Instruments, Silver Spring, MD, USA). A representative tumour-bearing slide was selected for each case by a board certified pathologist with a special interest in GEP-NET pathology (WW). Typical tumour areas from the centre of the lesion as well as from the invasive margins were marked on the respective H&E slides. Subsequently, three tissue cylinders of 1.5 mm diameter were punched from each tumour-bearing donor block and transferred to a tissue microarray paraffin block. In addition, from every corresponding donor block, one conventional 2 μm paraffin section was cut for Ki-67 staining.

As normal reference control, ten cases of pancreatic tissue without significant pathology were investigated for the expression of the respective pathway components. Normal tissue was evaluated on conventional paraffin sections. Tissue was taken from patients with pancreatic NETs well away from the tumour.

### Immunohistochemistry

Anti-mTOR antibody, anti-4EBP1, anti-4EBP1 phosphorylated at Thr70 (p-4EBP1), anti-eIF4E phosphorylated at Ser209 and anti-S6K phosphorylated at Thr389 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). For immunohistochemistry, 3 μm paraffin sections were cut and incubated with anti-mTOR (1:50), anti-4EBP1 (1:50), anti-p-4EBP1 (1:25), anti-p-S6K (1:100) and anti-p-eIF4E (1:50) antibodies. The omission of the primary antibody served as negative control.

Ki-67 staining was performed in a Benchmark XT autostainer (Ventana, Tuscon, AZ, USA) according to the manufacturer's protocol.

### Evaluation of staining of tissue slides

Staining of mTOR, 4EPB1, p-4EBP1, p-S6K and p-eIF4E in tumour tissue was scored by applying a semi-quantitative immunoreactivity scoring (IRS) system, as described previously (Darb-Esfahani *et al.* 2009). Briefly, category A documented the intensity of staining as 0 (no immunostaining), 1 (weak), 2 (moderate) and 3 (strong). Category B documented the percentage of immunoreactive cells as 0 (none), 1 (<10%), 2 (10–50%), 3 (51–80%) and 4 (>80%). Multiplication of categories A and B resulted in an IRS ranging from 0 to 12 for each individual case. The raw expression scores were used for correlation analysis. For correlation with clinicopathological variables, cases that showed any expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E (IRS 1–12) were scored as positive; cases without expression (IRS 0) were scored as negative.

### Statistical analysis

Statistical analyses were carried out with SPSS 16.0 and GraphPad Prism 4.0. The significance of correlations between mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E staining patterns and clinicopathological data was tested by Fisher's exact test and $\chi^2$ test for trends. The significance of correlations of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores in primary tumours and their corresponding lymph node and distant metastases was assessed by the Wilcoxon test for paired sample analysis. The correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores with each other and with proliferation indices was done by Spearman's

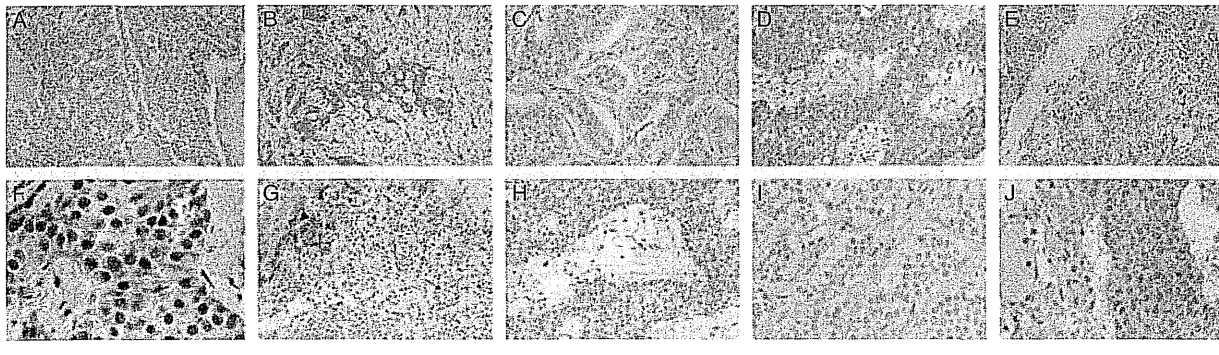


**Figure 1** mTOR, 4EBP, p-4EBP1, p-S6K and p-eIF4E expression patterns in gastroenteropancreatic neuroendocrine tumours. (A/B) mTOR expression in GEP-NET. (A) An mTOR-negative tumour is shown. (B) Tumour with strong cytoplasmic mTOR positivity. (C/D) 4EBP1 in GEP-NET. Neuroendocrine tumours with weak (C) and strong (D) expression of 4EBP1. (E/F) p-4EBP1 expression in GEP-NET. (E) A tumour with strong cytoplasmic and without nuclear expression is depicted. In contrast, the tumour in (F) showed moderate cytoplasmic and strong nuclear positivity. (G/H) p-S6K in GEP-NET. While the tumour (arrow) in (G) was essentially negative for p-S6K, the tumour in (H) showed strong expression of the phosphorylated protein. Note strong expression in liver parenchyma (arrowhead in G). (I/J) p-eIF4E in GEP-NET. (I) A tumour without expression of p-eIF4E is depicted, while the tumour in (J) was scored as positive.

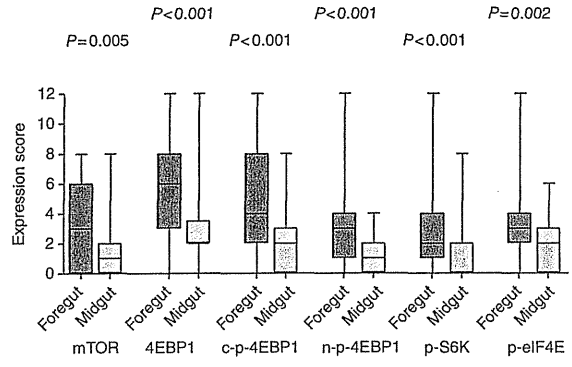


**Figure 2** Expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in dependence of tumour location. Expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E was higher in foregut than in midgut tumours. *P* values were calculated with the Mann–Whitney *U* test.

rank order correlation. Distribution of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores in dependence of tumour location was assessed by the Mann–Whitney *U* test. Differences in the percentages of Ki-67-positive cells in primary and metastatic tumours were investigated by the unpaired *t*-test and the Mann–Whitney *U* test.

