Molecular insights into endocrine resistance

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Introduction

Endocrine therapy with selective estrogen receptor modulators (SERMs) such as tamoxifen, or agents that lead to estrogen deprivation such as gonadotropin-releasing hormone (GnRH) agonists in premenopausal and aromatase inhibitors in postmenopausal women, are widely used in the treatment of all stages of breast cancer. Indeed, these are the most effective targeted therapies for breast cancer, and their use in the adjuvant setting for women with tumours that expresses estrogen receptor (ER) has been the single biggest contribution to the reduction in mortality over the last 15 years. However, resistance in ER-positive tumours to such treatments can be intrinsic occurring at first exposure (*de novo*), or develop over time after an initial response to endocrine therapy (acquired). Identification of the key molecular mechanisms involved in resistance to either tamoxifen or estrogen deprivation is important to allow development of an appropriate panel of biomarkers that may help predict response/resistance to specific treatments [1]. Ultimately such research is aimed at the development and application of new pharmaceutical agents targeted at the various molecular components of endocrine-resistance pathways, either with the goal of effective treatment of endocrine resistant disease, or more likely the prevention/delay of its development, thus, extending the benefit of endocrine therapy.

The article reviews recent progress in our understanding of endocrine resistance, in particular current knowledge about initial endocrine sensitivity in relation to ER biology in tumours. The potential mechanisms of acquired resistance to both tamoxifen and estrogen deprivation are then compared and contrasted, together with how such information could lead to more effective clinical strategies for maximising the benefit of endocrine therapy in the future.

ER molecular biology and *de-novo* endocrine resistance

The expression of ER in breast cancer cells has always been perceived to be the single most important determinant for endocrine response in breast cancer. Whilst it has become clear that tumours which do not express any ER are unable to respond to endocrine therapies and thus, exhibit primary '*de-novo*' resistance, evidence now exists that expression of ER alone in human breast carcinomas is insufficient to accurately predict response to endocrine therapy in the clinical setting. Thus, it is important to understand how estrogen and SERMs interact with ER, and to determine what additional factors other than ER expression alone may influence a given cellular response to ligand activation of the receptor.

Estrogen influences gene expression and cellular phenotype by diffusing into the cell and binding nuclear ER, which, in turn, activates receptor dimerisation, association with various co-activator and co-repressor proteins to a greater or lesser extent, respectively, and subsequent DNA binding of liganded ER within promoter regions of DNA upstream of estrogen-regulated target genes. Gene transcription is activated through two separate transactivation domains within ER, termed AF-1 in the amino-terminal A/B region and AF-2 in the carboxy-terminal E region (Fig. 1a) [2]. At its simplest level, the SERM tamoxifen functions as a competitive anti-estrogen to inhibit estrogen action. Tamoxifen-bound ER still dimerises and binds DNA, but the downstream effects are different as a result of the altered conformational shape of the tamoxifen–ER complex compared with estradiol. This results in a change in the receptor bound balance of co-activators and co-repressors such that tamoxifen-liganded ER may block gene transcription through the AF-2 domain, while AF-1 mediated gene transcription may still occur (Fig. 1b) [3]. This may explain the partial agonist activity of tamoxifen that is exerted in some tissues such as bone or endometrium, in addition to its ability to antagonise

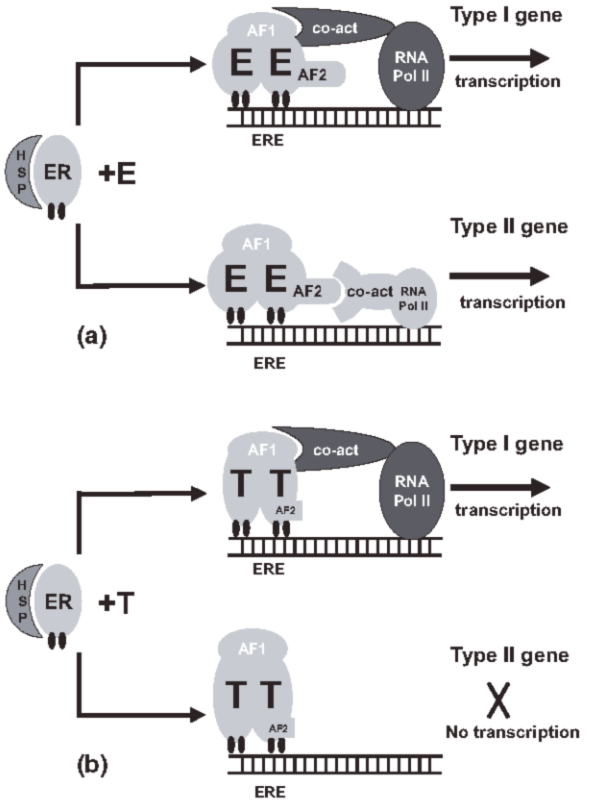

Fig. 1. (a) Mechanism of estradiol activation of ER–estradiol binding to ER leads to loss of Heat Shock Proteins (HSPs), dimerisation and phosphorylation of receptors, with conformational change leading to co-activator activation at both AF1 and AF2 sites – full agonist effect seen. (b) Mechanism of ER antagonism by SERMs – tamoxifen binding to ER leads to loss of HSPs, dimerisation and phosphorylation of receptors, but with different specific conformational change leading to co-activator activation at AF1 only, and not AF2 sites – therefore partial agonist effect seen.

estrogen-regulated gene transcription especially in the breast.

It has become clear that the molecular biology of ER in breast cancer is even more complex, and that several aspects other than mere expression of adequate levels of ER determine initial endocrine sensitivity in breast cancer cells. In addition to classical ER (now called ERα), a second ER was cloned (ERβ) which shares sequence homology within the DNA-binding domain [4], but which differs in that AF-1 activity is considerably less than with ERα [5]. Equally, ERβ lacks much of the C-terminal F domain of ERα, which may be an important region in determining an agonist response to tamoxifen [6]. The distribution in normal tissues of ERβ is different from ERα which implies a distinct physiological role, and some evidence has implicated increased ERβ expression as a mechanism for tamoxifen resistance in breast cancer [7]. It has also been established that in addition to the classical model of liganded ER which binds DNA at defined estrogen response elements (EREs) upstream of ER-regulated genes, alternative regulatory DNA sequences can become activated by ER. For example AP-1 response elements regulate several genes involved in cell proliferation, motility and apoptosis, and liganded ER may indirectly regulate AP-1 gene transcription through direct protein–protein interaction with AP-1 transcription factors (c-fos and c-jun). Tamoxifen was shown to be an agonist on AP-1 regulated genes with either ERα or ERβ [8], whereas estrogen liganded with ERβ inhibited AP-1 gene transcription [9]. Enhanced activation of AP-1 by tamoxifen may also be associated with tamoxifen resistance in models of breast cancer [10], and in tumours from breast cancer patients relapsing on tamoxifen [11]. Finally, the relative balance in a given cell type of co-activator and co-repressor proteins may also determine the given response of ER to a particular ligand. For example, over-expression of the co-activator SRC-1 has been shown to enhance the agonist stimulatory response to tamoxifen [12], while a reduction in level of the co-repressor N-CoR was associated with development of tamoxifen resistance in breast cancer xenografts [13]. Thus, expression of ERβ relative to ERα, enhancement of alternative pathways such as AP-1, or the relative balance of co-activator/co-repressor proteins could all account for initial differential sensitivity to endocrine therapy with tamoxifen in apparently ER-positive human breast carcinomas.

