Regulation of signaling phosphoproteins by epidermal growth factor and Iressa (ZD1839) in human endometrial cancer cells that model type I and II tumors

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Abstract

To understand how type I and II endometrial tumors uniquely respond to tyrosine kinase inhibitor treatments, we evaluated the signaling pathways of epidermal growth factor (EGF) receptor (EGFR) under the effects of EGF and Iressa (ZD1839, gefitinib) using Ishikawa H and Hec50co cells that model type I and II endometrial carcinomas, respectively. The cells were assayed for the expression of EGFR and both cell lines express an average of 100,000 EGFR per cell; however, Ishikawa H cells express higher levels of HER-2/neu compared with Hec50co cells (1.38 x 10^5 compared with 2.04 x 10^4, respectively). Using the Kinetworks multi-immunoblotting approach, which profiles 31 signaling phosphoproteins, the most striking result was that Hec50co cells show a higher number of basal phosphorylated sites compared with Ishikawa H cells. Furthermore, we identified targets of Iressa treatment in both cell lines. Iressa, at a dose of 1 μmol/L, blocked the autophosphorylation of EGFR in Ishikawa H and Hec50co cells with some distinctive effects on downstream effectors. Nevertheless, in both cell lines, EGF stimulated and Iressa blocked the major EGFR target mitogen-activated protein kinases extracellular signal-regulated kinase 1 and 2 equally. The high basal phosphorylation of numerous signaling molecules in Hec50co cells that were not inhibited by Iressa indicates that other growth factor pathways are active in addition to EGFR. We conclude that endometrial cancer cells that model type I and II carcinomas have the capacity to respond to EGFR inhibition as a therapeutic strategy; however, the response of the more aggressive type II tumors may be limited by the constitutive activation of other signaling pathways.

Introduction

Endometrial carcinoma, which arises from the uterine epithelial glands, is the most common gynecologic malignancy and the most curable one when it presents at an early stage (1–3). The median age at diagnosis is 63 years, and 75% of the patients with endometrial carcinoma are postmenopausal (2, 4, 5). Many risk factors have been reported, including excessive estrogen, obesity, nulliparity, treatment with tamoxifen, environmental factors, genetic factors, early menarche, and late menopause (1, 2, 6–20). Although curable at an early malignancy, advanced tumors are lethal. Approximately 7,000 women die in the United States each year of endometrial cancer. Unfortunately, no effective therapy is yet available for metastatic and recurrent disease; however, new agents targeting growth factor pathways are now under investigation in clinical trials.

The epidermal growth factor (EGF) receptor (EGFR) plays a key role in different cellular functions implicated in cancer development (11). The EGFR family consists of four distinct tyrosine kinase cell surface receptors that are expressed in the normal endometrium and may be over-expressed in endometrial cancers (12–17). These are EGFR, ErbB2 (HER-2/neu), ErbB3 (HER-3/EGFR3), and ErbB4 (HER-4/EGFR4). The receptors form homodimers and heterodimers at the cell surface. EGFR (molecular weight, 170 kDa) is encoded by the c-ErbB1 proto-oncogene. This receptor stimulates cell growth after ligand binding through its glycosylated extracellular domain (18–22). Unbound EGFR is constitutively phosphorylated at several threonine and serine residues, and binding of EGF induces autophosphorylation of EGFR on 14 tyrosine residues in the cytoplasmic domain. This event is followed by the downstream phosphorylation and dephosphorylation of signaling molecules that control diverse cellular functions, including the cell cycle, apoptosis, and proliferation (23–25).

