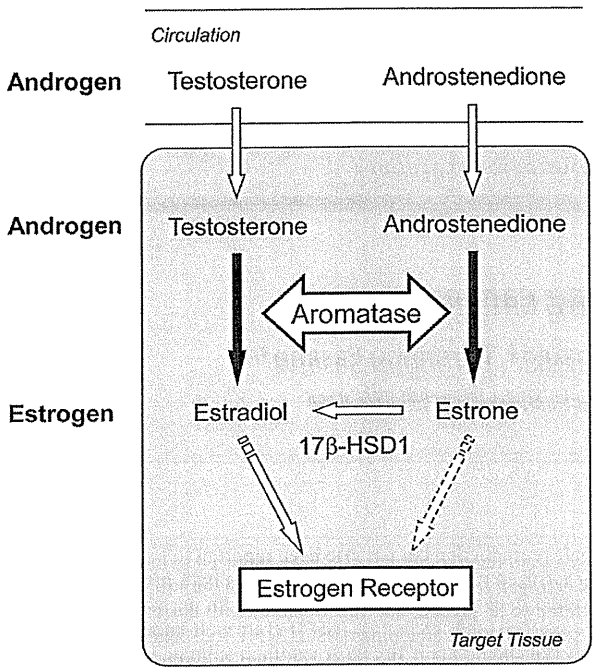

Fig. 1. Summary of intracrine of estrogens. Estrogen (estrone and estradiol) synthesized by aromatase binds to estrogen receptor (ER). The binding affinity of estrone is weaker than estradiol (Kuiper et al., 1997). Estrone is converted to estradiol by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1).

Estrogens immensely contribute to growth or invasion of breast carcinoma cells through binding to estrogen receptor (ER). Estrogens also play an important roles in several types of cancers had been considered as estrogen-independent such as tumors in esophagus (Hogan et al., 2009), stomach (Hogan et al., 2009), colon (Hogan et al., 2009; Sato et al., 2009), thyroid (Kawabata et al., 2003), kidney or renal cell carcinoma (Langner et al., 2004), oral cavity (Cheng et al., 2006), and bone or osteosarcoma (Dohi et al., 2008). Results of previous studies also demonstrated the ER expression in NSCLC (Niikawa et al., 2008). In addition, hormone replacement therapy (HRT) has also been reported to significantly decrease survival in women with lung cancer (Ganti et al., 2006). In premenopausal women, the ovary is a principle source of circulating estrogens but in postmenopausal women, the levels of circulating estrogens become markedly low compared to those in premenopausal women. However, intratumoral local synthesis of estrogens occurs in breast carcinoma tissues in postmenopausal patients (Sasano and Harada, 1998; Sasano et al., 2009). This "*intratumoral local synthesis*" is different from the classical concept of endocrinology such as "*autocrine*" and "*paracrine*" manners. *In situ* formation of biologically active estrogens at the sites of their actions from biologically inactive precursors in the circulation has been termed "*intracrine*" (Sasano and Harada, 1998; Sasano et al., 2009) as was elegantly demonstrated in *in situ* production of estrogens by Labrie (1991) and Labrie et al. (2003). Aromatase is a key enzyme in the estrogen synthesis involved in aromatization of C19 steroids such as androstenedione and testosterone into estrone and estradiol, respectively (Sasano and Harada, 1998; Sasano et al., 2009) (Fig. 1). In this review, we focused on the possible roles of estrogen and its synthesis pathway via aromatase in NSCLC.

2. Estrogens and NSCLC

Results of several clinical or epidemiological studies demonstrated an association between the physiological alterations of hormone status such as menstruation and pregnancy and relative risks of lung cancer. Koushik et al. (2009) reported that both menstruation and pregnancy were not necessarily associated with increased development of lung carcinoma in a population based case-control study carried out in Montreal including 422 women with lung cancer and 577 control populations. However, women who had non-natural menopause were demonstrated to have an increased risk of lung cancer development compared to women who had natural menopause (Koushik et al., 2009). Liu et al. (2005) reported the significant association between reproductive factors including hormone use and the risk of lung cancer development in a population based prospective study. In their study, self-administered questionnaires were distributed to 44,677 lifelong never-smoking women in 1990–1994 to assess menstrual and reproductive factors and hormone use. 8–12 years of subsequent follow-up studies revealed that 153 lung cancer cases were diagnosed. Among these women who developed lung cancer, those with either early age at menarche or late age at menopause had a significant increment in the risk of lung cancer compared to women with both late age, over 16 years old at menarche and early age, under 50 years old at menopause. Age at menopause, age at menarche, number of children, age at first live birth, breast feeding and use of hormones were, however, not significantly associated with a risk of lung cancer (Liu et al., 2005). In addition, women with induced menopause with experience of HRT had a significantly increased risk compared to naturally those with occurred menopause without female hormone usage (Liu et al., 2005). Slatore et al. (2010) evaluated a prospective cohort of 36,588 peri- and postmenopausal women recruited in Vitamins and Lifestyle (VITAL) study. Treatment of estrogen with progestin increased the risk of incident lung cancer in duration dependent manner, with an approximately 50% increased risk for use of ten years or longer (Slatore et al., 2010). Otherwise, in postmenopausal women recruited in Women's Health Initiative (WHI) trial, treatment of estrogen with progestin increased the number of death but not incidence of lung cancer (Chlebowski et al., 2009).

It is true that controversies existed in the literature regarding the relationship between lung cancer risk and previous use of exogenous hormones such as oral contraceptives and HRT (Payne, 2005). However, it is generally recognized that the disruption of physiological hormone status with environmental factors such as smoking may play an important role in the risks of lung cancer (Payne, 2005).

Recently we reported the intratumoral estrogen concentrations evaluated by liquid chromatography/electrospray tandem mass spectrometry in both carcinoma tissues and corresponding nonneoplastic tissues in 59 NSCLC cases (Niikawa et al., 2008). Intratumoral estradiol concentrations were significantly higher than the corresponding nonneoplastic tissues in this study (Fig. 2). The intratumoral concentration of estradiol was significantly positively correlated with tumor size and the Ki-67 labeling index of carcinoma cells in both ERα- or ERβ-positive cases (Niikawa et al., 2008). Ganti et al. (2006) reported the relationship between HRT and development of lung cancer in 498 women diagnosed with lung cancer. Overall survival was significantly higher in patients with no HRT compared with the patients who received HRT (86/498 cases, 17%) (Ganti et al., 2006). This appeared to be more pronounced in women with a smoking history. Therefore, these finding all suggest that estrogens are involved as the progression factors in NSCLC.

3. ERα and ERβ in NSCLC

The estradiol signals are in generally conveyed by the transcription factors, ERα and ERβ, which are encoded by distinct genes and are expressed in different tissues as well as in the same tissue at various levels (Gustafsson, 1999). ERα and ERβ are both expressed

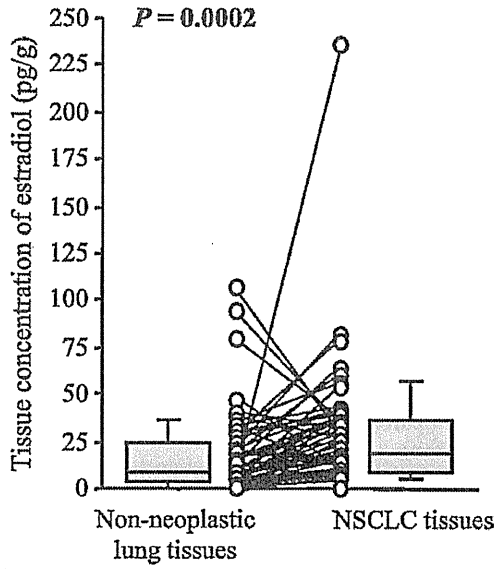

Fig. 2. Intratumoral concentration of estradiol in 59 NSCLC. Tissue concentration of estradiol in NSCLC and corresponding nonneoplastic lung tissues. Each value was shown in an open circle, and the paired values from the same patient were connected in a line. The grouped data are represented as box-and-whisker plots. The statistical analysis was done by a Mann–Whitney's *U* test. Reproduced from Niikawa et al. (2008) with permission of *American Association for Cancer Research*, Philadelphia, PA.

in various types of tissues including ovary, breast, CNS, bone, and kidney (Gustafsson, 1999). ERβ has been reported to be expressed in both normal lung and lung tumors and to be associated with biological function (Taylor and Al-Azzawi, 2000; Omoto et al., 2001). However, biological significance of estrogens including ERβ signaling pathway remains largely unclear in human normal lung tissues. Results of previous immunohistochemical studies of ERα and ERβ in human NSCLC are summarized in Table 1. ERα in NSCLC has been evaluated mostly by immunohistochemistry in previously reports (Omoto et al., 2001; Dabbs et al., 2002; Radzikowska et al., 2002; Schwartz et al., 2005; Kawai et al., 2005; Skov et al., 2008; Niikawa et al., 2008; Nose et al., 2009). Results of these reported studies demonstrated that relatively high rate (over 50%) of ERα positive cases were detected in NSCLC (Table 1). However, it is also true that several studies reported that there were no or low rate (under 10%) of ERα positive cases in NSCLC (Table 1). In the immunohistochemistry of lung carcinoma cases, mouse monoclonal ERα antibody, 6F11 is generally considered more sensitive compared to other ERα clone, ER1D5 (Sica et al., 2008). Very recently, SP1 clone, which is a rabbit monoclonal ERα antibody, was developed and has a significantly higher detection rate for the expression of ERα in lung cancer compared with either 1D5 or 6F11 clones (Gomez-Fernandez et al., 2010). Therefore, these controversies regarding ERα expression in NSCLC tissues described above may be due to the different anti-ERα antibodies employed (Table 1). In breast carcinoma tissues, immunoreactivity of ERα is detected in the nucleus but not in cytoplasm or membrane of carcinoma cells. In lung carcinoma tissues, several studies evaluated immunoreactivity of ERα in cytoplasm as well as in nuclei of carcinoma cells (Skov et al., 2008; Nose et al., 2009). ERα located in cellular cytoplasm is considered

Table 1
Summary of previous immunohistochemical studies of ERα and ERβ in lung carcinoma tissues.

