

New Mechanisms of Signal Transduction Inhibitor Action: Receptor Tyrosine Kinase Down-Regulation and Blockade of Signal Transactivation¹

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Abstract

The explosion of signal transduction research over the last 10 years has provided a unique insight into the complexity of these intricate pathways. Whereas intermediates of multiple signaling pathways have been identified, understanding their function and, in particular, the interactions between them has become a daunting task. The increasing evidence that many of these pathways can cross-talk with each other via signal transactivation inevitably raises the question of how cells determine specificity in signaling. Despite the mind-numbing complexity of these pathways, progress has been made in developing highly specific and potent signal transduction inhibitors (STIs). STIs show promise in the treatment of cancer in preclinical studies and are currently in a number of clinical trials. Whereas many of these agents were “rationally designed,” we barely understand their mechanisms of action. This review will highlight how recent studies using these STIs have elucidated novel mechanisms of STI action that may be used in the development of new therapeutic strategies for the treatment of cancer.

Introduction

The complexity of development and growth of organisms can in part be attributed to the diverse and dynamic interactions between hormones, growth factors, cell contact, and other external stimuli that coordinate cell fate through cell surface

receptors. The explosion of signal transduction research over the last 10 years has deciphered the basic signaling mechanisms of a number of these receptors (1).³ However, although great progress has been made in identifying signaling intermediates and some of their functions, we are far from a complete understanding of many of these pathways. The sequencing of the human genome has allowed the identification of different members of various signaling pathways. Indeed, 11.2% of genes whose function can be predicted are directly involved in signal transduction (2). This is, however, an underestimate of the total number of genes involved because 41.7% of the genes in the human genome have unpredicted function, and many genes are important in multiple functions, such as cell attachment and ECM⁴ genes, and are not classified as signal transduction categories. Our increased knowledge of the human genome has far outpaced our ability to understand how these proteins and their pathways interact and function. Despite this, it is clear that the understanding of the human genome and, more importantly the human proteome will have a major impact on medicine and pharmaceutical discovery (3).

One of the most interesting, yet daunting, aspects of signal transduction discovered in the last 5 years is the realization that very disparate pathways can interact at multiple levels—this is often referred to as signal cross-talk. A search of Medline showed that the term “cross-talk” or “cross talk” first appeared in 1991 and has been used in over 3500 articles since then. It has become clear that most pathways are so intricate and complex, having multiple layers of regulation, that a complete understanding of any single pathway will only be accomplished by taking into account its global networks of interactions. It is anticipated that computer modeling programs such as neural networks will play a major role in signal transduction research in the future (4, 5).

The elucidation of growth factor signaling pathways and the observation that these pathways are often altered in human cancer have led to the development of a number of highly specific inhibitors for these pathways (6). These new compounds are often referred to as STIs. This review will discuss the complexity of signal transduction and highlight how new STIs are not only proving useful in the treatment of cancer but are

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⁴ The abbreviations used are: ECM, extracellular matrix; STI, signal transduction inhibitor; ER, estrogen receptor; RTK, receptor tyrosine kinase; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptors; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; PDGFR, platelet-derived growth factor receptor; Prl, prolactin; GH, growth hormone; IRS, insulin receptor substrate; IGFBP, IGF-binding protein.

also elucidating novel mechanisms of signal transduction cross-talk.

Cross-Talk between Hormone Receptors and Growth Factors

An area of cross-talk that may have critical importance in breast cancer is interaction between hormone receptors and growth factors (7). The ER is a critical growth regulator in breast cancer and an excellent biological target for breast cancer prevention and treatment (8). Blockade of growth factor signaling represents a new and promising targeted biological therapy in breast cancer (9). Evidence that a RTK termed ErbB2 was amplified in breast cancer and that overexpression conferred a poor prognosis led to the pivotal development of inhibitors of ErbB2 receptor such as trastuzumab, which were successful in preclinical trials and are now used in breast cancer management (10).

Molecular studies have revealed that there are considerable interaction and cross-talk between hormone receptor and RTK signaling pathways (7). For example, the steroid hormone estrogen and the peptide growth factor IGF-I are both potent mitogens for a range of human breast cancer cell lines (11) and are both implicated in the growth and progression of breast cancer (12). Estrogen acts through a nuclear hormone receptor that upon activation increases transcription and expression of hormone-responsive genes (13). On the other hand, IGF-I acts through a transmembrane RTK (12) that signals via a series of phosphorylation events. Although the signal transduction pathways elicited by the two mitogens seem distinct, there appears to be considerable coordination and synergism between the two mitogens. Estrogen can increase IGF signaling, and, conversely, IGF-I can increase estrogen signaling (7). Recent studies have shown that these interactions can occur *in vivo*, with estrogen sensitizing the mammary gland to IGFs (14), and systemic IGF-I treatment of mice causing rapid activation of the ER (15).

Signal Transactivation

The original diagrams of linear signal transduction pathways have now been replaced with diagrams that start to incorporate the intricacies of signaling networks. Whereas many of the signals are transmitted in a linear fashion, there is now evidence that many connections may be made in a multidimensional manner. This type of signaling has recently been called "signal transactivation," and probably one of the best examples occurs within the EGFR pathway (16). The EGFR belongs to a family of tyrosine kinases termed EGFR/ErbB1, HER2/ErbB2/neu, HER3/ErbB3, and HER4/ErbB4 (we will use the term ErbB1–4). The four ErbB receptors can either homodimerize or heterodimerize in response to a number of different ligands (17). Dimerization results in receptor tyrosine phosphorylation that allows for the binding of downstream signaling molecules via Src homology-2 domain interactions. Different combinations of ErbB receptors can bind and activate different subsets of signaling intermediates, thus allowing signal diversification and specificity. Whereas many of these pathways have been extensively mapped and are becoming understood, recent evidence suggests that many other receptors can phosphorylate and activate ErbB receptors (Fig. 1) and that ErbBs may act as a conduit

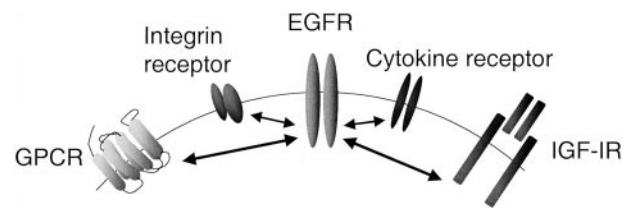


Fig. 1 Signal transactivation of ErbB receptors. ErbB receptors can be directly or indirectly activated by GPCRs, integrin receptors, cytokine receptors, or RTKs.

for multiple other signaling pathways (18).