Iressa (ZD1839, gefitinib; AstraZeneca Pharmaceuticals, Wilmington, DE and Cheshire, United Kingdom) is a potent, specific inhibitor of EGFR tyrosine kinase activity (Medical Research and Communications Group, Zeneca...
Pharmaceuticals Investigators Brochure, ZD1839, Edition 3, March 1999). The drug binds to the ATP-binding site on the EGFR kinase domain with a higher affinity than ATP itself and thereby inhibits receptor activation (26). Iressa inhibits the autophosphorylation of EGF-stimulated EGFR in a variety of EGFR-expressing human cancer cell lines. Inhibition lasts over 24 hours after the compound is removed from the culture medium. This compound is less active against HER-2/neu kinase; inhibition of non-EGFR-stimulated cell growth requires a 40-fold higher concentration of Iressa. At higher concentrations, Iressa inhibits EGFR2-HER-2/neu and other intracellular transmembrane tyrosine kinase receptors, such as vascular endothelial growth factor, fibroblast growth factor, platelet-derived growth factor, and insulin-like growth factor (26). Interestingly, some authors have speculated that coexpression of HER-2/neu with EGFR may increase tumor sensitivity to Iressa (27), and it is believed that the EGFR/HER-2/neu heterodimer may be the most active heterodimer within the EGFR family (28).

Iressa has been tested as a therapeutic agent against various malignancies in which the EGF/EGFR pathway is active, and it has recently been approved by the U.S. Food and Drug Administration for locally advanced or metastatic non–small cell lung cancer in the United States (26). Iressa has been reported to have antitumor activity in some clinical trials and results in longer survival time for a subset of patients, especially in patients with adenocarcinomas (29).

Iressa has now been used in a therapeutic trial for women with advanced endometrial cancer (Gynecologic Oncology Group Study 229C). Therefore, it is important to investigate how these types of endometrial cancers respond to such therapy. Endometrial cancers have been divided into two categories (30–32). Type I tumors are generally of the endometrioid subtype, are well differentiated, express high levels of estrogen receptor and progesterone receptor, and occur in a setting of estrogen excess unopposed by the differentiating effects of progesterone. The surrounding endometrial epithelium is hyperplastic, indicating the presence of high local levels of estrogen. Such tumors are predicted to have high EGF expression as a result of estrogen-induced EGF gene transcription (33). If EGF levels are high, such tumors may respond to EGFR blockade even with normal cellular levels of EGF. Therefore, the ligand, the receptor, or both may be overexpressed leading to inappropriate cellular proliferation, but in any of these circumstances Iressa may be an effective therapy. Type II tumors include poorly differentiated endometrioid, clear cell, and papillary serous subtypes. These are either primarily or secondarily resistant to hormone growth regulation and are more likely to be surrounded by atrophic nonmalignant endometrial epithelium. Such tumors sometimes down-regulate estrogen receptor and progesterone receptor expression and are poorly differentiated. We hypothesize that type II endometrial tumors, like estrogen receptor–negative breast cancer cells (34), maintain proliferation and have become hormone resistant by constitutively activating growth factor pathways, such as EGF/EGFR. Therefore, EGFR blockade may also be effective therapy for type II tumors. The studies reported herein were undertaken to assess the downstream pathways activated by EGF and blocked by Iressa in endometrial cells that model these tumor types and to further distinguish the cellular characteristics and targets that may have an effect on the drug performance.

Materials and Methods

Drugs and Chemicals

Iressa was provided by AstraZeneca Pharmaceuticals (Wilmington, DE and Cheshire, United Kingdom). The drug was dissolved in DMSO for all in vitro studies. EGF was purchased from Invitrogen (Carlsbad, CA). Antibodies against total and phospho-EGFR (Tyr1173), total and phospho-Akt (Ser473), total and phospho–extracellular signal-regulated kinase (ERK) 1/2 (Thr202/Tyr204), β-actin, and horseradish peroxidase–conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The protein ladder size standard was purchased from Bio-Rad (Hercules, CA). For cell surface receptor quantitation by flow cytometry, a standard curve was prepared using Quantum Simply Cellular Anti-Mouse IgG purchased from Bangslabs, Inc. (Fishers, IN).