References	Histo.	ERα			ERβ		
		Posi./Total	%	Antibody	Posi./Total	%	Antibody
Omoto et al. (2001)	Ad	0/20	0	ER1D5 (Dako)	17/20	85	[c]
	Sq	0/10	0		3/10	30	
Dabbs et al. (2002)	Ad	16/20	80	ER1D5 (Dako)	-		
	Bac	14/25	56		-		
Radzikowska et al. (2002)	Ad	0/18	0	ER1D5 (Dako)	-		
	Sq	1/14	7		-		
	Ad	1/18	6	6F11 (Novocastra)			
	Sq	0/14	0				
Schwartz et al. (2005)	[a]	0/94	0	ER1D5, 6F11	–		
	Ad	-			128/216	59	
	Bac	-			9/16	56	
	Adsq	-			4/4	100	
	Lc	-			5/9	56	MCA1974S (Serotec)
	Sc	-			7/14	50	
	Sq	-			11/13	85	
Kawai et al. (2005)	Ad	74/102	73		47/102	46	
	Sq	21/28	75	HC-20 (SantaCruz)	19/28	68	HC-150 (SantaCruz)
	Lc	1/2	50		1/2	50	
Skov et al. (2008)	Nsc	3/104	3	ER1D5 (Immunotec)	87/104	84	
	Nsc	57[b]/104	55				PPG5/10 (Dako)
Niikawa et al. (2008)	Ad	28/44	64	6F11 (Novocastra)	42/44	95	
	Sq	4/15	27		11/15	73	14C8 (GeneTex)
Nose et al. (2009)	Ad	0/447	0	HC-20 (SantaCruz)	329/447	74	
	Ad	377[b]/447	84				HC-150 (SantaCruz)

Histo., histologic types; Posi., number of positive cases; Total, number of total cases examined; Ad, adenocarcinoma; Sq, squamous cell carcinoma; Bac, bronchiolo-alveolar carcinoma; Adsq, adenosquamous cell carcinoma; Lc, large cell carcinoma; Sc, small cell carcinoma; Nsc, non-small cell carcinoma (histologic subtype was unknown). –, not examined.

[a] Histologic type was unknown.
[b] Cytoplasmic staining.
[c] Anti-ERβ chicken IgY polyclonal antibody.

to represent a variant form, which may lack part of NH2 terminus (Stabile et al., 2002). ER binding with estrogen undergoes conformational changes that allow receptor dimerization (ERα/ERα, ERα/ERβ, and ERβ/ERβ) and subsequent association of estrogen–ER complexes with specific estrogen response elements in DNA (genomic actions) (Evans, 1988). In addition, ER also regulates gene expression without direct binding to DNA (non-genomic actions) (Stabile et al., 2005; Curtis et al., 1996). Therefore, ER located in cytoplasm but not nucleus may be considered to exert non-genomic proliferation caused by mitogen-activated protein kinase (MAPK) pathway but it awaits further investigations for clarification.

Recently, we demonstrated both ERα (6F11 clone) and ERβ (14C8 clone) expression using immunohistochemistry in NSCLS (Niikawa et al., 2008). In this study, we evaluated relative immunointensity of nucleus ERs by *H* score in NSCLC and correlated the findings with those in the same number of breast carcinoma cells previously reported. The relative immunointensity of ERα was significantly lower in NSCLC than in that of breast carcinomas (Fig. 3A). Otherwise, ERβ immunointensity in NSCLC was significantly higher than that in breast carcinomas (Fig. 3B) (Niikawa et al., 2008). Subsequent *in vitro* studies demonstrated that estradiol significantly increased cell proliferation of NSCLC cell line, A549 transfected with ERα or ERβ, which was also significantly suppressed by selective ER modulators (SERMs) (tamoxifen and raloxifene) or ER inhibitor [fulvestrant (ICI182,780)] (Niikawa et al., 2008). These results all indicated that ERβ may play significantly roles such as cell proliferation or invasion in NSCLS compared to ERα in NSCLC. Many studies also examined ERβ status as well as ERα status using immunohistochemistry in human lung carcinoma tissues. ERβ has been reported to be expressed in approximately 30–100% of lung carcinoma cases (Table 1). However, it is important to note that several different criteria were employed in order to define "ERα and/or ERβ positive" NSCLC cases in these reported studies. Therefore, an establishment of immunohistochemical evaluation method of ERα and ERβ is required to establish anti-estrogen therapy as novel lung cancer therapeutics.

4. Aromatase in NSCLC

The aromatase P450 gene is located at chromosome 15 (15q21.2). This enzyme complex consists of two components including aromatase cytochrome P450 (aromatase) and NADPH–cytochrome reductase (reductase). Both aromatase and reductase are located in the endoplasmic reticulum (Fournet-Dulguerov et al., 1987). Human placental aromatase was reported to be associated with both the mitochondrial and microsomal compartments (Conley and Hinshelwood, 2001). Aromatase is expressed in numerous human tissues including ovary, placenta, bone, muscle, skin, and aorta (Miki et al., 2007a; Sasano et al., 2009). Intratumoral aromatase has been therefore regarded as the important target of breast cancer endocrine therapy. Aromatase inhibitors such as anastrozole, letrozole, and exemestane are clinically useful for reducing the progression of breast carcinomas especially in postmenopausal patients. Results of immunohistochemical analysis in human breast carcinoma tissues demonstrated that aromatase is predominantly detected in intratumoral stromal cells but not in parenchymal carcinoma cells (Suzuki et al., 2005; Miki et al., 2007b).

Results of previous immunohistochemical studies of aromatase in human lung carcinoma tissues are also summarized in Table 2. Immunohistochemistry for ERβ and aromatase in lung adenocarcinoma tissue were demonstrated in Fig. 4A and B. All of these reports demonstrated that immunoreactivity of aromatase was detected in carcinoma cells of approximately over 60–70% lung carcinoma cases (Pietras et al., 2005; Weinberg et al., 2005; Mah et al., 2007; Márquez-Garbán et al., 2009; Oyama et al., 2009). Results of laser capture microdissection/RT-PCR analysis in human lung adenocarcinoma tissues demonstrated that aromatase mRNA is predominantly detected in parenchymal carcinoma cells but not in intratumoral stromal cells (Miki et al., 2010). Mah et al. (2007) further examined the significance of aromatase expression in 422 lung carcinoma patients using immunohistochemical analysis. They reported that a relatively lower level of aromatase expression turned out to be a favorable predictor for survival in women especially older than 65 years with NSCLC in the early stage (stage I/II) (Mah et al., 2007). Abe et al. (2010) reported that the simultaneous expression of both estrogen receptor β and aromatase was significantly correlated with higher Ki-67 labeling index in carcinoma cells and younger age in female patients. In addition, Oyama et al. (2009) also reported that aromatase positive rate was significantly higher in tumor stage (T) 1 and T2 than T3 and T4 of lung carcinoma patients. In mice xenograft model of NSCLC, administration of aromatase inhibitor such as anastrozole

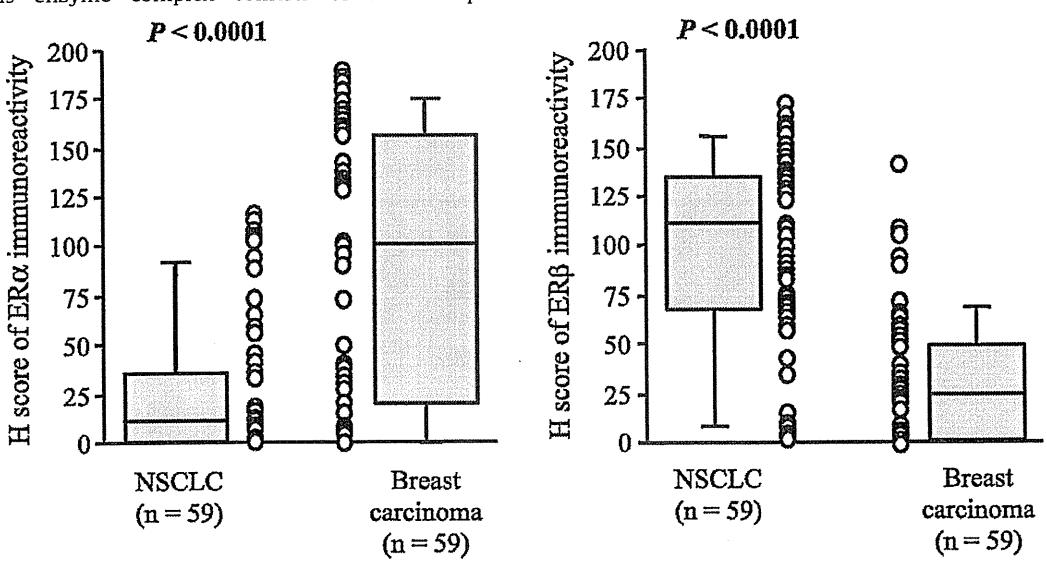

Fig. 3. Immunointensity of ERα (A) or ERβ (B) in NSCLC compared with breast carcinomas. Each value was represented as an open circle and the grouped data were shown as box-and-whisker plots. The statistical analysis was done by a Mann–Whitney's *U* test. Reproduced from Niikawa et al. (2008) with permission of *American Association for Cancer Research*, Philadelphia, PA.

Table 2
Summary of previous immunohistochemical studies of aromatase in lung carcinoma tissues.

	Histo.	No. of cases	Aromatase	
			Posi. (%) or score	Antibody
Pietras et al. (2005)	Nsc	Unknown	Unknown[a]	Unknown
Weinberg et al. (2005)	Ad	40	90%	C-16 (SantaCruz)
	Sq	8	88%	
	Adsq	1	100%	
	Bac	4	75%	
Mah et al. (2007)	Ad	951	1.49 ± 0.02[b]	C-16 (SantaCruz)
	Sq	409	1.56 ± 0.03[b]	
	Lc	125	1.49 ± 0.06[b]	
	Sc	24	0.99 ± 0.13[b]	
Márquez-Garbán et al. (2009)	[c]	10	Unknown[a]	C-16 (SantaCruz)
Oyama et al. (2009)	Ad	48	60%	Rabbit polyclonal (Oyama et al., 2009)
	Aq	30	70%	
Abe et al. (2010)	Ad	79	86%	#677 (Sasano et al., 2005)
	Sq	22	82%	
	Adsq	3	100%	
	Lc	1	0%	

Histo., histologic types; Posi., % of positive cases; Nsc, non-small cell carcinoma (histologic type was unknown.); Ad, adenocarcinoma; Sq, squamous cell carcinoma; Adsq, adenosquamous cell carcinoma; Bac, bronchiolo-alveolar carcinoma; Lc, large cell carcinoma; Sc, small cell carcinoma.; C-16, goat polyclonal antibody; #677, mouse monoclonal antibody.