The probability of differences in overall survival as a function of time was determined using the Kaplan–Meier method, with a log-rank test to probe for significance. *P* values $<0.05$ were considered significant.

## Results

### Expression patterns of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in GEP-NET

Cytoplasmic mTOR expression was found in 60 (61.2%) out of 98 tumours available for analysis. No nuclear immunostaining was observed. The intensity of immunostaining ranged from weak to strong and was fairly homogenous throughout a given tumour (Fig. 1). mTOR expression was significantly higher in foregut tumours than in midgut tumours ($P=0.005$, Fig. 2); this was also true when stage was included in the analysis (data not shown). There was no significant difference between mTOR expression in gastric, duodenal and pancreatic tumours ($P=0.096$, data not shown). However, while gastric and pancreatic tumours showed the same prevalence of mTOR positivity (~67%), duodenal tumours were less likely to be positive (16.7%).

Cytoplasmic 4EBP1 immunopositivity was noted in 91 (92.9%) out of 98 tumours investigated (Fig. 1). A very faint nuclear staining was detected in some cases, which might correspond to the nuclear localisation of the phosphorylated protein (see below). However, nuclear staining was too weak to allow for a quantitative evaluation of this staining pattern. Expression of 4EBP1 was significantly higher in foregut tumours than in their midgut counterparts ($P<0.001$, Fig. 2), which again was independent from tumour stage (data not shown). No significant differences in expression were found when gastric, duodenal and pancreatic tumours were compared ($P=0.591$, data not shown).

Phosphorylated 4EBP1 was located either in the cytoplasm or in the nucleus in 79 (79.8%) and 68 (68.7%) cases respectively (Fig. 1). Both cytoplasmic and nuclear positivity were significantly more likely to be found in foregut than in midgut tumours (Fig. 2, $P<0.001$ for both correlations). This finding was also valid after differences in stage were taken into account (data not shown). Gastric, duodenal and pancreatic tumours showed no significant differences in the expression of cytoplasmic ($P=0.443$) and nuclear p-4EBP1 ($P=0.105$). However, pancreatic tumours showed a lower percentage of positive cases for nuclear expression (67.7%) when compared with duodenal (83.3%) and gastric (100%) tumours (data not shown).

**Table 3** Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in gastroenteropancreatic neuroendocrine tumours

| | mTOR score | 4EBP1 score | Cytoplasmic p-4EBP1 score | Nuclear p-4EBP1 score | p-S6K score |
|---|---|---|---|---|---|
| 4EBP1 score | $r=0.322$ | | | | |
| | $P=0.001$ | | | | |
| Cytoplasmic p-4EBP1 score | $r=0.402$ | $r=0.632$ | | | |
| | $P<0.001$ | $P<0.001$ | | | |
| Nuclear p-4EBP1 score | $r=0.260$ | $r=0.406$ | $r=0.642$ | | |
| | $P=0.009$ | $P<0.001$ | $P<0.001$ | | |
| p-S6K score | $r=0.187$ | $r=0.443$ | $r=0.281$ | $r=0.239$ | |
| | $P=0.067$ | $P<0.001$ | $P=0.005$ | $P=0.019$ | |
| p-eIF4E score | $r=0.346$ | $r=0.646$ | $r=0.259$ | $r=0.162$ | $r=0.374$ |
| | $P=0.001$ | $P<0.001$ | $P=0.010$ | $P=0.113$ | $P<0.001$ |

Phosphorylated S6K (p-S6K) was exclusively found in the cytoplasm of tumour cells (Fig. 1). In total, 56.7% of tumours were positive for activated S6K to varying degrees (Tables 1 and 2). Similar to mTOR and 4EBP4, p-S6K expression was higher in foregut than in midgut tumours ($P<0.001$, Fig. 2) in a stage-independent manner. There was no significant difference in the expression of p-S6K between gastric, duodenal and pancreatic tumours ($P=0.786$, data not shown).

p-eIF4E was observed in 79.4% of tumours and varied considerably from case to case (Fig. 1, Tables 1 and 2). Again, expression was significantly higher in tumours from foregut when compared with tumours from midgut origin ($P=0.002$, Fig. 2). With respect to specific foregut locations, the number of positive cases did not show a relevant variation ($P=0.983$, data not shown).

Overall mTOR expression significantly correlated with 4EBP1, cytoplasmic and nuclear p-4EBP1 expression as well as with p-eIF4E expression ($P<0.01$ for all comparisons). The correlation coefficients ($r$) indicated a modest to fairly strong degree of interaction (Table 3). mTOR was associated with p-S6K as well; however, the association was weak ($r=0.187$) and failed to show statistical significance ($P=0.067$, Table 3).

As normal reference control, mTOR pathway component expression was investigated in a set of normal pancreatic tissues including adjacent stromal and inflammatory cells. These stainings revealed stable expression of several of the proteins in a distinct set of normal cells (e.g. lymphocytes). The respective results are summarised in Supplementary Table 1, see section on supplementary data given at the end of this article.

## Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with proliferation indices

Foregut tumours showed a higher proliferative activity than midgut tumours (mean foregut: 11% Ki-67-positive cells, mean midgut: 5% Ki-67-positive cells, $P=0.002$). This was also found when only stage IV tumours were compared ($P<0.001$).

