinoma which also contains invasive ductal epithelial component without mucin (26). This subtype is most predominant in postmenopausal women, with an average age of onset of 70 years (22,26). The vast majority of mucinous carcinomas are luminal A molecular subtype tumors; and the androgen receptors may be expressed at low levels, and HER2 is not amplified in these tumors (21,26). Mucinous carcinomas are normally treated with surgery and post-operative hormone therapy (26). These tumors have a favorable prognosis and less frequent lymphatic metastasis compared to IDC (22,26). Pure mucinous tumors have a better prognosis than mixed mucinous tumors and the overall 5 year survival rate ranges from 81%-94% (26).

1.4 Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is a rare subtype of breast cancer that occurs 2%-4% of breast cancer patients and IBC has also been known to occur in men (27,28). In women, IBC tends to have an earlier onset with Hispanic women having the youngest average age of onset at

50.5 years, followed by Black women at 55.2 years, and White women at 58.1 years, and IBC is notably more predominant in Black women than White women (27,28). IBC is an aggressive tumor, with a poor prognosis and the 5 year overall survival rate is 30%-40% (28). These tumors usually present with pain, erythema, and swelling of the affected breast and they can be HER2-enriched, HR+, or triple negative (27,28).

1.5 Treatment of HR+ Breast Cancer

A variety of therapies exist for treating breast cancer ranging from surgery, radiation, chemotherapy, and targeted therapies (29). Treatments for patients are dictated by stage, molecular subtype, and heritable genetic factors and are used in combination in order to enhance the treatment efficacy (29,30). Despite a myriad of treatment types available to patients, in too many cases, they fail and new therapies that help prolong overall survival and disease free survival and decrease recurrence are urgently needed.

1.5.1 Surgery

It is common for patients with breast cancer to undergo some type of surgery in order to treat the tumor (29). There are multiple types of surgeries that can be performed depending on progression of the disease. For many patients, chemotherapy, radiation, or targeted therapies may be used before surgery in order to shrink tumor size and make it easier to remove (29,31). In early stages a lumpectomy may be the most appropriate course of treatment if the tumor is still small in size (29). A lumpectomy is sometimes also referred to as a breast-conserving surgery because only the tumor and some surrounding normal tissue are removed. These procedures may also remove some of the lymph nodes if appropriate. Lumpectomies can be outpatient procedures with short recovery times, allowing patients to return to normal activities within 5 to 10 days. After a lumpectomy, most women will receive radiation therapy to help prevent cancer from

returning to the breast. In some cases chemotherapy or a targeted therapy may be indicated (29,31).

A mastectomy is a procedure in which the entire breast is removed and in some cases the breast's skin, nipple, and areola may be preserved. Lymph nodes from under the arm are typically removed and in some cases the lining over the chest muscle is also removed. Mastectomies can be performed on one or both breasts and if both breasts are removed it is called a double mastectomy (29,31). Double mastectomies may be performed for patients whose risk of developing breast cancer is high, such as someone with a robust family history of breast cancer or someone who has a *BRCA* mutation (29). A mastectomy may be accompanied by reconstructive surgery in which an implant or tissue from a different part of the body is used and a nipple and areola may be added through tattooing by a plastic surgeon (29,31). Mastectomies may be indicated for women with small breasts but a large area of cancer, cancer in more than one part of the breast, cancer under the nipple, or if the patient is unable to receive radiation therapy. Recovery from mastectomies takes longer than lumpectomies and is typically observed after 3 to 4 weeks or longer (6-8 weeks) if a patient opts for reconstructive surgery (31). In the case of a mastectomy it is still common for patients to need radiation, chemotherapy, or targeted therapies (29,31).

1.5.2 Radiation

Radiation therapy is frequently used to treat breast cancer patients and may be used both before and after surgery. Radiation before surgery is used to help shrink the tumor to facilitate surgical removal and kill any potential cancer cells remaining (29,31). The duration of radiation treatment depends on severity of the tumor. After a lumpectomy, radiation is performed daily for three to four weeks, although some patients may receive it for less than three weeks. After a

mastectomy or if there is evidence for tumors in the lymph nodes, radiation is usually prescribed daily for six weeks. Radiation may also be used for metastatic breast cancer cases as palliative care to reduce symptoms due to cancer spread throughout the body (29). Radiation therapy is only contraindicated in patients with germline *TP53* mutations, in these cases a mastectomy is recommended and patients with a high likelihood of locoregional recurrence may receive radiation if indicated. Patients with germline *TP53* mutations are believed to be less able to repair DNA damage due to radiation therapy and this could result in a greater risk for radiation-associated sequelae (32).

1.5.3 Chemotherapies for HR+ Breast Cancer

Chemotherapy is important for the treatment of breast cancer and in many breast cancers patients, chemotherapy is used to prevent recurrence in patients with stages I-III breast cancer (33). Because of the success of hormone therapies, chemotherapies are either used in combination with other drugs or as later line treatments in patients with HR+ breast cancer (34). Assessing risk and considering tumor burden and subtype is also important for selecting appropriate chemotherapy regimens (33,34). High-risk patients and patients who express lower levels of ER and PR may receive therapies that include both anthracycline followed by taxane (34,35). In patients with advanced breast cancer endocrine therapies considered best line of treatment unless endocrine resistance occurs in which case single agent chemotherapy would then be employed (33,34).

1.5.4 Hormone Therapies

Hormone therapies are beneficial for breast cancers patients that express hormone receptors which are vital for proliferation, growth, and survival among other pathways. By inhibiting these pathways, hormone therapies can mitigate the ability of tumors to thrive and possibly survive (33-36). A variety of hormone therapies exist that target the estrogen receptor,

including therapies that may interfere with ER signaling, induce ER degradation, or interfere with its production of its ligands (33-35).

1.5.4.1 Selective Estrogen Receptor Modulators

Targeting the estrogen receptor and pathways that affect the estrogen receptor have been important for treating breast cancer patients since most early stage breast tumors express the estrogen receptor (ER) (36). Selective estrogen receptor modulators or SERMs have been integral for treatment of ER+ breast cancer tumors and tamoxifen, a SERM, has been the first line of treatment for ER+ tumors for over 30 years (35,37). It is preferred for use in premenopausal women because of potential drug resistance, blood clots, and increased risk of endometrial cancer associated with tamoxifen treatment in postmenopausal women (35-37). These potential side effects however are decreased in premenopausal women (37). Tamoxifen acts as a prodrug that is rapidly metabolized in the liver, its metabolite, endoxifen, has a high affinity for binding and blocking ER action as an antagonist. Tamoxifen is generally used as an adjuvant treatment for five years and this treatment increases disease free survival (37). Fifteen years after diagnosis in ER+ patients that used adjuvant tamoxifen there is a decrease in mortality and there is a 50% decrease in recurrent breast cancer (32,27).

Tamoxifen may also be used as a chemopreventive agent and was the first drug to be designated as a preventative agent for any cancer. It is has been found to be efficacious in both premenopausal and postmenopausal women for cancer prevention but does not decrease mortality. Tamoxifen is generally recommended for women who are premenopausal, for women who have a high likelihood of developing cancer in the near term, and because of genetic or other established risk factors that do not include a previous personal history of breast cancer (37).

Raloxifene is a second generation SERM primarily used for treatment of osteoporosis, however, in the United States it is also approved as a preventative treatment for postmenopausal women for reducing the risk of invasive breast cancer (37-39). Unlike tamoxifen, raloxifene does not undergo metabolic activation and does not function as a prodrug (38). It competes with estrogen for binding to the estrogen receptor and has been found to have positive effects on biomarkers associated with breast cancer (37-39). Increased IGF-I and decreased IGF binding protein-3 (IGFBP-3) are associated with increased risk of breast cancer and increased sex hormone-binding globulin (SHBG) is associated with a decreased risk of breast cancer. Raloxifene treatment results in decreased IGF-I and increased IGFPB-3 and increased SHBG (38,39). Currently raloxifene treatment for breast cancer prevention is recommended for postmenopausal women 35 or older who have a greater than 1.7% chance of developing breast cancer in the next five years or have a history of LCIS (38). Raloxifene differs from tamoxifen in that it is only recommended for postmenopausal women and is not recommended for treatment of noninvasive breast cancers (38,39).

Toremifene was first approved in 1997 for treatment of metastatic breast cancer in postmenopausal women who are ER+ and in tumors with unknown ER status. Toremifene differs from tamoxifen by one chlorine atom and is not a prodrug like tamoxifen (40,41). Toremifene may be of benefit to patients with CYP2D6 mutations that complicate metabolism of tamoxifen, thus decreasing its efficacy (41). Toremifene and tamoxifen exhibit similar side effects and overall 5-year survival rates for both drugs are similar (40,41). Currently, toremifene is not approved for use in premenopausal women, and few clinical trials have taken place to determine clinical utility (41).

1.5.4.2 Selective Estrogen Receptor Degraders

Selective Estrogen Receptor Degraders, or SERDs, function differently than SERMs in that they bind to the estrogen receptor and induce degradation thereby preventing dimerization and activation of ER signaling pathways (40). Currently, only one SERD, fulvestrant, is approved for clinical treatment of breast cancer. However, lack of bioavailability of this drug coupled with its toxicity has led to efforts in identifying other SERDs (40,42). SERDs differ from SERMs in that they act as full antagonists, whereas SERMs may act as antagonists or agonists depending on the tissue. SERDs, such as fulvestrant, act by destabilizing helix 12 region of the estrogen receptor thereby dissociating it from the rest of the ligand binding domain. It is thought that fulvestrant binds to the estrogen receptor when it is in its monomeric conformation thus inhibiting dimerization and activating degradation through the ubiquitin-proteasome pathway (40).

1.5.4.3 Aromatase Inhibitors

Aromatase Inhibitors (AI) are used primarily for treating postmenopausal ER+ breast cancer patients and acts by reducing the amount of circulating estrogen by inhibiting its production (43). AIs inhibit the aromatase enzyme thereby blocking the conversion of androgens to estrogens (43-45). AIs are less efficacious in premenopausal women due to their inability to fully suppress ovarian estrogen production. Upon administration of an AI to premenopausal women, gonadotrophins increase, and this can result in induction of the ovarian aromatase promoter (43). AIs are effective in women that have been previously treated with tamoxifen and have undergone surgery for breast cancer (43,44). There are three generations of AIs, each generation is more effective and is accompanied by fewer side effects than the previous generation (43-45).

The first generation AI, aminoglutethimide, was an effective treatment but was poorly tolerated in women (43,44). It's major side effects included liver toxicity, inhibition of cortisol production, and hypothyroidism. Aminoglutethimide may be used as a second line treatment in tandem with hydrocortisone for patients who do not show any response to tamoxifen treatment, and it may also be a potential first line treatment for women that have had an ovariectomy (44). Second generation AIs, include fadrozole and 4-hydroxyandrostenedione (4-OHA) (43,44). Fadrazole is a non-steroidal AI that has efficacy similar to tamoxifen as a first line treatment for patients with advanced breast cancer and it is well tolerated and has fewer side effects, particularly in patients with a potential for thromboembolic events. 4-OHA is a steroidal AI and is also highly effective for treating patients with ER+ tumors but is ineffective in patients who have not received previous hormone therapy. 4-OHA may be effective in premenopausal women when combined with goserelin, which suppresses the production of estrogen (44).

Third generation AIs are currently in use and like second generation AIs are a mix of steroidal and non-steroidal (43,44). Third generation AIs are more selective than their predecessors, having less impact on interrelated steroidal pathways, as well as fewer side effects (44). Anastrozole and letrozole are non-steroidal and exemestane is steroidal. Anastrozole inhibits aromatase activity without affecting other hormones (43,44). Anastrozole has fewer treatment-related adverse events than tamoxifen and has a lower recurrence rate and significantly increased disease free survival when used as a first line treatment compared to tamoxifen (44).

Letrozole is another third generation AI that is a specific non-steroidal. It reduces estrogen biosynthesis by binding to the heme portion of aromatase. Letrozole is more potent than aminoglutethimide by at least two orders of magnitude and is more effective at improving response rates and time to progression (TTP) in postmenopausal women compared to tamoxifen.