Cells and Culture Conditions

Ishikawa H cells are well differentiated and representative of type I endometrial cancer (hormone receptor positive, endometrioid cell type; refs. 35, 36). Hec500c cells are poorly differentiated and representative of type II endometrial cancer (hormone receptor negative, may differentiate into a serous subtype in xenografted animal models; ref. 37). The cells were originally provided by Dr. E. Gurpide (New York University, New York, NY) and are grown in DMEM (Sigma-Aldrich, Inc., St. Louis, MO) with 10% fetal bovine serum (FetalPlex, Gemini Bio-Products, Woodland, CA), 2 mmol/L l-glutamine (Life Technologies, Grand Island, NY), and 1× antibiotic-antimycotic solution (Life Technologies). Before the cells were treated with Iressa, they were serum starved for 24 hours followed by incubation with 1 μmol/L Iressa in DMSO for 2 or 20 hours. In samples where EGF (30 ng/mL) was added, cells were incubated with EGF for 5 or 15 minutes before harvesting.

Flow Cytometry Analysis of EGFR and HER-2/neu

Cultured Hec500c and Ishikawa H cells were analyzed for cell surface EGFR and HER-2/neu expression using FITC-labeled mouse monoclonal antibody sc-120 FITC and sc-23864 FITC, respectively. Cells (1 × 10⁶) were blocked with 3% bovine serum albumin in PBS for 10 minutes at room temperature followed by incubation with 5 μg/mL antibody in PBS for 1 hour in the dark with rotation. After washing twice with PBS, cells were fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature followed by two washes with PBS. Fluorescence data were obtained using an Epics Elite ESP flow cytometer (Beckman Coulter, Fullerton, CA). A minimum of 4,000 cell
In vitro Kinase Activity Assay

Cells were treated with 1 μmol/L Iressa for 2 hours and stimulated by 30 ng/mL EGF for 5 minutes. Untreated (negative) and EGF-treated (positive) cells served as controls. Cells were washed with PBS before lysis in NP40 buffer [50 mmol/L Tris (pH 7.2), 150 mmol/L NaCl, 1% NP40, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL antipain, 2 μg/mL leupeptin, and 20 μg/mL aprotinin], and lysates were cleared by centrifugation (13,000 × g for 10 minutes at 4°C). The protein concentration of the supernatant was measured by the Bradford method (Bio-Rad). EGFR was immunoprecipitated with protein G-Sepharose beads (Amersham Biosciences, Piscataway, NJ) using anti-PY and EGFR antibodies (Santa Cruz Biotechnology) for 2 hours with continuous rotation at 4°C. Samples were then centrifuged and supernatants were aspirated. Pellets were washed twice with lysis buffer with continuous rocking to eliminate nonspecific binding followed by washes with HEPES buffer [25 mmol/L HEPES (pH 7.2), 150 mmol/L NaCl, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 μg/mL antipain and leupeptin] and kinase buffer [25 mmol/L HEPES (pH 7.2), 10 mmol/L MnCl2]. Pellets were then incubated with 10 μCi [γ-32P]ATP sample in kinase buffer for 4 minutes at 30°C. Samples were centrifuged and washed with kinase buffer. Pellets were suspended in 40 μL of 2× Laemmli buffer with β-mercaptoethanol and boiled for 5 minutes to detach the protein complex from the beads. Equal amounts of protein were loaded and separated by gradient SDS-PAGE (4–15%). Gels were stained with Coomassie blue, destained, dried, and analyzed by autoradiography.

Western Blot Analysis

Cells were lysed with extraction buffer [1% (v/v) Triton X-100, 10 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 50 mmol/L NaCl, 50 mmol/L NaF, 20 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 mmol/L Na3VO4] and subjected to three freeze/thaw cycles. The lysate was cleared by centrifugation at 20,000 × g for 10 minutes, and the protein concentration of the supernatant was measured by the method of Bradford. Equal aliquots of protein were separated by 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (BioScience, Dassel, Germany) and membranes were then probed with antibodies against EGFR, phospho-EGFR (Tyr1173), Akt, phospho-Akt (Ser473), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), or β-actin followed by horseradish peroxidase–conjugated secondary antibody. Signal was visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Western blotting detection reagents (Pierce, Rockford, IL).

