REVIEW

Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer

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Abstract

Tamoxifen has been the mainstay of hormonal therapy in both early and advanced breast cancer patients for approximately three decades. The availability of novel compounds such as aromatase inhibitors (AIs) and fulvestrant, with different mechanism of action, is changing the scenario of endocrine treatment of postmenopausal breast cancer patients. In this review article, we have summarized the current knowledge of the mechanisms of resistance to endocrine therapy, in order to derive information that might be useful for therapeutic intervention. We propose that resistance to endocrine therapy is a progressive, step-wise phenomenon induced by the selective pressure of hormonal agents, which leads breast cancer cells from an estrogen-dependent, responsive to endocrine manipulation phenotype to a non-responsive phenotype, and eventually to an estrogen-independent phenotype. In particular, evidence suggests for each 'action' introduced to block estrogen stimulation of breast cancer cells (i.e. treatment with anti-estrogen), there are one or more corresponding 'reactions' that tumor cells can use to escape our attempts to block their growth: estrogen hypersensitivity associated with increased transcriptional activity of estrogen receptor α (ERα) and/or increased non-genomic activity of ERα, estrogen supersensitivity, increased growth factor signaling, suppression of ERα expression and finally estrogen independence. Activation of growth factor signaling is involved in each step of this phenomenon, and might ultimately substitute estrogen in sustaining the growth and the survival of breast cancer cells. In this respect, results of pre-clinical and clinical studies with AIs, fulvestrant and signaling inhibitors sustain this hypothesis. More importantly, the knowledge of the mechanisms involved in the resistance of breast cancer cells to endocrine therapy offers potential for novel therapeutic strategies.

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Introduction

It has long been established that estrogen is involved in the pathogenesis of breast carcinoma, and that it sustains the growth of breast cancer cells that express the receptor for this hormone. Indeed, approximately 70% of breast cancer patients are positive for estrogen receptor (ER) or progesterone receptor (PgR) expression at diagnosis. These patients are therefore suitable candidates for hormonal therapy, which aims to block estrogen stimulation of breast cancer cells. This can be achieved by different approaches. In postmenopausal women, who are characterized by no longer exhibiting ovarian production of estrogen, ovarian suppression is not required, and medical therapy is based on the use of drugs that will: block the activity of ER such as tamoxifen or other selective estrogen receptor modulators (SERMs); induce destabilization and degradation of ER such as the selective estrogen receptor down-regulators (SERDs);

or reduce the production of estrogen in peripheral tissues and within the tumor using aromatase inhibitors (AIs).

Tamoxifen has been the mainstay of hormonal therapy in both early and advanced breast cancer patients for approximately three decades (Early Breast Cancer Trialist Group 1998, Gradishar 2004). As a matter of fact, tamoxifen was the first target-based agent directed against a growth-promoting pathway that entered clinical practice. Almost all patients with ER-positive tumors in Western countries have been treated with this drug either as adjuvant treatment following surgery or as first-line treatment for advanced disease. However, approximately 50% of patients with advanced disease do not respond to first-line treatment with tamoxifen. Furthermore, almost all patients with metastatic disease and approximately 40% of the patients that receive tamoxifen as adjuvant therapy experience tumor relapse and die from their disease. These findings strongly suggest that mechanisms of *de novo* or acquired resistance to tamoxifen occur in breast cancer patients, and that this phenomenon might largely affect the efficacy of this treatment.

Results of recent clinical trials suggest that AIs have an enhanced anti-tumor effect as compared with tamoxifen, and that they might be effective in patients that are resistant to tamoxifen (Gradishar 2004, Strasser-Weippl & Goss 2005). The rationale for use of these compounds in postmenopausal patients derives from the observation that estrogen in this set of patients is produced by aromatase in peripheral tissues and in the tumor (Johnston & Dowsett 2003, Lonning 2004). However, the response rate to these compounds is only slightly higher as compared with tamoxifen in patients with advanced breast cancer, and mechanisms of *de novo* or acquired resistance to these compounds clearly affect their efficacy. The availability of different novel drugs for hormonal therapy of breast cancer, the knowledge of the mechanisms that are employed by breast cancer cells to adapt to estrogen deprivation and the generation of novel compounds that can interfere with the growth factor-driven signaling pathways involved in resistance to anti-hormonal therapy, might enable novel strategies to be designed for therapeutic intervention in ER-positive breast cancer patients. The aim of this review article is indeed to summarize the current knowledge on the mechanisms of resistance of breast cancer cells to endocrine manipulation, and the results of most recent clinical trials with novel anti-hormonal drugs such as AIs and fulvestrant. More importantly, we propose a model that tries to summarize the steps involved in the progressive resistance to endocrine therapy that arises in breast cancer cells

following treatment with hormonal agents. We are aware that this model has some pitfalls, since it has been basically built on the results of pre-clinical studies. However, we believe that such provocative interpretation of current data on resistance to endocrine therapy might help to open the discussion on crucial points that we feel need to be addressed, and to develop novel therapeutic strategies in breast cancer patients.

Mechanism of action of ER

In order to discuss the mechanisms of resistance to hormonal therapy, we need briefly to describe the molecular pharmacology of ER. Actually, there are two different ERs, ERα and ERβ that are produced by distinct genes. The peculiar characteristics of ERβ will be briefly described in one of next paragraphs. If not otherwise specified, 'ER' will refer to 'ERα' in the following paragraphs. Binding of estrogen to ER induces activation of the receptor. In fact, ER dissociates from heat shock proteins, and undergoes conformational changes, dimerization and phosphorylation (Osborne & Schiff 2005). The activated ER binds to estrogen response elements (EREs) that are located upstream of estrogen-regulated genes. In this respect, it has been demonstrated that approximately 70% of estrogen-regulated genes are down-regulated following treatment with estradiol by using microarray analysis of gene expression in MCF-7 cells (Frasor *et al.* 2003). Many down-regulated genes are transcriptional repressors, or genes with anti-proliferative or pro-apoptotic function, whereas genes that induce cell proliferation are up-regulated. Two different domains, activating function-1 (AF-1) and AF-2, mediate positive regulation of gene expression by ER. AF-1 is at the N-terminus of the receptor, its function is regulated by phosphorylation and it is hormone-independent, whereas AF-2 is in the ligand-binding domain of the receptor and is hormone-dependent. The two activating domains act synergistically, although some gene promoters have been shown to be activated independently by AF-1 or AF-2 (Gronemeyer 1991, Osborne *et al.* 2001). Co-regulatory molecules that interact with the ER–ligand complex modulate the transcriptional activity of ER. In particular, the transcriptional activity of ER is enhanced by binding to the AF-2 domain of co-activators such as nuclear-receptor co-activator 1 (NCoA1 or SRC1), NCoA2 (TIF2) and NCoA3 (AIB1, TRAM1, RAC3 or ACTR) (McKenna *et al.* 1999, Leo & Chen 2000). These proteins form large complexes that enhance ER-driven transcription by different mechanisms
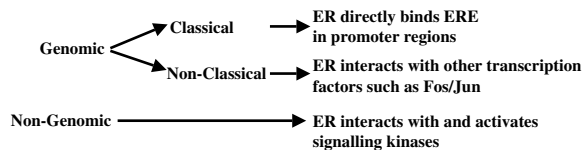
Figure 1 Modes of action of estrogen receptor (ER). ERE, estrogen response elements.

including recruitment of histone-acetyltransferase (HAT) at the promoter site. In contrast, co-repressor proteins such as nuclear-receptor co-repressor 1 (NCoR1) and NcoR2 influence ER-induced transcription at least in part by recruitment of histone-deacetylase complexes (Chen & Evans 1995, Horlein *et al*. 1995).

The genomic actions of ER are profoundly affected by tamoxifen binding. In fact, tamoxifen induces in ER a conformational change that prevents binding of co-activators and therefore blocks AF-2-induced transcription (Shiau *et al*. 1998). These findings explain the ability of tamoxifen to function as both an antagonist and an agonist of estrogen. In fact, tamoxifen blocks the transcription of genes that depend essentially on AF-2 for gene expression. However, in AF-1-dependent genes tamoxifen can function as an agonist (Tzukerman *et al*. 1994, McDonnel *et al*. 1995). In addition, it has been shown that tamoxifen interacts with co-repressors when bound to ER, and that this mechanism is involved in the suppression of transcription (Lavinsky *et al*. 1998, Schiff *et al*. 2003). Therefore, the availability of co-regulators might be the cause of the tissue-dependent effects of tamoxifen.

The above-described mechanism is referred as the 'classical mode' of action of ER and is directly related to its ability to regulate the expression of genes that have ERE elements in the promoter region (Fig. 1). However, different mechanisms of action of ER have been demonstrated. In particular, ER has been shown to interact with other transcription factors such as the Fos–Jun complex to regulate gene expression at alternative regulatory DNA sequences such as AP-1, SP-1 and other non-defined sites (non-classical mode) (Ray *et al*. 1997, Kushner *et al*. 2000, Safe 2001). Furthermore, the ability of membrane ERα to interact with and/or activate several kinases including the insulin-like growth factor-1 receptor (IGF-1R), Src, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR) and ErbB-2 has been demonstrated (non-genomic effects of ER) (Migliaccio *et al*. 1996, Kahlert *et al*. 2000, Sun *et al*. 2001, Chung *et al*. 2002, Wong *et al*. 2002, Schiff *et al*. 2004, Shou

et al. 2004). The cytoplasmic kinases can also phosphorylate co-activators that can modify ERα activity (Bunone *et al*. 1996, Font de Mora & Brown 2000, Campbell *et al*. 2001). Interestingly, it has been suggested that SERMs such as tamoxifen behave as estrogen agonists on these membrane effects of ERα (Schiff *et al*. 2004, Shou *et al*. 2004). However, membrane functions of ER depend on the levels of the above-mentioned kinases, and they might be modest in breast cancer cells that express low levels of tyrosine kinase receptors such as EGFR and ErbB-2.