More recently, the presence of alternative non-genomic mechanisms for ER action have been postulated as an alternative mechanism for initial resistance of ER-positive breast cancers to tamoxifen. Membrane-initiated signalling by ER is thought to occur by ER interacting with and/or activating several kinases including the insulin-like growth factor-1 receptor (IGF-1R), the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), and the adaptor proteins Src and Shc. These various interactions by estrogen-liganded ER can result in different cell survival and cell proliferative signals via the AkT (protein kinase B) and mitogen activated protein kinase (MAPK) pathways, respectively [14–16]. Furthermore, tamoxifen may exert agonist effects via interaction of membrane ER with peptide growth factor signalling (EGFR or HER-2), thus, stimulating rather than inhibiting cell growth [17]. This gives an additional role for ER in directly activating growth factor signalling at the membrane rather than via nuclear activation/transcription of growth factors to act in an autocrine loop. This non-genomic activity of ER may be facilitated by enhanced expression of several

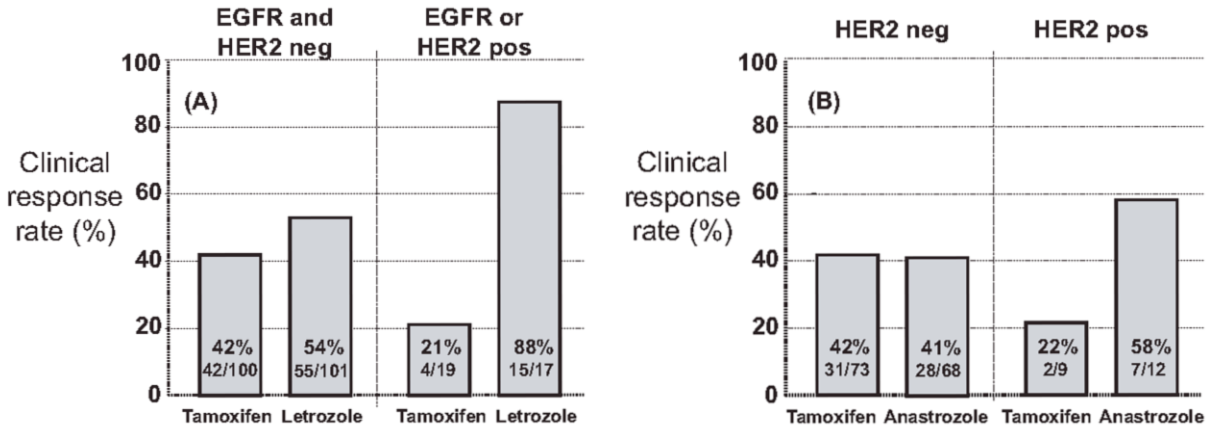

Fig. 2. (a) Objective tumour response rate in primary ER positive breast cancers to either neoadjuvant tamoxifen or letrozole dependent on co-expression of type I growth factor receptors EGFR or HER2 (data from ref. [20]). (b) Objective tumour response rate in primary ER positive breast cancers to either neoadjuvant tamoxifen or anastrozole dependent on co-expression of HER2 (data from ref. [21]).

adapter proteins that facilitate membrane ER interactions (MNAR/PELP1 and MTA1) [18]. Likewise, in ER-positive tumours with HER2 amplification, additional molecular cross-talk may occur following downstream activation by various HER-2 driven intracellular kinases (e.g. p38 MAPK, ERK1/2), which in tamoxifen-treated tumours may result in the phosphorylation of nuclear tamoxifen-liganded ER and associated co-activators, which, in turn, activates ER-dependent endocrine resistant growth. As such, existence of these bi-directional cross-talk pathways can result in tamoxifen stimulating the growth of these ER positive breast cancer cells.

In the clinical arena, several aspects of ER' s biology are now being evaluated to determine whether any given biomarker profile in an ER-positive tumour can help determine initial sensitivity or resistance to various endocrine therapies. Firstly, ER-positive tumours that co-express EGFR or HER-2 are known to be more resistant to tamoxifen in experimental models [17], as well as in several clinical studies [19]. In two neo-adjuvant studies of endocrine therapy in post-menopausal women, this profile may also predict for relative resistance to tamoxifen, yet greater sensitivity to estrogen-deprivation therapies with aromatase inhibitors (Figs. 2a,b) [20,21]. Firstly, the reason for this difference relates to reduced ligand activation of both nuclear and membrane ER by aromatase inhibitors (due to removal of estrogen), compared with activation of the interaction of membrane ER with kinase pathways in the presence of tamoxifen, in addition to HER2 activation of tamoxifen-liganded nuclear ER. Secondly, in ER-positive tumours high levels of the co-activator AIB1 in the presence of HER2 may also predict for an agonist response to tamoxifen, confirmed in the clinical setting by relative resistance to tamoxifen in patients with tumours that contained high levels of AIB1 [22]. Thus, levels of AIB1 and/or EGFR/HER2 may modulate response to tamoxifen in ER-positive breast cancer.

Likewise, co-expression of ER-regulated genes such as progesterone receptor (PR) has hitherto been associated with enhanced endocrine sensitivity, and PR has been used as an additional marker to predict for likelihood of a good response to endocrine therapy [23]. Evidence has been based on large clinical series utilising co-expression of PR with ER as a prognostic factor in series of patients treated with adjuvant tamoxifen. More recently, it has become clear that loss of PR in ER-positive tumours may be a distinct molecular event associated with activation of the EGFR/HER2 pathway, and, thus, may serve as an additional indirect marker for resistance to tamoxifen. There is recent evidence that there may be a differential initial clinical benefit between aromatase inhibitors and tamoxifen according to PR status of ER-positive tumours. A retrospective subgroup analysis in the ATAC adjuvant trial of anastrozole versus tamoxifen found that patients that were ER+PR+ or ER+PR− had a similar recurrence-free survival on anastrozole, but that patients that were ER+PR− faired much worse on tamoxifen than ER+PR+ patients. The co-segregation of growth factor receptor positivity with PR negativity may again be responsible for this differential benefit, and recent studies have reported that growth factor signalling through IGF-1R or EGFR/HER2 results in down-regulation of transcription of the PR gene, possibly via ER mediated AP1 transcriptional regulation [24,25]. In experimental models of tamoxifen resistance, both *de-novo* tamoxifen-stimulated ER-positive HER2-positive xenografts and acquired tamoxifen-resistant MCF-7

