Growth factor signalling and resistance to selective oestrogen receptor modulators and pure anti-oestrogens: the use of anti-growth factor therapies to treat or delay endocrine resistance in breast cancer

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Abstract

De novo insensitivity and acquired resistance to the selective oestrogen receptor modulator tamoxifen and the pure anti-oestrogen fulvestrant (faslodex) severely limit their effectiveness in breast cancer patients. This is a major clinical problem, since each year upward of 1 million women are dispensed anti-oestrogenic drugs. In order to investigate the phenomenon of anti-oestrogen resistance and to rapidly screen drugs that target the resistance mechanism(s), we have previously established several in vitro breast cancer models that have acquired resistance to anti-hormones. Such cells commonly develop an ability to proliferate after approximately 3 months of exposure to 4-hydroxytamoxifen or fulvestrant, despite an initial endocrine-responsive (i.e. growth-suppressive) phase. The current paper explores the role that growth factor signalling plays in the transition of oestrogen receptor-positive endocrine-responsive breast cancer cells to anti-oestrogen resistance or insensitivity and how we might, in the future, most effectively use anti-growth factor therapies to treat or delay endocrine-resistant states.

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Introduction

In oestrogen-sensitive breast cancer cells, it is widely believed that locally and distally produced growth factors engage intracellular signalling pathways that productively cross-talk with oestrogen receptor (ER) signalling elements to facilitate tumour cell growth, with anti-oestrogenic drugs acting to disrupt such events and promote growth inhibition and limited apoptosis (Nicholson & Gee 2000, Nicholson et al. 2001, 2004a,b). In the commonly used MCF-7 breast cancer cell line, growth factor signalling through the insulin-like growth factor-I (IGF-I) receptor (IGF-IR) is believed to extensively cross-talk with the ER to enhance its activity as a nuclear transcription factor, which in turn is believed to enhance the expression and activation of IGF-IR signalling components (Lee et al. 1997, Surmacz 2000). Although both tamoxifen and fulvestrant (faslodex) are thus able to efficiently reduce ER and IGF-IR signalling during their growth inhibitory phase (Wakeling et al. 2001), acquired drug-resistant growth inevitably occurs with evidence of re-established ER and growth factor signalling. Importantly, within the anti-oestrogen-resistant models, elevated or sustained growth factor signalling can eventually lead to endocrine insensitivity, where either ER signalling is circumvented or the ER is lost.
(Nicholson et al. 2004a,b). In our experience this process is often accompanied by morphological changes in the cells that resemble an epithelial mesenchymal transition (EMT). Certainly, altered patterns of growth factor signalling often promote more aggressive cell behaviour with increased growth rate, motility and invasiveness frequently characterising anti-hormone resistance and insensitivity (Hiscox et al. 2004, Nicholson et al. 2004a).

In light of the above, the current article examines aspects of our work associated with the transition of anti-oestrogen-responsive breast cancer cells to endocrine resistance and insensitivity and the therapeutic consequences of this knowledge.

**Growth factor signalling as a mechanism to promote anti-hormone failure**

It is now widely documented that the inappropriate activation of growth factor signalling cascades, either through an enhanced supply of growth factor ligands, or via up-regulation and increased activation of their target growth factor receptors or their recruited downstream signalling elements, can readily promote anti-hormone failure in breast cancer cells. As such, this phenomenon has been described for the over-expression of multiple growth factors and their receptors, including heregulins acting through HER3 and HER4 (Pietras et al. 1995, Lupu et al. 1996, Tang et al. 1996), epidermal growth factor and transforming growth factor (TGF)-α acting through the epidermal growth factor receptor (EGFR) (McClelland et al. 2001, Knowlden et al. 2003, Nicholson et al. 2004a), and IGF-I and -II acting through the IGF-IR (Guvakova & Surmacz 1997, Stephen et al. 2001), with HER2 contributing to anti-hormone failure either directly when overexpressed (Benz et al. 1993, Liu et al. 1995, Kurokawa et al. 2000), or indirectly through heterodimerisation with other erbB receptor family members (Knowlden et al. 2003).

