Substrate inhibition of 17 beta-hydroxysteroid dehydrogenase type 1 in living cells and regulation among the steroid-converting enzymes in breast cancers

Thèse

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Sous la direction de :

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RÉSUMÉ

Cette étude a permis de démontrer les fonctions et les mécanismes de la 17bêta-hydroxystéroïde déshydrogénase de type 1 (17β-HSD1) et de la stéroïde sulfatase (STS) au niveau du cancer du sein, y compris la cinétique moléculaire et cellulaire, la liaison du ligand étudiée par la titration de fluorescence, la régulation des stéroïdes et la régulation mutuelle entre les enzymes stéroïdiennes et les cellules cancéreuses du sein.

1), L'inhibition de la 17β-HSD1 par son substrat a été démontrée par la cinétique enzymatique au niveau cellulaire pour la première fois, soutenant ainsi la fonction biologique de l'inhibition produite par le substrat.

2), En tant qu'inhibiteur, la dihydrotestostérone (DHT) n'a pas affecté la concentration du substrat estrone (E1) à laquelle l'activité enzymatique a commencé à diminuer, mais certaines augmentations de vitesse ont été observées, suggérant une diminution significative de l'inhibition par le substrat.

3), Les résultats de la modulation de l'ARNm ont démontré que la transcription du gène codant la 17β-HSD7 diminuait en réponse à l'inhibition de la 17β-HSD1 ou au knockdown dans les cellules du cancer du sein par la modification estradiol (E2).

4), L'expression de la STS est stimulée par E2 de manière à générer une rétroaction positive, ce qui favorise la biosynthèse de E2 dans les cellules de cancer du sein.

5), L'inhibition conjointe de la STS et de la 17β-HSD7 pourrait bloquer leurs activités enzymatiques, diminuant ainsi la formation de E2, mais rétablissant la formation de DHT, réduisant de façon synergique la prolifération cellulaire et induisant l'arrêt du cycle cellulaire en G0 / G1.

6), Les 17β-HSD7 et STS synthétisent E2 et sont toutes deux régulées par E2. Ainsi, elles forment un groupe fonctionnel d'enzymes mutuellement positivement corrélées, l'inhibition de l'une peut réduire l'expression d'une autre, amplifiant ainsi potentiellement les traitements inhibiteurs.

7), Le recepteur estrogenique α ERα a été non seulement régulés à la baisse par E2, mais également réduits par la DHT grâce à l'activation des récepteurs aux androgènes (AR).

En conclusion, la 17β-HSD1 et la 17β-HSD7 jouent des rôles essentiels dans la conversion et la régulation des hormones sexuelles, et l'inhibition conjointe de la STS et de la 17β-HSD7 constitue une nouvelle stratégie pour le traitement hormonal des cancers du sein sensibles aux estrogènes.

Summary

Human 17beta-hydroxysteroid dehydrogenase type 1 (17β-HSD1), 17beta-hydroxysteroid dehydrogenase type 7 (17β-HSD7) and steroid sulfatase (STS) play a crucial role in regulating estrogen synthesis for breast cancer (BC). However, mutual regulation of enzymes and the interaction of these steroids (estrogens, androgens and their precursor dehydroepiandrosterone (DHEA)) are not clear. This study demonstrated the functions and mechanisms including kinetics at molecular level and in cells, ligand binding using fluorescence titration, regulation of steroids and mutual regulation between steroid enzymes in BC cells:

1) Substrate inhibition of 17β-HSD1 was shown for the first time by enzyme kinetics at the cell level, supporting the biological function of substrate inhibition.

2) As an inhibitor, dihydrotestosterone (DHT) did not affect the estrone (E1) substrate concentration at which the enzyme activity started to decrease, but some increases in velocity were observed, suggesting a corresponding decrease in substrate inhibition.

3) The mRNA modulation results demonstrated that 17β-HSD7 transcription decreased in response to 17β-HSD1 inhibition or knockdown in BC cells due to estradiol (E2) concentration decrease.

4) The expression of STS is stimulated by E2 in a positive-feedback manner which finally promotes E2 biosynthesis within BC cells.

5) The joint inhibition of STS and 17β-HSD7 could block the activities of these enzymes, thus decreasing E2 formation but restoring DHT formation, to synergistically reduce cell proliferation and induce G0/G1 cell cycle arrest.

6) 17β-HSD7 and STS can synthesize E2 and are all regulated by E2. Thus, they form a functional group of enzymes mutually positively correlated, inhibition of one can reduce the expression of the other, thereby potentially amplifying the inhibitory effects.

7) Estrogen Receptor α (ERα) is not only down-regulated by E2, but also reduced by DHT though androgen receptor (AR) activation.

In conclusion, 17β-HSD1 and 17β-HSD7 play essential roles in sex-hormone conversion and regulation, and the joint inhibition of STS and 17β-HSD7 constitutes a novel strategy for hormonal treatment of estrogen-receptor positive BC.

CONTENTS

List of Tables

List of Figures

List of Abbreviations

3β Adiol, 5α-Androstane-3β,17β-diol;

4-dione, 4-androstene-3,17-dione;

5-diol, androst-5-ene-3α, 17β-diol-FA, 5-diol fatty acid;

5-diol-s, androst-5-ene-3alpha,17beta-diol sulphate;

11β-HSD2,11β-hydroxysteroid dehydrogenase type 2;

17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1;

17β-HSD7, 17β-hydroxysteroid dehydrogenase type 7;

A-dione, 5α-androstane-3,17-dione;

ADT, epiandrosterone;

AR, androgen receptor;

AI, aromatase inhibitor;

ATCC, American Type Culture Collection;

BC, breast cancer;

DCC, dextran-coated charcoal;

DHEA, dehydroepiandrosterone;

DHEA-S, dehydroepiandrosterone-sulfate;

DMEM, Dulbecco's Modified Eagle Medium;

DHT, 5α-dihydrotestosterone;

E1, estrone;

E2, estradiol;

ECM, extra-cellular matrices;

EC_{50}, half maximal effective concentration;

E1-S, estrone-sulfate;

E2-S, estradiol-sulfate

EGF, epidermal growth factor;

ELISA, enzyme-linked immunosorbent assay;

ER+, estrogen receptor positive;

ERG, estrogen-responsive gene;

FBS, Fetal bovine serum;

HER, human epidermal growth factor receptor;

HPG axis, the hypothalamic pituitary ovarian axis;

INH1, 3-(3,17b-Dihydroxy-2-methoxy-estra-1,3,5(10)-trien-16b-ylmethyl)-benzamide, inhibitor of 17β-hydroxysteroid dehydrogenases type 1;

INH7, 17β-[(N-Alkyl)formamido]-4-methyl-4-aza-5r-androstan-3-Ones, inhibitor of 17β-hydroxysteroid dehydrogenases type 7;

INH80, 17β-(N-Decylformamido)-4-aza-5r-androstan-3-one, another inhibitor of 17β-hydroxysteroid dehydrogenases type 7;

IC_{50}, concentration inhibiting 50% of the activity;

NADPH, nicotinamide adenine dinucleotide phosphate;

NKCA, Natural killer cell activity;

PBLs, peripheral blood lymphocytes;

PGE2, prostaglandin estradiol;

PI, propidium iodide;

PgR+, progesterone receptor positive;

Q-RT-PCR, Quantitative real-time PCR;

RoDH-1, Ro dehydrogenase 1;

SERMs, selective estrogen receptor modulators;

siRNA, small inhibitory RNA;

STS, steroid sulfatase;

Sult2B1, sulfotransferase 2B1;

Testo, testosterone;

TLC, thin-layer chromatography;

TNM, tumor, lymph nodes and metastasis;

UGT1A1, uridine glucuronosyl transferase 1A1;

Ugt2b28, uridine glucuronosy transferase 2B28;

5-diol, androst-5-ene-3α, 17β-diol-FA, 5-diol fatty acid; 5-diol sulfate.

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FOREWORD

I fulfilled my doctoral study in Axe Endocrinologie et Néphrologie, CHU de Quebec Research Center affiliated to Laval University. My study involved several aspects of molecular and cellular biology using a variety of assessments at cell level and at molecular level. In particular, I studied the enzyme kinetics at both cell level and molecular level, with the mutual regulation between the enzymes. The present thesis includes all results obtained during my doctoral study and divided into three chapters.

Chapter 2: I am 1st auther of the first manuscript (Chapter 2) entitled “Substrate Inhibition of 17β-HSD1 in living cells and regulation of 17β-HSD7 by 17β-HSD1 knockdown”.

I finished most of the experimental work and wrote the paper.

List of co-authors: Hui Han, Jean-François Thériault, Guang Chen, Sheng-Xiang Lin.

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Chapter 3: I am 2nd author of the second manuscript (Chapter 3) entitled: Steroid sulfatase inhibition success and limitation in breast cancer clinical assays: an underlying mechanism.

I carried out significant amount of the experiments and was involved in writing this paper.

List of co-authors: Xiaoye Sang, Hui Han, Donald Poirier, and Sheng-xiang Lin.

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Chapter 4: I am the co-first author of the third manuscript (Chapter 4): Steroid-convertig enzymes: multiple mutual regulations contributing to breast cancer development, which was prepared for submission to Cancers.

I finished half of the experiments and wrote two parts of the manuscript.

List of co-authors: Xiaoye Sang[1], Hui Han[1], Tang Li, and Sheng-xiang Lin.

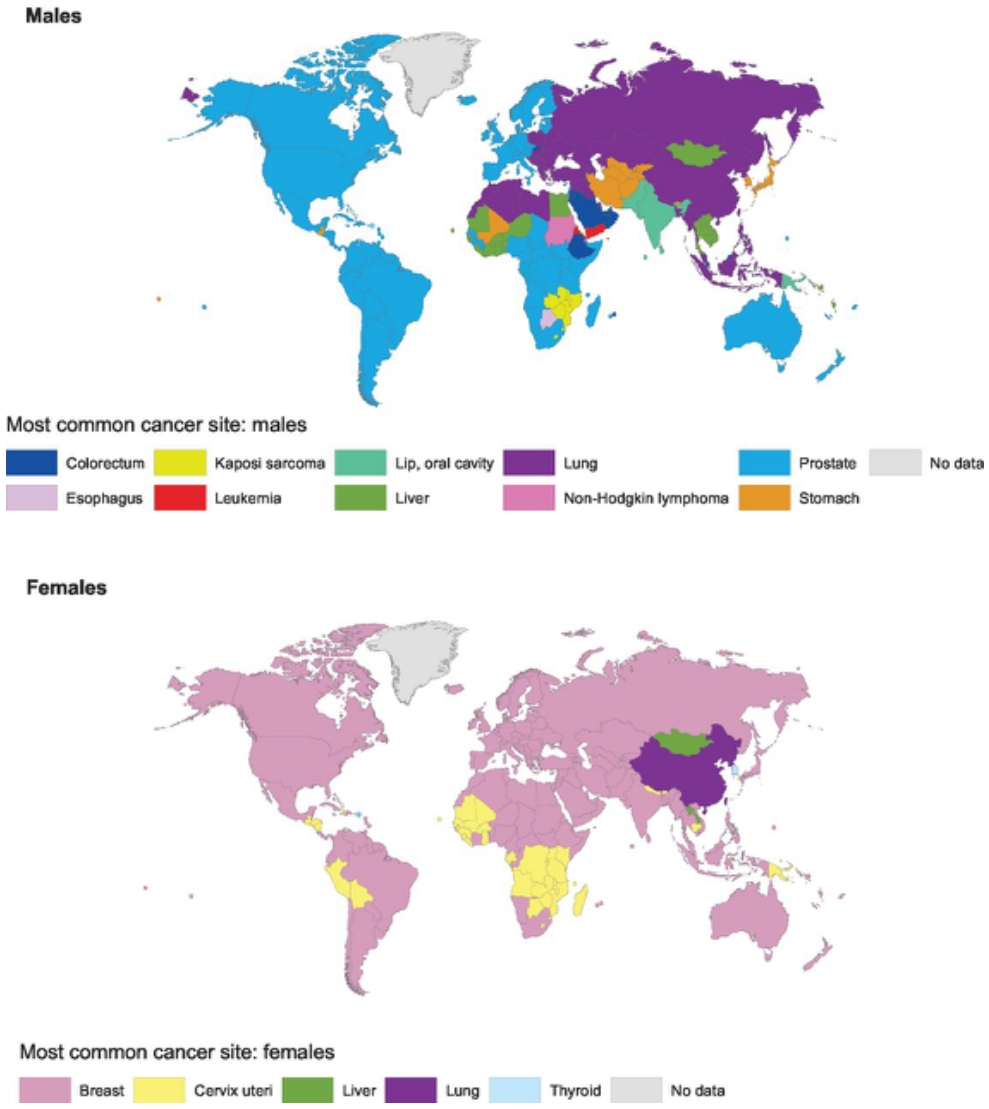
CHARPTER I

GENERAL INTRODUCTION

1.1 Breast cancer general introduction

Cancer, which is uncontrolled growth of abnormal cells, is more than 600,000,000 years old. It was found in dinosaur bones(1). Cancer is regarded as the major cause of morbidity and mortality in the world, with approximately 14 million new cases in 2012 (2) and 8.7 million cancer related deaths in 2015 (3). Breast cancer (BC) is the most frequent cancer among female in North America, Europe, Oceania, Latin America, the Caribbean, Africa, and also in most of Asia (4). In the USA, one in eight (12% of the women is affected by BC during their lives and it is the second most severe cancer that can kill women (5). Approximately 50% of BCs in USA happens in women older than 65 years (6). Among 40-49 years women, BC is the main cause to death (7). Women during pregnancy are more vulnerable to suffer BC compared with women who have never been pregnant (8).

Figure 1.1 Most commonly diagnosed cancers, 2012. (Compiled from GLOBOCAN 2012) (4).



The prevalence of BC in men is much lower than that of women. However, a large study (1998 -2007) found that men diagnosed with BC are more likely to die from the disease than women whereas the 5-year survival rate for women was 83% and it was 74% for men (9).

BC has been classified to subtypes by the estrogen receptor (ER) and human epidermal growth factor receptor-2 (HER-2). Several distinct subtypes have been

classified such as ER-positive and ER-negative. ER-positive tumors can be divided into two subtypes, luminal A (High expression of ER-related genes, low expression of the HER2 cluster of genes, and low expression of proliferation-related genes, such as Ki67) and luminal B (lower, but still expressing of ER-related genes, variable expression of the HER2 cluster, and higher expression of the proliferation cluster (high Ki67)). They are identified by expression of genes, also expressed by normal breast luminal epithelial cells. Their ER expression profile overlap, so they are called luminal A and B. Luminal B tumors signals a worse prognosis than luminal A tumors. Male were more likely to suffer from ER(+) tumors compared to female (88.3% vs. 78.2%)(9).

There are several subtypes that are characterized by low expression of hormone receptor-related genes (ER-negative), one of which is called the "HER2-enriched" subtype (HER2+/ER-) and another is called the "basal-like" subtype. HER2-enriched subtype means the HER2+/ER- subtype, which is characterized by high HER2 expression, and low expression of the luminal cluster (including luminal A and luminal B) (10). The basal-like subtype expresses many genes characteristic of normal breast basal epithelial cells. These tumors are typically ER-, PR- and HER2-negative on clinical assays. So they are also named “triple negative” (11).

Based on its origin, BCs can be classified into two categories: carcinoma and sarcomas. Carcinomas are cancers that are generated from the epithelial components of the breast tissue, and these components include the cells lining the lobule and terminal ducts. In normal conditions, these epithelial cells make milk for breast feeding. Sarcomas are rare cancers that are generated from the stromal components of the breast, and the component consists of myofibroblasts and blood vessel cells. The sarcomas include phyllodes tumors and angiosarcoma.

Carcinomas are the major form of all BCs and sarcomas only occupy less than 1% of primary BCs.

There are many subtypes of the vast group of carcinomas and the major division is between in situ and invasive. In situ carcinomas mean that the cancer cells exist and grow inside of the pre-existing normal lobule and duct without invading the breast tissue. However, invasive carcinomas are invading and growing outside of the normal lobule and duct, and they can metastasize to lymph nodes and other organs. About 80% of breast carcinomas are invasive ductal carcinomas, and nearly 10% of cases are invasive lobular carcinomas (12). The different subtypes have distinctive pathologic features. Different subtypes have different treatments and prognosis (13).

1.2 Clinical informations about breast cancer

1.2.1 Pathogeny and risk factors

Mechanisms leading to BC have not been completely demonstrated by now, but various factors can increase the risk. These factors include older age, female gender, personal or family history of BC, known genetic mutations, reproductive and hormonal factors, and other environmental factors such as exposure to radiation (14).

Family history of BC is a significant risk factor for BC. However, some research demonstrated that there were other important factors involved in inducing BC, because 8 out of 9 women's first-degree relatives did not suffer from BC (15).

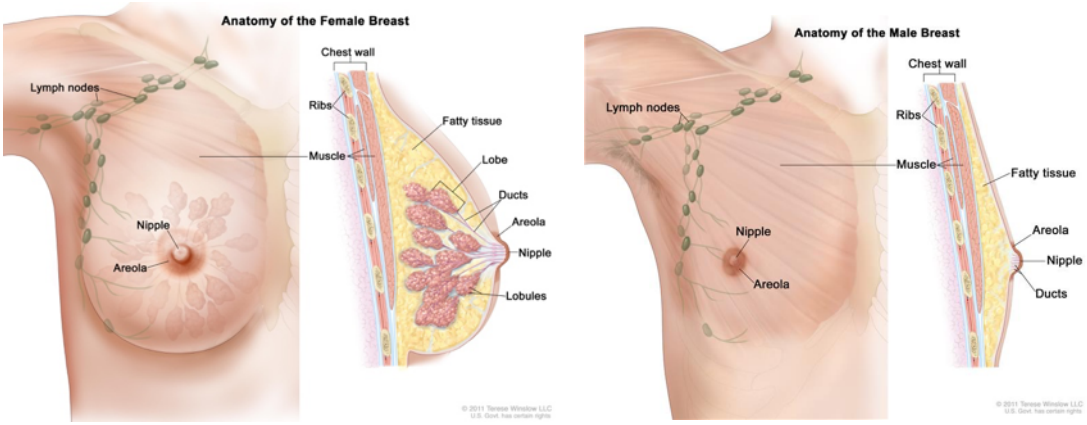
Estrogens play a major role in BC, and genetic factors also play important roles to increase the risk, such as BRCA1, BRCA2, p53 and PTEN (16).

The hypothalamic pituitary ovarian axis (HPG axis) alludes to the connection between the hypothalamus, pituitary gland, and gonads, which maturates during early life and adolescence, and it regulates production of ovarian hormones. Birth length, birth weight and adult height appear to be positively associated with HPG axis (17). Natural killer cells are involved in normal defense of tumor cell recognition and lysis (18). Natural killer cell activity (NKCA) has a limited but remarkable relationship with BC, especially related to objective BC risk, but not subjective BC risk (15). Objective risk is defined for getting BC based on scientifically established risk factors, which can predict the resultant health outcomes. Subjective risk means an individual's perception of her chance for developing BC based on her cognitive appraisal, includes psychological influence and behavioral factors that can affect health or health outcomes (15).

1.2.2 Symptoms

Most women do not present any symptoms until they were diagnosed by doctors. However, some masses can be palpated in breast or in the axilla by patient self-examination or doctor. Changes in skin and nipple of breast have been reported in BC, but not all the lesions belong to cancers. The skin changes include redness, scaling, dimpling and ulceration. Nipple changes include nipple scaling or new nipple reversion.

Figure 1.2 Anatomy of the female and male breasts.

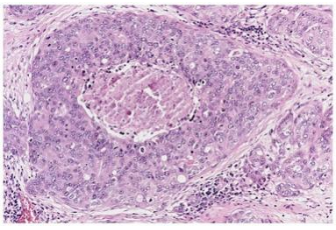
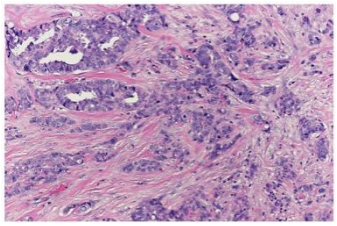
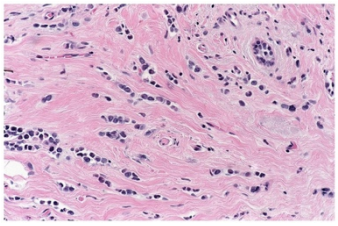


The Nipple, areola, lymph nodes, lobes, lobules, ducts, and other parts of the breast are shown. (From NATIONAL CANCER INSTITUE: http://www.cancer.gov/types/breast)

1.2.3 Diagnosis

Most of the BCs are first detected by abnormal screening imaging, such as mammography, magnetic resonance imaging (MRI) or ultrasound. These masses include benign tumors and malignant tumors. The tumor samples should be diagnosed by pathology if a mass is seemed malignant by the imagery. There are a few methods to obtain the samples for different types of BCs, including fine needle aspiration (FNA), core needle biopsy, and excisional biopsy. These samples will then be evaluated by a pathologist.

Figure 1.3 Types of Breast Cancers: Histologic examples of in situ & invasive carcinomas of the breast (19)

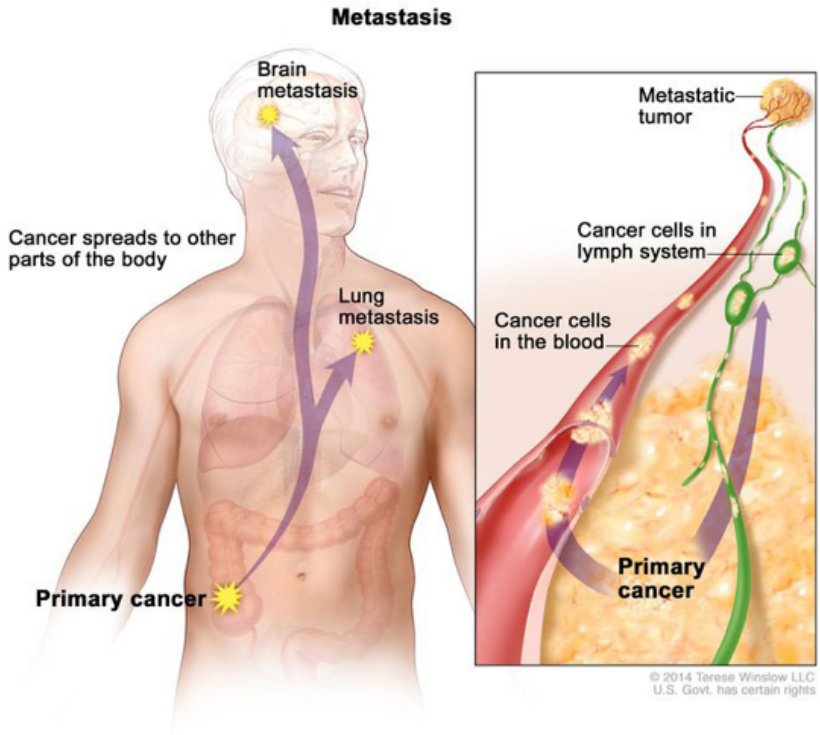
a.  b.  c. 

a. High grade ductal carcinoma in situ; b, Invasive ductal carcinoma; c. Invasive lobular carcinoma.

1.2.4 Stages of breast cancer

According to the TNM (tumor, lymph nodes and metastasis) classification of BC stages (AJCC (American Joints Committee on Cancer), 7th edition), BC is characterized according to the size of the tumor (T0: no evidence of primary tumor; Tis: carcinoma in situ; T1: tumor ≤2cm, T2: 2<tumor≤5cm, T3: tumor>5cm; T4:tumor any size tumor with direct extension to a) chest wall or b) skin, and whether or not the tumor has spread to the lymph node (NX: Regional lymph nodes cannot be removed, N0: no regional lymph node metastasis, N1: metastasis to movable ipsilateral axillary lymph nodes, N2: metastases in ipsilateral axillary lymph nodes fixed of matted (N2a) or met, only in clinically apparent ipsilateral mammary nodes without clinically evident axillary lymph nodes (N2b), N3: metastases in ipsilateral infraclavicular lymph nodes (N3a) or clinically apparent ipsilateral internal mammary lymph nodes (N3b) or ipsilateral supraclavicular lymph nodes (N3c), and finally whether the tumor has metastasized or not: Mx: Distant metastasis cannot be assessed, M0: no distant metastasis, M1: distant metastasis. As BC develops, stage is always expressed from scale 0 to Ⅳ. Stage 0 (TisN0M0); Stage Ⅰ: T1N0M0; Stage Ⅱ: T0~1N1M0, T2N0~M0, T3N0M0; Stage Ⅲ: T0~2N2M0, T3N1~2M0, T4N0~3M0, TXN3M0, Stage Ⅳ: TXNXM1. Men were more likely to grow larger tumors which were more likely to have spread to lymph nodes and distant metastasis (9). When cancer cells break away from where they first formed and traveled via blood and lymph system, they can cause metastatic tumors in other parts of the body. Both the primary tumor and metastatic tumor are the same type of cancer.

Figure 1.4 Cancer cells metastasis pathway.



In metastasis, cancer cells break away from where they first formed (primary cancer), travel through the blood or lymph system, and form new tumors (metastatic tumors) in other parts of the body. The metastatic tumor is the same type of cancer as the primary tumor. From: https://www.cancer.gov/about-cancer/understanding/what-is-cancer

1.2.5 Treatments

Surgery is still one of the main methods to treat BC, besides there are chemotherapy, endocrine adjuvant therapy, radiation therapy, immunotherapy and biotherapy recently (20).

After mastectomy, breast carcinoma can also recur in the chest wall or regional nodal basins, including the supraclavicular, axillary, and internal mammary regions. The function of radiation therapy is to eradicate the risk of recurrence and thus improve overall survival (21). From some studies, it was demonstrated that not only node-negative patients who have high risk disease (T3 or T4) can benefit from

radiation therapy, but also the patients with node-positive diagnosis (22). The radiation therapy for borderline patients may be renewed from some retrospective studies of node-negative patients (23–25). These studies considered a series of risk factors, such as patient age, larger tumor size, close invasion, omission of systemic therapy and high nuclear grade. Race and ethnicity are also believed to be as the important factors that affected to the prognosis. Black, Hispanic white and American Indian women often have advanced BC and have poorer survival rates compared with non-Hispanic whites (26).

Hormone receptor-positive BCs represent the majority of BCs in the world. Nearly 60% to 75% of BCs are estrogen receptor-positive (ER+), while progesterone receptor positive (PgR+) occupies about 65% (27). For hormone receptor-positive (ER+ and/or PR+) patients, endocrine therapy is considered as the most effective treatment. Patients who are positive for hormone receptors but are negative for Her2 are considered to have better prognostics than the patients whose tumors are negative for hormone receptors with Her2 over-expression (28).

After a BC diagnosis, physical activity may reduce the risk of death from this disease (29).

1.3 Hormone effects on breast cancer

In the etiology of BC, reproductive and hormonal factors play critical roles. With increasing concentrations of some sex hormones, the risk of BC increases statistically significantly. The hormones examined include: estrogens ((estradiol) E2, estrone (E1)), androgens (5α-dihydrotestosterone (DHT), androstenedione, dehydroepiandrosterone (DHEA), testosterone), and intermediate sex-hormones (estrone sulfate (E1-S), dehydroepiandrosterone sulfate (DHEA-S)) (30).

Estrogens help the proliferation of breast epithelium and androgens are involved in regulatory mechanisms for the growth of normal and malignant cells as judged from epidemiological and endocrine evidences. In premenopausal women, the estrogens are secreted mostly from ovaries. In postmenopausal women, because the cessation of ovarian function, estrogens stay at low concentration, and are mostly are produced either by utilizing aromatase from the local conversion of androstenedione to E1 in adipose and normal and malignant breast tissues (31), or by involving STS, which catalyzes the conversion of E1-S to E1 (32). Mean E2 in plasma decreases from 400 pM in premenopausal phase to 25 pM in postmenopausal phase. In BC tissue, the E2 concentration is about 10-20 times higher than that in the plasma, while the E1 concentration in breast tissue is 2-10 times higher than that in the plasma (33)(34). In BC tissue, the concentration of E2 was even as high as 10 nM, whereas that of E1 is 2.5nM (27,35).

E2 concentration in blood is different during various phases of the female as follows (36): when a woman (>18 years old) is in follicular phase of the menstrual cycle, E2 level is 70-510 pmol/L, during pre-ovulatory peak, it is 390-1480 pmol/L; during luteal phase, E2 is 70-600 pmol/L; during post-menopause, it is <130 pmol/L. The E2 level in a male is 50-200 pmol/L in blood. E2 is the most important female sex hormone which stimulates the growth of BC cells, and it is obligatory for the induction and progression of hormone-dependent BCs (37)(38). The ratio of E2 concentration and E1 concentration in vivo makes a significantly contribution to BC cell proliferation (39). The ratio was significant higher in postmenopausal women with a higher risk of BC than in premenopausal women (38)(40). It was found in our laboratory that the E2/E1 ratio is controlled both by 17β-HSD1 and 17β-HSD2, but type 1 is the most influential factor in cells compared with type 2 and cofactors (41).

Estrogen can induce cyclin D1 expression in mammary epithelial cells (42). In ERα positive BC cells proliferation can be stimulated by estrogen through activating the cyclin D1(CCND1) oncogene (43).

E1-S can be reactivated within breast cells by STS (44). Levels of E1-S and E2-S are also high in breast tumors (45). E1-S can not bind to ER directly, but when converted to E2 it can significantly increase progesterone receptor level (46) and pS2 protein (47), The latter can increase the production of cathepsin D, an estrogen-inducible protein (48)(49). From clinical evidence, DHEA-S can support breast tumor growth in postmenopausal women. After aromatase inhibitor therapy, serum DHEA-S concentrations of patients was significantly higher than those of patients with stable disease (50).

DHEA decreases 40% after menopause, and results in falling androgens and estrogens in peripheral target tissues. This can induce a series of medical problems at menopause, such as hot flushes, night sweats, vulvovaginal atrophy, bone loss and fractures, muscle loss, sexual dysfunction, loss of memory, loss of cognition and possibly Alzheimer's disease (51). In premenopausal women, E2 is mainly produced by gonads such as ovaries, and functions as a circulating hormone. After menopause, E2 and DHT are synthesized mostly from DHEA in peripheral tissues. Nearly 95% of the active estrogens and androgens remain in an inactivated form locally before being release into blood as functional metabolites (52).

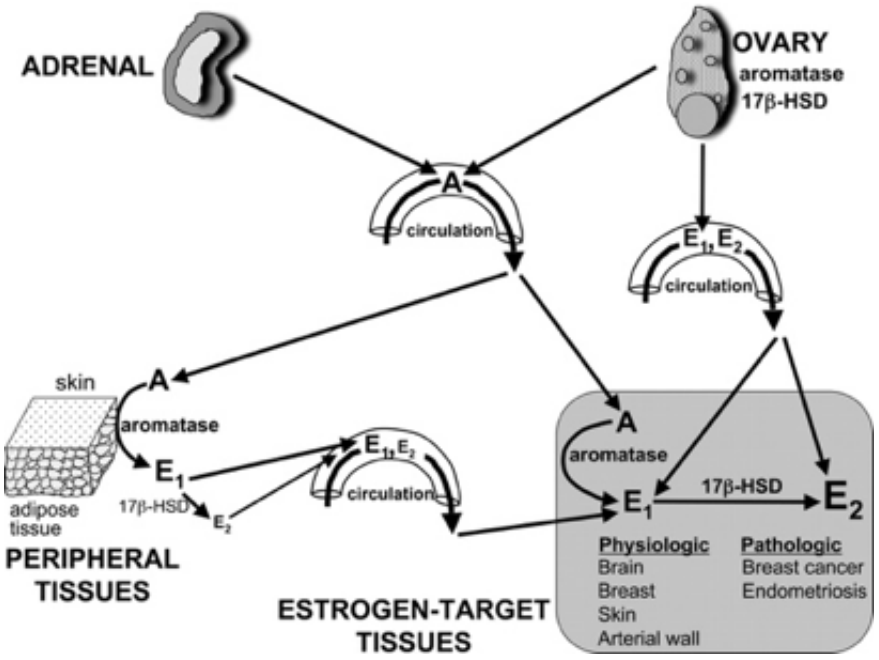
Approximately 75% of BCs are positive in androgen receptor (AR) (53). DHT has two or three fold higher affinity to AR than testosterone (54). In a series of *in vivo* studies, DHT was shown to inhibit BC cell proliferation using both BC cell lines and dimethylbenzanthracene- (DMBA)-induced mammary tumors in rats (55)(56).

The intratumoral concentration of DHT decreases from 259 pg/g to 200 pg/g, comparing the patients aged <70 years to those who are more than 70 years (57). DHT significantly decreases cell proliferation in the presence of E2 in T47D cells (39)(58).