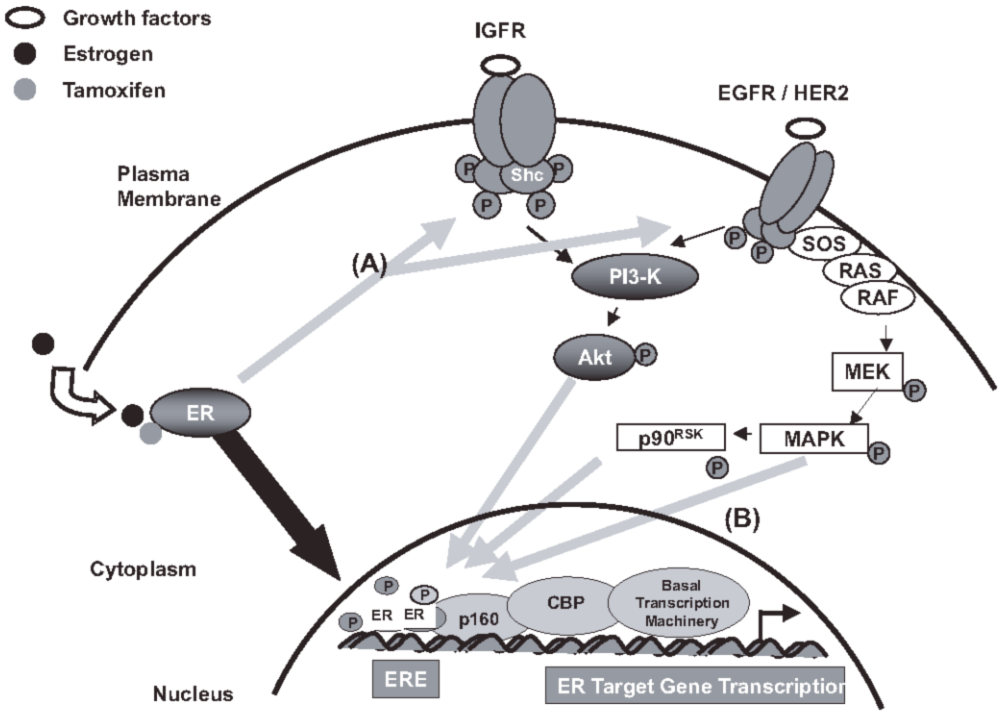

Fig. 3. Bidirectional cross-talk pathways that may be active in tamoxifen resistant breast cancer. (A) liganded ER can interact at the plasma membrane with adapter proteins such as Shc and the kinase domains of either IGF-1R and/or EGFR/HER2 to cause rapid non-genomic activation of peptide growth factor signalling, while (B) signalling from EGFR/HER2 or IGFR can activate either downstream MEK/MAPK, pp90^{rsk} or the PI3K–Akt pathways, all of which can phoshorylate nuclear co-activators and ER to initiate ER-dependent gene transcription and growth. In the presence of bi-directional cross-talk between ER and growth factor pathways, both tamoxifen and estrogen function as agonist ligands.

xenografts, levels of ER-regulated genes such as IGF-1, cyclin Di, bcl-2 and PgR switched to either being reduced or absent compared with parental tamoxifen sensitive xenografts [26]. While some have suggested that this biomarker profile (i.e. ER-positivity with either PgR-negative status and/or EGFR/HER2 positive status) could explain differences in initial endocrine sensitivity to tamoxifen relative to aromatase inhibitors [27], this remains to be confirmed in other large trials – indeed initial results from the BIG-198 study of letrozole versus tamoxifen have not demonstrated similar differences in outcome in ER positive tumours dependent on presence or absence of PgR expression [28].

Thus, our current knowledge of the molecular biology of the ER has provided new insights into potential mechanisms for *de-novo* resistance in ER-positive breast cancer. Experimental and clinical data have generated provocative hypotheses to explain initial resistance to tamoxifen relating either to membrane versus nuclear ER activity, bi-directional cross-talk with co-expressed peptide growth factor pathways (IGFR or EGFR/HER2) (Fig. 3), and potential biomarker profiles in relation to the presence or absence of ER-regulated genes such as PR. Further more-accurate profiling of ER-positive cancers may come from ongoing DNA-microarray studies, and preliminary data have suggested that molecular signatures may help predict optimal initial sensitivity to tamoxifen [29]. At the same time, more detailed experimental molecular studies have been conducted in the setting of acquired resistance to endocrine therapy, with some similar themes emerging as discussed below.

Mechanisms of acquired endocrine resistance

A clinically important observation is that patients whose breast cancer relapses after a prior response to tamoxifen have a good chance of a subsequent response to aromatase inhibitors [30]. Thus, the molecular mechanisms leading to resistance to tamoxifen do not always result in complete endocrine resistance in most patients. While estrogen deprivation therapy with aromatase inhibitors may be more effective than tamoxifen in the metastatic and adjuvant settings [31], eventually resistance to this approach also develops. What has been unclear is whether similar mechanisms

of resistance recently identified in tamoxifen-resistant tumours also account for acquired resistance to aromatase inhibitors. Our current understanding of resistance to each of these therapies is discussed below.

Acquired resistance to tamoxifen

An important early finding was that ER expression was lost at relapse in only about 15–25% of breast cancer patients who developed acquired tamoxifen resistance [32,33]. In some studies of human breast cancer, loss of ER expression has been associated with aberrant methylation of DNA at CpG islands, with evidence for chromatin inactivation by histone deacetylation resulting in ER gene silencing [34]. This has raised the possibility of utilising either demethylating agents or histone deacetylase (HDAC) inhibitors as therapeutic strategies to re-express ER and restore endocrine sensitivity [35]. However, such a mechanism has not been confirmed as a mechanism for acquired loss of ER expression in tamoxifen treated tumours. In the majority of tumours, ER expression appears to be retained, and the clinical evidence that response might still occur to further endocrine therapy suggests that tumour re-growth may still involve functional ER signalling. At one stage it was suggested that alterations in the structure and function of the ER protein might change its response to tamoxifen [36], although little evidence was found for significant mutations in the ER gene in patients with breast cancer [37–39]. An estrogen-hypersensitive ER mutant (lys303arg) has been reported in 20 out of 59 atypical breast hyperplasias and pre-invasive breast carcinomas that may result in enhanced binding of co-activators, thus, leading to an agonist response to tamoxifen or sensitization to the low levels of residual estrogen with aromatase inhibitors [40]. However, there are no reports for this mutation in tumours with acquired endocrine resistance to suggest that this represents a common mechanism.

By whatever means, it appears clear that ERα remains involved in the majority of tumours with acquired resistance. Tamoxifen permits receptor activation primarily because it still allows ER dimerisation and binding to DNA [41], and in the setting of acquired resistance it favours association with co-activators and transcription of estrogen-regulated genes. Clinical evidence for an agonist (rather than antagonist) response to tamoxifen in the acquired resistance setting was demonstrated in experiments whereby breast cancer cells from malignant pleural effusions taken from patients relapsing on tamoxifen were established in short-term clonogenic assays in soft agar, and shown to be stimulated equally by estrogen and tamoxifen [42]. In contrast, these cells when grown *ex-vivo* were growth inhibited by the steroidal anti-estrogen ICI182780 (fulvestrant), which is devoid of any agonist activity, and has been shown to bind and degrades ER in breast cancer cells. Subsequently, this has been supported by two clinical trials, which have shown efficacy for fulvestrant tumours from patients with acquired tamoxifen-resistance [43,44], again supporting the concept that ER survives and is functionally active in these resistant tumours.