Cross-Talk between ErbB Receptors and GPCRs. Perhaps the best evidence for signal transactivation of ErbB receptors comes from studies with GPCRs. Whereas GPCRs generally act through second messengers such as diacylglycerol and inositol triphosphate, many ligands for GPCRs (*e.g.*, bombesin) can also activate the MAPK pathway (18). Furthermore, this activation of MAPK has been shown to occur via GPCR activation of ErbB1 (19), and the fact that ErbB1 is involved in bombesin-mediated proliferation (20) further emphasizes this signal transactivation. There appear to be multiple mechanisms whereby GPCRs can activate ErbB1 signaling. First, GPCRs can activate kinases such as Src, which directly phosphorylate ErbB1 (21). Second, GPCRs can also regulate calcium influx. This regulation has been linked to ErbB1 activation and signaling (22), possibly via increased protein kinase C (23) or pyk2 (24) activity. In addition to these direct mechanisms of activation, GPCRs can indirectly cross-talk with ErbB receptors by activating metalloproteinase that cleaves heparin-bound EGF, which can then bind and activate ErbB1 (25).

Cross-Talk between ErbB and Growth Factor Receptors. The ErbB family members are able to associate not only with themselves and other family members, but also with other RTKs. PDGFR is another RTK that has a similar structure to ErbB1 (single molecule that passes through the cell membrane once) and acts in a similar manner to ErbB receptors. ErbB1 and PDGFR can physically interact (26), and EGF can increase phosphorylation and activation of PDGFR (27). In contrast, platelet-derived growth factor has been shown to cause threonine phosphorylation of ErbB1 and inhibit its function (28). IGF-IR is another RTK that, in contrast to ErbB receptors and PDGFRs, always exists as a heterotetramer of two α and two β subunits (29). As such, IGF-IR does not undergo ligand-mediated dimerization but does respond to IGF ligands by activation of intrinsic tyrosine kinase activity. IGF-IR activation can lead to cross-talk with ErbB receptors because recombinant IGF rapidly but indirectly activates ErbB1 and its downstream MAPK signaling pathway (30). The mechanism for cross-talk has not been identified but does not appear to involve c-Src or metalloproteinase-mediated activation of ErbB1, as occurs with GPCR. However, IGF-IR has been shown to physically associate with other ErbB receptors such as ErbB2 (31), an association that is regulated by heregulins and IGF-I. Interestingly, IGF-IR-null cells are not able to respond to EGF (32), suggesting that the interaction between these two receptors may be critical for EGF to mediate its full signaling response. IGF-IR signaling can

induce an EGFR-dependent autocrine loop (33), and it has been shown that EGF can cause phosphorylation and activation of IGF-IR (34).

Cross-Talk between ErbB Receptors and Cytokine Receptors. Cytokines such as Prl and GH act through transmembrane receptors that do not contain intrinsic kinase activity (35) but recruit cytoplasmic intermediates such as Janus tyrosine kinase (specifically JAK2; Ref. 36). GH and Prl are able to increase phosphorylation of ErbB1 both in cell lines and in mice (37). GH activation of JAK2 results in direct phosphorylation of tyrosine 1068 in ErbB1, which is a binding site for Grb2. Prl can also cause JAK2-mediated phosphorylation of ErbB2, and this may account in part for the ligand-independent activation of ErbB2 that is seen in many breast cancers (38). Indeed, blockade of autocrine Prl production in breast cancer cell lines can reduce ErbB2 signaling, and breast tumors that express both ErbB2 and Prl have a poor prognosis (38). Finally another cytokine, interleukin 6, has been shown to increase ErbB2 activation, signaling, and cell migration in breast cancer cell lines (39). The biological relevance of this interaction is currently under investigation.

Cross-Talk between ErbBs, Integrins, and the ECM. The ECM controls cell growth, differentiation, and survival by signaling through integrin receptors (40). Whereas these interactions were initially viewed as "housekeeping" functions, it has become apparent that integrins activate intricate intracellular signaling pathways that equal or exceed the complexity of the RTK pathways (41). Most important, in several systems, ECM and integrins are higher-order controlling elements of other signaling pathways. For instance, binding of primary mammary epithelial cells to laminin is required for both Prl (42) and insulin (43) signaling, which are essential mediators of cellular differentiation. ECM can also control RTK action in a number of cell culture systems (44), and ligation of integrins is essential for RTK-mediated signal transduction (45). Part of this effect may come through direct association of integrin receptors with RTKs and their signaling intermediates. For instance, $\alpha_v\beta_3$ integrin physically associates with PDGFR (46) and also with the cytoplasmic signaling intermediate IRS-1 (47). Furthermore, IRSs can be activated by integrin ligation even in the absence of RTKs (48). $\alpha_6\beta_4$ integrin associates with ErbB2 after EGF stimulation of breast cancer cells, and treatment of cells with an antibody that binds and activates α_6 integrin causes increased association between ErbB2 and $\alpha_6\beta_1$ and promotes cell proliferation and invasion (49). Several of the IGFbps have RGD sequence that is used in integrin receptor binding. IGFBP-1 can bind $\alpha_5\beta_1$ integrin and increase cell migration (50), but addition of excess recombinant IGFBP-1 can block IGF-I-mediated breast cancer cell motility (51).

