Mechanisms of tamoxifen resistance

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Abstract
The anti-oestrogen tamoxifen is the most commonly used treatment for patients with oestrogen-receptor (ER)-positive breast cancer. Although many patients benefit from tamoxifen in the adjuvant and metastatic settings, resistance is an important clinical problem. The target of tamoxifen in vivo is the ER. Over the last decade many advances have been made in our understanding of the biology of the ER which may help to explain how resistance to tamoxifen develops. Such mechanisms may include changes in the expression of ERα or ERβ, alterations in co-regulatory proteins, and the influences of cellular kinase signal transduction pathways. The experimental and clinical evidence supporting these mechanisms of tamoxifen resistance are discussed in this review.

Introduction
The anti-oestrogen tamoxifen is the most commonly used treatment for patients with oestrogen-receptor alpha (ER)-positive breast cancer. As an adjuvant therapy in early breast cancer tamoxifen improves overall survival, and its widespread use is thought to have made a significant contribution to the reduction in breast cancer mortality seen over the last decade (Early Breast Cancer Trialists’ Collaborative Group 1998, Peto et al. 2000). In previously untreated metastatic breast cancer, more than 50% of patients with ER-positive tumours achieve an objective response or tumour stabilisation with tamoxifen (Lippman & Allegra 1980, Paridaens et al. 1980, Campbell et al. 1981, Stewart et al. 1982, Ingle et al. 1991, Jaiyesimi et al. 1995). Tamoxifen may also have clinical utility as a preventative agent for hormone-dependent breast cancer (Cuzick et al. 2003). Despite the obvious benefits of tamoxifen in these treatment settings, almost all patients with metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen eventually relapse and die from their disease. The biological mechanisms underlying intrinsic (de novo) and acquired resistance to tamoxifen are therefore of considerable clinical significance. Better understanding of these mechanisms may suggest novel strategies to overcome tamoxifen resistance and make further improvements in breast cancer survival.

Oestrogen receptor mechanism of action
The target of tamoxifen in vivo is the ER, and levels of ER expression are the best predictor of benefit from tamoxifen (Early Breast Cancer Trialists’ Collaborative Group 1998). Therefore understanding the biology of the ER is critical to understanding how resistance to tamoxifen may develop.

The ER is a member of the nuclear receptor family of ligand-activated transcription factors. After entering the cell, oestrogen binds the ER, which dissociates from heat shock proteins and undergoes conformational changes, phosphorylation and dimerization before binding to the oestrogen response elements (ERE) upstream of oestrogen-dependent genes. This is referred to as the classical mode of action. ERs can also regulate gene expression without interacting with DNA directly, via other transcription factors such as the Fos/Jun activating protein-1 (AP-1) complex (non-classical mode) (Kushner et al. 2000, De Nardo et al. 2003, Schiff et al. 2003). Recent data also indicate that cytoplasmic membrane ER activity may interact directly with key growth-factor dependent kinases, although the biological significance of these non-genomic effects of ER is not fully understood. (Fig. 1).

The binding of ER to the ERE leads to alterations in transcription of oestrogen regulated genes. Recent data using microarray analysis to assess gene expression in the MCF7 cell line, suggests that the majority (70%) of
oestrogen-regulated genes are down-regulated by treatment with oestradiol (Frasor et al. 2003). Those genes down-regulated were shown to be transcriptional repressors, anti-proliferative and pro-apoptotic genes. Conversely there was up-regulation of positive proliferation regulators including genes involved in cell cycle progression (Frasor et al. 2003). Where up-regulation does occur, this is mediated by two distinct transactivation domains: Activating Function (AF)-1 (close to the N terminus) and AF-2 (in the ligand-binding domain). AF-1 activity is regulated by phosphorylation and is hormone-independent. AF-2 is integral to the ligand-binding domain (LBD) and therefore is hormone-dependent. AF-1 and AF-2 act synergistically in most cells, but certain gene promoters, sometimes as a result of their cellular context, can be independently transactivated by AF-1 or AF-2 alone (Gronemeyer 1991, Osborne et al. 2001). This promoter- or cell-dependence of ER-mediated transcription may be further modulated by the presence of co-regulatory proteins which are recruited to and interact with promoter-bound receptor–ligand complexes. Co-activators which enhance transcription and co-repressors which suppress transcription have both been described.

Co-activators include the p160 family which stimulate ER activity via interaction with AF-2. The three members are the nuclear-receptor co-activator 1 (NCoA1 also known as SRC1), NCoA2 (TIF2 or GRIP1) and NCoA3 (AIB1, TRAM1, RAC3 or ACTR) (McKenna et al. 1999, Leo & Chen 2000). Other coactivators include the SWI/SNF complexes, CREB-binding protein (CBP), p300/CBP-associated factor (PCAF), and the TRAP/DRIP/SMCC complex (Chen et al. 1997, Sudarsanam & Winston 2000, Ito & Roeder 2001, Vo & Goodman 2001). These proteins associate with each other and the general transcription machinery of the cell to form large complexes capable of synergistically activating oestrogen-driven transcription. Many also have histone-acetyltransferase (HAT) activity which leads to chromatin decondensation and increased rates of transcriptional initiation. Two co-repressor proteins have also been described: nuclear receptor co-repressor 1 (NCoR1) and NCoR2 (also known as SMRT) (Chen & Evans 1995, Horlein et al. 1995, Heinzl et al. 1997, Nagy et al. 1997). Co-repressors influence transcription at least in part by recruitment of histone-deacetylase complexes (HDAC) which lead to chromatin-condensation and decreased rates of transcriptional initiation.