By trend, Ki-67 staining was higher in nodal (mean primary: 3.1%, mean nodal metastasis: 4.2%) and distant metastases (mean primary: 3.3%, mean distant metastasis: 8.3%) when compared with the corresponding primary tumours. These differences were statistically significant in parametric tests for both comparisons ($P<0.001$) but only for the comparison of primary tumour and distant metastasis in non-parametric tests ($P=0.024$).

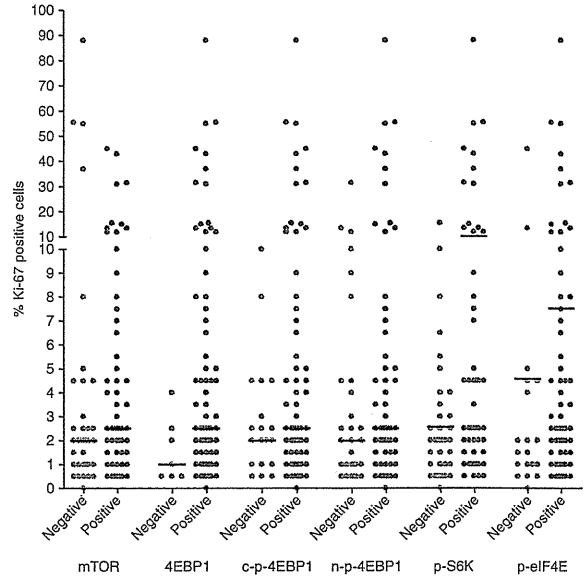


**Figure 3** Proliferative activity in dependence of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in GEP-NET. Proliferative activity was higher in those tumours with stronger expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E (details: see text).

Overall mTOR positivity was slightly but significantly higher in tumours with higher proliferative capacity ($r=0.213$, $P=0.038$). This correlation was also found for the expression of phosphorylated cytoplasmic and nuclear 4EBP1 ($r=0.238$, $P=0.020$ and $r=0.262$, $P=0.010$ respectively). Expression of 4EBP1 showed an even higher degree of correlation ($r=0.463$, $P<0.001$; Fig. 3). In addition, p-S6K ($r=0.364$, $P<0.001$) as well as p-eIF4E ($r=0.273$, $P=0.008$) expression was associated with higher proliferative capacity, as well (Fig. 3).

## Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with clinicopathological variables

In foregut, mTOR expression was significantly higher in tumours with distant metastasis ($P=0.035$; Table 1). No other correlations of the expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E with clinicopathological variables in either foregut or midgut tumours were evident (Tables 1 and 2).

## Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in the primary tumour and in corresponding lymph node and distant metastases

We investigated the expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in matched pairs of

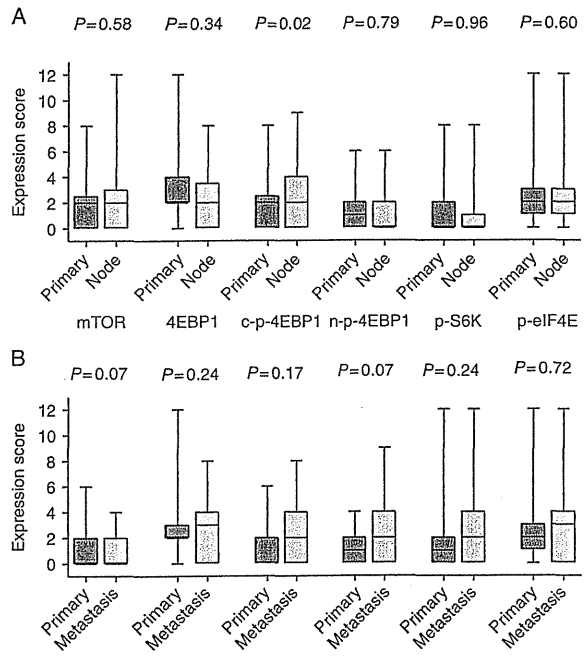


**Figure 4** Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in primary tumours and corresponding lymph node and distant metastases. While mTOR expression is a bit lower in distant metastatic tumours when compared with the corresponding primaries, activation levels of 4EBP1 are usually higher in nodal and distant metastases and expression levels of p-S6K and p-eIF4E were higher in distant metastases. *P* values were calculated with the Wilcoxon test.

primary tumours, nodal and distant metastases of GEP-NET (Fig. 4). There was a tendency towards lower mTOR expression in distant metastasis when compared with the respective primary tumours; however, this correlation was only of borderline significance ($P=0.07$). In addition, metastatic nodal (only cytoplasmic p-4EBP1) and distant tumour seeds usually showed slightly higher expression of phosphorylated 4EBP1, S6K and eIF4E when compared with the corresponding primary tumour, indicating higher activity of the mTOR pathway in metastatic tumours. However, this association was only found to be significant for cytoplasmic p-4EBP1 and nodal spread ($P=0.02$) and was of borderline significance for nuclear p-4EBP1 and distant spread ($P=0.07$).

### Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with survival

A probatory survival analysis in the homogenous subgroup of patients with stage IV midgut tumours ($n=39$) revealed that neither mTOR ($P=0.329$) nor 4EBP1 ($P=0.186$) or p-eIF4E ($P=0.521$) expression had an impact on NET-related death in univariate survival analysis in this group of patients (Fig. 5). Those patients whose tumours showed cytoplasmic p-4EBP1 expression had a trend towards longer disease-specific survival than those patients without activation of 4EBP1 ($P=0.055$). Interestingly, patients with activated S6K in their tumours had a significantly shortened disease-specific survival ($P=0.048$, Fig. 5). Neither grade ($P=0.764$) as a correlate for tumour aggressiveness nor treatment ($P=0.148$) had an impact on survival in this stage IV midgut patient cohort.