STIs

Since the early discovery that a viral oncogene was also a mutated RTK in human cancer, a wealth of literature has implicated RTKs in cancer pathogenesis (6). This has led to the development of a number of inhibitors of RTK signaling pathways. RTK signaling has been blocked by a number of mechanisms *in vitro* including (a) blocking ligand binding to the RTK (using binding proteins such as IGFBP-1 or antibodies that bind

the RTK), (b) inhibiting RTK expression (using antisense oligonucleotides), or (c) inhibiting RTK activity (using kinase-dead and dominant negative RTKs or small molecule inhibitors). Clinical development has occurred with antibodies that block receptor activation and also recently with small molecules that block the tyrosine kinase activity of the RTK (52).

Predicting Response to STIs. Because these STIs have been designed against specific targets and show very high affinity and specificity in *in vitro* systems, it would be logical to expect that expression of the target of a STI may predict the sensitivity of a cell to that STI. For instance, antiestrogens are only effective in breast cancer cells that express ER and have very little or no effect in ER-negative breast cancer cell lines (53). This finding is reproduced in the clinical situation, where ER is an excellent predictive marker for response to antiestrogens (54). However, a greater understanding of the molecular biology of ER action has shown that antiestrogens can have dramatically opposing effects in different tissues due to other modulators of ER action, including coregulators, other growth factor pathways, and other isoforms of ER (55).

With regard to STIs, it has been found that response of breast cancer cells to the selective ErbB1 tyrosine kinase inhibitor ZD1839 (Iressa) does not correlate with ErbB1 status (56, 57). On normal mammary epithelial cells (MCF-10A) that are dependent on EGF for growth, ZD1839 has a growth inhibitory IC_{50} of 0.02 μ M, consistent with its *in vitro* IC_{50} value. However, higher concentrations of 0.5–20 μ M are needed for growth inhibition in breast cancer cell lines. Possible explanations for this are that (a) ZD1839 is inhibiting other ErbB receptor heterodimers, (b) other pathways are modulating EGFR activation, or (c) alterations in other signal transduction pathways reduce the dependence of these cells on EGF. It is clear that more studies of this nature are needed to identify the markers that predict response to specific STIs. These studies will no doubt involve the use of newly developed phospho-specific antibodies to activated growth factor signaling molecules and the use of gene array technology to measure global gene expression patterns.

STI Blockade of Signal Transactivation. Whereas STIs have shown promise in preclinical settings and are currently in clinical trials, we poorly understand their mechanisms of action. However, it is expected that these STIs will act as novel tools for dissecting and understanding RTK signaling and cross-talk. For example, Prl secreted by breast cancer cells can act in an autocrine manner and cause phosphorylation of ErbB2 via JAK2 (38). We know this in part because blockade of this signal transactivation, using STIs against either Prl receptor, ErbB2, or JAK2, results in reduced ErbB2 phosphorylation and decreased proliferation.

We have shown that blockade of ER using antiestrogens or blockade of IGF-I signaling using a neutralizing binding protein (IGFBP-1) can have dramatic effects on both signaling pathways. Antiestrogens are very effective at eliminating both estrogen and IGF signaling and growth (7). Additionally, whereas IGFBP-1 is effective at inhibiting IGF signaling and growth *in vitro* and *in vivo*, it also blocks IGF-I activation of the ER and inhibits estrogen-mediated growth (58). Other groups have similar findings (59).

Another example of blockade of signal transactivation, this

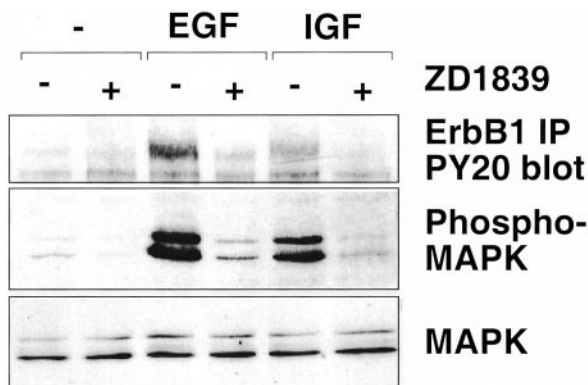


Fig. 2 ZD1839 inhibits IGF-I-mediated phosphorylation of ErbB1. Serum-starved FSK-7 cells were stimulated with EGF or IGF-I and treated with or without ZD1839 (1 μ M). ErbB1 was immunoprecipitated from cell lysates and immunoblotted for phosphotyrosine (*top panel*). Total cell lysates were immunoblotted for phospho-MAPK (*middle panel*) or total MAPK (*bottom panel*). This figure has been reproduced from Ref. 30 with permission.

time between ErbB1 and IGF-IR, has recently been shown using primary mammary epithelial cell cultures (30). In this system, EGF treatment results in phosphorylation of ErbB1 and MAPK, but phosphorylation also occurs when cells are treated with IGF-I (Fig. 2). The ErbB1 tyrosine kinase inhibitor ZD1839 completely blocks EGF signaling, as expected, but it also inhibits IGF-mediated phosphorylation of MAPK. This effect of ZD1839 is not through nonspecific inhibition of IGF-IR activation because phosphorylation of IGF-IR and its downstream signaling intermediate IRS-1 are not affected. The conclusion is that IGF interaction with IGF-IR causes phosphorylation of ErbB1, leading to activation of the MAPK pathway, and that the IGF-mediated activation of MAPK signaling can be blocked by inhibiting its cross-talk with ErbB1.