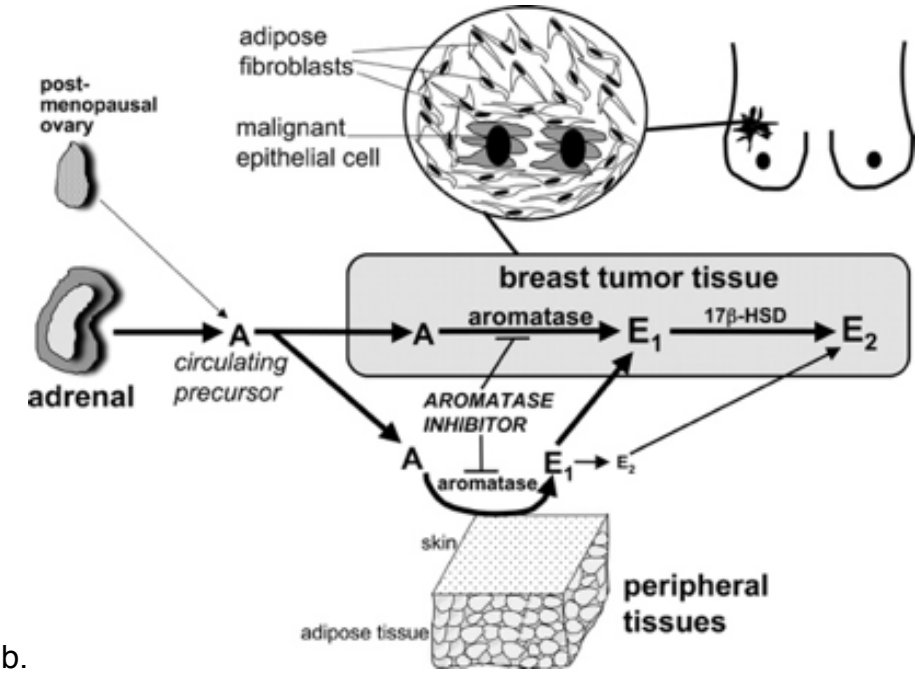
The androgen receptor antagonist hydroxyflutamide can reverse the DHT inhibition, this means androgens inhibit BC cells proliferation via an AR-mediated mechanism (59). From BC cell studies in vitro, it was demonstrated that androgens inhibit the proliferation of ZR-75-1 cells to 40%, and this effect can be reversed by hydroxyflutamide (55). DHT can inhibit cell proliferation and prolongs the G1 phase of the cell cycle around 40% in MCF7 and T47D cell lines (60).

Testosterone is the major circulation androgen secreted by the testis and then activated in muscle. It is an active androgen which binds to AR effectively, and it plays its role without the requirement of transformation into DHT (61).

Figure 1.5 Origins of estrogen in women, and tissue sources of estrogen in postmenopausal breast cancer.



a.



b.

a. Origins of estrogen in women. The biologically active estrogen E2 is produced in at least three major sites: 1) direct secretion from the ovary in reproductive-age women; 2) by conversion of circulating androstenedione (A) of adrenal and/or ovarian origins to estrone (E1) in peripheral tissues; and 3) by conversion of A to E1 in estrogen-target tissues. In the latter two instances, estrogenically weak E1 is further converted to E2 within the same tissue. The presence of the enzyme aromatase and 17-HSD is critical for E2 formation at these sites. E2 formation by peripheral and local conversion is particularly important for postmenopausal women and for estrogen-dependent diseases such as BC, endometriosis, and endometrial cancer. b. Tissue sources of estrogen in postmenopausal BC. This figure exemplifies the important pathologic roles of extra-ovarian (peripheral) and local estrogen biosynthesis in an estrogen-dependent disease in postmenopausal women. The estrogen precursor androstenedione (A) originates primarily from the adrenal in the postmenopausal woman. Aromatase expression and enzyme activity in extraovarian tissues such as fat increases with advancing age. The aromatase activity in skin and subcutaneous adipose fibroblasts gives rise to formation of systemically available E1 and to a smaller extent estradiol (E2). The conversion of circulating A to E1 in undifferentiated breast adipose fibroblasts compacted around malignant epithelial cells and subsequent conversion of E1 to E2 in malignant epithelial cells provide high tissue concentrations of E2 for tumor growth. The clinical relevance of these findings is exemplified by the successful use of aromatase inhibitors to treat BC. (62)

1.4 Enzymes related to breast cancer

There are many enzymes related to BC, and lots of substrates are involed. In the presence of more than one substrates for one enzyme, or at least two enzymes for one substrate, the metabolism will occur between the enzyme and the substrate possessing higher affinity.

1.4.1 17β-HSDs

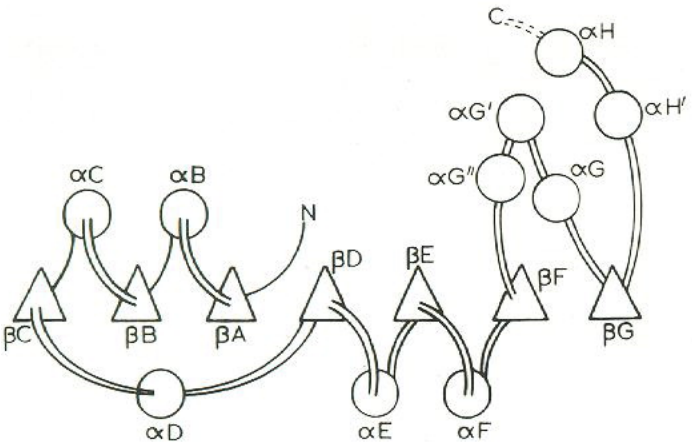
17beta-hydroxysteroid dehydrogenases (17β-HSDs) are the enzymes responsible for the reduction or oxidation of steroid hormones, fatty acids and bile acids in vivo with NAD(P)(H) as cofactors (31). 17β-HSDs catalyze the last step in the activation or the first step in the degradation of sex hormones, but poss very low identity between members (63). Fifteen types have been described to date, of which 14 types are found in human, and 12 isozymes of 17β-HSD have been cloned. All

types belong to short-chain dehydrogenases/reductases (SDR) superfamily, except the 17β-HSD5, which belongs to aldoketo-reductase family (31)(64). Thus, 17β-HSDs are important mediators in pre-receptor regulation of sex hormone action. Although 17β-HSDs show the major redox activity at 17β-position of the steroid, some 17β-HSDs can convert multiple substrates at multiple sites, like at the 3-position on the steroid ring. The multi-specificity of the enzyme was shown for several steroids (65–68). Oxidation of the C17 hydroxyl of DHT, testosterone, or E2 decreases the potency of these steroids. The reduction of the C17-keto group of 5α-androstane-3,17-dione, or E1 activates their biological steroid ability. In the biosynthesis of the most active estrogens and androgens, 17β-HSDs catalyze the reduction at the final step, to produce E2, 5-androstene-3β, 17-diol, testosterone and DHT (69), or the first step of their degradation, such as degrading DHT into 3β-diol.

Human 17β-HSD1 is the most well characterized member of 17β-HSDs and is a key enzyme to catalyze the conversion of E1 to E2. It has been studied since the 1950s (70), and it was known that the enzyme is expressed in gonads as well as in several peripheral tissues (71). It usually functions as a homodimer under native conditions. The rapid purification of homogeneous 17β-HSD1 revealed that the enzyme has very high specific activity as compared to former reports and laid down the basis for its crystallization (72). Dr. Lin' s laboratory was the first to crystallize and to determine the first three dimensional structure of any human steroid enzymes, i.e. the human 17β-HSD1 in 1995 (73)(74). This membrane-associated protein consists of 327 amino acids (75) organized as a homodimer of 34.5 kDa per subunit (72). The active site has two clefts: one binds the cofactor and the other binds the substrate. The segment (βA-βF) (as Fig.1.6) belongs to the Rossmann fold which binds NAD(P)H cofactors, while the segment (βD-βG) which

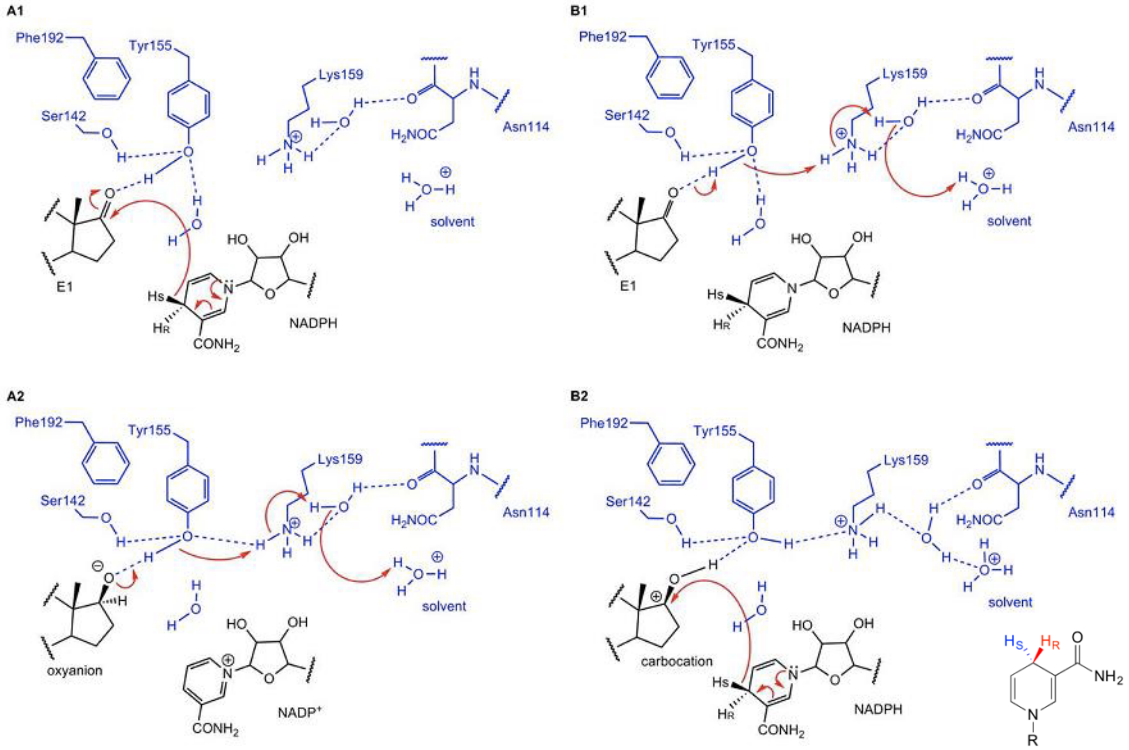
binds steroid partially belongs to the Rossmann fold (76). E2 can bind to estrogen receptors (ERα and ERβ) or to the G protein coupled membrane receptor (GPR30), followed with recruiting promoters of several genes which are related to cell proliferation, so as to stimulate the cell growth (76–78).

Figure 1.6 Folding topology of strands (triangles) and helices (circles) in 17β-HSD. (76)



Catalysis by 17β-HSD1 have been studied (62, 64,68,71) and two mechanisms have been proposed: a one-step mechanism with simultaneous transfer of hydride and proton (not shown) and a two stepwise mechanism. The latter differs by the intermediate formation of either an oxyanion or a carbocation, in Fig.1.7.

Figure 1.7 Two possible stepwise catalytic mechanisms for 17β-HSD1 (80).



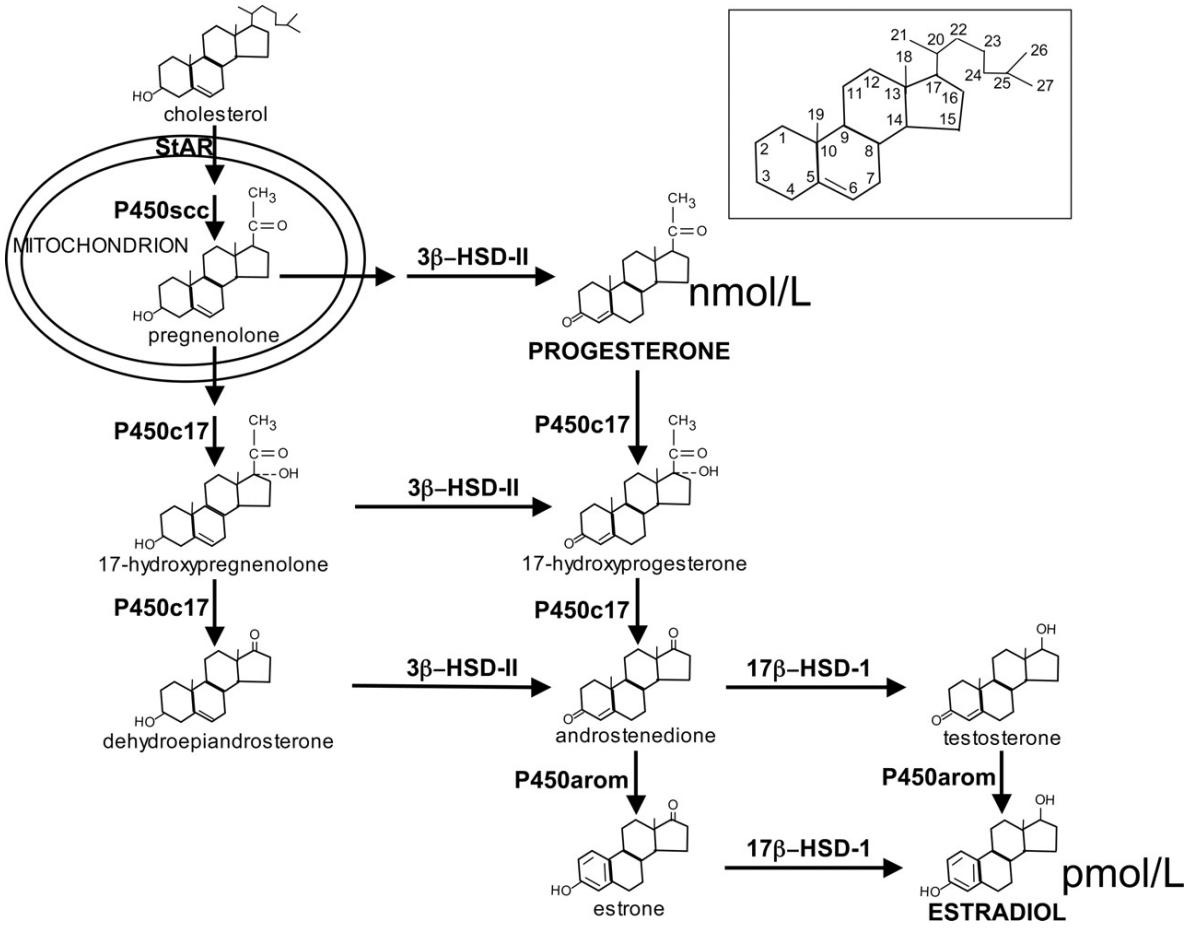
A) In the first step the *pro-S hydride of NADPH is transferred to the α-face of E1 at the planar C17 carbon (A1), resulting in an energetically favorable aromatic system; the resultant oxyanion is subsequently protonated by the acidic-OH group of Tyr155 (A2). (B) In the first step the keto oxygen of E1 is protonated by the acidic-OH of Tyr155 (B1); the resultant carbocation then accepts the pro-S hydride of NADPH at the α-face (B2). The proton relay is facilitated by a H-bond network involving Lys159, two water molecules and Asn114, an electrostatic interaction between the protonated side chain of Lys159 and the phenyl ring of Tyr155 (74) as well as T-stacking between Phe192 and Tyr155 (81). Hydrogen bonds are represented by dashed lines. For the sake of clarity π–π-interactions are not depicted.

*pro-S: If two identical substituents are attached to a sp3-hybridized atom, the descriptors pro-R and pro-S are used to distinguish between the two. Promoting the pro-R substituent to higher priority than the other identical substituent results in an R chirality center at the original sp3-hybridized atom, and analogously for the pro-S substituent

According to biochemical (82,83) and structural studies of the 17β-HSD1-E2-NADP complex (76,84–86), 17β-HSD1 belongs to an NADP(H)-preferring class of enzyme. However, it also has ability to bind NAD(H) cofactors in vitro. 17β-HSD1

catalyzes the conversions of E1 into E2 (87) and DHT into 3β-diol (88). 17β-HSD1 also catalyzes the reduction of DHEA into 5-androsteren-3β,17β-diol (5-diol) (89), a weaker estrogen that becomes more important after menopause (88)(90). 17β-HSD1 possesses some 3β-HSD activity because the concomitant 3β-reduction of DHT into 3β-diol and 17β oxidation to A-dione was reported (66). In kinetic studies of 17β-HSD1, it was demonstrated that the enzyme has 240 fold higher specificity towards E1 reduction than E2 oxidation at physiological pH using triphosphate cofactors, while the cofactor plays a considerable role of the specificity for estrogen reduction (87).

Figure 1.8 Steroidogenic pathway (62).



The biologically active steroids progesterone and estradiol are produced from cholesterol by a number of enzymes in the ovary. The key steps seem to be the entry of cholesterol into the mitochondrion facilitated by the StAR protein for progesterone production and the conversion of androstenedione to estrone catalyzed by aromatase (P450arom) for estrogen production. Please note that biologically active quantities of progesterone (nanomolar) are 100 to 1000 times higher than those of estradiol (picomolar). This underscores the fact that relatively very low quantities of aromatase enzyme and estradiol give rise to important biological functions.

In several studies, the patients whose 17β-HSD1 expressed highly will have obviously shortened disease free and overall survival in BC (32,91,92). These supports inhibition of 17β-HSD1 enzyme activity may be used for effective treatment of the hormone-dependent BC in postmenopausal patients. In Saloniemi's study (93), 17β-HSD1 plays a pivotal role in causing a significant amount of androgen inactivation in vivo. It means that inhibition of 17β-HSD1 may also have a function in female patients relating to androgenic dysfunction. In ER+ patients, a high ratio of 17β-HSD1/17β-HSD2 has a better prognosis in tumor, and low expression of 17β-HSD2 is related to a significantly higher recurrence rate (32). Some literature reports demonstrated that 17β-HSD1 is expressed in a high proportion (61-100%) of BC tissues (94). However, there are also some studies demonstrating that only 2% of BC cases expressed 17β-HSD1 , and 48% expressed 17β-HSD7, while 83% expressed 17β-HSD12 (95).

17β-HSD2 can activate 20α-progesterone to synthesize progesterone, but it can also be involved in the oxidation of retinoids(96).

17β-HSD5 is structurally an aldo-keto reductase, which is also a member of the SDR-family (97). In BC tissue, 3β-HSD, 17β-HSD3/5 and 5α-reductase 1 and 2 are involved in the formation of DHT. DHEA, which is the direct product of DHEA-S by STS, is converted into 4-dione by 3β-HSD, followed by conversion to testosterone

by 17β-HSD3/5. Then 5α-reductase 1 and 2 converts testosterone into DHT, which is the most potent androgen in tissue (98).

17β-HSD7 was first named as prolactin receptor-associated protein in rat (99). 17β-HSD7 has similar estrogenic function and androgen inactivating roles in BC cells, and DHT inactivation ability is stronger compared with 17β-HSD1. The major role of 17β-HSD7 may be as a 3-ketosteroid reductase in cholesterol biosynthesis, with reducing zymosterone at the 3-position to form zymosterol as suggested by the literature (100,101).

1.4.2 Aromatase

Aromatase (CYP7B1) plays an important role in E2 synthesis and it is a member of the cytochrome P450 enzyme superfamily (102). It also catalyzes the conversion of 4-dione and testosterone to E1 and E2, respectively (Figure 1.9). The gene CYP19 which encodes the aromatase enzyme is located on 15q21.1 (103). The cytochrome P450 CYP7B1 is believed to be the primary enzyme, which is responsible for the inactivation and elimination of 3β-Adiol (104)(105).

After menopause, aromatase is an important enzyme for the estrogen by converting androgens into estrogens. The enzyme is primarily found in adipose tissue, muscle, skin, benign and malignant BC (106), but it is also highly expressed in the placenta and in the granulosa cells of ovarian follicles, and the expression in these organs is related to cyclical gonadotropin stimulation (33). The increase of aromatase mRNA expression is closely related to the patients who had distant metastasis and local recurrence and/or survival time (92). Thus high aromatase expression carries poor outcome. Aromatase is known as an enzyme that is

regulated by cytokines, growth factors, steroids, and prostaglandin estradiol (PGE2) (107).

1.4.3 Steroid sulfatase (STS)

STS is responsible for the hydrolysis of aryl and alkyl steroid sulfates and it plays a critical role in regulating the formation of biologically active steroids. STS is the single enzyme that hydrolyze several sulfated steroids such as E1-S, DHEA-S and cholesterol sulfate (108)(109). STS is a transmembrane protein that is predominantly located in the endoplasmic reticulum, with smaller fractions in the Golgi cisternae and the trans-Golgi reticulum (110). The molecular mass of STS is approximately 65 kDa (111). STS belongs to a superfamily of 12 different mammalian sulfatases (112). The gene for the human STS is located on the distal short arm of the X-chromosome and maps to Xp22.3-Xpter. The gene is pseudo autosomal and escapes X-inactivation (113).

STS activity was first demonstrated in rat liver microsome in 1954 (114). At the molecular level, cytokines TNFα and IL-6 synergistically up-regulate STS enzyme activity through post-translational mediated effect rather than through any changes in gene transcription or mRNA stability in MCF-7 BC cells. However, they did not increase the activity separately. It is indicated that activation occurred independently of STS promoter and enhancer elements. The mechanism of increasing activity may be through posttranslational modification of cysteine to formyl glycine in the active site, or indirectly through variations in membrane fluidity or organic anion transporters, allowing enhanced intake of the hydrophilic substrate (115). The inflammatory cytokine IL-1β reduced the activity and mRNA expression of STS (116,117), while basic fibroblast growth factor and IGF-I increased STS activity (118). STS activity can be increased 50-fold greater in both pre- and

postmenopausal breast tumors compared with normal breast tissue (45). The highest activity was examined in placenta but only in placental syncytiotrophoblasts (119). In addition, some factors are likely to influence the activity of STS which regulate the extent of post-translational modification of cysteine-formyl glycine, glycosylation, and translocation at the endoplasmic reticulum (113).

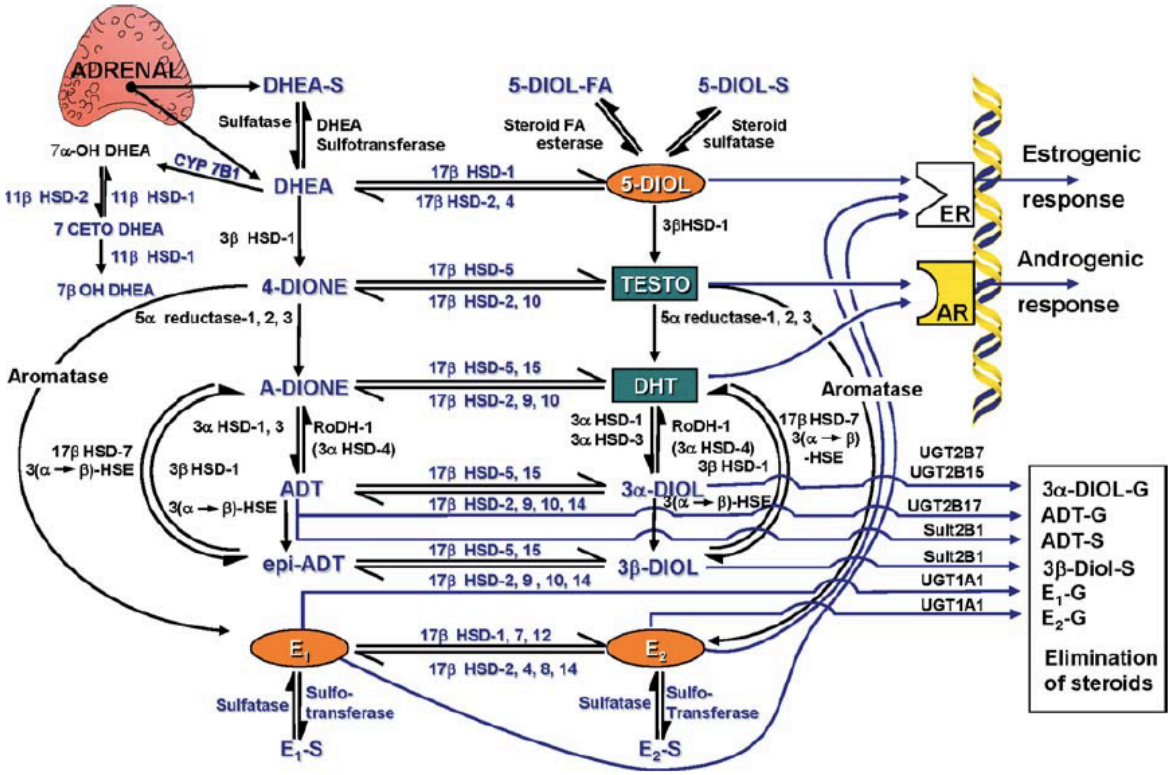
The placenta is the most abundant sources of STS in human, and STS also exists in other tissues, such as adrenal glands, ovary, testis, prostate, skin and brain, fetal lung, viscera, endometrium, peripheral blood lymphocytes, aorta, kidney and bone. The enzyme was found to be localized in the secretory cells of fallopian tubes. A larger number of positive cells were in tissues in the early luteal phase than in tissues in the follicular phase of the menstrual cycle (120). Amplified STS mRNA transcripts were detected at weak levels in adult lung, aorta, liver, thyroid, testis, uterus, and all fetal tissues (119). STS mRNA expression is higher in malignant breast tissues (1458 amol/mg/RNA) than nonmalignant tissues (536 amol/mg/RNA) (121).

Breast tissue of postmenopausal women can have ten times higher estrogen levels compared with plasma from the same patients (122). The hyper-estrogenic state is most likely caused by the fact that STS activity is at least 50 times higher in both premenopausal and postmenopausal breast tumors compared with normal breast tissues (123)(124), whereas aromatase expression is only found in 60-70% of BC tissues (125)(126) and that STS activity in BC is obviously higher than that of the aromatase (127). The increased STS could result in a 10-folds greater amount of E1 originating via the sulfatase route than via the aromatase pathway (123). In addition, real-time RT-PCR experiments have demonstrated that STS mRNA expression in malignant breast tissue was significantly higher (about 3 times) than

in normal tissues (121). Clinical studies also showed STS mRNA expression may be an indicator of recurrence in BC patients but this association and prognosis were applied only to ER+ tumors (128). A very interesting research showed that STS activity was elevated in the tissue from BC patients treated with AIs (129). All these results have the clear implications for the continued development of STS inhibitors.

STS mRNA expression is much higher than aromatase mRNA expression and enzyme activity of STS is considerably higher than that of aromatase (130). From Toshiaki's study, it was demonstrated that higher STS mRNA expression was implicated with lymph node metastasis more than without nodal involvement. High levels of STS mRNA involved to predict decreasing relapse-free survival as a continuous variable (131).

Figure 1.9 Human steroidogenic and steroid-inactivating enzymes in peripheral intracrine tissues.



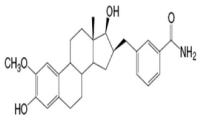
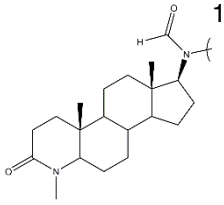
4-dione, androstenedione: A-dione, 5α-androstane-3,17-dione; ADT,epiandrosterone; E1, estrone; E1-S, estrone sulfate; E2, 17β-estradiol; E2-S, estradiol sulfate; 5-diol, androst-5-ene-3α, 17β-diol-FA, 5-diol fatty acid; 5-diol-s, androst-5-ene-3alpha,17beta-diol sulphate; HSD, hydroxysteroid dehydrogenase; testo, testosterone; RoDH-1, Ro dehydrogenase 1; ER, estrogen receptor; AR, androgen receptor; ugt2b28, uridine glucuronosy transferase 2B28; Sult2B1, sulfotransferase 2B1; UGT1A1, uridine glucuronosyl transferase 1A1.(132)

1.4.4 Enzyme inhibitors

The development inhibitors of 17β-HSD1 started slowly and the first inhibitors for drug purpose were designed in the 1980s by Covey's group (133). The first published review on inhibitors of 17β-HSD1 was in 1996 by Penning (134). MeO-CC-156 (RD579-25) is coming from a more recent series of 17β-HSD1 inhibitors with less estrogenic effects than previously developed inhibitors (94). INH1 has a methoxy group at 2-position and shows a significant inhibition with an IC_{50} value of

275 nM for the transformation of E1 to E2 (Table 1) (94). It binds specifically to 17β-HSD1 (94). GD-572-174B (EB-357-030) is one of the inhibitors of 17β-HSD7 (INH7), whose IC_{50} is 195nM for the transformation of E1 to E2 (135). From Yannick Laplante's paper (2009) (136), the type 1 inhibitor we used cannot influence the activity of 17β-HSD7 and it does not have estrogenic activity. INH7 has high specificity to bind to 17β-HSD7 (135). In recent years, there are some great progresses in the design and development of 17β-HSD inhibitors to treat various disorders, such as breast and prostate cancer and endometriosis (31)(137). 17β-HSD 1 inhibitors have been developed rapidly, especially in the relationship of inhibitory activity and estrogenicity. The first generation of non-estrogenic inhibitors active in vivo has been published more recently(69).

Table 1.1 Characteristics of 17β-HSD1 and 17β-HSD7 inhibitors used in this study. (138)

Compound	Targeting enzyme	Chemical structure	IC50 for E1~E2 conversion	IC50 for DHT~3β–diol conversion
MeO-CC-156 (INH1)[a]	17β-HSD type 1		275 ± 5 nM	None
EB-357-030 (INH7)[b]	17β-HSD type 7		195 ± 18 nM	230 ± 15 nM

None, data not shown.

a.IC_{50}, concentration of the compound inhibiting 50% of E1 to E2 conversion in T47D cells (94)(139).

b.IC_{50}, concentration of the compound inhibiting 50% of DHT inactivation in HEK293 cells overexpressing 17β-HSD7 (135).

Aromatase inhibitors (AIs) were first evaluated in an adjuvant setting by the Royal Marsden Hospital, London (140). They used to treat women with ER+ BC were first reported by the American Society of clinical Oncology (ASCO) in 2002 (141). Anastrozole was used as a first-line therapy, and letrozole is another aromatase inhibitor approved by the U.S. Food and Drug Administration (FDA). They reprent the future third-generation of AIs, highly potent, well-tolerated and nonsteroidal AIs. The third generation AIs (letrozole, anastrozole and exemestane) inhibit the peripheral conversion of adrenal androgens to estrogens, which then decreases the circulating E2 concentration to down regulate the growth of tumor (142), and all three inhibitors belong to nonsteroidal inhibitors.

Tamoxifen is one of the selective estrogen receptor modulators (SERMs), which is usually used to treat ER+ BC (143). It is confirmed that long-term treatment with adjuvant tamoxifen is more effective than a short duration treatment (144). According to ASCO committee recommendations in 2010, patients who used the combination of AIs and tamoxifen had lower the risk of BC recurrence compared with those who only took tamoxifen alone for 5 years, and the appropriate duration of adjuvant endocrine therapy should be 5 years (145). In 2014, ASCO recommended that premenopausal women should take tamoxifen for a total duration of 10 years if they received 5 years of adjuvant tamoxifen. For postmenopausal women who have already been treated by tamoxifen for 5 years, they could choose to continue tamoxifen or switch to AIs (27).

The hormone therapies have improved significantly with the research and clinical efforts of SERMs with tamoxifen and AIs like Letrozole (146). Unfortunately, the resistance to tamoxifen (147) and the associated potent induction of endometrial cancer (148) commonly occur in patients with progressive cancer. In addition, a

resistance to AIs has also been observed in clinical setting (149). Therefore, other enzyme inhibitors directed towards steroid biosynthesis are required.

The development of STS inhibitors started from 1990s (111,150–154) and had significantly progressed over the past ten years (155–159), but these inhibitors had initially an undesirable residual estrogenic activity. Some compounds arrived in preclinical stages but only one inhibitor was scheduled for clinical Phase I trial. Based on the chemical synthesis and biological activity studies, there are two families of inhibitor of second generation, including steroidal inhibitors and non-steroidal inhibitors. 667 Coumate (STX-64) is a tricyclic coumarin sulfamate and an irreversible inhibitor which showed a non-estrogenicity profile (160). It was the only first STS inhibitor that entered in a Phase I trial for treatment of hormone-dependent BC (44)(130). The steroid derivative EM-1913 is a non-estrogenic but potent steroidal irreversible inhibitor of STS (161).