In our in vitro studies, the resistant sub-lines that we have developed by prolonged exposure of endocrine-responsive MCF-7 breast cancer cells to anti-oestrogens uniformly express increased amounts of EGFR mRNA and protein (McClelland et al. 2001, Knowlden et al. 2003, Nicholson et al. 2004a). Thus, while EGFR immunostaining of the parental MCF-7 cells demonstrates only modest levels of this receptor, tamoxifen-or fulvestrant-resistant cells show up to 40-fold higher levels of EGFR membrane staining. We have also noted marked parallel increases in HER2 immunostaining in anti-oestrogen-resistant cells, again localised at the plasma membrane (Hutcheson et al. 2002, 2003, Knowlden et al. 2003). In each instance, the immunostaining gives the cells a cobblestone appearance, a feature of endocrine-insensitive clinical disease that also associates with aggressive cell behaviour (Nicholson et al. 1993, Gee et al. 2005). Consistent with this, our tamoxifen-resistant cells show increased growth rate, motility and invasiveness (Hiscox et al. 2004, Nicholson et al. 2004a).

The concept that overexpressed EGFR and HER2 play a role in the development of anti-oestrogen resistance is supported by data that demonstrate using immunofluorescence that the receptors co-localise and that they are heterodimerised with an increased basal level of activation following their immunoprecipitation (Knowlden et al. 2003). Because the tamoxifen-resistant variants also express several EGFR ligands (see below), each of which can further increase activation of EGFR and HER2 and induce additional growth responses, it is likely that the new growth signal originates from an EGFR-driven autocrine regulatory loop (Knowlden et al. 2003). Further assessment of EGFR/HER2 signalling in anti-oestrogen-resistant cells has shown that they recruit multiple signal transduction cascades that act to increase the activation of ERK1/2 mitogen-activated protein kinase (ERK1/2 MAPK), AKT and protein kinase C (PKCα and δ), key elements in the regulation of cell proliferation and survival signals (Bonni et al. 1999, Gibson et al. 1999, Stambolic et al. 1999, Campbell et al. 2001). Such signalling would act as a counterbalance to the anti-proliferative and pro-apoptotic effects of anti-oestrogens. Interestingly, complementary associations have previously been reported by other groups between acquisition of tamoxifen resistance by ER-positive breast cancer cells in vitro and increased ERK1/2 MAPK activation (Coutts & Murphy 1998, Kurokawa et al. 2000, Donovan et al. 2001, Oh et al. 2001) or AKT phosphorylation (Campbell et al. 2001, Kurokawa & Arteaga 2003). MAPK increases are also reported to contribute to the growth of ER-positive breast cancer cells during adaptation to long-term oestrogen deprivation (Jeng et al. 2000, Martin et al. 2003). Similarly, overexpression of PKCα in ER-positive breast cancer cells appears able to promote hormone-independent growth (Tonetti et al. 2000).

**Growth factor-mediated ER phosphorylation in anti-oestrogen-resistant breast cancer cells**

**ER phosphorylation in tamoxifen-resistant cells and its role in establishing an EGFR-driven autocrine growth regulatory loop**

Nuclear ER is often retained in cells with acquired tamoxifen resistance, and a key role for ER in such
cells is evidenced by obvious sensitivity at a signal transduction and growth level to inhibition by fulvestrant (Hutcheson et al., 2003, Nicholson et al., 2004a). This equates with fulvestrant responses observed in tamoxifen-resistant patients. Our previous laboratory studies have shown that the increased EGFR/MAPK signalling in tamoxifen resistance is central in promoting ER phosphorylation on serine 118 (and serine 167), thus allowing ligand-independent activation of nuclear ER (Britton et al., 2002). We have now shown that the EGFR/MAPK-induced Ser118 phosphorylation of ER promotes binding of the receptor to oestrogen response elements (EREs), as measured using ERE reporter gene constructs. It also enables recruitment of multiple co-activators to the ER/ERE, notably including p68 RNA helicase (D Britton, unpublished observations), a co-factor linked in the literature to ligand-independent (MAPK-driven) ER activation (Endoh et al., 1999). We have previously shown that several EGFR ligands (including TGFα) are expressed by our tamoxifen-resistant cells (Hutcheson et al., 2003, Nicholson et al., 2004a). However, we have now revealed by PCR (and by microarray analysis) that amphiregulin is the most abundant of these ligands, expressed at high levels in tamoxifen-resistant cells subsequent to its inhibition during the anti-hormone-responsive phase. Chromatin immunoprecipitation assays have shown that ER associates with part of the promoter of the amphiregulin gene, binding to a consensus ERE (D Britton, unpublished observations). In parallel with their anti-tumour effects, the regulation of amphiregulin mRNA can be reduced by inhibitors of the EGFR/MAPK pathway (gefitinib and PD184352 respectively) and ER signalling (fulvestrant). Treatment of the tamoxifen-resistant cells with a neutralising antibody to amphiregulin efficiently blocks EGFR/MAPK/ER signalling.