In primary mammary epithelial cell cultures, EGF and IGF-I are survival factors allowing cells to evade cell death. Interestingly, whereas ZD1839 is often thought of as an inhibitor of ErbB1-mediated cell growth, in primary mammary epithelial cells ZD1839 can block EGF-mediated protection from apoptosis by inhibiting phosphorylation of ErbB1 and activating the proapoptotic protein BAD (49). Importantly, ZD1839 is able to block IGF-I-mediated protection from cell death, substantiating the signaling cross-talk between IGF-I and ErbB1. This observation has important clinical implications for future approaches in which STIs are combined with other apoptosis-inducing agents (60).⁵ More studies are needed to fully understand the complex cross-talk between growth factor signaling pathways in both normal and neoplastic cells. These studies will have to define the mechanisms of action of these STIs and may predict potential toxic side effects and possible mechanisms of resistance.

STI-mediated Down-Regulation of RTKs. Careful characterization of STI action in cell lines has cast light on a

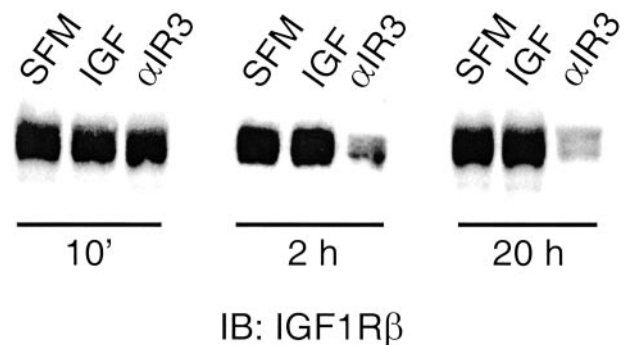


Fig. 3 Antibodies to IGF-IR cause receptor down-regulation. MCF-7 cells were serum starved overnight and then stimulated with IGF-I or α -IR3. Cells were harvested at various time points, and total cell lysates were immunoblotted for IGF-IR (β subunit).

novel mechanism of action that may unfold new avenues for designing alternative strategies to inhibit RTK function. Whereas RTK inhibitors were designed either to block ligand binding or receptor dimerization (*e.g.*, using monoclonal antibodies such as trastuzumab) or to block the tyrosine kinase activity (*e.g.*, by modifying the ATP binding site with CI-1033), it has been found that these strategies also result in down-regulation and proteasome degradation of ErbB2 (61). Whereas it is not unexpected that binding of an antibody at the cell membrane to ErbB2 puts the receptor in a unusual conformation that is recognized as abnormal by the cell and thus marked for degradation, it is unclear why an inhibitor of the tyrosine kinase activity would cause the same effect. Despite this, down-regulation of ErbB2 is clearly a very favorable outcome for blocking ErbB signaling because ErbB2 is a major signaling partner for the other ErbB family members. Interestingly, evidence suggests that ErbB2 may be such a potent signal transducer because after activation and endocytosis it is recycled to the cell surface, rather than being sent for degradation like ErbB1 (62). Blockade of ErbB2 function may disturb this process and thus block its potent activity. Whereas the mechanisms for trastuzumab- and CI-1033-mediated proteasome degradation are unclear, ErbB2 can also be targeted for proteasome degradation by geldanamycin, an ansamycin antibiotic that interferes with ATP binding to the Hsp90 chaperone and results in selective degradation of ErbB receptors (63). Citri *et al.* (61) showed that CI-1033 and geldanamycin act in an additive manner, using a common mechanism of chaperone-mediated degradation. This suggests that a common mechanism for ErbB2 degradation may be directly and specifically targeted.

Interestingly, antibodies that bind IGF-IR and block its signaling capacity also result in receptor down-regulation (Fig. 3). MCF-7 cells were treated with either IGF-I or a commercially available monoclonal antibody (α -IR3) that can block breast cancer cell growth *in vitro* and *in vivo* (64, 65). Neither IGF-I stimulation nor α -IR3 changed IGF-IR levels after 10 min, but after 2 h, α -IR3 caused a significant decrease in IGF-IR levels, whereas IGF-I did not. More studies are needed to define the biochemical mechanism of this reduction in IGF-IR levels, but the data suggest that down-regulation of RTKs may be a common feature after blocking receptor function. It is possible

⁵ <http://stke.sciencemag.org>.

that the antibodies force the RTK into an unnatural conformation that is then recognized by heat shock proteins, resulting in RTK ubiquitination and degradation. These *in vitro* studies simply that the agonist or antagonist biochemical properties of any such receptor binding antibody may not be important as long as binding results in down-regulation of cell surface receptor expression.

Targeted Down-Regulation of Signaling Intermediates.

The realization that STIs not only inhibit RTK action but also cause RTK down-regulation suggests that specifically targeting the mechanism of down-regulation may be a potential therapeutic strategy in breast cancer. Indeed, this is already being tested clinically with geldanamycin, an antibiotic that, as noted above, inhibits ATP binding of the chaperone Hsp90 and causes proteasomal degradation of many intracellular signaling intermediates including steroid receptors and RTKs (63). However, geldanamycin targets a relatively nonspecific form of protein degradation that may prove to be too nonspecific for therapeutic benefit.

Recent studies have shown that signal transduction pathways can initiate very specific targeted proteasomal degradation. Whereas the complete mechanisms are still to be elucidated, the SCF complex seems to be a key player in allowing phosphorylation-targeted ubiquitination of signaling intermediates (66). For instance, cell cycle components must be synthesized and degraded in a rapid and tightly controlled manner to allow cell cycle control. Many of the cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors undergo rapid proteasomal degradation after phosphorylation on specific residues (67). Inhibitors of the proteasome have been developed and are being used in clinical trials for treatment of a number of cancers (68, 69).