Figure 1 The genomic (classical and non-classical) and non-genomic actions of the ER

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The ligand bound ER complex in association with co-regulatory proteins cannot be regarded in isolation from other pathways in the cell. Both the ER itself and co-regulatory proteins can be subject to phosphorylation at multiple sites by numerous cellular kinases, raising the possibility of further influences on ER-mediated transcription. When oestrogen binds to ER there are complex interactions which effect the ultimate influence of oestrogen-binding on transcription of oestrogen-responsive genes (Fig 2).

Over recent years, data has accumulated to suggest that in response to its ligand, membrane- or cytoplasm-associated ER can interact directly with key growth-factor dependent kinases (Kelly & Levin 2001). This non-genomic action occurs rapidly following ligand-binding and is independent of gene transcription. The form in which the ER mediates these non-genomic effects and its cellular localisation requires further study. However using Western blotting, ERα has been shown to be present in the plasma membrane caveolae (Kim et al. 1999), and in further studies a 46 kD ER splice-variant has been identified, expressed in the plasma membrane (Li et al. 2003). Oestrogen-activated membrane ERα has been shown to activate the insulin-like growth factor-1 receptor (IGF-1R) cell signalling cascade by direct binding to IGF-1R, followed by its phosphorylation, a process that was found to be dependent on mitogen-activated protein kinase (MAPK) kinase activity (Kahlert et al. 2000). In addition, ligand activated membrane ER can phosphorylate and activate epidermal growth factor receptor (EGFR), and can interact directly with a number of other key signalling molecules and pathways including members of the Src family, matrix metalloproteinases, G-proteins and the regulatory subunit of phosphatidyl-inositol-3 OH kinase (PI3K) (Sun et al. 2001, Migliaccio et al. 2002, Wong et al. 2002, Razandi et al. 2003). Although these effects are initially independent of gene transcription, activation of key secondary signalling pathways may mean subsequent effects on ER transcriptional activity are observed (Sun et al. 2001, Wong et al. 2002). Therefore the genomic and non-genomic actions of the ER are not necessarily independent and under some circumstances may represent inter-related complementary pathways.

When tamoxifen binds to the ER it leads to different conformational changes to those observed when oestrogen binds. Oestrogen binds within the hydrophobic pocket of the LBD and it is sealed inside by helix 12. The ensuing conformational change activates AF-2. In contrast, studies examining the crystal structure of the ERα LBD when bound to 4-hydroxytamoxifen suggest

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**Figure 2** The cellular kinase pathways interacting with ER function (with thanks to Dr Lesley-Ann Martin)
that under these circumstances helix 12 is prevented from sealing the binding pocket (Shiau et al. 1998). This repositioning of helix 12 prevents the binding of co-activators and hence AF-2 transcription is prevented. Therefore tamoxifen inhibits AF-2 activation and functions as an antagonist on genes which rely on the AF-2 region for ER-mediated transcription. However, in genes where AF-2 function is redundant and transcription is driven by the AF-1 domain, tamoxifen may function as an agonist (Tzukerman et al. 1994, McDonnell et al. 1995).

The predominance of AF-1 or AF-2-activated genes in particular tissues (for example uterus and breast respectively) has led to the apparent tissue selectivity of tamoxifen in its agonist and antagonist activity and the derivation of the acronym SERM (selective oestrogen receptor modulator) to describe its mode of action. The potential for the agonist activity of tamoxifen to outweigh its antagonist effects has been a key focus of research on tamoxifen resistance.

When tamoxifen is bound to the ER it also interacts with co-repressor molecules which result in suppression of its agonist activity (Lavinsky et al. 1998). Therefore, altered availability or recruitment of co-regulators to the tamoxifen–ER complex probably contributes to the tissue dependence of tamoxifen effects, and may also underlie some tamoxifen resistance. The complexity of ER activation and tamoxifen interaction with ER provides several potential mechanisms by which resistance to tamoxifen might evolve.

Mechanisms of resistance

Loss of ERz expression/function

Since the effects of tamoxifen are primarily mediated through the ER, and the degree of ER expression is a strong predictor of responses to tamoxifen, loss of ER expression could confer resistance to therapy. Indeed, lack of ER expression is the dominant mechanism of de novo resistance to tamoxifen, with the majority of ER/PgR negative tumours not responding to anti-oestrogens (Lippman & Allegra 1980, Paridaens et al. 1980, Campbell et al. 1981, Stewart et al. 1982, Ingle et al. 1991, Jaiyesimi et al. 1995). We have previously used an immunohistochemical assay to determine ER status in 72 paired biopsies taken before treatment and at progression or relapse on tamoxifen (Johnston et al. 1995). Patients whose tumours were treated with primary tamoxifen and responded prior to developing acquired resistance, frequently (but not always) remained ER-positive; 16 out of 18 (89%) were ER-positive pre-treatment, and 11 out of 18 (61%) were positive on relapse. In a second similar series, tissue microarrays were constructed from biopsy samples taken pre-treatment and at relapse from patients treated with adjuvant tamoxifen. Of the 29 patients who were ER-positive pre-treatment, five (17%) became ER-negative at relapse (Dowsett et al. 2003). Therefore, although ER expression may be lost in some patients who develop acquired tamoxifen resistance, and be the mechanism of resistance in these patients, the majority still express ER at progression. In fact up to 20% of patients who have relapsed on tamoxifen respond to aromatase inhibitors or to the ER-down-regulator fulvestrant suggesting that the ER continues to regulate growth even in many tamoxifen-resistant patients (Howell et al. 2002, Osborne et al. 2002).