These findings suggest the existence of a self-propagating EGFR-driven autocrine growth regulatory loop in tamoxifen resistance in which several EGFR ligands are regulated by ligand-independent Ser118 ER phosphorylation. This appears to contrast the regulation of growth and Ser118 ER phosphorylation in MCF-7 cells, where comparative studies challenging with IGFs, the IGF-IR inhibitor AG1024, or the MAPK and phosphatidylinositol 3-kinase (PI3K) inhibitors PD184352 and LY294002 respectively, indicate very little impact of EGFR/MAPK signalling but dominant regulation of ER phosphorylation and ERE transcriptional activity by IGF-IR/PI3K/AKT signalling in anti-hormone-responsive cells.

**IGF-IR activation of EGFR/MAPK/ER**

Although initial responses of MCF-7 breast cancer cells to anti-oestrogenic drugs are associated with a reduction of IGF-IR signalling, we have recently noted that the EGFR-activated ER in tamoxifen-resistant variants promotes the expression of IGF-II, with a subsequent re-activation of IGF-IR signalling. Importantly, the IGF-IR, in addition to its direct effects on proliferation and survival signals, also appears to play a supportive role to maximise the efficiency of the EGFR to direct growth (Knowlden et al., 2003, Hutcheson et al., 2004). Thus IGF-II-driven activation of IGF-IR is able to promote the phosphorylation of the EGFR on tyrosine 845 by a c-src-dependent mechanism, while inhibitors of the IGF-IR and c-src block this phosphorylation (Hutcheson et al., 2004). Significantly this pathway also appears to contribute to the increased levels of ER phosphorylation seen in tamoxifen-resistant cells (and thereby their autocrine growth regulation of growth) since IGF-II primes ER phosphorylation on Ser118 and ERE activity in a manner that is blocked by gefitinib and IGF-IR inhibitors.

**Increased growth factor signalling reduces ER levels and activity to promote endocrine insensitivity**

Although, as established above, growth factor pathways can enhance ER phosphorylation, transcriptional activity and cell growth in a ligand-independent manner, paradoxically a decline in ER expression is also a possible outcome when growth factor signalling is extreme or sustained (Gee et al., 2004). Thus, we have recently shown that challenge of MCF-7 with specific exogenous growth factors (i.e. heregulins, IGFs), or further enhancing EGFR signalling with EGF-like ligands in tamoxifen-resistant cells, is able to generate cells refractory not only to the growth-inhibitory effects of tamoxifen, but also to multiple forms of anti-ER strategies including fulvestrant and oestrogen withdrawal (i.e. ‘complete endocrine insensitivity’), suggesting that extreme growth factor signalling is capable of dislocating growth from ER signalling (Hutcheson et al., 2003, Nicholson et al., 2004b).