The genomic and non-genomic mechanisms of action of ER are not mutually exclusive, and many interactions between these two pathways exist. For example, ER induces the expression of transcripts for both transforming growth factor α (TGFα) and amphiregulin (AR) (Saeki *et al*. 1991, Normanno *et al*. 1993). TGFα and AR are both able to bind and activate EGFR thus leading to activation of MAPK and AKT signaling (Salomon *et al*. 1995). Formation of EGFR/ErbB-2 heterodimers in cancer cells can also lead to activation of ErbB-2 that has no known ligands. MAPK and AKT pathways can be also activated by direct interaction of ERα with these kinases. In addition, ERα binds to caveolin-1 at the cell membrane and activates specific G proteins (Levin 2003). This phenomenon leads to activation of *src*, which in turn activates matrix metalloproteinases that cleave transmembrane precursors of heparin binding-EGF (HB-EGF), an EGFR ligand (Razandi *et al*. 2003). Therefore, both genomic and non-genomic activities of ER can lead to increased activation of EGFR and its downstream effectors.

Finally, it has been demonstrated that ERα can be phosphorylated and activated by different intracellular kinases, a process defined as ligand-independent activation (Schiff *et al*. 2003, Johnston 2005). In particular, ERα is phosphorylated at key positions (serine 118, serine 167 and threonine 311) in the AF-1 domain and in other domains following activation of MAPK/ERK, PI3K/AKT, p90rsk and p38 MAPK pathways, which occurs in response to various cytokines and growth factors — including ligands of EGFR or IGF-1R (Kato *et al*. 1995, Bunone *et al*. 1996, Joel *et al*. 1998, Campbell *et al*. 2001).

Mechanisms of resistance to hormonal therapy

Several different mechanisms have been hypothesized to be involved in the resistance of breast cancer cells to hormonal therapy (Table 1). These mechanisms have

Table 1 Potential mechanisms of resistance to endocrine therapy in breast carcinoma.

Loss of expression or altered function (mutations) of ERα
Lack of expression of PgR
Increased expression of ERβ
Metabolism of hormonal agents (CYP2D6 variants for tamoxifen)
Altered expression of co-regulators
Estrogen hypersensitivity
Estrogen supersensitivity
Increased growth factor signalling

been accurately described in excellent recent review articles, and to describe them in detail is beyond the purpose of this review (Johnston & Dowsett 2003, Santen *et al*. 2003, Nicholson *et al*. 2004, Ring & Dowsett 2004). Therefore, we will briefly summarize these findings in the next paragraphs in order to provide the reader with the basis for the discussion of the results of clinical trials. More importantly, we will try to draw a hypothesis that summarizes the main findings in this field, and that might be useful in the generation of novel therapeutic strategies.

Three different classes of agents are currently employed or are under investigation as anti-estrogen therapy: SERMs such as tamoxifen that have both antagonist and agonist activity; SERDs such as fulvestrant, which destabilize the receptor and induce its degradation; AIs that block the peripheral and intra-tumoral production of estrogen. As we will discuss later, the mechanisms of resistance to these three classes of compounds differ at least in part. Of course, there is a large body of literature on the resistance to tamoxifen whereas the information available for more recent drugs is limited. Therefore, we will consider each type of resistance mechanism in turn, and will try to discuss whether different types of agents might share the particular mechanism.

Alterations in ERα and PgR expression or function

Since expression of ERα is the main predictor of response to endocrine therapy, lack of expression of ER is clearly the main mechanism of *de novo* resistance to treatment with hormonal agents. In this respect, the absence of ERα gene expression has been associated with the aberrant methylation of its CpG islands in a significant fraction of breast cancers (Weigel & deConinck 1993, Ottaviano *et al*. 1994). Recent data indicate that chromatin inactivation mediated by histone deacetylation and DNA methylation

are indeed critical components of ERα silencing in human breast cancer cells (Parl 2003). *In vitro* studies have shown that DNA (cytosine-5) methyltransferase (DNMT1) interacts physically with either histone deacetylase 1 (HDAC1) or histone deacetylase 2 (HDAC2) and that co-treatment with DNMT1 and HDAC inhibitors can synergistically induce ERα gene expression in ERα-negative breast cancer cells (Robertson *et al*. 2000, Rountree *et al*. 2000, Yang *et al*. 2001). Recent findings suggest that pRb2/p130-multimolecular complexes involving HDAC and DNMT can be key elements in the regulation of ERα gene expression (Macaluso *et al*. 2003). Therefore, these proteins may be viewed as promising targets for the development of novel therapeutic strategies in the treatment of breast cancer, especially for those tumors that are ER negative (Davis *et al*. 2000, Yang *et al*. 2000, 2001).

It has also been hypothesized that loss of expression of ERα might be responsible for acquired resistance to tamoxifen. However, it has been demonstrated that patients with expression of ERα and sensitivity to tamoxifen, usually do not lose expression of ERα following development of resistance to tamoxifen. In fact, loss of ERα expression has been demonstrated only in 17–28% of patients with acquired resistance to tamoxifen (Johnston *et al*. 1995, Gutierrez *et al*. 2005). In addition, approximately 20% of tamoxifen-resistant patients will eventually respond to second-line treatment with AIs or fulvestrant (Howell *et al*. 2002, Osborne *et al*. 2002). These observations imply that the majority of patients with acquired resistance to tamoxifen still express ER.

Mutations of ERα might also affect the response to anti-estrogens. However, such mutations have rarely been found in human primary breast carcinomas, and therefore they are not likely to contribute significantly to resistance to agents that modulate the function of the receptor, such as SERMs. In addition, many of these mutations have been detected in patients that were clinically classified as ER negative (Herynk & Fuqua 2004). Fuqua's group reported the occurrence of a single amino acid substitution changing lysine 303 to arginine mutation in 20 out of 59 hyperplastic breast lesions (Fuqua *et al*. 2000). Such mutation leads to a hypersensitive ERα that shows enhanced binding of co-activators in the presence of low estrogen levels. However, the frequency of this mutation in primary breast carcinomas needs to be explored in a larger number of patients.

PgR expression has recently been shown to be associated with increased benefit from adjuvant tamoxifen (Bardou *et al*. 2003). In particular,

ER-positive/PgR-positive patients showed a significantly higher reduction of relative risk of recurrence and death as compared with ER-positive/PgR-negative patients. Interestingly, analysis of data from adjuvant treatment of postmenopausal breast cancer patients also shows that AIs are impressively more effective than tamoxifen in ER-positive/PgR-negative patients (Dowsett 2003). In this respect, it has been hypothesized that loss of PgR expression might reflect a sustained activation of growth factor signaling pathways (Osborne *et al.* 2005). In fact, it has been demonstrated that activation of the PI3K/AKT pathway — which might be determined by either IGF-1R or EGFR/ErbB-2 tyrosine kinases in breast cancer cells — downregulates the transcription of the PgR gene (Cui *et al.* 2003). Increased growth factor signaling might also lead to increased non-genomic activities of ERα, which are enhanced following tamoxifen-binding to the receptor, as we will discuss below. This hypothesis might explain the superiority of AIs over tamoxifen in this subset of patients.

ERβ

The role of ERβ in resistance to endocrine therapy has not been completely elucidated yet. In fact, it has been demonstrated that when ERβ is bound to tamoxifen, raloxifen or the anti-estrogen ICI 164 384, transcription of AP-1-dependent genes is increased (Paech *et al.* 1997). In addition, the levels of ERβ transcripts were found to be approximately 2-fold higher than ERα levels in tamoxifen-resistant patients as compared with tamoxifen-sensitive tumors (Speirs *et al.* 1999). Taken together, these findings might suggest a role for ERβ in the resistance to endocrine therapy. However, other studies have demonstrated that ERβ has a negative effect on ERα-promoted transcription (Hall & McDonnell 1999, Pettersson *et al.* 2000). Finally, a recent report has shown that the levels of ERβ mRNAs are not correlated with response or resistance to toremifene in breast cancer patients that received hormonal therapy as neoadjuvant treatment (Cappelletti *et al.* 2004). Therefore, no conclusions on the role of ERβ in the resistance to endocrine therapy can be drawn at this point.

Pharmacogenomic mechanisms

Metabolism of tamoxifen in agonistic metabolites might be involved in the resistance to this drug. In fact, a novel active metabolite of tamoxifen has recently been identified (4-hydroxy-*N*-desmethyl-tamoxifen or endoxifen) in patients that received adjuvant tamoxifen (Stearns *et al.* 2003). Interestingly, the

baseline levels of this metabolite were significantly higher in patients carrying the wild-type CYP2D6, a cytochrome P450 enzyme, as compared with women carrying a variant allele (*4, 6, 8). In patients with the wild-type allele the levels of endoxifen were significantly reduced by co-treatment with paroxetine, a selective serotonin reuptake inhibitor which is prescribed to alleviate tamoxifen-associated hot flashes and that can inhibit CYP enzymes. More recently, it has been shown that the recurrence-free survival of ER-positive breast cancer patients homozygous for the wild-type CYP2D6 allele was equal between tamoxifen-treated and tamoxifen-untreated patients (Wegman *et al.* 2005). In contrast, patients carrying at least one CYP2D6*4 allele showed a better outcome when treated with tamoxifen, as compared with non-treated patients. Taken together, these findings support a role for cytochrome P450 enzyme variants in regulating the response to tamoxifen.

Co-regulators

The transcriptional regulatory activity of ER is significantly influenced by the formation of multi-molecular complexes that comprehends either co-activators or co-repressors. In this respect, a recent study demonstrated that high levels of expression of the co-activator AIB1 are associated with a shorter disease-free survival (DFS) in patients receiving tamoxifen as adjuvant treatment (Osborne *et al.* 2003). This outcome might also be due to important interactions between AIB1 and ErbB-2 that we will describe later. Interestingly, in untreated patients high levels of AIB1 were associated with a better outcome. These findings support laboratory studies suggesting that high levels of co-activators might enhance the agonistic effect of tamoxifen and therefore contribute to tamoxifen resistance.

Co-repressors are usually recruited by ER when an antagonist such as tamoxifen is bound to it (Lavinsky *et al.* 1998, McKenna *et al.* 1999). Although *in vitro* studies have suggested that reduced levels of co-repressors might be associated with resistance to tamoxifen, no clinical data are available at this point that might sustain this hypothesis.

Adaptation to estrogen withdrawal

Pre-clinical and clinical findings strongly support the hypothesis that a major mechanism of resistance to endocrine therapy is the acquisition by breast cancer cells of an increased sensitivity to estrogen. As we will discuss later, the adaptation to estrogen withdrawal

might be involved in the resistance to both tamoxifen and AIs. However, different molecular mechanisms have been hypothesized to cause this phenomenon.