Mutations of the ER genes may lead to a functionally negative ER phenotype without loss of ER expression as determined by protein-based immunohistochemical assays. Using site-directed mutagenesis in the AF-2 region of the mouse ER, it is possible to reduce oestrogen-dependent transcriptional activation, without significantly affecting hormone and DNA binding (Mahfoudi et al. 1995). A mutation substituting aspartate for tyrosine at position 351 has been identified in a tamoxifen-stimulated cell line (Wolf & Jordan 1994). More recently substitution of aspartate with glycine at amino acid 351 in the ER has been shown in an experimental system to silence the agonist activity of 4-hydroxytamoxifen (MacGregor Schafer et al. 2000).

Although mutations in the ER which alter the effects of bound anti-oestrogens can be generated in vitro, and can be detected in some resistant cell lines, it is relatively uncommon to detect such mutations in patients (Karnik et al. 1994, Roodi et al. 1995). In a previous collaboration we screened 96 breast carcinomas for base mutations in the ER gene (Anderson et al. 1997). No somatic mutations were detected, although it should be noted that the primers used in the constant denaturant gel electrophoresis (CDGE) and the single-strand conformation polymorphism (SSCP) were located too close to the intron/exon boundaries to detect splice mutations. Zhang and colleagues screened 30 tumours from patients with metastatic breast cancer for mutations in the ER gene using SSCP (Zhang et al. 1997). Three mis-sense mutations were identified, one of which substituted tyrosine-537 in the ligand-binding domain for asparagine. This substitution conferred constitutive oestрадиол-independent transcriptional activity which was virtually unaffected by tamoxifen.

A single amino-acid substitution changing lysine 303 to arginine has been detected in 20 of 59 hyperplastic breast lesions (Fuqua et al. 2000). This mutation produced a hypersensitive receptor, with enhanced binding of co-activators in the presence of low oestrogen levels. Subsequently it has been found that this mutation alters the crosstalk between ERz and various ERz.
pathways that normally downregulate ER signalling (Cui et al. 2002). Such loss of regulation could theoretically enhance ER-mediated cell growth and contribute to the development of resistance.

In addition to genetic changes, epigenetic changes such as CpG island hypermethylation may cause transcriptional inactivation of the ER gene (Ottaviano et al. 2001). In a recent study the methylation status of 449 CpG dinucleotides in 177 candidate genes was analysed in 200 ER and/or progesterone receptor positive tumours from patients with recurrent breast cancer who had received tamoxifen (Martens et al. 2003). A predictive score was developed, based on promoter methylation status, and could be used to identify those likely to respond to tamoxifen. Further study of this approach in independent data sets should be undertaken in order to better confirm its validity.

So there is experimental data to suggest that ER mutations may contribute to tamoxifen resistance, and such mutations are detected, albeit infrequently, in clinical samples. However when they do occur their clinical contribution to tamoxifen resistance is not always apparent, and resistance certainly occurs in their absence.

**Altered expression of ERβ**

In 1996, a second ER was cloned from a rat prostate cDNA library (Kuiper et al. 1996) and subsequently the human homologue was cloned (Mosselman et al. 1996). The gene was named ERβ to distinguish it from the classical ER which was renamed ERα. ERβ occurs in several variant forms and is expressed in both normal and malignant breast tissue (Fuqua et al. 1999, Leygue et al. 1999, Palmieri et al. 2002). In a paper published by Roger et al. (2001) decreased expression of ERβ protein was observed in pre-invasive mammary tumours compared with normal or benign lesions, leading the authors to suggest a protective effect of ERβ against the mitogenic effects of oestrogen in mammary premalignant lesions (Roger et al. 2001).

ERβ is highly homologous to ERα in its DNA and LBDs, and binds oestradiol with similar affinity to ERα. There is conflicting data concerning the relative co-expressions of ERα and ERβ and their associations with established prognostic variables, endocrine responsiveness and survival (Dotzlaw et al. 1999, Jarvinen et al. 2000, Mann et al. 2001, Omoto et al. 2001, Iwase et al. 2003, Fuqua et al. 2003). However, it appears that in experimental systems the two ERs signal in different ways depending on the ligand and response element (Paech et al. 1997). Paech and colleagues transfected HeLa cells with either an ERα or ERβ expression plasmid that contained a luciferase gene under the transcriptional control of an ERE. Both ERα and ERβ showed the same pattern of transactivation with the ligands 17β-oestradiol, raloxifene, diethylstilboestrol (DES), tamoxifen and ICI 164,384. However when ligand-induced transactivation behaviour of ERα and ERβ at an AP-1 site was examined, different patterns of transactivation were seen. With ERα, all five ligands stimulated luciferase transcription at an AP-1 site. In contrast, when ligand activation of ERβ at an AP-1 element was assessed, treatment with DES and 17β-oestradiol did not increase luciferase transcription, but raloxifene, tamoxifen and ICI 164,384 did (Paech et al. 1997). Therefore in oestrogen target tissues where signalling from ER-dependent AP-1 elements occurs, ERα and ERβ will have different effects on transcription in response to ligand-binding.