Further supportive evidence for a negative impact on ER of exposure to extreme growth factor signalling can be drawn from several stable transfection studies in ER-positive breast cancer cells. Such studies demonstrate that growth factor signalling elements comprising the EGFR/HER2 pathway that share an ability to hyperactivate ERK1/2 MAPK, all act to impair ER function and promote ER loss when overexpressed.
in ER-positive breast cancer cells. In our own study, we have shown that constitutive up-regulation of MEK1 in MCF-7 cells leads to a substantial increase in MAPK activation, decreased ER level and marked loss of expression of the ER-regulated gene PR (R A McClelland, unpublished observations). Similarly, El-Ashry and colleagues have noted precipitous falls in ER mRNA and protein following transfection of constitutively active HER2, MEK1 (Δmek), Raf1 (Δraf) or ligand-stimulated EGFR into MCF-7 cells (Liu et al. 1995, Pietras et al. 1995, El-Ashry et al. 1997, Oh et al. 2001), all of which hyperactivate ERK1/2 MAPK. There is a parallel loss of oestrogen-mediated gene expression (e.g. pS2 and PR) and a marked suppression of activity of ERE-reporter gene constructs in transient transfection experiments that is not overcome by oestriol treatment. Not surprisingly, the severe impact of elevated growth factor signalling on ER expression and function associated with these transfection studies resulted in oestrogen independence and acquisition of anti-oestrogen resistance. Holloway et al. (2004) demonstrate that hyperactivated ERK1/2 MAPK is able to down-regulate ER via substrates including the transcription factor nuclear factor kappa B (NFκB), which is markedly increased in activity in the various transfection models and is inhibited by abrogating ERK1/2 MAPK signalling. Other systems indicate that NFκB activation may occur via ERK1/2 MAPK-mediated induction of autocrine growth factors (e.g. heparin-binding EGF). Overexpression of AKT, PKCα and AP-1 components have all been linked with decreased ER function, loss of ER and endocrine-resistant states (Tzukerman et al. 1991, Smith et al. 1999). Interestingly, many of these parameters do appear to be elevated de novo in multiple ER-negative cells, for example EGFR, ERK1/2 MAPK activity (Biswas et al. 2000). Activated NFκB also appears to be prominent in tumours bearing HER2 overexpression (Biswas et al. 2004) and has also been associated with elevated AKT activity (de Graffenried et al. 2004). Moreover, marked overexpression of EGFR and extreme downstream signalling (e.g. ERK1/2 MAPK activity) has invariably been associated with ER negativity, as well as with adverse clinicopathology, metastasis and shortened relapse-free survival in breast cancer patients (Nicholson et al. 1994, Gee et al. 2001, 2005).

**Growth factor signalling and faslodex resistance: a progression towards ER negativity**

Excitingly, 2004 saw the launch of fulvestrant in advanced breast cancer patients, where it has proven effective in those who have developed resistance to tamoxifen, and it is also being used following aromatase inhibitor failure. This event underpins the need to not only further establish its most appropriate position in therapeutic sequencing, but also to understand fulvestrant-resistance mechanisms in order to extend its clinical utility. To this end, we have now developed two acquired fulvestrant-resistant models based on their exposure time to fulvestrant. The cells have been used for signalling and microarray studies.

‘Early’ fulvestrant-resistant cells

We have previously reported on an early passage fulvestrant-resistant MCF-7 variant (passage 40) that grows relatively slowly, shows a rounded cell morphology and exhibits very low ER (McClelland et al. 2001). These cells are EGFR-positive, utilise EGFR/MAPK signalling for growth and are thus gefitinib-sensitive. In such cells ER levels are fully recoverable on removal of the drug and they regain full ER signalling and oestrogen growth sensitivity (McClelland et al. 2001).