With the aim to reduce estrogen concentration by the sulfatase pathway, STX-64 is a first generation STS inhibitor, which has been tested and showed to display no estrogenicity (162). After in vitro studies and in vivo studies, STX-64 was the first STS inhibitor tested in women with BC. When STX-64 was tested at doses of 5 mg on nine women and 20 mg on five woman subjects, the STS activity in peripheral blood lymphocytes and tumor tissues was inhibited by over 90%, demonstrating the potency of this class inhibitor (44). Furthermore, a Phase II clinical trial was performed by the pharmaceutical group Ipsen Ltd. However, there is no report about its success and paper published. In a Phase II endometrial cancer trial STX-64 did not achieve desired results for patients that had demonstrate stable disease for 6 months nor was it deemed that STX-64 would prove superior to megestrol

acetate, a progesterone derivative commonly administered to patients (Ipsen press release) (163).

1.5 Molecular pathways of breast cancer

In BC pathogenesis, many studies recently focused on molecular pathways. Mutation in oncogenes, pro-oncogenes and tumor suppressor genes has been regarded as potential elements, especially DNA amplification and DNA deletion. NF-κB plays a wide range of activities in apoptosis, cell survival, cell proliferation pathways, cell adhesion and angiogenesis. These functions include enhancing tumor cell invasion and angiogenesis, increasing expression of proto-oncogenes such as c-myc and cyclin D1 which directly stimulate proliferation (164). Ras signaling pathway is commonly activated in breast tumor progression, followed by recruiting of abundant downstream effector molecules, such as PI-3K, Raf serine kinase, GRB associated-binding protein (GAP) and Ras-related protein (Ral) (165).

Hormone therapy or oral contraception may proliferate the existing quiescent tumor cells, and may result in the increase of breast tumor development risk. Some polymorphisms can also increase the risk of estrogen-associated BC, such as G478T, A908G and C975C (166). Growth factor and cytokine receptors can be induced by progestin at the cell surface, which can be involved in the regulation of several intracellular elements, including Stat 5, and by potentiating mitogen-activated protein kinase (MAPK) and Janus kinase. These regulations are through increasing the levels and altering their subcellular compartmentalization at cytoplasmic level (167).

1.6 Breast cancer cell lines

In vitro studies confirmed that MCF-7, T47D, ZR-75-1 and MFM-223 cell lines are receptors positive, including androgen, estrogen, and progesterone receptors (168).

MCF-7 cell line originates from a 69-year-old Caucasian woman with breast malignant tumor (169). MCF-7 cell line was demonstrated in 1975 as a human BC cell line with estrogen, androgen, progesterone, and glucocorticoid receptors (170). This cell line is an excellent model for studying the mechanisms of the four steroid in relation to BC cells, especially for estrogen.

T47D cell line was originally isolated from a 54-year-old female patient with an infiltrating ductal breast carcinoma (171). Both T47D and MCF-7 are ER+ cell lines (172)(173), have similar characteristics, and possess some significant differences in 17β-HSD1 expression. The 17β-HSD1 mRNA in T47D cells is 697 thousands copies of mRNA/μg, which is much higher than in MCF-7 cells (87 thousands copies of mRNA/μg) (172).

Series cell lines ZR-75-1 expresses ER, PR and AR (174). MFM-223 cell line is characterized by high androgen and low estrogen and progesterone receptor levels (168).

AR levels are 12200±1321 sites per cell for the MCF-7 cells and 19450±2134 sites per cell for the T47D cells. For ZR75-1, MDA-MB-451 and LNCaP cell lines, AR levels are calculated to 45030±2216, 69540±2634 and 78455±2111 sites per cell, respectively. For immunocytochemical analysis of cells stained positively for AR, the percentages are 24.0±7.1, 29.5±6.4, 75.7±7.5, 88.1±2.5 and 94.0±6.5 in MCF-7, T47D, ZR75-1, MDA-MB-451 and LNCaP cell lines (59).

1.7 Aims of study

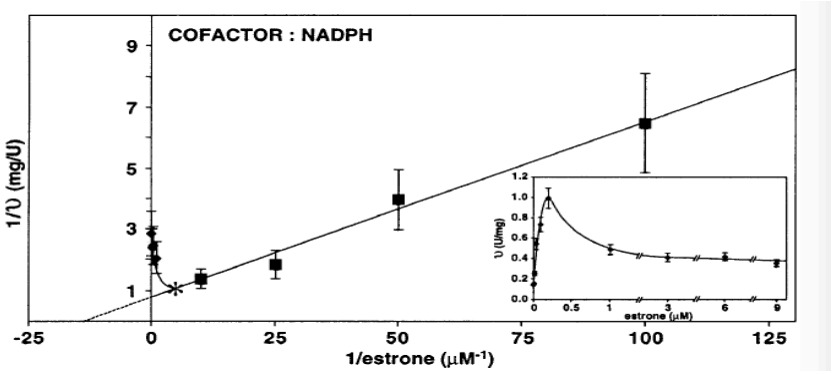
1.7.1 Background

Our group has been studying steroid-converting enzymes involved in BC with various approaches, including enzyme kinetics, structural biology, functional studies at the molecular level and cell level. Enzyme kinetics and binding affinity will be tested and the values can be well correlated. Comparing the K_D values with K_m can help in the understanding of steroid binding and conversion catalyzed by the enzyme (89). Steroid binding affinity to the enzyme (K_D) can demonstrate the interaction, while the K_m value, also called the apparent binding constant, obtained from steady state kinetics can support the binding study (87).

Inspection of the Henri-Michaelis-Menten equation shows that K_m is equivalent to the substrate concentration that yields half-maximal velocity. 17β-HSD1 kinetics have been studied using purified enzyme (83,89). The affinity is variable with different cofactors, such as $NADP^+$, NADPH, NAD^+ and NADH. 17β-HSD1 has the highest specificity for estrogen activation with NADPH. K_m, V_{max}, k_{cat} and k_{cat}/K_m of 17β-HSD1 for estrone reduction with NADPH are 0.03±0.01μM, 2.5±0.5 U/mg, 2.9±0.4 s^{-1}, 96±10 $s^{-1}(\mu M)^{-1}$, and for estradiol oxidation with NADP are 4.6±1.0μM, 1.8±0.2 U/mg, 2.0±0.2 s^{-1},0.4±0.1 $s^{-1}(\mu M)^{-1}$ (83). Anne Gangloff et al. published that substrate inhibition for estrone concentrations above 0.2μM, the following values were obtained: K_m=0.08 μM, k_{cat}=1.6 s^{-1}, specificity=20 $\mu M^{-1}\cdot s^{-1}$ and K_i=1.3 μM (Fig.1.10). 17β-activity and 3β-activity of 17β-HSD1 are comparable and the values are shown in Table 1.2 (66).

Substrate inhibition exists in the reaction using purified 17β-HSD1 enzyme at molecular level.

Figure 1.10 Lineweaver–Burk plot for oestrone reduction catalysed by 17β-HSD1 utilizing NADPH as the cofactor (89).



The reaction mixture contained 50 mM Tris/HCl, pH 7.5, 20 μM NADPH, 0.05 mg/ml BSA, 2% ethanol and oestrone at 37 °C (n=2). The insert shows the v versus s plot for the same reaction. The data obtained in the ascending part of the curve (<0.2 μM oestrone) were processed using ENZFITTER and the following values were obtained : K_m=0.07±0.01 μM, k_{cat}=1.5±0.1 s^{-1}, therefore specificity=21 $\mu M^{-1} \cdot s^{-1}$. Encompassing the complete range of concentrations of oestrone and using the substrate inhibition equation (see the text), the data were further analysed with the program Leonora and the following values were obtained : K_m=0.08 μM, k_{cat}=1.6 s^{-1}, specificity=20 $\mu M^{-1} \cdot s^{-1}$ and K_i=1.3 μM. U, units.

When kinetics could demonstrate how fast of the reaction proceed, affinity could show how strong is the complex formed. The dissociation constants (K_D) of substrate binding can be measured by equilibrium dialysis (83). K_D of 17β-HSD1 and E2 and E1 were 4.7±0.9 μM, 11.0±1.0 μM respectively, in the absence of cofactors.

Table 1.2 K_m, V_{max}, k_{cat}/K_m (87) and K_D of 17β-HSD1 for substrates.

Varied ligand	Fixed ligand	K_m μM	V_{max} U/mg	k_{cat} s^{-1}	k_{cat}/K_m $\mu M^{-1}\cdot s^{-1}$
Estrone	NADPH	0.03±0.01	2.5±0.5	2.9±0.4	96±10
	NADH	0.36±0.16	7.1±0.9	8.0±1.5	24.5±8
Estradiol	NADP	4.6±1.0	1.8±0.2	2.0±0.2	0.4±0.1
	NAD	1.7±0.5	5.8±0.7	6.6±0.8	3.9±1.4

Note. Data represent the average (±SD) of three measurements.

	Estrone	Estradiol	DHT	DHEA
K_m (μM)	0.07±0.01 (NADPH)(89)	4.6± 1.0(NADP)(87)	26± 6(NADP)(89)	24± 4(NADPH)(89)
K_D(μM)	11±1.0 (no cofactor)(87)	4.7± 0.9(no factor)(87)		

Our previous results showed E2 and DHT play vital roles in BC cell proliferation, while 17β-HSD1, 17β-HSD7 are the critical enzymes in E2 synthesis. On the other side, 17β-HSD7 is regulated by E2 concentration reversely (133,168).

In phase I trial, it is possible to inhibit STS activity by over 90% in peripheral blood lymphocytes (PBLs), which have a high level of STS activity (37). However, only modest effects of sulfatase inhibition were obtained in the phase II clinical study (38-41). When sulfatase is inhibited, both E2 and DHT decreased in BC cells, while the critical role of DHT inactivation by 17β-HSD7 has been demonstrated by our laboratory in both in vitro and in vivo studies (42).

1.7.2 Hypotheses

1、Substrate inhibition of 17β-HSD1 by E1 may play a role in living cells.

2、Reductive enzyme 17β-HSD7 may be regulated by 17β-HSD1, STS, aromatase inhibition via E2 modification.

3、Combined inhibition of STS and other reductive 17β-HSDs such as 17β-HSD7 could inhibit BC cell proliferation more significantly.

1.7.3 Objectives

To explore the function and mechanisms of enzymes related to BC for further biomedical applications.

1、 Determine if substrate inhibition of 17β-HSD1 by E1 plays a role in living cells.
2、 Characterize the possible regulation between steroid enzymes. In consideration of the interaction between 17β-HSD7 and 17β-HSD1, 17β-HSD7 and aromatase, 17β-HSD7 and STS respectively, QRT-PCR will be performed to study the mRNA level of 17β-HSD7 after different treatment in varying cell lines.
3、 Verify the cellular effects of the STS inhibitor on sex-hormone levels and cell cycle progression. Cell proliferation, DHT and estradiol concentrations will be examined after STS inhibition.

In order to get a better understanding of the roles of the main enzymes synthesizng sex-hormones, and the interaction of the enzymes, the thesis will present detailed studies at molecular level and cell level.

CHAPTER II

Substrate Inhibition of 17β-HSD1 in living cells and regulation of 17β-HSD7 by 17β-HSD1 knockdown

2.1 Résumé

L’inhibition de la 17β-hydroxystéroide déshydrogénase type 1 (17β-HSD1) par son substrat a d’abord été démontrée par la cinétique enzymatique au niveau cellulaire, soutenant ainsi la fonction biologique de l’inhibition du substrat. En tant qu’inhibiteur, la 5α-dihydrotestosterone (DHT) n’a pas affecté la tendance de la vitesse de la réaction catalysant l’estrone (E1) a l’estradiol (E2) à laquelle l’activité enzymatique a commencé à diminuer, mais certaines augmentations de vitesse ont été observées, suggérant une diminution significative de l’inhibition par le substrat. Les résultats de la modulation de l’ARNm ont démontré que la transcription de la 17β-HSD7 diminuait en réponse à l’inhibition de la 17β-HSD1 ou son knockdown dans les cellules du cancer du sein par l’intermédiaire de la modification de la concentration de l’estradiol (E2).

2.2 Abstract

This study addresses first the role of human 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) in breast cancer (BC) cells. The enzyme has a high estrone (E1)-activating activity that is subject to strong substrate inhibition as shown by enzyme kinetics with purified enzyme. We used BC cells to verify this phenomenon in living cells, and 5α-dihydrotestosterone (DHT) demonstrated some inhibition of estrogen activation at both the molecular and cellular levels. The presence of DHT did not change the tendency toward substrate inhibition for E1 conversion, but shifted the inhibition toward higher substrate concentrations. We then followed the concentrations of estradiol (E2) and performed Q-RT-PCR measurements of reductive 17β-HSDs after 17β-HSD1 inhibition. Knockdown and inhibition of 17β-HSD1 produced reduction in E2 levels and the down-regulation of another reductive enzyme 17β-HSD7, thus “amplifying” the reduction of E2 by the 17β-HSD1 modulation itself. The critical positioning of 17β-HSD7 in sex-hormone-regulation as well as the mutual regulation of steroid enzymes via E2 in BC, are clearly demonstrated.

Our study demonstrates that fundamental enzymology mechanisms are relevant in living cells. We also demonstrated the central role of 17β-HSD7, supporting it as a novel target for BC.

2.3 Article 1: Substrate Inhibition of 17β-HSD1 in living cells and regulation of 17β-HSD7 by 17β-HSD1 knockdown

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Hui Han finished most of the experimental work and wrote the paper;
Jean-François Thériault repeated some experiments in kinetics and helped to check the language and grammar;
Guang Chen gave some suggestions and advices;
Sheng-Xiang Lin has supervised this work and finalized the manuscript.

Key words: breast cancer, reductive 17β-hydroxysteroid dehydrogenases, enzymology, enzyme substrate inhibition, estradiol regulation of 17β-hydroxysteroid dehydrogenase type 7.

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1. Introduction

Breast cancer (BC) is the most frequent cancer affecting women and is one of the top prevalent cancers in humans (1,2). It is the fifth-highest cause of cancer death (6.1%) (3), but yielding the second-highest mortality rate in female cancers (13.6 %) (4). Reproductive and hormone factors play critical roles in the etiology of BC and it has a statistically significant higher risk with increasing concentrations of estradiol (E2) (5-8). E2 is produced by enzymes such as 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) (8-22), 17β-hydroxysteroid dehydrogenase type 7 (17β-HSD7) (23,24), aromatase (25), steroid sulfatase (STS) (26) and 17β-hydroxysteroid dehydrogenase type 12 (27) in some tissues. Approximately 75% of BCs are estrogen-receptor positive (ER+) in postmenopausal women (6).

17β-HSD1 catalyzes the conversions of estrone (E1) into E2 (28) and 5α-dihydrotestosterone (DHT) into 3β-diol (29). 17β-HSD1 also catalyzes the reduction of dihydroepiandrosterone (DHEA) into5-androsteren-3β, 17β-diol (5-diol) (30), a weaker estrogen that becomes more important after menopause (29)(31).DHT can be bound in 17β-HSD1 (32), refer to Figure 2.1.d.It has now been shown that estrone conversion and DHT degradation catalyzed by 17β-HSD1 synergistically stimulate ER+ BC (33). Inhibition of 17β-HSD1 is regarded as an intracrine strategy for the treatment of estrogen-dependent diseases (34-41). Also, DHT has been used as an inhibitor to reduce the effective concentration of substrate for the enzyme involved in the final steps of E2 production (30)(33); consequently, it may play a pro-apoptotic effect in a BC cell line (42). DHT can inhibit cell proliferation and prolongs the G1 phase of the cell cycle (43). 17β-HSD7 also catalyzes the conversions of E1 to E2, and DHT to 3β-diol more efficiently than 17β-HSD1 (44). Furthermore, enzymes such as 17β-HSD1 and STS, may be clinically significant targets for endocrine

therapy in BC patients (45). In our previous study(46), cell viability in T47D and MCF-7 cells decreased more significantly after knocking down both 17β-HSD1 and 17β-HSD7 than knocking down one of them, and cell cycle progression was impeded to enter the S phase from G0-G1.

Substrate inhibition of 17β-HSD1with E1 has been proved using the purified enzyme (30). There is no report demonstrating if substrate inhibition plays a role in living cells. Also, there is no research directly showing if 17β-HSD1 activity in estrone conversion was influenced by DHT concentration in kinetic study. In BC, E2 plays an important role, including regulating 17β-HSD7 expression (47)(48). However, the mutual relation between the two enzymes has not been reported so far. In this study, T47D and MCF7 cells have been chosen as the most commonly used ER+ and progesterone receptor-positive (PR+) BC cell lines (49,50). After studying 17β-HSD1 activity and affinity, it is of great interest to know if there is a relationship between of the functions of the type 1 and 7 enzymes besides their own catalyses. Previous work demonstrated that the joint inhibition of these two enzymesis favorable to decrease E2 concentration and cells proliferation (46). We would like to verify if this is only due to the addition of the effects of the two enzymes or because they have an inter-relationship in affecting the cell proliferation. This investigation will facilitate in depth understanding of the mechanism of sex-hormone converting enzymes in breast cancer, eventually facilitating the development of new approaches to BC therapy.

2. Materials and methods

2.1 Enzyme purification and characterization

The 17β-HSD1 enzyme used for kinetic studies was purified from fresh human placenta tissue as described previously (51,52). The buffer solution contained 40mMTris-HCl, pH 7.5, 1mM EDTA, 50% glycerol, 0.01mg/ml BSA, and 0.4mM DTT. One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol product in 1 min under the experimental condition. The enzyme concentration was determined by a standard Bradford assay. The 17β-HSD1 enzyme used for affinity studies was purified from transfected insect cells (52).

The enzyme activity was assayed by spectrophotometric measurement of NADP reduction, indicated by the absorbance increase at 340nm at room temperature. The reaction mixture contained 25μM estradiol, 0.5mM NADP in 50mM NaHCO3–Na2CO3 at pH 9.2 as buffer. The blank contained the reaction mixture without the enzyme.17β-HSD1 catalyzes the following reaction at the molecular level: E2 +NAD(P)+⇋ E1 +NAD(P)H+H+, while with significant higher specificity using NADPH and NADP as cofactors (20). In cells, the principal reaction is E1 to E2 conversion using NADPH (12)(30)(45).

2.2 Cell culture

T47D and MCF-7 cells were both originally purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). All cells were maintained in culture flasks (75cm2 growth area) at 37°Cunder a 5% CO2 humidified atmosphere. T47D cells were grown in RPMI-1640(ATCC) medium with 10% fetal bovine serum (FBS), Antibiotic-Antimycotic, high-glucose and 7.5μg/ml insulin. MCF-7 cells were maintained in DME low-glucose medium (Sigma, Saint-Louis, MO, USA) with 10% FBS and penicillin. T47D and MCF-7 cells were cultured with phenol red-free medium containing 5% dextran-coated charcoal (DCC)-FBS for 24h prior to the experiment.

2.3 SiRNA synthesis and transfections

The sense and antisense sequences of three 17β-HSD1 siRNA were designed according to a previous study (Table 2.1 in (33)). Triplex siRNA of 17β-HSD1 and control siRNA were synthesized and purified by HPLC by Gene Pharma (Shanghai, China). Knockdown experiments were carried out by transfection with 100nM mixed 17β-HSD1 siRNA or 100nM control siRNA in duplicates using 0.2 µl/well Lipofectamine 2000 in 96-well plates, and 2.5 µl/well Lipofectamine 2000 in 6-well plates. Lipofectamine 2000 transfection reagent was purchased from Thermo Fisher Scientific (Burlington, Ontario, CA). SiRNA was removed after transfection for 4h, and replaced by charcoaled medium.

The 17β-HSD1 inhibitor (Meo-cc-156, INH1) (41) and the 17β-HSD7 inhibitor (EB-357-030, INH7) (44) were provided byDr. Donald Poirier (CHUQ-CHUL Research Center), refer to Table 2.2.

2.4 Steady-state kinetic studies

2.4.1 Kinetic studies at the molecular level

The initial velocity was evaluated by the quantity of the product formed per minute using radiolabeled substrate([C14]-E1) with less than 10-20% substrate consumption. The enzyme was diluted in a buffer containing 40mM Tris-HCl, 1mM EDTA, 0.2mM DTT, 20% glycerol and 0.05mg BSA (28).

The reaction mixture contained 20µM NADPH in 50 mMTris/HCl, pH 7.5, 0.05 mg/ml BSA and 2% ethanol (30). The reaction mixture contained various concentrations of E1 and various concentrations of DHT at 37.0±5 °C. For substrate inhibition studies, the substrate (E1) concentrations were from 0.01µM to 8µM and the DHT

concentrations were 0 and 20μM. For the DHT inhibition study, the substrate (E1) concentrations varied from 0.01μM to 0.08μM, whereas the inhibitor (DHT) concentrations varied from 1μM to 80μM. Steroids were separated by the standard Thin Layer Chromatography (TLC) protocol (28). The TLC plates were analyzed by Typhoon 9200 from GE Healthcare with Image Quant TL software. Enzyme activities were expressed as μmol/min/mg or simply U/mg. Independent experiments were performed in duplicate for the purified native enzyme.

2.4.2 Kinetic simulation in cells:

To evaluate the enzyme mechanism in BC cells, 1 $\times 10^4$T47D cells were cultured in charcoal-treated medium in 24-well plates for 24 h prior the experiments, with [C^{14}] labelled E1. The mRNAs for 17β-HSD1 and 7, are highly expressed in T47D cells, whereas those for STS and aromatase are expressed at low levels (53). Type 7 inhibitor (INH7) was added with final concentration of 1.95μM (10-fold IC_{50}), in the reaction mixture containing different concentrations of E1 and DHT. The medium was collected at 4 different time points and steroids were extracted from medium by adding 4 volume of ether with a standard TLC protocol (28). One unit of enzyme activity in cells is defined as the amount of enzyme that catalyzed the formation of 1pmol product in 1 h per 1 $\times 10^4$ cells under incubation in CO2.

2.5 Fluorescence measurements:

Static fluorescence spectroscopy was performed on a Fluorolog-3-21 JOBIN YVON-SPEX equipped with a temperature-regulated cell compartment. A 0.3cm×0.3cm square quartz cuvette with a sample volume of 180μl was used to perform measurements. The excitation and emission wavelengths were 280 nm and 340nm, respectively. To minimize the enzyme photobleaching, a small excitation-slit width

of 2 nm was chosen, while a large emission-slit width of 16 nm was chosen for an adequate signal-to-noise ratio. A water bath was used to maintain the temperature of the sample compartment at 25±0.1°C. Fresh enzyme solution (1μM) was used for each ligand or enzyme concentration on the titration curve. The DHT concentration used in the fluorescence titration assay was from 4μM to 100μM and that of DHEA varied from 20μM to 400μM.

2.6 Western blot

T47D cells were seeded in six-well plates at 2.5×10^5 cells per well in charcoal-treated medium and transfected with 100nM17β-HSD1-specific siRNA (Table 2.1Ai) (mixed siRNA1, siRNA2, and siRNA3) or control siRNA using Lipofectamine 2000. Twenty four hours after transfection, total proteins were extracted from cells with RIPA (Sigma, Saint-Louis, MI, USA) containing a protease inhibitor cocktail (Calbiochem, Merck, Germany). Proteins were quantified using Bradford assay, and equal amounts of proteins from each sample were separated on a 12% SDS–polyacrylamide geland then electro blotted onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS–Tween 20 for 1 h at room temperature. The membranes were then incubated overnight at 4°C in blocking buffer containing a 1:3,000 dilution of the primary anti-17β-HSD1 mouse monoclonal antibody (Sigma-Aldrich, MO, USA SAB1403946). A 1:2,500 dilution of monoclonal anti-α-tubulin mouse antibody produced in the mouse (Sigma-Aldrich T6074) was used as the loading control. The membranes were washed and incubated for 1 h with respective horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 2,000 times. Protein signals were visualized with Western Lightning Plus-ECL (PerkinElmer, MA, USA).

2.7 Quantitative real-time PCR (Q-RT-PCR)

T47D and MCF-7 cells were seeded in six-well plates at 2.5×10^5 cells per well in charcoal-treated medium and subjected to different treatments. Cells were transfected with 100nM17β-HSD1-specific mixed siRNA or control siRNA using Lipofectamine 2000 after 24 h of culture. The transfection medium was replaced by fresh charcoal-treated medium 4 h post-transfection. After 24h culture, INH1 (described in 2.3) was added into the well and the final concentration of INH1 was 2.75μM (ten times of the IC_{50}). Duplicate wells were prepared and experiments were performed three times. Cell incubations were continued for 12-, 24- or 48-h periods. Total RNA was extracted from cells using EZ-10 DNA away RNA Mini-Preps Kit (Bio Basic Canada INC, Ontario, CA) and transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Lithuania) for RT-PCR. To purify the transcribed cDNA, EZ-10 Spin Column PCR Products Purification Kit (Bio Basic Canada INC, Ontario, CA) was used. We then performed Q-RT-PCR using Fast Start Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) to evaluate the expression level of the 17β-HSD7. The forward and reverse primers for 17β-HSD7 were 5′TCC ACC AAA AGC CTG AAT CTC TC and5′ GGG CTC ACT ATG TTT CTC AGG A. For the 18s housekeeping gene, sequences 5′ ACG GAC CAG AGC GAA AGC ATT and5′ TCC GTC AAT TCC TTT AAG TTT CAG CT were used as forward and reverse primers respectively (11). The specificity of the primers has been tested by PCR and their products have been verified by sequencing service provided by the Genome sequencing platform of CHUL. Gene bank entries: 18s ribosomal 5 (RNA5): NR_ 003286.1, 17β-HSD7: NM_016371. Data were analyzed using a multivariate Gaussian linear regression including the housekeeping gene 18s and a random effect.

2.8 Determination of E2 level

T47D and MCF7 cells (5× 10^3 per well) were cultured in 96-well plates with charcoaled medium for 24 h. Dependent on the experiment, the media were then refreshed with100nM type 1 siRNA or with 2.75μM INH1. The media were collected after a 48-h treatment period and immediately frozen at –20°C until analysis. Four wells were prepared for each treatment and the experiment was repeated three times. E2 levels were determined according to the Estradiol EIA Kit (Cayman Chemical, USA). E2 levels were analyzed by a plate reader (SpectraMax 340PC; Molecular Devices, Sunnyvale, CA) at 412nm.

2.9 Equations:

Steady-state kinetic studies (30):

With the substrate-inhibition equation:

$$v = \frac{V \cdot [\mathrm{s}]}{K_\mathrm{m} + [\mathrm{s}]\left(1 + \frac{[\mathrm{s}]}{K_\mathrm{i}}\right)}$$

Fig.2.1.a, 1.c and Table 2.1B were obtained by Graphpad Prism 7.0.

Fluorescence titration to study ligand binding (54):

The equilibrium dissociation constant (K_D) was calculated using the Eadie plot (Fersht, 1985):

$\Delta F = \Delta F_{max} - K_D \cdot (\Delta F/[L])$.

ΔF refers to the fluorescence change at the ligand concentration [L] and ΔF_{max} corresponds to the fluorescence change when all protein molecules are in complex with ligands.

3. Results

3.1 Substrate Inhibition

3.1.1 Steady-state kinetics in the absence and presence of DHT at the molecular level

The convex shape of the curve, obtained for the activity dependence of 17β-HSD1 with the cognate substrate E1, did reflect standard Michaelis–Menten kinetics at low E1 concentration, but showed significant substrate inhibition for concentrations of E1 above 0.4μM. Using E1 as a substrate in the presence of 20 μM DHT as an inhibitor, the curve also showed substrate inhibition for concentrations of E1 above 0.4μM (Fig.2.1.a). When the substrate concentration was less than 0.1 μM, the enzyme activity in the presence of the inhibitor (20μM DHT) was lower than that in the absence of DHT (Fig,2.1.a & b). When the substrate concentration was tested over 0.2μM, the enzyme activity in the presence of DHT was slightly higher than that observed in its absence (Fig.2.1.a).

The data obtained in the presence or absence of DHT in the ascending part of the curve (<0.1μM estrone) could reflect Michaelis–Menten behavior. Whereas the data were plotted from the substrate inhibition equation indicated in 2.9, the following values were obtained with Graphpad Prism 7.0(see the Appendix, Table 2.1B, Fig.2.1.a): K_m = 0.111± 0.033μM, k_{cat}= 0.376± 0.026 s^{-1}, therefore specificity = 3.39$\mu M^{-1}\cdot s^{-1}$ and K_i= 2.91 ± 0.87μM. In the presence of 20 μM DHT, the following values were obtained by Graphpad Prism 7.0: K_m = 0.191± 0.062μM, k_{cat}= 0.442± 0.035s^{-1}, specificity = 2.31 $\mu M^{-1}\cdot s^{-1}$ and K_i= 2.49± 0.85μM.

3.1.2 Substrate inhibition plays a role in T47D cells

The results demonstrated that reaction velocity increased as E1 went up from zero to 0.65 μM reaching a maximal velocity similar to the case of molecular study, the velocity then went down when E1 further increased from 0.65 μM and up. This is clearly like substrate inhibition though it cannot be considered as a formal kinetic study, as the reactant and inhibitor concentrations may not be identical in and out of the cells (Fig.2.1.c). The following values can be estimated according to the present data: K_m of 17β-HSD1 in E1 to E2 steady-state kinetics was 0.141±0.057μM and Ki for the substrate inhibition can be 1.78±0.81μM. There was no significant difference from the values obtained using the purified enzyme and that estimated with the cell media.

3.2 DHT inhibition on E2 synthesis

3.2.1 The effects of DHT inhibition on 17β-HSD1 activity during E2 synthesisat the molecular level

Estrone conversion by 17β-HSD1 decreased in the presence of increased DHT concentrations: the K_m value increased for E1 activation in the presence of increasing DHT. K_i of DHT was 19.5±0.34μM with purified enzyme (Figures 2.2.a&b).

3.2.2 The effects of DHT inhibition on E2 synthesis in T47D cells

Cells were cultured under the same conditions as detailed for the kinetic study. Using E1 as the substrate with different concentrations of DHT (5 to 40μM) as the inhibitor, the enzyme activity decreased as the DHT concentration increased. The K_m value of E1 activation increased as the DHT concentration increased, and an "K_i" can be estimated at 14.2±0.4μM (Figures2.c&d).

3.3 DHT and DHEA binding to 17β-HSD1

Fluorescence spectroscopy was used to examine the affinity between 17β-HSD1 and DHT, and between 17β-HSD1 and DHEA (Fig.2.3).

From the fluorescence titration, K_D of 17β-HSD1 for DHT was 24.3±4μM. K_D of 17β-HSD1 for DHEA was 57.9±7μM (Table 2.1Ci), indicating the binding of these steroids with 17β-HSD1, which are weaker than that of estrogens (28).

3.4 Down-regulation of 17β-HSD1 suppresses 17β-HSD7 expression

3.4.1 17β-HSD7 expression regulated by 17β-HSD1 inhibition in T47D cells

We have recently studied 17β-HSD7 inhibition in ER+ BC cells. It has been demonstrated that 17β-HSD7 inhibition led to the decrease of its expression by a feedback system via estradiol modification (47,48). We were curious to verify whether the expression of 17β-HSD7 may also be modulated in response to INH1 and type 1 siRNA treatment.

Inhibition or knock down of 17β-HSD1 can down-regulate the E2 concentration in T47D cells (Figure 2.4.b). Type 1 siRNA knocked down 17β-HSD1 effectively in T47D cells (Fig.2.4.a). Messenger RNA for 17β-HSD7 was quantified by Q-RT-PCR after type 1 siRNA or INH1 treatments. The results demonstrated that 17β-HSD7 mRNA decreased from 130 thousand copies of mRNA per μg RNA to 84 thousand copies per μg RNA (dropped to about 64%) at 24 h and to 64 thousand copies of mRNA per μg RNA (to nearly 49%) at 48h after knocking down 17β-HSD1 (Figure 2.4.c), in parallel with the E2 concentration decrease (Fig.2.4.b).

We also use INH1 (10 $\times IC_{50}$) to inhibit enzyme activity, 17β-HSD7 transcription decreased from 130 thousand copies of mRNA per μg RNAto 68 thousand copies

per μgRNA (to about 52%) at 12h and to 32 thousand copies of mRNA per μg RNA (to approximately 24%) at 48 h (Figure 2.4.d). According to these results, inhibition or knock down of 17β-HSD1 reduced17β-HSD7 transcription via E2 decrease. Type 7 expression is significantly controlled with 17β-HSD1 modulation via E2 concentration modification (47,48).

In the T47D cell study, the results demonstrated that the use of 17β-HSD1 siRNA and INH1 decreased the level of 17β-HSD7 mRNA. We compared the function of type1 siRNA and INH1 on 17β-HSD7 mRNA,17β-HSD7 mRNA was more reduced by INH1 (to 24% at 48 h) than type 1 siRNA (to 49% at 48 h), under the experimental conditions.