It is also interesting to note that using RT-PCR, median ERβ mRNA levels were approximately 2-fold higher than ERα levels in tamoxifen-resistant tumours compared with tamoxifen-sensitive tumours (Speirs et al. 1999). However more work is required to establish whether altered expression of ERβ and its variants are important components of tamoxifen resistance.

**Endocrine adaptation**

In pre-menopausal women receiving tamoxifen, oestrogen levels rise significantly above normal levels (Ravdin et al. 1988). Elevated oestradiol levels could thereby limit ER saturation by tamoxifen and contribute to resistance. However elevated oestradiol levels have not been reported as being related to acquired tamoxifen resistance in pre-menopausal women and in post-menopausal women, changes in oestrone and oestradiol levels are not seen with tamoxifen treatment. Therefore this is not likely to be a dominant mechanism of resistance.

**Pharmacologic tolerance**

A common mechanism of drug resistance is the emergence of reduced intracellular concentrations of drug as a result of decreased influx or increased efflux; the latter is often mediated by the membrane pump P-glycoprotein. We have previously reported serum and intra-tumoural tamoxifen concentrations in 51 patients who had de novo resistance to primary tamoxifen, acquired resistance after an initial response or had relapsed during adjuvant tamoxifen (Johnston et al. 1993). There were no significant differences in serum tamoxifen concentrations between the groups with tamoxifen-resistant breast cancer. In contrast, significantly lower intra-tumoural tamoxifen levels were observed in the acquired resistance group than in the de novo resistance group. In the adjuvant relapse group of tumours, the median level was marginally higher than in the acquired resistance group and was not significantly different from the de novo group.
Therefore this study suggests that acquired, but not de novo resistance to tamoxifen may be associated with reduced intra-tumoural concentrations in the presence of maintained serum levels. The mechanism responsible for altered tamoxifen accumulation is not understood, although P-glycoprotein is probably not responsible (Clarke et al. 1992). Sensitivity to tamoxifen could also be affected by a reduction in the intracellular availability of tamoxifen due to the presence of intracellular anti-oestrogen binding sites (Pavlik et al. 1992). The extent to which such a mechanism contributes to clinical resistance is not known. The fact that tamoxifen and its metabolites saturate the ER by more than 99.9% in post-menopausal women (Dowsett & Haynes 2003) suggests that only exceptionally large changes in the disposition of tamoxifen could lead to resistance.

Increased metabolism of tamoxifen to agonistic metabolites is a further potential resistance mechanism (Osborne et al. 1991). Tamoxifen is metabolised in man to N-desmethyltamoxifen and 4-hydroxytamoxifen. N-desmethyltamoxifen is the major metabolite detected in the serum; although 4-hydroxytamoxifen has a greater affinity for the ER than tamoxifen, the levels in serum are very low. In one model of resistance, oestrogenic metabolites would compete with anti-oestrogenic metabolites for ER activation. However it has been proposed that very large increases in oestrogenic metabolites would be required to overcome intratumoural anti-oestrogen levels (Clarke et al. 2001), and serum levels of the major metabolites of tamoxifen have been shown to remain constant over several years of therapy (Fahey et al. 1989).

However in a recent study a further active metabolite was identified (4-hydroxy-N-desmethyltamoxifen) which was present in the blood at higher concentrations than 4-hydroxytamoxifen (Stearns et al. 2003). The generation of this metabolite was found to be dependent on CYP2D6, and patients carrying the wild-type CYP2D6 allele who were treated with paroxetine had a decreased plasma concentration of 4-hydroxy-N-desmethyltamoxifen. (Paroxetine is a selective serotonin re-uptake inhibitor which is sometimes co-administered with tamoxifen in order to ameliorate hot flushes). In addition, women with a variant CYP2D6 allele receiving tamoxifen also had lower plasma concentrations of the metabolite (Stearns et al. 2003). These results indicate that both pharmacogenomic effects and pharmacological interactions may alter the metabolism and potentially the efficacy of tamoxifen.