Richard Santen's group have established an *in vitro* model of long-term estrogen deprivation (LTED) by culturing MCF-7 cells in estrogen-free medium to mimic the effects of ablative endocrine therapy (Santen *et al.* 2003). These cells adapted to grow in very low levels of estrogen that derive from plastic tissue culture dishes. The hypersensitivity of these cells was confirmed by the ability of estrogen to stimulate their proliferation at concentrations four-log lower as compared with wild-type MCF-7 cells (Masamura *et al.* 1995). In this model, Santen and co-workers found that the non-genomic ERα functions are significantly enhanced. In particular, LTED produced a 4- to 10-fold increase in the levels of ERα in MCF-7 cells, and the levels of ERα translocated at the plasma membrane are elevated. Growth factor signaling is significantly increased in LTED cells. Following estrogen treatment, ERα rapidly associates and phosphorylates Shc, an adaptor protein that is involved in tyrosine kinase receptor signaling. In this regard, association of ERα, Shc and the IGF-1R has been demonstrated in LTED cells (Song *et al.* 2004). In addition, these cells show increased activation of both *src* and the *ras/raf*/MEK/MAPK signaling pathways that appear as key events in Erα-induced signaling in LTED cells (Song *et al.* 2002*a,b*). However, the demonstration that treatment of LTED cells with fulvestrant blocks MAPK activation, suggests that MAPK is a downstream effector of the ERα-induced non-genomic signaling activated in these cells (Song *et al.* 2002*a*, Santen *et al.* 2003). Increased activation of the PI3K/AKT pathway has also been demonstrated in this model of LTED (Yue *et al.* 2003). In this respect, activation of both MAPK and AKT has been associated with resistance to endocrine therapy and a worse outcome in breast cancer patients (Gee *et al.* 2001, Perez-Tenorio *et al.* 2002). Additional studies from Santen's group did not exclude the possibility that an increase in basal levels of transcription of ERα-regulated genes might have a role in adaptive hypersensitivity, although it does not represent the main mechanism involved in this phenomenon.

A different mechanism of resistance to LTED has been hypothesized by Mitch Dowsett's group (Johnston & Dowsett 2003). In their model, LTED of MCF-7 cells initially produces a phase of estrogen hypersensitivity with cells showing increased levels of expression of ERα and responding to concentrations of estrogen below 10^{-13} M (Chan *et al.* 2002). In contrast with the findings of Santen, the model developed by Dowsett showed an enhanced transcriptional activity of ERα that is associated with an increase in the activation of growth factor pathways, which in turn trans-activate ERα. Interestingly, after prolonged culture in the absence of estradiol (80 weeks), ERα appears to function independently from exogenous estradiol. This independent state has been hypothesized to be due to a supersensitivity of LTED to residual estrogen present in the medium (Chan *et al.* 2002, Martin *et al.* 2003). The estrogen-supersensitive breast cancer cells, defined as LTED-I, were maintained in insulin-containing medium, and the removal of insulin resulted in significant reduction of their growth and restoration of a hypersensitivity state. In this regard, the observations that LTED-I cells express elevated ERα, that the transcriptional activity of EREs is significantly higher as compared with wild-type cells and that their growth is significantly inhibited by fulvestrant suggest that ERα has a key role in the insulin-dependent growth of these cells. Indeed, it has been shown that estrogen-supersensitive cells show increased levels of phosphorylation of ERα at serine 118, a site that can be phosphorylated by different intracellular kinases. Dowsett's group has also demonstrated that supersensitive cells show an increased IGF-1R and ErbB-2 signaling, which is associated with increased MAPK activation (Martin *et al.* 2003). Blockade of either MAPK or EGFR/ErbB-2 signaling produced significant reduction in both proliferation and ERα transcription in LTED-I. However, inhibition of either MEK/MAPK or PI3K/AKT signaling did not block phosphorylation of ERα at serine 118, suggesting that additional kinases might be involved in this phenomenon. In conclusion, Dowsett's data suggest that different growth factor signaling pathways enhance ERα genomic activity in LTED-I cells.

More recently, an *in vitro* model of resistance to estrogen withdrawal (MCF-7X cells) has been developed by Nicholson and co-workers (Nicholson *et al.* 2004). Interestingly, these cells did not show any evidence of estrogen hypersensitivity. However, MCF-7X cells carried a functional ERα, and their growth was inhibited by fulvestrant, implying that the ERα pathway is still involved in the proliferation of these cells. The growth of MCF-7X cells was found to be mainly supported by the PI3K/AKT pathway (Nicholson *et al.* 2004). However, no activation of EGFR/ErbB-2 or IGF-1R signaling was demonstrated in these cells, suggesting that the phenomenon of adaptation to estrogen withdrawal might occur without increased sensitivity to estrogen or activation of classical growth factor receptors.

We might speculate that estrogen-hypersensitive cells obtained following mid-term estrogen deprivation might represent a model of tamoxifen resistance, whereas LTED-I cells that have been obtained following longer estrogen deprivation and that show supersensitivity to residual estrogen are more likely to represent a model of resistance to AIs. Indeed, several findings suggest that the process of adaptive hypersensitivity obtained by estrogen withdrawal and the acquired resistance to tamoxifen resulting from long-term exposure to this drug share some common features. In fact, it has been demonstrated that following long-term exposure to tamoxifen (>5 years) the growth of tamoxifen-resistant MCF-7 xenografts in nude mice is inhibited by estrogen (Yao *et al.* 2000). More recently, Richard Santen's group have confirmed that long-term exposure of MCF-7 xenografts to tamoxifen induces hypersensitivity to estrogen (Berstein *et al.* 2004). A common feature of tamoxifen-resistant models *in vivo* is that their growth is stimulated by tamoxifen (Gottardis & Jordan 1988, Osborne *et al.* 1991). In contrast, pure anti-estrogens such as fulvestrant inhibit the growth of these xenografts, thereby confirming that mechanisms of adaptation to tamoxifen include the enhanced agonistic effects of this drug (Gottardis *et al.* 1989, Osborne *et al.* 1994). As we will discuss below, the agonistic effect of tamoxifen in these resistant tumors is probably due to the enhanced growth factor signaling that is associated with acquired resistance to this drug (Schiff *et al.* 2003, 2004, Nicholson *et al.* 2004). In particular, both genomic and non-genomic activities of the tamoxifen–ERα complex are involved in this phenomenon. In fact, phosphorylation and activation of ERα by intracellular kinases leads to increased transcriptional activity of the receptor, increased production of estrogen-regulated growth factors such as TGFα and AR, and increased growth-factor-driven signaling; therefore reinforcing the above-described loop that will finally drive cell proliferation.

The phenomenon of acquired resistance to AIs has also recently been addressed by using MCF-7 cells stably transfected with human aromatase gene (MCF-7Ca) (Long *et al.* 2004a, Brodie *et al.* 2005). MCF-7Ca tumors of mice treated with letrozole initially regressed but gradually resumed growth and had doubled in volume by week 21 of treatment (Brodie *et al.* 2005). Interestingly, it was found that letrozole-resistant tumors did not respond to either tamoxifen or fulvestrant as second-line therapy (Long *et al.* 2004a). These findings are in apparent contrast with the above-mentioned reports from LTED *in vitro* experiments that suggested activity of fulvestrant in cells that had

undergone adaptive supersensitivity, a condition that *in vitro* resembles resistance to AIs (Johnston & Dowsett 2003). In this regard, previous reports from the same group showed that aromatase-overexpressing xenografts were sensitive to treatment with fulvestrant when tumors were transplanted in a different mouse and treatment with letrozole was suspended for a short period prior to treatment with fulvestrant (Long *et al.* 2002). In the subsequent report, letrozole-resistant tumors were immediately treated with fulvestrant. In addition, mice were switched off letrozole before treatment with fulvestrant (Long *et al.* 2004a, Brodie *et al.* 2005). In this respect, it has been shown that in LTED cells fulvestrant is effective in the presence of low concentrations of estrogen, whereas its efficacy is reduced if cells are treated with higher levels of estrogen. In agreement with these findings, treatment of MCF-7Ca xenografts with a combination of fulvestrant and letrozole resulted in tumor regression that was sustained for a significantly longer period as compared with fulvestrant or letrozole alone (Brodie *et al.* 2005). This observation supports the hypothesis that patients with resistance to AIs should receive fulvestrant in addition to these compounds, rather than fulvestrant alone. The Study of Faslodex, Exemestane and Arimidex (SoFEA) trial, will clinically address this question.

Preliminary data on the molecular characteristics of letrozole-resistant tumors are available. Although the levels of ER were decreased by 50% in letrozole-resistant tumors as compared with control tumors, PgR expression was modestly increased implying active ER signaling in these cells. Indeed, the levels of phosphorylation of ERα in serine 167 were significantly increased in letrozole-resistant tumors, suggesting ligand-independent activation of ER (Brodie *et al.* 2005). In addition, the levels of expression of ErbB-2 and the adaptor protein Grb-2, as well as the levels of phosphorylation of Shc and MAPK, were also increased in tumors growing on letrozole. Taken together, these preliminary findings suggest that acquired resistance to letrozole involves activation of growth factor signaling that might induce ligand-independent activation of ERα. Interestingly, these phenomena are similar to those observed in MCF-7 cells following prolonged LTED and development of estrogen supersensitivity.

Growth factor signaling in resistance to endocrine therapy

The above-summarized data clearly demonstrate that growth factor signaling plays an important role in

the adaptation of breast cancer cells to estrogen withdrawal. However, growth factor signaling is involved in both *de novo* and acquired resistance of breast cancer cells to endocrine manipulation through different mechanisms that we will discuss in this section.

The main mechanism of *de novo* resistance of breast cancer cells to endocrine therapy is loss of expression of ER. In this regard, the levels of expression of EGFR, ErbB-2 and TGFα are generally higher in ER-negative breast cancer as compared with ER-positive tumors (Normanno *et al.* 1994). Evidence suggests that activation of growth factor signaling might indeed reduce ERα expression and/or function. For example, treatment with EGF, IGF-I, TGFβ or phorbol myristate acetate (TPA) reduces the levels of ERα mRNA and protein in MCF-7 cells (Martin *et al.* 1995, Stoica *et al.* 1997, 2000*a,b*). Increased signaling through EGFR, PI3K/AKT, PKA and PKC is involved in this phenomenon. Furthermore, heregulin, which can activate both EGFR and ErbB-2 through formation of heterodimers with either ErbB-3 or ErbB-4, has been shown to depress ERα or its transcriptional activity (Mueller *et al.* 1995, Tang *et al.* 1996). Analogously, transfection of constitutively active ErbB-2, Raf1 or MEK resulted in significant reduction in the expression of ERα mRNA and protein, and in marked suppression in the transcription of estrogen-regulated genes, events leading to the development of an estrogen-independent phenotype (Liu *et al.* 1995, Pietras *et al.* 1995, El-Ashry *et al.* 1997, Oh *et al.* 2001). Taken together, these data suggest that growth factor signaling might contribute to transcriptional repression of ERα expression in breast cancer cells, thus resulting in resistance to endocrine treatment.