3.4.2 17β-HSD7 regulation by 17β-HSD1 inhibition in MCF-7 cells

According to our research results, 17β-HSD7 transcription can be regulated by 17β-HSD1 modulation in T47D cells. We would like also to show if this occurs in other BC cell lines. In the MCF-7 cells, INH1 can down regulate the E2 concentration (Fig.2.4.b), it is consistent with a former study (46). After MCF-7 cells were treated with INH1, messenger RNA for 17β-HSD7 was quantified by Q-RT-PCR. This demonstrated that 17β-HSD7 transcription decreased from 143 thousands copies of mRNA per μg RNA to 125 thousand copies of mRNA per μg RNA (to almost 87%) at 12 h and to 77 thousand copies of mRNA per μg RNA (to about 54%) at 48 h using INH1 ($10\times IC_{50}$) as shown in Figure 2.4.e.

4. Discussion

This is a new report that demonstrates substrate inhibition of 17β-HSD1, and DHT inhibition of the E1 to E2 conversion play a role in living cells. Fig.2.1.c showed significant substrate inhibition for concentrations of E1 above 0.4 μM in cells, similar

to the results of Gangloff et al. at the molecular level (30). Substrate inhibition for E1 to E2 conversion was reported (30) to have a K_i of 1.3μM at the molecular level, and these kinetic constants were obtained: K_m=0.08 μM, k_{cat}=1.6 s^{-1}, specificity=20 $\mu M^{-1} \cdot s^{-1}$. In the present study, it was demonstrated that substrate inhibition phenomena of 17β-HSD1 was also demonstrated in cells (Figure 2.1.c). The substrate inhibition also exists in the presence of DHT at the molecular level (Figure 2.1.a). The reduction velocity increased to a maximal level then decreased with further increase of E1. When E1 was present at a low concentration (<0.1μM), DHT played a crucial role as an inhibitor (Figure 2.1.b) because DHT compete with E1 to bind on 17β-HSD1 (55-57). With DHT as an inhibitor, E1 still showed significant substrate inhibition when its concentration was higher than 0.4μM. As an inhibitor, DHT did not affect the E1 substrate concentration at which the enzyme activity started to decrease, but some increase in velocity was observed, suggesting a corresponding decrease in substrate inhibition. As E1 increased, DHT played an additional role that "stimulated" the enzyme activity when the E1 concentration was over 0.2 μM. Furthermore, this suggests that DHT may affect the substrate inhibition of E1, probably via the destabilization of the "dead-end complex" (58) formed between the enzyme, E1 and the cofactor (30).

In T47D cells, a substrate inhibition is observed when the E1 concentration was above 0.65μM (Figure 2.1.c). This demonstrated that cells could adjust E2 synthesis by 17β-HSD1 to prevent accumulation of elevated E2 concentrations. The K_i is 1.78± 0.81μM in cell research, whereas under similar conditions but at the molecular level, a K_i of about 2.91± 0.87 μM was obtained. 17β-HSD1 is the most active enzyme in the synthesis of E2 and the latter exerts predominantly proliferative effects in breast carcinoma (33).

Reportedly, DHT can play an anti-proliferative role on the mitogenic effect of estrogens in BC cells (59,60). The E2 concentration in breast cancer tissue is about 10-20 times higher than that in the plasma and the E1 concentration in breast tissue is 2-10 times higher than thatin the plasma (61). The peak concentration of E2 was 10nM in breast cancer tissue, whereas that of E1 is 2.5nM in breast tissue (62,63). Our studies were performed as close as to the physiological conditions. It suggests that cells can adjust their E2 concentration, with a strong control on the excessive increase of E2.

In a previous study (28), K_D of 17β-HSD1 for E1 was evaluated as 11.0±1.0 μM, and the K_D value of 17β-HSD1 for E2 was 4.7±0.9 μM (Table 2.1Cii), in the absence of cofactors. The steroid hormones (DHT, DHEA and E2) occupy essentially the same binding site on 17β-HSD1 but with different interactions and orientation (32)(64). The results show that the steroids with strongest affinities to 17β-HSD1 are the estrogens, whereas DHT and DHEA have significantly weaker affinities (Tables 2.1C). From the fluorescence titration, Fig.3 demonstrated the affinity between 17β-HSD1 and DHT, and between 17β-HSD1 and DHEA. In our previous study (55), the K_m value of DHT reduction by 17β-HSD1 was 32 ± 9μM with NADPH as the cofactor in steady-state kinetic studies. The affinities of 17β-HSD1 should be very different between DHT, DHEA and E1. In addition, these affinity values in Table 2.1Ci are in good agreement with the pseudo-affinity represented by the K_m of DHT inactivation by Gangloff *et al*. (55).

Previous studies demonstrated down-regulation of 17β-HSD7 expression by a low E2 concentration (47,48). The 17β-HSD1 and 17β-HSD7 are the principal enzymes synthesizing E2 in the sulfatase pathway (7). Inhibition of 17β-HSD1 leads to a decrease in E2 concentration, and we found 17β-HSD1 inhibition affects 17β-HSD7

expression. Our report is the first study to directly demonstrate such regulation. The mRNA modulation results demonstrated that 17β-HSD7 transcription decreased in response to INH1 or 17β-HSD1 knockdown in T47D cells via E2 modification (Fig.2.4.b,c, and d). Similar results were obtained using MCF-7 cells (Figure 2.4.b and e). T47D cell line originally isolated from a pleural effusion of a 54-year-old female patient who was an infiltrating ductal breast carcinoma patient (65). MCF-7 cell line originated from a 69-year-old Caucasian woman with breast malignant tumor (66). However, the 17β-HSD1 mRNA in T47D cells is 697 thousands copies of mRNA/μg RNA, which is much higher than in MCF-7 cells (87 thousands copies of mRNA/μg RNA) (53). Both T47D cells and MCF-7 cells are ER(+) cell lines (50)(53), have similar characteristics, possessing some significant differences in 17β-HSD1 expression. INH1 can inhibit more than 80% activity of 17β-HSD1 (41), while knockdown efficiency of type 1 siRNA on 17β-HSD1 protein level was 53% (46). Therefore, INH1 decreased more the E2 concentration (Fig.2.4.b), and then the modulation of 17β-HSD7 mRNA transcription was stronger in T47D cells (to 24% in 48 h) (Fig.2.4.d) than in MCF-7 cells (to 54% in 48 h) (Fig.2.4.e). 17β-HSD1, the main enzyme synthesizing E2, can regulate 17β-HSD7 transcription through modifying E2 concentration.

Conclusion

The present study may facilitate further research in enzyme mechanisms in living cells, such as the positive and negative cooperativity, similar to the substrate and ligand inhibition we have demonstrated in living breast cancer cells. Enzyme self- and mutual regulations may also exist in other proteins and cells. If we can inhibit or knockdown 17β-HSD1, both of the two important enzymes 17β-HSD1 and 7 will be down regulated, and then E2 decrease becomes more obvious by double effects

(46). Our research revealed the detailed mechanism study in breast cancer, facilitating the finding of targets for BC and contributing to ER(+) BC treatment.

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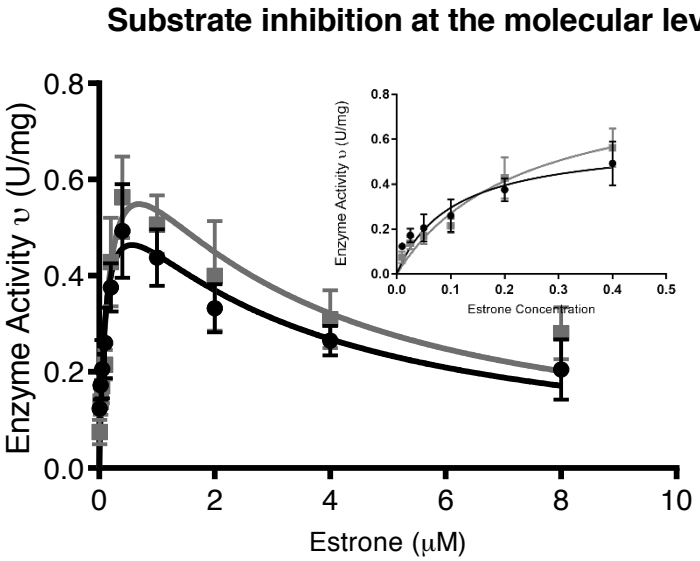
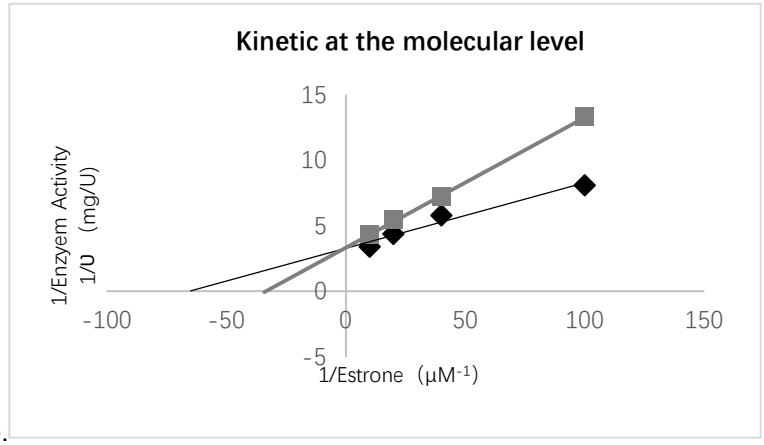
FIGURES

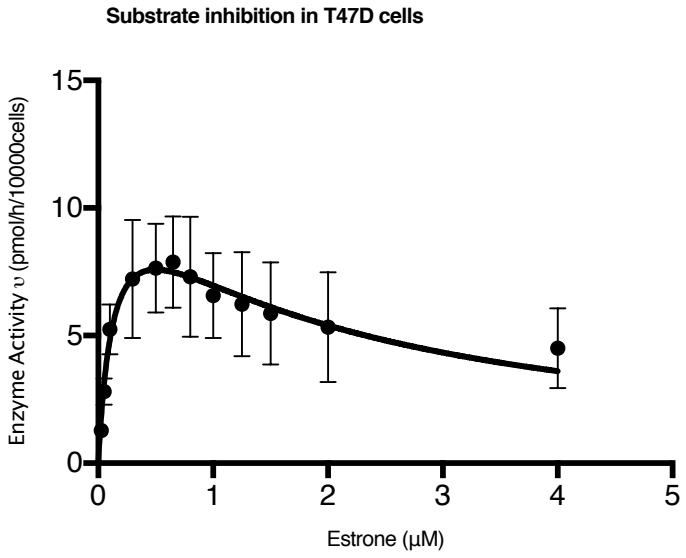
Figure 2.1 Estrone reduction catalyzed by 17β-HSD1 in the absence and presence of DHT

a.

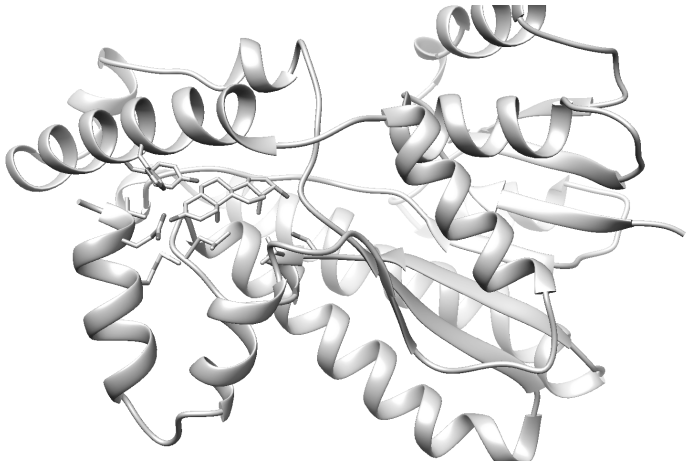


b.





c.



d.

(a, b). Kinetics utilizing NADPH as the cofactor. The symbol (◆) corresponds to the absence of DHT as inhibitor, and the symbol (■) corresponds to the presence of 20 μM DHT as inhibitor. In the absence of DHT, the following values were obtained by Graphpad Prism 7.0: K_m = 0.111 ± 0.033 μM, k_{cat} = 0.376 ± 0.026 s^{-1}, therefore specificity = 3.39 $\mu M^{-1} \cdot s^{-1}$ and K_i = 2.91 ± 0.87μM. In the presence of 20 μM DHT, the following values were obtained by Graphpad Prism 7.0: K_m = 0.191 ± 0.062μM, k_{cat} = 0.442 ± 0.035 s^{-1}, specificity = 2.31 $\mu M^{-1} \cdot s^{-1}$ and K_i = 2.49 ± 0.85μM.

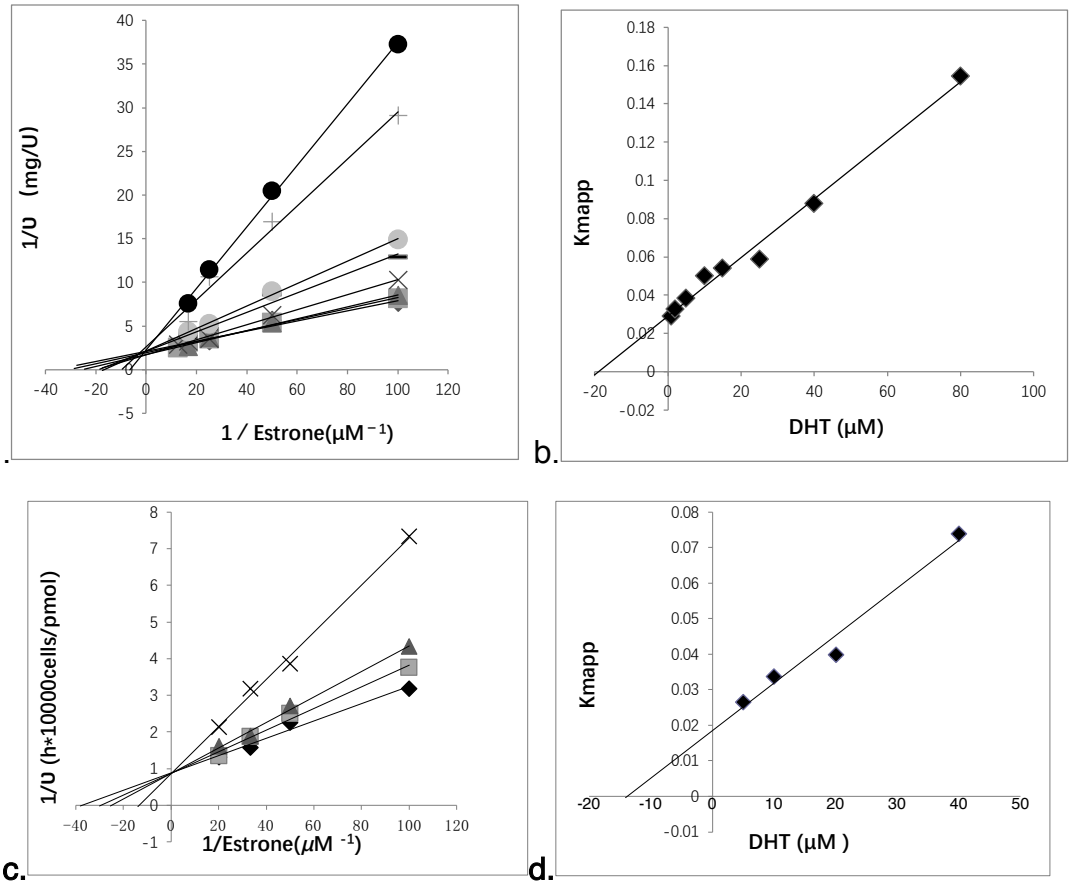
(b). Line weaver-Burk plot comparing effect of DHT in E1 reduction catalyzed by 17β-HSD1 E1 concentration less than 0.1 μM was used to verify the linear part of E1 reduction. The symbol (◆) corresponds to the absence of DHT as inhibitor, and the symbol (■) corresponds to the presence of 20 μM DHT as inhibitor.

(c). Enzyme Kinetic studies of 17β-HSD1 in T47D cells. Significant substrate inhibition was observed in T47D cells when the substrate (estrone) concentration was above 0.65μM. The

concentration of INH7 added was 10-fold of the respective $IC_{50}$to block the 17β-HSD7 activity. The following values were estimated by Graphpad Prism 7.0: K_m=0.141± 0.057μM, K_i=1.78 ± 0.81μM.

(d). Structure of the 17β-HSD1-DHT complex. The different orientations at the substrate “entry-loop” are highlighted by grayscale (17β-HSD1-DHT, PDB code: 1DHT;). Structure from: Han Q*et al.* & Lin SX, J Biol Chem. 2000,275:1105.

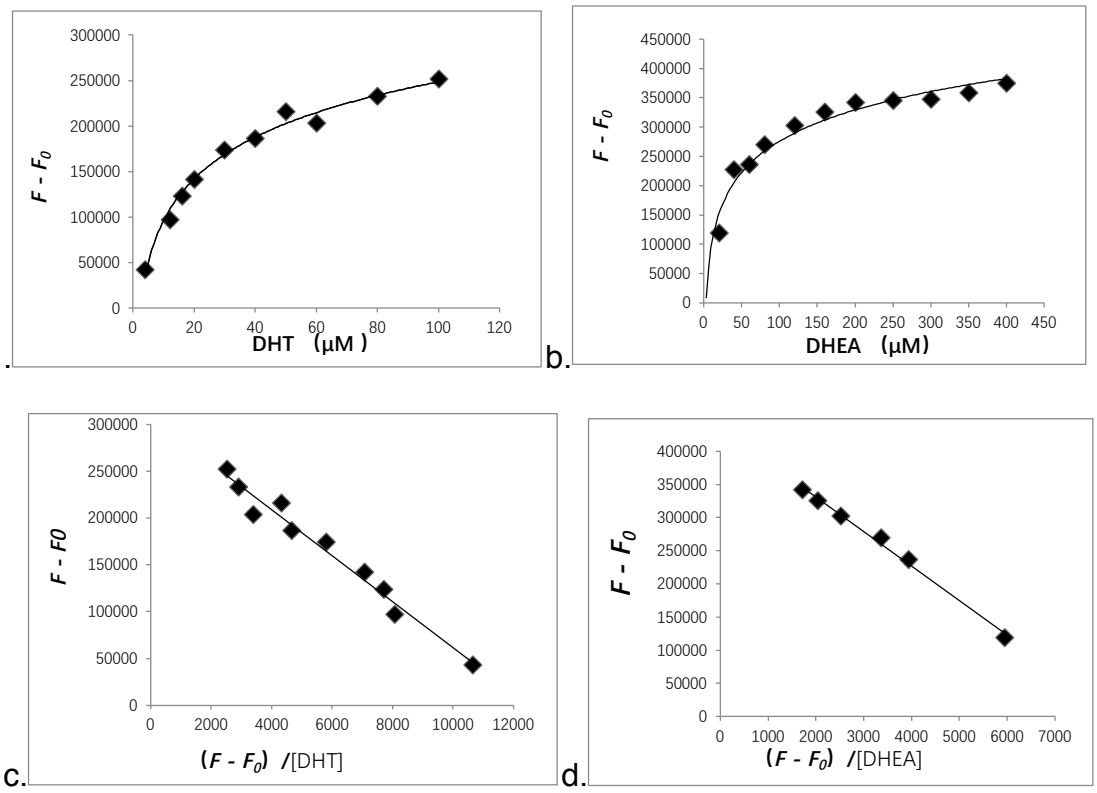
Figure 2.2 Inhibition study on the conversion of E1 to E2 by 17β-HSD1 at the molecular and cellular levels



(a, b), at the molecular level. a. Line Weaver-Burk plot of E1 reduction catalyzed by purified 17β-HSD1 in the presence of various fixed concentrations of DHT. b. It shows the K_{mapp} versus s plot for the same reaction. The experiment was carried out with DHT = 1μM (◆), DHT = 2μM (■), DHT = 5μM (▲), DHT = 10 μM(×), DHT = 15μM (-), DHT = 20μM (●), DHT = 40μM (+), and DHT = 80 μM(●). The data were obtained with different DHT concentrations: K_i of DHT = 19.5 μM.

(c, d), in T47D cells. Line weaver-Burk plot of E1 reduction in the presence of various fixed concentrations of DHT. The experiment was carried out with DHT = 5 μM (◆), DHT = 10 μM (■), DHT = 20 μM (▲), and DHT = 40 μM(×).INH7 was added with the final concentration of 1.95μM.The plot of apparent K_m versus [DHT] on the right shows the (-K_i) value of DHT was the intersection point on the [DHT] axis, and a K_i 14.2μM was obtained.

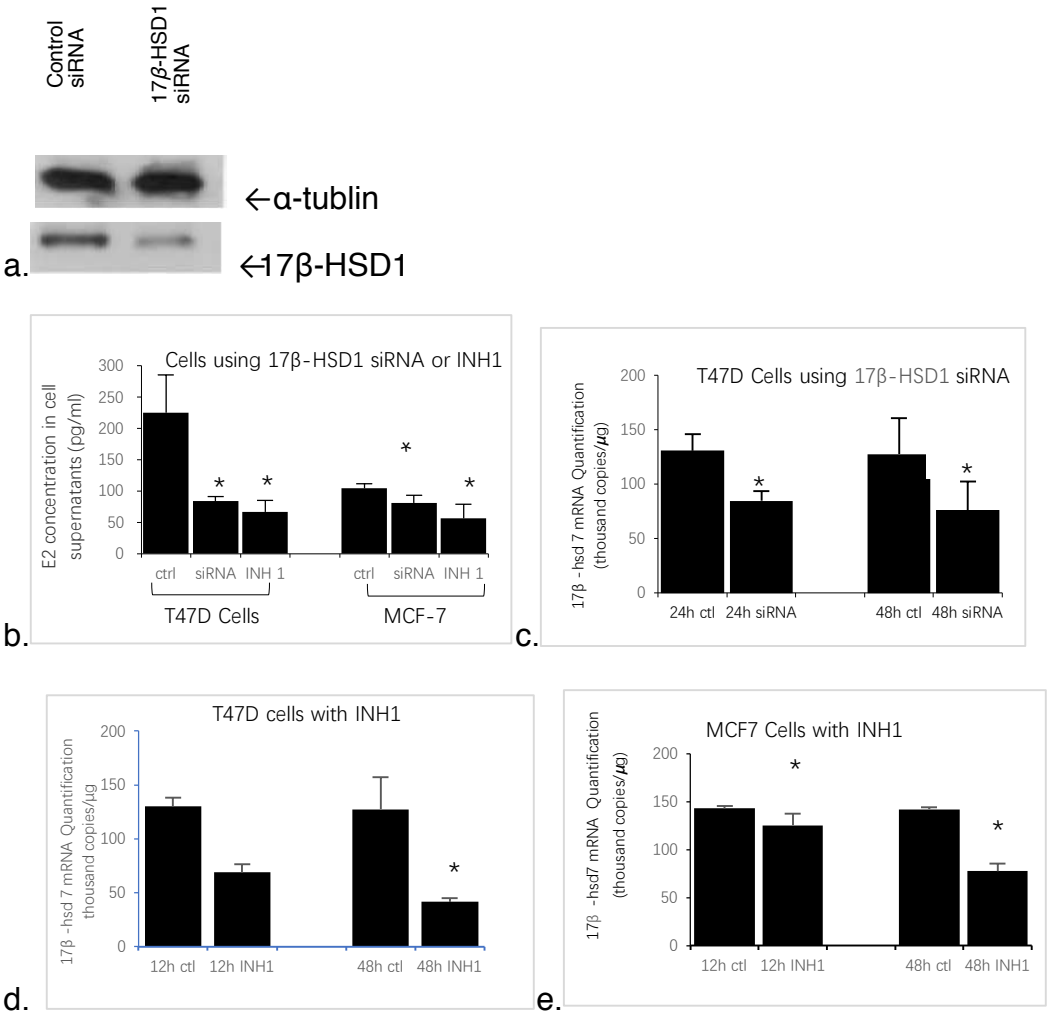
Figure 2.3 Fluorescence titration of 17β-HSD1 for DHT and DHEA



a,b: Relative fluorescence intensity modification of17β-HSD1 *vs.* ligand concentration demonstrated in the titration;

c,d: Eadie Plots to obtain the binding constant. In (c), we obtained K_D of 17β-HSD1 for DHT was 24.3 ± 4 μM. In (c), we obtained K_D of 17β-HSD1 for DHEA was 57.9 ± 7 μM.

Figure 2.4 17β-HSD7 mRNA modulation by 17β-HSD1 inhibition in BC cell lines.



(a) Western blot showing 17β-HSD1 expression 24 hours after transfection of T47D cells with 17β-HSD1 siRNA (mixed siRNA 1, 2 and 3) or control siRNA in T47D cells.

(b) Estradiol concentrations were quantified by the Estradiol EIA Kit in T47D cell supernatants and in MCF-7 cell supernatants. Cells were cultured in charcoaled medium for 48 hours.

(c) 17β-HSD7 mRNA copies using 17β-HSD1 siRNA in T47D cells. The Q-RT-PCR results showed that 17β-HSD1 siRNA significantly suppressed 17β-HSD7 expression after 24 and 48 hours incubation.

(d) 17β-HSD7 mRNA copies after INH1 treatment in T47D cells. The results showed that INH1 substantially suppressed 17β-HSD7 expression after 12 and 48 hours incubation.

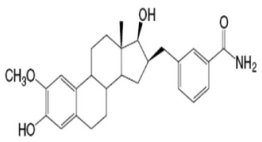
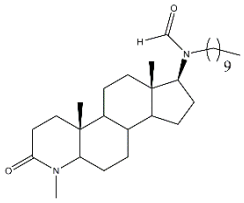
(e) 17β-HSD7 mRNA copies after INH1 treatment in MCF-7 cells. The results showed that INH1 substantially suppressed 17β-HSD7 expression after 12 and 48 hours incubation.

17β-HSD7 mRNA copies were quantified by RT-PCR comparing with the standards cDNA curves and we used 18s as housekeeping gene. All experiments were repeated three times and by duplicate. Error bars represent standard deviation. *$p < 0.05$ analyzed by a two-way ANOVA *vs*. control.

Table 2.1 siRNA sequences for 17β-HSD1

SiRNA name	Sense sequence (5′–3′)	Antisense sequence (3′–5′)
siRNA1	GCUGGACGUGAAUGUAGUA[dT][dT]	UACUACAUUCACGUCCAGC[dT][dT]
siRNA2	GCCUUUCAAUGACGUUUAU[dT][dT]	AUAAACGUCAUUGAAAGGC[dT][dT]
siRNA3	CCACAGCAAGCAAGUCUUU[dT][dT]	AAAGACUUGCUUGCUGUGG[dT][dT]

Table 2.2 Characteristics of 17β-HSD1 and 17β-HSD7 inhibitors used in this study. (138)

Compound	Targeting enzyme	Chemical structure	$IC_{50}$of E1~E2	$IC_{50}$of DHT~3β-diol
Meo-cc-156 (INH1)[a]	17β-HSD type1		275 ± 5 nM	None
EB-357-030 (INH7)[b]	17β-HSD type7		195 ± 18 nM	230 ± 15 nM

None, data not shown.

a.IC_{50}, concentration of the compound inhibiting 50% of E1 to E2 conversion in T47D cells (Laplante et al., 2008).

b.IC_{50}, concentration of the compound inhibiting 50% of DHT inactivation in HEK293 cells overexpressing 17β-HSD7 (Bellavance et al., 2009).

Table 2.3 Data obtained in the ascending part of the curve on E1 to E2 conversion by 17β-HSD1 (<0.1 μM estrone)

	K_m（μM）	k_{cat} (s^{-1})	specificity ($\mu M^{-1} \cdot s^{-1}$)	K_i(μM)
0 μM DHT	0.111± 0.033	0.376± 0.026	3.39	2.91±0.87
20 μM DHT	0.191± 0.062	0.442± 0.035	2.31	2.49± 0.85

Note. Data represent the average (±SD) of three measurements. Values were calculated by Graphpad Prism 7.0.

Table 2.4 The equilibrium dissociation constant of 17β-HSD1 for substrates

Ligand	DHT	DHEA
K_D (μM)	24.3 ± 4	57.9 ± 7

Table 2.5 The dissociation constants of 17β-HSD1 for estradiol and estrone (87)

Ligand	Estradiol	Estrone
K_D (μM)	4.7 ± 0.9	11.0 ± 1.0

Note. Data represent the average (±SD) of three measurements.

CHAPTER III

STEROID SULFATASE INHIBITION SUCCESS AND LIMITATIONS IN BREAST CANCER CLINICAL ASSAYS: AN UNDERLYING MECHANISM

3.1 Résumé

Introduction : La stéroïde sulfatase (STS) est détectable dans la plupart des cancers du sein hormono-dépendants (CS). *Objectifs :* Après avoir traité les cellules MCF-7 et T47D avec deux inhibiteurs de la STS (STX64 et EM1913), on a mesuré la prolifération cellulaire, le cycle cellulaire et les concentrations d'estradiol (E2) et de la 5α-dihydrotestostérone (DHT). *Résultats* : Des comparaisons ont été faites avec des inhibitions de 17β-hydroxystéroïdes déshydrogénases réductrices (17β-HSDs). Des études de prolifération cellulaire ont montré que la synthèse de l'ADN a légèrement diminuée (environ 20%), accompagnée d'un taux allant jusqu'à 6,5% des cellules dans la phase G0 / G1 et de la réduction de l'expression de la cycline D1. Les concentrations d'E2 et de DHT ont diminué respectivement de 26% et 3%. Cependant, la supplémentation en DHT a produit une augmentation significative (environ 35,6%) de l'effet antiprolifératif de l'inhibition du STS. Cette étude a clarifié les rôles de l'E2 et de la DHT qui mènent une réduction dans l'effet de l'inhibition de la STS par rapport à l'inhibition de 17β-HSD7. *Conclusion* : Le traitement combiné des inhibiteurs de la STS avec les inhibiteurs de la 17β-HSD pourrait être prometteur pour le CS hormono-dépendante.

3.2 Abstract

Steroid sulfatase (STS) is detectable in most hormone-dependent breast cancer (BC). After MCF-7 and T47D cells were treated with two STS inhibitors (STX64 and EM1913), cell proliferation, cell cycle, and the concentrations of estradiol (E2) and 5α-dihydrotestosterone (DHT) were measured. Comparisons were made with inhibitions of reductive 17β-hydroxysteroid dehydrogenases (17β-HSDs). Cell proliferation studies showed that DNA synthesis was modestly decreased (approximately 20%), accompanied by an up to 6.5% in cells in the G0/G1 phase and cyclin D1 expression reduction. E2 and DHT concentrations were decreased by 26% and 3% respectively. However, supplementation of DHT produced a significant increase (approximately 35.6%) in the anti-proliferative effect of STS inhibition. This study has clarified the different roles of E2 and DHT by leading to a reduction in the effect of STS inhibition when compared with 17β-HSD7 inhibition. This suggests combined treatment of STS inhibitors with 17β-HSD inhibitors could hold promise for hormone-dependent BC.

3.3 Article 2: Steroid sulfatase inhibition success and limitation in breast cancer clinical assays: an underlying mechanism

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Xiaoye Sang performed most of the research works and wrote this paper ;
Hui Han carried out a significant amount of the experiments and was involved in writing this paper, the expersiments include: EM1913 and STX64 suppressed cell proliferation providing DHEA-S; and EM1913 and STX64 decreased E2 and DHT biosynthesis in MCF-7 and T47D;
Donald Poirier gave some important advices and supplied enzyme inhibitors ;
ShengXiang Lin supervised the research and finalized the manuscript.

Key words: 5α-dihydrotestosterone supplement, Steroid sulfatase, estradiol, reductive 17β-hydroxysteroid dehydrogenases.

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1. Introduction

Breast cancer (BC) is the most frequently occurring cancer in women, accounting for approximately 10% of cancer incidence worldwide. Furthermore, it is diagnosed in one million women each year (1,2).

A large proportion of BCs are initially estrogen-dependent (3), including 60% of BC cases in premenopausal women and 75% of cases in postmenopausal women (4). Estrogens are synthesized from precursor steroids by steroidogenic enzymes in peripheral tissues in an intracrine manner in postmenopausal women (5,6). Estradiol (E2), the most potent estrogen, is suspected to play an important role in carcinogenesis by stimulating cell proliferation through binding to estrogen receptors (ER) and activating down-stream transcriptions (7).