Several groups investigated the pathways involved in ER activation in tamoxifen-resistant disease have been investigated by several groups. Emerging evidence suggests that peptide growth factor pathways cross-talk with ER and its transcription machinery, and might be involved in phosphorylation and activation of ER [45,46] (Fig. 3). The MAPK/ERK pathway (which may be activated by upstream growth factors such as HER-2 and EGFR), the AKT/PI-3 kinase pathway (which may be activated by the insulin-like growth factor pathway) and the p38 MAPK pathway (activated by stress or various cytokines) can all phosphorylate ER at key positions (i.e., ser 118, ser 167, tyr 131) in the AF-1 and other domains of the receptor. Because tamoxifen can still bind and partially activate ER, cells that co-express ER and HER-2/EGFR may demonstrate an ' agonist' response to tamoxifen due to tamoxifen-liganded ER being phosphorylated by these various intracellular kinases. There is also emerging evidence that in these cells ER may be bound to the plasma membrane, and that tamoxifen-liganded ER may interact and directly activate peptide growth factor receptors at the cell surface [17,47] (Fig. 3). By contrast, complete estrogen deprivation in these cells would prevent ER activation (membrane or nuclear DNA bound) due to removal of the majority of available ligand for ER; thus, effectively abrogating any cross-talk activation of ER signalling that may exist due to peptide growth factor pathways. Again, this could explain why tamoxifen-resistant tumours may be preferentially more sensitive to estrogen-deprivation therapies with aromatase inhibitors, or ER downregulation by fulvestrant.

HER-2 may also activate one of the major co-activators involved in ER-mediated gene transcription [22,48]. Growth factor signalling can activate ER via phosphorylation and activation of the co-activator AIB1 (also know as SRC-3), and evidence suggests that high levels of AIB1 may reduce the antagonist effects of tamoxifen. Previous studies have shown that breast cancer cells that co-express ER and HER2 are

less responsive to tamoxifen [49], and recent clinical data suggest that co-expression of AIB1 with HER2 predicted for a worse outcome in patients treated with tamoxifen after surgery [22]. These basic studies of ER activation led to consideration that some of these molecular pathways might explain resistance to endocrine therapy with tamoxifen.

Experimental models and clinical studies have now suggested that these growth factor receptor pathways become up-regulated and over-expressed in breast cancer cells that acquire resistance to tamoxifen during prolonged exposure. Enhanced expression of EGFR and subsequent downstream MAPK activation has been found in MCF-7 breast cancer cells that become resistant over time to tamoxifen, with evidence that co-treatment with the EGFR receptor tyrosine kinase inhibitor ZF1839 (gefitinib) may prevent or delay this resistance by blocking this signalling pathway [45,46]. More recently, changes have been reported in intracellular signalling in clinical samples from breast cancer patients taken before and at the time of relapse on adjuvant tamoxifen several years later [33]. In tumours with retained ER expression (the majority), there was enhanced expression of HER-2 in some patients, with evidence that, in these tumours, expression of the stress-activated kinase p38 MAPK was enhanced [33]. These clinical and laboratory data support a concept that, over time, breast cancer cells utilise alternative intra-cellular signalling pathways to enhance and activate ER signalling, and that this allows cells to escape from their initial endocrine therapy with tamoxifen. As discussed in more detail below, strategies to block these signalling pathways from the outset by co-treatment with an EGFR tyrosine kinase inhibitor such as gefitinib in addition to tamoxifen have been utilised as a means to prevent development of endocrine resistance.

Acquired resistance to estrogen deprivation

Randomised trials in the metastatic and adjuvant settings have shown that estrogen deprivation with aromatase inhibitors is more effective than tamoxifen [31], and can circumvent some of the resistance pathways described above that are specific to tamoxifen by removing all available ligand for ER. However, it is known that in-time hormone-sensitive ER positive breast carcinomas treated with aromatase inhibitors will also acquire resistance and start to regrow. To date, there have been few clinical/laboratory studies that have investigated resistance to aromatase inhibition. Site-directed mutation can produce an aromatase enzyme that is resistant to inhibition by some (but not all) aromatase inhibitors [50], although such mutations have not been found in human breast carcinomas [51]. Evidence has existed for some time that in pre-menopausal patients who respond and relapse after estrogen withdrawal by ovarian suppression with Luteinising-Hormone Releasing Hormone (LHRH) agonists, the tumours can respond to further suppression of estrogen levels at the time of relapse by the addition of an aromatase inhibitor to the LHRH agonist [52]. These clinical data would suggest that acquired resistance to estrogen deprivation in breast cancers may be overcome by a further stepwise reduction in circulating levels of estrogens in these patients, implying that resistant cancer cells must retain partial hormone sensitivity despite initial adaptation to, and re-growth in, a low-estrogen environment.

In the laboratory, data from several groups now support a concept of adaptation to a low-estrogen environment with an enhanced sensitivity to estrogen as a means of escape from estrogen deprivation therapies. While wild-type MCF7 breast cancer cells respond maximally to doses of estradiol of $\sim 10^{-11}$–10^{-10}M, so-called long-term estrogen deprived (LTED) cells adapt and instead respond maximally at $\sim 10^{-13}$M [53–56]. In part, this may be caused by an absolute increase in levels of ER protein, but in some models may also be related to an increase in re-location of ER to the plasma membrane in association with Shc and IGF-1R [57]. In these cells, it has been shown that following treatment with estradiol, ER-alpha will rapidly associate with Shc, an adapter protein involved in tyrosine kinase signalling. In addition, there is an increase in activation of both *src* and the *ras/raf*/MEK/MAPK signalling pathway [58,59], and treatment with fulvestrant is able to block MAPK activation suggesting that ERα is functioning upstream of MAPK in a non-genomic membrane associated fashion [58].

There is undoubtedly an increase in growth factor signalling in ER-positive breast cancer cells that acquire resistance to LTED, through the HER1/2 MEK/MAPK pathway [60] and the PI3K/Akt pathway [61]. While non-genomic activation by ER of growth factor signalling may be one mechanism as described above, there is also evidence for enhanced cross-talk between the various growth factor receptor signalling pathways and ER itself in a similar fashion to that described for tamoxifen resistance above. In experimental models of acquired resistance to LTED, ER-alpha may become phosphorylated and activated by a number of different intracellular kinases, including mitogen-activated protein kinases (MAPKs) downstream of HER2/HER3, the kinase pp90RSK, and the insulin-like growth factor (IGF)/

Table 1
Targeted intracellular small molecule inhibitors that are in development for breast cancer treatment, either as monotherapy for metastatic disease (including endocrine resistance), or in combination with endocrine therapy in ER-positive breast cancer (either as first-line therapy for metastatic disease, or as neoadjuvant therapy)

Intracellular target	Drug	Phase of development/clinical setting
EGFR	Gefitinib (Iressa)	Phase II metastatic and neoadjuvant endocrine combinations
	Erlotinib (Tarceva)	Phase II metastatic Rx
	EKB-569	Phase I/II
EGFR/Her2	Lapatinib (GW572016)	Phase III metastatic endocrine combinations
	AEE-788	Phase I
Pan-erbB	Canaternib (CI-1033)	Phase II metastatic Rx
Farnesyltransferase	Tipifarnib (Zarnestra)	Phase II/III metastatic endocrine combinations
	Lonafarnib (Sarasar)	Phase II metastatic endocrine combinations
	AZD3409	Phase I
mTOR	Everolimus (RAD-001)	Phase III metastatic and neoadjuvant endocrine combinations
	Temsirolimus (CCI-779)	Phase III metastatic endocrine combinations
PI3-Kinase	LY294002	Preclinical
IGFR-1	AG1024	Preclinical
Raf	BAY-43-9006	Phase I
MEK 1/2	PD-0325901	Preclinical
	AZD6244	Phase I
Src	AZD0530	Phase I
Cyclin-dependent kinase	AZD5438	Preclinical
NFkB	Bortezomib (Velcade)	Phase II

AKT pathway [62,63]. ER-mediated gene transcription was enhanced up to ten-fold in cells that acquire resistance to LTED, and was abrogated by a number of different approaches to interrupt intracellular signalling, including the EGFR tyrosine kinase inhibitor gefitinib, the MEK inhibitor UO126, in addition to the ER downregulator fulvestrant which degrades estrogen receptor [56]. This would suggest that ER was functioning downstream (rather than upstream) of these signalling pathways.