Protein Extraction for Kinetworks Immunoblotting

Protein was extracted according to Kinetworks guidelines (Kinexus, Victoria, British Columbia, Canada). Briefly, cells were harvested and washed twice with PBS. Protein was extracted using 0.5% Triton X-100 lysis buffer with sonication for 10 seconds on ice to rupture the cells. Cell extracts were then ultracentrifuged for 30 minutes at 100,000 × g. The supernatant was transferred to fresh tubes, and protein concentrations were measured promptly by Bradford assay according to the manufacturer’s protocol. For each sample, protein (400 μg) was transferred to a fresh 1.5 mL Eppendorf screw cap vial and mixed with 4× sample buffer [125 mmol/L Tris-HCl (pH 6.8), 4% (w/v) SDS, 50% (v/v) glycerol, 0.08% (w/v) bromophenol blue, and 5% β-mercaptoethanol] in a ratio of 1:4 sample to buffer. Samples were then boiled for 4 minutes, and vials were secured with parafilm and sent to Kinetworks.

Western Blot Analysis by the Kinetworks Approach

The Kinetworks multi-immunoblotting approach was used to evaluate the expression of signaling proteins downstream of EGFR in Ishikawa H and HeLa endometrial cancer cell lines. The methodology has been described elsewhere (refs. 38, 39; Kinexus Bioinformatics Corp. Web site). Briefly, the assay is based on SDS-polyacrylamide minigel electrophoresis with 20-lane multi-immunoblotters using different primary antibodies. This method allows specific and semiquantitative identification of multiple signaling proteins at the same time (40, 41).

To assess the effects of EGF and Iressa on signaling, cells were divided into four groups: control (vehicle alone), EGF (30 ng/mL) for 5 minutes, Iressa (1 μmol/L; AstraZeneca Pharmaceuticals, London, United Kingdom) for 20 hours, and Iressa plus EGF. Multiprotein immunoblotting was done according to the manufacturer’s instructions using the Kinetworks Phospho-Site Screen 1.3, which tracks the abundance of the following phosphoprotein targets: Adducin a (S724), Adducin c (S662), CDK1 (Y15), CREB (S133), ERK1 (T202/Y204), ERK2 (T185/Y187), GSK3α (S21, Y279), GSK3β (S9, Y216), JNK/SAPK (T183/Y185), JUN (S73), MEK1/2 (S217/S221), MEK3/6 (S189/S207), MSK1 (S376), MK1 (S896), p38 MAPK (T180/Y182), p70S6K (T389), PKBa/Akt (T308/S473), PKCa (S657), PKCa/a (T638/T641), PKCd (T507), PKCe (S729), PKR (T451), RAF1 (S259), RB1 (S780, S807/S811), SMAD1 (S463/S465), SRC (Y418/Y429), STAT1 (Y701), STAT3 (S727), and STAT5 (Y694). By this method, phosphosites on 31 proteins are identified. Cellular protein extracts (400 μg) were resolved on a 13% single-lane SDS-polyacrylamide minigel and transferred to a nitrocellulose membrane. Using a 20-lane multiblotter (Bio-Rad), the membrane was incubated with different mixtures of up to three antibodies per lane that react with a distinct subset of phosphorylated cell signaling proteins of known molecular masses, as listed above. After further incubation with a mixture of relevant horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology), the blots were developed using Enhanced Chemiluminescence Plus reagent (Amersham Pharmacia, Piscataway, NJ).

1 http://www.kinexus.ca.
relative density of each phosphoprotein band was determined compared with control cells. The results were reported as the percent of binding compared with the control, and peptide densities that varied ±25% from the controls were considered to be legitimate signaling targets, which could be reliably detected using this method.