However, letrozole did not appear to increase patient survival compared to tamoxifen. Side effect profiles differ, but some studies suggest that letrozole has a more favorable adverse event profile compared to tamoxifen, however, compared to tamoxifen, letrozole treatment increases the risk of fractures (44).

Exemestane is the only steroidal AI and causes a long-term decrease in plasma and urinary estrogen levels due to its ability to irreversibly bind the aromatase enzyme (44). Exemestane can be used for treatment of early-stage breast cancer or late stage breast cancer (46). In early stage breast cancer cases, switching from tamoxifen to exemestane has been associated with increased occurrence of disease-free survival, but not overall survival (43). Like the other third generation AIs, exemestane, increases risk for bone fracture when compared to tamoxifen (44,46). It has been reported that previous treatment with tamoxifen may reduce the potential for fracture in women later treated with AIs and patients on AI treatment are also advised to take vitamin D or prescribed bisphosphonates to prevent bone mineral loss (43). Overall AIs are well tolerated, with hot flashes being the most common adverse event (43,45,46).

1.5.5 Other Therapies for HR+ Breast Cancer

1.5.5.1 CDK4 and CDK6 Inhibitors

CDK4 and CDK6 inhibitors are currently approved for patients with HR+/HER2- advanced breast cancer (33,35,47). These inhibitors act at the G1-to-S cell cycle checkpoint. This checkpoint is controlled D-type cyclins, which are commonly over expressed in HR+ breast cancer and they activate CDK4 and CDK6. Thus inhibition of CDK4 and 6 prevents progression of the cell cycle leading to cell cycle arrest (47). Currently three CDK4/6 inhibitors are approved: Palbociclib, ribociclib, and abemaciclib (35,47). Palbociclib is approved as a first line treatment in tandem with an AI for treating postmenopausal women with advanced breast cancer and second-line treatment with fulvestrant in patients with advanced breast cancer and any

menopausal status. Ribociclib was approved as a first line treatment in combination with letrozole for postmenopausal women with advanced stage breast cancer. Abemaciclib was approved as a first line treatment in combination with AIs for treating postmenopausal women with advanced breast cancer and as a second line treatment in combination with fulvestrant. Abemaciclib may also be used as a single agent as a third line or later treatment for treating women and men who have not received prior therapy with a CDK4/6 inhibitor. Palbociclib, ribociclib, and abemaciclib have never been directly compared in clinical studies and are therefore considered to have equivalent efficacies. Currently these drugs are only approved for treating postmenopausal women and studies involving premenopausal women are ongoing. CDK4/6 inhibitors have been generally found to be well-tolerated and their safety profiles do not indicate that one drug is more favorable than another. Current studies are investigating combinations of CDK4/6 inhibitors with PI3K inhibitors and immunotherapies such as PD-1 and PD-L1 inhibitors; assessing CDK4/6 inhibitors in early stage breast cancer; and assessing effectiveness of CDK4/6 inhibitors in combination with anti-HER2 therapies (47).

1.5.5.2 HER2-Targeted Therapies

For women who express both ER and HER2, HER2-targeted therapies are advantageous if HER2 is overexpressed, and HER2-targeted therapies are recommended for HER2-overexpressing tumors regardless of ER status (48). The antibody, trastuzumab was the first of these HER2-targeted therapies developed in the 1990s that interfere with HER2 signaling by inhibiting dimerization, causing receptor internalization or degradation. This results in inhibition of PI3K-AKT signaling and growth promoting pathways and increases antibody-dependent cellular cytotoxicity (ADCC) (49). Trastuzumab along with chemotherapy is considered the first line of treatment of both early stage and late stage breast cancers that overexpress HER2 (48,49).

Pertuzumab, like trastuzumab, is a monoclonal antibody that binds extracellularly to HER2 (49,50). Pertuzumab binds in a different location, domain II, than trastuzumab which binds in domain IV. It acts by preventing dimerization of HER2 and HER3 thus decreasing downstream kinase signaling pathways such as PI3K/Akt and inducing ADCC. Pertuzumab has lower efficacy compared to trastuzumab. The combination of pertuzumab, trastuzmab, and doecetaxel is highly effective when used as a first line treatment for metastatic breast cancer and increases progression free survival without increasing cardiac toxicity (50).

Lapatinib is a small molecule EGFR and HER2 inhibitor approved as a first line treatment for metastatic breast cancer cases that are HER2+ and ER+. Lapatinib may also be used in combination with capecitabine in patients who have already tried trastuzumab and anthracyclines. Unlike trastuzumab or pertuzumab, lapatinib may be efficacious for treating trastuzumab-resistant patients because of its ability to bind intracellularly to wild type and mutant HER2. Lapatinib may also be more beneficial for some patients because of its ability to inhibit both EGFR and HER2. For patients whose breast cancer has metastasized to the brain, lapatinib can be a viable treatment option due to its ability to cross the blood-brain barrier. Lapatinib also is less cardiotoxic than trastuzumab, although it is still recommended that clinicians evaluate patients for potential cardiotoxic effects. Lapatinib can also be administered orally whereas trastuzumab and pertuzumab are administered intravenously (51).

Neratinib which was first approved in 2017 is a small molecule irreversible inhibitor of EGFR, HER2, and HER4 that improves 2 year invasive disease free survival rate after treatment with chemotherapy and trastuzumab-based adjuvant therapy (49,52). Neratinib is taken orally, requiring 6 pills a day, and this may reduce compliance due to pill burden. It also must be taken with food and antacids and may require additional medication to treat diarrhea which is a side

effect of neratinib. Like lapatinib, neratinib is able to cross the blood-brain barrier and may be effective for treating HER2 breast cancer that has metastasized to the brain; however, clinical studies have yet to be done to confirm this possibility. Clinical trials have also reported that patients who receive a year of trastuzumab followed by a year of neratinib experience less cardiac-related incidents compared to patients that have received trastuzumab treatment for two years (52).

1.5.5.3 mTOR/PI3K Inhibitors

Overactivation of mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinases (PI3K) is found in many cancers, and there is evidence that activation of the mTOR/PI3K pathway promotes anti-estrogen resistance (36,53,54). Phosphorylation of S167 on ESR1 via p70S6K which is regulated by mammalian target of rapamycin complex 1 (mTORC1) induces activation of ESR1 and phosphorylation of ESR1 at S104/S106 by mTORC1 induces transcription of ESR1-target genes (54). Currently everolimus plus exemestane is approved for treatment of postmenopausal women with HR+/HER2- breast cancer that is locally advanced, unresectable, or metastatic after treatment with letrozole or anastrozole has failed (36,54,55).

PI3K promotes estrogen receptor activity and mutations to PI3K mediate resistance to endocrine therapy (36,55). Clinical trials with PI3K inhibitors are still early in development and ongoing at this time. Thus far pan-PI3K inhibitors have been found to not significantly improve breast cancer patients survival and these inhibitors exhibit high toxicity, and difficulties with treatment compliance have resulted in termination of clinical trials. Even selective PI3K inhibitors lack significant efficacy and significant adverse event profiles associated with these inhibitors have hindered continued patient participation in clinical trials. It has been suggested that PI3K inhibitors may have more promise in treating metastatic or recurrent disease due to

greater dependence on the PI3K pathway for survival of late but not early stage tumors. Currently the FDA approves alpelisib, a selective PI3K inhibitor, in combination with fulvestrant for women with *PIK3CA* mutations with HR+/HER2-, advanced or metastatic breast cancer following an FDA approved test for the mutation (55). For women with HR+ breast tumors approximately 34% of these patients will have altered *PI3KCA* expression (53) It has also been noted that the presence of PIK3CA mutations have yet to be identified as a predictor of outcome or response to endocrine treatment (55).

1.5.5.4 PARP1 Inhibitors

Poly (adenosine diphosphate [ADP]-ribose polymerases, or PARPs, are a family of enzymes that function in DNA repair, gene transcription, chromatin architecture, and apoptosis in normal human cells. PARP1, plays an important role in single-stranded DNA base-exicision repair and is also the most abundant of the PARP enzymes. By inhibiting PARP single strand DNA breaks occur, leading to double strand breaks that can ultimately be repaired, unless the mechanism for double-strand breaks is impaired which would induce apoptosis and cell death (36,56). This is the case in cells with BRCA1 and BRCA2 mutations where remediating double-strand breaks is impaired. Individuals with BRCA1 mutations have a high incidence for developing TNBC and many studies evaluating PARP1 inhibitors have focused on patients that carry either BRCA mutation (36). In 2018 olaparib and talazoparib were approved for HER2-locally advanced or metastatic breast cancer with germline *BRCA1/2* mutations (56).

1.5.6 Potential Targets for HR+ Breast Cancer Therapies

Current therapies for treating breast cancer have improved survival, prolonged life, and improved quality of life for many women, but even with these treatments some women do not have viable therapeutic options. Certain types of breast cancer, like TNBC, are aggressive and current treatments are considered less than ideal. Targeted treatments such as tamoxifen and

trastuzumab are highly effective, but resistance and recurrence are not uncommon problems in women treated with these drugs. The current landscape of new drug development has often focused on new targeted therapies, which have proven beneficial for treating patients with HR+ and HER2-overexpressed breast cancers. Many new targets have been identified, and some have proven more promising than others at providing potentially viable treatments for breast cancer (36).

1.5.6.1 bcl-2 Inhibitors

B-cell lymphoma 2 (bcl-2) is a protein regulator of apoptosis and high expression of bcl-2 protects tumor cells in many cancers, including breast cancer, from cell death (53,57). One bcl-2 inhibitor, Venetoclax, is a first of its class drug and is currently approved for certain leukemias (53,57,58). Venetoclax has undergone phase I and phase I/II clinical trials for breast cancer. Bcl-2 is an estrogen-responsive gene and is overexpressed in approximately 80% of ER+ breast tumors (58). In preclinical studies and phase I clinical trials combination of venetoclax and tamoxifen showed antitumor activity including in heavily pretreated patients (53,58). Patients with ER mutations were observed to have prolonged stable disease and tumor response to treatment (58).

1.5.6.2 Histone Deacetylase (HDAC) Inhibitors

Aberrant alterations of histones are associated with multiple cancers, including breast cancer (59-62). HDACs are integral to chromatin remodeling and epigenetics and play a role in modifying histones which regulate interactions between nucleosomes. Both HDACs and histone acetyltransferases (HATs) affect lysine acetylation of histones, with HDACs removing acetyl groups and HATs adding them. HATs generally promote transcriptional activation, whereas HDACs remove acetyl groups and promote transcriptional repression and gene silencing. Increased levels of histone deacetylation is linked to cancer and affects well characterized

oncogenes and tumor-suppressor genes, such as: p53, RUNX3, STAT3, beta-catenin, ER, Myc, EKLF, GATA family, HIF-1 alpha, MyoD, NF-κB, or Foxp3 (59). In breast cancer, high expression of HDAC1 is associated with HR+ breast tumors. However, high expression of HDAC1 and HDAC6 has been found to be a good prognostic factor for ER+ IDCs. HDAC1 and HDAC6 may be independent prognostic factors for breast cancer, and HDAC1 for ER+ breast cancers in particular (61).

Currently a number of HDAC inhibitors are approved for the treatment of cancer. Panobinostat is approved for treating multiple myeloma and Vorinostat is approved for treating cutaneous T-cell lymphoma, and both are under investigation for their potential in treating breast cancer (36,59). Entinostat, is another HDAC inhibitor which is selective for targeting class I HDACs: HDAC1, HDAC2, and HDAC3; whereas Panobinostat and Vorinostat are considered pan-HDAC inhibitors that target class I, class II, and class IV HDACs. Currently, HDAC Inhibitors have not yet been approved for treating breast cancer in the United States. Entinostat has undergone phase III clinical trials combined with endocrine therapy. Phase II studies have been performed analyzing a multitude of HDAC inhibitors as monotherapies and in combination with chemotherapy (59).