[a] Only the detection in carcinoma cells.
[b] *H* score with modify.
[c] Histologic type was unknown.

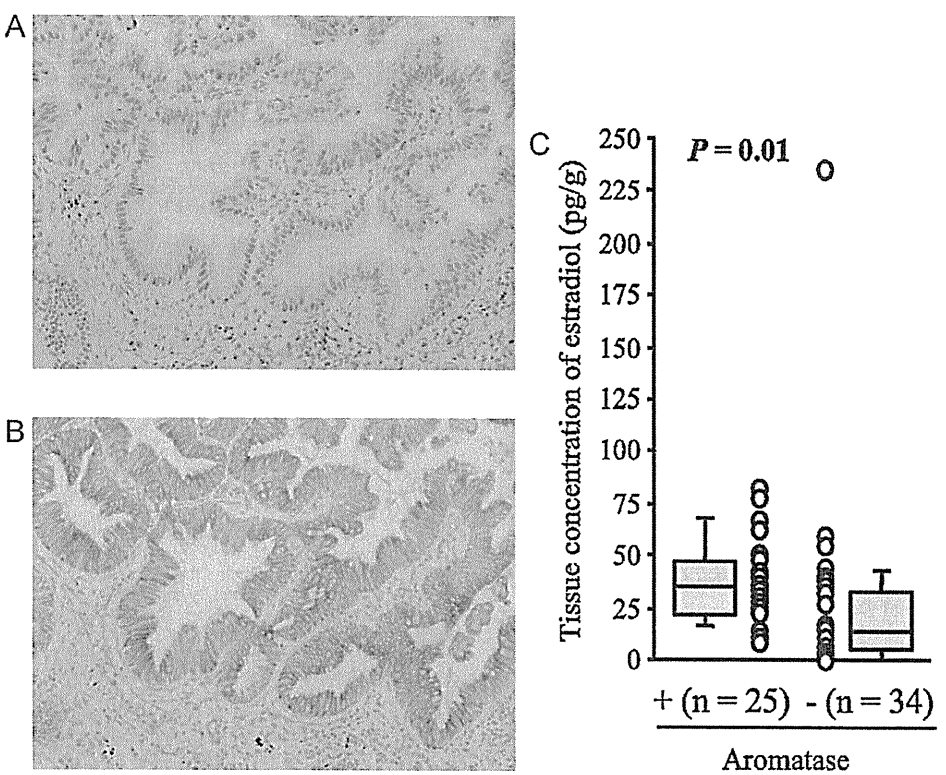

Fig. 4. Immunohistochemistry for ERβ (A) and aromatase (B) in lung adenocarcinoma. Monoclonal antibody for ERβ (14C8) was purchased from GeneTex Inc., San Antonio, TX (Niikawa et al., 2008; Abe et al., 2010). The aromatase monoclonal antibody #677 was raised against native recombinantly expressed human aromatase protein (Sasano et al., 2005; Miki et al., 2007b). (C) Association between intratumoral concentration of estradiol and aromatase in the NSCLC. Values of each case were represented as an open circle and the grouped data were shown as box-and-whisker plots. Expression of aromatase was evaluated by RT-PCR analyses. Positive case (+) of aromatase mRNA expression was detected as a specific single band by RT-PCR. The statistical analysis was done by a Mann–Whitney's *U* test. Reproduced from Niikawa et al. (2008) with permission of *American Association for Cancer Research*, Philadelphia, PA.

(Weinberg et al., 2005) and exemestane (Márquez-Garbán et al., 2009) elicited pronounced inhibition of tumor growth *in vivo*. Cell proliferation of aromatase expressing NSCLC cell lines induced by testosterone or androstenedione treatment was significantly suppressed by aromatase inhibitor such as anastrozole (Weinberg et al., 2005) and letrozole (Niikawa et al., 2008). Results of our previous report demonstrated that the intratumoral estradiol concentration in NSCLC was significantly associated with aromatase mRNA (Fig. 4C) but not with 17β-hydroxysteroid dehydrogenase type 1 or type 2 mRNA (Niikawa et al., 2008). In breast carcinoma patients, recent comparisons of aromatase inhibitors with selective ER modulator, tamoxifen demonstrate that aromatase inhibitors generally resulted in significantly increased response rates and greater durations of response (Howell and Dowsett, 2004). These findings also encourage the use of aromatase inhibitors in NSCLC patients in near future.

5. The cross talk between estrogen and EGF signals

The epidermal growth factor receptor (EGFR) family, EGFR, HER2, HER3, and HER4, regulates many developmental, metabolic and physiological processes. The activation of receptor tyrosine kinase leads to the autophosphorylation of the intracellular domain of EGFR, and the phosphotyrosine residues that are formed act as a binding site for various adapter molecules, which result in the activation of the cell growth signaling pathways including Ras/MAPK pathway (Gazdar, 2009). Treatment with the EGFR tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, results in dramatic antitumor activity in a subset of patients with NSCLC. Stabile et al. (2005) reported that EGFR protein expression was up-regulated in response to anti-estrogens *in vitro*, and that ERβ expression was decreased in response to EGF and was increased in response to gefitinib. These findings all suggest that increased EGFR signal might be caused by depletion of estrogen signals induced by anti-estrogen receptor or anti-aromatase therapeutics. In the analysis using lung cancer xenograft model, anti-tumor effects of the combination therapy of gefitinib and fulvestrant were demonstrated by pathological evidence of increased apoptosis/decreased cell growth compared with individual treatment (Stabile et al., 2005). Traynor et al. (2009) reported the results of pilot study of gefitinib and fulvestrant in the treatment of postmenopausal women diagnosed as NSCLC. No significant results were obtained in this small number of NSCLC patients (22 cases) but they reported that combination therapy with EGFR TKI and ER inhibitor was well tolerated. Phase II clinical trials of combination therapy erlotinib and Fulvestrant versus erlotinib alone in NSCLC patients (ClinicalTrials.gov Identifier; NCT00100854 and NCT00592007) are currently underway. In addition, a phase II randomized trial of Fulvestrant and anastrozole as consolidation therapy in postmenopausal women with advanced NSCLC (ClinicalTrials.gov Identifier; NCT00932152) is to be scheduled. A better understanding of NSCLC growth pathway including EGFR and/or ER signaling is important for the treatment of NSCLS especially postmenopausal women.

6. Conclusion

In breast cancer, estrogen signals including aromatase pathway has been well-examined as the important target of the anti-estrogen therapy. Several types of drugs such as SERMs (tamoxifen) and aromatase inhibitors (letrozole, anastrozole, and exemestane) are employed for blockade of intratumoral estrogen signal in breast carcinoma tissue. A better understanding of common pathway of estrogen signal and intracrine manner of estrogen between lung and breast cancers may lead to novel therapeutic approaches on lung cancer patients. Lung carcinoma-related factors, which are EGFR pathway and smoking history, may be implicated estrogen receptor signal or estrogen synthesis/metabolism in lung carcinoma cells. Although the further examinations are required for understanding of estrogen signal in lung cancer, human lung carcinoma considered being one of the estrogen-dependent carcinoma and aromatase plays a pivotal role in the intratumoral estrogen synthesis.

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Review

Steroid sulfatase inhibitors: Promising new tools for breast cancer therapy?

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ABSTRACT

Inhibition of aromatase is currently well-established as the major treatment option of hormone-dependent breast cancer in postmenopausal women. However, despite the effects of aromatase inhibitors in both early and metastatic breast cancer, endocrine resistance may cause relapses of the disease and progression of metastasis. Thus, driven by the success of manipulating the steroidogenic enzyme aromatase, several alternative enzymes involved in steroid synthesis and metabolism have recently been investigated as possible drug targets. One of the most promising targets is the steroid sulfatase (STS) which converts steroid sulfates like estrone sulfate (E1S) and dehydroepiandrosterone sulfate (DHEAS) to estrone (E1) and dehydroepiandrosterone (DHEA), respectively. Estrone and DHEA may thereafter be used for the synthesis of more potent estrogens and androgens that may eventually fuel hormone-sensitive breast cancer cells. The present review summarizes the biology behind steroid sulfatase and its inhibition, the currently available information derived from basic and early clinical trials in breast cancer patients, as well as ongoing research.

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Contents

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1. Introduction

Manipulation of estrogen synthesis and action has been used successfully for the treatment of hormone-dependent breast cancer for several decades [1]. While the antiestrogen tamoxifen was the gold standard of treatment during the 80s and early 90s, aromatase inhibitors (AIs) became 1st line therapy for metastatic, hormone-dependent breast cancer in postmenopausal women in the late 90s. Recently, aromatase inhibitors were also established as the 1st choice in estrogen receptor (ER) positive, postmenopausal early breast cancer [2]. Thus, estrogen suppression has turned out to be in general a more effective way to treat hormone-dependent breast cancer in selected patients compared to ER-blockade by selective estrogen receptor modulators (SERMs). However, in spite of all these advances, still many patients experience relapse of their breast cancer disease and all patients with metastatic, ER-positive breast cancer will have a progressive disease after a certain period of clinical benefit. Several studies have suggested conserved estrogen dependency of tumors following progression on first-line and even second-line endocrine therapies [1,3,4]. Thus, selective estrogen receptor downregulators (SERDs) like fulvestrant or steroidal aromatase inactivators like exemestane are currently used as (second-) third-line endocrine therapies in selected patients with MBC. However, other, non-cross-resistant therapies are urgently needed to give those patients an alternative treatment option prolonging the time-period without chemotherapy.