Co-activators

AIB1 (also known as SRC3, TRAM1, NCoA3, RAC3, ACTR and p/CIP) is an ER co-activator which is overexpressed in over 50% of breast tumours (Anzick et al. 1997). It is also highly expressed in the MCF7 breast cancer cell line, and is essential for the growth of these cells in vitro and also when assessed in a mouse xenograft model (List et al. 2001). Under these circumstances AIB1 appears to have a rate-limiting role in hormone-dependent human breast tumour growth, and in cultured cells AIB1 enhances the agonist activity of tamoxifen (Webb et al. 1998). In a recent report AIB1 expression was assessed by Western blot analysis in 187 patients who had received adjuvant tamoxifen, and 119 patients who received no adjuvant therapy (Osborne et al. 2003). In the untreated patients, high AIB1 levels were associated with a better outcome when patients in the top quartile for AIB1 expression were compared with those in the lower three quartiles. In contrast, in the tamoxifen-treated patients, high AIB1 expression was associated with a worse disease-free survival when regarded as a continuous variable (hazard ratio 1.43, 95% CI 1.03 to 1.97; P = 0.031). Furthermore there may be important interactions between AIB1 and HER2 which may explain this outcome (see below). These data support the laboratory studies suggesting that high levels of ER co-activators may enhance the oestrogen agonist activity of tamoxifen and contribute to tamoxifen resistance. In contrast, data from our own laboratory did not show increases in mRNA expression of the co-activators RIP140, TIF-1 or SUG-1 in 19 tamoxifen-resistant breast tumour samples when compared with six tamoxifen treated or 21 untreated tumours (Chan et al. 1999). Experimental data suggest that overexpression of other co-activators such as SRC-1 may be able to enhance oestrogen-stimulated expression of target genes, and enhance the agonist activity of 4-hydroxytamoxifen (Tzukerman et al. 1994, Smith et al. 1997). However to date there is no evidence of overexpression of SRC-1 in clinical samples taken from patients with tamoxifen-resistant breast tumours.

Co-repressors

When co-repressors are recruited to the ER, they form multi-subunit repressor complexes which include HDACs, facilitating chromatin condensation and inhibition of gene transcription (Lavinsky et al. 1998, McKenna et al. 1999). The co-repressors are usually only recruited when an antagonist, such as tamoxifen, is bound to the ER, and under these circumstances result in a repression of its agonist activity. A study published by Lavinsky and co-workers confirmed that NCoR only weakly associates with ERα in the absence of ligand, but did so avidly in the presence of the mixed anti-oestrogen trans-hydroxyta-
moxifen (Lavinsky et al. 1998). When NCoR activity was blocked using purified Ig G against NCoR, trans-hydroxytamoxifen was converted into an agonist in MCF7 cells. In further studies MCF7 cells were implanted into nude mice which were then treated with tamoxifen. NCoR levels (assayed on whole-cell extracts of the tumours) declined in many of the tumours that acquired resistance to the anti-proliferative effects of tamoxifen, relative to tumours retaining a response to the drug. Taken together, these data raise the possibility that progressive reductions in co-repressor activity during tamoxifen therapy may enhance the agonist effects of tamoxifen on the ER contributing to resistance. In a further clinical study there were no differences in the levels of the co-repressor SMRT mRNA in cohort of 19 tamoxifen-resistant breast tumour samples, compared with 21 untreated tumours but this study did not assess NCoR levels (Chan et al. 1999).

Cellular kinase/signal transduction pathways

Oestrogen receptor biology cannot be regarded in isolation from other intracellular signalling pathways. Over the last few years, a considerable body of evidence has emerged to suggest that there are multiple regulatory interactions between the ER, growth factor and other kinase signalling pathways. Hence growth factor receptor pathways may up-regulate and stimulate growth independently of the ER, or could communicate via cross-talk with the ER and thereby affect cell growth and patterns of resistance.

Peptide growth factor signalling pathway

Numerous studies suggest that cross-talk occurs between ER and growth factor receptor pathways, such as the EGFR/HER2 family and insulin-like growth factor receptor (IGFR) family. The ER can be phosphorylated at the serine-118 position within AF-1 by the MAPKs ERK1 and ERK2, which are downstream components of the HER2 signalling pathway (Fig. 2) (Kato et al. 1995). This enhances the sensitivity of the ER to ligand and potentially leads to ligand-independent activation (Bunone et al. 1996). Serine-167 in AF-1 is also phosphorylated by a component of the kinase-mediated growth factor signalling pathway, in this case ribosomal S6 kinase (RSK) which is itself activated by ERK1 and ERK2 (Joel et al. 1998). Therefore increased ERK activity could potentially contribute to resistance to endocrine therapy. Indeed, ERK1/2 expression and activity are increased in several breast cancer cell-line models of endocrine resistance (Coutts & Murphy 1998, Shim et al. 2000), and increased ERK 1/2 activity (assessed by phosphorylated MAPK immunostaining) correlates with shorter duration of response to endocrine therapy in clinical breast cancer (Gee et al. 2001).

In addition to activating ER directly, kinase-mediated growth factor signalling may also modulate ER activity indirectly by enhancing the activity of co-activators and attenuating co-repressor activity (Lavinsky et al. 1998, Font de Mora & Brown 2000). These effects are most likely to be achieved by the phosphorylation of co-regulators, which may in turn effect their nuclear sublocalisation (Hong & Privalsky 2000, Wu et al. 2002). Therefore, it is possible that up-regulation of tyrosine-kinase growth factor receptor pathways during tamoxifen treatment may lead to loss of oestrogen-dependence and tamoxifen resistance.