HDAC inhibitors have shown promising results in preclinical settings, but when translated to clinical setting, results have been less conclusive. Entinostat has shown promise in combination therapies for both HR+ and HER2+ cancers, with favorable results when combined with AIs or HER2-targeted therapies. Vorinostat, has not shown efficacy as a monotherapy, but has a favorable toxicity profile and is well-tolerated and has potential in combination therapies. Vorinostat has undergone phase II clinical trials to assess its efficacy in combination therapies with tamoxifen and trastuzumab (36,59). Panobinostat exhibits strong anticancer activities in

preclinical studies on breast cancer models. There are currently very few clinical trials involving Panobinostat and they are predominately early, in phase I. Phase II studies are planned to assess Panobinostat and letrozole in patients with postmenopausal metastatic breast cancer. Although HDAC inhibitors have been available for a long time, their role as anticancer drugs is still being defined. For breast cancer, HDAC inhibitors have shown promise in combination therapies in clinical settings, and further clinical trials are needed to confirm their potential benefits (59).

1.5.6.3 HSP90

Heat Shock Protein 90 (HSP90) is a chaperone protein that assists in folding and stabilization of proteins. Many of the proteins that use HSP90 are important for cancer cell propagation, thus suggesting it may be a drug target for treating cancer. Breast cancers that express high levels of HSP90 exhibit higher nuclear grade, tumors are larger, there is a likelihood of lymph node involvement, and higher expression of HER2 and ER (36). A number of clinical trials have been conducted with HSP90 inhibitors, thus far finding particular efficacy among HER2-overexpressing phenotypes. Development of second generation HSP90 inhibitors has helped to reduce liver toxicity initially associated with these inhibitors in clinical trials as well as improving oral bioavailability and drug metabolism (63).

1.5.6.4 IGF and IGFR Inhibitors

Insulin-like growth factor (IGF) inhibitors have been investigated as potential therapeutic targets, particularly for ER+ breast cancers. IGF-I and IGF-II are important for development of bone and skeletal muscle and their receptors help to regulate cell functioning. In certain types of cancers, including breast cancer, overexpression of IGF and IGF-receptors (IGF-R), may make those pathways a suitable drug target (36). A number of strategies have been employed to target the IGF-R axis including monoclonal antibodies targeting the receptor, tyrosine kinase inhibitors, and ligand neutralizing strategies; this final strategy has been the least investigated in a clinical

setting. Monoclonal antibodies targeting IGF1R have predominately produced negative results in phase III clinical trials producing negative off-target effects such as hyperglycemia and hyperinsulinemia that have led to patient discontinuation and trial termination (64).

1.5.6.5 PD-1/PD-L1 Inhibitors

Breast cancers that are particularly aggressive, may be most likely to benefit from immunotherapies. Those with activated T regulatory cells (Treg) and those that have an inflamed phenotype, with high levels of dendritic cells and CD8+ T cells in the tumor microenvironment, would be potential candidates for immune therapies, however these may make up a small percentage of breast cancer phenotypes, and have thus far only found success in TNBC subtype (65). In 2019 the FDA granted accelerated approval for atezolizumab in combination with nab-paclitaxel for women with locally advanced or metastatic TNBC who cannot undergo surgery and are PD-L1-positive (65,66). Atezolizumab is being investigated for HR+ breast cancers in combination with other therapies that are already approved like tamoxifen and some that are not currently approved for breast cancer such as Entinostat and bevacizmab (67-69).

In July 2021 the FDA approved pembrolizumab, a monocolonal antibody that targets PD-1, for treatment of women with high-risk early-stage TNBC. It was approved for treatment in combination with chemotherapy as a neoadjuvant and as a single agent adjuvant treatment after surgery (70). In addition to TNBC patients, pembrolizumab has undergone clinical trials for patients with luminal breast cancers and showed only modest efficacy. Clinical trials for women with ER+ status, have focused on women with metastatic disease, are high-risk, or endocrine-resistant. Avelumab is another monoclonal antibody in clinical trials for breast cancer treatment. Avelumab targets PD-1/PD-L1 interaction but not PD-1/PD-L2 interactions and unlike other antibodies displays ADCC cytotoxicity. Treatment with avelumab has shown modest efficacy in

clinical trials for treatment of breast cancer, with patients with TNBC showing slightly better outcomes than those with other types of breast cancer (65).

1.5.6.6 Selective Androgen Receptor Modulators

Anabolic steroids such as testosterone historically were used to treat breast cancer; however due to virilization, they are no longer indicated for treatment (71,72). Although anabolic steroids have fallen out of favor as a treatment, interest in targeting the androgen receptor (AR) has remained due to its high expression in breast cancers and preclinical evidence that targeting AR would be beneficial (71-73). AR is more highly expressed than either ER or PR, and is highly expressed in TNBC, therefore recent approaches have been to target the AR using selective AR modulators (SARMs) (71,72). AR expression is a positive prognostic factor in breast cancer and upregulation of AR is associated with increased antitumor activity (71,73). SARMs do not exhibit the same virilizing side effects as anabolic steroids and have demonstrated increase in lean body mass and bone mass in postmenopausal women during clinical trials making them favorable for clinical use (71). Currently clinical trials are ongoing for the SARM, Enobosarm, which has shown favorable results in phase II clinical trials and has exhibited significantly increased quality of life measurement as well as a tolerable side effect profile (71,72). Planning for phase III clinical trials underway for patients with ER+/AR+/HER2- metastatic breast cancer (72).

1.5.6.7 Selective Estrogen Receptor Covalent Antagonists

A new class of endocrine therapies referred to as selective estrogen receptor covalent antagonist (SERCA) has been described in recent years (74). This antagonist functions by targeting a cysteine residue (C530) that is unique to ER and not found on other hormone receptors. H3B-5942 is a SERCA that has demonstrated ESR1 antagonism by inducing a unique antagonist conformation (74,75). Research is ongoing to identify a potential second generation of

SERCA compounds that could improve potential fallout from H3B-5492 such as resistance due to mutation at C530 (75). H3B-5942 has undergone phase I/II clinical trial and has demonstrated a manageable safety profile and antitumor efficacy as a single agent in patients with metastatic ER+ breast cancer (76).

1.5.6.8 TROP-2 Inhibitors

Trop-2 is a transmembrane protein highly upregulated in some cancers and associated with poorer outcomes for some cancers. Phosphorylation of Trop-2 is likely involved in the release of intracellular calcium which can activate Raf and NF-κB pathways (77,78). Trop-2 increases cyclin D1 and cyclin E and can bind when bound to cyclin D1 acts as an oncogene to promote cell cycle progression. Trop-2 has also been found to induce MAPK signaling which upregulates a number of genes involved in angiogenesis, proliferation, apoptosis, invasion, and metastasis (78). In 2021, the FDA approved sacituzumab govitecan for patients with TNBC that metastatic or unresectable and locally advanced and have received two or more lines of prior systemic therapies (79). Phase II and phase III clinical trials evaluating sacituzumab govitecan in HR+ breast tumors are ongoing and are evaluating sacituzumab as a monotherapy or in combination with Pembrolizumab or chemotherapeutics. All trials are assessing use of sacituzumab govitecan in patients with metastatic breast cancer (80-82).

1.5.6.9 VEGF Inhibitors

Vascular endothelial growth factor (VEGF) is responsible for angiogenesis, in normal tissue it supports blood vessel formation and maintenance of newly formed blood vessels, but in cancer supports tumor growth. In animal models estrogen is known to modulate angiogenesis, and estrogen has been shown to increase VEGF expression, while estrogen withdrawal has been shown to decrease VEGF expression (83). High VEGF expression is associated with early recurrence and resistance to hormone therapy (84). The monoclonal antibody, bevacizumab was

previously approved by the FDA for treatment of metastatic breast cancer but was revoked in 2010 because the benefits of treatment did not outweigh the risk (83). Despite its removal, clinical trials for bevacizumab in HR+ breast cancer are ongoing (67,85-87).

1.6 Resistance to Endocrine Therapies

Resistance to therapy whether it be a targeted therapy or systemic therapy is commonly observed when treating breast cancer (88). For ER+ breast cancer patients efficacy of endocrine treatment relies on ER status and signaling which may change over the course of treatment (89). Resistance to endocrine therapies may either be de novo or acquired, and observations in clinical settings usually denote between these two categories, although additional subcategories have been suggested (90,91). Currently there is no exact definition of endocrine therapy resistance in breast cancer, and most data is derived from preclinical studies rather than clinical trials (90). A better understanding of therapy resistance may also help to design new therapies that are more effective for treating drug-resistant tumors (91).

1.6.1 De novo Resistance

Estrogen receptor alpha (ESR1) is viewed as the main contributor of tumor growth and survival in ER+ breast cancers (90). However, there is evidence that ESR1 is not the only survival pathway for these tumors, and de novo resistance can arise from other survival pathways (90,91,93). De novo resistance may take two forms: resistance to all endocrine therapies and resistance to some endocrine therapies while still sensitive to others (90,91). Receptor tyrosine kinases (RTKs), the family of receptors that include HER2 and EGFR, phosphorylate ER and its co-regulators directly, and are implicated in endocrine therapy resistance (89-93). Overexpression of either HER2 or EGFR in ER+ breast cancers is commonly associated with increased de novo resistance to antiestrogenic therapies (93). Phosphoinositide 3-kinase (PI3K) is an omnipresent signal transduction pathway that interacts with RTKs and plays an important

role in cell growth and survival among other functions (90-93). PI3K also appears to mediate endocrine therapy resistance (89-93). De novo resistance to tamoxifen may arise due to polymorphisms in CYP2D6 which prevent tamoxifen metabolism into the more active 4-hydroxytamoxifen metabolite (90,92). This polymorphism commonly occurs in White women at a rate of 7%-8% and in approximately 1% of Asian women with breast cancer (90).

1.6.2 Acquired Resistance

Acquired mutations occur after an initial response to a treatment that declines in its effectiveness followed by subsequently shorter responses to other endocrine therapies (90,91,93). Various mechanisms of acquired resistance exist in which the ER+ tumors gradually shift away from dependence on ER and estrogen for growth and survival (90-92). These pathways can include a decrease or loss of ER during the course of treatment; loss of PR which is associated with poorer outcomes; upregulation of HER2 which becomes the main driver of growth for the tumor (90-93). It also includes the pathways previously described for de novo resistance for RTKs and PI3K. Activation of RTKs may also include insulin-like growth factor 1 receptor (IGF1R), which like other RTKs, leads to increased activation of downstream mediators resulting in increased cellular proliferation and decreased ER dependence (89-93). IGF1R initiates the IGF1R-IGF1 signaling axis which can enhance the signaling of other RTKs and can mediate endocrine therapy resistance through mitogen-activated protein kinase (MAPK) and PI3K signaling (89-92). Activating mutations in PI3K, loss of expression of PTEN, a suppressor of the PI3K pathway, and increased upstream signaling from RTKs can lead to dysregulation of PI3K (90-93). This dysregulated signaling is associated with increased protein kinase B (Akt) activity and expression and high Akt expression has also been associated with endocrine resistance (93). mTOR, which is responsible for phosphorylation of ER is further implicated in the PI3K/Akt/mTOR pathway and is associated with downregulation ER and promotion of cell

growth and proliferation (89,93). Cell cycle regulators also appear to play a role in endocrine therapy resistance as aberrant expression of these regulators has been associated with resistance (89-92). Positive regulators that are overexpressed such as Myc, cyclin E1, and cyclin D1 can propel endocrine resistance by activating CDKs which are important during the G1 phase and by decreasing expression of p21 and p27, which act as negative regulators. Decreased expression of p21 and p27 is also implicated in endocrine therapy resistance (90-92).

1.6.2. Activating Mutations to ESR1

Unlike other mechanisms of endocrine resistance, mutations to ESR1 can provide a role for ER-dependent growth in the absence of estrogen. These mutations are generally found in the LBD of ESR1 and confer ligand independent constitutive activity (90,92,93). It is rare that mutations to ESR1 occur in the primary tumor, and this is observed in less than 1% of patients; however, for patients with recurrent metastatic breast cancer that have had endocrine treatment, these mutations occur at a high incidence (91,92). It is estimated that ESR1 mutations occur in 20%-40% of cases and women with metastatic breast cancer and women previously treated with AIs have higher rates of mutant ESR1 expression. Poorer clinical outcomes have been associated with patients with these mutations compared to those expressing wild-type ER and typical treatments used are less efficacious in these patients (89,90).