Inhibition of steroid sulfatase (STS) represents such a novel approach blocking the synthesis of a variety of steroids that have the potential to stimulate growth of human breast cancer (Fig. 1). Currently only one type of steroid sulfatase (also referred to as aryl sulfatase C) is known in humans, hydrolysing both aryl (estrone sulfate, E1S) as well as alkyl (DHEAS) steroid sulfates. Important STS crystallization and X-ray crystallographic studies carried out by Ghosh [5] have for the first time identified essential information about STS architecture and catalytic residues present at the active site. In particular, a catalytic cysteine residue, strictly conserved in all sulfatases, is posttranslationally modified into a formylglycine (FGS75). This is further hydrated to form hydroxyformylglycine. It is suggested that the mechanism of sulfate hydrolysis involves covalent attachment of the sulfate from the substrate to the hydroxyformylglycine. Similarly, irreversible inhibition of STS with compounds such as EMATE or Irosustat (i.e., arylsulfamates), involves mechanism-based irreversible inhibition of STS by suicide substrates such as EMATE [6].

As a consequence of steroid sulfatase inhibition, both estrogen and androgen synthesis will be reduced simultaneously. The relevance of steroid sulfatase in human breast cancer is underlined by findings of several studies suggesting steroid sulfatase mRNA in ER-positive breast cancer to be an independent prognostic indicator predicting relapse-free survival, with higher levels of expression being associated with a poor prognosis. Most interesting, Chanplakorn et al. recently showed increased steroid sulfatase and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) immunoreactivity following neoadjuvant therapy with the aromatase inactivator exemestane, suggesting a role for steroid sulfatase in the adaptation processes during therapy with AIs [7].

In contrast, aromatase mRNA levels have not been associated with breast cancer prognosis so far. As high levels of steroid sulfatase activity have been detected in most breast cancers and with convincing evidence for active uptake of sulfates into breast cancer cells via a specific organic anion transporter (organic anion transporter polypeptide B, OATP-B), this pathway may be a major contributor to the well-known elevated estrogen levels in ER-positive human breast cancer tissue [8].

While 60–80% of all postmenopausal breast cancers are classified as ER-positive, the androgen receptor (AR) is co-expressed in up to 80% of the patients. In addition, the AR is still found in many patients with an ER/PGR-negative disease. These findings indicate that human breast cancer cells might be stimulated by androgens via the AR in the absence of ER/PGR.

The STS pathway is also responsible for the production of another steroid with estrogenic properties, namely 5-androstenediol (Adiol), from DHEAS and subsequent reduction of DHEA by 17β-HSD1. Adiol, although an androgen, can bind to the ER and has been shown to stimulate the proliferation of a number of ER-positive breast cancer cells in an ER-dependent manner. Despite its lower affinity for the ER, the 100-fold higher concentration of this hormone has led to the speculation that it may have equally efficacious estrogenic properties to estradiol. This might be the case particularly under clinical situations when patients are treated with aromatase inhibitors, estradiol synthesis has been suppressed by >99% to undetectable levels, but at the same time the tumors have become sensitised to very low estrogen concentrations [9]. Adiol has been shown to stimulate tumor growth even in the presence of an AI and Billich et al. [10] demonstrated that inhibition of steroid sulfatase blocked DHEAS-stimulated growth of MCF-7 breast cancer cells; the same effect was not achieved by the use of an aromatase inhibitor thus highlighting that the generation of Adiol from DHEAS occurred totally independent from the aromatase-pathway. This is of clinical significance because in postmenopausal breast cancer patients treated with AIs, unrestricted production of Adiol can occur via the steroid sulfatase pathway and may promote tumor progression.

Motivated by the findings presented here, several inhibitors of steroid sulfatase have been developed. These drugs have been shown to be very potent inhibitors of steroid sulfatase activity in vivo and are currently being tested in early clinical trials for the treatment of human breast cancer. The theoretical background, basic endocrine findings as well as clinical experience with these compounds available so far will be summarized in the following chapters.

2. Estrogen and androgen synthesis in breast cancer tissue (general introduction)

The origin and manipulation of estrogen levels in human breast cancer tissue has been the subject of intensive research [11–13]. It is currently believed that both uptake from the circulation as well as local synthesis in the tumor contribute to the local estrogen concentrations in a particular breast tumor [8]. Beside steroid sulfatase, a network of different enzymes is involved in human estrogen synthesis and metabolism (Fig. 1). Most of hormone-responsive breast tumors express three major enzyme systems [i.e., aromatase/CYP19, STS and 17β-HSD] that are responsible for the local formation of E2. Aromatase is a cytochrome P450 (CYP450). It interacts with NADPH-cytochrome P450 reductase and converts androgens (mainly androstenedione and minor testosterone) into estrogens (mainly E1 and minor E2). After E1 is synthesized by aromatase, it can be converted to E1S (mainly in liver) by the catalysis of estrogen sulfotransferase [14]. Through circulation, E1S can be then stored in tissues, including breast tumors. Steroid sulfatase catalyzes the hydrolysis of E1S to E1, which is subsequently reduced to E2 by 17β-HSD1. 17β-HSDs are a group of enzymes that catalyze dehydrogenation of 17-hydroxysteroids in steroidogenesis. 17β-HSD1 is the best studied isozyme and remains an important enzyme for E2 production because it can use E1 as a substrate from both aromatase and sulfatase pathways, and it principally synthesizes E2 using reduced nicotinamide adenine dinucleotide (NADPH) as a cofactor [15].

In addition to estrogen uptake and synthesis, the expression of the ER has been suggested as a major factor influencing on estrogen disposition in human breast cancer [16].

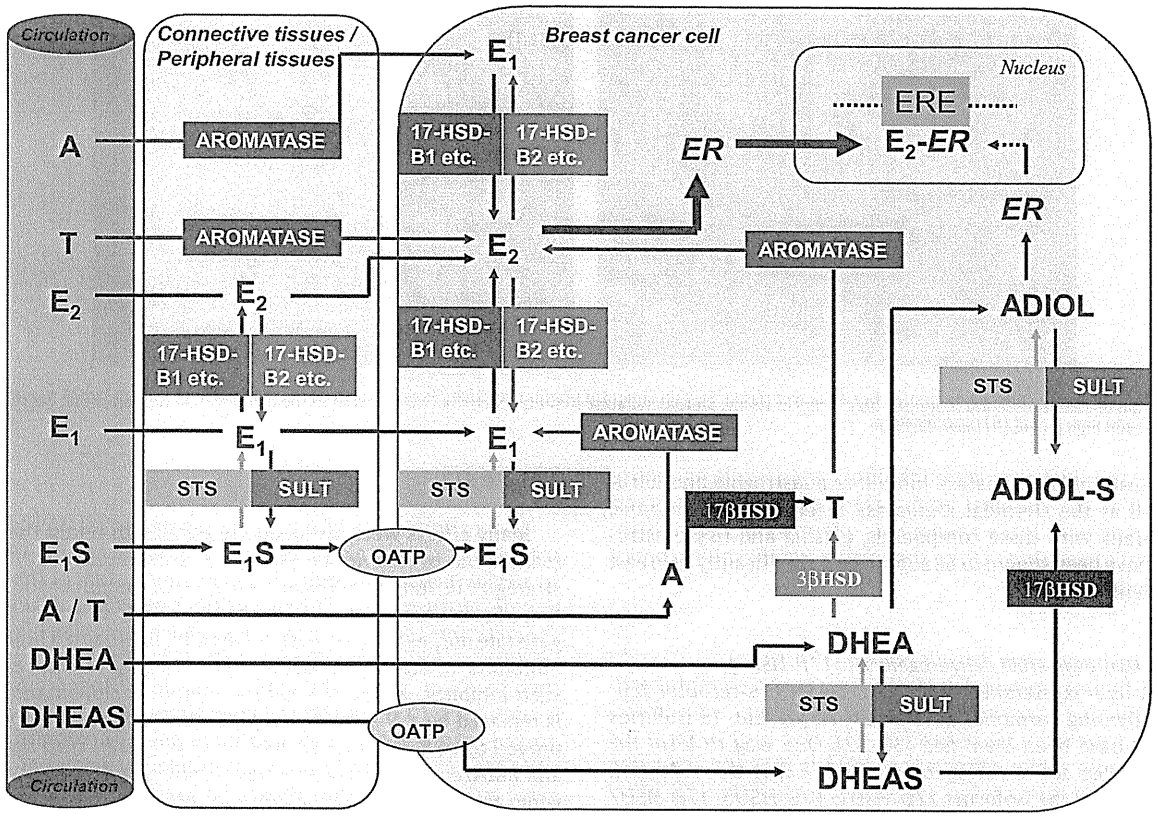

Fig. 1. Major pathways of estrogen and androgen synthesis in human breast cancer tissue. A, androstenedione; T, testosterone; E1, estrone; E2, estradiol; E1S, estrone sulfate; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; 17-β-HSD, 17β-hydroxysteroid dehydrogenase; STS, steroid sulfatase; SULT, steroid sulfotransferase; ER, estrogen receptor; ERE, estrogen receptor response element; ADIOL, androstenediol; ADIOL-S, androstenediol-sulfate.

2.1. Steroid sulfatase in breast cancer tissue (detection, expression and regulation)

Steroid sulfatase activity has been reported to be higher in breast cancer tissues than that in normal breast tissues as has been stated above. In addition, the enzymatic activity of steroid sulfatase is detected in the great majority of human breast tumors [17,18], although Evans et al. [18] reported no significant association between steroid sulfatase activity and clinical parameters such as time to recurrence or overall survival time in breast cancer patients. Therefore, the analysis of steroid sulfatase enzymatic activity could be the gold standard in determining the status of steroid sulfatase in individual patients with breast cancer. However, rather laborious procedures of this enzymatic assays as well as requirement of frozen tissue specimens have made it difficult to be applied in a wide scale fashion for routine clinical practice. mRNA expression of steroid sulfatase could be evaluated in breast carcinoma tissues and results were usually correlated with those of enzymatic activities [13]. Utsumi et al. [19] reported that patients with high mRNA levels for steroid sulfatase were associated with an increased risk of recurrence after surgery. However, the analysis of mRNA in clinical specimens is usually associated with similar problems described above. Therefore, it then becomes important to apply more practical methods of evaluating the steroid sulfatase status in individual breast cancer patients.