Following menopause, E2 is predominantly produced from adrenal precursors such as dehydroepiandrosterone (DHEA), 4-androstene-3,17-dione (4-dione) and estrone-sulfate (E1-S) by several steroidogenic enzymes (8). Two pathways are important for local E2 production in target tissues: the “sulfatase pathway,” in which biologically inactive steroid sulfates are the source for E2 (E1-S to E1 and DHEA-S to DHEA), and the “aromatase pathway”, which transforms androgens into estrogens (9). 17β-HSDs are integral to both pathways and convert estrone (E1) into potent E2 (10-12). A consequence of the up-regulation of steroidogenic enzymes is the generation of an estrogen-rich microenvironment that sustains tumor growth and proliferation (13,14). Therefore, steroid sulfatase (STS), aromatase and reductive 17β-HSDs involved in the last step of E2 biosynthesis are primary targets for the blockade of E2 production (15).

Androgens are synthesized in breast cancer from adrenal precursor such as DHEA or 4-dione, which are the metabolites in the sulfatase pathway. In contrast with E2, which predominantly contributes to the breast carcinoma growth through binding to the ER, the non-aromatic androgen dihydrotestosterone (DHT), displayed 5~10 times higher affinity towards androgen receptor (AR) than Testosterone (16) and exerted anti-proliferative effects via activating $P21^{waf1/cip1}$ or inhibiting Cyclin D1 in vitro (17,18). Furthermore, about 80% of ER positive breast cancers were found to co-express AR (19). A systematic meta-analysis demonstrated that co-expression of AR in female breast tumour is associated with a better prognosis and outcome (20).

According to extensive studies, STS activity can be detected in most breast tumors (21). This enzyme belongs to the family of arylsulfatases of the sulfatase superfamily, which catalyzes hydrolysis of the sulfate bond present in various endogenous and exogenous substrates (22). Steroid sulfatase performs a central role in the formation of active sex steroid hormones, as it is the key enzyme hydrolyzing steroid sulfates, including E1-S into E1 and DHEA-S into DHEA (23,24). Therefore, STS was proposed to play an important role in regulating hormones and was considered a potential target for the treatment of hormone-dependent BC.

The development of STS inhibitors began in the nineties (25-31) and accelerated during the decade following 2000 (32-35). Most compounds were in preclinical testing but one inhibitor was scheduled for clinical phase I trial. STX64 (also known as Irosustat, Table 1) belongs to the first-generation of STS inhibitors and was shown to be a potent STS inhibitor in placenta (in vitro). It also blocked the ability of E1-S to stimulate the growth of carcinogen-induced mammary tumors in

ovariectomized mice (36). The results from phase I trial have shown it is possible to inhibit STS activity by over 90% in peripheral blood lymphocytes (PBLs), which have a high level of STS activity (37). However, only modest effects of sulfatase inhibition were obtained in the phase II clinical study (38-41).

This study helps to clarify observations from several investigations of steroid metabolism in ER+ BC cell lines and addresses a possible explanation for the modest results of the sulfatase inhibitor (Irosustat) in the phase II clinical study. When sulfatase is inhibited, both E2 and DHT may be decreased in BC cells as DHEA-S is the common intracrine source for estrogens and androgens in post-menopausal women. Thus, it is essential to verify the cellular effects of the STS inhibitor on sex-hormone levels and cell cycle progression. Furthermore, while the critical role of DHT inactivation by 17β-HSD7 has been demonstrated by our laboratory in both in vitro and in vivo studies (42), it is fundamentally important to understand the impact of the cellular DHT concentration with regard to the cell cycle and cell proliferation (17,18). Such comparative contributions will be analyzed in light of the in vitro study with the respective enzyme inhibitors and may help us to design the best strategy for therapeutic targeting of estrogen-dependent BC.

2. Materials and Methods

2.1. Inhibitors and chemicals

Two STS inhibitors were used in this study: the non-steroidal STS inhibitor STX64 or Irosustat (IC_{50}: 8 nM) (43) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). The 17β-HSD7 inhibitor INH80 (IC_{50}: 200nM) (44,45) and steroidal inhibitor EM1913 (IC_{50}: 0.024 nM) without estrogenicity were synthesized in the Laboratory of Medicinal Chemistry (CHU de Québec-CHUL) as reported (46).

Stock solutions were prepared by dissolving the compounds in ethanol. Dilution to the desired concentrations was performed using culture medium (Table 3.1). The culture medium for MCF-7 was Dulbecco's Modified Eagle's Medium-Low glucose which was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). The medium for T47D was Gibco RPMI Medium 1640 (1×), purchased from Thermo Fisher scientific Canada.

2.2. Cell Culture

The ER+ cell lines MCF-7 and T47D, both of which express STS, were obtained from the American Type Culture Collection, and propagated according to a previously described protocol (47). Both culture media were phenol red-free. Fetal bovine serum (FBS) (PAA, Etobicoke, ON, Canada) was stripped with 2% dextran-coated charcoal (Sigma, Saint-Louis, MI, USA) as before (47) and added to the protocol medium to a final concentration of 10%. Cells were plated in this charcoal-treated medium for 24 h, then the medium was replaced by fresh medium containing the desired concentrations of substrates: DHEA-S (1 μM) or E1-S (0.5 nM) (48-50). The substrate concentrations were close to the intracellular physiological concentrations found in BC cells. Cells plated in only with charcoal-treated medium were used as the baseline control (w/o).

Cell Proliferation Measurement: An assay for DNA content with CyQUANT assay kit

The CyQUANT cell proliferation kit (Molecular Probes, Invitrogen, ON, Canada), which directly measures cellular nucleic acid content, was used to quantify the anti-proliferative effects of inhibitors. Different cells types were individually plated into 96-well plates at a density of 5×10^3 cells per well. These were treated with

inhibitors at the desired concentrations for 4 days in the presence of substrates. Culture medium was renewed every two days. After the desired treatment periods, the plates were washed with PBS and frozen at –80°C for at least 24 h. The testing procedure was conducted according to the manufacturer's introductions. Cell proliferation in response to the substrates DHEA-S or E1-S was recorded as 100%. Data were reported as a percentage (%) by comparing DNA synthesis of inhibitor-treated groups vs. control group (100%). Each condition was performed in quintuplicate and experiments were repeated three times.

2.3. Cell cycle analysis by flow cytometry

Further investigation of cell proliferation inhibition was conducted by cell cycle analysis with flow cytometry using the Click-iT EdU assay kit and propidium iodide (PI) (Molecular Probes, Invitrogen, ON, Canada). The incorporation of EdU was monitored as a parameter for deoxyribonucleic acid synthesis during the S-phase. Cells were plated in 6-well plates at a density of 2.5×10^5 cells per well, and were treated with inhibitors at the desired concentrations for 4 days. Medium was renewed every 2 days. On the fourth day, the cells were harvested and labeled with EdU for 4 h, followed by fixation with 70% ethanol and permeabilization with 0.2% Triton X-100. The total DNA content was labeled with PI. Samples were analyzed by flow cytometry with a BD FACS Canto II instrument (BD Bioscience, San Jose, CA, USA). The percentage of cells in each phase: G0/G1, S and G2/M was calculated with Multi Cycle AV software (Phoenix Flow Systems, San Diego, CA, USA). Data were recorded and reported as the percentage of living cells (G0/G1, S and G2/M = 100%). Each condition was conducted in triplicate, and experiments were repeated three times.

2.4. Determination of E2 and DHT levels

Cells were plated in 96-well plates at a density of 5×10^3 cells per well and treated with individual STS inhibitors at the desired concentrations for 4 days, in the presence of substrates. Medium was renewed every 2 days. On the fourth day, the cell culture supernatants were pooled and frozen at –80°C. Meanwhile, the protein in the cells of each group were extracted with RIPA buffer (Sigma, Saint-Louis, MI, USA) containing a protease inhibitor cocktail (Calbiochem, Merck, Germany). Then the contents of protein were measured with Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Canada) according to the manufacturers' instructions. The E2 and DHT concentrations were determined by "Estradiol EIA Kit" (Cayman Chemical, Ann Arbor, MI, USA; the cross reactivity towards E2 was 100%) and DHT ELISA Kit (Alpha Diagnostic International, San Antonio, TX, USA; The cross-reactivity towards DHT was 100%). The procedures were conducted according to the manufacturers' instructions. Duplicate wells were prepared for each condition. Absorbance was determined at 412 nm for E2 and 450 nm for DHT. Finally, the E2 and DHT concentrations of each group was standardized to protein by dividing protein concentration. Each condition was conducted in triplicate and experiments were repeated three times.

2.5 qRT-PCR

MCF-7 or T47D cells were seeded in sex-well plates at 2.5×10^5 cells per well in charcoal-treated medium. 24h later, the cells were treated with individual STS inhibitors or INH80 at the desired concentrations for 4 days, in the presence of substrates. Medium was renewed every 2 days. On the fourth day, the cells were harvested and total RNA was extracted with the RNeasy mini kit on-column DNase (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Determination of mRNA levels was carried out using 18S as the normalization

genes. The expression of gene's mRNA was determined by qRT-PCR, performed by the Q-PCR platform of the Quebec Genomics Center (CHU du Quebec, Canada). mRNA quantity in response to the substrates DHEA-S or E1-S was recorded as 100%. Data were reported as a percentage (%) by comparing mRNA quantity of inhibitor-treated groups vs. control group (100%) according to the literature before (51). The target gene were using primers 5'-acg ccc tcc cag tgt gca aat-3' and 5'-aat ctg tgt tgt gag ccg agg-3' (10). Each condition was conducted in triplicate and experiments were repeated three times.

2.6. Western blot

The CyclinD1 protein expression was determined by western blot. MCF-7 and T47D cells were treated with 10×IC_{50} STX64 or EM1913 for 4 days in the presence of 0.5 nM E1-S. Total proteins were extracted with RIPA buffer (Sigma, Saint-Louis, MI, USA) containing a protease inhibitor cocktail (Calbiochem, Merck, Germany). Equal amounts of protein were separated by 15% SDS-PAGE. Primary antibodies used were anti-cyclin D1 (ab134175) and anti-actin (A5316) (Sigma, Saint-Louis, MI, USA). Secondary antibodies included goat-anti-rabbit IgG-HRP (sc-2004) and goat-anti-mouse IgG-HRP (sc-2005) (Santa Cruz Biotechnology, CA, USA). Blots were visualized by enhanced chemi-luminescence (ECL plus Western Blotting Detection Kit, PerkinElmer, MA, USA) and quantified with NIH ImageJ software. The relative quantity of protein expression was determined by the ratio between the signals from the protein of interest and β-actin. Each condition was conducted in triplicate and experiments were repeated three times.

2.7. Statistical analysis

Multiple comparisons were performed by one-way ANOVA. The Student's t-test was used for the comparison of two groups. Data were presented as means ± SD. Statistically significant differences were determined by $P< 0.05$, $P< 0.01$ and $P< 0.001$.

3. Results

3.1 EM1913 and STX64 suppressed cell proliferation providing DHEA-S or E1-S

Here, DHEA-S and E1-S were provided as starting steroids to determine the inhibition of the STS pathway. Proliferation of MCF-7 cells in the presence of DHEA-S was reduced by 14.5% with 80 nM STX64 (10× IC_{50}) vs 17.8% with 0.24 nM EM1913 (10× IC_{50}). In the presence of E1-S, the cell proliferation was suppressed by 17.3% with 80 nM STX64 (10× IC_{50}) vs. 19.0% with 0.48 nM EM1913 (10× IC_{50}) (Figure 3.1.A). Proliferation of T47D cells treated with DHEA-S was decreased by 13.3% with 10 × IC_{50} STX64 vs. 16.9 % with 10× IC_{50} EM1913. With E1-S, cell proliferation declined by 16.5% with 10× IC_{50} STX64 vs. 20.8% with 10× IC_{50} EM1913 (Figure 3.1.B).

In comparison, in the presence of 0.5 nM E1-S, the 17β-HSD7 inhibitor INH80 reduced cell proliferation by 27.8% in MCF7 and 25.4% in T47D cells (Figure 3.1.C). Data of MCF-7 cells or T47D cells just treated with STX64, EM1913 or INH80 were also provided in Appendix figures (Appendix Figure 3.10).

Furthermore, inhibition of 17β-HSD1 induced 17.6% cell proliferation suppression in MCF-7 and suppressed cell proliferation by 27.6% in T47D (Table 3.2 and Table

3.3). These results demonstrate that STS inhibition does reduce epithelial BC proliferation, but at a modest level.

3.2 EM1913 and STX64 decreased E2 and DHT biosynthesis

To study the contribution of the STS pathway to the metabolism of E2 and DHT, these two sex-hormone concentrations were quantified after STS inhibition. The E2 and DHT levels in the supernatants of MCF-7 and T47D cell culture media were determined by ELISA and standardized to protein (from Figure 3.2.A to 3.2.D). In MCF-7, cells in the presence of DHEA-S, STX64 and EM1913 reduced the E2 quantity/µg protein of cells from 2.064 ± 0.559 nmol/µg to 1.805 ± 0.614 nmol/µg (to 87.5%) and from 2.064 ± 0.559 nmol/µg to 1.721 ± 0.639 nmol/µg (to 83.4%) respectively. The DHT quantity/µg protein of cells was decreased from 4.516 ± 0.968 nmol/µg to 4.159 ± 0.616 nmol/µg (to 92.1%) and 4.135 ± 0.525 nmol/µg (to 91.5%) by STX64 and EM1913, respectively. With E1-S as a substrate, the E2 quantity/µg protein of cells was reduced from 7.416 ± 0.892 nmol/µg to 6.018 ± 0.598 nmol/µg (to 81.1%) and from 7.416 ± 0.892 nmol/µg to 5.442 ± 0.322 nmol/µg (to 73.4%) by STX64 and EM1913 respectively. The DHT level declined from 0.762 ± 0.160 nmol/µg to 0.706 ± 0.140 nM (to 92.7%) and from 0.762 ± 0.160 nmol/µg to 0.743 ± 0.151 nmol/µg (to 97.5%) (Figure 3.2.A and Figure 3.2.B).

In T47D, cells in the presence of DHEA-S, the E2 quantity/µg protein of cells was reduced from 4.516 ± 0.968 nmol/µg to 3.942 ± 0.616 nmol/µg (to 87.3%) and from 4.516 ± 0.968 nmol/µg to 3.947 ± 0.525 nmol/µg (to 87.4%) respectively (Ctrl vs 10 × IC_{50} STX64 or EM1913). However, the DHT quantity/µg protein of cells increased from 2.796 ± 0.171 nmol/µg to 2.989 ± 0.187 nmol/µg (to 106.9%) with STX64 and from 2.796 ± 0.171nmol/µg to 2.955 ± 0.182 nmol/µg (105.7%) with EM1913,

showing no-significant modifications. When E1-S was provided, the STX64 and EM1913 decreased the E2 level from 7.158 ± 0.767 nmol/µg protein of cells to 6.316 ± 0.631 nmol/µg protein of cells (to 88.2%) and from 7.158 ± 0.767 nmol/µg protein of cells to 5.983 ± 0.471 nmol/µg protein of cells (to 83.5%) respectively. The DHT level was reduced from 0.297 ± 0.158 nmol/µg protein of cells to 0.270 ± 0.132 nmol/µg protein of cells (to 90.9%) and from 0.297 ± 0.158 nmol/µg protein of cells to 0.281 ± 0.122 nmol/µg protein of cells (to 94.6%), respectively (Ctrl vs 10 × IC_{50} STX64 or EM1913) (Figure 3.2.C and Figure 3.2.D).

In comparison, the E2 level was reduced to 75.6% in MCF7 and 72.3% in T47D cells in response to 17β-HSD7 inhibition. However, the DHT concentration was significantly restored, showing a 3.16- fold increase in MCF-7 and a 3.06-fold increase in T47D cells (Figure 3.3.A and 3.3.B).

Under similar conditions, INH1 suppressed the E2 concentration by 24.7% with modest up-regulation of DHT (15.7%) in MCF-7 cells. Moreover, INH1 decreased the E2 concentration by 58.7% in T47D cells, without significant activation of DHT (6.9%) (Table 3.2 and Table 3.3).

These results indicate that the STS inhibitors block both E2 and DHT biosynthesis at their physiological concentrations. This differs from 17β-HSD7 inhibition whereby the cellular estradiol concentration is decreased while the DHT concentration is increased, synergistically down-regulating BC cell proliferation. Sulfatase inhibition with reduction of both E2 and DHT limit the effect on cell proliferation reduction, due to the stimulatory effect of DHT decrease at the physiological concentration (11)(42)(52). This suggests an inherent mechanism of reduced proliferation decrease with STS inhibition.

3.3 STX64, EM1913 and INH80 suppressed pS2 mRNA expression

Estrogen activity is mediated by its cognate receptor (ER), and the mechanism involves the induction of the receptor activation by E2: by binding to ER, E2 induces ER conformational changes that allows its interaction with specific response element known as estrogen response elements (EREs). This may ultimately lead to the expression of an estrogen-responsive gene (ERG) as well as the stimulation of cell growth and proliferation by altered expression of genes responsible for controlling cell cycle and proliferation (53)(54). The breast cancer estrogen-inducible protein (pS2), which is strongly expressed in breast cancer but barely expressed in breast, was chosen in this study to examine whether STS or 17β-HSD7 can modulate the expression of an ERG (10)(55).

In MCF-7, in the presence of DHEA-S, the pS2 mRNA was decreased to 89.2 ± 6.7% and 87.7 ± 9.3% with 10 × IC_{50} STX64 and 10 × IC_{50} EM1913 respectively. With substrate of E1-S, STX64 and EM1913 reduced pS2 mRNA to 84.4 ± 3.2% and 82.3 ± 3.4%. Under similar conditions, 10 × IC_{50} INH80 decreased pS2 mRNA to 75.5 ± 4.0% (Figure 3.4.A). Similar conditions can be observed in T47D, the pS2 was significantly down-regulated by inhibitors of sulfatase or INH80 (Figure 3.4.B). These results showed that the sulfatase and 17β-HSD7 were suppressed by sulfatase inhibitors or INH80, leading to E2 reduction.

3.4. EM1913 and STX64 arrest the cell cycle in G0/G1 phase by suppressing cyclin D1.

An EdU incorporation assay combined with Propidium Iodide (PI) was performed to investigate the cytostatic effects of STX64 or EM1913. In the presence of DHEA-S, 80 nM STX64 arrested 6.2% of MCF-7 cells in the G0/G1 phase (87.8% ± 1.7% *vs*.

81.6 ± 1.2% Ctrl) and cells in the S-phase decreased by 3.9% (5.2% ± 0.2% *vs* 9.1% ± 0.6% Ctrl). With EM1913 (0.48 nM), the percentage of cells in G0/G1 increased by 6.5% (88.1% ± 1.3% *vs* 81.6 ± 1.2% Ctrl) and the percentage of cells in S-phase declined by 4.1% (5.0% ± 0.2% *vs* 9.1% ± 0.6% Ctrl). In the presence of E1-S, there was a 4.7% increase in cells arrested in the G0/G1 phase by STX64 (73.8% ± 1.2% *vs*. 69.1% ± 1.8% Ctrl), and cells in the S-phase were decreased by 3.2% (5.8% ± 0.3% *vs* 9.0% ± 0.6% Ctrl). In the presence of EM1913, there was a 5% increase in cells in the G0/G1 phase (74.1% ± 1.7% *vs* 69.1% ± 1.8% Ctrl) with a 4.9% decline of cells in the S-phase (4.1% ± 0.8% *vs* 9.0% ± 0.6% Ctrl) (Figure 3.5).

Following a similar protocol, in the presence of 0.1nM E1, and with inhibition of 17β-HSD7, 8.15% of cells were arrested in the G0/G1 phase, and the proportion of cells in the S-phase decreased by 7.5%. In contrast, INH1 displayed less potency with regard to G0/G1 arrest (3.65%) and S phase cell suppression (3.35%) (42) (Table 4). Thus, arrest in G0/G1 phase by sulfatase inhibition is inferior to that resulting from 17β-HSD7 inhibition yet greater than that caused by inhibition of 17β-HSD1.

Similar results were observed in in T47D cells (Figure 3.6). In the presence of DHEA-S, STX64 and EM1913 arrested 5.1% and 6.0% of cells in the G0/G1 phase and cells in S-phase were decreased by 3.1% and 2.9% respectively. In the presence of E1-S, 3.3% of cells in the G0/G1 phase were arrested by STX64, and cells in the S-phase were decreased by 2.1%. The effect of EM1913 was to produce a 4.4% increase in cells arrested in the G0/G1 phase and a 2.4% decline in cells in the S-phase.

Under similar conditions, in the presence of 0.1nM E1, with inhibition of 17β-HSD7, 8.3% of cells arrested in the G0/G1 phase and the number of cells in the S phase was reduced by 3.7%, INH1 arrested 4.6% cells in G0/G1 and decreased the number of cells in the S phase by 3.4%.

Thus, sulfatase inhibition had a reduced effect on the cell cycle than 17β-HSD7 inhibition did but an increased effect than 17β-HSD1 inhibition in MCF-7 and T47D cells. These results suggest that inhibitions producing only a reduction in E2 without significant DHT up-regulation can result in fewer cells arrested in the G0/G1 phase and a reduction in the number of cells in the S phase, which is associated with modest inhibitory effects. Conversely, INH7 could reduce E2 and also restore DHT, resulting in a significant arrest of cells in the G0/G1 phase and reduction of cells in the S phase (Table 3.4).

The cell cycle checkpoint is monitored by the Cdk4-cyclin D1 complex, and the inhibition of sulfatase induced cell cycle arrest in the G0/G1 phase with fewer cells in S phase. Therefore, a western blot was conducted to confirm the expression of cyclin D1. When MCF-7 cells were treated with STX64 or EM1913, the expression of cyclinD1 was decreased by 11.9% and 14.3% respectively (Figure 3.7.A). Similar effects were observed in T47D cells. STX64 and EM1913 reduced the cyclinD1 protein expression by 12.5% and 13.7% respectively (Figure 3.7.B). Under similar conditions, in the presence of 0.1nM E1, 17β-HSD7 inhibition induced a 1.5-fold down-regulation of cyclin D1 in MCF-7 (42). These observations indicate that sulfatase inhibition arrests the cell cycle in G0/G1 by regulation of cyclin D1.

3.5. Effect of DHT supplementation on breast cancer cell proliferation after STS inhibition

After comparing sulfatase inhibition with that of 17β-HSD1 and 17β-HSD7, we evaluated the impact of DHT on sulfatase inhibition in BC cells. MCF-7 and T47D cells were cultured for 1 day in steroid-deprived medium (dextran-coated charcoal-treated medium). The medium was then replaced with charcoal-treated medium supplemented with 0.5nM E1-S and 10 × IC_{50} EM1913, and different concentrations of DHT were supplied for 4 days. Proliferation was evaluated to determine whether the restoration of DHT could enhance the anti-proliferative effects of sulfatase inhibition. Cells cultured in the presence of DHT in steroid-deprived medium were used as a comparison. Previous reports suggested that androgen inhibits the estrogenic stimulated growth of estrogen-dependent tumors in the presence of estrogens (56-58).

The results showed that DHT had no significant effect on cell proliferation in steroid-deprived medium. However, proliferation of MCF-7 cells in the presence of E1-S was reduced by 27.3% by a combination of EM1913 and 0.01nM DHT (Figure 3.8.A), in comparison with 17.8% by EM1913 alone. Moreover, EM1913 coupled with 1nM DHT induced a 35.6% reduction in proliferation, which was significantly higher than that observed for group EM1913 (18.2%, Figure 3.8.B). Proliferation of T47D cells in the presence of EM1913 with both DHT concentrations (0.01 and 0.1nM) was decreased by 28.7% and 23.5%, respectively. These anti-proliferative effects were significantly enhanced than the T47D cells exposed to only EM1913 (18.2%) (Figure 3.8.C and 3.8.D). These results strongly suggest that restoration of DHT can significantly improve the anti-proliferative effects of sulfatase inhibition. This agrees with our recent observation

that synergistic estradiol reduction and DHT restoration can promote marked BC cell growth inhibition (42).

3.6. The joint inhibition of sulfatase and 17β-HSD7 significantly suppressed proliferation by reducing E2 and restoring DHT

As the anti-proliferative effects of sulfatase inhibition can be improved by restoration of DHT, we analyzed the combination inhibition of sulfatase and 17β-HSD7 as the 17β-HSD7 inhibition can restore DHT.

In this study, E1-S was used as substrate. In MCF-7, the cell proliferation was decreased by 33.6% vs. 36.4% when cells were treated with 10× IC_{50} INH80 and STX64 or EM1913 respectively (Fig. 3.9.A). When treated with 10× IC_{50} INH80 and STX64, the E2 nmol/µg protein of cells was reduced from 7.517± 0.877 nmol/µg to 5.485 ± 0.981 nmol/µg (to 73.0%) (Figure 3.9.B). On the other hand, the DHT nmol/µg protein of cells was increased from 0.802 ± 0.180 nmol/µg to 2.622± 0.435 nmol/µg (to 3.3 folds) (Figure 3.9.C). Similarly, 10× IC_{50} INH80 and EM1913 produced a reduction of E2 to 5.171 ± 0.738 nmol/µg protein of cells (to 68.8%) (Figure 3.9.B) and up-regulation of DHT to 2.706 ±0.527 nmol/µg protein of cells (to 3.4 folds) (Figure 3.9.C).

Under similar conditions, in T47D, with 10× IC_{50} INH80 and STX64 vs. 10× IC_{50} INH80 and EM1913, the cell proliferation was decreased by 33.0% vs. 35.0% respectively (Fig. 3.9.A), the E2 quantity/µg protein of cells was reduced from 7.169 ± 0.772 nmol/µg to 4.625± 0.832 nmol/µg (to 64.5%) and to 4.513± 0.728 nmol/µg (to 63.0%) respectively (Figure 3.9.B). On the contrary, the combination of INH80 and sulfatase inhibitors increased the DHT concentrations to about 3.1 folds (Figure 3.9.C).

It is indicated that the combination of INH80 and sulfatase inhibitors showed more significant inhibition than the single application of INH80 or sulfatase inhibitors.

4. Discussion

4.1. Mechanism of STS pathway for sex-hormone synthesis

In our study, inhibition of STS activity resulted in reductions in estrogen and androgen concentrations in vitro studies. The E2 concentration was reduced by approximately 26%, and the DHT level was slightly decreased by less than 10%. I*n vitro* studies suggest that cell proliferation was related to cytostatic effects, which is induced by anti-estrogen agents in ER+ BC cells (12). These anti-estrogen agents were associated with cell cycle arrest in the G0/G1 phase due to estrogen blockade, resulting in a reduction in the relative proportion of cells synthesizing DNA during the S phase (13).

CyclinD1 is an estrogen-responsive gene that is suggested to work as an oncogene with an important pathogenic role in ER + BC (129). Furthermore, DHT-activated androgen receptor (AR) represses transcription of cyclin D1 in ER + BC cells (18). The blockade of E2 formation can inhibit cyclin D1, producing cell cycle arrest in G0/G1. .

In BC tissue, 3β-HSD, 17β-HSD3/5 and 5α-Reductase 1/2 are involved in the formation of DHT. Dehydroepiandrosterone (DHEA), which is produced directly from DHEA-S by sulfatase, is converted into 4-dione by 3β-HSD, followed by conversion to testosterone (Testo) by 17β-HSD3/5. 5α-Reductase 1/2 subsequently converts Testo into DHT, which is the most potent androgen found in tissue (59). Dihydrotestosterone has been reported to exert an anti-proliferative effect via AR by activating $^{p21waf1/cip1}$ and inhibiting cyclin D1 (17,18). Therefore, an

emerging concept for BC treatment involves the joint targeting of E2 reduction and DHT restoration (60).

Furthermore, previous studies suggested that the high level of 3α-diol formed in MCF-7 and T47D cells was caused by the presence of other enzymes that principally reduce DHT into 3α-diol in these two cell lines (61). However, DHT is more preferentially reduced to 3β-diol than 3α-diol. The 3α-diol can be converted back to DHT via 3α-hydroxysteroid oxidase activity, but the formation of the 3β-diol metabolite is irreversible (62,63). Therefore, it is possible that 3β-diol could be the primary products of DHT reduction. Moreover, it has been shown that 3β-diol possesses some low estrogenicity, that induces BC cell proliferation and shows binding to ERα and ERβ rather than AR (64,65).

The physiological concentration of DHT in BC cells is maintained at the nanomolar level (66,67). This concentration maintenance is important to limit hormone-dependent BC cell growth because the addition of physiological concentration of DHT to BC cell culture decreases the proliferation of these cells in the presence of estrogen (17,68,69).

Furthermore, Genome-wide screen have identified high-affinity estrogen response elements (EREs) (70) and androgen response elements (AREs) (71) in the promoter of AR, meaning that AR can be positively regulated by E2 and DHT. According to studies of Sang XY et al, When MCF-7 cells were in presence of 10 × IC_{50} EM1913 for 48h, the mRNA of AR was decreased to 75.8%. Under similar conditions, in T47D, the AR mRNA expression was reduced to 79.1% (unpublished results).

When STS is inhibited, both E2 and DHT levels were decreased, thus the AR expression was also down-regulated. A systematic meta-analysis demonstrated that co-expression of AR in female breast tumor is associated with a better prognosis and outcome (20). Therefore, the fact that the anti-proliferative effect declines to below 20% can be related to the reduction of DHT, which can lead to the down-regulation of AR.

4.2. Comparisons of the effects of sulfatase and 17β-HSDs inhibition

Several important reductive enzymes activate estrogen, including 17β-HSD7, 17β-HSD1 and 17β-HSD12. The 17β-HSD7 and 17β-HSD1 enzymes are involved in the final step of conversion from E1 to E2. Comparison of our study with that of 17β-HSD7 inhibition, revealed that MCF-7 cell proliferation decreased by around 20.8% by EM1913, which was significantly less than the reduction observed in response to treatment with INH80 (approximately 28%) (Table 3.2). Meanwhile, the DHT level following sulfatase inhibition was decreased by about 3%. Conversely, the level of DHT increased by 3.16-fold in the presence of INH80. When the DHT level was restored to 0.1nM in the culture medium, the anti-proliferative effect of EM1913 increased to 31.5%, which is similar to that observed in response to INH80 inhibition. This strongly suggests that the reduction of both estrogen and androgen following sulfatase inhibition may be responsible for the decreased cell proliferation effect by STS inhibition, which resulted in limited results of clinical phase II trial of Irosustat. Combined inhibition of sulfatase and 17β-HSD7 may be a promising therapy for ER+ BC treatment, with enhanced E2 reduction in addition to DHT restoration.

The estrogen level decreased significantly in response to 17β-HSD1 inhibition, but there was no significant regulation of DHT. This accounts for the anti-proliferative

effect of INH1 (18%) in MCF-7 cells. However, because of the high expression of 17β-HSD1 in T47D, cell proliferation was significantly suppressed by 17β-HSD1 inhibition (35%) (Table 3.3) (42).

5. Conclusion

Single blockade of the STS pathway decreases both E2 and DHT concentrations, inducing a modest inhibition of ER + BC cell proliferation. Restoration of DHT can significantly enhance the suppression of cell growth induced by sulfatase inhibition. Thus, combined inhibition of STS with other 17β-HSDs such as 17β-HSD7 would be a more beneficial strategy through the dual regulation of estrogen and androgen. Additional cellular and in vivo studies are required to better understand whether such combinations offer advantages over the use of a single-agent therapy.