The effects of the growth factor signalling pathways on the ER are bi-directional. When bound to ligand, ER can activate growth factor receptors and their downstream kinases, and signalling molecules apparently by direct interaction at the cell membrane (Kelly & Levin 2001). A direct physical association between ERz and IGFR leads to activation of IGFR and the downstream ERK1/2 MAPK pathways (Kahler et al. 2000). This interaction is blocked by the addition of the pure anti-oestrogen ICI 182,780 (fulvestrant) and by the MAPK inhibitor PD 98059. Therefore, ligand bound ER is capable of rapid activation of IGFR and its downstream signalling cascade. ERz also appears to interact directly with HER2, and membrane-bound ER transactivates EGFR by phosphorylation (Chung et al. 2002, Razandi et al. 2003). Recent data also suggest that progesterone can interact with the IGFR pathway by induction of insulin receptor substrate-2 (IRS-2) mRNA levels (Cui et al. 2003). Short-term progestin treatment was also found to increase binding of IRS-2 to Grb-2 and the PI3K regulatory sub-unit p85, and lead to enhanced ERK and AKT activation, demonstrating that cross-talk between endocrine and growth factor receptor pathways occurs at several levels.

There is a considerable body of experimental evidence to implicate these pathways and their cross-talk in de novo resistance to tamoxifen. Transfection of HER2 cDNA into the oestrogen sensitive MCF7 cell line can generate transfecteds (MCF/HER2-18) which express the HER2 receptor at a level 45-fold higher than the parental cell line (MCF7) or control transfected cells (MCF neo-3) (Benz et al. 1993). When the three cell lines are implanted into ovariectomised nude mice, no tumours were produced in the absence of oestrogen. In oestrogen-supplemented mice, the MCF/HER2-18 xenograft grew most rapidly. When oestrogen was stopped and tamoxifen started, MCF7 and MCF neo-3 tumour growth ceased immediately, whilst MCF/HER2-18 tumours continued to grow (Benz et al. 1993). These data suggest that HER2-transfected MCF7
cells are oestrogen-dependent but tamoxifen-resistant. Similar experiments carried out by Pietras et al. (1995) confirmed that HER2 transfectants were tamoxifen-resistant, but in vitro growth was found to be oestrogen-independent (Pietras et al. 1995). These latter studies also demonstrate that overexpression of HER2 or its activation with heregulin leads to the down-regulation of ER, increase in ligand-independent ER phosphorylation and transcriptional activation. Furthermore when the MAP-kinase inhibitor U0126 was added to MCF7 cells transfected with HER2 it was found to restore the inhibitory effect of tamoxifen on cell proliferation (Kurokawa & Arteaga 2001). These experiments provide evidence that overexpression of HER2 may be a primary mechanism of de novo resistance to tamoxifen.

There is also evidence from cell lines that overexpression of HER2/EGFR growth factor receptor pathways may contribute to acquired resistance to endocrine therapies. Continuous culture of MCF7 cells with either tamoxifen or the pure anti-oestrogen Fulvestrant has been shown to generate sublines which can tolerate the presence of these agents, and grow at rates equivalent to their parental lines (McClelland et al. 2001, Knowlden et al. 2003). Under these circumstances of anti-oestrogen resistance, parallel increases in EGFR and HER2 protein have been observed. In addition sensitisation to the effects of the EGFR tyrosine kinase inhibitor, gefitinib, have also been reported (Nicholson et al. 2001). It is therefore possible that acquired resistance to anti-oestrogens is facilitated by increases of EGFR/HER2 expression enabling stimulation of proliferation via this hormone-independent pathway.

There is therefore pre-clinical evidence to support the presence of parallel inter-communicating growth stimulatory pathways in breast cancer cells. The relative activity of these pathways in an individual cell may be an important determinant of the efficacy of a treatment targeting a single pathway. The existence of cross-talk between the pathways adds a further level of complexity. Such a model would predict that increases in growth factor receptor pathway signalling would potentiate the ER pathway, which in turn would reactivate growth factor signalling pathway leading to further transcriptional activation of oestrogen-responsive genes and enhanced cross-talk. It is therefore possible to envisage that enhanced growth factor signalling pathway activity may circumvent tamoxifen inhibition of growth and contribute to endocrine resistance. The possibility of such a mechanism of resistance has important clinical implications as it infers that HER2-targeted therapy with trastuzumab and the EGFR tyrosine-kinase inhibitor gefitinib may be useful agents either to prevent or to circumvent tamoxifen resistance. However is there clinical evidence that overexpression of such pathways may contribute to resistance?

In the adjuvant setting several studies suggest that patients overexpressing HER2 may derive relatively less benefit from adjuvant tamoxifen (Carlomagno et al. 1996, Dowsett 1999, Knoep et al. 2001, De Placido et al. 2003). However this is not a universal finding and studies in both the adjuvant and metastatic settings have methodological limitations because they are retrospective, have small numbers of patients and use inconsistent methods to assess HER2 status (Berry et al. 2000, Stal et al. 2000), reviewed in (Ring & Dowsett 2003). However studies undertaken in the neoadjuvant setting, have the substantial advantage that outcome data are based on objective responses to treatment, and ER and HER2 can be assessed directly in the lesion being measured. In our own studies, the effect on proliferation (measured by Ki67 levels) of hormone therapy was assessed at 2 and/or 12 weeks in biopsies from 115 patients with primary ER-positive breast cancer (Dowsett et al. 2001). (The patients in this analysis had participated in one of 3 neoadjuvant trials and had received tamoxifen, idoxifene, anastrozole or vorozole.) It was found that patients with tumours which overexpressed or amplified HER2, had an impeded anti-proliferative response to endocrine therapy, and that this was likely to be translated into clinical response, but the value of this observation was reduced by the mixture of endocrine agents included in the analysis.