Immunohistochemical evaluation using archival or 10% formalin-fixed and paraffin embedded tissue specimens have been in general considered ideal in this point. Various attempts have been made in immunohistochemical analysis of steroid sulfatase in clinical materials of breast cancer patients. Saeki et al. [20] reported the presence of steroid sulfatase immunoreactivity in carcinoma cells in 22 out of 25 cases (88.0%). Suzuki et al. further evaluated immunolocalization of steroid sulfatase in 113 cases of human breast invasive ductal carcinoma using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) [21]. Steroid sulfatase immunoreactivity was detected in carcinoma cells in 84 out of 113 carcinoma cases (74.3%), respectively, which was also associated with mRNA levels determined by RT-PCR analysis. This immunohistochemical detection kit is currently available for detection of steroid sulfatase immunoreactivity using the same primary antibody above [21].

Steroid sulfatase immunoreactivity was detected in cytoplasm of carcinoma cells as shown in Fig. 2. In addition, the combined analysis of micro-dissection/RT-PCR analyses demonstrated that both steroid sulfatase protein and mRNA were detected only in carcinoma or parenchymal cells, which is consistent with results of immunohistochemistry. In addition, steroid sulfatase immunoreactivity in these carcinoma cells was positively associated with tumor size of the patients.

2.2. Other steroidogenic enzymes in breast cancer tissue

2.2.1. Aromatase (CYP19)

The enzyme aromatase is encoded by the human CYP19 gene (P450 arom), a member of the cytochrome P_{450} superfamily, localized on the long arm of chromosome 15 (15q21) [22]. Aromatization of C_{19} steroid precursors is the rate-limiting step in estrogen synthesis in humans and is regulated by the use of 10 tissue-specific promoters [23,24]. Aromatase inhibition is currently the dominating treatment option for postmenopausal, hormone dependent breast cancer suitable for endocrine manoevres [25]. Three compounds, all belonging to the "third generation" of drugs are in use:

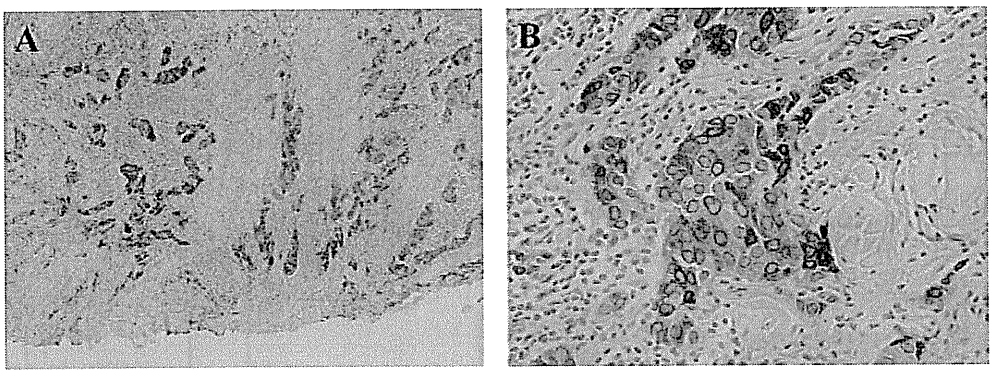

Fig. 2. Detection of steroid sulfatase in human breast cancer tissue. Steroid sulfatase immunoreactivity was detected in the cytoplasm of human breast cancer cells using low power (A) and high power (B) magnification.

the two nonsteroidal aromatase inhibitors anastrozole and letrozole as well as the steroidal aromatase inactivator exemestane. During therapy with these compounds, plasma and tissue estrogen levels have been shown to be suppressed significantly in breast cancer patients [26–28].

2.2.2. 17β-Hydroxysteroid dehydrogenases (17β-HSDs)

The 17β-hydroxysteroid dehydrogenases are pre-receptor activating/inactivating hormones in vivo [29]. At present, 15 isoforms of 17β-HSD have been identified [30–32]. One way to form the biologically most active estrogen estradiol (E2) is the reduction of estrone (E1) by the isoforms 17β-HSD1, 17β-HSD5, 17β-HSD7 and 17β-HSD12 (Fig. 1). While 17β-HSD1 has been suggested to play the major role in humans [33], recent data published by Haynes et al. showed a significant positive correlation of intratumor E2 levels with 17β-HSD7 only [16]. High mRNA levels of 17β-HSD5 have been shown to be related to a significantly higher risk of late relapse in ER-positive patients remaining recurrence-free later than 5 years after diagnosis [34]. Selective inhibitors of 17β-HSD isoforms have been synthesized with the goal to investigate their potential in breast cancer therapy [35,36]. STX 1040, a selective inhibitor of 17-HSD1, was recently reported to be efficacious in vivo in a breast cancer xenograft model [37]. In a separate study, Husen et al. reported the inhibitory activities of their inhibitors in a MCF-7 (17-HSD1) model in immunodeficient mice [38].

3. Sulfatase inhibitors

3.1. STX 64 (667 Coumate, BN83495, Irosustat)

The first specifically designed and synthesized steroid sulfatase inhibitor was estrone methylthiophosphonate (E1-MTP), an E1S-surrogate which possessed modest inhibitory properties. Extensive structure-activity relationship studies led to the identification of estrone 3-*O*-sulfamate (EMATE) as the first ever mechanism-based irreversible inhibitor of STS (Fig. 3, **1**). Unexpectedly, however, EMATE was found to have potent estrogenic properties, being 5 times more estrogenic than ethinylestradiol in rodents on oral application. This undesirable property stimulated the development of non-steroidal mimics which led to the discovery of STX 64, the only steroid sulfatase inhibitor that has entered into a phase I trial to date (Fig. 3, **2**). STX 64 is a non-steroidal agent with a tricyclic coumarin scaffold. It was shown to be devoid of estrogenic activity, as tested in an ovariectomised rat uterotrophic assay, and showed excellent efficacy in various in vivo tumor models [39].

3.2. STX 213

Many efforts were also made in parallel to retain the steroidal scaffold but overcome the estrogenic drawbacks of EMATE. These strategies included modification of its ring system or the introduction of substituents at various positions of its steroidal scaffold to generate non-estrogenic derivatives which remained highly potent inhibitors of steroid sulfatase. STX 213 (Fig. 3) represents one such inhibitor, where the natural steroid cyclopentanone D-ring is replaced by a *N*-substituted piperidine-2,6-dione ring. STX 213 proved to be 8-fold and 18-fold more potent *in vitro* than STX 64 and EMATE respectively and was completely devoid of any estrogenic activity. It was thus chosen for preclinical development as a second-generation steroid sulfatase inhibitor. The most significant distinction of the second generation STS inhibitor was its prolonged duration of steroid sulfatase inhibition. The time to recover 50% of rat liver steroid sulfatase activity ($t_{1/2}$) was around 3 days for STX 64 but 10 days for STX 213 when tested at a single oral dose of each inhibitor of 10 mg/kg [40,41].

3.3. Other steroid sulfatase inhibitors

In the past decade many other steroid sulfatase inhibitors have been identified including 6-[2-(adamantylidene)hydroxybenzoxazole]-*O*-sulfamate (AHBS, Fig. 3, **4**) [42] and KW-2581 (Fig. 3, **5**) [43]. Recently, a novel dual sulfatase–antiestrogen inhibitor, SR16157 (Fig. 3, **10**), has completed preclinical toxicity and PK evaluation in dogs and has excellent bioavailability and favourable safety profile [44]. However, to date, all highly active and irreversible steroid sulfatase inhibitors incorporate the phenol sulfamate ester pharmacophore required for potent steroid sulfatase inhibition, and first identified in EMATE.

4. Clinical experience with steroid sulfatase inhibitors in breast cancer patients

Clinical experience with sulfatase inhibitors is still limited. However, recently Stanway et al. published the results of a phase I, single-arm, open-label, study of the non-steroidal sulfatase inhibitor STX 64 (667 Coumate; BN83495, Irosustat) [45]. Briefly, 14 postmenopausal patients suffering from either metastatic or locally advanced breast cancer patients were enrolled in this study. The patients were heavily pretreated with antiestrogens, aromatase inhibitors, other endocrine options and with several lines of chemotherapy (median: 2). STX 64 was given in two different doses. A 5 mg daily dose was given to nine patients while five patients received a 20 mg daily dose. Steroid sulfatase activity in human tumor samples was inhibited at the 5 and 20 mg doses by