Results

EGFR and HER-2/neu Surface Expression on Ishikawa H and Hec50co Cells

EGFR and HER-2/neu surface expression levels were measured in the Ishikawa H and Hec50co cells using flow cytometry. Results show that comparable levels of EGFR are expressed on the surface of both cell lines (Fig. 1; 1.14 × 10^5 for Hec50co cells and 9.64 × 10^4 for Ishikawa H cells). However, HER-2/neu levels were approximately seven times higher in Ishikawa H cells (1.38 × 10^5) compared with Hec50co cells (2.04 × 10^4).

Effects of Iressa on EGFR Kinase Activity and Autophosphorylation on Tyr1173 in Ishikawa H and Hec50co Cells

Iressa blockade of EGFR phosphorylation was assayed using Western blotting and in vitro kinase activity approaches. Cells were treated with EGF for 15 minutes with or without previous Iressa treatment for 20 hours. EGFR autophosphorylation was measured by Western blotting using an antibody raised against phospho-EGFR Tyr1173. We did a timetable and found that EGFR was maximally phosphorylated at 15 minutes. We employed 1

Figure 1. Flow cytometry analysis of cell surface EGFR and HER-2/neu on Ishikawa H and Hec50co cells. Untreated cells were labeled with EGFR antibody (right), labeled with HER-2/neu antibody (middle), or unlabeled (left).
of EGFR on tyrosine residues in the presence of EGF, which could be blocked by Iressa. The kinase data for Hec50co cells are shown in Fig. 2C; similar results were obtained for Ishikawa H cells (data not shown).

**Kinexus Multiphosphoprotein Signaling Analysis**

To evaluate the expression of a large number of signaling and kinase proteins downstream of EGFR in endometrial cancer cells, Kinetworks protein profiling was done using multi-immunoblotters with antibodies against 31 phosphoproteins. Examples of the immunoblots are presented in Fig. 3 for Hec50co cells and in Fig. 4 for Ishikawa H cells. Each of the 20 lanes on the blot was probed with a cocktail of primary antibodies against chosen phosphoproteins, and each band on the blot identifies a specific phosphorylation site on the protein. In each panel, A shows the baseline control phosphorylation of the targets, B shows the effect of EGF treatment, C shows Iressa treatment, and D shows EGF plus Iressa. At baseline (Fig. 3A), Hec50co cells show detectable phosphorylation of 24 of the 31 possible phosphoproteins detected on the immunoblots. These include Adducin α, Adducin γ, CDK1, CREB, PKBa (Akt), PKCa, PKCa/b, ERK1, ERK2, GSK3α, GSK3β, JUN, MEK1/2, MEK3/6, MKS1/2, NR1, p38 MAPK, p70S6K, PKCe, PKR, RAF1, RB1, etc. (Table 1). In Ishikawa H cells, fewer downstream phosphoproteins (17) were identified at baseline (Fig. 4A). These include SRC, PKBa (Akt), GSK3α, GSK3β, CDK1, CREB, ERK1, ERK2, NR1, MEK1/2, JUN, RAF1, PKCa, PKCa/b, PKCe, STAT3, and JNK/SAPK (Table 1).

We were particularly interested in the phosphorylation sites that were augmented by EGF and inhibited by Iressa. In Hec50co cells, EGF treatment for 5 minutes induced the phosphorylation of the following peptides compared with control: ERK1, ERK2, JUN, MEK1/2, MKS1/2, p70S6K, PKBa (Akt1), PKCa/b, PKCe, RAF1, RB1, and STAT3. Of these, Iressa (1 μmol/L for 20 hours) down-regulated only ERK1, ERK2, MEK1/2, and RB1 phosphorylation to below the control level. In Ishikawa H cells, phosphorylation of ERK1 and ERK2 was up-regulated by EGF. ERK1 and ERK2 activation was down-regulated by Iressa in both EGF-treated and untreated (baseline phosphorylation level) cells (Figs. 3, 4, and 5). The effects of Iressa in combination with EGF on the 31 phosphoproteins were diverse; some phosphoproteins were up-regulated, whereas others were down-regulated (Table 1).

