Prospects for Combining Hormonal and Nonhormonal Growth Factor Inhibition

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Abstract
In patients with estrogen receptor (ER)-negative disease or ER+ hormone-resistant disease, the dominant influence on tumor cell growth is growth factors, e.g., epidermal growth factor (EGF), heregulins, and insulin-like growth factors acting through specific receptor tyrosine kinases at the cell surface. This superfamily of ligand-activated growth factor receptors triggers cascades of biochemical signals that influence tumor cell motility, invasiveness, angiogenesis, and survival, as well as proliferation. In breast tumors, expression of epidermal growth factor receptor (EGFR) and/or erbB2 is associated with poor prognosis; the therapeutic utility of blocking these receptors has been established using trastuzumab (Herceptin), a monoclonal antibody that blocks erbB2 signaling. An alternative therapeutic approach is offered by small molecule inhibitors of EGFR-TK, exemplified by ZD1839 (Iressa), a potent and selective EGFR-TK inhibitor. Resistance to tamoxifen is associated with up-regulation of the EGFR-TK pathway and mitogen-activated protein kinase activity is substantially increased in tamoxifen-resistant MCF-7 cells. ZD1839 treatment of tamoxifen-resistant MCF-7 cells blocks mitogen-activated protein kinase activity. Furthermore, treatment of wild-type MCF-7 cells with tamoxifen and ZD1839 prevents development of tamoxifen resistance. These data suggest the potential clinical utility of ZD1839 in tamoxifen-resistant breast cancer and support the possibility of preventing resistance by the early use of combination ZD1839 with antiestrogenic agents such as tamoxifen or ICI 182,780.

Introduction
The dominant role that estrogens play in the establishment and progression of breast cancer is well established and, after the successful use of the antiestrogen tamoxifen in the treatment of all stages of the disease, new treatment strategies to reduce estrogen-stimulated tumor growth are being introduced. These include potent and selective aromatase inhibitors and, most recently, the pure antiestrogen, ICI 182,780 (fulvestrant, Faslodex; Fig. 1; Ref. 1). Whereas endocrine therapy has been very successful, those patients who respond to first-line and succeeding endocrine treatments usually relapse and develop endocrine-resistant disease. There are also a large number of patients of which their disease is endocrine-resistant at diagnosis. Thus, there remains a critical need to understand the factors that are dominant in controlling tumor growth in patients with de novo or acquired resistance to endocrine treatment. The first clue to which other growth control pathways might supercede (or complement) estrogen-mediated growth emerged from screening tumors for expression of EGFR and other members of the erbB family, for example erbB2 (HER2/neu; Refs. 2, 3). Such studies established: (a) that expression of EGFR and/or erbB2 predicts a poor prognosis and is negatively correlated with expression of the ER and response to endocrine therapy; and (b) that the erbB family represent a potential therapeutic target (4–6). Delineation of the signal transduction cascade of mitogens in the EGF and IGF families through the RTK-ras-MAPK pathway to control transcription (7) provided the first signposts on the road to understanding how estrogen- and growth factor-mediated signaling might interact.

The developing understanding of the role of growth factors led directly to drug discovery initiatives targeted at the RTK pathway. The recent introduction of trastuzumab, an antibody that binds to the extracellular domain of HER2/neu and blocks erbB2 signaling, provided clinical proof of the hypothesis that inhibition of this pathway could provide effective treatment of breast cancer (8). As an alternative, other investigators have targeted selective, small molecule inhibitors of the kinase activity of EGFR-TK (9–11) and a number of potent TKIs, e.g., ZD1839 (Iressa; Fig. 2; Ref. 12) and OSI-774 (13) have entered clinical trials.

Inhibition of Tumor Growth
Antiestrogens. Estrogens bind to ERs with high affinity and specificity, and induce receptor dimerization, nuclear localization, and binding to specific DNA sequences in the control regions of estrogen-sensitive genes to directly stimulate breast tumor growth (14). Antiestrogens competitively inhibit binding of estradiol to ER and, in the case of partial agonists like tamoxifen, toremiphene, and raloxifene, attenuate but do not