The involvement of ErbB-2 in *de novo* resistance of breast cancer cells to tamoxifen has long been hypothesized (Benz *et al.* 1993, Pietras *et al.* 1995). More recently, Shou *et al.* (2004) demonstrated that in the presence of low levels of estrogen tamoxifen acts as an agonist for MCF-7/HER2-18 cells. In these cells, tamoxifen as well as estrogen induces activation of EGFR/ErbB-2 signaling, which leads to activation of both MAPK and AKT signal transduction pathways. These intracellular kinases phosphorylate and functionally activate both ERα and the co-activator AIB1. Furthermore, in MCF-7/HER2-18 cells treatment with tamoxifen increased the expression of estrogen-regulated genes, nearly as well as estrogen itself. This phenomenon is due to the ability of the tamoxifen–ERα complex to recruit co-activators such as AIB1 rather than co-repressors in ErbB-2-over-expressing cells. Interestingly, all these phenomena

could be blocked by treatment with the EGFR-tyrosine kinase inhibitor gefitinib, suggesting that EGFR/ErbB-2 signaling is directly involved in the growth-promoting activity of tamoxifen in ErbB-2-overexpressing cells. In this respect, gefitinib was highly effective in inhibiting the tamoxifen-induced growth of MCF-7/HER2-18 cells, whereas it had little effect on estrogen-induced growth. The above-mentioned findings are in agreement with clinical observations indicating that tumors that co-express ErbB-2 and AIB1 have a poor outcome when treated with tamoxifen (Osborne *et al.* 2003). Furthermore, preliminary studies have shown that patients that express either ErbB-2 or EGFR are relatively resistant to tamoxifen, whereas they are sensitive to AIs (Dowsett *et al.* 2001, Ellis *et al.* 2001).

Experimental evidence provided by Robert Nicholson's group has demonstrated that EGFR/ErbB-2 signaling is involved in the acquired tamoxifen resistance of breast cancer cells (Nicholson *et al.* 2004). This group has established an *in vitro* tamoxifen-resistant model derived from MCF-7 cells. These cells showed increased levels of expression of EGFR and ErbB-2, increased activation of EGFR/ErbB-2 heterodimers and increased phosphorylation of MAPK, AKT and nuclear ERα in serine residues 118 and 167 (Britton *et al.* 2003, Hutcheson *et al.* 2003, Knowlden *et al.* 2003*a*, Jordan *et al.* 2004). The growth of these cells was significantly inhibited by treatment with either gefitinib or trastuzumab (Gee *et al.* 2003). Interestingly, phosphorylation of ERα and ERα-induced transcription was increased by exogenous EGF-like peptides and was blocked by treatment with gefitinib. The enhanced transcriptional activity of ERα produced an increased synthesis of TGFα and AR that sustains the EGFR/ErbB-2 autocrine loop operating in tamoxifen-resistant cells (Hutcheson *et al.* 2003). An involvement of IGF-1R signaling in tamoxifen-resistant cells has also been shown. In fact, it has recently been reported that IGF-II induces an increase of both IGF-1R and EGFR activation in tamoxifen-resistant cells (Knowlden *et al.* 2003*b*, Hutcheson *et al.* 2004). Taken together, these findings suggest that enhanced growth factor signaling, which induces both genomic and non-genomic activities of ER, is the main mechanism of acquired resistance to tamoxifen. Notably, these molecular mechanisms are similar to those observed in estrogen-hypersensitive cells. Interestingly, MCF-7 cells adapted to grow in fulvestrant showed an increased EGFR signaling, suggesting that growth factor signaling also plays a central role in the resistance to this novel compound (McClelland *et al.* 2001). In agreement with these
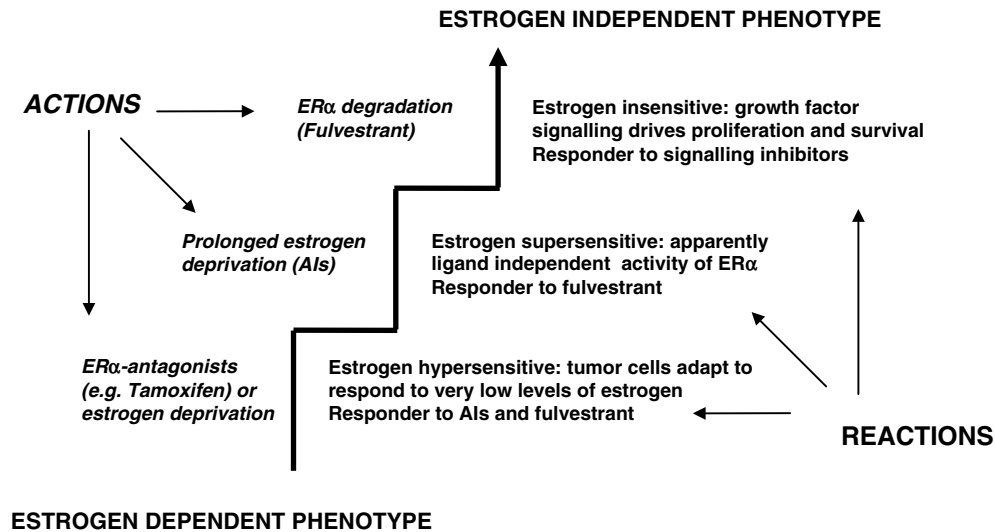
in vitro findings, increased levels of expression of ErbB-2 and increased activation of p38-MAPK have been described in patients with acquired resistance to tamoxifen (Gutierrez *et al.* 2005). Interestingly, activation of p38-MAPK was observed in MCF-7 xenografts treated with estrogen deprivation plus tamoxifen, but not with estrogen deprivation alone, suggesting that p38-MAPK activation is a peculiar phenomenon of resistance to tamoxifen. In addition, preliminary results of a phase-II clinical trial of gefitinib in tamoxifen-resistant advanced breast cancer patients suggest clinical activity of anti-EGFR agents in this subset of patients (Robertson *et al.* 2003*a*). However, larger clinical trials are needed to confirm these preliminary findings.

Activation of growth factor signaling might also lead to the development of an estrogen-independent phenotype as an ultimate escape mechanism from the anti-tumor activity of anti-estrogen drugs. In this respect, the development of an estrogen-independent phenotype appears to be a complex phenomenon. For example, overexpression of TGFα, EGFR or, more recently, the EGF-CFC protein CRIPTO-1 in estrogen-responsive breast cancer cells was not able to induce an estrogen-independent phenotype (Clarke *et al.* 1989, Valverius *et al.* 1990, Normanno *et al.* 2004*a*). In contrast, overexpression of ErbB-2 or of heregulin, which can induce ErbB-2 transactivation by binding to ErbB-3 and ErbB-4, resulted in estrogen-independent growth and resistance to anti-estrogen (Liu *et al.* 1995, Pietras *et al.* 1995, Tang *et al.* 1996, Atlas *et al.* 2003). In addition, constitutive activation of signaling molecules downstream of tyrosine kinase receptors such as MEK or *raf*-1 also resulted in estrogen independence and resistance to anti-estrogens, as above described (El-Ashry *et al.* 1997, Oh *et al.* 2001). The reduced ability of EGFR to induce suppression of ERα expression and estrogen independence as compared with ErbB-2 could be due to the low levels of activation of EGFR in the absence of exogenous ligands (Oh *et al.* 2001). Taken together, these data suggest that prolonged and sustained activation of growth factor signaling pathways might finally lead to the development of an estrogen-independent phenotype. Suppression of ERα expression through the mechanisms that we have above described might have a role in this phenomenon. In this regard, Nicholson *et al.* (2004) have recently reported that fulvestrant-resistant cells lost expression of ERα following prolonged exposure to anti-estrogen. Expression of ERα did not resume in fulvestrant-resistant cells following removal of the drug, and it was associated with the development of an estrogen-independent

phenotype. Interestingly, these authors reported that fulvestrant-resistant cells were more prone to develop an ER-negative phenotype as compared with tamoxifen-resistant cells or cells that were long-term cultured in the absence of estrogen. These observations suggest that the development of a specific phenotype might depend on the type of endocrine agent that is employed.

Trying to summarize the mechanisms of resistance to endocrine manipulation: the action–reaction hypothesis

As described in the above paragraphs, several different mechanisms are involved in the resistance of breast cancer cells to endocrine manipulation. Such mechanisms can lead breast cancer cells from an estrogen-dependent phenotype — which responds to endocrine manipulation — to a non-responder phenotype, and eventually to an estrogen-independent phenotype. However, such phenotypic modifications are not spontaneous but are induced in breast cancer cells by the selective pressure of hormonal agents. In fact, compelling evidence suggests that for each 'action' carried out with the aim of blocking estrogen stimulation of breast cancer cells (i.e. treatment with anti-estrogen), there are one or more corresponding 'reactions' that tumor cells can use to escape our attempts to block their growth: estrogen hypersensitivity associated with increased transcriptional activity of ERα and/or increased non-genomic activity of ERα; estrogen supersensitivity; increased growth factor signaling; suppression of ERα expression; and finally estrogen independence. Although the 'paths' that tumor cells can follow to escape hormonal treatment are various and may depend on different factors, what we may hypothesize is that this action–reaction rule leads to a step-wise increase in the ability of breast cancer cells to escape endocrine manipulation (Fig. 2). Indeed, untreated ER-positive breast carcinomas are likely to be sensitive to treatment with any hormonal agent, i.e. tamoxifen, AIs and fulvestrant. The majority of breast cancer patients have been treated up to now with tamoxifen. Breast cancer cells that acquire resistance to tamoxifen show phenotypic characteristics that are similar to the initial adaptive hypersensitivity described by Santen *et al.* (2003) and Dowsett (2003). In these cells growth factor signaling is increased and tamoxifen might behave as an agonist. Pre-clinical and clinical observations suggest that the growth of tamoxifen-resistant tumors can be blocked by either AIs, which produce a more pronounced and prolonged estrogen deprivation, or by fulvestrant that