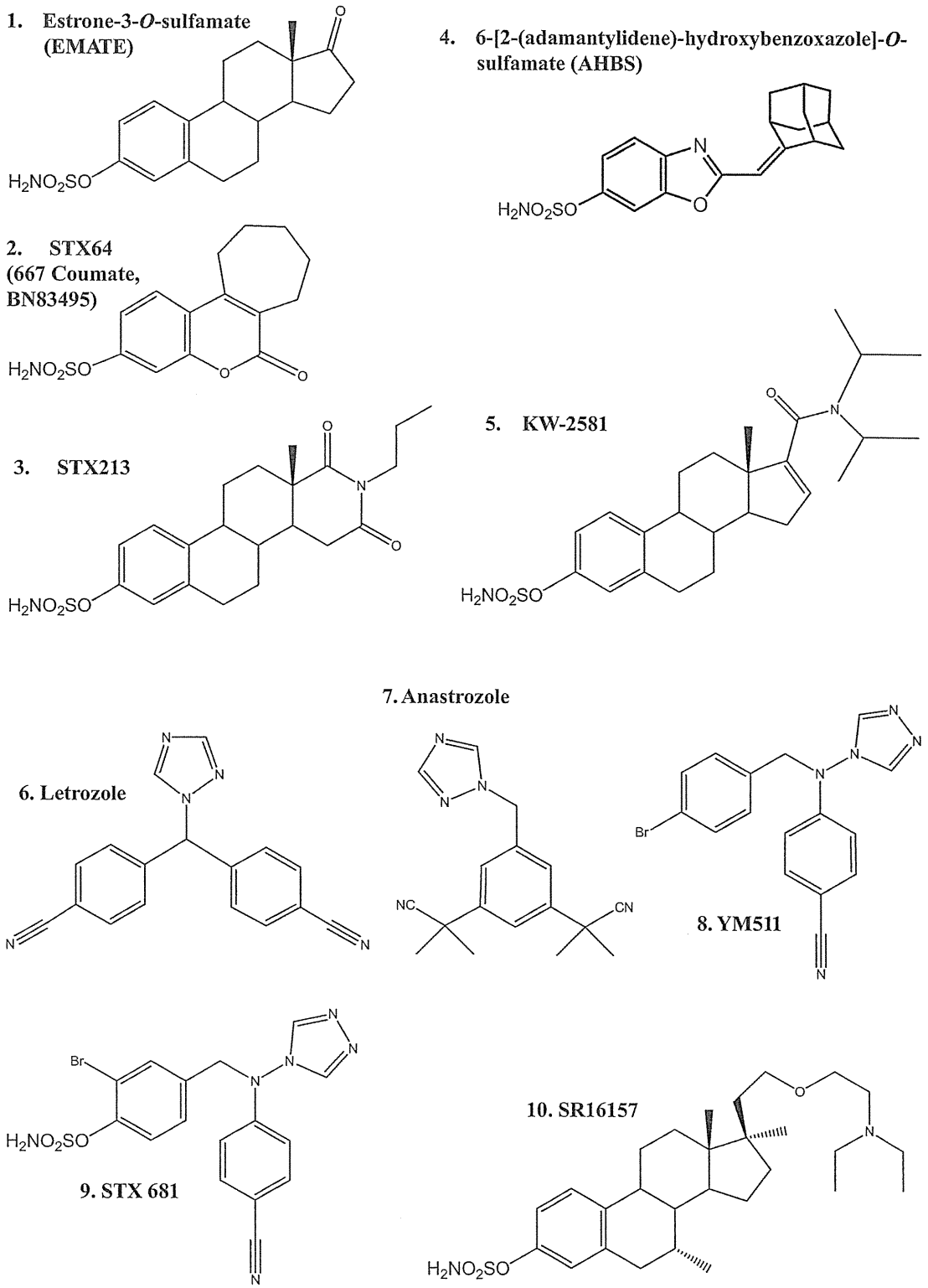

Fig. 3. Structures: selected steroid sulfatase inhibitors (compounds **1–5**), aromatase inhibitors (compounds **6–8**), a dual aromatase–sulfatase inhibitor ("DASI"; compound **9**), as well as a dual sulfatase–antiestrogen inhibitor (compound **10**).

99% (median) with both doses. In addition, plasma median concentrations of E1 were decreased at the 5 and 20 doses by 55% and 42%, respectively, with E2 plasma levels decreasing by 47% and 41%, respectively. Concerning plasma androgens, the levels of DHEA, androstenedione (A), and testosterone (T) were decreased by 52%, 63%, and 46%, respectively during therapy with STX 64. Although not a primary endpoint of this study, several patients experienced a stable disease for 2.75 to 7 months during therapy with STX 64 [45].

A further dose-escalation study in 29 patients has been completed recently [46]. The optimum dose was determined to be 40 mg of STX 64 per patient per day in tablet form.

5. Dual sulfatase/aromatase inhibitors

Since aromatase is needed for the synthesis of estrogens that are then converted into estrogen sulfates by estrogen sulfotransferase, hormone-dependent breast cancer may be more effectively treated by dual inhibition of aromatase and steroid sulfatase. A new design strategy was explored that involves introducing the aromatase inhibitory pharmacophore into a template that has been designed primarily for sulfatase inhibition [47]. A series of compounds that can inhibit both aromatase and sulfatase have been developed based on the structure of estrone 3-sulfamate, a typical estrone sulfatase inhibitor [48]. In contrast, a series of single agent dual aromatase-sulfatase inhibitors that are sulfamate derivatives of nonsteroidal AIs, including letrozole and anastrozole, have been successfully developed [49–51]. The design of these dual aromatase–sulfatase inhibitors shares a common strategy; that is, to engender the sulfatase inhibitory pharmacophore into an established aromatase inhibitor with minimal structural change incurred to the original scaffold in order to retain and maximize aromatase inhibition. At the same time, possible negative pharmacological interactions between several aromatase and sulfatase inhibitors given in concert could be avoided. It is also reasoned that resistance to drugs targeting two different enzymes is not likely to develop simultaneously. Thus, *D*ual *A*romatase-*S*ulfatase *I*nhibitors (DASIs) have been developed engendering the steroid sulfatase inhibitory pharmacophore into established aromatase inhibitors with minimal structural changes otherwise. At this stage, DASIs are available based on the triazoles letrozole (Fig. 3, **6** [52], anastrozole (Fig. 3, **7**) [53], and YM511 (Fig. 3, **8**) [51], in addition to alternative AIs characterized by their biphenyl templates [54].

STX 681 (Fig. 3, **9**) is a YM511-based DASI that has been shown to have in vivo activity. Using a xenograft nude mouse model, Foster et al. demonstrated that STX 681 completely inhibited the growth of MCF-7_{AROM} and MCF-7_{STS} tumors [55]. The authors conclude that targeting both the aromatase enzyme and the sulfatase enzyme at the same time has the potential to become a novel treatment strategy of hormone-dependent breast cancer (HDBC).

6. Sulfatase inhibitors: ongoing research and future aspects

Given the potency of this new class of sulfamate-based steroid sulfatase inhibitors, the large volume of preclinical data available on the use of steroidal and non-steroidal STS inhibitors in a variety of hormone-dependent cancer models and, given the encouraging results obtained in two phase I studies completed with BN83495 (STX 64) it will be important to carry out clinical trials to assess its efficacy in different clinical settings as well as in non-cancer disease indications. While clinical studies are planned to investigate the effect of BN83495 in women with ER-positive early breast cancer, the compound is currently in further clinical development for advanced endometrial cancer (phase II) as well as in phase I evaluation for castrate-resistant prostate cancer in North America. Additional trials will examine whether combining BN83495 with an AI or LHRH antagonist will improve response rates.

As the biological role of steroid sulfatase is also implicated in several disorders of the skin (acne, psoriasis, hirsuitism) and in memory function, BN83495 may find use in such non-cancer diseases [6].

7. Conclusions

Inhibition of steroid sulfatase is one promising new approach to develop alternative treatment strategies for hormone-sensitive breast cancer. In contrast to aromatase inhibition alone, suppressing plasma and tissue estrogen synthesis, sulfatase inhibition causes both estrogen and androgen depletion simultaneously. Early clinical findings suggest that breast cancer patients with progressive disease while on therapy with aromatase inhibitors, may experience a new response when treated with a steroid sulfatase inhibitor as monotherapy. Most interesting, upregulation of steroid sulfatase has recently been confirmed in breast cancer patients treated with an aromatase inhibitor, suggesting steroid sulfatase to be possibly involved in adaptation to estrogen deprivation and/or endocrine resistance. Phase I-II trials involving sulfatase inhibitors are now initiated to study the influence of these compounds on intra-tumor steroid levels and enzyme activity. Moreover, compounds inhibiting aromatase and sulfatase activity at the same time (DASIs) have been developed.

While sulfatase inhibition certainly is one of the most promising new treatment strategies for hormone-sensitive breast cancer, its role in daily praxis is currently unclear. Ongoing trials will investigate the potential of these drugs either as monotherapy or in combination with established drugs. Finally, the identification of biological relevant tumor markers that might serve as predictive factors (like steroid sulfatase activity in human cancer tissue, normal tissue, hair etc.) is urgently requested to allow the use of these drugs in groups of patients with a high chance for clinical responses.

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Review

Aromatase in human lung carcinoma

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ABSTRACT

Lung cancer is the leading cause of cancer mortality in both women and men worldwide but gender differences exist in their clinical and biological manifestations. In particular, among life time non-smoker, female are far more likely to develop lung carcinoma than male. Recent studies demonstrated that estrogens are synthesized *in situ* in both male and female lung cancers through aromatase, suggesting that sex steroid may contribute to the pathogenesis and development of lung carcinoma. In addition, human lung carcinomas have been recently demonstrated to be frequently associated with expression of estrogen receptors in both male and female patients and a lower expression of aromatase was reported to be associated with better prognosis. Preclinical studies further demonstrated that aromatase inhibitor (AI) suppressed the lung tumor growth both *in vitro* and *in vivo*. These findings all suggest a potential role of intratumoral aromatase in biological behavior of non-small cell lung cancer (NSCLC), the most common form of human lung malignancy. Therefore, AIs may become viable therapeutic options for disease management in NSCLC patients but further studies are definitely required to obtain a better understanding of the potential roles of intratumoral aromatase expression as a predictive biomarker for clinical outcome in these NSCLC patients.

Contents

1. Introduction

Lung cancer remains the leading cause of cancer mortality worldwide. It is true that tobacco smoking still remains its prime cause among both men and women [1,2] but among non-smoking-associated lung cancer patients, it is also true that women are more likely than men to develop lung carcinomas, especially adenocarcinomas [3,4]. In addition, both estrogen receptors (ERs) and aromatase were reported to be present in human lung tumors [5–16]. These findings all suggest a possible role of estrogens in biological behavior of human lung cancer as summarized in Tables 1 and 2. In particular, a lower expression of intratumoral aromatase was reported to be associated with better prognosis of NSCLC patients especially among postmenopausal female [13,17]. Therefore in this brief review, we focused on possible roles of intratumoral aromatase and will propose aromatase inhibitors as potential future therapeutic option in lung cancer.

2. Estrogenic effects on human lung cancer

Results of previously reported studies all suggested that estrogens could play an important role in lung cancer development in some cases [5,18–25]. Estrogens mainly exert its effects via two different distinctive estrogen receptor (ER) subtypes identified as ERα and ERβ [26,27]. Estrogens stimulate cell proliferation in non-small cell lung carcinoma (NSCLC) cell lines to a far greater extent than in non-neoplastic lung fibroblasts [18]. Estrogens also promoted the transcription of estrogen-responsive genes in NSCLC cells expressing endogenous ERs [19]. These genomic actions were demonstrated to be induced only by ligands specific to ERβ and not

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Table 1
Summary of previous studies on ERs expression in lung carcinoma tissues.