In women that develop somatic mutations to ESR1, the efficacy of endocrine therapies is greatly reduced (89,90). It has been postulated that the conformational changes in ESR1 that occur due to these mutations can enable insensitivity to endocrine therapies through constitutive activation of the receptor. When wild-type (WT) ER is not ligand bound the LBD is inactive and interacts heat shock proteins, such as HSP90, preventing dimerization (94). If estrogen binds ESR1, the HSPs are lost, the ER dimerizes, and helix 12 folds over the ligand binding pocket

(LBP) to form a hydrophobic groove that binds with coactivators, such as steroid receptor coactivators (SRC). In contrast, tamoxifen binds to the LBD and helix 12 is prevented from forming an active conformation by blocking the hydrophobic groove where coactivators would normally bind (94,95).

Activating ESR1 mutations are generally found in the LBD between amino acids 304-554 and most mutations occur between amino acids 534-538. These mutations help maintain an *apo*-conformation for ESR1, allowing it to remain active in the absence of ligand (90,92-94). Many of these mutations appear to provide this critical function by modulating the h11-12 loop (94-96). Mutations at Y537, and Y537S are among the most common mutations observed, and they have been extensively studied as mechanistic models of endocrine resistance (94,97,98). Other mutations at Y537 such as Y537C and Y537N have been described in patients (94,95,97,98). D538G is the most common mutation and has been observed in approximately 20% of patients previously treated with AIs for metastatic breast cancer. Mutations also appear at L536, S463P, and E380Q, among other locations, but are generally found less frequently than mutations at Y537 and D538 (94,97,98).

The tyrosine at 537 is the most frequently observed activating ESR1 mutation (94,97). The Y537S mutation exhibits high endocrine therapy resistance and exhibits nearly full constitutive activity and occurs more than any other mutation at Y537 (94,95,97,99). In vivo studies show that higher doses of endocrine therapies are required for Y537S ESR1 expressing cells to fully inhibit the receptor (94,99). These characteristics are less prominent for tumors expressing the Y537N mutation and even more so for the Y537C mutation (94,97). X-ray crystallography has been used to investigate how the Y537S mutation is able to maintain its high constitutive activity (94). A strong hydrogen bond between S537 and D351 appears in ESR1

with the Y537S mutation thus facilitating an agonist conformation (94,99). This differs from WT ESR1 which appears to experience strain in the agonist conformation due to aqueous exposure of hydrophobic residues spanning 533-536 due to hydrogen bonding between Y537 and N548 (94).

The D538G mutation has constitutive activity comparable or slightly less than Y537S (94-98). D538G differs from WT in that it changes the loop conformation at the beginning of helix 12 which allows enhanced side chain packing of hydrophobic residues (94). Similar to the Y537S mutation, D538G disrupts normal hydrogen bonding at Y537 and N348 while having weaker hydrogen bonding between G538 and D351 compared to S537 and D531 (94,99). The D538G mutant is able to bind SRC3 without the presence of estrogen (99). Previous *in vitro* and *in vivo* studies have indicated that, like the Y537S mutant, D538G mutation is less sensitive to fulvestrant, and a higher amount of fulvestrant is required to inhibit ESR1 compared to WT ESR1 in breast cancer cells (98,99).

Mutations at L536 are found in less than 1% of patients with AI-treated metastatic breast cancer (94). L536P is found to be the most common of L536 mutation followed by L536R and L536H (97). The ESR1-L536 mutant exhibit less constitutive activity than previously described mutations, although in some cases, such as ESR1-L536R, resistance to endocrine therapies is also observed (94,97). While X-ray structures are not available for mutations at this site it has been proposed that the changing of hydrophobic leucine to less hydrophobic or polar residues may allow for improved arrangement of the hydrophobic side chains (94).

S463P is a mutation found in a loop between helix 9 and helix 10 and it is not entirely clear how this mutation confers constitutive activity or endocrine resistance, even though like the Y537S, ESR1-S463P exhibits high stability in the presence or absence of ligand (94,98). It has been suggested that the proximity of the loop to the dimer interface affects the stability of dimers

and that the changing of the residue from serine to proline could favor an activated LBD that would release HSPs bound to the protein. This mutation is found in approximately 2% of patients with metastatic breast cancer treated with AIs (94). While the S436P mutation confers lower constitutive activity and doesn't show as much resistance to endocrine therapies and *in vitro* studies with this mutation show only slight resistance to fulvestrant, the S463P mutation has been reported to drive estrogen independent growth similarly to or better than other mutations (94,98). Due to its ability *in vitro* to lack coactivator binding and promote activity, S463P may also promote estrogen independent activity through mechanisms yet to be described (98).

Mutations at E380 are the third most common type of ESR1 mutation found in breast cancer, occurring in around 5% of patients with metastatic breast cancer treated with AIs (94,97). E380Q confers endocrine resistance in clinical settings, however there is a discrepancy with results of cell based assays that have shown more modest resistance as well as modest constitutive activity (94). In some studies the mutation does not exhibit significantly increased ligand independent activity compared to WT ESR1 and the E380Q mutation promotes estrogen hypersensitivity rather than ligand independent activity (97,100). Structural information about this mutation is lacking, but it has been proposed that changes in the aa residue at E380Q, which is near the C-terminal of helix 12, eliminates a negative charge repulsion that normally occurs between E380 and E542 and D545 of helix 12. The lack of repulsion would thus facilitate formation of the active conformation without ligand binding (94). E380Q confers less fulvestrant resistance and may also have yet to be determined mechanisms that drive hormone independent activity (98).

1.6.3 SERDs for the Treatment of Patients Expressing ESR1 Mutations

For patients with activating ESR1 mutations, therapies that directly target the estrogen receptor rather than estrogen receptor signaling provide modest therapeutic efficacy. Fulvestrant is the treatment of choice for women expressing these mutations despite the less than ideal pharmacokinetic properties and efficacy against certain ESR1 mutations. These deficiencies have led to the development of SERDs with more favorable properties. Fulvestrant is administered via intramuscular injection (IM) and the amount of fulvestrant needed limits its effectiveness due to lack of bioavailability, while also making IM the only route of administration currently available for fulvestrant (101,102). Oral administration is considered to be more effective than intramuscular injection and could potentially lead to enhanced receptor knockdown and potentially a more rapid clinical response (102).

AZD9833 is an oral SERD developed by AstraZeneca that is currently in phase IIb and phase III clinical trials. Preclinically, it demonstrated antitumor efficacy similar to fulvestrant (103-105). Current phase III trials are investigating AZD9833 plus Palbociclib versus anastrozole plus Palbociclib for ER+/HER2- patients who have not previously had systemic treatment for advanced disease (105,106). The primary endpoint of the study will be progression-free survival while secondary endpoints will be overall survival, length of second progression-free survival period, objective response, time to chemotherapy, and changes in quality of life (105). Phase II clinical trials AZD9833 are evaluating AZD9833 as a monotherapy and comparing its efficacy to fulvestrant (104,106,107).

AZD9496 is an oral SERD that is a potent inducer of ESR1 degradation and showed increased inhibition of tumor growth when administered in combination with a PI3K inhibitor or a CDK4/6 inhibitor in preclinical studies (102,108,109). During phase I clinical trials AZD9496

demonstrated an acceptable safety profile, was well-tolerated, and prolonged disease stabilization (108). AZD9496 was discontinued by AstraZeneca and is no longer listed in their pipeline (106).

SAR439859, or amecenestrant, is an orally bioavailable SERD developed by Sanofi (110,111). SAR439859 is distinct from SERDs because of its fluoropropyl pyrrolidinyl side chain. Like other SERDs, SAR439859 induces ESR1 degradation and at subnanomolar concentrations. Like fulvestrant, it also is inhibited by MG132, a proteasome inhibitor, which blocks ESR1 degradation (111). There are currently five clinical trials ongoing for SAR439859 and they include trials in phases I, II, and III. It is being investigated as monotherapy and in combination with other drugs such as Palbociclib, alpelisib, everolimus, and abemaciclib. It is being studied in postmenopausal women with ER+ breast cancer and women with advanced or metastatic ER+ breast cancer (112-116).

RAD-1901, or elacestrant, is an orally bioavailable SERD and unlike other SERDs, it is able to cross the blood-brain barrier (101,117-119). At low doses it may act as an agonist, but at higher doses RAD-1901 has been found to antagonize and degrade ESR1 (119). Preclinical studies had reported that RAD-1901 showed antitumor activity in models resistant to CDK4/6 inhibitors and fulvestrant, and in models that contained either Y537S or D538G mutations (101,118). RAD-1901 had undergone phase III clinical trials for men and postmenopausal women who have previously tried one or two lines of endocrine therapy for advanced or metastatic breast cancer (117). However, due to vasomotor symptoms, RAD-1901 was discontinued (120).

2. SHORT CHAIN FATTY ACIDS AS INHIBITORS OF BREAST CANCER

Approximately 70% of all diagnosed breast cancer cases express estrogen receptor α (ERα, ESR1) and 17β-estradiol-mediated activation of ESR1 induces patterns of gene expression that are important for breast tumor growth and survival (121, 122). Established therapies for ERαexpressing tumors include antiestrogens such as tamoxifen that block ESR1-mediated responses and aromatase inhibitors that decrease estrogen synthesis and the combination of antiestrogen plus aromatase inhibitors are highly effective for treating patients with ESR1-positive tumors (123, 124). Despite the success of endocrine therapies some patients develop resistance to this therapeutic regimen, and this is due in part to expression of constitutively active ESR1 mutants (95, 99, 126, 127). Most of the mutations are observed in amino acids in the ligand binding domain of ESR1 and the most frequent mutants are D538G and Y537S which are constitutively active (98). Endocrine-resistant ER-positive breast cancer patients have a poor prognosis and are treated with the antiestrogen Fulvestrant which also induces degradation of wild-type and mutant ER. Fulvestrant is a prototypical selective ER degrader or downregulator (SERD), however, the clinical effectiveness of this compound is limited due to poor oral bioavailability and toxic side effects (128-131). There is considerable ongoing research and clinical evaluation of novel SERDs that target ER degradation. For example, AZD9496 is non-steroidal ER antagonist and SERD being developed for treatment ER+ advanced breast cancer and this agent shows promising preclinical and clinical results (102, 132, 133).

SERDs such as Fulvestrant interact directly with ER and activate proteasome dependent degradation of the receptor, however, there are many other examples of pathways resulting in decreased expression of ESR1 (134). For example, the potent AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces inhibitory AhR-ESR1 crosstalk which results AhR-

dependent downregulation of ER in breast cancer cells and this is also accompanied by AhR degradation (135, 136). This pathway is unidirectional since E2 does not induce AhR degradation but activates proteasome-dependent degradation of ESR1 in breast cancer cells. In addition, there is also a report showing that ligand activated PPARγ induces ESR1 degradation (137). Histone deacetylase (HDAC) inhibitors are being developed for treatment of breast and other cancers and there is evidence that structurally-diverse HDAC inhibitors induce ESR1 degradation in breast cancer cells (138-144). Short chain fatty acids (SCFAs) such as butyrate, propionate and acetate are produced in the gut by microbial degradation of high fiber diets and SCFAs also exhibit activity as HDAC inhibitors (145-147). We hypothesized that SCFAs, like other HDAC inhibitors would also downregulate ERα and thereby act as SERDs and be effective for treating endocrine-resistant ESR1 positive breast cancers. This hypothesis was confirmed, and this study shows that SCFAs induce degradation of wild-type and mutant ESR1 in MCF-7 and T47D breast cancer cells. Moreover, in an in vivo athymic nude mouse orthotopic model bearing MCF-7-ESR1-Y537S cells butyrate inhibits tumor growth and downregulates mutant ERα in the tumors. These results support future studies on development of high fiber diets that are converted by intestinal microorganisms into SCFAs as a novel dietary approach for delivering SERD activity to enhance effectiveness of current chemotherapies for endocrine-resistant breast cancer.