## Discussion

In this study, we report a differential expression of mTOR, 4EBP1, phosphorylated 4EBP1, phosphorylated S6K and phosphorylated eIF4E in a large cohort of GEP-NET. Expression levels of mTOR as well as activation of its downstream targets were higher in foregut tumours than in midgut tumours, indicating a higher activity of the mTOR pathway in the former. This increase in activity was accompanied by a higher proliferative capacity of foregut tumours when compared with midgut tumours. Foregut tumours with distant metastases showed strong mTOR expression, and metastatic tumours in general showed slightly higher mTOR pathway activation indicated by enhanced phosphorylation of 4EBP1 as well as by enhanced phosphorylation of S6K and eIF4E. Interestingly, those stage IV midgut patients with activated S6K had a reduced disease-specific survival, while this was not true for other downstream effectors or mTOR itself.

The detection of p-4EBP1 in the nucleus by us and other groups both *in vitro* and *in vivo* is interesting (Zhou *et al.* 2004, Castellvi *et al.* 2006, Rojo *et al.* 2007, Rong *et al.* 2008). It has been demonstrated that the target of 4EBP1, eIF4E, has functions as a nuclear regulator of the export of several RNAs involved in proliferation and cell growth (Culjkovic *et al.* 2007). The presence of 4EBP1 in the nucleus has been proposed to provide a means to regulate the release of eIF4E from the nucleus and may thus prevent the untimely export of eIF4E bound mRNAs (Missiaglia *et al.* 2010). The relevance of this mechanism with respect to carcinogenesis has to be elucidated.

Recently, researchers have begun to focus on the mTOR pathway in GEP-NET, since treatment of metastasized NETs with the mTOR inhibitor everolimus in combination with octreotide showed promising results in phase II clinical studies (Yao *et al.* 2008*b*, 2010). In addition, the mTOR pathway plays a central role in the tumourigenesis of familial cases as well as in the sporadic cases of NETs. The notion that this pathway is of importance in this tumour entity has

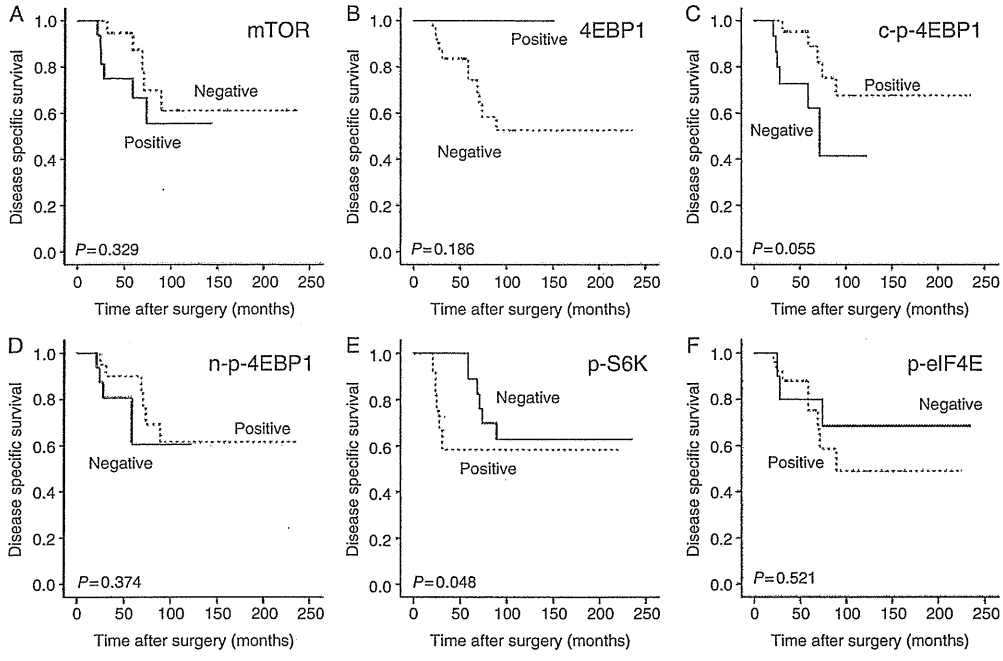


**Figure 5** Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with survival in stage IV midgut NET. No significant differences in disease-specific survival were observed in dependence of mTOR (A) and 4EBP1 (B), cytoplasmic (C) and nuclear (D) p-4EBP1 as well as p-eIF4E (F) positivity. In contrast, patients whose tumours expressed phosphorylated S6K (E) had a reduced disease-specific survival time. *P* values were calculated with a log-rank test.

further been substantiated by results of a high-throughput RNA expression analysis of pancreatic NETs in which the upstream inhibitors of mTOR, TSC2 and PTEN were found to be downregulated (Missiaglia *et al.* 2010). In addition, mTOR inhibition by rapamycin has been shown to significantly reduce NETs cell growth *in vitro* and *in vivo* (Moreno *et al.* 2008). This might be due to an induction of growth arrest in $G_0/G_1$ phase and enhanced apoptosis (Zitzmann *et al.* 2007). Furthermore, it has been proposed that deactivation of the AKT–mTOR kinase axis is responsible for this effect (Grozinsky-Glasberg *et al.* 2008). These *in vitro* results are in line with our findings that mTOR expression as well as downstream activation of 4EBP1, eIF4E and S6K correlates with proliferation in GEP-NET.