In a study conducted by Ellis et al. (2001) 337 post-menopausal women who were ER- and/or PgR-positive on pre-treatment biopsy were randomly assigned to either primary letrozole or tamoxifen (Ellis et al. 2001). In 237 women a biopsy was also available for central testing of HER2/ EGFR. In the ER-positive tamoxifen-treated patients the response rate was 21% for those who expressed EGFR and/or HER2 and 42% in those who were EGFR- and HER2- negative, although this difference did not reach statistical significance (P = 0.095). For all ER-positive and/or PgR-positive patients the response rate was higher for letrozole compared with tamoxifen (60% vs 41%, P = 0.004). However a novel finding was that in ER-positive patients who were also EGFR- and/or HER2-positive, the response rate was significantly higher to letrozole than to tamoxifen (88% vs 21%, P = 0.0004) (Fig. 3). In contrast there was no significant difference between response rates to letrozole and tamoxifen in the ER-positive, EGFR and HER2 negative patients (54% vs 42%, P = 0.078 > ). In the IMPACT study 330 post-menopausal women with operable breast cancers were randomised to receive 3 months of neoadjuvant anastrozole, tamoxifen or the combination (Smith et al. 2004). Thirty-four of 239 (14%) per protocol treated ER-positive patients were HER2-positive. In these patients the response rate to
anastrozole (58%) was higher than that to tamoxifen (22%) although this did not reach statistical significance ($P = 0.09$). The suggestion from these studies is that women with HER2 positive tumours are differentially more likely to respond to aromatase inhibitors than to tamoxifen, a conclusion that is potentially highly important but bearing in mind the small sample sizes must be regarded as preliminary and will require confirmation.

Studies such as that reported by Ellis et al. (2001) concentrate on whether de novo overexpression of HER2 contributes to tamoxifen resistance. However many tumours are not resistant from the outset but rather exhibit an initial response or period of stabilisation before acquiring resistance to treatment. It is therefore a possibility that HER2 may contribute to acquired resistance by becoming overexpressed during the period of endocrine treatment, and thereby providing an alternative pathway for autonomous tumor growth. At our institution HER2 status was assessed in 155 patients who were progressing on tamoxifen treatment (Newby et al. 1997).

Figure 3 Clinical responses according to EGFR/HER2 status in patients treated with neoadjuvant endocrine therapy.
Marginaly higher levels of HER2 positivity were present in samples taken from patients who were progressing on tamoxifen compared with untreated tumors. However in the 61 patients in whom paired pre-treatment and relapse specimens were available, there was no significant increase in HER2 positivity. In contrast, in a second study tissue microarrays were constructed from biopsy samples taken pre-treatment and at relapse from patients who had received adjuvant tamoxifen (Dowsett et al. 2003). Paired data were available from 39 patients, of whom 29 were ER-positive. Of these 29 ER-positive patients, three were HER2-positive pre-treatment and remained so at relapse. However a further three patients who were HER2-negative pre-treatment became HER2 positive at relapse. This second (but smaller) study therefore supports the hypothesis of acquisition of tamoxifen resistance by HER2 overexpression in a proportion of patients.

The studies reviewed show that the contribution of HER2 overexpression to tamoxifen resistance is by no means certain. However as has already been discussed high levels of the co-activator AIB1 can reduce the antagonist effects of tamoxifen, and kinase-mediated growth factor signalling may enhance the activity of co-activators such as AIB1 (Font de Mora & Brown 2000). Therefore, it is possible that in tumours with high levels of AIB1 which overexpress HER2, AIB1 may be further activated by signalling downstream of HER2 and the anti-tumour effectiveness of tamoxifen be reduced because of enhanced oestrogen-agonist activity. In this way, the assessment of AIB1 levels might complement and enhance the predictive value of HER2 (Schiff et al. 2003). There is clinical evidence to support this hypothesis (Osborne et al. 2003). One hundred and eighty seven patients treated with tamoxifen and 119 with no adjuvant systemic therapy were studied. It was found that only those tumours expressing both high AIB1 and high HER2 had a worse outcome with tamoxifen, whereas those patients with tumours with high HER2 or AIB1 had a good disease-free survival (Osborne et al. 2003). These data support the hypothesis that increased signalling through EGFR/HER2 may activate MAPK in turn activating ER and AIB1. In the presence of a phosphorylated ER and high levels of activated AIB1, the agonist activity of tamoxifen may be enhanced and resistance develop.