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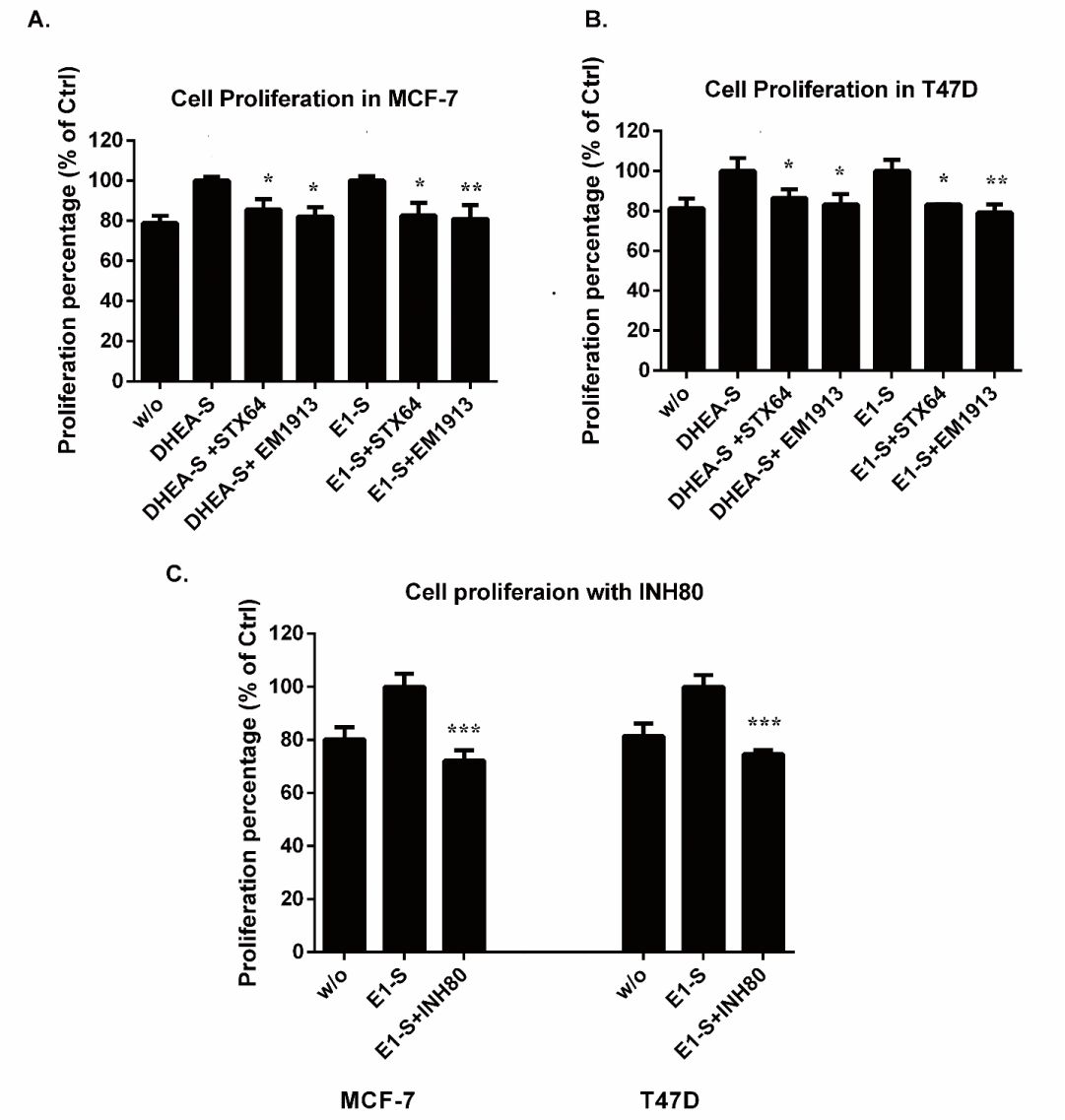
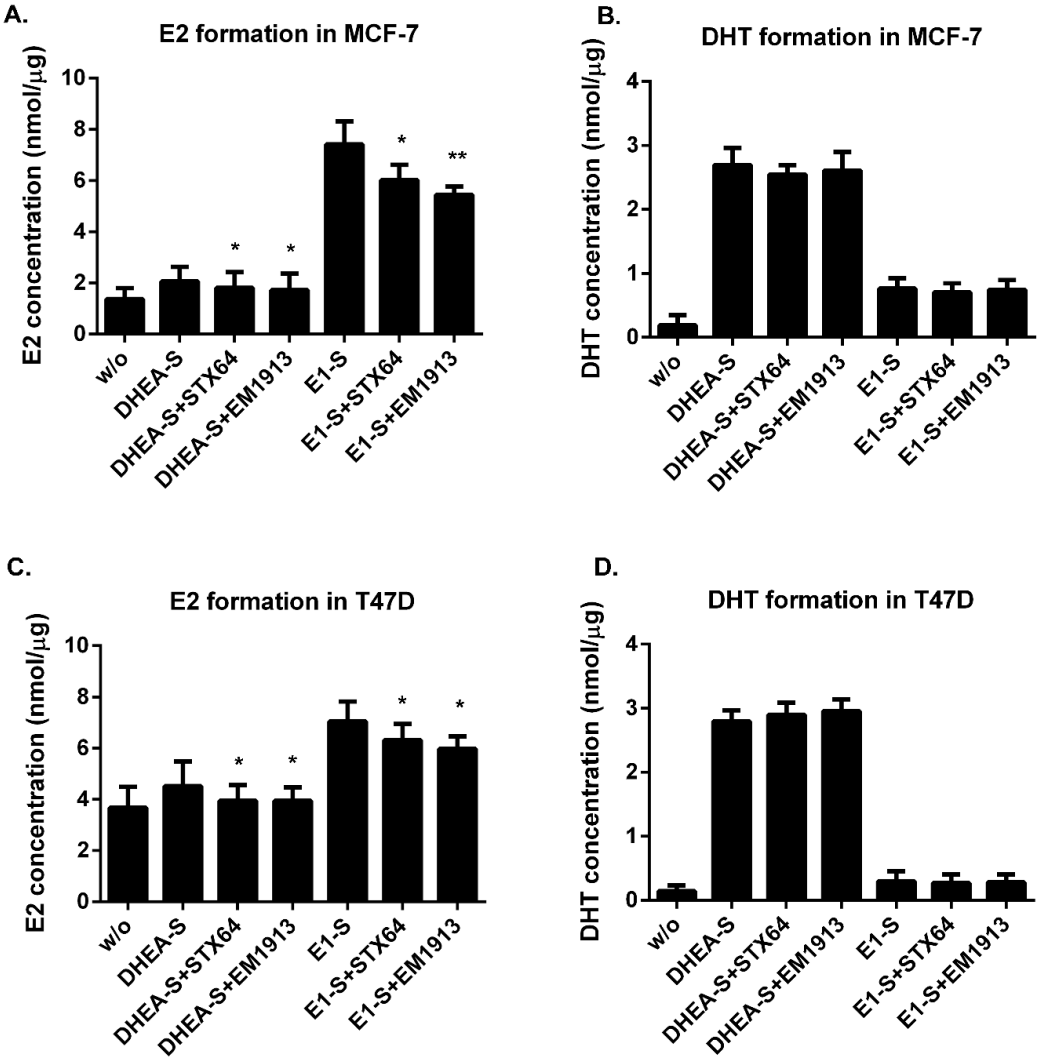
Legends and Figures

Figure 3.1 Cytostatic effect of STX64, EM1913 and INH80 in MCF-7 and T47D cells



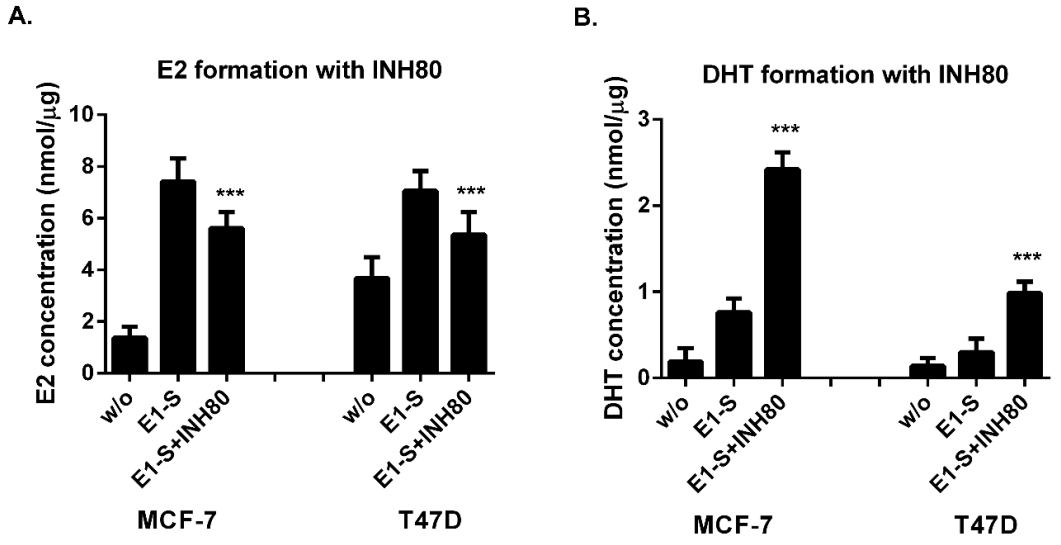
Cell proliferation assay with the Cyquant kit in MCF-7 (Figure A and C) and T47D cells (Figure B and C). DHEA-S or E1-S was used as substrate for sulfatase inhibition, cells treated with STX64 or EM1913, respectively. Cells were treated with INH80 in the presence of E1-S for 17β-HSD7 inhibition. Data are reported as % of DNA synthesis vs control (100%). Each point represents the mean of four replications (mean ± SD). Statistical significance by one-way ANOVA (Figure 1-A and Figure B) and Student's t test (Figure C): * $P < 0.05$ *vs.* control (Ctrl); ** $P < 0.01$ *vs.* control (Ctrl); *** $P < 0.001$ *vs.* control (Ctrl).

Figure 3.2: E2 and DHT formation in MCF-7 and T47D cells treated with STX64 or EM1913.



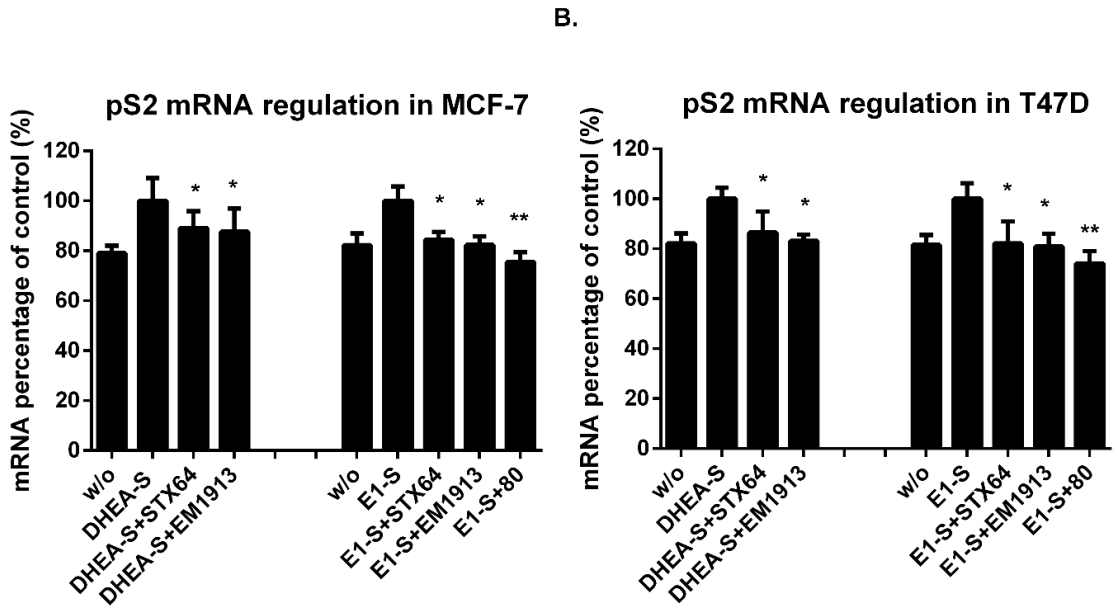
E2 and DHT formation in MCF-7 (A, B) and T47D (C, D) cells treated with STX64 or EM1913. DHEA-S and E1-S were used as substrates respectively. Cell culture supernatants were collected for determination of E2 and DHT by ELISA kit. Each bar represents the mean of three replications (mean ± SD). Statistical significance by one-way ANOVA: * $P < 0.05$ vs. control (Ctrl); ** $P < 0.01$ *vs*. control (Ctrl).

Figure 3.3: E2 and DHT formation with 17β-HSD7 inhibitor INH80 in MCF-7 and T47D cells



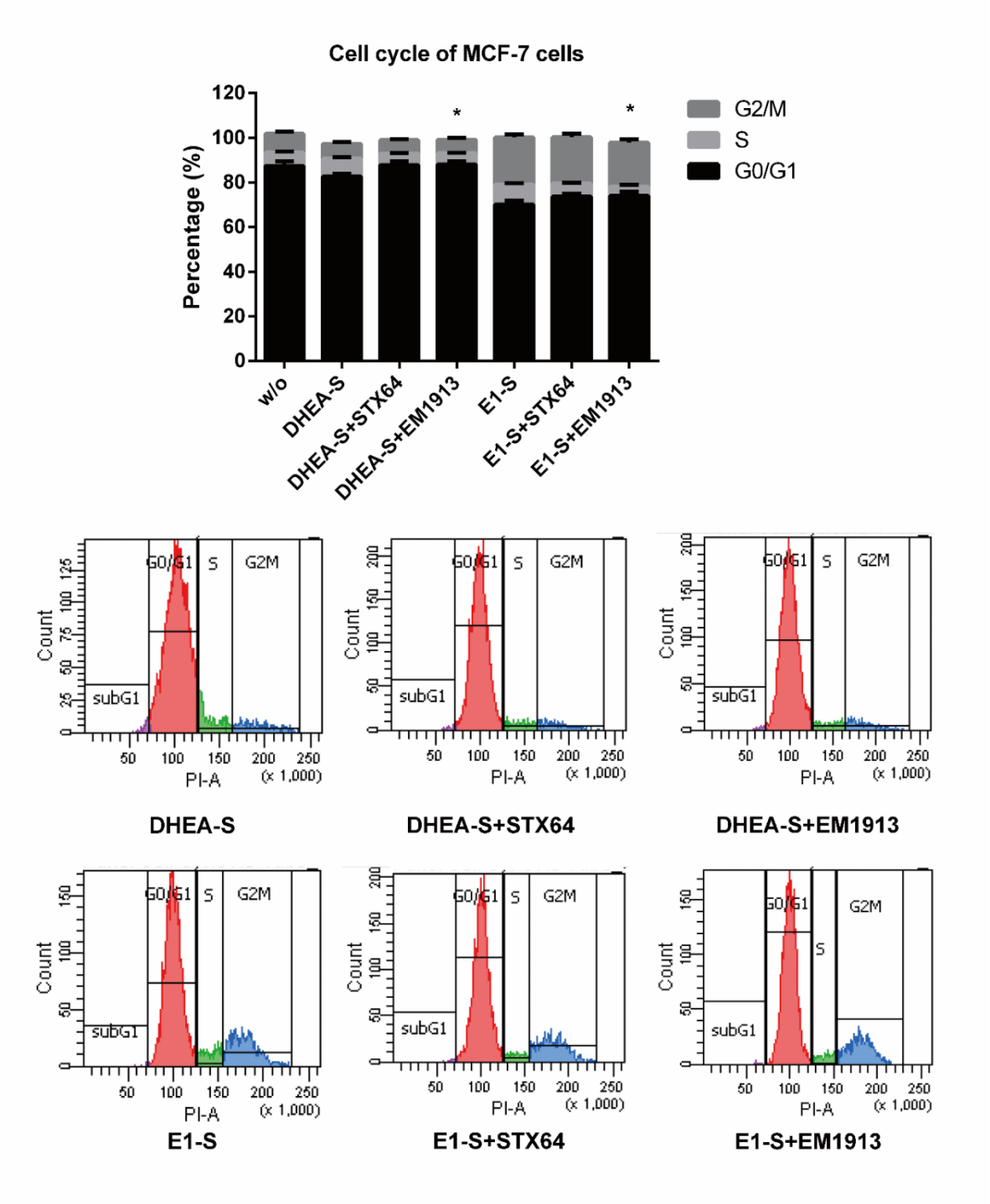
E2 (A) and DHT (B) formation with 17β-HSD7 inhibitor INH80 in MCF-7 and T47D cells. E1-S was used as substrate. Cell culture supernatants were collected for determination of E2 and DHT by ELISA kit. Each bar represents the mean of three replications (mean ± SD). Statistical significance by Student's t test: *** $P < 0.001$ *vs.* control (Ctrl).

Figure 3.4 pS2 mRNA expression in MCF-7 cells or T47D cells



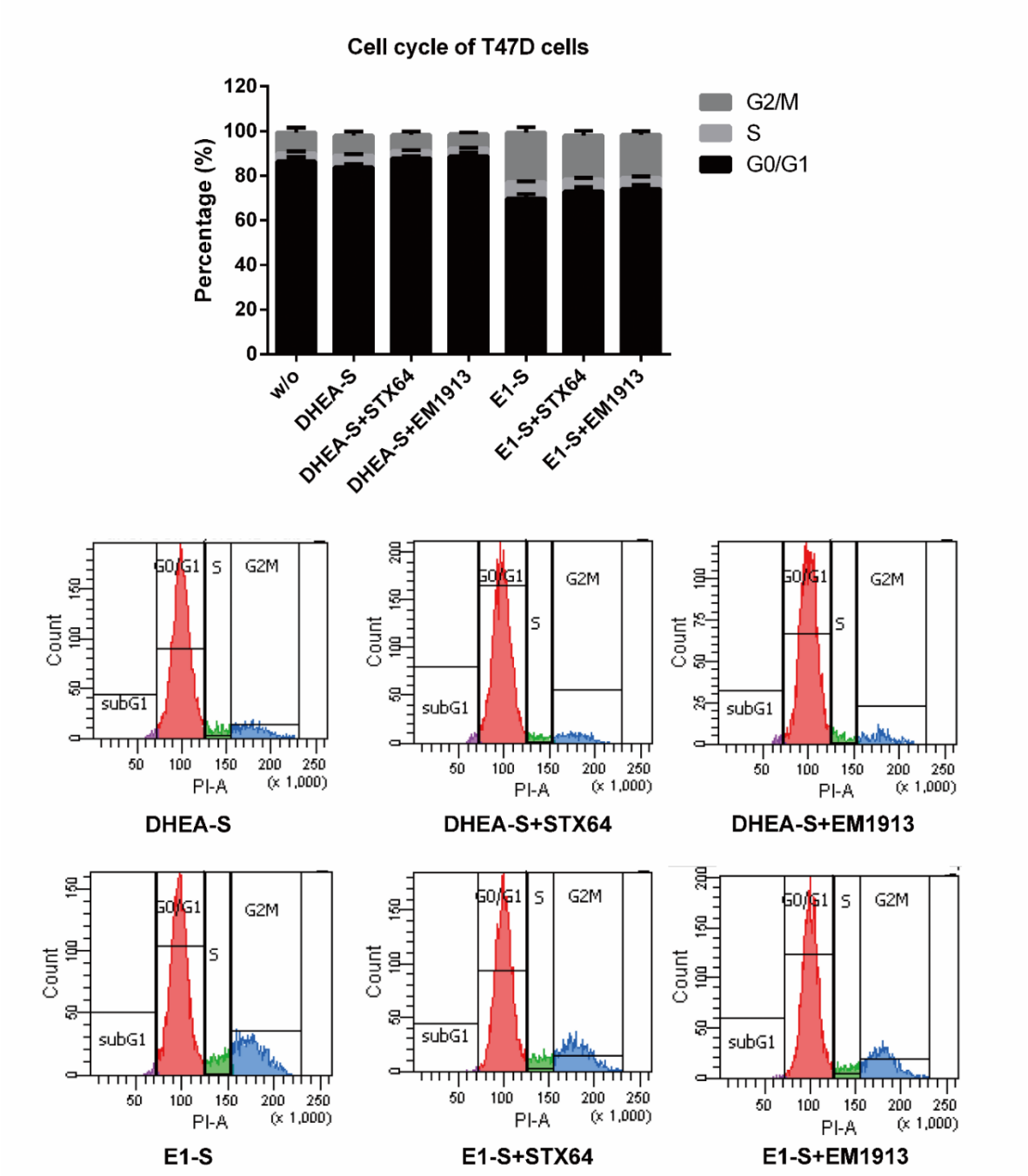
pS2 mRNA expression in MCF-7 cells (A) or T47D cells (B). DHEA-S or E1-S was used as substrate for sulfatase inhibition, cells treated with STX64 or EM1913, respectively. Cells were treated with INH80 in the presence of E1-S. mRNA level was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by one-way ANOVA: * $P < 0.05$ *vs* control (Ctrl). ** $P < 0.01$ *vs*. control (Ctrl).

Figure 3.5 Cell cycle analysis of MCF-7 treated with STX64 or EM1913



DHEA-S and E1-S were used as substrates, respectively. Data are presented as % of living cells (G0/G1, S and G2/M cells = 100%). Each number represents the mean of experiments carried out in triplicate (mean ± SD). Statistical significance by one-way ANOVA: * $P < 0.05$ *vs.* control (Ctrl). Flow cytometry figures represent the distribution of each phase of the cell cycle after treatment.

Figure 3.6 Cell cycle analysis of T47D treated with STX64 or EM1913



DHEA-S and E1-S were used as substrates respectively. Data are presented as % of living cells (G0/G1, S and G2/M cells = 100%). Each number represents the mean of experiments carried out in triplicate (mean ± SD). Statistical significance was determined by one-way ANOVA. Flow cytometry figures represent the distribution of each phase of the cell cycle after treatment.

Figure 3.7 Cyclin D1 protein expression determined by western blot in MCF-7 and T47D treated with STX64 and EM1913

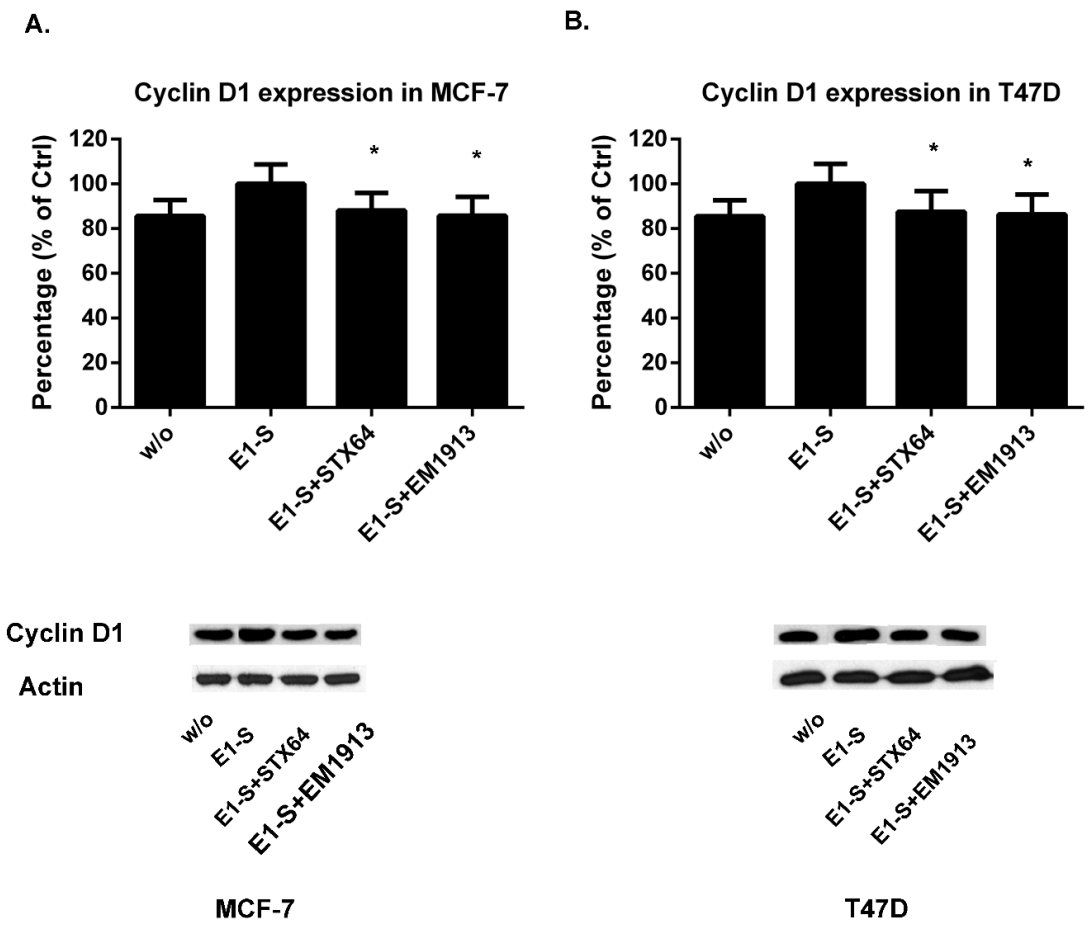
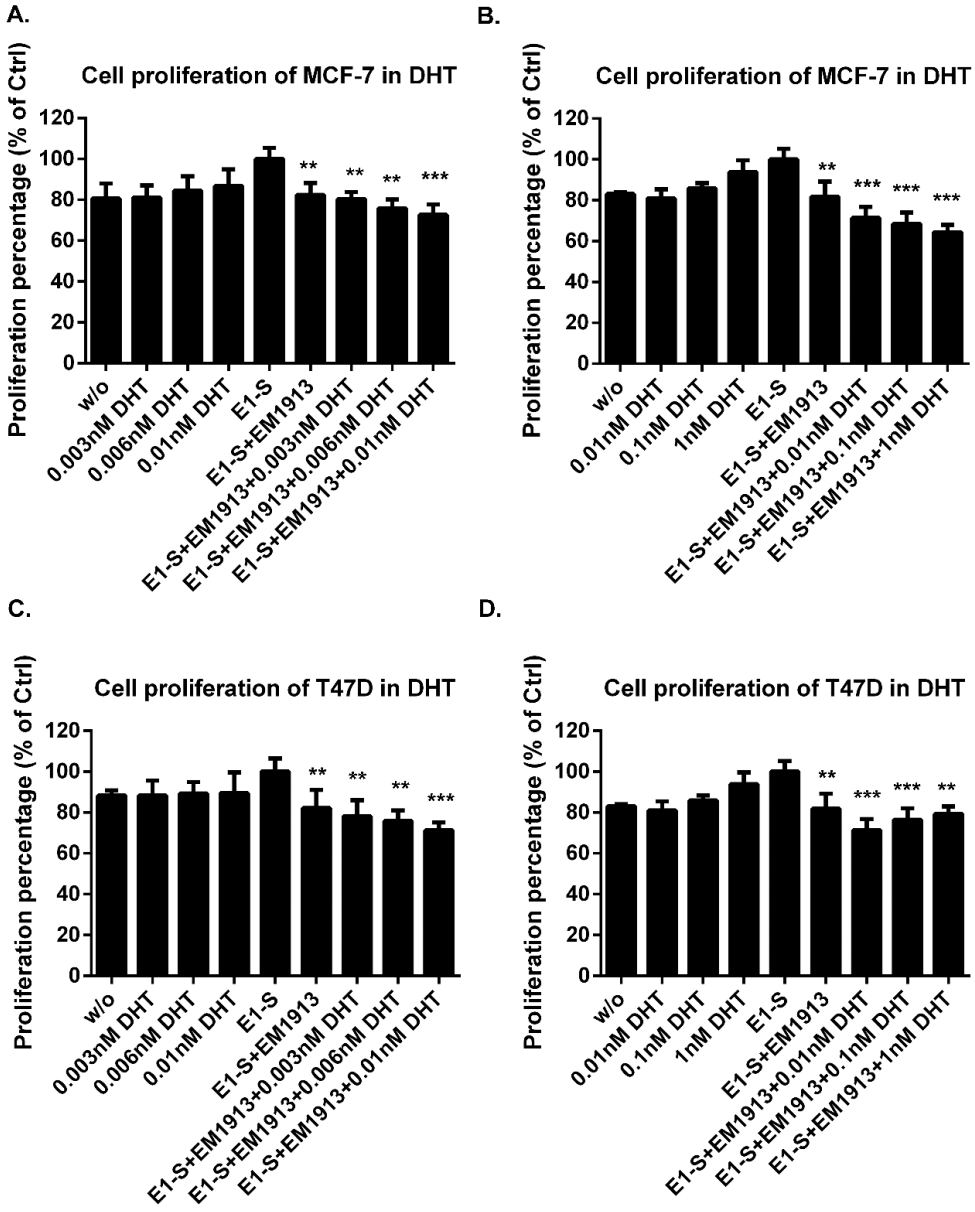


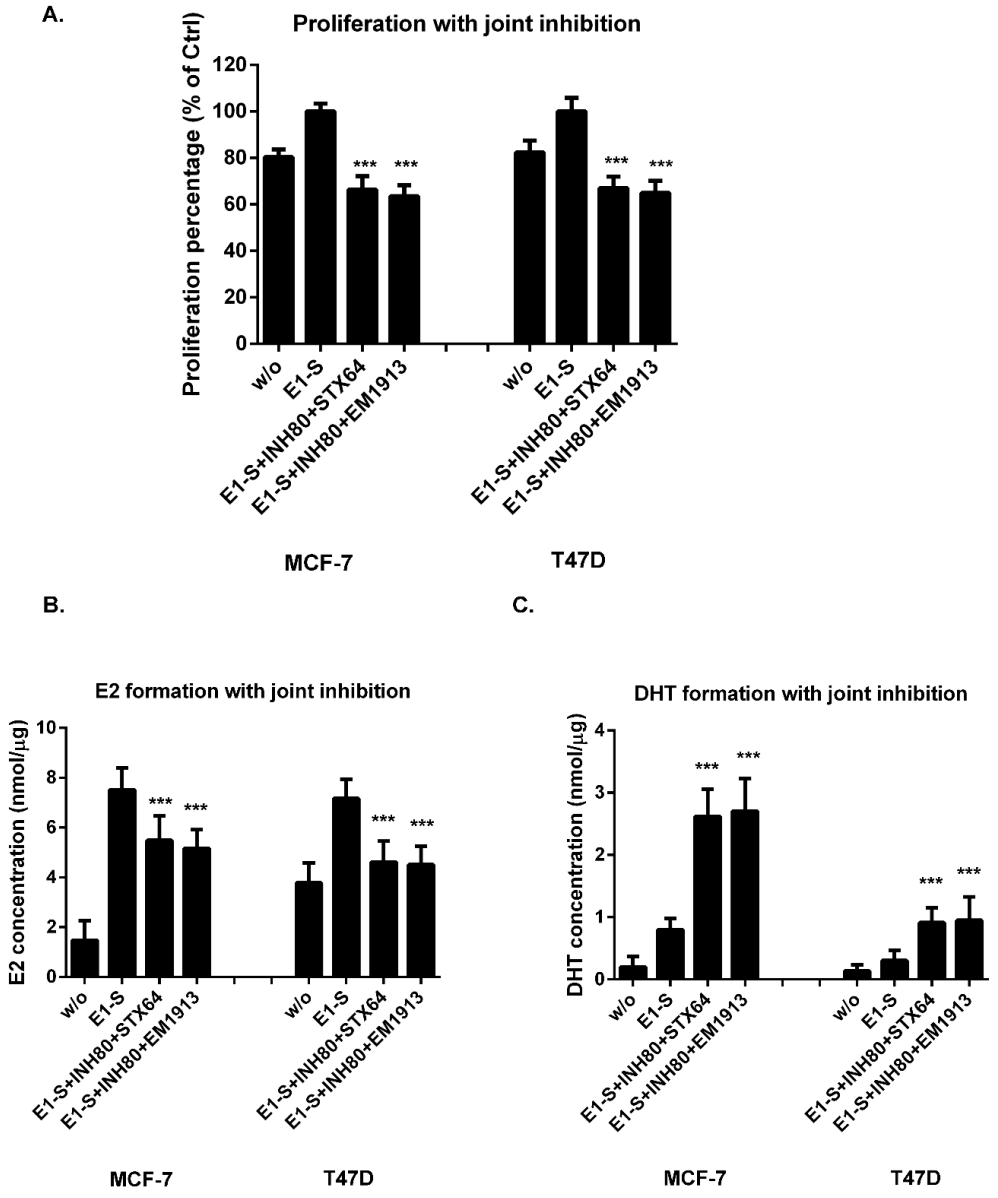
Figure 7: Cyclin D1 protein expression determined by western blot in MCF-7 (A) and T47D (B) treated with STX64 and EM1913. Data are reported as the mean ± SD (n=3) of the individual experiments. Statistical significance by Student's t test: * $P < 0.05$ *vs.* Ctrl.

Figure 3.8: Cell proliferation assay performed with the Cyquant kit for MCF-7 and T47D cells.

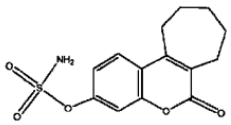
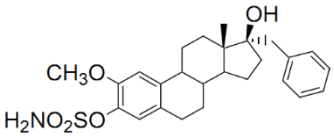
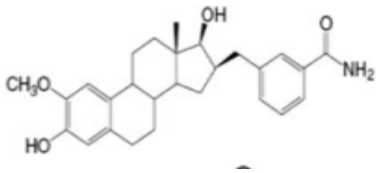
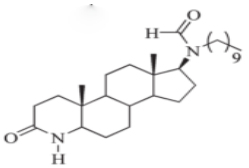


Cell proliferation assay performed with the Cyquant kit for MCF-7 (A and C) and T47D (B and D) cells. 0.5 nM E1-S was provided as a substrate. DHT concentrations varied from 0.003nM to 0.01nM (A and B) and from 0.01nM to 0.1nM (C and D). Data are reported as % of DNA synthesis *vs* Ctrl (100%). Each point represents the mean of experiments carried out as four replications (mean ± SD). Statistical significance by one-way ANOVA: ** $P < 0.01$ vs Ctrl; *** $P < 0.001$ *vs* Ctrl.