Thus, once again it would appear that the ER remains an integral part of signalling, even following failure of long-term estrogen-deprivation therapies. In an analogous fashion to tamoxifen resistance, emerging data exist for resistance to LTED to support bidirectional interaction between ER and growth factor signalling, either non-genomic ER activation of growth factor receptors at the plasma membrane, or intracellular activation of classical genomic ER in the nucleus by various intracellular kinases driven by upstream growth factor pathways (similar to Fig. 3). At present, there are no clinical data to substantiate these recent findings in experimental models of resistance to LTED, and studies of these pathways in tumours from patients who relapse after therapy with aromatase inhibitors are clearly needed. However, hope now exists that by learning which intracellular signalling pathways are operative in this process, logical combinations of signal transduction inhibitors (STIs) can de devised to target key molecular pathways implicated in development of resistance, thus, enhancing the benefit of current endocrine therapies with aromatase inhibitors through prevention or significant delay in the acquisition of endocrine resistance.

Molecularly targeted therapies to treat/prevent endocrine resistance

As outlined above, recent research into the molecular mechanisms of endocrine resistance has revealed various growth factor pathways and proteins involved in the signal transduction cascade that become activated and utilised by breast cancer cells to bypass normal endocrine responsiveness [64]. These intracellular pathways represent attractive targets for pharmacologic intervention with small molecule signal transduction inhibitors (STIs) that target aberrantly or excessively expressed proteins. Several drugs are in active development for breast cancer including type-1 growth factor tyrosine kinase inhibitors, farne-

syltransferase inhibitors, MEK inhibitors, and mTOR antagonists (Table 1).

Pre-clinical effects of STIs in breast cancer

Enhanced expression of EGFR and HER2, together with subsequent downstream activation of MAPK/ERK signalling pathways, has been found in breast cancer cells that become resistant over time to endocrine therapy either with tamoxifen [46] or long-term estrogen deprivation [56,65]. Treatment with various STIs has been used in preclinical models in an attempt to overcome this resistance by blocking upregulated signalling pathways [66]. For example, in MCF-7 cells that developed resistance to tamoxifen, gefitinib (which targets the internal tyrosine kinase domain of EGFR) and trastuzumab (which blocks the external domain of HER2) were effective at reducing downstream ERK1/2 MAPK signalling and inhibiting cell growth [46]. EGFR and HER2 heterodimerize in the resistant cells, such that targeting either one of the receptors can be an effective therapy. Of note, hormone-sensitive cells (in which neither receptors are overexpressed) were unaffected by either gefitinib or trastuzumab therapy. Similar data have been reported by other groups in tamoxifen-resistant HER2-transfected MCF-7 cells with AG1478, a HER2 tyrosine kinase inhibitor, and with trastuzumab [67, 68]. Likewise, in cells resistant to LTED, growth and ER-mediated gene transcription was abrogated by a number of different intracellular approaches to interrupt signalling, including the tyrosine kinase inhibitor gefitinib, the MEK inhibitor UO126, and the ER downregulator fulvestrant that degrades residual estrogen receptor [56]. Several groups have shown that these different pharmacological approaches may also inhibit the growth of breast cancer xenograft tumours *in vivo* [17,67].

Small molecules have been developed against other intracellular signalling and cell survival pathways that become activated in hormone-resistant breast cancer. Akt (or PKB) is a serine/threonine kinase that promotes cell survival and is activated in response to many different growth factors, including insulin, insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF). The mammalian target of rapamycin (mTOR) is a downstream effector of the PI3K/Akt signalling pathway that activates p70S6 kinase and 4E-binding protein-1, which, in turn, regulate transition through the G1/S phase of the cell cycle. Approaches to targeting these cell survival pathways have included either specific PI3K inhibitors such as LY294002, or rapamycin analogues such as temsirolimus (CCI-779) or everolimus (RAD-001) that target mTOR. Breast cancer cell lines with activated Akt (e.g., via loss of the regulatory PTEN tumour suppressor gene) are especially sensitive to mTOR antagonism [69].

Enhanced effects for STIs in combination with endocrine therapy

Several pre-clinical reports have implied that, in hormone-sensitive ER-positive breast cancer, STIs as monotherapy may have only a minimal effect on tumour growth, especially if cells lack the activation and critical dependence on the various signal transduction pathways. Emerging evidence suggests that adaptive changes occur during prolonged endocrine therapy, in particular upregulation of growth factor signalling. Thus, strategies to combine endocrine with STI therapies have been used as a means to prevent development of resistance and improve therapeutic efficacy. *In vitro*, combined tamoxifen and gefitinib provided near complete inhibition of phosphorylated ERK1/2 MAPK and Akt, together with greater G0/G1 cell cycle arrest and suppression of the cell-survival protein bcl-2 than that observed with just tamoxifen [70]. In particular, combined therapy prevented the acquired expression of EGFR/MAPK signalling and the subsequent resistance that occurred after 5 weeks in tamoxifen-alone treated cells.

For established hormone-resistant HER2-positive breast cancer, the strategy of combined STIs and endocrine therapy may also be more effective than using STIs alone [67]. *In vivo*, gefitinib and tamoxifen provided maximal growth inhibition and significantly delayed the growth of HER2 positive MCF-7 xenografts compared with gefitinib alone [17]. Moreover, similar effects were seen with gefitinib combined with estrogen deprivation, which provided greater inhibition of growth and substantially delayed acquired resistance compared with estrogen deprivation alone [71]. A synergistic effect has also been reported for trastuzumab combined with tamoxifen in ER-positive/HER2-positive BT-474 breast cancer cells, with enhanced accumulation of cells in G0/G1 and reduction in S phase of the cell cycle compared with either therapy alone [72]. Recently, the dual EGFR/HER2 inhibitor lapatinib has been shown to cooperate with tamoxifen to provide more rapid and profound cell cycle arrest than either therapy alone in hormone resistant cells [73]. The two drugs together caused a greater reduction in cyclin D1, together with a greater increase in the kinase inhibitor p27 and cyclin E-cdk2 inhibition in various tamoxifen-resistant