3. MATERIALS AND METHODS

3.1 Cell lines, antibodies, and reagents

Breast cancer cell lines, MCF-7 and T-47D, were kindly provided by Dr. Steffie Oesterreich, University of Pittsburgh, Department of Pharmacology and Chemical Biology and by Dr. Ben Ho Park of Vanderbilt University School of Medicine (Nashville, TN). Cells were grown and maintained at 37°C in the presence of 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) or RPMI-1640 Medium with 10%FBS. DMEM, FBS, and trypsin were purchased from Gibco. Estrogen Receptor α (D6R2W), HDAC6 (D2E5), Acetyl-Histone H3 (Lys9/Lys14), Acetyl-Histone H3 (Lys27), Acetyl-Histone H4 (Lys8) antibodies were purchased from Cell Signaling (Boston, MA); HDAC1 (10E2) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); βActin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Sodium butyrate, sodium propionate, and sodium acetate were purchased from Sigma Aldrich. HDAC inhibitors Entinostat, Vorinostat, and Panobinostat were purchased from LC Laboratories (Woburn, MA).

3.2 Cell Viability Assay

Cells were plated in a 12 well plate at a density of 100,000 per well with DMEM containing 10% FBS. After 24 hours, cells were treated with DMSO and containing different concentrations of butyrate, propionate, or acetate with DMEM containing 2.5% FBS for 24 hours. After treatment with SCFAs, cells were washed with 100 µL of Hank's balanced salt solution (HBSS) and trypsinized with 100 µL. Once cells detached, 900 µL of DMEM containing 2.5% FBS was added. Cells were counted using a cell counter.

3.3 Measurement of Apoptosis (Annexin V staining)

Cells were seeded in a 6 well plate at a density of 200,000 per well with DMEM containing 10% FBS. After 24 hours, cells were treated with either DMSO or butyrate,

propionate, or acetate for 24 hours. Cells were stained and analyzed by flow cytometry using Annexin V staining kit according to the manufacturer's protocol by Invitrogen (Grand Island, NY).

3.4 Western blot analysis

MCF-7 and T47-D cells were seeded at a density of 200,000-300,000 in 6 well plates and allowed to attach for 24 hours. Cells were treated with either DMSO or various concentrations of butyrate, propionate, and acetate. Whole cell proteins were extracted using RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate, and 0.1% SDS with protease and phosphatase inhibitor cocktail from Gen Depot. Protein concentrations were measured using Bradford assay and equal amounts of protein were separated in either 8%, 10%, or 15% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 hour with either 5% BSA or 5% skim milk. Membranes were incubated with primary antibodies overnight at 4°C and incubated with corresponding HRP-conjugated secondary IgG antibodies for 3 hours or overnight in 5% skim milk. Cell signaling antibodies were used at a ratio of 1:1000 in 5% BSA, SCBT antibodies were used at a ratio of 1:500 in 5% BSA; Sigma Aldrich antibodies were used at a ratio of 1:50,000 in 5% skim milk. Immune-reacted proteins were detected with chemiluminescence reagent.

3.5 Small Interfering RNA Interference Assay

Cells were seeded in 6 well plates at a density of 50,000 cells per well in DMEM supplemented with 10% FBS. After 24 hours, cells were transfected with 100 nM of each siRNA for 6 hours using OptiMEM I Reduced Serum Medium (Gibco) and Lipofecatamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Protein was extracted using RIPA lysis buffer after 72 hours post transfection incubation and western blot analysis was performed.

The siRNA used to perform this assay were purchased from Sigma Aldrich and are: SASI_Hs01_00079968 (HDAC1 #1), SASI_Hs01_00079964 (HDAC1 #2), SASI_Hs01_00048982 (HDAC6 #1), SASI_Hs02_00340796 (HDAC6 #2).

3.6 Xenograft Study

Female athymic nu/nu mice (4-6 weeks old) were purchased from Charles River Laboratory (Wilmington, MA). MCF-7 Y537S cells (5×106) were harvested in 100 μL of DMEM and suspended in ice-cold Matrigel (1:1 ratio) and orthotopically injected into the mammary fat pad of the mice. After two weeks of tumor cell inoculation, mice were divided in to two groups of 7 animals each. The first group received 100 μL of vehicle (corn oil), and the second group of mice received an oral gavage of 200 mg/kg/day of sodium butyrate in corn oil for three weeks. All mice were weighed once a week over the course of treatment to monitor changes in body weight and tumor volume was measured. After three weeks of treatment, mice were sacrificed, and tumor weights were determined. All animal studies were carried out according to the procedures approved by the Texas A&M University Institutional Animal Care and Use Committee.

3.7 Statistical Analysis

All of the experiments were repeated a minimum of three times. The data are expressed as the mean ± standard deviation (SD) and significant differences between treatment groups compared to the untreated control were determined by students t-test. Data with p-values less than 0.05 were considered statistically significant.

4. RESULTS

4.1 Inhibition of growth and downregulation of WT and mutant ESR1 by SCFAs

The ability of a cancer therapeutics ability to decrease tumor growth is an important part of its function. In order to test this, MCF-7 cells expressing wild-type ESR1 and mutants Y537S and D538G were generated using recombinant adeno-associated virus technology using AAV vectors for both mutants (148). In T47D breast cancer cells, mutants of ESR1 were generated using CRISPR-Cas9 genome editing (148). The effects of short chain fatty acids: butyrate, propionate, and acetate were investigated for their effects on proliferation of MCF-7 cells expressing both wild-type and mutant ESR1. In MCF-7 cells expressing ESR1-WT, ESR1-D538G, and ESR1-Y537S 0.5-5 mM of butyrate inhibited growth (Fig. 1A). Butyrate treatment also led to downregulation of ESR1-WT, ESR1-D538G, and ESR1-Y537S (Fig. 1B). In T47D cells expressing both WT and mutant ESR1, higher concentrations of butyrate were needed to decrease proliferation. Concentrations of 100 mM and greater were observed to decrease tumor growth in T47D cells with ESR1-WT, ESR1-D538G, and ESR1-Y537S (Fig. 1C). However, butyrate decreased both WT and mutant ESR1 expression at lower concentrations, 2 mM or greater, in T47D cells (Fig. 1D).

MCF-7 cells treated with propionate also showed decreased proliferation at concentrations greater than 10 mM and this was observed with downregulation of WT and mutant ESR1 at similar concentrations (Fig. 2A-B). Like butyrate in T47D cells, no decrease in proliferation was observed in T47D cells with the exception of ESR1-D538G which showed a decrease in proliferation at 100 mM and greater, while concentrations of 40-50 mM downregulated WT and mutant ERS1 expression in T47D cells (Fig. 2C-D).

Figure 1

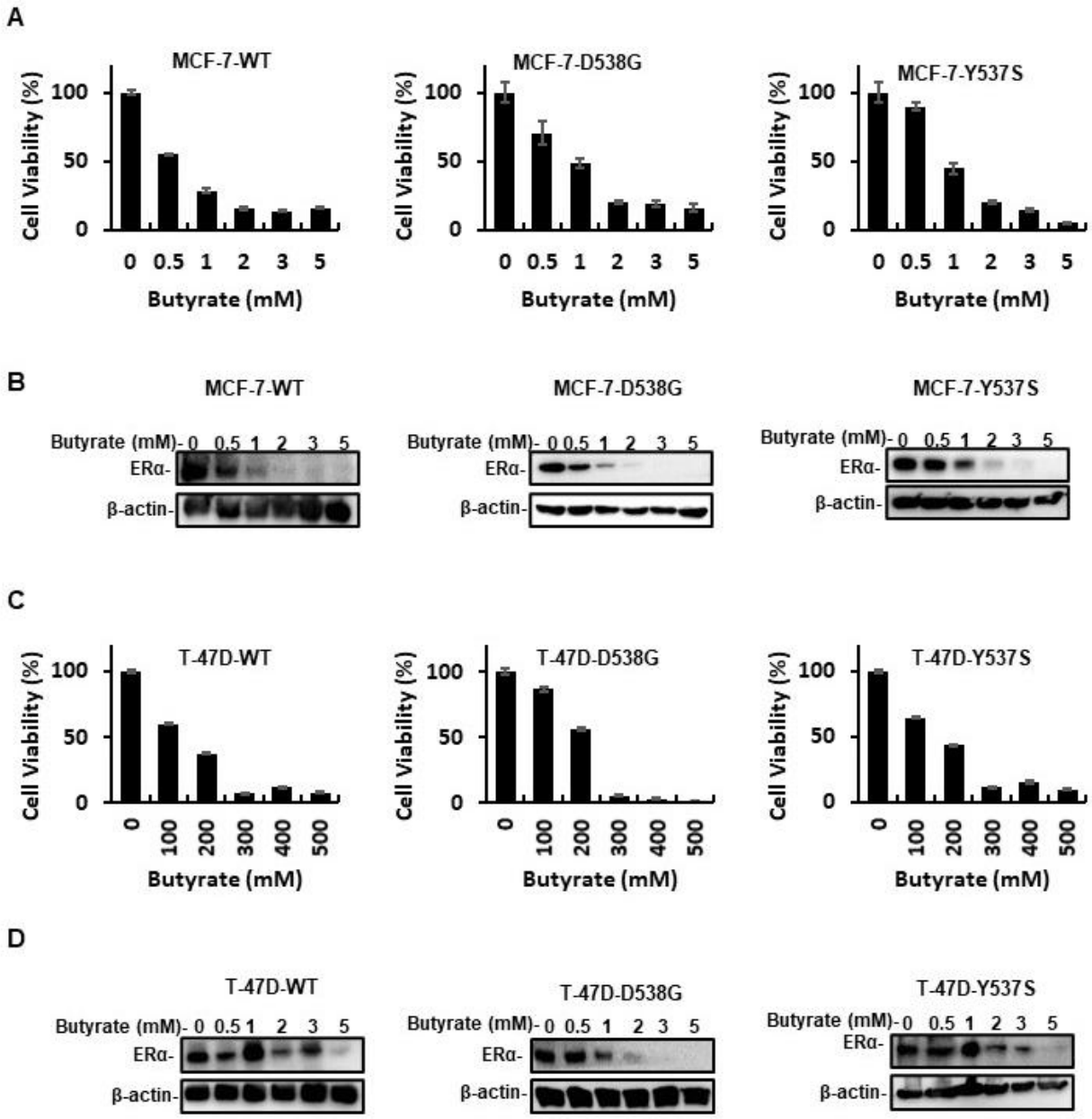

Figure 1. Effects of butyrate on breast cancer cell growth and ER downregulation. MCF-7 cells expressing wild-type and mutant ERα were treated with different concentrations of butyrate and effects on cell viability (A) and ERα downregulation (B) were determined as outlined in the Methods. T47D cell expressing wild-type and mutant ERα were treated with

different concentrations of butyrate and effects on cell proliferation (C) and ERα downregulation (D) were determined as outlined in the Methods. Results (B and D) are means ± SD for at least 3 determinations and significant ($P<0.05$) inhibition is indicated (*).