‘Late’ fulvestrant-resistant cells

Our Cell Culture Unit has now developed a much later passage of fulvestrant-resistant cells (passage 70) for study. In these cells, we have observed that the elevated growth factor signalling sustained during continued treatment (detectable as focally increased EGFR expression and phosphorylation, with parallel nuclear-activated MAPK) is associated with a markedly increased cell growth rate, a more angular de-differentiated morphology (reflective of EMT), increased motility/invasiveness and complete loss of ER expression, phosphorylation and transcriptional activity (Nicholson et al. 2004b). Significantly, unlike the earlier passage cells, the long-term fulvestrant-resistant MCF-7 variants are unresponsive to oestrogens on anti-hormone removal and significant levels of ER and its function are not recovered even after several months of drug withdrawal. Clearly, the progression from anti-hormone-responsive cells to resistance/insensitivity can ultimately involve loss of steroid hormone receptors. We are currently investigating the mechanism(s) associated with generation of this ER-negative phenotype and determining whether it can be reversed by novel therapeutic approaches (Gee et al. 2004). Such study is important not only with regards to determining the potential phenotypes evolving during fulvestrant treatment, but also to further understand the ER-negative state. ER negativity is an important clinical feature in breast cancer, apparent in 30% of specimens on presentation and in
some ER-positive tumours at the time of tamoxifen relapse (Gee et al. 2005). Moreover, in the clinic ER negativity is associated with poor prognosis, precluding response to all categories of anti-hormones and associating with a more aggressive, proliferative phenotype (Nicholson et al. 1993), features of our later passage fulvestrant-resistant cells.

With regards to the growth-factor-driven mediator of ER down-regulation in our late passage fulvestrant-resistant cells, ongoing microarray, RT-PCR and transfection studies have revealed increased NFκB levels and activity, in part under EGFR/MAPK regulation. Interestingly, our initial studies suggest that the ER gene has become methylated during prolonged fulvestrant exposure, preventing its expression. By collaboration with Dr M Widschwendter (Innsbruck University, Austria) we are utilising highly sensitive ‘methyl-light’ PCR technology to examine CpG island promoter methylation and the role of methylation in gene suppression in fulvestrant resistance. Based on our emerging experimental data, it may be feasible to manipulate growth factor signalling (by application of appropriate anti-growth factor strategies) to reinstate the ER-positive better-prognostic phenotype in ER-negative cells. Furthermore, since drugs are now available that can efficiently reverse epigenetic silencing (e.g. the DNA methyl transferase inhibitor aza-2-deoxycytidine, and histone deacetylase inhibitors such as trichostatin), their application in our tumour models may also prove valuable in regenerating ER expression and endocrine response, paving the way for similar strategies in patients. We are currently exploring these concepts in vitro, where our preliminary data indicate ER can be recovered in de novo ER-MDA-MB-231 breast cancer cells using an aza-2-deoxycytidine/trichostatin strategy.

**Therapeutic targeting of growth factor signalling to treat/delay/prevent the development of endocrine resistance**

The above studies clearly establish that anti-oestrogen-resistant growth and aggressive growth behaviour can proceed in either an ER-dependent manner, where increased growth factor signalling is harnessed to the ER to facilitate growth factor signalling, or an ER-independent manner, where the importance of ER is superseded by sustained growth factor signalling onto proliferation and survival pathways. In the latter instance, sustained non-ER-directed signalling could arise from paracrine or endocrine sources of growth factors, through additional oncogenic events or the reprogramming of the cells, as with the long-term faslodex-treated cells. Whatever the mechanism, both ER-dependent and -independent activation of growth factor signalling should be susceptible to anti-growth factor strategies.

**Mono-therapies in anti-oestrogen-resistant cells**

Multiple signal transduction inhibitor agents have now been examined in our models of tamoxifen and faslodex resistance, including inhibitors of EGFR/HER2/IGF-IR/MAPK and AKT and shown to be growth inhibitory (Nicholson et al. 2004a,b). Additionally, faslodex blocks tumour cell growth in our model of acquired resistance to tamoxifen, confirming the importance of ER signalling (Hutcheson et al. 2003). Characteristically, over a 3 week period, anti-hormone-resistant growth can be inhibited by 70-90% by signal transduction inhibitors, with responses lasting several months. Thus in the case of gefitinib, one of the more effective agents, inhibition of tumour growth is almost complete at 3 weeks and resistant growth is not observed for 4-6 months. Unfortunately, the outcome of all mono-therapies tested to date is drug resistance and this is sometimes characterised by an increased aggressive behaviour with cells showing an increased growth rate, motility and invasiveness (Jones et al. 2004, 2005). Studies to address the reasons for incomplete responses and the development of drug resistance point to (i) an ability of anti-hormones and anti-oestrogens to induce compensatory signals that act to limit their anti-proliferative and pro-apoptotic actions (Nicholson et al. 2004a) and (ii) a plasticity of tumour cells that allows them to switch between growth signalling pathways (Jones et al. 2005). As such, we have noted that gefitinib, while blocking EGFR signalling in tamoxifen-resistant cells, paradoxically promotes IGF-IR signalling, allowing the cells to tolerate the drug, with increased IGF-IR signalling subsequently re-coupling to ER and becoming the resistant mechanism (Jones et al. 2004, 2005, Knowlden et al. 2004).