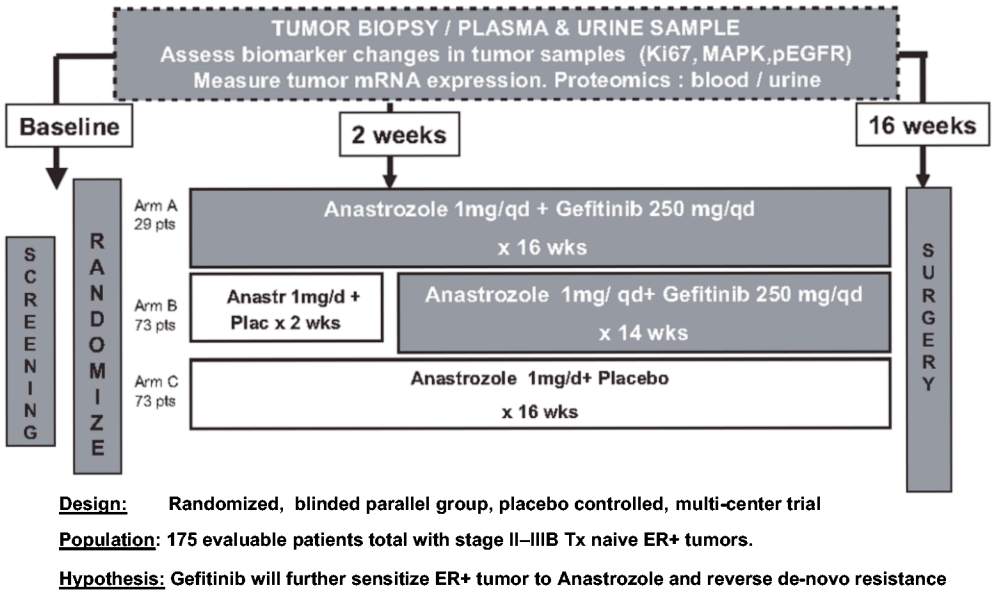

Fig. 4. Schema for a neo-adjuvant trial of anastrozole with/without gefitinib in ER-positive primary breast cancer, with the primary biological endpoint being an enhanced reduction in cell proliferation (Ki-67) for the combination as assessed in paired tumour biopsies taken pre-therapy, at 2 weeks, and at surgery after 16 weeks therapy.

breast cancer cell lines. Lapatinib was able to restore tamoxifen-sensitivity in EGFR or HER2 expressing cells, whilst *in vivo*, combined lapatinib and tamoxifen caused maximal regression of HER2 over-expressing tamoxifen-resistant MCF-7 xenografts [73].

Other STIs that have only a minimal effect on hormone-sensitive breast cancer may also be more effective when combined with endocrine therapy. The farnesyltransferase inhibitor (FTI) tipifarnib inhibits the growth of a number of human breast cancer cells lines *in vitro* [74], but only produced a modest cytostatic effect on hormone-sensitive MCF-7 xenograft growth *in vivo* [75]. In contrast, when tipifarnib was combined with tamoxifen or estrogen deprivation therapy, combined treatment induced significantly greater tumour regression compared with either endocrine therapy alone [76]. Three other groups have since reported similar findings and have suggested either a synergistic [77] or an additive anti-tumour effect [78]. One recent study implied an additive effect on G0/G1 cell-cycle arrest with enhanced inactivation of cyclin E/Cdk2 complexes and decreased phosphorylation of pRb [79].

Finally, a similar rationale has emerged to support the combination of mTOR antagonists with either tamoxifen or an aromatase inhibitor in preclinical models of ER-positive hormone-sensitive and resistant breast cancer [80,81]. The estrogen-dependent growth of both wild-type MCF7 and aromatase-expressing (MCF7/Aro) breast cancer cells was inhibited in a dose-dependent manner by the mTOR antagonist everolimus (RAD-001), suggesting that mTOR signalling is required for the estrogen-dependent proliferation of these cells. In subsequent experiments the combination of letrozole and everolimus produced maximal growth inhibition with clear evidence for additive/synergistic effects [80]. Others have shown that MCF-7 cells expressing a constitutively active Akt were able to proliferate under reduced- estrogen conditions and were resistant to the growth inhibitory effects of tamoxifen, *in vitro* and *in vivo* in xenograft models [81]. However, co-treatment with the mTOR inhibitor temsirolimus (CCI-779) inhibited mTOR activity and restored sensitivity to tamoxifen, primarily through induction of apoptosis, thus, suggesting that Akt-induced tamoxifen resistance may in part be mediated by signalling through the mTOR pathway.

With each of these small molecule STIs, clinical trials are in progress to assess not only evidence for efficacy in breast cancer patients, but also whether a combined approach of an STI with an endocrine agent is an effective strategy to enhance the benefit of endocrine therapy (Table 1) [82]. In particular, there are now several trials assessing the efficacy of combinations of small molecule tyrosine kinase inhibitors (TKI) such as gefitinib and lapatinib with either tamoxifen or aromatase inhibitors in the second-line endocrine-resistant and first-line hormone-sensitive setting. Biomarker studies in the pre-surgical setting are also being utilized as an alternative approach to

investigate whether combined endocrine/STI therapy is an effective clinical strategy (Fig. 4).

Conclusions

Substantial progress has been made in recent years in understanding some of the molecular mechanisms involved in *de novo* and acquired endocrine resistance. Laboratory and clinical data support the concept that, over time, breast cancer cells utilise alternative intracellular signalling pathways to enhance and activate ER, and that this then allows cells to escape from their initial endocrine therapy. As these pathways are elucidated, strategies are emerging to block these signalling pathways from the outset by co-treatment with various STIs. *In vitro* and *in vivo*, data now exist to show that this approach may delay resistance, and this will now be tested in ongoing randomised controlled trials in the clinic.

References

1 Johnston SR, Dowsett M. Aromatase inhibitors for breast cancer: lessons from the laboratory. *Nat Rev Cancer* 2003, **3**(11): 821–831.

2 Kumar V, Green S, Stack G, *et al.* Functional domains of the human estrogen receptor. *Cell* 1987, **51**, 941–951.

3 Tzukerman MT, Esty A, Santisomere D, *et al.* Human estrogen receptor transcriptional capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol* 1994, **8**, 21–30.

4 Kuiper GG, Enmark E, Pelto-Huikko M, *et al.* Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996, **93**, 5925–5930.

5 Cowley SM, Parker MG. A comparison of transcriptional activation by ER alpha and ER beta. *J Steroid Biochem Mol Biol* 1999, **69**(1–6): 165–175.

6 Montano M, Muller V, Trobaugh A, *et al.* The carboxy-terminal F domain of the human estrogen receptor; role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol* 1995, **9**, 814–825.

7 Spiers V, Parkes AT, Kerin MJ, *et al.* Coexpression of estrogen receptor alpha and beta; poor prognostic factors in human breast cancer. *Cancer Res* 1999, **59**, 525–528.

8 Webb P, Lopez GN, Uht RM, Kushner PJ. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol* 1995, **9**, 443–456.

9 Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, *et al.* Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 1997, **277**, 1508–1510.

10 Schiff R, Reddy P, Coronado E, *et al.* Development of tamoxifen-stimulated growth in-vivo is associated with changes in AP-1 activity. *Breast Cancer Res Treat* 1997, **46**, A347.

11 Johnston SRD, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M, *et al.* Increased activator protein-1 DNA binding and c-jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. *Clin Cancer Res* 1999, **5**, 251–256.

12 Smith CL, Nawaz Z, O' Malley BW,*et al.* Coactivator and co-repressor regulation of the agonist/antagonist activity of the mixed antiestrogen 4-hydroxytamoxifen. *Mol Endocrinol* 1997, **11**, 657–666.

13 Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen T-M, Schiff R, *et al.* Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci USA* 1998, **95**(6): 2920–2925.

14 Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, Grohe C. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. *J Biol Chem* 2000, **275**(24): 18447–18453.

15 Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 2000, **407**(6803): 538–541.

16 Levin ER. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. *Mol Endocrinol* 2003, **17**(3): 309–317.

17 Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, *et al.* Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* 2004, **96**(12): 926–935.