**Effects of EGF and Iressa on Proteins Downstream of EGFR Signaling**

To validate the Kinetworks approach and to further study the essential pathways downstream of EGFR signaling, we did Western blotting on Hec50co and Ishikawa H cells using specific antibodies targeted against total or phosphorylated proteins, including PKBa/Akt, ERK1, and ERK2 (Fig. 5). These proteins were chosen because they are important effectors downstream of EGFR signaling. Baseline expression of total and phospho-ERK1/2 are comparable between Hec50co and Ishikawa H cells (Fig. 5). Furthermore, phospho-ERK1/2 are similarly induced by EGF and inhibited by Iressa in both cell lines (Fig. 5). These data

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**Figure 3.** Phosphoproteins tracked in Hec50co cells. A, untreated. B, treated with 30 ng/mL EGF for 5 min. C, treated with 1 μmol/L Iressa for 20 h. D, treated with Iressa (20 h) and EGF (5 min). Bands that are indicative of specific phosphoproteins: 1, NR1; 2, Adducin α; 3, Adducin γ; 4, SRC; 5, CDK1; 6, PKCa; 7, PKCa/b; 8, MEK3/6; 9, p70S6K; 10, ERK1; 11, ERK2; 12, PKCe; 13, STAT3; 14, JUN; 15, Raf1; 16, PKBa (Akt; left, T308; right, S473); 17, MEK1/2; 18, PKR; 19, GSK3α (left, S21; right, Y279); 20, GSK3β (left, S9; right, Y216); 21, p38; 22, Rb1 (left, S780; right, S807/S811); 23, MEK1/2; 24, CREB.
confirm the Kinexus findings. Also in Fig. 5, it can be seen that total and phospho-Akt is significantly enriched in Ishikawa H cells compared with Hec50co cells. These findings correlate with the loss of PTEN in Ishikawa H cells and its continued expression in Hec50co cells. However, our immunoblot results showed down-regulation of phospho-Akt on Ser473 with Iressa in Hec50co (Fig. 5), whereas the Kinexus data indicate no change (Table 1). This discrepancy may be due to a slightly more sensitive Western blotting approach done in our laboratory compared with the Kinetworks multi-immunoblots.

Discussion

Endometrial carcinoma is an understudied disease, although it is the most common gynecologic malignancy. Endometrial cancers have been divided into two categories (type I and II) based on histologic differences, unique patterns of tumor suppressor expression, and clinical outcome variables. Type I cancers are more often well differentiated, of endometrioid subdifferentiation, and associated with estrogen excess. Type II tumors are more often high-grade endometrioid, serous, or clear cell subtypes that are more aggressively and difficult to treat.

In the present studies, Ishikawa H and Hec50co cells were used as models for type I and II human endometrial cancer, respectively. Ishikawa H cells were derived from a moderately differentiated endometrioid carcinoma. These cells form glands in tissue culture and continue to express estrogen and progesterone receptors (all consistent with type I cancer). The patient was cured of her disease, also consistent with a type I cancer. Further, the cells harbor a mutation or deletion of PTEN, which is classic for type I cancer. Finally, the cells form glandular structures when placed in the xenografted mouse model. On the other hand, Hec50co cells were clearly derived from a type II endometrial cancer. They do not form glands in tissue culture or in the animal model; they originated from an advanced metastatic lesion from the peritoneal cavity of a patient who died of her disease. Most importantly, the cells have the capacity to subdifferentiate to a papillary serous phenotype in the mouse model as reported previously (37).