**Combination therapies in anti-oestrogen-resistant cells**

In light of the above, we have examined various combination therapies in an attempt to more completely inhibit ER and growth factor signalling in anti-oestrogen-resistant cells and thereby improve anti-tumour responses.

**Fulvestrant and gefitinib**

The existence of an EGFR/MAPK/ER-driven growth loop in tamoxifen-resistant cells has led us to examine the hypothesis that the co-targeting of ER and EGFR signalling might more efficiently break the autocrine...
loop than either agent alone and thereby promote a more worthwhile growth inhibition. In order to address this we have examined the ability of fulvestrant and gefitinib to block ER/EGFR signalling and growth, when used alone and in combination. As previously described, mono-therapies of fulvestrant and gefitinib are effective strategies to reduce EGFR/MAPK/ER signalling and promote growth inhibition. Significantly, however, neither treatment entirely abolished MAPK or Ser118 ER phosphorylation and a significant level of residual signalling was observed. In contrast, the combination of fulvestrant and gefitinib more completely inhibited the phosphorylation of these signalling molecules and promoted a more extensive growth inhibition. Indeed, extension of the growth studies to 3 months clearly demonstrates that while tumour cell re-growth is evident in the mono-therapy arms of the experiment, indicating the initiation of drug resistance, the combination of fulvestrant and gefitinib continues to show a far superior growth inhibition. This principle appears to apply to the targeting of ER and growth factor signalling elements in breast cancer cells resistant to oestrogen deprivation (Staka et al. 2005).

**Gefitinib and AG1024**
The capacity of the IGF-IR to improve the efficiency of EGFR signalling in tamoxifen-resistant cells (Hutcheson et al. 2004, Knowlden et al. 2004) and the ability of gefitinib to aid IGF-IR activity during the gefitinib growth inhibitory phase (see above and Knowlden et al. 2004) has led us to examine the hypothesis that co-targeting of these growth factor receptors might be more efficacious than the use of either agent alone. Initial experiments indicate this to be the case with the IGF-IR inhibitor AG1024 improving the sensitivity of tamoxifen-resistant cells to gefitinib and delaying the occurrence of gefitinib resistance (Nicholson et al. 2004a).

**Anti-hormones and gefitinib in endocrine-responsive cells**
Previous studies from our laboratory have shown that treatment of endocrine-responsive cells with gefitinib efficiently improves the inhibitory activity of the anti-oestrogens tamoxifen and fulvestrant, and severely delayed, and in some instances prevented, the development of resistance (Gee et al. 2003, Nicholson et al. 2004a). We hypothesise that since anti-hormones induce EGFR expression, early signalling from this receptor stimulates a survival pathway which reduces anti-hormone-mediated cell kill. Thus, the anti-hormone-treated cells, although initially growth inhibited, survive and eventually establish a resistant state driven by the EGFR pathway, an event abrogated by co-treatment with gefitinib (Gee et al. 2003).

**Conclusions**
Our studies demonstrate that increased growth factor signalling is a potent mechanism for the promotion of ER-dependent and -independent forms of anti-oestrogen resistance and aggressive behaviour. A variety of anti-growth factors and signal transduction inhibitors have been shown to inhibit the growth of such cells, thus demonstrating a clinical potential for these agents (See Johnson et al. 2005, Agrawal et al. 2005). Significantly, however, combination therapies appear considerably more effective than mono-therapies in both anti-oestrogen-responsive and -resistant cells and parallel the superior anti-tumour effects of combination therapy in resistance to oestrogen withdrawal (Staka et al. 2005) and anti-growth factors (Jones et al. 2005).

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