ESTROGEN INDEPENDENT PHENOTYPE

ACTIONS → *ERα degradation (Fulvestrant)*

Estrogen insensitive: growth factor signalling drives proliferation and survival Responder to signalling inhibitors

Prolonged estrogen deprivation (AIs)

Estrogen supersensitive: apparently ligand independent activity of ERα Responder to fulvestrant

ERα-antagonists (e.g. Tamoxifen) or estrogen deprivation

Estrogen hypersensitive: tumor cells adapt to respond to very low levels of estrogen Responder to AIs and fulvestrant

REACTIONS

ESTROGEN DEPENDENT PHENOTYPE

Figure 2 The action–reaction hypothesis: at each 'action' aiming to block estrogen stimulation of breast cancer cells (i.e. treatment with anti-estrogen), there are one or more corresponding 'reactions' that tumor cells can use to escape our attempts to block their growth. AIs, aromatase inhibitors.

destabilizes ERα. However, deprivation of estrogen for a longer period leads to the development of an estrogen-supersensitive phenotype; this phenotype has been described by Dowsett's group and is likely to represent a model of resistance to AIs. In these cells, activation of both EGFR/ErbB-2 and IGF-1R signaling occurs. However, estrogen-supersensitive cells are still growth inhibited by treatment with fulvestrant, demonstrating a role of ERα in the growth of these cells. In this regard, findings from Nicholson's group suggest that long-term exposure to fulvestrant might lead to loss of expression of ERα and development of an estrogen-independent phenotype in breast cancer cells. In any case, the development of an estrogen-independent phenotype is the ultimate mechanism of resistance to hormonal therapy, and it most probably occurs independently from the type of hormonal agent following long-term treatment. In fact, activation of different growth factor-driven signaling pathways accompanies each step of the development of resistance to endocrine manipulation. Growth factor signaling down-regulates both ERα and PgR transcription, and can substitute for estrogen in supporting the growth and the survival of breast cancer cells.

Of course, breast cancer cells might follow different 'paths' in developing a resistant or an estrogen-independent phenotype, depending on the type of hormonal agent, on the genetic background and other, as yet undefined, factors. For example, the observation that only a subset of tamoxifen-resistant patients respond to treatment with AIs implies that some of the tumors progressing on tamoxifen therapy may have acquired some degree of estrogen-independent growth. It is also important to note that this model has been hypothesized by using information deriving mainly from *in vitro* studies. In this respect, the pharmacokinetics of *in vitro* studies is 'simplified' since tumor cells are directly exposed to pharmacologically active concentrations of drugs. Furthermore, other variables such as the metabolism of drugs are not taken into account in such models. Of course, experiments in animals are more informative. However, the pharmacokinetics and the pharmacodynamics of many drugs in mice are not superimposable to humans.

Finally, our model is consistent with the observation that agents that are active in advanced disease and in resistant tumors might not be as active in first-line therapy since they function by blocking mechanisms that develop during acquisition of resistance. For example, fulvestrant is able to block the growth of both estrogen-hypersensitive and -supersensitive cells. In clinical trials fulvestrant is not more effective than tamoxifen as a first-line therapy, whereas it is as effective as AIs as a second-line treatment of advanced breast cancer and it is also active in AI-resistant patients, as we will discuss below. In this respect, it has been hypothesized that tamoxifen might be more effective than fulvestrant in first-line therapy because the tamoxifen–ER complex might compete with other transcription factors in binding to estrogen-regulated genes, therefore blocking their function (McDonnell

2005). This phenomenon does not occur for fulvestrant or similar compounds that destabilize ER. However, in advanced disease the agonist effects of tamoxifen increase; and in this setting fulvestrant, which has no agonistic effects, is more effective. An additional example derives from clinical and pre-clinical experiences with EGFR-tyrosine kinase inhibitors such as gefitinib. The results of clinical trials of gefitinib in breast cancer are disappointing, with a disease control rate of approximately 10% (Normanno *et al.* 2004*b*). In contrast, preliminary results of a phase-II trial of gefitinib in tamoxifen-resistant patients suggest a much higher disease control rate in this specific subset of patients (Robertson *et al.* 2003*a*). We have described the mechanisms involved in this phenomenon above. This is a clear example of selection of EGFR-dependent cells following treatment with tamoxifen. However, EGFR signaling might contribute to tumor growth and resistance to endocrine therapy in untreated patients as well. In this regard, the results of a randomized phase-II trial of gefitinib plus placebo versus gefitinib plus anastrozole as neoadjuvant therapy in ER-positive and EGFR-positive breast cancer patients have recently been published (Polychronis *et al.* 2005). Expression of EGFR occurs in approximately 30% of ER-positive patients, and it has been reported to be associated with resistance to tamoxifen (Salomon *et al.* 1995, Dowsett *et al.* 2001, Ellis *et al.* 2001). In this specific subset of patients, gefitinib was able to reduce by 92.4% the Ki67 labeling index and to induce partial responses in 14/28 patients. This phenomenon was also associated with a significant reduction in the levels of phosphorylation of ER at serine 118. The combination of anastrozole plus gefitinib produced a greater reduction in Ki67 labeling index as compared with gefitinib alone, and partial remissions in approximately 55% of the patients. These results clearly demonstrate that blockade of EGFR in ER-positive tumors that express this receptor might lead to significant reduction of tumor growth.

As we will describe in next paragraphs, our hypothesis is largely confirmed by the results of clinical trials in breast cancer patients. More importantly, this evidence is the basis for novel therapeutic approaches for breast carcinoma with combinations of anti-hormones and signal transduction inhibitors.

Clinical trials with novel anti-estrogen drugs

In the next paragraphs, data coming from recent clinical trials of hormonal treatment of breast cancer, both in the metastatic and adjuvant settings, are summarized. In both settings, the major changes are due to the availability of third-generation AIs that have been brought into large phase-III trials for use as adjuvant treatment following the promising results reported in metastatic disease. In addition, in the metastatic setting, fulvestrant — the only SERD available for treatment of patients — is being studied to establish its optimal position in the strategy of hormonal treatments.

Metastatic disease

There are three third-generation AIs available for clinical use: the non-steroidal anastrozole and letrozole, and the steroidal AI exemestane. These drugs have been registered for treatment of advanced breast cancer following the results of randomized phase-III clinical trials. These trials can be broadly divided into two sets. The first set includes trials of second-line therapy, dedicated to patients who progressed after tamoxifen treatment (Buzdar *et al.* 1998, 2001, Dombernowsky *et al.* 1998, Kaufmann *et al.* 2000). In these trials, AIs were compared with megestrol acetate; outcomes were never worse for AIs and in some cases (overall survival (OS) with anastrozole, time to progression with exemestane, response rate with letrozole) were significantly improved. More importantly, third-generation AIs showed advantages in comparison with tamoxifen in first-line treatment clinical trials. Anastrozole, indeed, was compared with tamoxifen in two trials (Bonneterre *et al.* 2000, Nabholtz *et al.* 2000); while in one of these there was no difference between anastrozole and tamoxifen for all study end-points, in the other anastrozole significantly prolonged time to progression. Letrozole produced higher response rate and longer time to progression than tamoxifen in the largest trial of this group, including more than 900 patients (Mouridsen *et al.* 2003). Finally, exemestane resulted in higher response rate and longer time to progression in a randomized phase-III trial of the European Organization for Research and Treatment of Cancer (EORTC; Paridaens *et al.* 2004). Overall, AIs were slightly more effective than tamoxifen in metastatic breast cancer; however, the wide use of tamoxifen in an adjuvant setting has rendered AIs the most useful drugs for the treatment of this stage of disease.

A much lower number of trials have been performed for the clinical development of fulvestrant. In a small phase-II trial, the drug had shown activity with an objective response in 7 out of 19 patients (37%) with advanced breast cancer, resistant to tamoxifen (Howell

et al. 1996). Following this result, the efficacy of fulvestrant has been compared with anastrozole in two randomized phase-III trials, dedicated to post-menopausal women with advanced breast cancer which had progressed after prior endocrine therapy (Howell *et al.* 2002, Osborne *et al.* 2002). The results of these trials demonstrate that fulvestrant is well tolerated and is at least as effective as anastrozole. A prospectively planned, combined analysis of data from those two clinical trials showed that median time to progression was 5.5 months and 4.1 months, and overall response rates were 19.2 and 16.5% for fulvestrant and anastrozole respectively (Robertson *et al.* 2003*b*). Based on these results, fulvestrant has recently been approved by the US Food and Drug Administration and the European Agency for the Evaluation of Medicinal Products (EMEA) for the treatment of hormone-receptor-positive, metastatic breast cancer in postmenopausal women progressing on prior anti-estrogen therapy. However, non-inferiority of fulvestrant could not be demonstrated in a randomized trial versus tamoxifen with 587 patients at their first-line hormonal treatment for metastatic breast cancer (Howell *et al.* 2004). Median time to progression was 6.8 and 8.3 months for fulvestrant and tamoxifen respectively, and objective response rates were 31.6 and 33.9% respectively.