1 Presented at the First International Conference on Recent Advances and Future Directions in Endocrine Therapy for Breast Cancer, June 21–23, 2001, Cambridge, MA.
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3 The abbreviations used are: EGFR, epidermal growth factor receptor; ER, estrogen receptor, EGF, epidermal growth factor; IGF, insulin-like growth factor; RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase; TKI, TK inhibitor; VEGF, vascular endothelial growth factor; DCIS, ductal carcinoma in situ.
completely block estrogen-mediated transcription (15). The pure antiestrogen, ICI 182,780, which differs from the selective estrogen receptor modulators in having no estrogen-like, partial agonist activity, binds ER with much lower affinity than tamoxifen, but blocks receptor dimerization, nuclear localization, and transcriptional activation of ER. As a consequence of the disruption of ER function, ICI 182,780 accelerates the loss of ER protein from breast cancer cells (Fig. 3) and completely blocks estrogen action (16–18). ICI 182,780 is more effective than tamoxifen in preventing tumor growth in nude mice bearing xenografts of MCF-7 human breast cancer cells; its antitumor effect against established xenografts lasts twice as long as that of tamoxifen, and ICI 182,780 inhibits the growth of MCF-7-derived tumors with acquired resistance to tamoxifen treatment (19–21). Clinical studies have demonstrated that ICI 182,780 down-regulates ER in breast cancer patients (22) and provides effective treatment of patients who have relapsed after tamoxifen treatment (23, 24).

**Tyrosine Kinase Inhibitors.** ZD1839 (Fig. 2) is a potent and selective inhibitor of EGFR tyrosine kinase activity and of EGF-stimulated cell growth (25, 26). In *in vitro* kinase substrate phosphorylation assays, ZD1839 inhibited EGFR kinase activity, whereas it had minimal activity against the closely related erbB2 kinase, VEGF receptor kinases, or serine-threonine kinases (Table 1). ZD1839 markedly inhibited the autophosphorylation of EGF-stimulated EGFR in KB oral squamous, A549 lung, DU145 prostate, and HT29 colorectal carcinoma cells *in vitro* (IC$_{50}$ = 0.16 – 0.8 μM; Fig. 4).

ZD1839 selectively inhibited EGF-stimulated growth of the human squamous tumor cell line KB; inhibition of serum-stimulated growth required a 50-fold higher concentration of ZD1839 (Table 2). Selectivity was also demonstrated using human umbilical vein epithelial cells, which proliferate in response to several different growth factors. ZD1839 inhibited EGF- but not fibroblast growth factor- or VEGF-stimulated cell growth and had no effect on ras transformed fibroblasts (Table 2).

Studies with a range of human tumors, grown as xenografts in nude mice, showed that once daily oral treatment with ZD1839 inhibits tumor growth in a dose-dependent manner and, in combination with cytotoxic agents, provides an additive or synergistic antitumor effect (27, 28). ZD1839 inhibits the proliferation of breast cancer cells (10, 29, 30), of ductal carcinoma *in situ* (31), and of normal breast epithelium (32, 33). Among the large number of EGF-TK inhibitors described in the literature, ZD1839 is most advanced in clinical development. Early clinical trials demonstrated that Iressa is relatively well tolerated and provided encouraging signs of clinical efficacy in patients with lung and prostate cancer (34, 35). Phase III drug registration trials of Iressa in non-small-cell lung cancer are in progress (36).

**Prospects forCombining Antiestrogen and Tyrosine Kinase Inhibitor Treatment**

There are many levels at which the ER- and RTK-mediated signaling pathways for breast tumor cell growth may interact; these include growth factor-induced transcriptional activation by phosphorylation of ER or transcriptional coactivators, and estrogen-induced changes in the synthesis of growth factors, their binding proteins or receptors, or in the activity of downstream components of their signaling pathways (37, 38). Studies with breast cancer cells have revealed several means by which such interactions might be exploited in future clinical trials by inhibiting ER and erbB signaling either sequentially or concurrently.

**Clinical Trials.** ZD1839 (Iressa) is a potent and selective, oral active EGFR tyrosine kinase inhibitor (EGFR-TKI) that inhibits tumor xenograft growth in vivo. *In vivo* models of human breast cancer xenografts demonstrate that ZD1839 is effective in treating both hormone-responsive and hormone-refractory tumors. These xenograft models are useful in studying the development of resistance to treatment, including endocrine and tyrosine kinase inhibitors. Among preclinical studies, ZD1839 inhibited tumor xenograft growth in vivo in breast cancer patients (22) and provides effective treatment of patients who have relapsed after tamoxifen treatment (23, 24).