References	Cases	Female/male	Histology	ER posi. %	ERα posi. %	ERβ posi. %	Principle findings
[36]	64	26/38	Ad Sq Pd Lc Bac	10091100100100	------	------	ERs were expressed only in cancerous tissue and not in normal pulmonary tissue of the human lung.
[37]	52	16/36	Ad Sq Lc Bac SCLC	06000	------	------	ERs were expressed more abundantly in lung tumors from women than from men.
[6]	30	8/22	Ad Sq	--	00	8530	ERβ, but not ERα, could play important roles in human lung.
[7]	45	27/18	Ad Bac	8056	--	--	ER expression in lung adenocarcinomas was dependent upon the antibody clones that were used
[8]	32	15/17	Ad Sq	--	67	--	
[9]	278	214/64	AdBacAdsqLcSCLCSq	------	000000	5956100565085	Nuclear ERβ was expressed more frequently in men than women with lung adenocarcinomas and was associated with better clinical outcome
[10]	132	56/76	Ad Sq Lc	---	737550	466850	ERα expression and absence of ERβ expression were associated with a poorer prognosis in NSCLC patients.
[11]	301	127/174	Ad Sq Adsq	---	---	5041–	ERβ overexpression was a positive prognostic marker among stage II and III NSCLC patients.
[12]	104	33/71	Nsc Nsc	--	355*	84	ERβ in a lung tumor was a positive prognostic factor for men with NSCLC.
[13]	59	26/33	Ad Sq	--	6427	9573	Estrogen could be locally produced in NSCLC mainly by aromatase.
[14]	447	187/260	Ad Ad	--	084*	74	Prognostic significance of nuclear ERβ was limited to NSCLC patients with an EGFR mutation.
[15]	317	167/150	Ad Sq	--	0–480–33	22–9816–98	ERα expression was associated with EGFR mutations in lung adenocarcinomas.
[16]	105	38/67	Ad Sq Adsq	---	100	736866	ERβ and aromatase expression was highly concordant in NSCLC patients.

Table 2
Summary of previous studies on aromatase expression in lung carcinoma tissues.

References	No. of cases	Female/male	Methodology	Histology	Aromatase posi. % 'or' score	Principle findings
[5]	NS	NS	IHC	Nsc	NS	Aromatase was significantly expressed in lung carcinoma tissues.
[75]	53	33/20	IHC	AdSqAdsqBac	90 88 100 75	Aromatase was present and biologically active in human NSCLCs.
[17]	442	NS	IHC	Ad Sq Lc Sc	1.49 ± 0.021.56 ± 0.031.49 ± 0.06.99 ± 0.13	Lower levels of aromatase predicted a greater chance of survival in women 65 years and older.
[13]	59	26/33	RT-PCR	Nsc	–	Aromatase expression was significantly associated with intratumoral estradiol concentration.
[73]	10	NS	IHC	Nsc	–	Aromatase was expressed in NSCLC tissue and aromatase inhibitor reduced tumor growth in NSCLC xenograft.
[54]	78	NS	IHC	Ad Aq	60 70	Aromatase expression in NSCLC was independent of any clinical and pathological parameter except tumor stage.
[16]	105	38/67	IHC	Ad Sq Adsq Lc	86 82 100 0	Aromatase expression was significantly associated with ERβ expression in NSCLC patients.
[64]	9	3/6	RT-PCR/IHC	Ad Sq	100 0	Aromatase was expressed only in carcinoma cells but not in stromal cells.

Posi. %, percentage of positive cases; Nsc, non-small cell carcinoma (histological type unknown); Ad, adenocarcinoma; Sq, squamous cell carcinoma; Adsq, adenosquamous cell carcinoma; Bac, brochio-alveolar carcinoma; Lc, large cell carcinoma; SCLC, small cell lung carcinoma; Pd, poorly differentiated carcinoma; *, cytoplasmic staining; –, not examined.

by ligands specific to ERα [20]. In addition to genomic actions, estrogenic actions may occur at the cell surface in NSCLC cells involving mainly membrane/cytoplasmic pools of ERs [5,19–23]. In breast carcinoma cells, ERs utilize the membrane epidermal growth factor receptor (EGFR) to rapidly signal through various kinase cascades, i.e. mitogen activated protein kinase (MAPK) and/or protein kinase B (PKB/Akt) [28]. NSCLC cells demonstrated similar transactivation of EGFR on administration of exogenous estrogens which ultimately resulted in MAPK activation [21]. Many other investigators also reported a similar activation of MAPK and/or Akt on 17β-estradiol treatment in NSCLC cells, but without the transactivation of EGFR [5,19,20,22,23]. However, these non-genomic actions in NSCLC cell lines have not been well characterized compared to those in breast carcinoma cell lines and further investigations are required for further clarification [29–34]. Among estrogens, 17β-estradiol treatment has been demonstrated to result in significantly enhanced cell proliferation in various lung carcinoma cell lines [5,19–23]. In addition, 17β-estradiol exposure stimulated the growth of lung carcinoma xenografts [21,24,25]. Whether estrogen exerts its effects in NSCLC primarily through genomic or non-genomic signaling pathway has, however, still remained in dispute. In conclusion at this juncture, NSCLC is reasonably considered a novel estrogen target tissue.

3. Expression of ERs in lung carcinoma cells

Both ERα and ERβ have been reported to be expressed in variety of tissues including ovary, breast, CNS, bone and kidney [35]. Earlier studies on the presence of ERs in lung tumor focused only on the classical ERα, then termed simply as ER. ERβ expression in human lung carcinoma has been examined by various investigators but patterns of both ERα and ERβ in NSCLCs using immunohistochemistry were highly inconsistent varying from "0 to 100% for ERα" and "30 to 100% for ERβ" as summarized in Table 1. Earlier studies demonstrated that ERs were expressed only in lung tumor cells and not in normal lung tissue with a much higher frequency in female patients [36,37]. However, ERs, particularly ERβ, was then demonstrated to be expressed and to be functional both in normal and cancerous lung tissue of both [6,21,38,39]. Previously, only ERα was considered as tumor promoter, whereas ERβ was believed to inhibit tumorigenesis due to the absence of its expression in ovarian, breast, and cervical cancers, when compared to normal tissue [40]. However, results of recent studies demonstrated that ERβ can function as a tumor promoter in the absence of ERα expression [41–45]. Many NSCLC cell lines lacking ERα but expressing ERβ demonstrated similar tumor promoting features [19–21,24,25]. In contrast to the previously reported results of both *in vitro* and *in vivo* studies in NSCLC cell lines, those of several reported immunohistochemical analysis of NSCLC patients all indicated that the status of ERβ immunoreactivity was associated with better clinical outcome especially in male patients [9,11,12]. In addition, the presence of ERα and the absence of ERβ expression in lung tumor tissues were also reported to be associated with poor prognosis in NSCLC patients [10]. Recently this reported prognostic significance of ERβ could be limited to the NSCLC patients with EGFR mutations [14]. In addition, EGFR mutations, which were more frequently reported in Japanese patients with lung adenocarcinoma than those in non-Japanese patients [46], were associated with ERα expression [15] whereas ERβ expression was associated with aromatase expression in NSCLC patients [16]. These reports all suggest a possible functional correlation between ER expression with either aromatase expression or/and EGFR mutations in NSCLC patients. However, it is also important to note that several different criteria and antibodies were employed to define "ERα and/or ERβ" positivity in these studies and a comparison using standard immunohistochemical method has to be explored of status and clinical significance of ERs in NSCLC patients.

4. Estrogen synthesis in lung carcinoma

The upward trend of lung cancer deaths among female non-smokers was confined to elderly women [3]. In breast cancer patients 74% of the newly diagnosed patients were also post-menopausal [47]. Risks of developing estrogen dependent cancer, i.e. breast and endometrial cancer, and conceivably ovarian cancer, for postmenopausal women increase significantly with serum concentrations levels of estrogens [48–50]. In premenopausal women, the ovary is the principle source of circulating estrogens [51,52]. However, it is also true that a large proportion of estrogens in women (approximately 75% before menopause, and close to 100% after menopause) are produced in peripheral hormone-target tissues through aromatase from abundantly present circulating precursor adrenal androgens [53]. Aromatase was reported to be expressed frequently in both male and female patients with human lung carcinoma, Table 2. Aromatase expression was also significantly associated with intra-tumoral estrogen concentration in NSCLCs [13]. A lower expression of aromatase was associated with better prognosis of NSCLC patients especially among post-menopausal female [17]. In addition, aromatase expression was significantly associated with ERβ expression [16] and tumor stage [54] in two different reported studies. These findings all indicated the possible importance of elevated *in situ* estrogen concentrations through aromatase in NSCLC patients. However, other enzymes, i.e. 17β-hydroxysteroid dehydrogenase (17β-HSD) isozyme, steroid sulfatase (STS) and estrogen sulfotransferase (EST), also play pivotal roles in intra-tumoral estrogen production [55,56]. 17β-HSD type1 was expressed more frequently than 17β-HSD type2, which suggested further dependency of NSCLC on *in situ* estrogen production [13]. There have been no studies available on possible roles of STS and EST in NSCLC patients. In addition, among NSCLC patients intratumoral concentration of estradiol was significantly higher in men than postmenopausal women [13] and males frequently co-express ERs and aromatase [16]. Therefore, ER pathway would be expected to be targeted in males with NSCLC as well, especially if local estrogen production is present via aromatase but further investigations are required for clarification.

5. Regulation of aromatase in lung cancer

Among estrogen-dependent tumors, breast cancer tissues and endometrial cancer tissue expresses aromatase primarily in stromal cells adjacent to tumors cells and to a much lower degree in carcinoma cells [57–59,61,63]. However, among breast cancer tissues some studies demonstrated a more intense aromatase staining in the malignant epithelia [60,62]. In contrast, aromatase was detected predominantly in parenchymal/carcinoma cells in human lung carcinoma tissues [64]. Aromatase expression was much lower in NSCLC cell lines than breast carcinoma cell lines [62,64]. However, the level of aromatase expression in NSCLC tissues, where aromatase was detected in about 60–70% of NSCLC cases, was much higher than NSCLC cell lines [64], as summarized in Table 2. In human breast carcinomas, aromatase activity was predominantly confined to stromal cells which was reported to be regulated by various factors derived from breast carcinoma cells, such as prostaglandin E_2 (PGE_2) and cytokines including interleukin (IL)-1, IL-6, IL-11 and tumor necrosis factor (TNF)-α [65–68]. However, results of recent *in vitro* studies in both breast and endometrial carcinoma cell lines did demonstrate an increment in aromatase activity in carcinoma cells following the stimulus form stromal cells in co-culture system [62,63]. In addition, recent studies using the

Table 3
Summary of previous studies on use of estrogen down modulators in lung carcinomas.