We have investigated the proteasomal degradation of IRS-1, a signaling intermediate that is regulated by hormone receptors and has ominous prognostic significance in breast cancer (70). Cell line studies have shown that, after activation, IRS-1 is ubiquitinated and then degraded by the proteasome (71). Importantly, the signal that mediates this degradation is the phosphatidylinositol 3'-kinase/Akt/mTOR pathway (72, 73), and the signal may actually be direct phosphorylation of IRS-1 on serine/threonine residues by one of these signaling proteins (74, 75). Interestingly, IRS-1 is upstream of the phosphatidylinositol 3'-kinase/Akt/mTOR pathway, and so down-regulation of IRS-1 represents a rapid and potent negative feedback mechanism for shutting off this pathway. Additional studies on the biology of IRS-1 have revealed that its level is hormonally regulated in the normal mammary gland.⁶ In particular, we have shown that IRS-1 proteins decline dramatically when the mammary gland undergoes involution (76). This decrease occurs within 6 h and occurs in the absence of changes in mRNA levels, suggesting that this represents protein turnover or degradation, similar to observations in cell culture.

Blockade of IRS-1 action in breast cancer may be difficult because IRS-1 has no intrinsic kinase activity, and interrupting protein-protein interactions with small molecules is technically

challenging. In contrast, lowering IRS-1 levels by stimulating degradation may be an ideal mechanism for inhibiting IRS-1 action. We are performing *in vitro* and *in vivo* studies to better understand the molecular pathways that are critical for IRS-1 degradation, with the goal of designing small molecules that will enhance IRS-1 degradation. Whereas phosphorylation-directed degradation probably uses common mechanisms for destroying proteins, *e.g.*, the 26S proteasome, we believe that specificity is obtained through the combination of unique phosphorylation motifs and E3 proteins, thus allowing highly specific phosphorylation-targeted proteasome degradation for each signaling intermediate. Perhaps we can harness the power of naturally occurring negative feedback systems to stimulate degradation pathways that will allow the selective degradation of certain signaling intermediates.

Summary

STIs show promise in preclinical studies and are currently in a number of clinical trials. Whereas many of these agents were "rationally designed," we barely understand their mechanisms of action. Part of this is due to our limited understanding of cross-talk among receptor systems and the key pathways necessary for cell growth. For example, trastuzumab has clinical activity only in a minority of patients that overexpress the HER2 target. The reasons for *de novo* resistance are not understood and must be elucidated if we are to optimally use these new strategies. A greater understanding of signaling interactions and networks will be needed to better predict how inhibitors will affect neoplastic cell growth and death, and it is anticipated that careful *in vitro* studies with these specific high-affinity inhibitors will elucidate novel mechanism of signal cross-talk that will then lead to the development of new inhibitors that will block these new interactions. Once identified *in vitro*, these findings can be tested in clinical settings to make rational treatment choices for cancer patients

Acknowledgments

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Open Discussion

Dr. Adrian V. Lee: The theory is that the IGF-I activates the IGF receptor, which then transphosphorylates the EGFR and causes EGFR signaling. If you give Iressa (ZD1839), you block EGFR signaling, which shows it's a specific blocker, but it also takes out the IGF signal as well.

Dr. Kent Osborne: Does it take out the IGF receptor or just the IGF downstream signaling?

Dr. Lee: That is a very critical question. It has practically zero effect on IGF receptor or IGF signaling downstream. So this is really proof of IGF receptor transphosphorylation of EGFR. Previous data came from transfection studies, but now in mammary epithelial cells, it's been shown that IGF activates the EGF pathway, and Iressa can wipe that out.

Dr. Carlos Arteaga: But that suggests that that cross-talk still requires the EGFR kinase, and so the activation of the receptor by IGF-I. Is that your interpretation?

Dr. Lee: That depends on whether that receptor is only taking out the kinase or whether it's a down-regulated receptor.

⁶ A. V. Lee and D. L. Hadsell, manuscript in preparation.

These inhibitors also cause down-regulation and destruction of the receptor.

Dr. Arteaga: Iressa doesn't. Just to underscore that these small molecules are not "me too" drugs, we have done similar experiments with OSI-774 and Iressa, and there's really no EGFR or HER2 down-regulation from the cell surface whatsoever. So there is something unique about this inhibitor that is doing that.

Dr. Steven Come: Well, I think now you see why we're anxious to go ahead and put Iressa into a clinical trial. It's looking better and better.

Dr. Arteaga: The Eastern Cooperative Oncology Group Phase II trial of Herceptin plus Iressa in HER2-overexpressing metastatic disease is accruing. The rationale is based on preclinical data indicating that when you block both receptors at the same time, you induce very profound apoptosis in culture, and there is also synergistic activity in xenograft and transgenic models. We have some data using a human mouse mammary tumor virus HER2 transgenic nude mouse from Genentech showing that tumors as big as 2 cm³ disappear with the combination of Herceptin and OSI-774, which is similar to Iressa. With either drug alone, there's little or no effect. So tumor cells may be smart, but they're not that smart. It would be logical that the first compensatory mechanism they use would be receptors of the same network, and that would be the EGFR. That could have been anticipated from the biochemistry of the EGFR/HER2 network.

Dr. Stephen Johnston: If HER2 is really critical and you take out HER2, the tumor cells couldn't get around it. If HER2 is really the prominent dimerization partner, then maybe they have to switch to another network.

Dr. Mitch Dowsett: The data you presented are provocative and also pretty discouraging in terms of applying these agents in the metastatic setting because of these multiple escape pathways.

Dr. Lee: We talked about that at last year's meeting, about whether metastatic disease is the right setting for testing these agents. That's a classic finding with Iressa working really well on normal cells and then having a different IC₅₀ in tumor cells. So maybe that's why these agents should be tested in the neoadjuvant setting. We understand why chemotherapy works in the metastatic setting, because it's completely nonspecific. In targeting a very defined pathway, either we're going to have to find the predictive biomarkers, which again is going to take time, or we have to try it in the adjuvant setting before any data come out.