![Figure 1.](image)

**Figure 1.**

- High affinity for ER
- No agonist activity
- Estrogen receptor (ER) down-regulator
- Active in tamoxifen-resistant cells

![Figure 2.](image)

**Figure 2.**

- Selective, potent, oral active EGFR tyrosine kinase inhibitor (EGFR-TKI)
- Inhibits tumor xenograft growth in vivo
- In Phase III trials for NSCLC

MCF-7 human breast cancer cells exposed to long-term antiestrogen treatment develop resistance to their growth inhibitory effects, modeling the development of resistance to treatment in patients. One mechanism underlying the acquisition of resistance to tamoxifen, or to ICI 182,780, is increased expression of EGFR, erbB2, and of transforming growth factor α. These cells have a complete autocrine EGFR growth signaling pathway and elevated MAPK activity (30). Such cells show a much higher degree of sensitivity to growth inhibition by ZD1839 compared with wild-type MCF-7 cells, and treatment with ZD1839 blocked phosphorylation of MAPK, indicative of effective blockade of the EGFR signaling pathway (30). In studies with ICI 182,780, MCF-7 cells escaped from growth inhibition after a 3-month treatment period; however, McClelland *et al.* (30) showed that in cells treated with the combination of ICI 182,780 and ZD1839, growth was arrested for a period in

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excess of 6 months, with significant cell loss via both reduced proliferation and increased cell death. These studies make two predictions: (a) that some patients with acquired resistance to antiestrogen treatment may be prime candidates for treatment with inhibitors of EGFR signaling; and (b) that combination treatments with inhibitors of ER- and EGFR-mediated signaling may provide more effective or longer-lasting tumor remission. These predictions are currently being tested in nude mouse xenograft studies using tamoxifen, ICI 182,780, and ZD1839.

Initial studies of combined blockade of ER and erbB signaling in xenografts of MCF-7 cells transfected with HER-2/neu and representative of the 20–30% of human breast cancers that overexpress HER-2/neu, provided encouraging data (39, 40). Tumors derived from such cells are completely resistant to tamoxifen, but treatment with a monoclonal antibody to HER-2/neu together with tamoxifen proved as effective as tamoxifen treatment of wild-type cells not transfected with HER-2/neu (39), and a combination of tamoxifen and a HER2 kinase inhibitor prevented tumor growth (40). Similar studies with breast cancer cells expressing ER and HER-2 showed that anti-HER2 antibodies enhanced the growth inhibitory effect of ICI 182,780 (41). In breast cancer cells expressing both EGFR and HER2, low concentrations of ZD1839 inhibited cell growth, colony formation, and HER2 phosphorylation, and ZD1839 was effective in cells resistant to treatment with a HER2 antibody (30, 42). These data, together with the demonstration that ZD1839 but not trastuzumab decreased epithelial proliferation in DCIS (43), suggest that ZD1839-induced growth inhibition acts through inhibition of EGFR-HER2 heterodimer signaling as well as through EGFR-TK inhibition.

Conclusions

New data emerging from studies of the crosstalk between the ER- and growth factor-mediated signal transduction pathways have demonstrated how the dominance of each pathway may differ among normal, DCIS, and transformed mammary epithelia. Recent studies indicating that antiestrogen treatment may affect these interactions promise to open up an array of novel treatment options. The availability of new inhibitors, for example ICI 182,780, which accelerates the destruction of ER, and antibody or small molecule inhibitors of erbB signaling (e.g., trastuzumab and ZD1839), has made an important contribution to elucidation of this crosstalk and holds out great promise for future treatment if this understanding can be translated to the clinic. A major focus for future clinical studies will be to determine the optimal sequencing or combination of established and new treatments, perhaps tailored to the individual and the stage of the disease. Some immediate priorities suggested by the cell and animal model data discussed here are to determine the efficacy of ZD1839 (and other RTK inhibitors) alone in all stages of the disease, particularly in those patients whose tumors express EGFR, with or without co-overexpression of HER2, or who have already been treated with antiestrogens. The studies of

Table 1  Selectivity of ZD1839 in enzyme assays

<table>
<thead>
<tr>
<th>Kinase</th>
<th>ZD1839 IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine kinases</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>0.027a and 0.033b</td>
</tr>
<tr>
<td>erbB2</td>
<td>&gt;3.7</td>
</tr>
<tr>
<td>KDR</td>
<td>&gt;3.7</td>
</tr>
<tr>
<td>c-flt</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Serine-threonine kinases</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>&gt;100</td>
</tr>
<tr>
<td>raf</td>
<td>&gt;10</td>
</tr>
<tr>
<td>MEK-1</td>
<td>~10</td>
</tr>
<tr>
<td>ERK-2</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

a Expressed kinase domain of EGFR.
b Membrane preparation of EGFR from A431 cells.
normal breast and DCIS suggest that there may be a place for TKIs in breast cancer chemoprevention. The observations that erbB inhibition may restore sensitivity to antiestrogens and that a combination of antihormones and erbB inhibitors may radically slow development of endocrine resistance and increase apoptosis open up intriguing questions for future trials.