References	Animal/cells	Methodology	Treatment	Drug	Principle findings
[18]	Mice/H23	Tumor xenograft	ER blocker	ICI 182,780	ER blocker inhibited the tumor growth.
[19]	273T	Gene array	ER blocker	ICI 182,780	ER blocker inhibited the E2 responsive gene expression.
[5]	NIH-H23	Cell prolifertion	Anti-estrogen EGFR inhibitor	Faslodex Gefitinib	Combination therapy with both EGFR-TKI and anti-estrogen was far more effective.
[21]	A549 273T Mice/201T	Cell proliferation Apoptosis assay Tumor xenograft	ER blocker EGFR inhibitor	ICI 182,780 Gefitinib	Combination therapy with both EGFR-TKI and ER blocker was far more effective than either blocker alone against NSCLCs.
[75]	A549/H23 Mice/A549	Cell proliferation Tumor xenograft	Aromatase inhibitor	Anastrozole	Aromatase inhibitor suppressed the tumor growth both *in vitro* and *in vivo*.
[23]	Mice/A549	Tumor xenograft	Anti-estrogen EGFR inhibitor	Faslodex Erlotinib	Combination therapy with both EGFR-TKI and anti-estrogen was far more effective than either blocker alone against NSCLCs.
[13]	ERα over. A549 ERβ over. A549	Cell proliferation	ER blocker Anti-estrogen Anti-estrogen Aromatase inhibitor	ICI 182,780 Tamoixifene Raloxifene Letrozole	Cell proliferation caused by testosterone was significantly inhibited by the addition of the letrozole in both A549 + ERα and A549 + ERβ cells.
[73]	H23 Mice/H23	Cell proliferation Tumor xenograft	ER blocker DNA damage/Apoptosis Aromatase inhibitor ER blocker EGFR inhibitor	ICI 182,780 Cisplatin Exemestane ICI 182,780 vandetanib	Combination therapy with both cisplatin and aromatase inhibitor was far more effective than either blocker alone. Combination therapy with both EGFR-TKI and ER blocker was far more effective than either blocker alone.
[70]	H23/A549	Cell number	Aromatase inhibitor	Exemestane	Exemestane treatment alone reduced cell number of NSCLC cell lines.
[71]	A549 H1650	Cell proliferation	Anti-estrogen EGFR inhibitor	Tamoixifene Gefitinib	Combination therapy with both EGFR-TKI and anti-estrogen was far more effective than either blocker alone.
[64]	LK87	Cell proliferation	ER blocker AR blocker Aromatase inhibitor	ICI 182,780 Flutamide Letrozole	Combined treatment with testosterone and androgen receptor blocker caused enhanced proliferative effect which was abrogatedby treatment with either ER blocker or aromatase inhibitor.

co-culture methodology to simulate *in vivo* stromal–carcinoma cell interactions in NSCLCs, stromal stimulus were reported to result in increased aromatase expression in NSCLC cells [64]. These stromal derived factors were identified as cytokines, i.e. interleukin-6 and oncostatin M [64]. Several investigators demonstrated that soluble factors, derived from lung carcinoma cells induced differentiation and cell proliferation of fibroblastic stromal cells [69]. Therefore, aromatase-inducible cytokines secreted from stromal cells of human lung carcinoma tissues may also be under the control of an interaction with carcinoma cells in the lung cancer microenvironment. However, further investigations are needed to clarify the mechanisms between various cytokines produced as a result of carcinoma–stromal interactions and aromatase induction in NSCLC cells.

6. Potential application of aromatase inhibitor therapy in lung cancer patients

The importance of estrogens in NSCLC is crucial and provides a strong rationale to evaluate anti-tumor activities of estrogen down modulators in lung cancer. However, abrogation of estrogen signaling resulted in either upregulation or activation of epidermal growth factor receptor (EGFR) protein suggesting that the EGFR pathway becomes activated when estrogen is depleted in NSCLC cells [21,70,71]. Similarly ERβ expression was increased on treatment with EGFR tyrosine kinase inhibitor (EGFR-TKI) in NSCLC cells [21,71]. This bi-directional crosstalk between EGFR signaling and estrogen signaling in NSCLCs suggested that combining or simultaneous therapies to target both the pathways is most reasonably considered the most beneficial antitumor effects in the patients with NSCLC. Many reports, both *in vitro* and *in vivo*, have demonstrated that combination therapy with EGFR-TKI along with estrogen down modulators resulted in enhanced anti-tumor activity than either treatment alone in NSCLCs, Table 3. In addition, results of a clinical study on 22 postmenopausal female NSCLC patients demonstrated that combination treatment with both ER blocker, fulvestarnt, and EFGR-TKI, geftinib, was well tolerated [72]. Phase II clinical trial for combination therapy with erotinib, an EGFR-TKI, and fulvestant, an ER blocker, versus erlotinib alone in NSCLC patients are also underway (ClinicalTrails.gov Identifier; NCT00100854 and NCT00592007). Exemestane, an irreversible steroidal inactivator, either alone [70] or in combination with cisplatin [73], a standard chemotherapy in NSCLC patients [74], demonstrated significant anti-tumor effects in two separate studies. Both letrozole and anastrozole, reversible steroidal inactivators, demonstrated similar anti-tumor activity in NSCLCs [13,64,75]. In breast cancer patients aromatase inhibitor generally results in significantly increased response rates and greater duration of response than selective ER modulator (SERM), i.e. tamoxifen [76]. In addition, patients assigned to exemestane displayed a trend of lower incidence of subsequent primary lung cancer compared to those maintained on tamoxifen [77]. These results all suggest that aromatase inhibitors could be more viable therapeutic option than SERMs for NSCLC patients in the future. As an initial step, phase II randomized trial of fluvestrant and anastrozole as consolidation therapy in postmenopausal women with advanced NSCLC is to be scheduled (ClinicalTrial.gov Identifier; NCT00932152). Both male and female NSCLC patients express ERs and aromatase, and cell lines derived from both sexes respond to estrogens, anti-estrogens, and aromatase inhibitors therefore therapeutic treatments with aromatase inhibitors would benefit all patients, not just women.

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Short communication

LKB1 expression is inhibited by estradiol-17β in MCF-7 cells

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ABSTRACT

The liver kinase B1 (LKB1) is encoded by the *STK11* gene and acts as a tumour suppressor and a regulator of energy homeostasis. LKB1 expression is reduced in primary breast tumours compared to normal breast epithelium. Although its expression in primary tumours does not appear to correlate with estrogen receptor (ER) status, it is differentially expressed in breast cancer cell lines where ER-negative cells have lower LKB1 expression than ER-positive cells. The present study aimed to examine the effects of estradiol on LKB1 expression and activity in the ER-positive breast cancer cell line MCF-7. Results demonstrate that estradiol causes a dose-dependent decrease in LKB1 transcript and protein expression and consistent with this, a significant decrease in the phosphorylation of the LKB1 target AMPK ($P \leq 0.05$). In order to assess whether effects of estradiol were due to effects on ERα binding to the *STK11* promoter, ChIP was performed. Results demonstrate that ERα binds to the *STK11* promoter in a ligand-independent manner and that this interaction is decreased in the presence of estradiol. Moreover, *STK11* promoter activity is significantly decreased in the presence of estradiol ($P \leq 0.05$). LKB1 transcript and IHC score were assessed in primary tumours of 18 patients and demonstrated no significant correlation with ER status ($n = 18$). Our results thereby provide a mechanism whereby LKB1 is decreased in ER-positive breast tumours.

1. Introduction

The observation that liver kinase B1 (LKB1), encoded by the *STK11* gene, can cause G1 cell growth arrest when over-expressed in breast cancer cells led to an increased interest in the newly identified tumour suppressor [1]. Interestingly, breast cancer cell lines have differential expression of LKB1 depending on their estrogen receptor (ER) status. The human breast cancer cell line MCF-7, which is ER-positive, expresses LKB1, whereas ER-negative cell lines, such as MDA-MB-435 and MDA-MB-231, have a reduced LKB1 expression [2]. AMP-activated protein kinase (AMPK) is now recognized as a master regulator of energy homeostasis and is tightly regulated by endocrine signals, including leptin, adiponectin, estradiol and phytoestrogens [3–5]. LKB1 activates AMPK by directly phosphorylating its α-catalytic subunit at Thr172. The activation of AMPK in liver and adipocytes results in decreased lipogenesis and increased fatty acid oxidation. Interestingly, a high rate of lipogenesis is essential for the proliferation of many tumour cells including breast cancer cells [6], suggesting that LKB1/AMPK must be down-regulated in breast cancer cells to allow this process to go forward. The present study aimed to examine the effect of estradiol on LKB1 expression and activity in the human breast cancer cell line MCF-7 and to relate these findings to expression in clinical samples.

2. Materials and methods

2.1. Plasmids

The LKB1prom reporter construct was generated by amplifying a 3998 bp fragment of the *STK11* promoter located −3002 to +996 using primers LKB1prom-F: 5′-ACT TTG GAA ATT CAG TGT GTA GGG CA-3′ and LKB1prom-R: 5′-CAA CAA AAA CCC CAA AAG GA-3′ from BAC clone #RP11-50C6 (BAC PAC Resources, Children's Hospital Oakland Research Institute). Further PCR using primers LKB1prom-XhoI-F: 5′-CGG GAA TCT CGA GAC TTT GGA AAT TCA GTG TGT AGG GCA-3′ and LKB1prom-HindIII-R: 5′-AAA GCG CAA

Abbreviations: LKB1, liver kinase B1; STK11, serine-threonine kinase 11; ER, estrogen receptor; AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipitation.

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