Figure 3.9: In vitro studies of joint inhibition of sulfatase and 17β-HSD7.



In vitro studies of joint inhibition of sulfatase and 17β-HSD7. Cell proliferation assay performed with the Cyquant kit for MCF-7 and T47D cells (Figure A). Data are reported as % of DNA synthesis vs Ctrl (100%). Each point represents the mean of experiments carried out as four replications (mean ± SD). E2 formation (Figure B) and DHT formation (Figure C) in MCF-7 and T47D cells treated with STX64 or EM1913. 0.5nM E1-S was used as substrates. Cell culture supernatants were collected for determination of E2 and DHT by ELISA kit. Each bar represents the mean of three replications (mean ± SD). Statistical significance by one-way ANOVA: *** $P < 0.001$ vs Ctrl.

Table 3.1: Inhibitor characteristics			
Compound	**Targeting enzyme**	**Chemical structure**	**IC_{50}***
STX64	STS		8nM* (43)
EM1913	STS		0.024nM* (46)
E2B-Methoxy (INH1)	17β-HSD1		275nM[a] (42)
INH80	17β-HSD7		195nM[a] (44)

(*) IC_{50}: concentration of the compound inhibiting 50% of substrate transformation in HEK293 over-expressing STS.

(a) IC_{50}, concentration of the compound inhibiting 50% of E1 to E2 conversion in T47D cells (45)

Table 3.2: Inhibition effects of vital reductive 17β-HSDs in MCF-7

Enzyme inhibition	Steroids formation		Proliferation (%)
	E2(%)	DHT(%)	
Control	100	100	100
Inhibition by sulfatase	73.4	97.5	81.0
Inhibition by 17β-HSD1	75.3	115.7	82.4
Inhibition by 17β-HSD7	75.6	316	72.2

Abbreviations: 17β-HSD1, 17beta-hydroxysteroid dehydrogenases type 1; 17β-HSD7, 17beta-hydroxysteroid dehydrogenases type 7;

Table 3.3: Inhibition effects of vital reductive 17β-HSDs in T47D

Enzyme inhibition	Steroids formation		Proliferation (%)
	E2(%)	DHT (%)	
Control	100	100	100
Inhibition by sulfatase	83.5	94.6	79.2
Inhibition by 17β-HSD1	41.3	106.9	72.4
Inhibition by 17β-HSD7	72.3	306	74.6

Abbreviations: 17β-HSD1, 17beta-hydroxysteroid dehydrogenases type 1; 17β-HSD7, 17beta-hydroxysteroid dehydrogenases type 7;

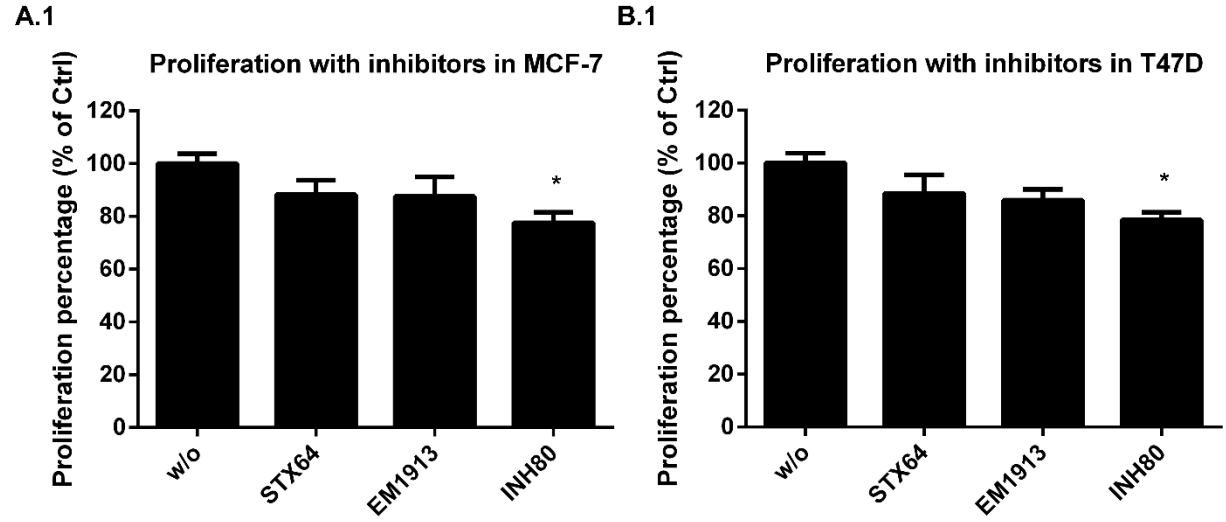
Table 3.4: The comparison of cell cycle with inhibitions of principal enzymes in MCF-7

Enzyme inhibition	MCF-7		
	G0/G1 (%)	S (%)	G2M (%)
Inhibition by sulfatase	4.0 (with E1-S)	4.9	1.4
Inhibition by 17β-HSD1	3.65	3.35	2.15
Inhibition by 17β-HSD7	8.15	7.5	1.35

Abbreviations: 17β-HSD1, 17beta-hydroxysteroid dehydrogenases type 1; 17β-HSD7, 17beta-hydroxysteroid dehydrogenases type 7;

Appendix

Figure 3.10: Cell proliferation assay with the Cyquant kit in MCF-7 and T47D cells



Cell proliferation assay with the Cyquant kit in MCF-7 (A.1) and T47D cells (B.1). Charcoal-treated medium were used as the baseline control (w/o). Cells treated with STX64, EM1913 or INH80, respectively. Data are reported as % of DNA synthesis vs control (100%). Each point represents the mean of four replications (mean ± SD). Statistical significance by one-way ANOVA (Figure 3.1.A and Figure 3.1.B) and Student's t test (Figure 3.1.C): * $P < 0.05$ vs. control (Ctrl).

CHAPTER IV

STEROID-CONVERTING ENZYMES: MULTIPLE MUTUAL REGULATIONS CONTRIBUTING TO BREAST CANCER DEVELOPMENT

4.1 Résumé

Introduction : Les 17β-hydroxystéroïdes déshydrogénases réductrices (17β-HSDs) et la 11β-hydroxystéroïde déshydrogénase 2 (11β-HSD2) jouent un rôle crucial dans la régulation de la synthèse des œstrogènes pour le cancer du sein (BC). *Objectifs* : Dans cette étude, les cellules MCF-7 et T47D ont été traitées avec des inhibiteurs de la 17β-HSD1, la 17β-HSD7, l'aromatase ou la stéroïde sulfatase (STS), puis des taux d'ARNm de la 17β-HSD7, de la STS, du récepteur des œstrogènes alpha (ERα), du récepteur androgénique (AR) et de la 17ß-HSD2 ont été déterminés par qRT-PCR. *Résultats* : Nos résultats démontrent que la 17β-HSD7, la STS et la 11β-HSD2 peuvent synthétiser l'estradiol (E2) et qu'elles sont toutes régulées par l'E2. Ainsi, elles forment un groupe fonctionnel d'enzymes mutuellement corrélées positivement et l'inhibition de l'une de ces enzymes peut réduire l'expression d'une autre. En outre, l'ERα a non seulement été régulés à la baisse par l'E2, mais également été réduite par la dihydrotestostérone (DHT) par l'activation du AR. *Conclusion* : En résumé, notre conclusion est en accord avec les statistiques sur l'expression de ces enzymes et ces récepteurs relatifs dans un grand nombre d'échantillons cliniques, fournissant une base pour la conception de la thérapie endocrinienne combinée pour la le cancer du sein.

4.2 Abstract

Reductive 17β-hydroxysteroid dehydrogenases (17β-HSDs) and 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) play a crucial role in regulating estrogen synthesis for breast cancer (BC). In this study, MCF-7 and T47D cells were treated with inhibitors of 17β-HSD1, 17β-HSD7, aromatase or steroid sulfatase (STS), then mRNA levels of 17β-HSD7, STS, estrogen receptors α (ERα), androgen receptor (AR) and 17β-HSD2 were determined by qRT-PCR. Our results demonstrate 17β-HSD7, STS and 11β-HSD2 can synthesize estradiol (E2) and are all regulated by E2. Thus, they form a functional group of enzymes mutually positively correlated, inhibition of one can reduce the expression of another, thereby potentially amplifying the inhibitory treatments. Furthermore, ERα were not only down-regulated by E2, but also reduced by dihydrotestosterone (DHT) though AR activation. In summary, our conclusion agrees with statistics on the expression of these enzymes and relative receptors in a large number of clinical samples, providing a basis for the design of combined endocrine therapy for BC.

4.3 Article 3: Steroid-converting enzymes: multiple mutual regulations contributing to breast cancer development

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Xiaoye Sang performed half of the experiments and wrote more than half of the manuscript;
Hui Han finished an important part of the experiments and wrote two parts of the manuscript, the experiments include: 1. 17β-HSD7 mRNA level was suppressed by respective inhibition of aromatase, sulfatase in MCF-7 and T47D and the E2 and DHT concentration test after inhibition of aromatase and type 7 in cells of MCF-7 and T47D; 2. Sulfatase mRNA was suppressed by inhibition of 17β-HSD7 in MCF-7 and T47D;
Tang Li gave some references supports and helped to solve machine problems;
Sheng-xiang Lin supervised the whole work and finalized the manuscript.

Key words: 17β-HSD7, sulfatase, aromatase, 11β-HSD2, estrogen receptor, androgen receptor, mutual regulation

1. Introduction

Most breast cancers (BCs), including 75% of post-menopausal cases and 60% of pre-menopausal cases are initially estrogen-dependent, where the sex-hormone estradiol (E2) plays an important role in cancer initiation and progression (1-4). The sex-hormones and estrogen receptor (ER) complex can mediate the activation of protooncogenes and oncogenes, and nuclear proteins in addition to other target genes. Consequently, the enzymes which modulate the intracellular concentrations of active estrogens could be targets for endocrine therapy for BC (5).

Several enzymes such as aromatase, steroid sulfatase (STS), and reductive 17β-hydroxysteroid dehydrogenases (17β–HSDs) (type 1 and type 7) are fundamentally involved in E2 formation (6-9). There is a great deal of interest in generating antagonists of these enzymes (10-13). However, there is limited information concerning how these enzymes modulate the expression of each other to promote cancer cell progression.

E2 is known to enhance cellular division in target cells by binding to its specific protein receptor called the ER (14). In general, the ER plays vital roles in the progression of malignant BC by interacting with estrogen response elements (EREs) contained in the promoter region of specific genes. The subsequent regulation of gene expression results in the biological effects of estrogen (15).

Compared with ER, the androgen receptor (AR) has an even higher expression, approximately 80% in BC cells (16). A systematic meta-analysis demonstrated that co-expression of AR in female breast tumours is associated with a better prognosis and outcome (17). Moreover, metabolites of DHT such as 5α-androstane-3β,17β-diol (3β-diol) displayed weak estrogenic potency towards MCF-7 cells through

binding to ER (18), and this was thought to be a possible mechanism of inducing aromatase inhibitor resistance (19,20).

In addition, studies have shown that glucocorticoids modulate aromatase activity and can affect the estrogen synthesis rate in breast carcinoma cells (21). In glucocorticoid target tissues, the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) modulates intracellular hormone levels, converting 11-hydroxycorticosteroids, cortisol and corticosterone to their 11-keto metabolites and 11-dehydrocorticosterone (22). Isoforms of 11β-HSD, 11β-HSD1 and 11β-HSD2 have been identified in mammals. 11β-HSD 2 colocalizes with the mineralocorticoid receptor in human BC specimens (23). Furthermore, inhibition of 11β-HSD2 activity in BC cells in vitro has been shown to enhance the antiproliferative effect of glucocorticoids (24). It is also indicated that 11β-HSD activity varies during the menstrual cycle according to the secretion levels of E2 and progesterone (25,26). Despite growing interest in the biological roles of 11β-HSD, only limited data exist relating to factors regulating its expression.

The aim of this investigation was to evaluate the interactive regulations of expression of principal steroid-converting enzymes in two BC epithelial cell lines (MCF-7 and T47D). It was also of interest to determine how they modulated receptors such as ER and AR to promote BC proliferation. Following respective inhibition of each steroid-converting enzyme, qRT-PCR was used to determine the gene expression of enzymes such as sulfatase and receptors such as ERα or AR. Western blot was used to verify sulfatase protein expression. It was revealed that sulfatase, 17β-HSD7, 11β-HSD2, and AR could be down-regulated by suppressing any enzyme involved in E2 synthesis. On the other hand, 17β-HSD1 was down- regulated by E2. This knowledge will help us further explore the mechanism of endocrine enzyme

interactions in BC, with the goal of designing and developing more efficient therapies for hormone-dependent BC.

2. Materials and Methods

2.1 Inhibitors and chemicals

Several inhibitors were used in this study: the STS inhibitor EM1913 (IC_{50}: 0.024 nM) without estrogenicity; 17β-HSD1 inhibitor INH1 (IC_{50}: 275 nM) and 17β-HSD7 inhibitor INH80 (IC_{50}: 195 nM) were synthesized in the Laboratory of Medicinal Chemistry (CHU de Québec-CHUL) as reported (27-29). The aromatase inhibitor (AI) letrozole (IC_{50}: 0.7 nM） was purchased from Selleck Chemicals (Houston, TX, USA). Stock solutions were prepared by dissolving the compounds in ethanol. Dilution to the desired concentrations was performed using culture medium (Table 1).

2.2 Cell Culture

The estrogen receptor positive (ER+) cell lines MCF-7 and T47D, were obtained from the American Type Culture Collection, and propagated according to a previously described protocol (32). Both culture media were phenol red-free. Fetal bovine serum (FBS) (PAA, Etobicoke, ON, Canada) was stripped with 2% dextran-coated charcoal (Sigma, Saint-Louis, MI, USA) according to the literature (32) and added to the protocol medium to a final concentration of 10%. Cells were plated in this charcoal-treated medium for 24 h, then the medium was replaced by fresh medium containing the desired concentrations of inhibitors. Cells cultivated in charcoal-treated medium were used as the baseline control (control).

2.3 Determination of E2 and DHT levels

Cells were plated in 96-well plates at a density of 5×10^3 cells per well and treated with $10 \times IC_{50}$ aromatase inhibitor (letrozole) for 4 days. Medium was renewed every 2 days. On the fourth day, the cell culture supernatants were extracted and frozen at –80°C. The E2 and DHT concentrations were determined by "Estradiol EIA Kit" (Cayman Chemical, Ann Arbor, MI, USA; the cross reactivity towards E2 was 100%) and DHT ELISA Kit (Alpha Diagnostic International, San Antonio, TX, USA; the cross-reactivity towards DHT was 100%) according to the manufacturers' instructions. Absorbance was determined at 412 nm for E2 and 450 nm for DHT. Each condition was tested in triplicate and experiments were repeated three times.

2.4 qRT-PCR

The mRNA level of steroidogenic enzymes and receptors was determined by qRT-PCR. After treatment for 24 or 48 h, the total RNA was extracted from cells with the RNeasy mini kit on-column DNase (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The measures of mRNA levels were done with 18S as the normalization gene. The procedures were performed by the Q-PCR platform of the Quebec Genomics Center (CHU du Quebec, Quebec, Canada). The total mRNA levels were expressed as thousands of mRNA copies/μg total RNA. The primers used for each gene were listed in Table 2. Each condition was conducted in triplicate and experiments were repeated three times.

2.5 Western blot

MCF-7 and T47D cells were treated for 2 days in the presence of E2 or 4 days with inhibitors. Total proteins were extracted with RIPA buffer (Sigma, Saint-Louis, MI, USA) containing a protease inhibitor cocktail (Calbiochem, Merck, Germany).

Equal amounts of protein were separated by 15% SDS-PAGE. Primary antibodies used were anti-Sts1 antibody (ab197027) and anti-actin (A5316) (Sigma, Saint-Louis, MI, USA). Secondary antibodies included goat-anti-rabbit IgG-HRP (sc-2004) and goat-anti-mouse IgG-HRP (sc-2005) (Santa Cruz Biotechnology, CA, USA). Blots were visualized by enhanced chemi-luminescence (ECL plus Western Blotting Detection Kit, PerkinElmer, MA, USA) and quantified with NIH ImageJ software. The relative quantity of protein expression was determined by the ratio between the signals from the protein of interest and β-actin. Each condition was conducted in triplicate and experiments were repeated three times.

2.6 Statistical analysis

Multiple comparisons were performed by ANOVA. The Student's t-test was used for the comparison of two groups. Data were presented as means ± SD. Statistically significant differences were determined by $P < 0.05$, $P < 0.01$ and $P < 0.001$.

3. Results

3.1 17β-HSD7 mRNA level was suppressed by respective inhibition of aromatase, STS and 17β-HSD1

The predominant function of enzyme 17β-HSD7 in sex-hormone regulation has been studied in vitro and in vivo (30,33). It is suggested that 17β-HSD7 is a unique enzyme, which is positively-regulated by its reaction product, E2. Such feedback mechanism increases the local E2 concentration and promotes the growth of estrogen-dependent BC (34). The sulfatase and aromatase pathways constitute two important pathways for E2 synthesis. Thus, we studied the regulatory effects of STS inhibition and aromatase inhibition on 17β-HSD7.

STS was inhibited by 0.24 nM EM1913 (10 × IC_{50}) for 24 h and 48 h, then the 17β-HSD7 mRNA level was determined by qRT-PCR. Expression of 17β-HSD7 mRNA was significantly reduced at 48 h (to 78.3%) in MCF-7 cells (Figure 4.1.A). However, markedly lower reduction of 17β-HSD7 mRNA was observed in T47D cells. (Figure 4.1.B).

Letrozole (7 nM; 10 × IC_{50}) was used to study the relationship between AI and 17β-HSD7 transcription. In MCF-7 cells, 17β-HSD7 mRNA was decreased to 23.6% at 48 h (Figure 4.1.C). A greater reduction in 17β-HSD7 mRNA level was observed in T47D cells, with a concomitant decrease in E2 concentration (Figure 4.1.D & Figure 4.1.E). On the other hand, when MCF-7 cells were treated with 10 × IC_{50} Letrozole or INH80, the DHT level increased by 20% and 3.2 times respectively (Figure 4.1.F).

After treatment with 10 × IC_{50} INH1 for 48 h under the similar conditions, 17β-HSD7 mRNA in MCF-7 cells and T47D cells was suppressed by 46.1% and 46%, respectively (35). 10 × IC_{50} inhibitor for 17β-HSD7 reduced 17β-HSD7 protein expression by 42% in MCF-7 cells (30).

Moreover, the E2 and DHT levels following inhibition of each enzyme was also evaluated in MCF-7 cells (Table 4.3). These results collectively suggested that 17β-HSD7 was down-regulated by inhibition of enzymes activating E2.

3.2 STS mRNA and protein expression was suppressed by inhibition of 17β-HSD7, 17β-HSD1 and sulfatase

In this experiment, MCF-7 and T47D cells were treated with the respective inhibitors INH1, INH80 and EM1913 for 24 h and 48 h, and the mRNA levels were determined by qRT-PCR.

In MCF-7 cells, STS mRNA level was more significantly decreased by inhibition of 17β-HSD7 than by 17β-HSD1. And cells showed the least reduction in STS mRNA level following inhibition of STS (Figure 4.2.A).

Different results were observed in T47D cells. Inhibition of 17β-HSD7 reduced STS mRNA level to 67.9% at 48 h. However, in the presence of INH1, STS mRNA level was more significantly decreased. In addition, STS inhibition by 10 × IC_{50} EM1913 only reduced STS mRNA level to 78.8% at 48 h (Figure 4.2.B).

Western blot analysis was conducted to verify the variation in STS protein expression. Cells were treated with respective inhibitors for 48 h, and STS protein level was determined by western blot. In MCF-7 cells, STS protein was decreased to 77.3% and to 83.1% by 10 × IC_{50} INH80 and 10 × IC_{50} INH1, respectively. Furthermore, 10 × IC_{50} EM1913 suppressed STS protein expression to 85.5%. (Figure 4.2.C). In comparison, in T47D cells, 10 × IC_{50} INH80 and 10 × IC_{50} INH1 decreased STS protein level to 80.0% and 74.7%, respectively. Expression of STS protein was reduced to 84.1% by 10 × IC_{50} EM1913 (Figure 4.2.D).

These results indicated that the sulfatase mRNA and protein could be regulated by 17β-HSD1, 17β-HSD7; when these enzymes were inhibited, STS enzyme was also down-regulated.

3.3 E2 induces higher expression of STS mRNA and protein

In order to better understand the regulatory pathway mechanisms of key enzymes involved in E2 synthesis, the up-regulation by E2 requires clarification. A previous study demonstrated that E2 exerts feedback on the STS pathway in normal and cancerous human breast tissues (36). In this study, qRT-PCR was used to analyze the enzyme mRNA after MCF-7 and T47D cells were treated with different

concentrations of E2. In MCF-7 cells, the STS mRNA levels were increased 1.4 folds by 0.1 nM E2 and 1.8 folds by 1 nM E2 (Figure 4.3.A). In T47D cells, sulfatase mRNA was up-regulated 1.9 folds by 0.1 nM E2 and 2.4 folds by 1 nM E2 (Figure 4.3.B).

Furthermore, the expression of STS was also confirmed by western blot. Treatment with 0.1 nM E2 produced a 1.36-fold and 1.32-fold up-regulation of STS protein in MCF-7 and T47D cells, respectively, and the expression of STS was increased 1.7 folds and 1.82 folds by 1 nM E2 in MCF-7 and T47D cells, respectively (Figure 4.3.C and Figure 4.3.D). Overall, these results indicated E2 induced a significant increase in STS mRNA and protein expression.

3.4 11β-HSD2 mRNA was suppressed by inhibition of 17β-HSD7, 17β-HSD1, aromatase and STS

As 11β-HSD2 has been indicated to regulate glucocorticoids and to affect the rate of estrogen synthesis in BC, the regulation of 11β-HSD2 was also examined. In MCF-7 cells, the 11β-HSD2 mRNA level was significantly decreased at 48 h (to 70.6%) following inhibition of 17β-HSD7. When 17β-HSD1 was suppressed, a diminished reduction in 11β-HSD2 mRNA level was detected. However, 11β-HSD2 mRNA showed the most significant reduction at 48 h (to 58.9%) when aromatase was inhibited. Reduced suppression could be observed in response to STS repression (Figure 4.4.A).

In T47D cells, 17β-HSD7 repression decreased the 11β-HSD2 mRNA level significantly to 71.1% at 48 h. However, 17β-HSD1 inhibition reduced 11β-HSD2 mRNA levels to 63.4% at 48 h. Suppression of aromatase showed the most

significant suppression of 11β-HSD2 mRNA. Reduced inhibition was observed in response to STS inhibition (to approximately 83.1%) (Figure 4.4.B).

The above results indicated that 17β-HSD7, STS and 11β-HSD2 were regulated by E2-converting enzymes, which is coincident with the fact that estrogen-receptor elements (ERE) were detected in the promoters of these enzymes (see discussion).

3.5 ERα mRNA was up-regulated by inhibition of 17β-HSD7, 17β-HSD1, aromatase and STS but suppressed by E2 and DHT

The E2/ER interaction plays a crucial role in the progression of BC. Thus, we also evaluated ERα mRNA expression in response to regulation of E2 synthesizing enzymes. In MCF-7 cells, in response to aromatase inhibition, the ERα mRNA level showed the most significant up-regulation. A reduced increase in ERα mRNA expression was observed in response to 17β-HSD1 and STS inhibition. There was no significant modification to ERα levels following 17β-HSD7 inhibition (Figure 4.5.A).

Similar results were observed in T47D cells. The modulations of ERα mRNA levels by inhibition of 17β-HSD1 and aromatase were more significant. There was no significant regulation in response to treatment with INH80 (Figure 4.5.B).

To analyze the mechanism of ERα regulation, the modification of ERα mRNA expression in the presence of 0.1 nM E2 and different concentrations of DHT was also determined. In the presence of 0.1 nM E2, the mRNA level of ERα was decreased to 83.8% in MCF-7 and was further reduced by supplementation of 0.1 nM or 1 nM DHT (Figure 4.5.C). In T47D cells, ERα was down-regulated to 84.3%

by 0.1 nM E2 and was decreased further by supplementation of 0.1 nM DHT or 1 nM DHT (Figure 4.5.D).

It is suggested that E2 suppresses the expression of ERα in BC (37,38), our results revealed that supplementation of DHT in the presence of E2 further decreases ERα. In MCF-7 cells, in response to inhibition of aromatase, the E2 concentration was decreased most significantly. Thus, the ERα was most significantly up-regulated. 17β-HSD7 reduced E2 more significantly than 17β-HSD1 and STS, but 17β-HSD7 could restore DHT to 3.15 folds (Table 4.3), so the modulation of ERα mRNA in response to 17β-HSD 7 inhibition was lower than that in response to inhibition of 17β-HSD1 and STS.

It has been suggested that regulation of ER by androgens and estrogens shows tissue and organ specificity and modulation of receptor mRNA levels depends on several physiological factors (39). Highly-expressing ER breast cells may show less involution than ER-negative cells (triple-negative BC) because ER-positive BC cells are more likely to respond well to hormone therapy (40).

3.6 Androgen receptor mRNA was suppressed by inhibition of 17β-HSD1 and STS, but increased by inhibition of aromatase and 17β-HSD7

As androgen has been shown to consistently inhibit proliferation of estrogen-induced BC through binding to androgen receptors (41-45), AR mRNA expression was also studied. In MCF-7 cells, the inhibition of 17β-HSD1 and STS reduced AR mRNA expression. However, the AR mRNA level was slightly increased following inhibition of aromatase. When 17β-HSD7 was inhibited, AR mRNA was significantly increased 1.4 folds at 48 h (Figure 6-A).

In T47D cells, the level of AR mRNA was decreased by INH1 and EM1913 (Figure 4.6.B). However, following treatment with INH80, the level of AR mRNA was increased 1.3 folds at 48 h (Figure 4.6.B).

Previous studies suggested that DHT reduced BC proliferation by binding to AR and promoting AR expression (46). Inhibition of 17β-HSD1 produced a reduction in E2, but no significant regulation of DHT, so AR was down-regulated following E2 reduction (see Discussion). However, when we inhibited 17β-HSD7, E2 was reduced, but DHT was significantly increased (Table 4.3), showing an up-regulation of AR. Aromatase converts androgens to estrogens: when inhibited, androgen conversion into E2 is prevented so E2 is reduced, leaving residual androgen at higher level. Therefore, there is no significant regulation of AR expression. These results indicated that the expression of AR is regulated by both DHT and E2, which interactively promote BC growth.

4. Discussion

Local estradiol production plays a vital role in the development and progression of hormone-dependent BC (47-49). The enzymes such as aromatase, STS, 17β-HSD1 and 17β-HSD7 produce E2, interacting with ER to promote the development of hormone-dependent BC. Several studies have been conducted to determine gene regulation of 17β-HSDs in the presence of E2 and DHT (50,51). However, it is essential to determine the mutual regulation of these enzymes and their relationships with ER and AR expression, as this is important for designing more efficient therapies for the treatment of BC.

Previous studies demonstrated down-regulation of 17β-HSD7 by a low E2 concentration (30,34,35). A 185-bp region of the 17β-HSD7 promoter was

identified that confers regulation by E2 in MCF-7 cells. This region is devoid of a classical ERE but contains a nuclear factor (NF1) site that is essential for E2 action. Thus, E2 stimulates the recruitment and DNA binding of NF1 to this region of the 17β-HSD7 promoter (34). In our studies, 17β-HSD7 was down-regulated by inhibition of STS and aromatase. When these two enzymes were inhibited, E2 concentration was reduced, leading to a reduction in 17β-HSD7 expression.

Furthermore, STS, 11β-HSD2 and AR were also down-regulated when antagonists of E2 formation were provided, which means these enzymes have an estrogenic effect. The estrogenic effect of estrogens is conducted through activation of the ER, which recognize specific EREs near the targeted genes. Genome-wide screens have identified high-affinity EREs in the promoters of several steroid enzymes (Table 4.4) (52). Ligand binding (E2 or other estrogens) is essential for ERα to maximize its binding affinity to ERE in vivo (53) and the presence of ligand can also stabilize the ER/ERE complex (54). Therefore, when E2 concentration was reduced, the expression of STS, 17β -HSD7, 11β-HSD2 and AR was also down-regulated.

As a result of differences from the enzymes in the above group, E2 level negatively affects 17β-HSD1 expression, leaving this enzyme as a more independent target for estrogen-dependent BC (50,55).

On the other hand, the gene expression regulation effects of ERs are greatly influenced by cross-talk between ERs and other nuclear transcription factors (56). Therefore, each enzyme was not regulated to the same level.

Furthermore, the efficiency of ER/ERE transcriptional activation may not be consistent between cell types, which can be affected by various cell-specific factors

(57). In our studies, MCF-7 cell line was originally isolated from a 69-year-old Caucasian woman with a malignant breast tumour (58) and T47D cell line originated from a pleural effusion from a 54-year-old female patient who presented with an infiltrating ductal breast carcinoma (59). Differences in enzyme gene expression exist for these two cell lines (32). Thus, the level of enzyme regulated in MCF-7 and T47D cells varies.

ERα mRNA was suggested to be down-regulated by estrogen by inhibition of ER gene transcription at early time points and by a post-transcriptional effect on receptor mRNA at later time points (60-62). When STS, aromatase, 17β-HSD7 or 17β-HSD1 was inhibited, E2 concentration was reduced, inducing an up-regulation in ERα mRNA.

Furthermore, the identification of androgen response elements (ARE) in a human genome-wide analysis was performed by Wilson et al. using ChIP-Seq pattern, database knowledge, and position site specific matrix models. ERα, AR and 17β-HSD7 were positively regulated by both ARE full and half sites (63) (Table 5). Thus, 0.1 nM E2 suppressed ERα mRNA, which was further reduced following supplementation with DHT. This could be in response to activation of AR by DHT, suppressing ERα activity (46,64,65).

Studies showed although there is a gradient of clinical response to hormone therapy with ER expression in BC, this gradient is skewed such that tumors containing even a small proportion of ER-positive cells respond much better than ER-negative tumors, and nearly as well as tumors with high levels of ER (40,66). Therefore, therapy to decrease E2 could reduce the E2/ ERα interactions to a lower level, which further down-regulates the expression of other E2-synthesis enzymes.

In addition, in the presence of ERE and ARE on the promoter of AR, the AR expression was up-regulated by INH80 that decrease E2 but increase androgens.

5. Conclusions

In conclusion, 17β-HSD7, STS and 11β-HSD2 can synthesize E2 and are regulated by E2, thus forming a functional group of steroid enzymes that are mutually positively correlated. Inhibition of one enzyme can suppress the expression of another, which can potentially amplify future treatment. 17β-HSD1 is not modulated by E2, so this enzyme should be a more independent target for inhibition in the treatment of estrogen-dependent BC. ERα was up-regulated when E2 was reduced but was down-regulated following supplementation of DHT. The above experiments in BC cells are in agreement with statistics relating to the expression of steroid-converting enzymes and relative receptors in a large number of clinical samples, providing a basis for the design of combined endocrine therapy for BC.

Declaration of interest

The authors have no conflict of interest to declare.