Dr. Dowsett: The problem with a lot of these agents is a lack of long-term safety data because the metastatic studies are often quite short.

Dr. Kathleen Pritchard: But you have to take them into the adjuvant setting without long-term safety data anyway, so the question always is whether there's enough justification to take that big experiment with lots of patients when you don't know what the data are.

Dr. Come: The best thing by far, at least in this country, would be some sort of inter-SPORE (Specialized Program of Research Excellence) cooperation to do basically what Dr. Dowsett is doing. Once you resolve the institutional review board issues, involve several institutions that have the capacity

to do these studies, in which we decide we're going to get two pathologic specimens on each patient, that we're going to use these biomarkers to make meaningful measurements in that window. So then we'd be able to select what to go forward with in an adjuvant trial.

Dr. Eric Winer: I think it's the institutional review board and the safety issues that impede us.

References

- Gough, N. R., and Ray, L. B. Mapping cellular signaling. *Science's STKE*, 2002: EG8, 2002.
- Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., *et al.* The sequence of the human genome. *Science (Wash. DC)*, 291: 1304–1351, 2001.
- Venter, J. C. Genomic impact on pharmaceutical development. *Novartis Found. Symp.*, 229: 14–15, 2000.
- Arkin, A. P. Synthetic cell biology. *Curr. Opin. Biotechnol.*, 12: 638–644, 2001.
- Levchenko, A. Computational cell biology in the post-genomic era. *Mol. Biol. Rep.*, 28: 83–89, 2001.
- Zwick, E., Bange, J., and Ullrich, A. Receptor tyrosine kinase signalling as a target for cancer intervention strategies. *Endocr. Relat. Cancer*, 8: 161–173, 2001.
- Lee, A. V., Cui, X., and Oesterreich, S. Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. *Clin. Cancer Res.*, 7 (Suppl.): 4429s–4435s, 2001.
- Osborne, C. K., Zhao, H., and Fuqua, S. A. Selective estrogen receptor modulators: structure, function, and clinical use. *J. Clin. Oncol.*, 18: 3172–3186, 2000.
- Baselga, J. New therapeutic agents targeting the epidermal growth factor receptor. *J. Clin. Oncol.*, 18: 54S–59S, 2000.
- Baselga, J., Albanell, J., Molina, M. A., and Arribas, J. Mechanism of action of trastuzumab and scientific update. *Semin. Oncol.*, 28: 4–11, 2001.
- Cullen, K., Yee, D., Sly, W., Perdue, J., Hampton, B., Lippman, M., and Rosen, N. Insulin-like growth factor receptor expression and function in human breast cancer. *Cancer Res.*, 50: 48–53, 1990.
- Sachdev, D., and Yee, D. The IGF system and breast cancer. *Endocr. Relat. Cancer*, 8: 197–209, 2001.
- Gross, J. M., and Yee, D. How does the estrogen receptor work? *Breast Cancer Res.*, 4: 62–64, 2002.
- Chan, T. W., Pollak, M., and Huynh, H. Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure antiestrogen ICI 182,780. *Clin. Cancer Res.*, 7: 2545–2554, 2001.
- Klotz, D. M., Hewitt, S. C., Ciana, P., Raviscioni, M., Lindzey, J. K., Foley, J., Maggi, A., DiAugustine, R. P., and Korach, K. S. Requirement of estrogen receptor- α in insulin-like growth factor-1 (IGF-1)-induced uterine responses and *in vivo* evidence for IGF-1/estrogen receptor cross-talk. *J. Biol. Chem.*, 277: 8531–8537, 2002.
- Gschwind, A., Zwick, E., Prenzel, N., Leserer, M., and Ullrich, A. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene*, 20: 1594–1600, 2001.
- Yarden, Y., and Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.*, 2: 127–137, 2001.
- Prenzel, N., Fischer, O. M., Streit, S., Hart, S., and Ullrich, A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr. Relat. Cancer*, 8: 11–31, 2001.
- Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.*, 16: 7032–7044, 1997.