Figure 2

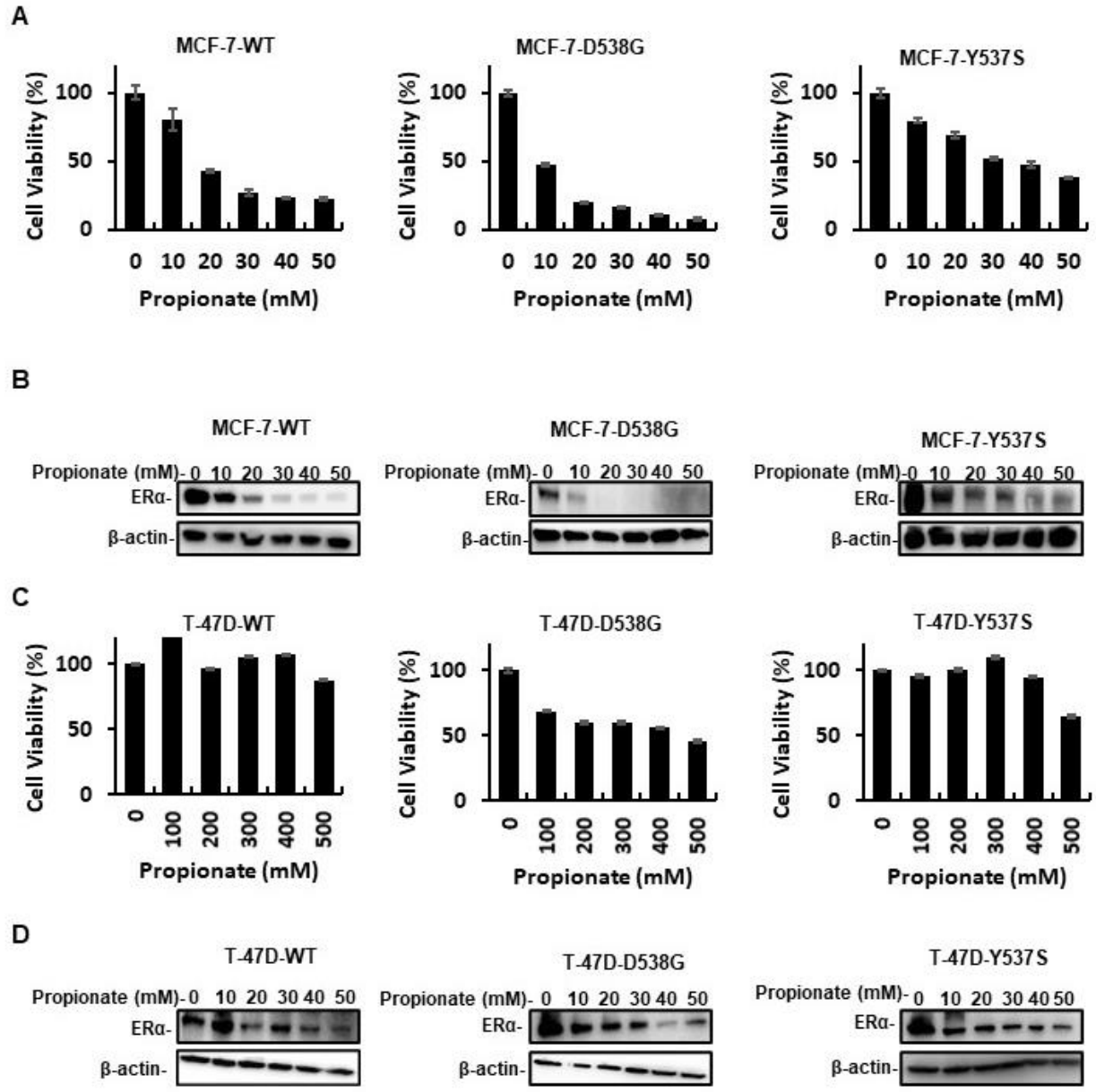

Figure 2. Effects of propionate on breast cancer cell growth and ER downregulation. MCF-7 cells expressing wild-type and mutant ERα were treated with different concentrations of propionate and effects on cell proliferation (A) and ERα downregulation (B) were determined as outlined in the Methods. T47D cell expressing wild-type and mutant ERα were treated with different concentrations of propionate and effects on cell proliferation (C) and ERα downregulation (D) were determined as outlined in the Methods. Results (B and D) are means ± SD for at least 3 determinations and significant ($P<0.05$) inhibition is indicated (*).

In MCF-7 cells, acetate inhibited growth between 30-50 mM or greater in cells expressing WT and mutant ESR1 (Fig 3A). Downregulation of ESR1 was not observed in MCF-7 cells expressing WT-ESR1 and downregulation of ESR1 in cells expressing ESR1-D538G was deemed insignificant, however, downregulation of ESR1 occurred in cells expressing ESR1-Y537S (Fig. 3B). In T47D cells Acetate inhibited growth at concentrations greater than 100 mM in WT and mutant ESR1 T47D cells, lower concentrations of acetate were observed to decrease ESR1 expression (Fig. 3C-D). This trend was the opposite of was noted for T47D cells treated with butyrate and propionate where lower concentrations were needed for growth inhibition compared to downregulation of ESR1. MCF-7 cells were used for further assessment of SCFAs-induced functional characterization for WT and mutant ESR1.

Figure 3

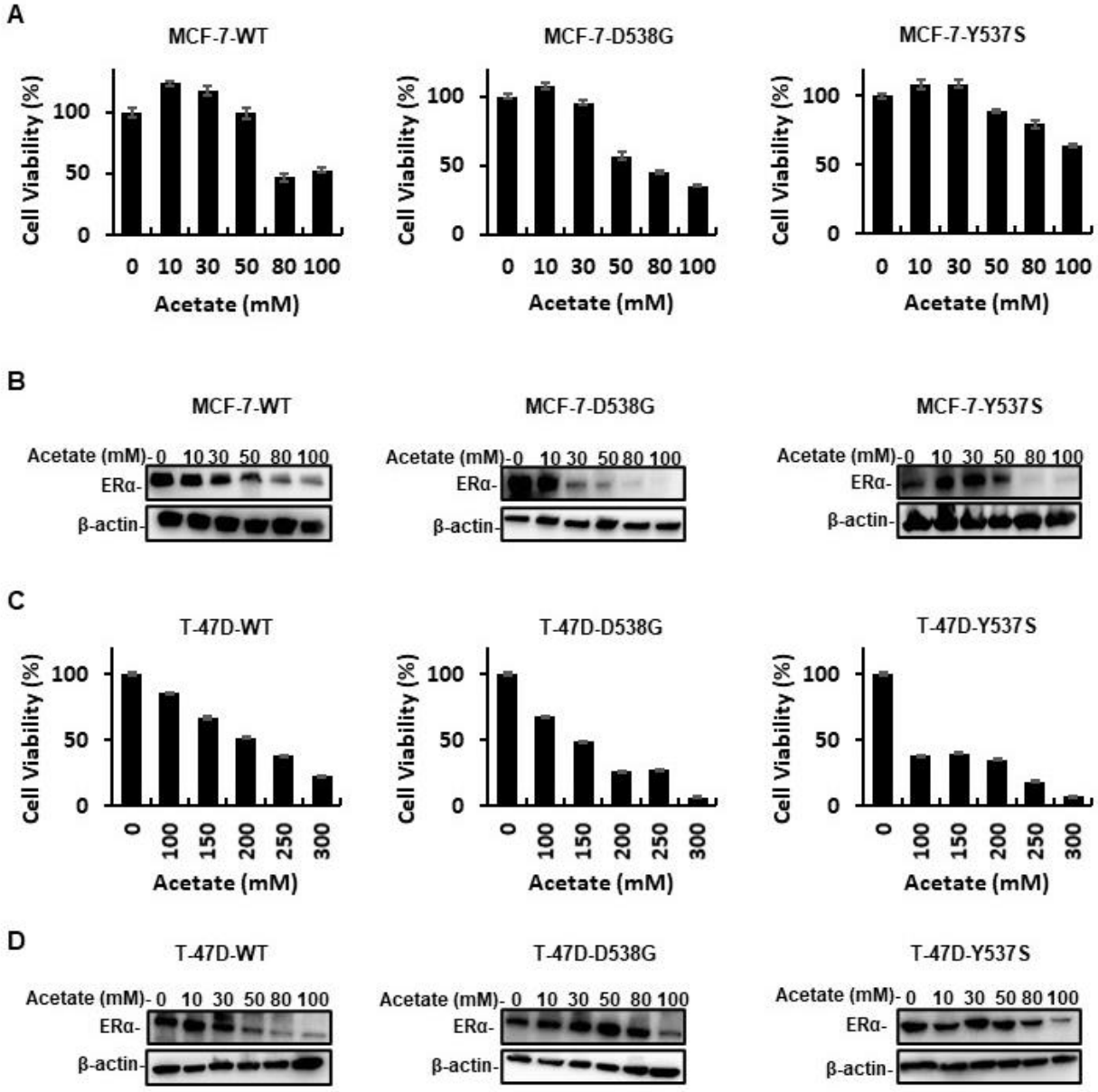

Figure 3. Effects of acetate on breast cancer cell growth and ER downregulation. MCF-7 cells expressing wild-type and mutant ERα were treated with different concentrations of propionate and effects on cell proliferation (A) and ERα downregulation (B) were determined as outlined in the Methods. T47D cell expressing wild-type and mutant ERα were treated with different

concentrations of propionate and effects on cell proliferation (C) and ERα downregulation (D) were determined as outlined in the Methods. Results (B and D) are means ± SD for at least 3 determinations and significant ($P<0.05$) inhibition is indicated (*).

4.2 Annexin V Staining

In cells expressing ESR1-WT, acetate at 100 mM and propionate at 50 mM induced apoptosis, but not butyrate (Fig. 4). A different pattern were observed in mutant ESR1 cell lines. In cells containing ESR1-D538G mutation 5 mM of butyrate induced apoptosis, but propionate and acetate were not observed to have induced apoptosis (Fig. 4). Conversely, cells containing ESR1-Y537S displayed apoptotic activity when treated with butyrate and propionate, but not acetate (Fig. 4). The effects of SCFAs were cell context-dependent and different from patterns observed in growth inhibition and downregulation of ESR1 by SCFAs.

Figure 4

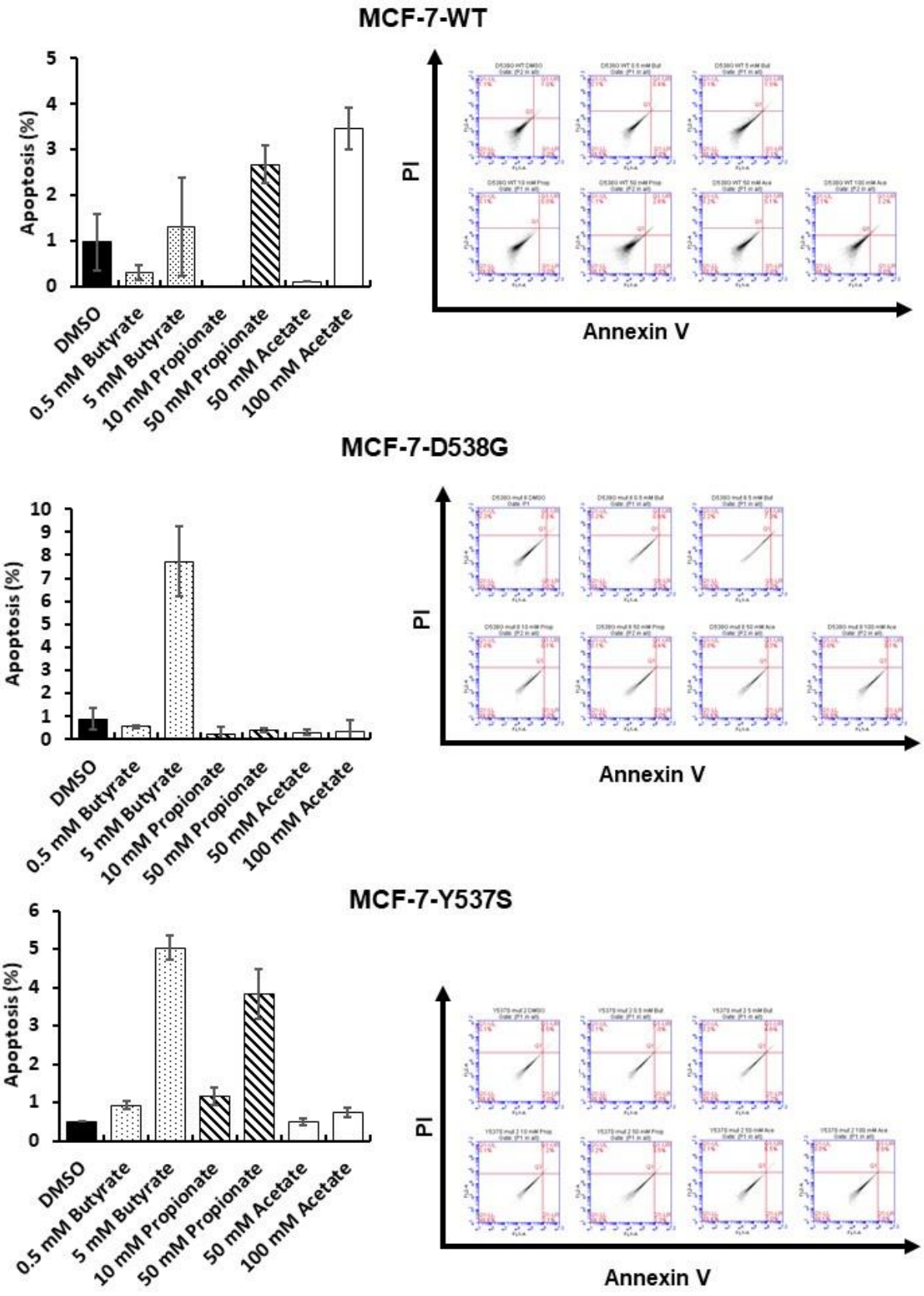
MCF-7-WT
Apoptosis (%)
0
1
2
3
4
5
DMSO
0.5 mM Butyrate
5 mM Butyrate
10 mM Propionate
50 mM Propionate
50 mM Acetate
100 mM Acetate
PI
Annexin V
MCF-7-D538G
Apoptosis (%)
0
1
2
3
4
5
6
7
8
9
10
DMSO
0.5 mM Butyrate
5 mM Butyrate
10 mM Propionate
50 mM Propionate
50 mM Acetate
100 mM Acetate
PI
Annexin V
MCF-7-Y537S
Apoptosis (%)
0
1
2
3
4
5
6
DMSO
0.5 mM Butyrate
5 mM Butyrate
10 mM Propionate
50 mM Propionate
50 mM Acetate
100 mM Acetate
PI
Annexin V