Most recently, in analogy to our work in GEP-NET, a large study on the expression of mTOR pathway components in lung NETs has been published in this journal (Righi *et al.* 2010). The authors reported an overexpression of p-4EBP1 in high-grade tumours, in contrast to p-mTOR and p-S6K, which were strongly expressed in low-grade tumours. In addition, in one recently published study on gastrointestinal NETs, phosphorylated mTOR, p-4EBP1 and p-S6K expression as well as several other factors were used to subclassify NET into novel potentially biological important subgroups (Iida *et al.* 2010). However, a correlation of the respective proteins with clinico-pathological variables and outcome has not been reported. Besides this, just one study on the expression of p-mTOR, which included only 20 GEP-NET (Shida *et al.* 2010) and in which the authors reported enhanced p-mTOR expression in poorly differentiated tumours, has been published. In our study, we did not find a straightforward correlation of either grouped mTOR expression or mTOR activity (as indicated by phosphorylation of 4EBP1) with tumour grade. However, we found an association of the expression of these proteins with the proliferation index, which in the novel grading scheme for GEP-NET is the central classifier for tumour grade.

mTOR expression and activity have been evaluated in a broad variety of human tumours, including most of the major tumour types, namely endometrial (Darb-Esfahani *et al.* 2009), esophageal (Boone *et al.* 2008), renal (Campbell *et al.* 2008), colorectal (Tampellini *et al.* 2007), prostate (Kremer *et al.* 2006), liver (Sahin *et al.* 2004), breast (Zhou *et al.* 2004, Rojo *et al.* 2007), lung (Anagnostou *et al.* 2009) and ovarian (Noske *et al.* 2008) cancer as well as glioblastoma (Pelloski *et al.* 2006). In all tumour entities, mTOR was either upregulated and/or activated in the tumour tissue when compared with the

corresponding tissue of origin. In addition, in some tumour entities, mTOR activity was linked to compromised patient prognosis. However, an association of the activated mTOR pathway with a better patient prognosis has been reported (Noske *et al.* 2008, Anagnostou *et al.* 2009) as well. In one study on bronchial NETs, no prognostic impact of mTOR pathway components was reported (Righi *et al.* 2010). We found that although mTOR expression itself was not associated with differences in patient prognosis, the detection of activated S6K confers a poor prognosis in stage IV midgut NETs. However, since this very homogenous subgroup of patients comprised only 39 cases, our results with respect to a possible impact of p-S6K positivity on survival must clearly be confirmed in much larger study cohorts.

In summary, we found that expression and activity of mTOR were strongly dependent on primary tumour location and metastatic status in GEP-NET. Expression as well as activation of mTOR pathway components was associated with enhanced proliferative capacity. Since everolimus, a small molecule targeting mTOR, proved to be effective in this tumour type and since it has been shown that response to other mTOR inhibitors may vary in dependence of expression and/or activity of the target, we suggest an investigation of mTOR expression profiles and phosphorylation of downstream targets in future clinical trials with this inhibitor.

## Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0126.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Review

# Suppression of estrogen actions in human lung cancer

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ABSTRACT

Estrogen plays a critical role in female reproduction but has also been reported to have important roles in various target tissues expressing estrogen receptor (ER) α and/or ERβ in both male and female. ERs especially ERβ have been demonstrated to be present and functional in both normal human lung and its disorders including cancer. Non-small cell lung carcinomas (NSCLCs) are well-known to be composed of heterogeneous groups. Squamous cell carcinoma is the most common subtype in men, but adenocarcinoma is the most common histologic subtype in women. Therefore, sex steroid hormones such as estrogens have been considered to play some roles in NSCLC. In particular, results of several epidemiological analyses pointed out the association between physiological or artificial alterations of hormone status such as menstruation and postmenopausal administration of hormone replacement therapy and lung cancer risks or its development especially in female subjects. In NSCLC tissues, intratumoral estrogen synthesis via aromatase, which is a key enzyme in the estrogen synthesis involved in aromatization of androgens into estrogens, has recently become of clinical interest as a possible target of therapy. Therefore, in this review, we focused on the potential of an endocrine therapy in NSCLC using clinically available inhibitors of estrogen and aromatase actions.



**Contents**



## 1. Introduction

Lung cancer is currently the most frequently diagnosed major cancer all over the world and the most common cause of cancer death in the world. Almost all lung cancers are carcinomas. Lung carcinoma is histologically classified into small cell carcinoma and non-small cell lung carcinoma (NSCLC). NSCLC is further composed of heterogeneous groups such as adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Small cell carcinomas comprise about 20% of cases and large cell undifferentiated carcinomas approximately 9% (Prkin et al., 2004). In other histological types, the proportions differ by sex: squamous cell carcinomas comprise 44% of lung cancers in men but 25% in women, while adenocarcinomas comprise only 28% of the cases in men but 42% in women (Prkin et al., 2004). In adenocarcinomas, although most cases are detected in smokers, it develops more frequency than any other histologic type of lung carcinoma individuals, particularly women, among those who have never smoked (Prkin et al., 2004). Bronchioloalveolar carcinoma is a subset of lung adenocarcinoma but is associated with distinct clinical presentation, tumor biology, response to therapy, and prognosis or clinical outcome compared to other subtypes of NSCLC (Raz et al., 2006). Bronchioloalveolar carcinoma disproportionately influences women, never-smokers, and Asians and is characterized by proliferation along alveolar septa without evidence of stromal, vascular, or pleural invasion (Raz et al., 2006). It is true that more men than women developed lung cancer, and more men than women die from the disease (Payne, 2005).