Based on the consideration that treatment of metastatic disease is palliative and that for patients with ER-positive tumors it is reasonable to exploit the potential activity of all available endocrine treatments, the choice of the optimal sequence is of clinical relevance (Piccart *et al.* 2003, Gradishar 2004). This choice is, of course, affected by which treatment has been given in the adjuvant setting. Currently, because most metastatic patients with ER-positive tumors have received adjuvant tamoxifen and the use of fulvestrant is not yet widespread, the choice is practically limited to AIs, with some attempts at sequencing a non-steroidal AI (anastrozole or letrozole) and the steroidal exemestane, particularly in cases of patients who respond to the first line. The introduction of fulvestrant is actually opening new scenarios. The two above-reported clinical trials comparing fulvestrant with anastrozole support the hypothesis that its efficacy is comparable with that of anastrozole in the treatment of patients resistant to tamoxifen. In support of its use before AIs there are also data showing that AIs still retain some activity after progression on second-line fulvestrant (Cheung *et al.* 2002, Vergote *et al.* 2003). However, it should be emphasized that such activity is quite low with a lower than 10% rate of partial responses and about 40% rate of clinical

benefit, including stable disease lasting more than 24 weeks. This observation is apparently in contrast with pre-clinical findings suggesting that resistance to fulvestrant is associated with activation of EGFR/ErbB-2 signaling and estrogen independence (McClelland *et al.* 2001, Nicholson *et al.* 2004). However, it is conceivable that at suspension of treatment with fulvestrant, some patients might return to a hypersensitive state in which AIs are active. Furthermore, resistance to fulvestrant might be due to different mechanisms that might alter the intra-tumoral concentration of the drug or its activity. For example, it has been recently demonstrated that an intact NEDD8 pathway that leads to ERα ubiquitination and degradation is essential for the anti-proliferative activity of fulvestrant (Fan *et al.* 2003). Therefore, in the latter hypothesis, tumor cells might become resistant to fulvestrant and be still sensitive to anti-estrogen therapy with AIs. Finally, more recent clinical data support the hypothesis that AI-resistant tumors might be still sensitive to fulvestrant with a clinical benefit rate of about 30% (Ingle *et al.* 2004, Perey *et al.* 2004). In conclusion, although some interesting indications have been reported, convincing randomized clinical trials addressing the relative efficacy of different sequences of hormonal treatment in metastatic breast cancer are lacking.

Adjuvant treatment

Following the encouraging results of trials in metastatic disease, the three third-generation AIs have been pushed into phase-III trials of adjuvant treatment. The results of some of these trials are already available, whereas some studies are still ongoing. Based on results of the Oxford Overview (Early Breast Cancer Trialist Group 1998), all trials include tamoxifen given for 5 years as standard treatment and comparator (Fig. 3). Owing to the significant but relatively small advantage observed in clinical trials conducted in patients with metastatic disease, all adjuvant trials are planned to detect small advantages; thus, also in consideration of the relatively low rate of events expected for early breast cancer patients with ER-positive tumors, all the studies have planned to enroll an extremely high number of patients. Thanks to the large sample size, the number of events required for *a priori* planned analyses (either definitive or *ad interim*) might be reached in quite a short time following the end of enrolment. Thus, although none of these trials can be criticized for the statistical methodology that was applied, there is room to consider that clinical maturity of data could not have always been reached
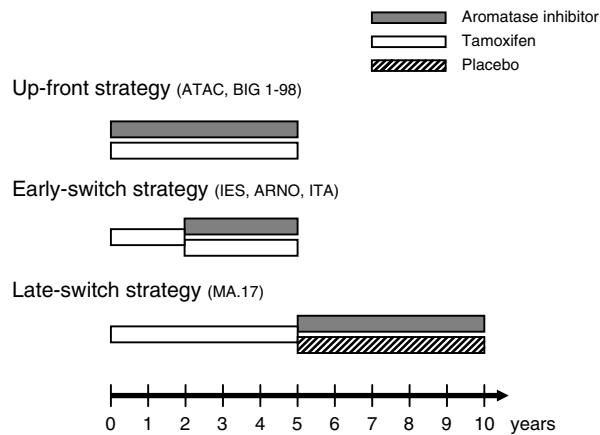
Figure 3 Different strategies employed in clinical trials of AIs in early breast cancer.

at the moment of releasing analysis results. This consideration, of course, becomes important in the presence of statistically significant results that can, in principle, change the patterns of clinical practice.

All the trials that have been reported as of February 2005 have produced positive results for AIs, with the exception of the trials using anastrozole + tamoxifen combined treatment; the results for this treatment were not better than tamoxifen alone in the Arimidex or Tamoxifen Alone or in Combination (ATAC) trial (Baum *et al.* 2002). In this trial, anastrozole given alone for 5 years was more effective than tamoxifen; at the most recent update (Howell *et al.* 2005), after a median follow-up of 68 months (limiting the analysis to 5216 patients with positive hormone receptors), the absolute advantage in DFS for those receiving anastrozole is 2.8% at 5 years, with an overall hazard ratio of 0.83 (95% confidence interval (CI), 0.73–0.94; $P = 0.005$). Interestingly, the size of absolute DFS gain in this study is progressively increasing, going from 1.7% to 2.4, 2.8 and 3.7% in the third, fourth, fifth and sixth year of follow-up respectively. If such a trend is confirmed after longer observation, this phenomenon will be similar to that observed in clinical trials of tamoxifen versus no treatment, where the reduction of the annual odds of recurrence is maintained over a period of time extending about 5 years after the end of active treatment. No effect of anastrozole has yet been seen on OS in the ATAC trial; however, it should be noted that 40% of deaths (331 out of 831) reported in the study were due to causes other than cancer, as a consequence of the patients' age.

A comparison similar to that of the ATAC trial has recently been reported with letrozole, at the 2005

St Gallen Breast Cancer Consensus Conference (BIG 1-98 Collaborative Group 2005). In the Breast International Group (BIG) 1-98 trial four arms are planned: the standard treatment (tamoxifen for 5 years); and three experimental treatments, namely letrozole for 5 years, tamoxifen for 2 years followed by letrozole for 3 years, and letrozole for 2 years followed by tamoxifen for 3 years. Data have been presented for the head-to-head comparison of tamoxifen versus letrozole, summing up patients in the single-drug arms and those in the sequential arms, the latter being censored at the date of drug switch. The *a priori* planned primary core analysis, done with 779 events out of 8010 patients and a median follow-up of 26 months, has shown an absolute 2.6% improvement in DFS at 5 years, corresponding to a hazard ratio of 0.81 (95% CI, 0.70–0.93; $P = 0.003$). As in the ATAC trial, the absolute difference increases during years of follow-up, going from 1.5% at 3 years to 2.2 and 2.6% at 4 and 5 years. As far as OS is concerned, the rate of deaths is probably still too low (4.1% with letrozole and 4.8% with tamoxifen, $P = 0.18$) to observe significant effects.

Exemestane has been studied within the Intergroup Exemestane Study (IES) that was designed to test whether switching to exemestane, after 2–3 years of tamoxifen therapy, was more effective than continuing tamoxifen therapy for the remainder of the 5 years of treatment (Coombes *et al.* 2004). The study included 4742 patients and data were released after the second interim analysis following the recommendation of the Independent Data and Safety Monitoring Committee. With a median follow-up of 31 months and 449 events (recurrence, contralateral breast cancer or death without cancer), there is an absolute advantage in DFS at 3 years after randomization (approximately 5 years after surgery) of 4.7%, with an adjusted hazard ratio of 0.67 (95% CI, 0.56–0.82; $P < 0.001$). No significant effect has been observed yet for OS (hazard ratio 0.89; 95% CI, 0.67–1.13; $P = 0.41$), as for the above-reported trials. Similar results have been reported in two trials with anastrozole that employed a 'switch' approach. In the Italian Tamoxifen Arimidex (ITA) trial, a study with 448 patients enrolled, a 0.35 (95% CI, 0.21–0.63) hazard ratio of recurrence was found favoring the sequential treatment with tamoxifen followed by anastrozole as compared with tamoxifen alone (Boccardo *et al.* 2003). In the combined ABCSG8/ARNO95 trial with 3224 patients, a hazard ratio of recurrence of 0.60 (95% CI, 0.44–0.81; $P = 0.0009$) favoring anastrozole following 2 years of tamoxifen versus tamoxifen has recently been reported (Jakesz *et al.* 2004).

A truly innovative strategy, the so-called 'extended adjuvant treatment' has been tested in the MA.17 trial in which patients who completed 5 years of adjuvant tamoxifen were randomized to receive letrozole or placebo for 5 additional years (Goss *et al.* 2003). This strategy differs from prolongation of adjuvant tamoxifen beyond the fifth year, which has produced negative results and is no longer considered as a treatment option in clinical practice, although the results of some large clinical trials are still awaited. Data from the MA.17 trial were released after the first interim analysis, with 5187 patients enrolled, a median follow-up of 2.4 years and 207 events for DFS analysis. According to protocol plans, a great benefit in DFS, for patients receiving letrozole as compared with those receiving placebo, was the reason for early disclosure of blinding. At 4 years of follow-up (thus at about 9 years from surgery) letrozole produced a 6% difference in DFS (Goss *et al.* 2003); this was slightly reduced to 4.8% in a subsequent updated analysis with 247 events (Goss *et al.* 2004). As far as OS is considered, no significant difference has been seen to date in the whole study group. Following its early closure, the MA.17 study is continuing offering to patients who reach the end of 5 years of letrozole (10 years of adjuvant treatment overall) the option of being randomized to a further 5 years of letrozole or placebo.

All the above adjuvant trials have been reported with subgroup analyses, planned or unplanned, that might be of interest to generate hypotheses for future studies. Such hypotheses, if verified, could help inform the selection of the optimal patients for AI adjuvant treatment and the choice among different available inhibitors. In both the ATAC and ABCSG8/ARNO95 trials (Dowsett 2003, Jakesz *et al.* 2004) the advantage of anastrozole over tamoxifen seems larger in the subgroup of patients with tumors that express ER but not PgR. In the IES study with exemestane (Coombes *et al.* 2004), and in the MA.17 (Goss *et al.* 2004) and BIG 1-98 (BIG 1-98 Collaborative Group 2005) studies, both with letrozole, such a trend is not evident. Another interesting suggestion is that the effect of AIs may be different according to whether patients had or had not received adjuvant chemotherapy before starting adjuvant treatment. Indeed, the effect of anastrozole seems similar to tamoxifen in the ATAC study among patients who had previously received adjuvant chemotherapy. However, it is reasonable that the small number of ER-negative patients who were enrolled in the ATAC trial could represent about one-third of the patients in the subgroup who received adjuvant chemotherapy, and this phenomenon might dilute the effect of anastrozole as compared with tamoxifen, because both drugs are ineffective against ER-negative tumors. In contrast, in the BIG 1-98 trial, the letrozole effect is even more evident (hazard ratio 0.70 versus 0.85) among patients who had received previous adjuvant chemotherapy. In the IES study, the effect of exemestane appears completely independent of previous chemotherapy. However, it must be considered that adjuvant chemotherapy is usually given to ER-positive patients only in the presence of other negative prognostic factors; therefore, such analyses should always be multivariate to understand whether the considered factor has a predictive value *per se* or just because it is correlated with other risk factors.