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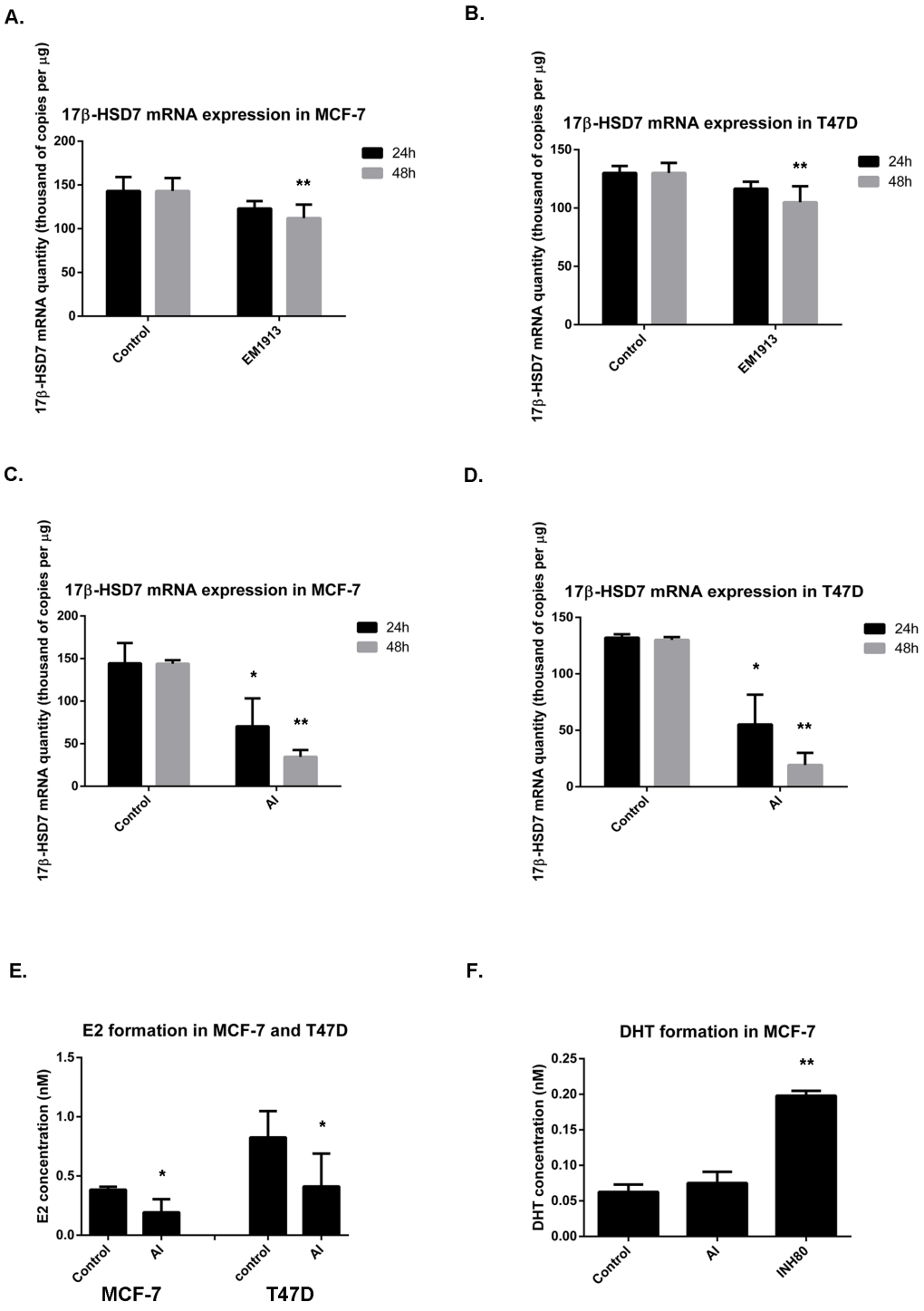
Figures and Legends

Figure 4.1 Regulation of 17β-HSD7 by steroid sulfatase inhibition or aromatase inhibition



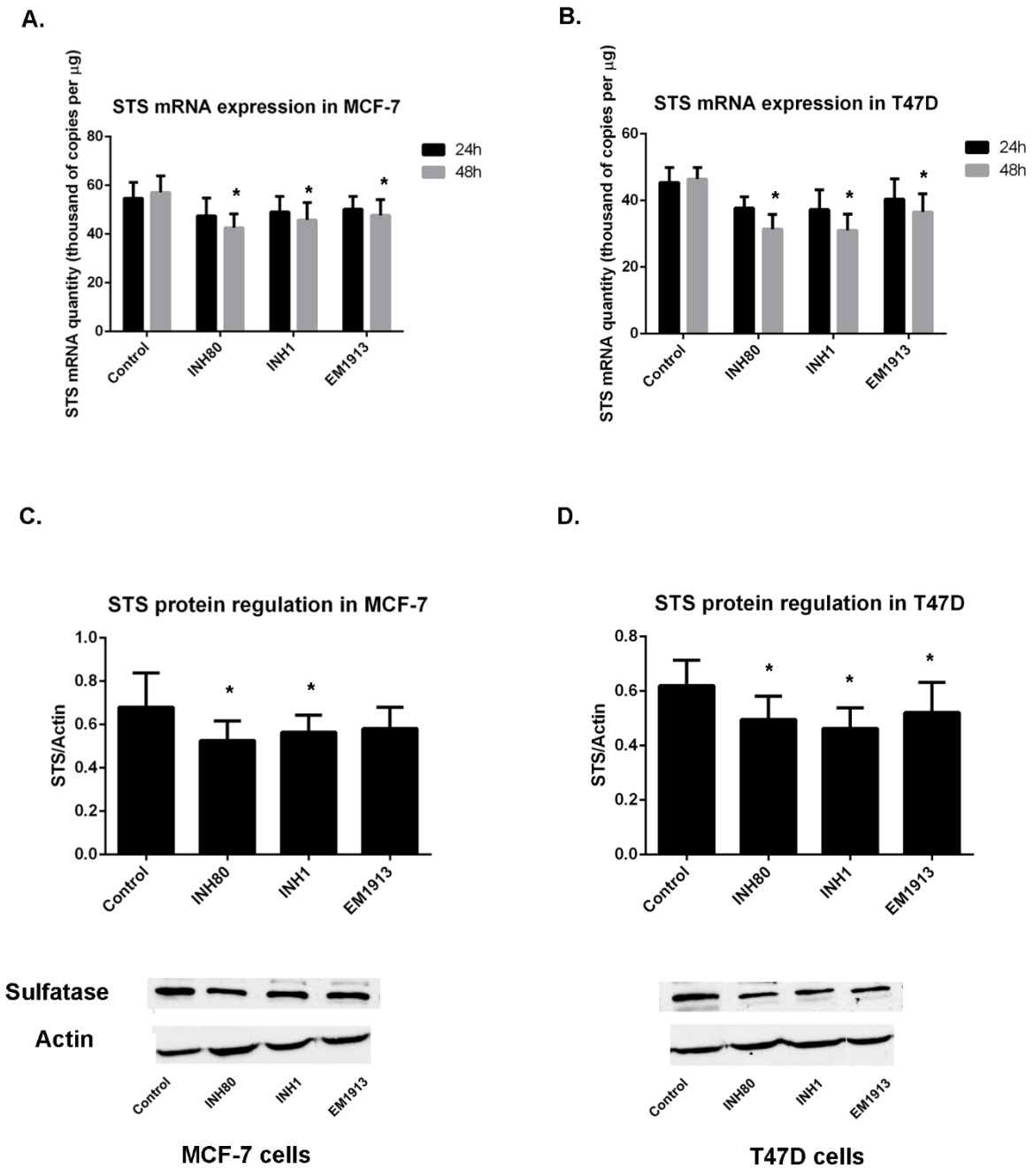
A and B: 17β-HSD7 mRNA expression after treatment with EM1913 for 48 h in MCF-7 cells (A) or T47D cells (B) was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by Student's test: ** $P < 0.01$ *vs*. control (Ctrl).

C and D: 17β-HSD7 mRNA expression after treatment with letrozole for 24 h or 48 h in MCF-7 cells (C) or T47D cells (C) was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by Student's test: * $P < 0.05$ *vs*. Ctrl; ** $P < 0.01$ *vs*. control (Ctrl).

E: E2 formation in MCF-7 cells and T47D cells treated with letrozole. Cell culture supernatants were collected for determination of E2 by ELISA kit. Each bar represents the mean of experiments carried out as five replications (mean ± SD). Statistical significance by Student's test: * $P < 0.05$ *vs*. Ctrl.

F: DHT formation in MCF-7 cells treated with letrozole and INH80. Cell culture supernatants were collected for determination of DHT by ELISA kit. Each bar represents the mean of experiments carried out as five replications (mean ± SD). Statistical significance by ANOVA: ** $P < 0.01$ *vs*. control (Ctrl).

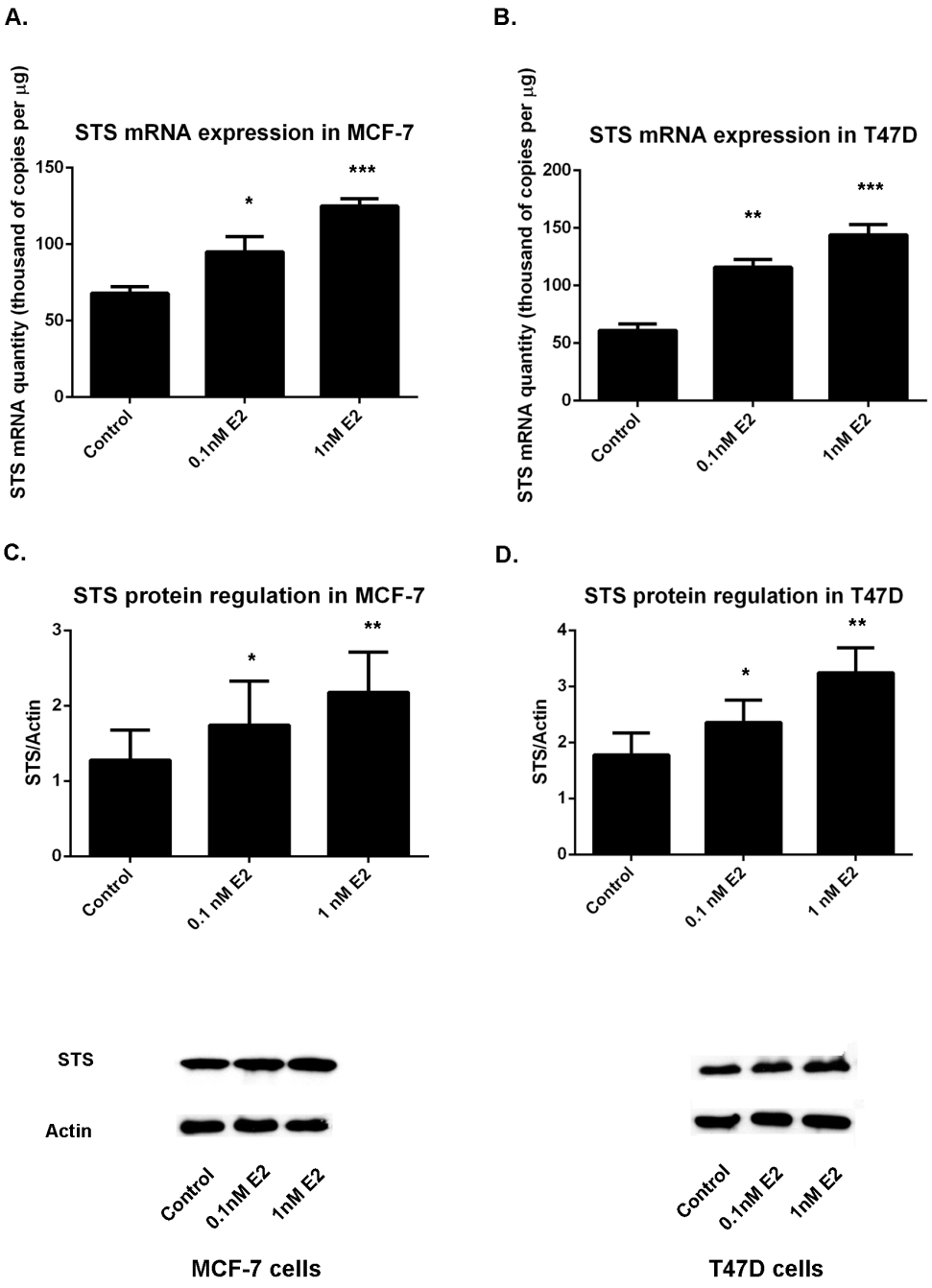
Figure 4.2 Regulation of STS mRNA and protein level by inhibition of 17β-HSD7, 17β-HSD1, aromatase and STS



A and B: STS mRNA expression after treatment with INH80, INH1 or EM1913 for 24 h and 48 h in MCF-7 cells (A) or T47D cells (B). mRNA level was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by ANOVA: * $P < 0.05$ *vs*. control (Ctrl).

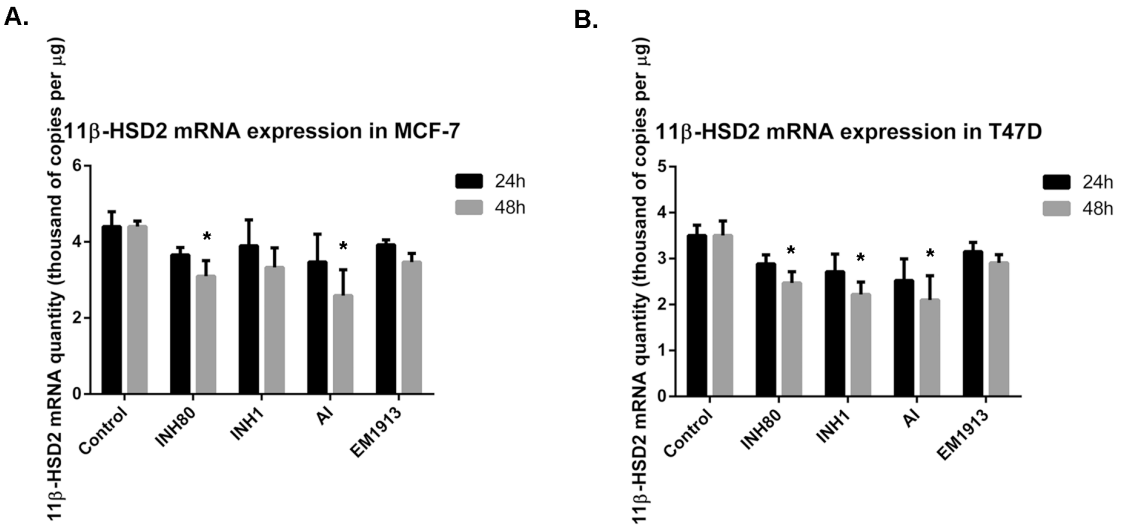
C and D: STS protein expression after treatment with different inhibitors for 48 h were determined by western blot in MCF-7 cells and T47D cells. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by Student's test: * $P < 0.05$ *vs*. control (Ctrl).

Figure 4.3 Regulation of STS mRNA and protein by E2



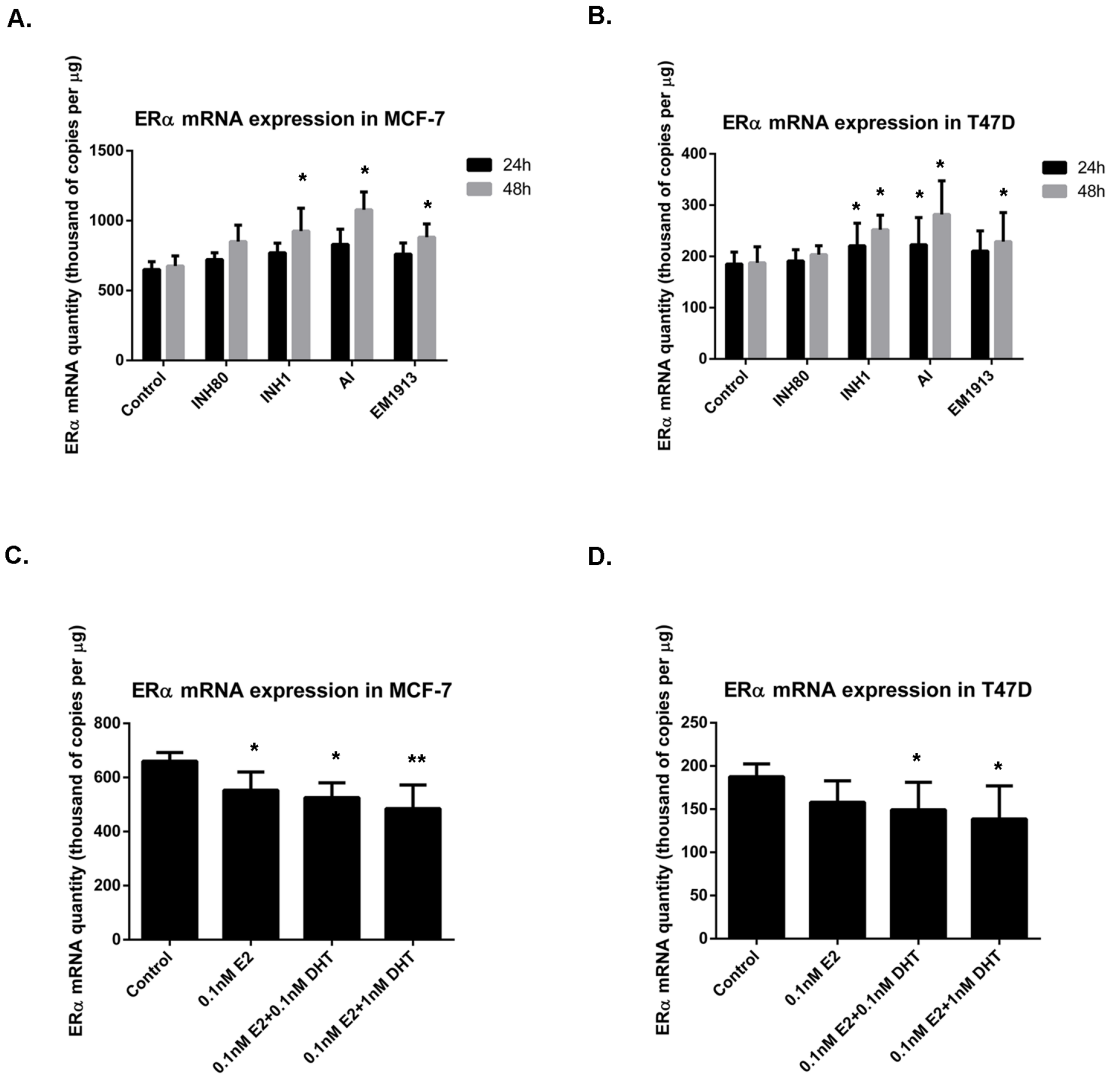
STS mRNA expression and protein expression after treatment with different concentrations of E2 (0.1 nM and 1 nM) for 48 h. mRNA level was determined by qRT-PCR (Figure A and B) and western blot (Figure C and D) in MCF-7 cells and T47D cells. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by Student's test: * $P < 0.05$ *vs*. control (Ctrl); ** $P < 0.01$ *vs*. control (Ctrl); *** $P < 0.001$ *vs*. control (Ctrl).

Figure 4.4 Regulation of 11β-HSD2 mRNA by inhibition of 17β-HSD7, 17β-HSD1, aromatase and STS



A and B: 11β-HSD2 mRNA expression after treatment of INH80, INH1, letrozole or EM1913 for 24 h and 48 h in MCF-7 cells (A) or T47D cells (B). mRNA level was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by ANOVA: * $P < 0.05$ *vs*. control (Ctrl).

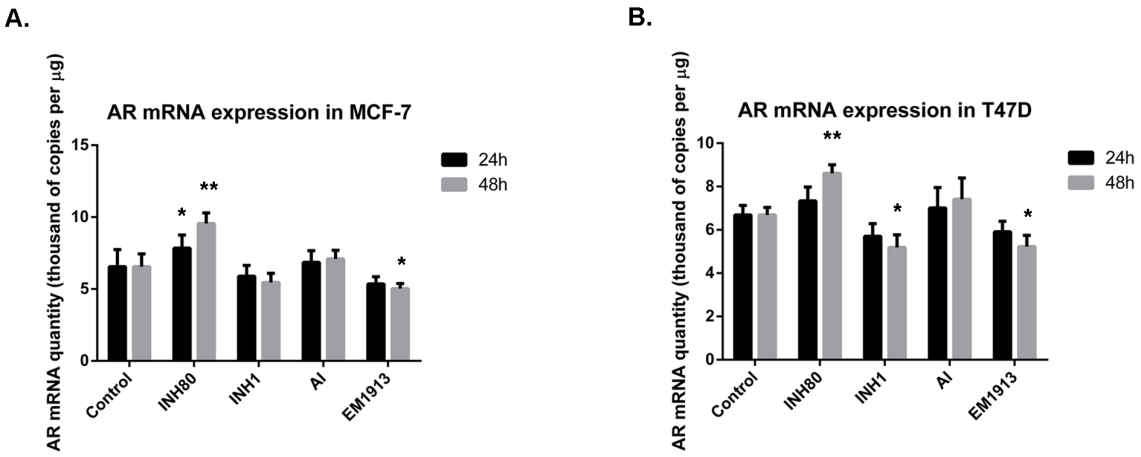
Figure 4.5 ERα regulation mRNA by inhibition of 17β-HSD7, 17β-HSD1, aromatase, STS, E2 and DHT



A and B: ERα mRNA expression after treatment of INH80, INH1, letrozole or EM1913 for 24 h and 48 h in MCF-7 cells (A) or T47D cells (B). mRNA level was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by ANOVA: * $P < 0.05$ *vs*. control (Ctrl).

C and D: ERα mRNA expression after treatment of 0.1nM E2 without or with supplement of different concentration of DHT for 48 h in MCF-7 cells (C) or T47D cells (D). mRNA level was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by ANOVA: * $P < 0.05$ *vs* control (Ctrl); ** $P < 0.01$ *vs*. control (Ctrl).

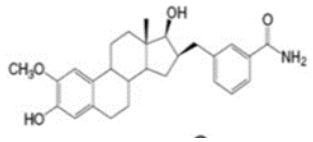
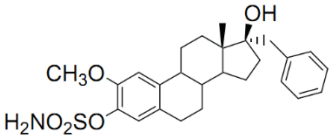
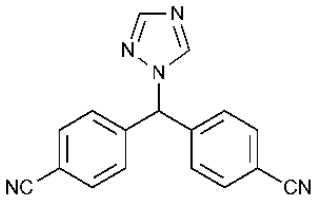
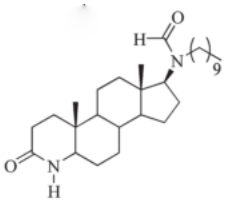
Figure 4.6 Regulation of AR mRNA by inhibition of 17β-HSD7, 17β-HSD1, aromatase and STS



A and B: AR mRNA expression after treatment with INH80, INH1, letrozole or EM1913 for 24 h and 48 h in MCF-7 cells (A) or T47D cells (B). mRNA level was determined by qRT-PCR. Data are reported as the mean ± SD (n = 3) of the individual experiments. Statistical significance by ANOVA: * $P < 0.05$ *vs*. control (Ctrl); ** $P < 0.01$ *vs*. control (Ctrl).

Tables

Table 4.1: Inhibitor characteristics

Compound	Targeting enzyme	Chemical structure	IC_{50}*
E2B-Methoxy (INH1)	17β-HSD1		275nM (30)
EM1913	STS		0.024 nM (27)
Letrozole	Aromatase		0.7 nM (31)
INH80	17β-HSD7		195 nM (29)

(*) IC_{50}: concentration of the compound inhibiting 50% of substrates transformation in HEK293 over-expressing STS

Table 4.2 Primers for steroidogenic enzymes

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
STS	AGC CCT AAT CCT GAC CCT TTT CTT GG	CCG CCT CCA CCG TTA GCC TCT
HSD11B2	GTC TCT TGA CTG GCT CAA GAA TTA GG	GTG GCA ATT GGG AAG TAC AGT ACA T
ESR1	TGC AAA ATC TAA CCC CTA AGG AAG TG	CTC CCA GTA CCC ACA GTC CAT CTC
AR	AAG ACG CTT CTA CCA GCT CAC CAA	TCC CAG AAA GGA TCT TGG GCA CTT
HSD17B7	TCC ACC AAA AGC CTG AAT CTC TC	GGG CTC ACT ATG TTT CTC AGG C
18S	AGC GAA AGC ATT	TCC GTC AAT TCC TTT AAG TTT CAG CT

Abbreviations: STS, gene name of steroid sulfatase; HSD11B2, gene name of 11beta-hydroxysteroid dehydrogenases type 2; ESR1, gene name for ERα; AR, gene name of androgen receptor; HSD17B7, gene name of 17beta-hydroxysteroid dehydrogenases type7.

Table 4.3 Inhibition effects of vital reductive 17β-HSDs enzymes and aromatase in MCF-7

Enzyme inhibition	Steroid regulation	
	E2(%)	DHT (%)
Control	100	100
Inhibition of sulfatase	73.4 [a]	97.5 [a]
Inhibition by 17β-HSD1	75.3 [a]	115.7 [a]
Inhibition by 17β-HSD7	75.6 [a]	316 [a]
Inhibition by aromatase	60.1	116.7

Reference [a]: Xiaoye Sang et al, Steroid sulfatase inhibition success and limitation in breast cancer clinical assays: an underlying mechanism; J. Steroid Biochem, 2018 (received)

Table 4.4: Steroid enzymes with promoters containing high-affinity EREs

Symbol	Gene ID	ERE sequence	ERE Position	Dist. Gene-ERE
STS	412	TGGTCACAGTGGCCT	7214353	-5103
HSD11B2	3291	GGCTCAGGCTGACCA	64722727	-8406
HSD17B7	51478	AGGTCAACTTGACAC	162792563	1861
HSD17B7	51478	AGGTCATTGTGAGCA	162785603	-5099
AR	367	GGGTAGAAATGACCT	67543433	-599

Abbreviations: STS, gene name of steroid sulfatase; HSD11B2, gene name of 11beta-hydroxysteroid dehydrogenases type 2; AR, gene name of androgen receptor; HSD17B7, gene name of 17beta-hydroxysteroid dehydrogenases type7.

Table 4.5: Steroid enzymes with promoters containing high-affinity AREs

Symbol	Gene ID	ARE sequence	ARE Position	Dist. Gene-ARE
STS	412	CGTACAATCTGTTCT	7324354	-177102
STS	412	AGCACAAGATGTTCA	7391767	-244515
HSD17B7	51478	AGAACATGCGGTGCT	162751907	38795
HSD17B7	51478	AGAGCAGGCTGTCCT	162754513	38189
HSD17B7	51478	AGAGCACATTGTTTT	162802378	-11676
HSD17B7	51478	AGAGCACATTGTTTT	162802380	-11678
AR	367	AGAACCCTCTGTTCT	67539003	5029
ESR1	2099	AGAACAGCCTGGTCC	151984068	-329920
ESR1	2099	AAAAGACAATGTTCT	151814205	-160057

Abbreviations: STS, gene name of steroid sulfatase; ESR1, gene name for ERα; AR, gene name of androgen receptor; HSD17B7, gene name of 17beta-hydroxysteroid dehydrogenases type7.

CHARPTER V

DISSCUSSION AND CONCLUSION

5. Discussion:

5.1 General discussion

The results presented in this thesis have been widely discussed above (Chapters 2, 3, and 4). Here, we will highlight the main points of the previous discussion in order to explain the relationship between these chapters and the roles of the enzymes, including 17β-HSD1, 17β-HSD7, STS, aromatase and 11β-HSD2. In kinetic study, we demonstrated the 17β-HSD1's characteristics at the molecular level and in cells and showed DHT effect on E2 synthesis. As we know, 17β-HSD7 is significantly regulated by E2 (175)(138). We also demonstrated that 17β-HSD7 was regulated by the inhibition of other enzymes, including 17β-HSD1、aromatase、STS via E2 concentration modification. 17β-HSD7 and STS can synthesize E2 while they are regulated by E2, thus forming a functional group of steroid enzymes that are mutually positively correlated. STS mRNA and protein expression were down-regulated by E2 reduction, while STS inhibition can block E2 formation but induced DHT formation. Combined inhibition of STS and 17β-HSD7, can induce synergistically cell proliferation.

5.2 17β-HSD1 kinetics

Our studies demonstrate substrate inhibition of 17β-HSD1, and DHT inhibition on the E1 to E2 conversion in living cells. Fig.2.1 showed significant substrate inhibition for concentrations of E1 above 0.4 μM in cells, similar to that of Gangloff et al. at the molecular level (89). In the present study, it was demonstrated that substrate inhibition phenomena with 17β-HSD1 was also demonstrated in cells (Figure 2.1.c) or even in the presence of DHT using purified enzyme (Figure 2.1.a). The reduction velocity of E1 to E2 increased to a maximal level then decreased

with further increase in E1. When E1 was present at a low concentration (< 0.1 μM), DHT played a crucial role as an inhibitor (Figure 2.1.b) because DHT compete with E1 to bind on 17β-HSD1 (66,177,178). When E1 concentration was higher than 0.4 μM, E1 still showed significant substrate inhibition with DHT as an inhibitor. DHT did not affect the E1 substrate concentration at which the enzyme activity started to decrease, but some increase in velocity was observed, suggesting a corresponding decrease in substrate inhibition. As E1 increased, DHT played an additional role that "stimulated" the enzyme activity when the E1 concentration was over 0.2 μM. Furthermore, this suggests that DHT may affect the substrate inhibition of E1, probably via the destabilization of the "dead-end complex" (179) formed between the enzyme, E1 and the cofactor (89).

5.3 17β-HSD7 expression modulated by other enzymes

Previous studies demonstrated down-regulation of 17β-HSD7 expression by a low E2 concentration (138)(175). Aromatase, 17β-HSD1, 17β-HSD7 and STS are the principal enzymes synthesizing E2 in the sulfatase pathway. Inhibitions of aromatase, 17β-HSD1 or STS lead to a decrease in E2 concentration, and the inhibitions affect 17β-HSD7 expression. Our report is the first study to directly demonstrate such regulation.

The mRNA modulation demonstrated that 17β-HSD7 gene transcription decreased in response to INH1 or 17β-HSD1 knockdown in T47D cells and MCF-7 cells via E2 modification (Fig 2.4.b, c, and d), while 17β-HSD7 gene transcription decreased in response to letrozole through the same way. However, the expression of 17β-HSD1 in T47D is much higher than in MCF-7 cells (172), therefore, INH1 decreased more the E2 concentration (Fig.2.4.b), and then the modulation of 17β-HSD7 mRNA transcription is stronger in T47D cells. In BC cells, STS expression is

stimulated by E2 in a positive-feedback manner which finally promotes E2 biosynthesis. Therefore, STS mRNA and protein expression were down-regulated by E2 reduction. 17β-HSD1, aromatase and STS, the main enzymes synthesizing E2, can regulate 17β-HSD7 transcription through modifying E2 concentration.

5.4 STS inhibition

The expression of cyclin D1 is an estrogen-responsive gene which is suggested as an oncogene with an important pathogenic role in ER+ BC (129). Furthermore, Cyclin D1 has been recently identified as a novel DHT response element by the AR in ER+ BC cells (180). The blockage E2 can inhibit Cyclin D1, resulting in cell cycle arrest in G0/G1 phase.

DHT has been reported to provide an anti-proliferation effect via the androgen receptor (AR) by activating$^{p21waf1/cip1}$ and inhibiting cyclinD1(60,180). Therefore, a conception for the BC treatment has emerged through the joint targeting of reduction of E2 and restoration of DHT (63).

However, androgen reduction effect can also be observed from STS inhibitors, which had an inhibition of estrogen synthesis. The fact that the anti-proliferative effect of STS inhibition compared to other reductive enzyme declines to below 20% can be related to the reduction of DHT, which can lead to the down-regulation of AR.

5.5 Mutual regulations between steroids and their converting enzymes

17β-HSD7, STS can synthesize E2 while they are regulated by E2 simultaneously, thus forming a functional group of steroid enzymes that are mutually positively correlated. The inhibition of the dual functional 17β-HSD7 reducing E2 but

restoring DHT has been studied and demonstrated in our group (138)(181). The importance of dual-targeting has also been recognized as a potential therapeutic approach for hormone-dependent BC. From studies in vitro, our results indicated that with DHT restoring, the anti-proliferation effects have been enhanced significantly in both MCF7 and T47D cells. The synergistic effect of inhibitors could block the activities of STS and 17β-HSD7, thus reducing E2 formation but inducing DHT restoration, to synergistically induce G0/G1 cell cycle arrest. Therefore, the joint inhibition of STS and 17β-HSD7 can block E2 and induce a decrease in cyclin D1, and restoring DHT, which can result in a joint effect on cell proliferation reduction.

6. Conclusion:

The present study may facilitate further research in enzyme mechanisms in living cells, similar to the substrate and ligand inhibition we have demonstrated in living BC cells. Enzyme self- and mutual regulations may also exist in other proteins and cells. If we can inhibit or knockdown 17β-HSD1, STS or aromatase, more than one enzymes can be down regulated (such as 17β-HSD7, STS) via E2 decline, and then E2 concentration can decrease become obviously by double effects (58). Inhibition of one enzyme can suppress the expression of another, which can potentially amplify future treatment.

The single blockage of STS pathway could decrease E2 and DHT concentrations, inducing a modest inhibition for ER+ BC cell proliferation. And the joint inhibition of STS and 17β-HSD7 could block E2 formation and DHT inactivation. The sex hormone modulations synergistically suppressed BC growth. Therefore, we provide the demonstration that joint inhibition of STS and 17β-HSD7 would be a more beneficial target through the dual regulation of estrogen and androgen. Future in vivo studies and clinical trials will be required to determine whether such combinations offer advantages over the use of single-agent therapy. Our research revealed the detailed mechanism in BC and facilitated the finding of targets for BC, eventually contributing to BC treatment.

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