PI3K cell survival pathway
ER activity may also be influenced by the phosphatidylinositol-3-OH kinase (PI3K) pathway. When activated by tyrosine kinase receptors in response to growth factor stimulation, PI3K catalyses the formation ofPIP3. One of the downstream targets of this pathway is the serine/threonine protein kinase AKT, whose activation promotes cellular proliferation and anti-apoptotic responses (Datta et al. 1999, Clark et al. 2002). There is evidence that ERα can bind in a ligand-dependent manner with the p85α regulatory subunit of PI3K, leading to the activation of AKT and subsequent downstream effects (Simoncini et al. 2000). AKT is also a target of several other receptor-stimulated pathways including IGF-1R, EGFR and HER2 (Clark et al. 2002). However the relationship with ER is reciprocal, in that PI3K activates AKT which phosphorylates the ER at serine-167 resulting in ligand-independent activation (Campbell et al. 2001).

The potential clinical importance of the relationship between the PI3K cell survival pathway and ER-mediated signalling has been demonstrated in two laboratory studies (Campbell et al. 2001, Clark et al. 2002). Clark and colleagues measured tamoxifen-induced apoptosis in different cell lines with and without the PI3K pathway inhibitor LY294002 (Clark et al. 2002). The addition of LY294002 to tamoxifen was found to significantly increase the pro-apoptotic effects of tamoxifen, particularly in the cell line with the highest endogenous levels of AKT activity. Campbell and colleagues transfected MCF7 cells with AKT reducing the inhibition of cell growth by tamoxifen, suggesting that high expression of AKT could be associated with tamoxifen resistance but there are no clinical data to address this (Campbell et al. 2001).

Stress-activated protein kinase/c-junNH2 terminal kinase pathway.
The ER protein may interact with the stress-activated protein kinase/c-junNH2 terminal kinase pathway either by binding with the Activating Protein-1 (AP-1) transcription complex or possibly by direct activation of ER by p38 MAPK.

AP-1 is a complex of proteins composed of Jun and Fos family members which dimerize and bind to DNA at AP-1 response elements (Angel & Karin 1991). AP-1 transcriptional activity is increased by increased abundance of any of the components or by their phosphorylation by the Jun NH2-terminal kinases (JNKs) or stress activated protein kinases (SAPKs). These enzymes are activated by cellular stresses including oxidative stress (Kyriakis et al. 1994). It has been reported that the development of tamoxifen resistance in MCF7 cells is accompanied by increased AP-1 DNA binding (Dumont et al. 1996). These findings have been replicated in a panel of 30 primary human breast tumours with acquired tamoxifen resistance, compared with 27 untreated controls (Johnston et al. 1999). Similarly, but using a mouse xenograft model, Schiff and colleagues showed that tamoxifen-resistant tumours, compared with oestrogen-treated tumours, had increased AP-1 dependent transcription and phosphorylated c-Jun and JNK levels (Schiff et al. 2000). In addition, they found that the conversion to a
resistant phenotype was associated with an increase in oxidative stress (as measured by increases in superoxide dismutases and glutathione-S-transferase). It has been known for some years that tamoxifen can induce intracellular oxidative stress, and these data would be consistent with a model where tamoxifen-induced oxidative stress leads to activation of JNK and increased AP-1 activity (Schiff et al. 2000, Clarke et al. 2001). Such a chain of events could potentiate the agonistic effects of tamoxifen at AP-1 sites (Webb et al. 1995).

Activation of the p38 MAPK pathway has been reported to occur in response to a number of extracellular stimuli including growth factors, cytokines, physical and chemical stresses (New & Han 1998). The downstream targets of p38 MAPK include further protein kinases and transcription factors. In cell lines expressing the ER, 4-hydroxytamoxifen has been shown to activate the p38 MAPK pathway and to induce apoptosis (Zhang & Shapiro 2000). Under these circumstances inhibition of the p38 signalling pathway greatly reduces the ability of 4-hydroxytamoxifen to induce apoptosis. Moreover, in cell lines selected for resistance to 4-hydroxytamoxifen there is a correlation between ability to activate the p38 pathway and ability of this agent to induce apoptosis (Zhang & Shapiro 2000). In a series previously mentioned, tissue microarrays were constructed from biopsy samples taken pre-treatment and at relapse from patients treated with adjuvant tamoxifen (Dowsett et al. 2003). In those patients who were ER-positive and HER2 positive at relapse there was uniformly high expression of p38 MAPK, suggesting that in this subset of patients, activation of ER may have occurred by this route.

Summary and conclusions

Over the last few years it has become apparent that ER transcriptional effects are not just determined by the ligand, but also by complex interactions between co-regulatory molecules and multiple associated cell-signalling pathways. These interactions provide several plausible mechanisms by which breast cancer cells may circumvent a reliance on oestrogen-stimulated growth and become hormone-independent and tamoxifen-resistant. Altered levels of the expression of elements in these signalling pathways and co-regulatory molecules are common accompaniments in experimental models of endocrine resistance. Emerging clinical data suggests that such alterations may also play a part in resistance to tamoxifen in a clinical setting. A number of pharmacological agents targeting these pathways are available in the clinic or are in development. Further confirmation in clinical samples that the pathways described are determinants of tamoxifen resistance may allow the circumven-

tion or prevention of tamoxifen resistance using rational therapeutic approaches. It will be important to consider that there may be multiple resistance pathways that are highly important in some patients but are irrelevant in others.

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