20. Santiskulvong, C., Sinnett-Smith, J., and Rozengurt, E. EGF receptor function is required in late G₁ for cell cycle progression induced by bombesin and bradykinin. *Am. J. Physiol. Cell Physiol.*, *281*: C886–C898, 2001.
21. Biscardi, J. S., Maa, M. C., Tice, D. A., Cox, M. E., Leu, T. H., and Parsons, S. J. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr⁸⁴⁵ and Tyr¹¹⁰¹ is associated with modulation of receptor function. *J. Biol. Chem.*, *274*: 8335–8343, 1999.
22. Zwick, E., Wallasch, C., Daub, H., and Ullrich, A. Distinct calcium-dependent pathways of epidermal growth factor receptor transactivation and PYK2 tyrosine phosphorylation in PC12 cells. *J. Biol. Chem.*, *274*: 20989–20996, 1999.
23. Tsai, W., Morielli, A. D., and Peralta, E. G. The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *EMBO J.*, *16*: 4597–4605, 1997.
24. Soltoff, S. P. Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled P2Y₂ receptor. Phorbol ester or [Ca²⁺]_i elevation can substitute for receptor activation. *J. Biol. Chem.*, *273*: 23110–23117, 1998.
25. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature (Lond.)*, *402*: 884–888, 1999.
26. Liu, P., and Anderson, R. G. Spatial organization of EGF receptor transmodulation by PDGF. *Biochem. Biophys. Res. Commun.*, *261*: 695–700, 1999.
27. Habib, A. A., Hognason, T., Ren, J., Stefansson, K., and Ratan, R. R. The epidermal growth factor receptor associates with and recruits phosphatidylinositol 3-kinase to the platelet-derived growth factor β receptor. *J. Biol. Chem.*, *273*: 6885–6891, 1998.
28. Bagowski, C. P., Stein-Gerlach, M., Choidas, A., and Ullrich, A. Cell-type specific phosphorylation of threonines T654 and T669 by PKD defines the signal capacity of the EGF receptor. *EMBO J.*, *18*: 5567–5576, 1999.
29. LeRoith, D. Insulin-like growth factor receptors and binding proteins. *Bailliere's Clin. Endocrinol. Metabol.*, *10*: 49–73, 1996.
30. Gilmore, A. P., Valentijn, A., Wang, P., Ranger, A. M., Bundred, N., O'Hare, M. J., Wakeling, A., Korsmeyer, S. J., and Streuli, C. H. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin like growth factor receptor. *J. Biol. Chem.*, *277*: 27643–27650, 2002.
31. Balana, M. E., Labriola, L., Salatino, M., Movsichoff, F., Peters, G., Charreau, E. H., and Elizalde, P. V. Activation of ErbB-2 via a hierarchical interaction between ErbB-2 and type I insulin-like growth factor receptor in mammary tumor cells. *Oncogene*, *20*: 34–47, 2001.
32. Coppola, D., Ferber, A., Miura, M., Sell, C., D'Ambrosio, C., Rubin, R., and Baserga, R. A functional insulin-like growth factor I receptor is required for the mitogenic and transforming activities of the epidermal growth factor receptor. *Mol. Cell Biol.*, *14*: 4588–4595, 1994.
33. Wang, D., Patil, S., Li, W., Humphrey, L. E., Brattain, M. G., and Howell, G. M. Activation of the TGFα autocrine loop is downstream of IGF-I receptor activation during mitogenesis in growth factor dependent human colon carcinoma cells. *Oncogene*, *21*: 2785–2796, 2002.
34. Burgaud, J. L., and Baserga, R. Intracellular transactivation of the insulin-like growth factor I receptor by an epidermal growth factor receptor. *Exp. Cell Res.*, *223*: 412–419, 1996.
35. Goffin, V., and Kelly, P. A. The prolactin/growth hormone receptor family: structure/function relationships. *J. Mammary Gland Biol. Neoplasia*, *2*: 7–17, 1997.
36. O'Shea, J. J., Gagina, M., and Schreiber, R. D. Cytokine signaling in 2002: New surprises in the Jak/Stat pathway. *Cell*, *109* (Suppl.): S121–S131, 2002.
37. Yamauchi, T., Ueki, K., Tobe, K., Tamemoto, H., Sekine, N., Wada, M., Honjo, M., Takahashi, M., Takahashi, T., Hirai, H., Tushima, T., Akanuma, Y., Fujita, T., Komuro, I., Yazaki, Y., and Kadowaki, T. Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. *Nature (Lond.)*, *390*: 91–96, 1997.
38. Yamauchi, T., Yamauchi, N., Ueki, K., Sugiyama, T., Waki, H., Miki, H., Tobe, K., Matsuda, S., Tsushima, T., Yamamoto, T., Fujita, T., Taketani, Y., Fukayama, M., Kimura, S., Yazaki, Y., Nagai, R., and Kadowaki, T. Constitutive tyrosine phosphorylation of ErbB-2 via Jak2 by autocrine secretion of prolactin in human breast cancer. *J. Biol. Chem.*, *275*: 33937–33944, 2000.
39. Badache, A., and Hynes, N. E. Interleukin 6 inhibits proliferation and, in cooperation with an epidermal growth factor receptor autocrine loop, increases migration of T47D breast cancer cells. *Cancer Res.*, *61*: 383–391, 2001.
40. Juliano, R. L. Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. *Annu. Rev. Pharmacol. Toxicol.*, *42*: 283–323, 2002.
41. Schwartz, M. A., and Ginsberg, M. H. Networks and crosstalk: integrin signalling spreads. *Nat. Cell Biol.*, *4*: E65–E68, 2002.
42. Edwards, G. M., Wilford, F. H., Liu, X., Hennighausen, L., Djiane, J., and Streuli, C. H. Regulation of mammary differentiation by extracellular matrix involves protein-tyrosine phosphatases. *J. Biol. Chem.*, *273*: 9495–9500, 1998.
43. Farrelly, N., Lee, Y. J., Oliver, J., Dive, C., and Streuli, C. H. Extracellular matrix regulates apoptosis in mammary epithelium through a control on insulin signaling. *J. Cell Biol.*, *144*: 1337–1348, 1999.
44. Lee, Y. J., and Streuli, C. H. Extracellular matrix selectively modulates the response of mammary epithelial cells to different soluble signaling ligands. *J. Biol. Chem.*, *274*: 22401–22408, 1999.
45. Zheng, B., and Clemmons, D. R. Blocking ligand occupancy of the α_vβ₃ integrin inhibits insulin-like growth factor I signaling in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA*, *95*: 11217–11222, 1998.
46. Schneller, M., Vuori, K., and Ruoslahti, E. α_vβ₃ integrin associates with activated insulin and PDGFR receptors and potentiates the biological activity of PDGF. *EMBO J.*, *16*: 5600–5607, 1997.
47. Vuori, K., and Ruoslahti, E. Association of insulin receptor substrate-1 with integrins. *Science (Wash. DC)*, *266*: 1576–1578, 1994.
48. Shaw, L. M. Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the α₆β₄ integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion. *Mol. Cell Biol.*, *21*: 5082–5093, 2001.
49. Falcioni, R., Antonini, A., Nistico, P., Di Stefano, S., Crescenzi, M., Natali, P. G., and Sacchi, A. α₆β₄ and α₆β₁ integrins associate with ErbB-2 in human carcinoma cell lines. *Exp. Cell Res.*, *236*: 76–85, 1997.
50. Jones, J. I., Gockerman, A., Busby, W. H., Jr., Wright, G., and Clemmons, D. R. Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the α₅β₁ integrin by means of its Arg-Gly-Asp sequence. *Proc. Natl. Acad. Sci. USA*, *90*: 10553–10557, 1993.
51. Zhang, X., and Yee, D. Insulin-like growth factor binding protein-1 inhibits breast cancer cell motility. *Cancer Res.*, *62*: 4369–4375, 2002.
52. Baselga, J., and Norton, L. Focus on breast cancer. *Cancer Cell*, *1*: 319–322, 2002.
53. Oesterreich, S., Zhang, P., Guler, R. L., Sun, X., Curran, E. M., Welshons, W. V., Osborne, C. K., and Lee, A. V. Re-expression of estrogen receptor α in estrogen receptor α-negative MCF-7 cells restores both estrogen and insulin-like growth factor-mediated signaling and growth. *Cancer Res.*, *61*: 5771–5777, 2001.
54. Osborne, C. K. Steroid hormone receptors in breast cancer management. *Breast Cancer Res. Treat.*, *51*: 227–238, 1998.
55. Osborne, C. K., Schiff, R., Fuqua, S. A., and Shou, J. Estrogen receptor: current understanding of its activation and modulation. *Clin. Cancer Res.*, *7* (Suppl.): 4338s–4342s, 2001.
56. Moasser, M. M., Basso, A., Averbuch, S. D., and Rosen, N. The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven sig-