The tyrosine kinase inhibitor Iressa is being used in a therapeutic trial for women with advanced endometrial cancer (Gynecologic Oncology Group Study 229C). Therefore, it is important to understand how different types of endometrial cancers respond to such therapy. We hypothesized that both types may benefit from blocking EGF/EGFR signaling with Iressa for distinct reasons (see Introduction). The downstream signaling pathways activated by EGF and blocked by Iressa were assessed in each cell line to further distinguish the cellular characteristics that may have an effect on drug performance.

The present studies aimed to investigate the effects of 1 μmol/L Iressa, the approximate biological concentration of Iressa in the blood achieved after treating patients with standard daily therapy, on Hec50co and Ishikawa H cells, which are representative of type I and II human endometrial cancers, respectively. We profiled Iressa and EGF effects by tracking 31 phosphoproteins. A global analysis of

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2 Dai and Leslie, unpublished results.
the phosphorylation status of multiple effector proteins downstream of EGFR and other growth factor receptors was done using the Kinetworks multi-immunoblot approach. Kinetworks immunoblotting is an important new tool because it gives the investigator a global analysis of the phosphorylation state of multiple signaling peptides in a single experiment. This provides a snapshot of the signaling pathways activated and their potential relationship to each other within one convenient analysis. Of course, the most important findings from such a global experiment should be independently confirmed using standard immunoblotting, which must be targeted to a relatively small number of proteins for each experiment. In addition, the Kinetworks experiments are hypothesis generating. One does not have to have a preconceived idea of the signaling pathways affected before performing the experiment as would be necessary for immunoblotting. We found that phospho-ERK1/2 was a major target for Iressa blockade (Fig. 5). Hec50co cells showed more robust baseline phosphorylation of targets compared with Ishikawa H cells (Figs. 3 and 4; Table 1). This finding suggests that multiple signaling pathways are active in these cells in addition to EGFR/EGFR. The noted difference in the overall robust phosphorylation status of pathways in our cells has been reported previously in the literature for hormone-resistant breast cancer cells (42). Despite these differences in baseline phosphorylation, the phosphorylation of the mitogen-activated protein kinase cascade (ERK1 and ERK2) was inhibited by Iressa in both cell lines (Figs. 3, 4, and 5). Down-regulation of ERK1 and ERK2 phosphorylations occurred in Hec50co cells just as it did in Ishikawa H cells.

### Table 1. Phosphoproteins regulated in Hec50co and Ishikawa H cells detected by Kinetworks Phospho-Site Screen 1.3 multiblotting approach

<table>
<thead>
<tr>
<th>Kinetworks Phospho-Site Screen 1.3</th>
<th>Phosphosites regulated in Hec50co</th>
<th>Phosphosites regulated in Ishikawa H</th>
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<tr>
<td>Untreated</td>
<td>EGF</td>
<td>Iressa</td>
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<td>Adducin a (S724)</td>
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<td>Adducin g (S662)</td>
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<td>CDK1 (Y15)</td>
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<td>CREB (S133)</td>
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**NOTE:** √, detected basic phosphoprotein level; ↑, increase; ↓, decrease; ←, no change. Arrows indicate ~25% change from the control in two separate experiments and multiple arrows indicate 2-fold change or more within the treatment categories.
cell lines, whereas total and phospho-Akt is significantly enriched in 5 min), 1 Hec50co and Ishikawa H cells. Cells were treated with 30 ng/mL EGF Iressa blocks the phosphorylation of ERK1/2 in both cells and Akt (Ser473) distribution of progesterone receptors A and B in normal and malignant 3. Arnett-Mansfield RL, DeFazio A, Mote PA, Clarke CL. Subnuclear in vitro kinase activity assays, respectively, and AstraZeneca Pharmaceuticals for providing Iressa for the present studies.

Acknowledgments

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References


Molecular Cancer Therapeutics

Regulation of signaling phosphoproteins by epidermal growth factor and Iressa (ZD1839) in human endometrial cancer cells that model type I and II tumors

Lina Albitar, Laura L. Laidler, Rony Abdallah, et al.


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