Figure 4. SCFAs induced Annexin V staining MCF-7 cells expressing wild-type and mutant ERα. MCF-7 cells expressing wild-type ERα (A), ERα-D538G (B) and ERα-Y537S (C) were treated with different concentrations of SCFAs and Annexin V staining was determined as outlined in the Methods. Results are expressed as means ± SD for at least 3 determinations per treatment group and significant ($P<0.05$) inhibition is indicated (*).

4.3 HDAC inhibitors downregulate WT and mutant ESR1 and enhance histone acetylation

The effects of the HDAC inhibitors: Panobinostat, Vorinostat, and Entinostat were characterized in MCF-7 cells for ESR1 downregulation and histone acetylation (Fig. 5). All of the HDAC inhibitors were observed to downregulate both WT and mutant ESR1 (Fig. 5 A-C). Panobinostat was observed to decrease ESR1 in the nM range, with concentrations of 0.5-1 nM required for ESR1-WT, but higher concentrations, up to 5 nM, required to downregulate ESR1-D538G and ESR1-Y537S (Fig. 5A). Vorinostat and Entinostat required doses in the μM range to downregulate both WT and mutant ESR1 (Fig. 5B). Histone acetylation was observed to increase following treatment with each of the HDAC inhibitors (Fig. 5D). Differences were observed in intensity and effects did appear to be concentration dependent with higher concentrations of HDAC inhibitors inducing greater histone acetylation than lower concentrations.

Figure 5

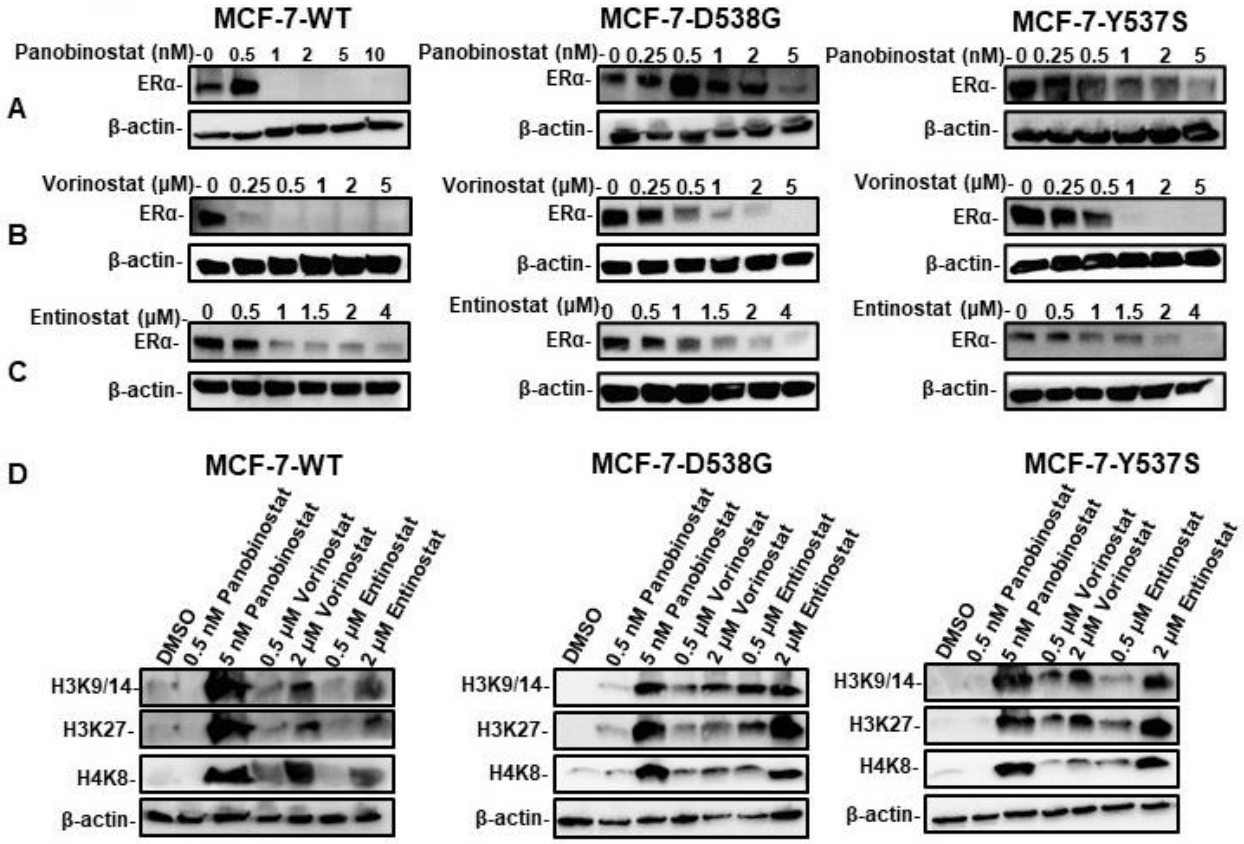

Figure 5. HDAC inhibitors downregulate wild-type and mutant ERα. MCF- cells expressing wild-type and mutant ERα were treated with Panobinostat (A), Vorinostat (B) and Entinostat (C) for 24 hours and whole cell lysates were analyzed by western blots. (D) Treatment with the HDAC inhibitors and effects on histone acetylation were also determined is outlined in A-C and in the Methods.

4.4 SCFAs inhibit HDAC activity and role of HDACs in downregulation of ESR1

SCFAs were assessed for their HDAC inhibitory properties, by assessing histone acetylation of H3K9/14, H3K27, and H4K8 in WT and mutant ESR1 MCF-7 cells (Fig. 6). Butyrate most consistently induced histone acetylation, while results for propionate and acetate varied and appeared to be dependent on treatment and cell line ESR1-WT MCF-7 cells showed

increased acetylation with butyrate and propionate treatment, while very modest effects were observed with acetate treatment even at the higher dose (80 mM) (Fig. 6A-C). Butyrate and propionate induced acetylation of histone H3K9/14, H3K27, and H4K8, while modest increases were seen with acetate treatment (Fig. 6A-C). In cells expressing ESR1-Y537S, butyrate increased H3K9/14, H3K27, and H4K8, while unpronounced effects were observed for propionate and acetate treatment (Fig. 6C).

The effects of knockdown of individual HDACs were to assess the linkage of HDAC knockdown to ESR1 downregulation (Fig. 6D-F). RNA interference was used to knockdown the HDACs of interest. The individual HDACs were selected due to their high expression in breast cancer cell lines. In cells expressing ESR1-WT, ESR1-D538G, and ESR1-Y537S, HDAC1 or HDAC6 led to downregulation of ESR1. Results for knockdown were variable based on quantitation of the data, but siHDAC1 and siHDAC6 oligonucleotides still showed significant decreases in ESR1 (Fig. 6D-F). The effects of SCFAs on HDAC1 or HDAC6 expression were observed in WT and mutant ESR1 MCF-7 cells (Fig. 6G-I). Only acetate (80 mM) significantly decreased expression of ESR1-WT (Fig. 6G). In cells expressing ESR1-D538G, 5 mM of butyrate and 20 mM of propionate decreased HDAC6 expression, while acetate displayed no effects on HDAC6 expression, and HDAC1 expression was not decreased by any treatment (Fig. 6H). In cells expressing ESR1-Y537S, butyrate and propionate decreased HDAC1 and HDAC6 expression, while acetate did not affect expression of either HDAC (Fig. 6I).

Figure 6

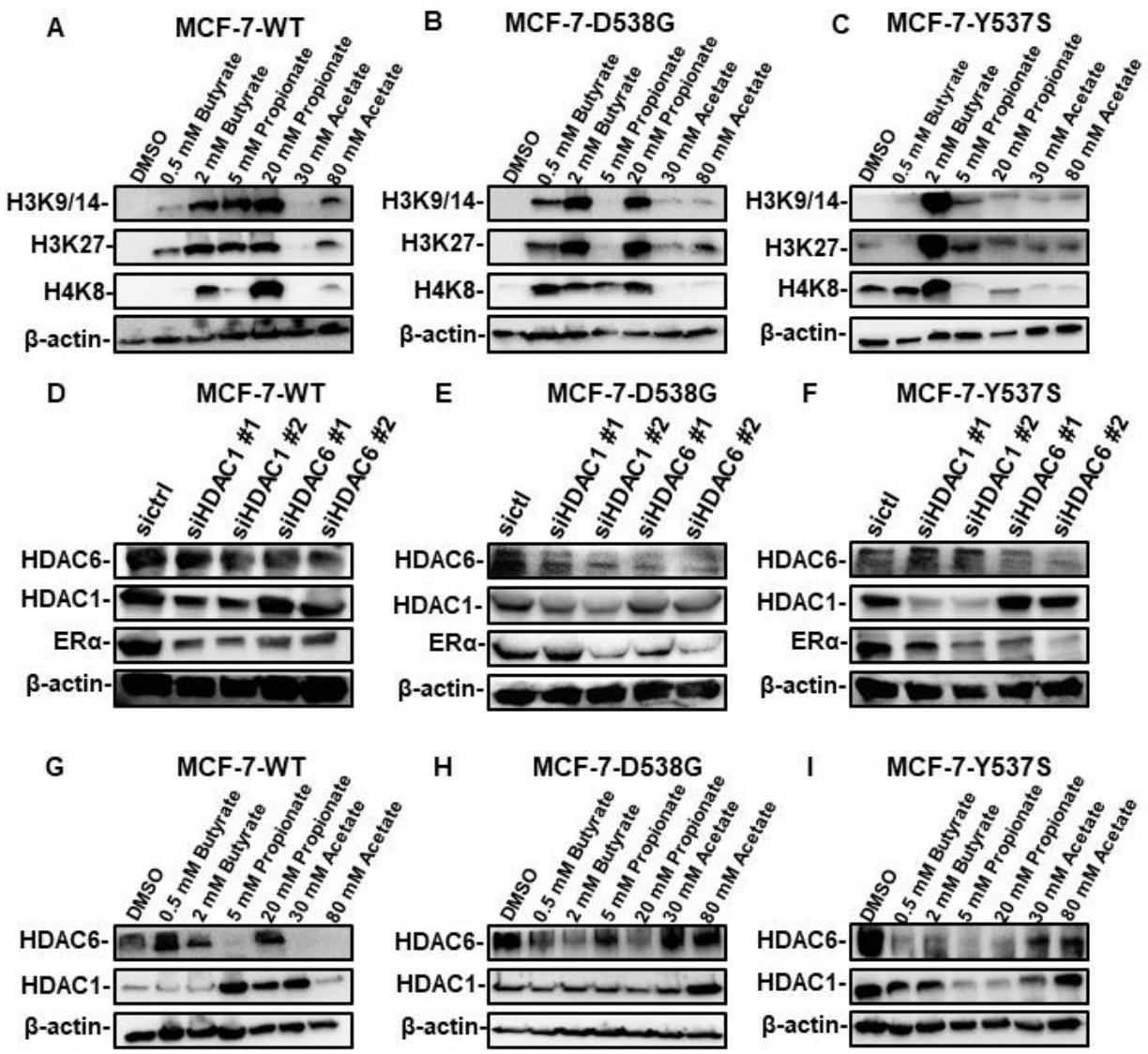

Figure 6. Effects of SCFAs on HDAC1 and HDAC6 expression. MCF-7 cells expressing wild-type ERα (A), ERα-D538G (B) and ERα-Y537S (C) were treated with SCFAs for 24 hours and whole cell lysates were analyzed by western blots for changes in histone acetylation and effects on total H3 and H4 are also given. MCF-7 cells expressing wild-type ERα (D), ERα-D538G (E) and ERα-Y537S (F) were transfected with oligonucleotides targeted against HDAC6 or HDAC1 and after 72 hours whole cell lysates were analyzed by western blots.