18 Osborne CK, Schiff R. Estrogen-receptor biology: continuing progress and therapeutic implications. *J Clin Oncol* 2005, **23**(8):1616–1622.

19 Mass R. The role of HER-2 expression in predicting response to therapy in breast cancer. *Semin Oncol* 2000, **27**(6 Suppl 11): 46–52; discussion 92–100.

20 Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicke F, *et al.* Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. *J Clin Oncol* 2001, **19**, 3808–3816.

21 Smith I, Dowsett M. Comparison of anastrozole vs tamoxifen alone and in combination as neoadjuvant treatment of estrogen receptor-positive (ER+) operable breast cancer in postmenopausal women: the IMPACT trial. *Breast Cancer Res Treat* 2003, **82**, S6; abstr 1.

22 Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, *et al.* Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2003, **95**(5): 353–361.

23 Bardou VJ, Arpino G, Elledge RM, Osborne CK, Clark GM. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *J Clin Oncol* 2003, **21**(10): 1973–1979.

24 Cui X, Zhang P, Deng W, Oesterreich S, Lu Y, Mills GB, *et al.* Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer. *Mol Endocrinol* 2003, **17**(4): 575–588.

25 Petz LN, Ziegler YS, Schultz JR, Nardulli AM. Fos and Jun inhibit estrogen-induced transcription of the human progesterone receptor gene through an activator protein-1 site. *Mol Endocrinol* 2004, **18**(3): 521–532.

26 Massarweh S, Osborne CK, Wakeling AE, Schiff R. Tamoxifen resistance in a breast cancer xenograft model coincides with a switch from an ER+/PgR+ to an ER+/PgR- phenotype accompanied by EGFR/HER2 activation. *Breast Cancer Res Treat* 2004, **82**, S18; abstr 33.

27 Osborne CK, Shou J, Massarweh S, Schiff R. Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clin Cancer Res* 2005, **11**(2 Pt 2), 865s–870s.

28 Thurlimann BJ, Keshaviah A, Mouridsen H, Mauriac L, Forbes JF, Paridaens R, *et al.* BIG 1-98: Randomized double-blind phase III study to evaluate letrozole (L) as adjuvant endocrine therapy for postmenopausal women with receptor-positive breast cancer. *Proc Am Soc Clin Oncol* 2005, **23**, 6s (abstr 511).

29 Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, *et al.* A Multigene Assay to Predict Recurrence of Tamoxifen-Treated, Node-Negative Breast Cancer. *N Engl J Med* 2004, **351**(27): 2817–2826.

30 Mouridsen HT, Ellemann K, Mattsson W, Palshof T, Daehnfeldt JL, Rose C. Therapeutic effect of tamoxifen versus tamoxifen combined with medroxyprogesterone acetate in advanced breast cancer in postmenopausal women. *Cancer Treat Rep* 1979, **63**, 171–175.

31 Smith IE, Dowsett M. Aromatase inhibitors in breast cancer. *N Engl J Med* 2003, **348**(24):2431–2442.

32 Johnston SRD, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, *et al.* Changes in estrogen receptor, progesterone receptor and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res* 1995, **55**, 3331–3338.

33 Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J, Allred DC, *et al.* Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. *J Clin Oncol* 2005, **23**(11): 2469–2476.

34 Parl FF. Multiple mechanisms of estrogen receptor gene repression contribute to ER-negative breast cancer. *Pharmacogenomics J* 2003, **3**(5): 251–253.

35 Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE. Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. *Cancer Res* 2001, **61**(19): 7025–7029.

36 Wolf DM, Jordan VC. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. *Breast Cancer Res Treat* 1994, **31**(1): 129–138.

37 Karnik PS, Kulkarni S, Liu XP, Budd GT, Bukowski RM. Estrogen receptor mutations in tamoxifen-resistant breast cancer. *Cancer Res* 1994, **54**(2): 349–353.

38 Andersen TI, Wooster R, Laake K, Collins N, Warren W, Skrede M, *et al.* Screening for ESR mutations in breast and ovarian cancer patients. *Hum Mutat* 1997, **9**(6): 531–536.

39 Dowsett M, Daffada A, Chan CM, Johnston SR. Oestrogen receptor mutants and variants in breast cancer. *Eur J Cancer* 1997, **33**(8): 1177–1183.

40 Fuqua SA, Wiltschke C, Zhang QX, Borg A, Castles CG, Friedrichs WE, *et al.* A hypersensitive estrogen receptor-alpha mutation in premalignant breast lesions. *Cancer Res* 2000, **60**(15): 4026–4029.

41 Johnston SRD. Acquired tamoxifen resistance in human breast cancer; potential mechanisms and clinical implications. *Anticancer Drugs* 1997, **8**, 911–930.

42 DeFriend DJ, Anderson E, Bell J, Wilks DP, West CM, Mansel RE, *et al.* Effects of 4-hydroxytamoxifen and a novel pure antioestrogen (ICI 182780) on the clonogenic growth of human breast cancer cells in vitro. *Br J Cancer* 1994, **70**(2): 204–211.

43 Howell A, Robertson JF, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, *et al.* Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* 2002, **20**(16): 3396–3403.

44 Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, *et al.* Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol* 2002, **20**(16): 3386–3395.

45 McClelland RA, Barrow D, Madden T-A, Dutkowski CM, Pamment J, Knowlden JM, *et al.* Enhanced Epidermal Growth Factor Receptor Signaling in MCF7 Breast Cancer Cells after Long-Term Culture in the Presence of the Pure Antiestrogen ICI 182,780 (Faslodex). *Endocrinology* 2001, **142**(7): 2776–2788.

46 Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, *et al.* Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* 2003, **144**(3): 1032–1044.

47 Stoica GE, Franke TF, Wellstein A, Czubayko F, List HJ, Reiter R, *et al.* Estradiol rapidly activates Akt via the ErbB2 signaling pathway. *Mol Endocrinol* 2003, **17**(5): 818–830.

48 Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* 2000, **20**(14): 5041–5047.

49 Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, *et al.* Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat* 1993, **24**(2): 85–95.

50 Kao YC, Cam LL, Laughton CA, Zhou D, Chen S. Binding characteristics of seven inhibitors of human aromatase: a site-directed mutagenesis study. Cancer Res 1996, **56**, 3451–3460.

51 Sourdaine P, Parker MG, Telford J, Miller WR. Analysis of the aromatase cytochrome P450 gene in human breast cancers. *J Mol Endocrinol* 1994, **13**, 331–337.

52 Dowsett M, Stein RC, Coombes RC. Aromatization inhibition alone or in combination with GnRH agonists for the treatment of premenopausal breast cancer patients. J Steroid Biochem Mol Biol. 1992, **43**, 155–159.

53 Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *J Clin Endocrinol Metab* 1995, **80**, 2918–2925.

54 Santen RJ. Adaptive hypersensitivity to estradiol: potential mechanism for secondary hormonal responses in breast cancer patients. *J Steroid Biochem Mol Biol* 2001, **79**, 115–125.

55 Chan CMW, Martin L.-A, Johnston SRD, Ali S, Dowsett M. Molecular changes associated with acquisition of estrogen hypersensitivity in MCF-7 breast cancer cells on long-term estrogen deprivation. *J Steroid Biochem Mol Biol* 2002, **81**, 333–341.