Due to the relatively good prognosis of ER-positive breast cancer patients and the long duration of adjuvant endocrine treatment, an important issue to consider is the toxicity of AIs. Typically, side effects can be divided in two groups: those appearing during treatment and those that could also potentially be expected after treatment cessation, with a variable time lag. To date we have little information on the latter group of side effects because the median follow-up time of all published trials is still quite short (ranging from 2.5 to 5 years); however, the picture for short- or medium-term side effects is substantially clearer. Gynecological side effects (including vaginal bleeding and discharge) are usually reduced with AIs as compared with tamoxifen; in the ATAC trial, the one with longest follow-up, there was also a significant reduction of the incidence of endometrial cancer, a feared — although rare — side effect of tamoxifen. Reduced levels of estrogens induced by AIs have detrimental effects on bone, as demonstrated by an increase in arthralgia, osteoporosis and clinical fractures in all the studies with the exception of the MA.17 trial, where letrozole is compared with placebo and not with tamoxifen. This supports the hypothesis that the detrimental effects of AIs on bone are particularly evident when they are compared with tamoxifen because the latter has positive effects on bone thanks to its site-specific estrogen-agonistic mechanism of action. Another important issue is that of cardiovascular side effects. Overall, thromboembolic vascular side effects are reduced with AIs. However, all three studies comparing AI with tamoxifen report a higher, although not significant, rate of cardiac events. For example, in the BIG 1-98 study 20 deaths due to stroke or cardiac events have been reported with letrozole as compared with 7 in the tamoxifen arm (Thurlimann *et al.* 2005). Of course, it must be underlined that the follow-up in adjuvant trials is still too short to derive

definitive conclusions, and that cross-trial comparisons of different AIs is not correct and should not be done.

A quality of life analysis has been performed in a subgroup of patients participating in the ATAC study (Fallowfield *et al*. 2004); while global scores of the applied measures did not vary significantly between the anastrozole and tamoxifen arms, the pattern of patient-reported side effects showed significant differences. For example, while vaginal discharge, irritation and bleeding are reduced with anastrozole, vaginal dryness is significantly worsened — together with pain or discomfort with intercourse; in addition, a significant loss of interest in sex is reported. Further quality of life analyses are required and consideration of such effects should constitute part of the information to be given to patients who are faced with a treatment choice of an endocrine adjuvant treatment for breast cancer.

In conclusion, thanks to the large clinical trials reported to date, it is clear that the addition of AIs — in various forms — to the adjuvant treatment of breast cancer does improve patients' outcomes. However, there are still open questions that need to be addressed in order to choose the best strategy of treatment. First, we do not know whether different AIs vary in their efficacy and safety because there are no head-to-head comparisons in the adjuvant setting, and data are also scanty in the metastatic setting. Secondly, different strategies have been used (AI upfront, early and late switch from tamoxifen to AI) but the results of direct comparisons are not available yet. Thirdly, subgroup analyses have produced discordant results that cannot be explained because of the lack of direct comparisons among AIs. Finally, important questions, such as possible interactions with ErbB-2, have not been addressed at all.

Hormonal treatment in ErbB-2-positive breast cancer patients

Conflicting results have been reported up to now on the role of ErbB-2 in regulating the sensitivity of ER-positive breast cancer patients to tamoxifen and, more generally, to endocrine therapy. However, it should be considered that variability of many key aspects (such as the technique used for ErbB-2 evaluation or the baseline characteristics of patients) render indirect comparisons among studies extremely difficult. In addition, the proportion of ER-positive patients among the ErbB-2-positive patients is approximately 10%. As reported by Knoop *et al*. (2001), in order to find a significant hazard ratio of 1.4 for the interaction between administration of tamoxifen

and ErbB-2 expression in ER-positive patients, approximately 2000 events would be required in a study comparing tamoxifen with no treatment. None of the studies summarized below had that power and such a trial will never be performed because a comparison of tamoxifen versus no treatment, in patients with ER-positive tumors, would be now considered unethical.

The first evidence of a potential negative correlation between expression of ErbB-2 and response to tamoxifen came from the Gruppo Universitario Napoletano (GUN)-1 study which evaluated ErbB-2 over-expression in 145 out of 308 node-negative breast cancer patients, randomly assigned to receive 2 years of tamoxifen ($n = 59$) or no adjuvant treatment ($n = 86$). Adjuvant tamoxifen was associated with an improved DFS and OS in ErbB-2-negative patients, but with a worse DFS and OS in patients with ErbB-2-positive tumors (Carlomagno *et al*. 1996, De Placido *et al*. 2003). In agreement with these findings, Stal *et al*. (2000) found that ErbB-2-positive patients did not receive further benefit from 5 years of tamoxifen compared with 2 years of treatment, whereas prolonged treatment produced significant benefit in ErbB-2-negative patients. However, other studies came to different conclusions. Berry *et al*. (2000) examined the interaction between ErbB-2 expression and tamoxifen effectiveness in patients with ER-positive, node-positive disease treated with adjuvant cyclophosphamide, doxorubicin and fluorouracil (Cancer and Leukemia Group B 8541). In this protocol, tamoxifen assignment was not randomized, but was at the physician's discretion. Approximately half of the 651 ErbB-2-positive patients received tamoxifen. The reduction in risk of disease recurrence or death resulting from tamoxifen was similar in negative or positive ErbB-2 patients, and the interaction between tamoxifen and ErbB-2 status was not significant in multivariate analysis. However, all patients in this study received chemotherapy, and this could have masked the impaired efficacy of hormonal treatment. In the Danish Breast Cancer Cooperative Group's 77c protocol (Knoop *et al*. 2001), 1716 postmenopausal patients with a high risk of recurrence were randomly assigned to tamoxifen (868 women) or to observation (848 women). Multivariate analysis demonstrated no increased risk of recurrence after treatment with tamoxifen for ErbB-2-positive patients. However, in this study tamoxifen was given for only 1 year, which has now been proven to be inferior to 2-year and 5-year treatment. Furthermore, ErbB-2-positive patients were combined with those positive for EGFR and, despite this combining, the number of

ErbB-2- or EGFR-positive patients receiving tamoxifen was only 51.

Several retrospective analysis of studies with tamoxifen in advanced breast cancer have shown a worse outcome for patients expressing high levels of ErbB-2 as compared with ErbB-2-negative patients, although this evidence was not confirmed in all studies (Wright *et al*. 1992, Elledge *et al*. 1998, Houston *et al*. 1999, Arpino *et al*. 2004). However, this phenomenon confirms a worse prognosis for ErbB-2-expressing patients treated with tamoxifen, rather than a predictive role of ErbB-2 in the response to this drug.

The availability of novel anti-estrogenic drugs such as AIs has instigated investigations into the efficacy of these drugs compared with tamoxifen in patients with overexpression of ErbB-2. A study from Lipton *et al*. (2003) suggested that letrozole is superior to tamoxifen as first-line treatment, independent of serum ErbB-2 levels. Furthermore, Ellis *et al*. (2001), in a secondary analysis of data collected in a trial of neoadjuvant endocrine therapy, comparing letrozole versus tamoxifen, found that response rate was significantly higher for letrozole compared with tamoxifen in the subgroup of patients with EGFR- and/or ErbB-2-positive tumors (88 versus 21%; $P = 0.0004$). Furthermore, ErbB-2-positive tamoxifen-treated tumors exhibited a lower response rate than ErbB-2-negative tamoxifen-treated tumors (17 versus 40%; $P = 0.045$). In contrast, the response to the AI was not significantly influenced by ErbB-2 status, with response rates of 53% for ErbB-2-negative tumors and 69% for ErbB-2-positive tumors. Unfortunately the relatively small sample size means that these results are not conclusive.

In conclusion, although a general consensus has not yet been reached, the prevalent message coming from these studies is that ErbB-2-positive patients may show resistance to treatment with tamoxifen but not with AIs. Consistent with this hypothesis, the majority of panelists involved in the last St Gallen Consensus Conference agreed to take ErbB-2 status into account when choosing endocrine treatment.

Integration of endocrine therapy and signal transduction inhibitors

Since increased growth factor signaling is involved in both *de novo* and acquired resistance of breast cancer cells to endocrine therapy, the use of signal transduction inhibitors in the treatment of ER-positive patients represents one of the most promising therapeutic approaches. In this respect, drugs that are able to block the different signaling pathways involved in resistance to hormonal therapy (EGFR, ErbB-2, IGF-1R, ras/raf/MEK/MAPK, PI3K/AKT) are in advanced clinical development. The results of preclinical studies suggest that these drugs might be effective in both hormone-sensitive and hormone-resistant breast cancer patients. In fact, different reports have shown that additive or synergistic effects are obtained when ER-positive breast cancer cells are treated with a combination of endocrine therapy and signal transduction inhibitors. For example, additive or synergistic effects of farnesyl transferase inhibitors when combined with tamoxifen or AIs in ER-positive breast cancer cells have been demonstrated (Johnston *et al*. 2002, Ellis *et al*. 2003, Long *et al*. 2004b). Similar results were obtained with combinations of mammalian target of rapamycin (mTOR) antagonists and estrogen deprivation therapy with letrozole in pre-clinical models (Rudloff *et al*. 2004). A synergistic anti-tumor effect has been reported for trastuzumab when combined with tamoxifen in ER-positive ErbB-2-over-expressing BT-474 breast cancer cells, although this combination did not induce apoptosis (Argiris *et al*. 2004). However, Ropero *et al*. (2004) found that this combination is additive at high levels of cell kills, whereas it is antagonistic at an effect level of 30% or lower. In addition, the efficacy of this combination was found to be dependent on the schedule of treatment, with simultaneous treatment yielding the highest anti-tumor activity. Finally, combined treatment of MCF-7 cells with tamoxifen and the EGFR-tyrosine kinase inhibitor gefitinib was more effective in inhibiting proliferation, promoting apoptosis and eliminating bcl-2 as compared with tamoxifen alone (Gee *et al*. 2003). Interestingly, combined treatment with gefitinib and tamoxifen prevented the occurrence of resistance to tamoxifen mediated by increased EGFR and MAPK signaling that is observed following treatment with tamoxifen alone. Taken together, these findings suggest that combinations of signal transduction inhibitors might be useful in upfront treatment of ER-positive breast cancer in order to improve the efficacy of hormonal therapy and to prevent the occurrence of resistance.