MCF-7 cells expressing wild-type ERα (G), ERα-D538G (H) and ERα-Y537S (I) were treated with SCFAs by western blots as outlined above and in the Methods.

4.5 Butyrate inhibits tumor growth *in vivo*

SCFAs were investigated *in vivo*, with butyrate serving as a model and administered via drinking water at 150 mM as previously described (Fig. 7). After 21 days, butyrate had significantly inhibited an increase in tumor volume of athymic nude mice bearing MCF-7 cells and expressing ESR1-Y537S (Fig. 7A-B). In addition to tumor size, tumor weights were assessed and showed decreased weight in butyrate-treated mice compared to controls (Fig. 7C). Tumor lysates were prepared for western blot analysis, and lysates displayed decreased expression of ESR1-Y537S, HDAC1, HDAC6, and increased expression of histone acetylation markers, H3K9/14 and H4K8 (Fig. 7E).

Figure 7

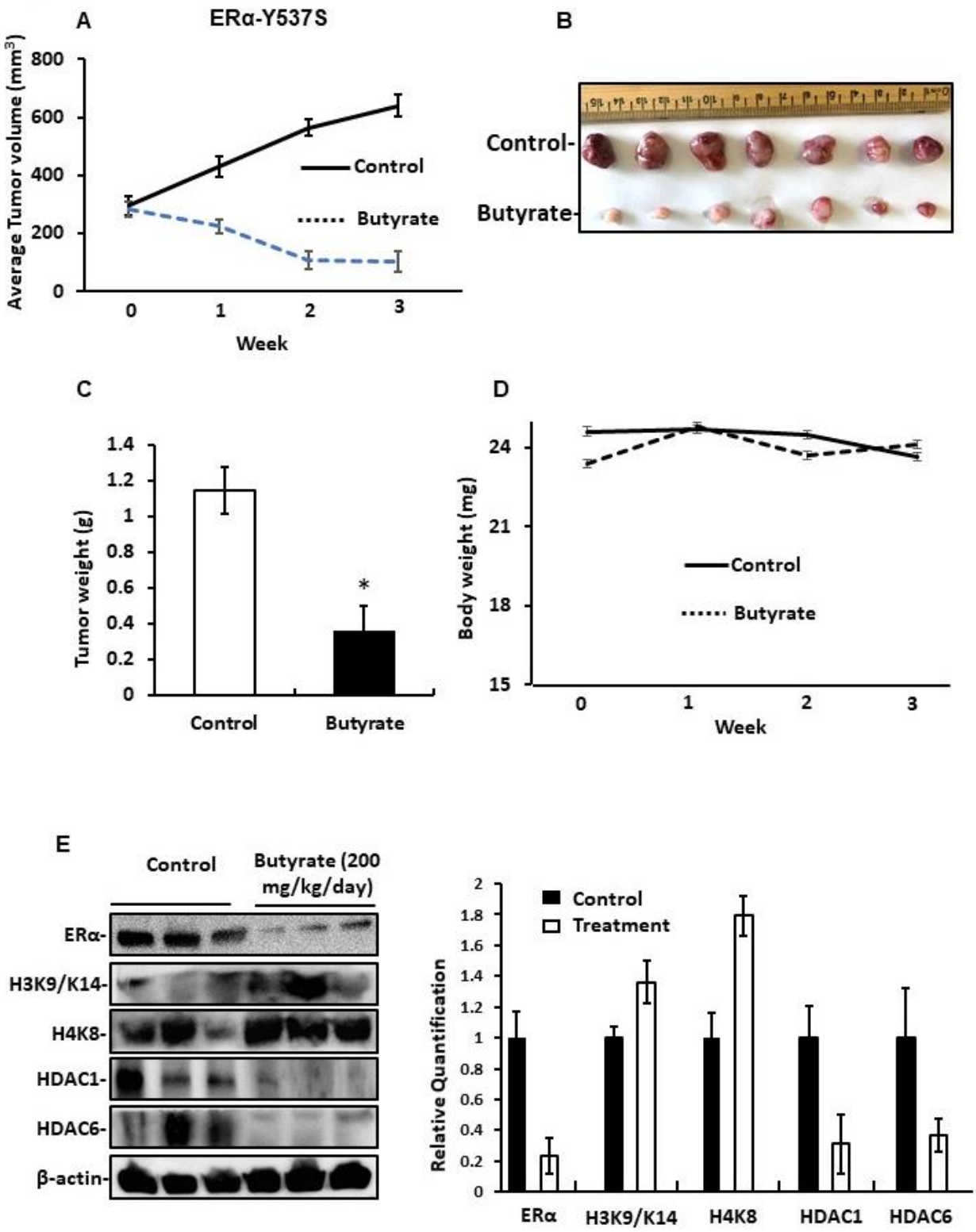
A
ERα-Y537S
Average Tumor volume (mm3)
800
600
400
200
0
0
1
2
3
Week
Control
Butyrate
B
Control-
Butyrate-
C
Tumor weight (g)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Butyrate
D
Body weight (mg)
24
21
18
15
0
1
2
3
Week
Control
Butyrate
E
Control
Butyrate (200 mg/kg/day)
ERα-
H3K9/K14-
H4K8-
HDAC1-
HDAC6-
β-actin-
Relative Quantification
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Treatment
ERα
H3K9/K14
H4K8
HDAC1
HDAC6

Figure 7. In vivo studies using MCF-7 (ERα-Y537S) cells and in an orthotopic model effects of oral butyrate. Athymic nude mice bearing MCF-7 (ERα-Y537S) cells orthotopically were administered butyrate in the drinking water for 3 weeks and effects of butyrate on tumor volumes (A, B), tumor weight (C) and whole-body weight (D) compared to controls were determined. For select tumors, lysates were obtained and analyzed by western blots (E) and effects on expression of selected proteins was determined by western blots as outlined in the Methods. Protein levels were quantitated relative to β-actin and levels in the control group were set at 1.0. Significant ($P<0.05$) decreases are indicated.

5. DISCUSSION

Fiber-rich diets have been shown to play a protective role in maintaining gut health by aiding microbial-induced metabolism of SCFAs such as butyrate, propionate, and acetate. SCFAs have been shown to exhibit anticancer activities in ER+ and ER- breast cancer cells by inhibition of cell proliferation, survival, and migration and invasion (149,150-156). Previous studies have indicated that butyrate and propionate act as HDAC inhibitors and have observed that HDAC inhibitors induce downregulation of ESR1 (139-144, 147). In this study we have also observed that they act as HDAC inhibitors in breast cancer cells, while acetate exhibited modest HDAC inhibitory effects.

MCF-7 and T47D cell lines expressing both ESR1-WT and constitutively active mutants ESR1-D538G and ESR1-Y537S were used to evaluate whether SCFAs acted as SERDs (148). MCF-7 and T47D cells were treated with butyrate, propionate, and acetate, and MCF-7 cells were found to be more responsive to treatment by individual SCFAs. Butyrate and propionate were each found to downregulate ESR1 expression for both WT and mutant ESR1, while the effects of acetate were more variable with ESR1-WT and ESR1-D538G showing growth inhibition at concentrations that did not display downregulation of ESR1 (Fig. 1-3). In T47D cells, butyrate and propionate were found to inhibit growth at far higher concentrations than those used in MCF-7 cells and those concentrations were higher than those found to downregulate ESR1 expression suggesting that other factors are contributing to growth (Fig. 1-2). Thus, we focused on MCF-7 cells expressing WT and mutant ESR1 as a model for further investigation of SCFAs as SERDs.

Previous reports have indicated that HDAC inhibitors are able to downregulate ESR1 (139-144). We tested and confirmed this using HDAC inhibitors Panobinostat, Vorinostat, and

Entinostat and showed that they decreased ESR1-WT, as well as ESR1-D538G and ESR1-Y537S (Fig. 5A-C). We then analyzed whether SCFAs and HDAC inhibitors would increase histone acetylation in MCF-7 cells expressing either WT or mutant ESR1 (Fig. 5D; Fig. 6A-C). We found that histone acetylation was varied depending on drug treatment and cell line. In particular, it was noted that acetate did not induce histone acetylation of either H3K9/14, H3K27, or H4K8 in cells expressing ESR1-Y537S despite downregulation of ESR1 (Fig. 6C), suggesting that SERD-like activity of SCFAs and HDAC inhibitors is due to pathways other than histone acetylation.

Previous studies have indicated that butyrate downregulated HDAC expression in lung cancer and mouse neural cells in culture and HDAC inhibitor LAQ824 was shown to decrease ESR1 and HDAC6 in MCF-7 cells (157, 158). Studies have also observed that HDACs stabilize ESR1 and knockdown of HDAC6 via RNA interference decreased ESR1 expression in MCF-7 cells (140). We observed that knockdown of either HDAC1 or HDAC6 by RNA interference decreased expression of ESR1-WT, ESR1-Y537S, but not ESR1-D538G (Fig. 6D-F). The effects of SCFAs on the expression of HDAC1 and HDAC6 was variable and were dependent on treatment and cell line (Fig. 6G-I). MCF-7 cells expressing ESR1-Y537S showed downregulation of HDAC1 and HDAC6 when treated with butyrate and propionate, and acetate modestly decreased HDAC6, but not HDAC1 (Fig. 6I). Our results suggest that SCFA-induced downregulation of HDACs mediates SERD-like activity targeting ESR1-Y537S. *In vivo* studies using mouse xenografts bearing MCF-7 cells expressing ESR1-Y537S showed that butyrate supplemented in the diet decreased tumor growth as well ESR1-Y537S, HDAC1, and HDAC6 (Fig. 7), which was observed *in vitro* studies with butyrate treatment in this cell line.

The SCFAs butyrate, propionate, and acetate have displayed SERD-like activity by inhibiting growth in MCF-7 cells expressing WT and mutant ESR1 and downregulation of ESR1. HDAC inhibitors Panobinostat, Vorinostat, and Entinostat displayed similar results. Downregulation of WT and mutant ESR1 may be associated with the HDAC inhibitory properties of SCFAs. SCFA-induced downregulation of HDAC1 and HDAC6 may contribute to the SERD-like activity of SCFAs in MCF-7 cells expressing ESR1-Y537S. We have observed that SCFAs exhibit SERD-like activity and could be a new class of SERDs for treatment of patients with endocrine resistant ER+ breast cancer.

6. CONCLUSION

We have assessed that SCFAs exhibit SERD-like activity in breast cancer cells with WT ESR1 and those with mutant ESR1. SCFAs behaved similarly to small molecule HDAC inhibitors by increasing histone acetylation and downregulating ESR1 expression. We have found that SCFAs exert a different response depending on the compound and cell line and further research is indicated to identify why these differences occur. SCFAs, butyrate and propionate, may downregulate ESR1 via downregulation of HDAC1 and HDAC6 in MCF-7 cells expressing ESR1-Y537S. These data suggest that HDAC inhibitors display SERD-like activity and may be a potential new class of SERDs.

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