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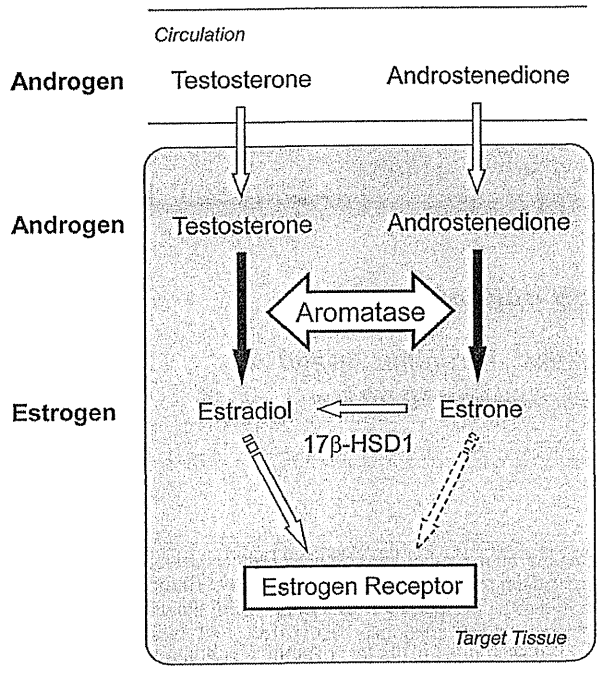


**Fig. 1.** Summary of intracrine of estrogens. Estrogen (estrone and estradiol) synthesized by aromatase binds to estrogen receptor (ER). The binding affinity of estrone is weaker than estradiol (Kuiper et al., 1997). Estrone is converted to estradiol by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1).

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

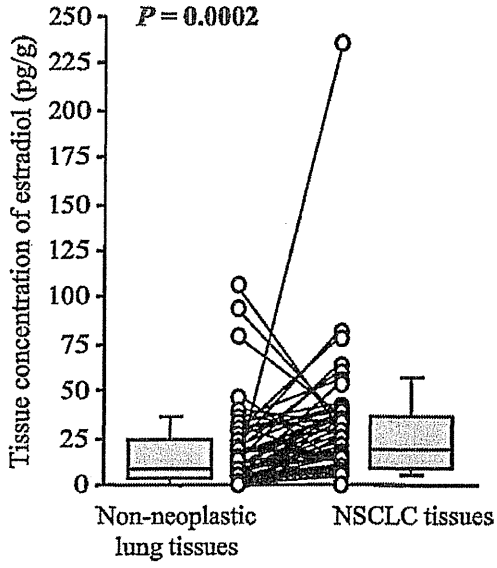


**Fig. 2.** Intratumoral concentration of estradiol in 59 NSCLC. Tissue concentration of estradiol in NSCLC and corresponding nonneoplastic lung tissues. Each value was shown in an open circle, and the paired values from the same patient were connected in a line. The grouped data are represented as box-and-whisker 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 | MCA1974S (Serotec) |
| | Bac | - | | | 9/16 | 56 | |
| | Adsq | - | | | 4/4 | 100 | |
| | Lc | - | | | 5/9 | 56 | |
| | Sc | - | | | 7/14 | 50 | |
| | Sq | - | | | 11/13 | 85 | |
| Kawai et al. (2005) | Ad | 74/102 | 73 | HC-20 (SantaCruz) | 47/102 | 46 | HC-150 (SantaCruz) |
| | Sq | 21/28 | 75 | | 19/28 | 68 | |
| | Lc | 1/2 | 50 | | 1/2 | 50 | |
| Skov et al. (2008) | Nsc | 3/104 | 3 | ER1D5 (Immunotec) | 87/104 | 84 | PPG5/10 (Dako) |
| | Nsc | 57[b]/104 | 55 | | | | |
| Niikawa et al. (2008) | Ad | 28/44 | 64 | 6F11 (Novocastra) | 42/44 | 95 | 14C8 (GeneTex) |
| | Sq | 4/15 | 27 | | 11/15 | 73 | |
| Nose et al. (2009) | Ad | 0/447 | 0 | HC-20 (SantaCruz) | 329/447 | 74 | HC-150 (SantaCruz) |
| | Ad | 377[b]/447 | 84 | | | | |

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

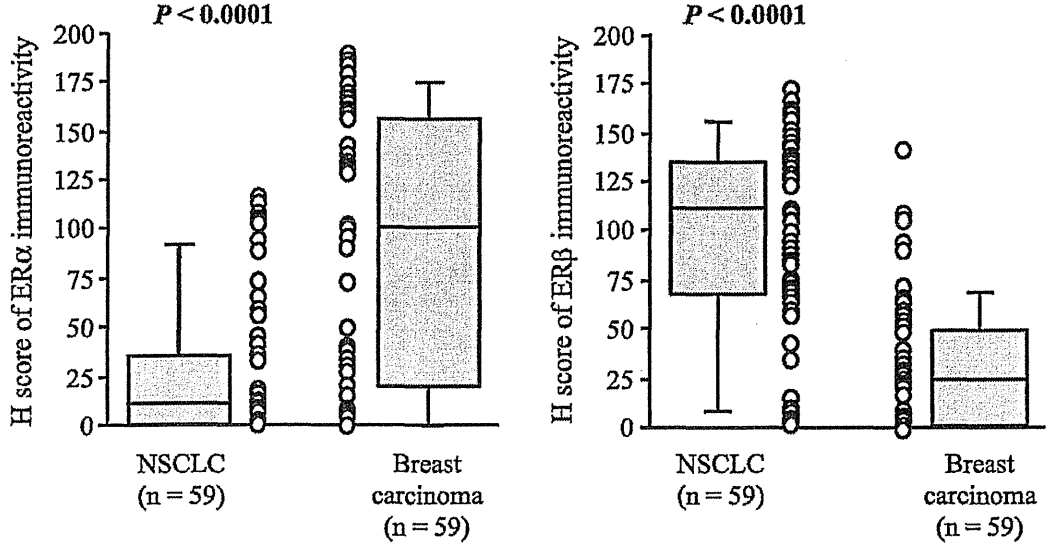


**Fig. 3.** Immunointensity of ERα (A) or ERβ (B) in NSCLC compared with breast carcinomas. Each value was represented as an open circle and the grouped data were shown as box-and-whisker plots. The statistical analysis was done by a Mann–Whitney's *U* test. Reproduced from Niikawa et al. (2008) with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