Signal transduction inhibitors also have a role in the treatment of hormone-resistant breast cancer. In fact, enhanced activity of anti-EGFR and anti-ErbB-2 agents in tamoxifen-resistant breast cancer cells has been previously demonstrated (Knowlden *et al*. 2003*a*). Furthermore, evidence suggests that in hormone-resistant cells combined treatment with signal transduction inhibitors and endocrine therapy may be more effective as compared to treatment with signal transduction inhibitors alone. For example, Carlos

Arteaga's group reported that treatment of ErbB-2-overexpressing tamoxifen-resistant MCF-7 cells with the ErbB-2 inhibitor AG1478 or the MAPK inhibitor U0126 restored the inhibitory effect of tamoxifen on ER-mediated transcription and cell proliferation (Kurokawa *et al.* 2000). More recently, Shou *et al.* (2004) have shown that treatment with gefitinib eliminated tamoxifen's agonist activity and restored its anti-tumor activity both *in vitro* and *in vivo* in ErbB-2-overexpressing MCF-7 cells (MCF-7/HER2-18). In addition, by using the same *in vivo* model, Osborne's group demonstrated that gefitinib delays development of acquired resistance to estrogen deprivation in mice treated with gefitinib and estrogen withdrawal (Massarweh *et al.* 2002).

The above-mentioned pre-clinical findings led to the development of several clinical trials of combinations of endocrine therapy and signal transduction inhibitors in ER-positive breast cancer patients. The ongoing phase-II and -III clinical trials with such combinations have recently been reviewed in an exhaustive article by Stephen Johnston (2005). These trials employ combinations of trastuzumab, EGFR-tyrosine kinase inhibitors, farnesyl transferase inhibitors and mTOR inhibitors with either tamoxifen, AIs or fulvestrant. Some of these trials are in the post-tamoxifen setting, although randomized trials in patients that have not been pretreated with endocrine therapy are being conducted. More importantly, in two of these trials (the tamoxifen $\pm$ gefitinib Astrazeneca 0225 phase-II trial and the letrozole $\pm$ lapatinib GSK EGFR30008 phase-III study), biological studies have been undertaken with the aim of finding molecular markers that might predict response to therapy. Hopefully, these studies and additional studies of neoadjuvant therapy will provide important information for patient selection.

Encouraging preliminary results have recently been disclosed from a trial testing the combination of trastuzumab and letrozole in ErbB-2-positive and hormonal receptor-positive patients with advanced breast cancer (Wong *et al.* 2003, Ellis 2004). The majority of patients (22/26) had received previous tamoxifen therapy in an adjuvant or metastatic setting. Treatment with trastuzumab and letrozole produced a complete response rate of 9% (2/22), with an overall objective response rate of 27% (complete and partial responses 4/22) and a clinical benefit rate of 64% (complete and partial responses plus stable disease 14/22). Interestingly, all responding patients had remissions lasting longer than 1 year, with two patients having remission for more than 2 years. With a median follow-up of 70 weeks (range 12–170 weeks),

the median time to disease progression was 31 weeks (range 15–47 weeks), and 43% of patients were free from progression at 1 year. However, a significant number of patients experienced early progressive disease, and this suggests overlapping resistance mechanisms. One suggested hypothesis is that defects in G1 checkpoint controls may determine a 'pan-resistance' to all anti-growth-factor strategies (Cariou *et al.* 2000).

More recently, results of a phase-II clinical trial of gefitinib versus gefitinib plus anastrozole have been published (Polychronis *et al.* 2005). As described above, combined treatment produced a more significant reduction of Ki67 labeling, as compared with gefitinib alone, in patients with ER-positive/EGFR-positive breast carcinoma.

Finally, an additional approach to treatment of endocrine-resistant breast cancer might be represented by the use of combinations of anti-EGFR and anti-ErbB-2 agents. In this regard, we have previously demonstrated that combined treatment of breast cancer cells that co-express EGFR and ErbB-2 with gefitinib plus trastuzumab results in a synergistic anti-tumor effect (Normanno *et al.* 2002). Similar findings were obtained by independent research groups (Moulder *et al.* 2001). Following these results, a phase-I/-II clinical trial of trastuzumab plus gefitinib in breast cancer patients with ErbB-2-expressing tumors has been completed (Arteaga *et al.* 2004). In the phase-I study, patients were treated with trastuzumab (2 mg/kg per week) plus gefitinib at two dose levels: 250 and 500 mg/day. At the highest dose, two out of three patients developed grade-3 diarrhoea. Therefore, the phase-II study was conducted using gefitinib at 250 mg/day. Few responses were observed and only in previously untreated patients (2/28), and time to progression appeared shorter than that previously reported with trastuzumab alone. These results led the investigators to conclude that further use of combinations of trastuzumab plus EGFR-tyrosine kinase inhibitors is not justified. However, we believe that the results of the phase-II study could have been flawed by the dose of gefitinib, which at 250 mg/day is lower than the 500 mg/day usually considered a full dose. In fact, equivalence of the 250 and 500 mg doses has been suggested by phase-II trials of gefitinib in non-small-cell lung cancer, a disease where the majority of patients responding to gefitinib carry a mutation of the EGFR-tyrosine kinase domain associated with increased sensitivity to gefitinib. Since EGFR mutations have not been found in breast cancer, the 250 mg dose could be too low to be active. This dose was chosen because of the unexpected

toxicity observed at the 500 mg level. However, a phase-I trial of the EGFR-tyrosine kinase inhibitor erlotinib plus standard-dose trastuzumab in women with ErbB-2-positive metastatic breast cancer yielded different results. Trastuzumab could be combined with 150 mg/day erlotinib, the maximum tolerated dose of the drug, without significant diarrhea, and two partial responses were observed (Britten *et al.* 2004). Therefore, the full daily dose of erolotinib was chosen for a phase-II trial of this combination in breast cancer. Finally, grade-3 diarrhea occurred in 10% of trastuzumab-resistant breast cancer patients treated with the dual EGFR/ErbB-2 inhibitor lapatinib, a frequency similar to that observed with pure EGFR-tyrosine kinase inhibitors (Blackwell *et al.* 2004). Interestingly, 46 and 24% of the patients treated with lapatinib were progression-free at 8 and 16 weeks respectively following the start of the treatment (Blackwell *et al.* 2004). These findings suggest that the contemporary blockade of EGFR and ErbB-2 does not result in increased intestinal toxicity, and that chance could have played a relevant role in the toxicity observed by Arteaga *et al.* (2004). In addition, the above-mentioned preliminary results obtained with either the erlotinib plus trastuzumab combination or lapatinib suggest that this approach might result in significant anti-tumor activity in heavily pre-treated breast cancer patients.

Conclusions and perspectives

The above-mentioned findings demonstrate that a number of questions still need to be addressed in order to select the best therapeutic strategy in post-menopausal patients with ER-positive breast cancer in both advanced and adjuvant settings. In particular, current opinion for the majority of clinicians is that patients with advanced breast cancer should be treated with AIs as first-line treatment, since these drugs have shown higher activity and a better toxicity profile as compared with tamoxifen. However, the difference in terms of time to progression between tamoxifen and AIs is marginal. In addition, fulvestrant showed, at best, an efficacy similar to tamoxifen in previously untreated patients. These observations suggest that differences among these drugs in untreated advanced disease are not impressive. In other words, untreated breast cancer is a disease that is extremely sensitive to treatment with any hormonal drugs. Conversely, AIs and fulvestrant are active in a proportion of tamoxifen-resistant patients. According to our results, this observation might imply that long-term control of tumor growth could be obtained, in at least a

proportion of patients, by using a sequence of tamoxifen, AIs and fulvestrant. In this respect, clinical data suggest that treatment with fulvestrant might either precede or follow AIs. Starting from the preclinical observations that we have summarized, the sequence AI–fulvestrant should be preferred, because treatment with fulvestrant might be associated with an increased incidence of development of an estrogen-independent phenotype. Of course, randomized clinical trials comparing different strategies are necessary to address these questions.

In the adjuvant setting, AIs have shown a greater efficacy as compared with tamoxifen, although long-term effects and toxicity have not been sufficiently studied yet. Positive results of clinical trials with AIs given after 2 or 5 years of treatment with tamoxifen are consistent with suggestions coming from the *in vitro* studies that we summarized in the above proposed model. In addition, the sequential schedule could also be convenient in terms of reduction of side effects and on economical grounds. However, it must be emphasized that head-to-head comparisons of sequential schedules with upfront AIs are needed in order to choose between the two strategies. In this regard, the biological characteristics of the tumor could be important in assisting the clinical decision. For example, they might be useful to identify patients at risk of early relapse, who might benefit from AIs as a first-line treatment. Finally, the widespread use of AIs in the adjuvant setting that is foreseen in the next few years implies that patients that will have tumor recurrence will be not suitable for treatment with such compounds. Therefore, the activity of fulvestrant and tamoxifen needs also to be addressed in patients with resistance to AIs.

It is likely that the next step in the development of novel therapeutic approaches for ER-positive patients is represented by the use of combinations of hormonal agents and signal transduction inhibitors. In this respect, preliminary results from clinical trials seem to indicate that ER-positive breast cancer is a more promising field for the development of such agents as compared with ER-negative patients. Indeed, activation of growth factor-driven signal transduction pathways is clearly involved in both *de novo* and acquired resistance to hormonal treatment. The role of these agents in the adjuvant setting needs to be explored. In particular, signal transduction inhibitors could increase the efficacy of endocrine therapy by preventing the occurrence of ER-negative tumors.

Finally, the knowledge of the molecular mechanisms involved in the suppression of ER expression and in

the development of an ER-negative phenotype might allow novel therapeutic approaches in ER-negative patients. In fact, drugs potentially able to revert the mechanisms involved in the suppression of the expression of ERα, such as HDAC inhibitors or de-methylating agents, are currently in clinical trials. In this respect, studies in our laboratories are ongoing to assess whether induction of expression of ERα in breast cancer cells might at least in part restore an estrogen-dependent phenotype. In this respect, evidence also suggests that growth factor signaling is also involved in the suppression of ERα expression in breast cancer cells. Therefore, combinations of agents capable of inducing expression of ERα in ER-negative breast cancer cells, anti-estrogenic drugs and signal transduction inhibitors might represent a novel therapeutic approach in breast cancer patients. Such a combination of target-based agents might prove efficient in blocking tumor growth, allowing at least a delay in the need for chemotherapy in selected patients.

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