Open Discussion

Dr. Carlos Arteaga: There are preliminary data from lung cancer trials that suggest that Iressa induces antitumor responses, but EGF receptor antibodies do not, implying that Iressa might be nonspecifically blocking something else in addition to the EGF receptor kinase. Can you tell us about other possible targets that you know of or that you may be looking for?

Dr. Alan Wakeling: We are not specifically looking for another kinase, which could be important to the activity of the drug. When the project was set up in the lab, I made a conscious decision that I wasn't going to express the 20 different kinases known at that time, put them all in test tubes, and test my compounds against them. We decided to do that assay effectually in vivo, so we selected a cell line that grows well in serum that has been partially depleted of growth factors. When we added exogenous EGF or TGF-α to those cells to drive the EGF kinase, proliferation was doubled. So we had the experiment to look at activity. You compare IC₅₀ plus or minus EGF. In that scenario, Iressa IC₅₀ is 2 logs different. If you go to concentrations that are in the range 1–5 μM, which are achieved in terms of gross circulating concentrations, other effects can come in. For example, you see direct inhibition of HER2 kinase in vitro with 5 μM.

Dr. Kent Osborne: I think the observation that even cells with low EGF receptor are affected is very important for breast cancer. For some reason, many people think that EGF receptor is overexpressed in breast cancer. In fact, normal breast epithelial cells have much higher EGF receptor than breast cancers do.

Dr. Anthony Howell: The normal breast is inhibited quite dramatically by Iressa, as you would predict from what you've said. The thinking about prevention with these types of compounds is really quite interesting and challenging.

Dr. Wakeling: The big issues are the dose and tolerance. The Phase III non-small cell lung cancer trials are looking at two different doses of drug, 500 mg or 250 mg once daily. The dose-limiting toxicity with the drug is gut and skin.

Dr. Howell: But we've actually no idea what dose of drug might be required to be effective. Let's say it's 50 mg/day, not 500. Almost nobody will experience significant side effects at that dose. Until you get up to a dose of 150 mg/day, you see almost no side effects.

Dr. Adrian Lee: The data you showed on the delay of tamoxifen resistance using Iressa—do you think that's specific for Iressa, or will any cell cycle inhibitor get that effect?

Dr. Wakeling: I can't answer the question. We haven't tried. We are of course asking what's switched on in doubly

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**Table 2** Selectivity of ZD1839 in cell proliferation assays

<table>
<thead>
<tr>
<th>Cells</th>
<th>Stimulation</th>
<th>ZD1839 IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>EGF</td>
<td>0.054</td>
</tr>
<tr>
<td>KB</td>
<td>SERUM</td>
<td>8.8</td>
</tr>
<tr>
<td>HUVEC</td>
<td>EGF</td>
<td>0.03–0.1</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Fibroblast growth factor</td>
<td>1–3</td>
</tr>
<tr>
<td>NIH 3T3 (morphology)</td>
<td>H-ras</td>
<td>&gt;10</td>
</tr>
<tr>
<td>NIH 3T3 (proliferation)</td>
<td>H-ras</td>
<td>~5</td>
</tr>
</tbody>
</table>
resistant cells, and one of the obvious focuses of attention there is the IGF-1 receptor, the insulin receptor substrate pathway, and how that relates to Akt.

**Dr. James Ingle:** Could you tell us your thoughts on fulvestrant’s mechanisms of action in the IGF system?

**Dr. Wakeling:** It’s as you classically might expect, an inhibition of the agonist effects of estrogen on that pathway.

**Dr. Osborne:** As I recall, Iressa didn’t do very much in wild-type cells. You had a lot of inhibition with tamoxifen, and you don’t have any more inhibition with Iressa. It wasn’t until you got to the tamoxifen-resistant cells that Iressa really wiped proliferation out. So that may be the answer. In other words, initially the cancer is not dependent on the growth factor pathway.

**Dr. Wakeling:** The IC₅₀ for Iressa in wild-type MCF-7 growing in 5% serum is about 2 μM, which is at the high end compared with other breast cancer cell lines. They most often come in at under 1 μM.

**Dr. Steven Come:** I’m curious if you know how fast these changes occur. You showed the wild-type cells and then you showed expression at the time of resistance. Have you looked at the wild-type cells right after exposure to antiestrogens and before clinical resistance develops?

**Dr. Wakeling:** The time scale of these events is a very important question. We’re looking at that now. It’s too early to give a specific comment on what is the sequence and timing of the change.

**References**


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