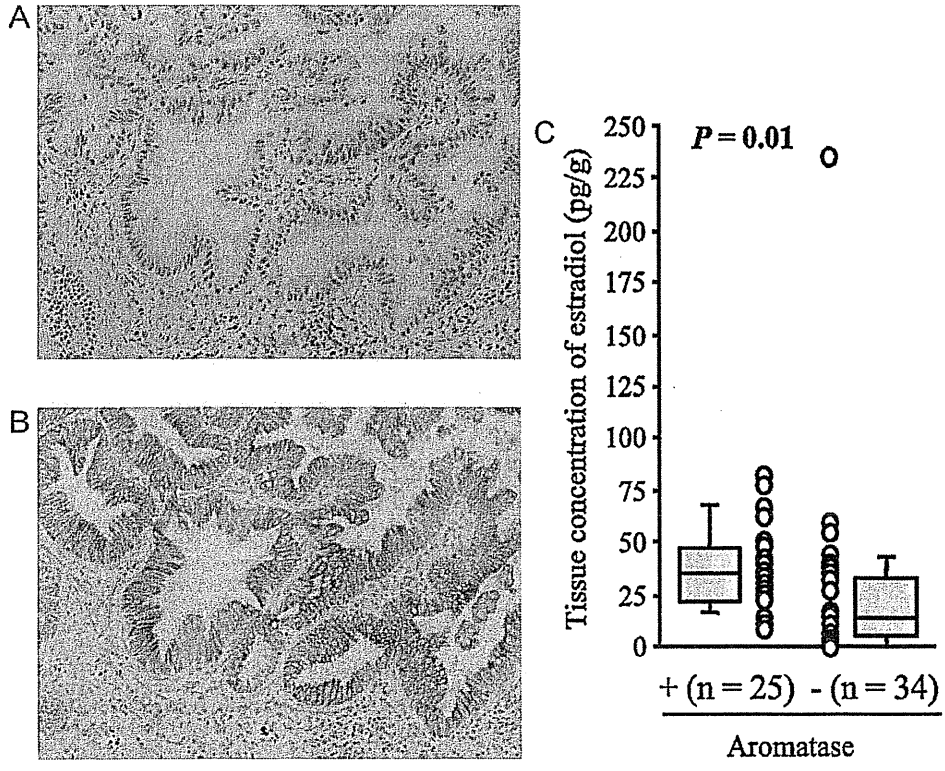


**Fig. 4.** Immunohistochemistry for ERβ (A) and aromatase (B) in lung adenocarcinoma. Monoclonal antibody for ERβ (14C8) was purchased from GeneTex Inc., San Antonio, TX (Niikawa et al., 2008; Abe et al., 2010). The aromatase monoclonal antibody #677 was raised against native recombinantly expressed 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].

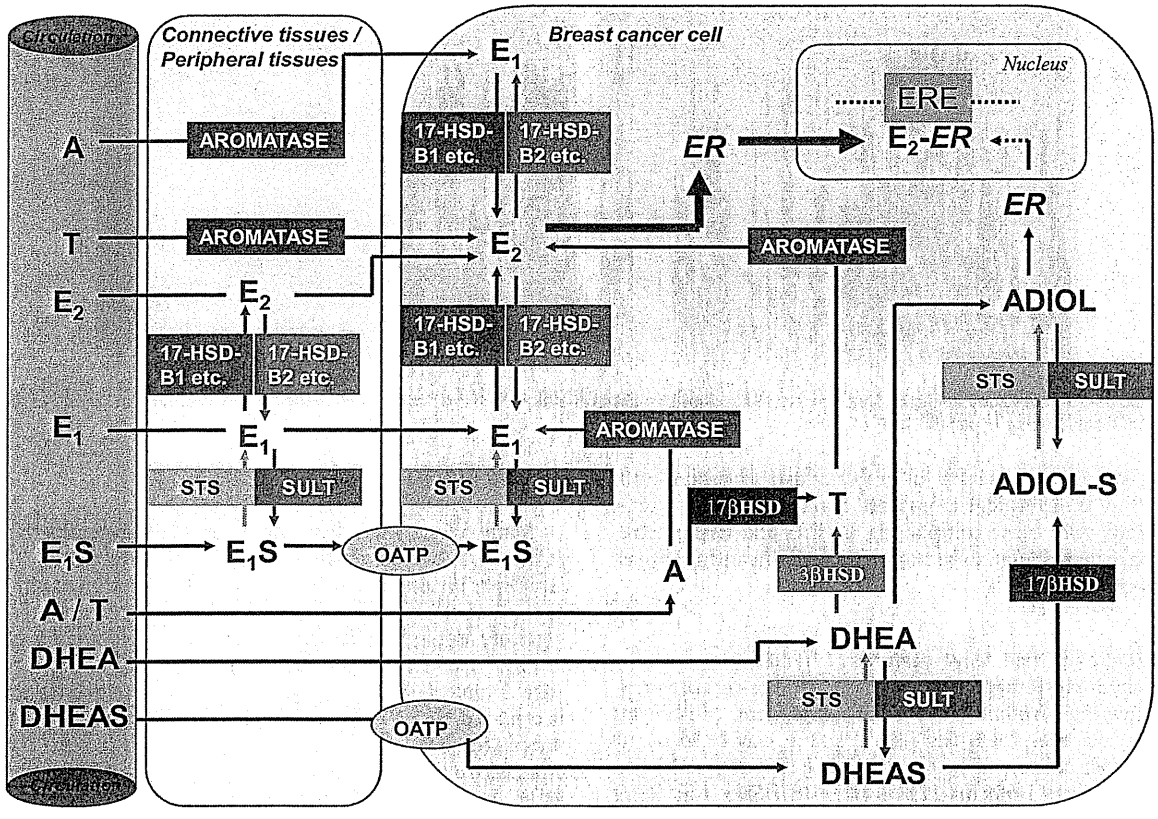


**Fig. 1.** Major pathways of estrogen and androgen synthesis in human breast cancer tissue. A, androstenedione; T, testosterone; E1, estrone; E2, estradiol; E1S, estrone sulfate; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; 17-β-HSD, 17β-hydroxysteroid dehydrogenase; STS, steroid 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:

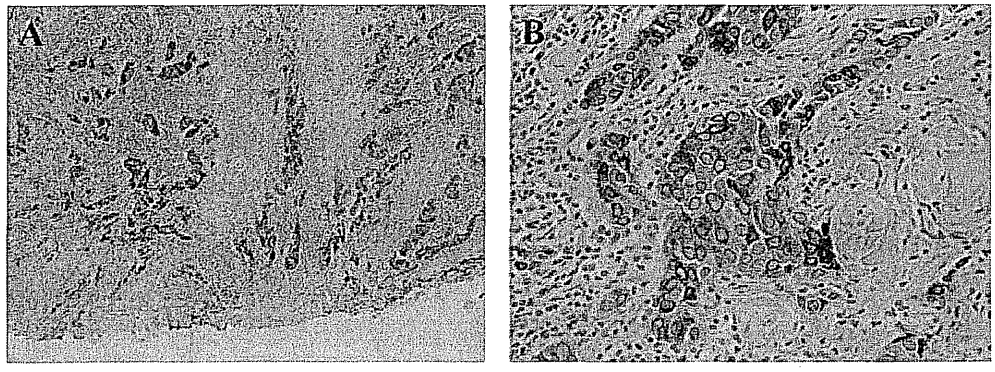


**Fig. 2.** Detection of steroid sulfatase in human breast cancer tissue. Steroid sulfatase immunoreactivity was detected in the cytoplasm of human breast cancer cells using low power (A) and high power (B) magnification.

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

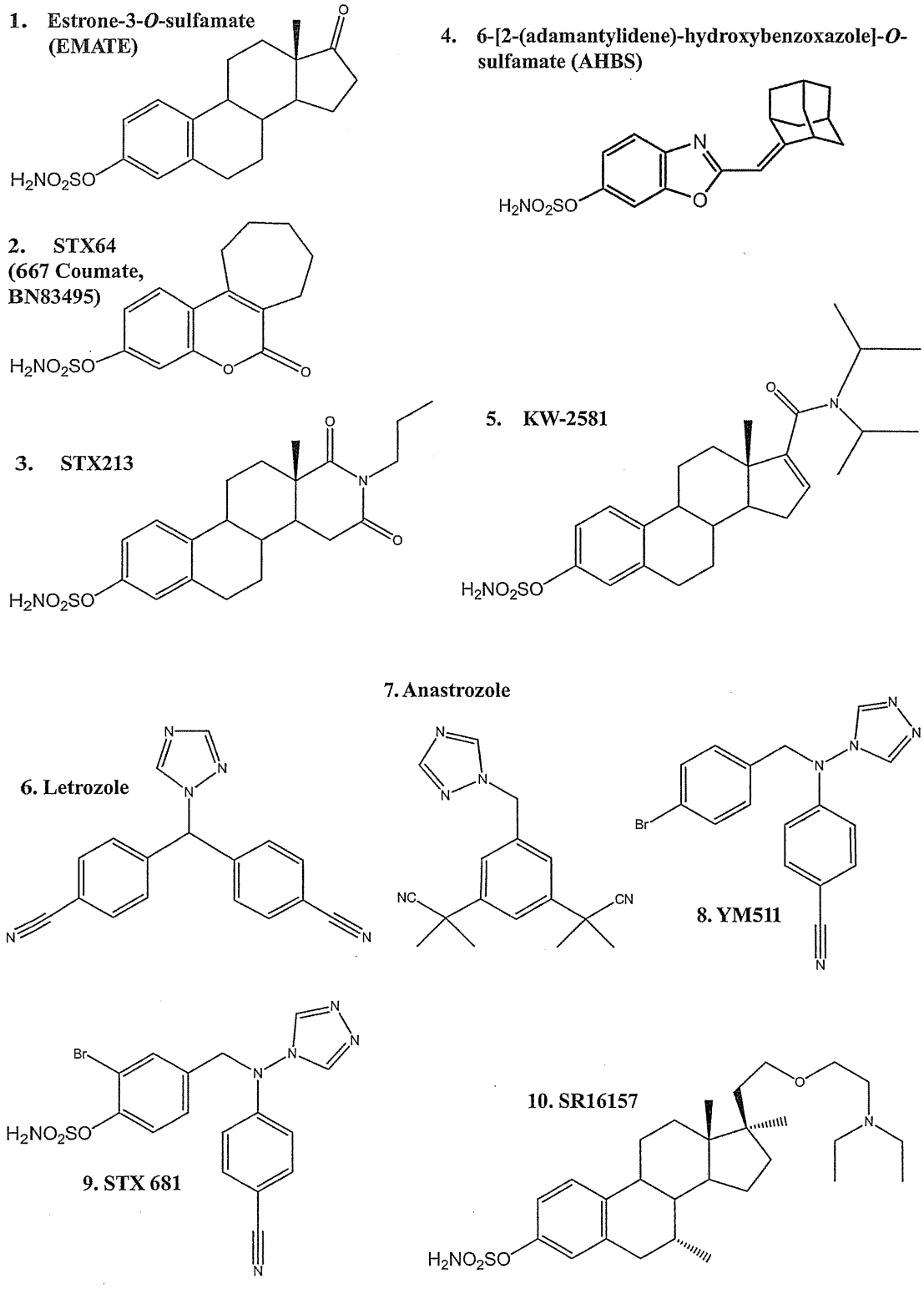


**Fig. 3.** Structures: selected steroid sulfatase inhibitors (compounds **1–5**), aromatase inhibitors (compounds **6–8**), a dual aromatase–sulfatase inhibitor ("DASI"; compound **9**), as well as a dual sulfatase–antiestrogen inhibitor (compound **10**).

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].