56 Martin L-A, Farmer I, Johnston SRD, Ali S, Marshall C, Dowsett M. Enhanced ER alpha, ERB2 and MAPK signal transduction pathways operate during the adaptation of MCF-7 cells to long-term estrogen deprivation. *J Biol Chem* 2003, **278**, 30458–30468.

57 Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R, Santen RJ. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. *Proc Natl Acad Sci USA* 2004, **101**, 2076–2081.

58 Song RX, Santen RJ, Kumar R, Adam L, Jeng MH, Masamura S, and Yue W. Adaptive mechanisms induced by long-term estrogen deprivation in breast cancer cells. *Mol Cell Endocrinol* 2002, **193**, 29–42.

59 Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ. Linkage of rapid estrogen action to MAPK activation by ER alpha-Shc association and Shc pathway activation. *Mol Endocrinol* 2002, **16**, 116–127.

60 Nicholson RI, Staka C, Boyns F, Hutcheson IR, Gee J. Growth factor-driven mechanisms associated with resistance to estrogen deprivation in breast cancer; new opportunities for therapy. *Endocrine Relat Cancer* 2004, **11**, 623–641.

61 Yue W, Wang JP, Conaway MR, Li Y, Santen RJ. Adaptive hypersensitivity following long-term estrogen deprivation; involvement of multiple signal pathways. *J Steroid Biochem Mol Biol* 2003, **86**, 265–274.

62 Jeng M-H, Yue W, Eischeid A, Wang J-P, Santen RJ. Role of MAP kinase in the enhanced cell proliferation of long-term estrogen deprived human breast cancer cells. *Breast Cancer Res Treat* 2000, **62**, 167–175.

63 Stephen RL, Shaw LE, Larsen C, Corcoran D, Darbre PD. Insulin-like growth factor receptor levels are regulated by cell density and by long-term estrogen deprivation in MCF-7 human breast cancer cells. *J Biol Chem* 2001, **276**, 40080–40086.

64 Nicholson, RI, McClelland, RA, Robertson, JRF, Gee, JMW. Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer. *Endocrine Relat Cancer* 1999, **6**, 373–387.

65 Jeng, MH, Yue, W, Eischeid, A, Wang, JP, Santen, RJ. Role of MAP kinase in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. *Breast Cancer Res Treat* 2000, **62**, 167–175.

66 Nicholson, RI, Hutcheson, IR, Knowlden, JM, Jones, HE, Harper, ME, Jordan, N, *et al.* Nonendocrine Pathways and Endocrine Resistance Observations with Antiestrogens and Signal Transduction Inhibitors in Combination. *Clin Cancer Res* 2004, **10**, 346S–354.

67 Kurokawa, H, Lenferink, AEG, Simpson, JF, Pisacane, PI, Sliwkowski, MX, Forbes, JT et al. Inhibition of HER2/neu (erbB-2) and Mitogen-activated Protein Kinases Enhances Tamoxifen Action against HER2-overexpressing, Tamoxifen-resistant Breast Cancer Cells. *Cancer Res* 2000, **60**, 5887–5894.

68 Witters, L, Engle, L, Lipton, A Restoration of estrogen responsiveness by blocking the HER-2/neu pathway. *Oncol Rep* 2002, **9**, 1163–1166.

69 Yu, K, Toral-Barza, L, Discafani, C, Zhang, WG, Skotnicki, J, Frost, P, Gibbons, JJ mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. *Endocrine Relat Cancer* 2001, **8**, 249–258.

70 Gee, JM, Harper, ME, Hutcheson, IR, Madden, TA, Barrow, D, Knowlden, JM, *et al.* The antiepidermal growth factor receptor agent gefitinib (ZD1839/Iressa) improves antihormone response and prevents development of resistance in breast cancer in vitro. *Endocrinology* 2003, **144**, 5105–5117.

71 Massarweh, S, Shou, J, Mohsin, SK, Ge, M, Wakeling, AE, Osborne, CK, Schiff, R Inhibition of epidermal growth factor / HER2 receptor signaling using ZD1839 (Iressa) restores tamoxifen sensitivity and delays resistance to estrogen deprivation in HER2-overexpressing breast tumors. *Proc Am Soc Clin Oncol* 2002, **21**, 33a (A130).

72 Argiris, A, Wang, CX, Whalen, SG, DiGiovanna, MP. Synergistic interactions between tamoxifen and trastuzumab (Herceptin). *Clin Cancer Res* 2004, **10**, 1409–1420.

73 Chu, I, Blackwell, K, Chen, S, Slingerland, J The dual ErbB1/ErbB2 inhibitor lapatinib (GW572016) cooperates with tamoxifen to inhibit both cell proliferation and estrogen-dependent gene expression in antiestrogen resistant breast cancer. *Cancer Res* 2005, **65**, 18–25.

74 End, DW, Smets, G, Todd, AV, Applegate, TL, Fuery, CJ, Angibaud, P, *et al.* Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 in vivo and in vitro. *Cancer Res* 2001, **61**, 131–137.

75 Kelland, LR, Smith, V, Valenti, M, Patterson, L, Clarke, PA, Detre, S, *et al.* Preclinical antitumor activity and pharmacodynamic studies with the farnesyl protein transferase inhibitor R115777 in human breast cancer. *Clin Cancer Res* 2001, **7**, 3544–3550.

76 Johnston, SRD, Head, J, Valenti, M, Detre, S, Dowsett, M Endocrine therapy combined with the farnesyltransferase inhibitor R115777 produces enhanced tumour growth inhibition in hormone-sensitive MCF-7 human breast cancer xenografts in-vivo. *Breast Cancer Res Treat* 2002, **76**, A245.

77 Ellis, CA, Vos, MD, Wickline, M, Riley, C, Vallecorsa, T, Telford, WG, Zujewskil, J, Clark, GJ Tamoxifen and the farnesyl transferase inhibitor FTI-277 synergize to inhibit growth in estrogen receptor-positive breast tumor cell lines. *Breast Cancer Res Treat* 2003, **78**, 59–67.

78 Long, BJ, Liu, G, Marrinan, CH, Black, S, Gheyas, F, Nomeir, A, *et al.* Combining the farnesyl transferase inhibitor (FTI) lonafarnib (SCH66336) with antiestrogens and aromatase inhibitors results in enhanced growth inhibition of hormone-dependent human breast cancer cells and tumor xenografts. *Proc Am Assoc Cancer Res* 2004, **45**, A3868.

79 Doisneau-Sixou, SF, Cestac, P, Faye, JC, Favre, G, Sutherland, RL Additive effects of tamoxifen and the farnesyl transferase inhibitor FTI-277 on inhibition of MCF-7 breast cancer cell-cycle progression. *Int J Cancer* 2003, **106**, 789–798.

80 Rudolf, J, Boulay, A, Zumstein-Mecker, S, Evans, DB, O' Reilly, T, Lane, HA The mTOR pathway in estrogen response: a potential for combining the rapamycin derivative FAD001 with the aromatase inhibitor letrozole in breast carcinomas. *Proc Am Assoc Cancer Res* 2004, **45**, A5619.

81 DeGraffenried, LA, Friedrichs, WE, Russel, DH, Donzis, EJ, Middleton, AK, *et al.* Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt activity. *Clin Cancer Res* 2004, **10**, 8059–8067.

82 Johnston SRD. Combinations of endocrine and biological therapies; present status of therapeutic and pre-surgical studies. *Clin Cancer Res* 2005, **11**, 889s–899s.