- naling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res.*, *61*: 7184–7188, 2001.
57. Kurokawa, H., and Arteaga, C. L. Inhibition of erbB receptor (HER) tyrosine kinases as a strategy to abrogate antiestrogen resistance in human breast cancer. *Clin. Cancer Res.*, *7* (Suppl.): 4436s–4442s, 2001.
58. Lee, A. V., Weng, C. N., Jackson, J. G., and Yee, D. Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *J. Endocrinol.*, *152*: 39–47, 1997.
59. Nickerson, T., Huynh, H., and Pollak, M. Insulin-like growth factor binding protein-3 induces apoptosis in MCF7 breast cancer cells. *Biochem. Biophys. Res. Commun.*, *237*: 690–693, 1997.
60. MacKeigan, J. P., Collins, T. S., and Ting, J. P. MEK inhibition enhances paclitaxel-induced tumor apoptosis. *J. Biol. Chem.*, *275*: 38953–38956, 2000.
61. Citri, A., Alroy, I., Lavi, S., Rubin, C., Xu, W., Grammatikakis, N., Patterson, C., Neckers, L., Fry, D. W., and Yarden, Y. Drug-induced ubiquitylation and degradation of ErbB receptor tyrosine kinases: implications for cancer therapy. *EMBO J.*, *21*: 2407–2417, 2002.
62. Yarden, Y. Biology of HER2 and its importance in breast cancer. *Oncology (Basel)*, *61* (Suppl. 2): 1–13, 2001.
63. Munster, P. N., Marchion, D. C., Basso, A. D., and Rosen, N. Degradation of HER2 by ansamycins induces growth arrest and apoptosis in cells with HER2 overexpression via a HER3, phosphatidylinositol 3'-kinase-AKT-dependent pathway. *Cancer Res.*, *62*: 3132–3137, 2002.
64. Arteaga, C. L., and Osborne, C. K. Growth inhibition of human breast cancer cells *in vitro* with an antibody against the type I somatomedin receptor. *Cancer Res.*, *49*: 6237–6241, 1989.
65. Arteaga, C. L., Kitten, L. J., Coronado, E. B., Jacobs, S., Kull, F. C., Jr., Allred, D. C., and Osborne, C. K. Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. *J. Clin. Investig.*, *84*: 1418–1423, 1989.
66. von Arnim, A. G. A hitchhiker's guide to the proteasome. *Science's STKE*, *2001*: PE2, 2001.
67. Obaya, A. J., and Sedivy, J. M. Regulation of cyclin-Cdk activity in mammalian cells. *Cell Mol. Life Sci.*, *59*: 126–142, 2002.
68. Shah, S. A., Potter, M. W., and Callery, M. P. Ubiquitin proteasome inhibition and cancer therapy. *Surgery*, *131*: 595–600, 2002.
69. Adams, J. Development of the proteasome inhibitor PS-341. *Oncologist*, *7*: 9–16, 2002.
70. Lee, A. V., Guler, B. L., Gooch, J. L., and Yee, D. IGF-I mediated degradation of insulin receptor substrate-1 is mediated by the 26S proteasome and blocked by phosphatidylinositol 3'-kinase inhibition. *Mol. Cell. Biol.*, *20*: 1489–1496, 2000.
71. Sun, X. J., Goldberg, J. L., Qiao, L. Y., and Mitchell, J. J. Insulin-induced insulin receptor substrate-1 degradation is mediated by the proteasome degradation pathway. *Diabetes*, *48*: 1359–1364, 1999.
72. Egawa, K., Nakashima, N., Sharma, P. M., Maegawa, H., Nagai, Y., Kashiwagi, A., Kikkawa, R., and Olefsky, J. M. Persistent activation of phosphatidylinositol 3-kinase causes insulin resistance due to accelerated insulin-induced insulin receptor substrate-1 degradation in 3T3-L1 adipocytes. *Endocrinology*, *141*: 1930–1935, 2000.
73. Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P. M., Olefsky, J. M., and Kobayashi, M. A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. *Mol. Endocrinol.*, *14*: 783–794, 2000.
74. Pederson, T. M., Kramer, D. L., and Rondinone, C. M. Serine/threonine phosphorylation of IRS-1 triggers its degradation: possible regulation by tyrosine phosphorylation. *Diabetes*, *50*: 24–31, 2001.
75. Li, J., DeFea, K., and Roth, R. A. Modulation of insulin receptor substrate-1 tyrosine phosphorylation by an Akt/phosphatidylinositol 3-kinase pathway. *J. Biol. Chem.*, *274*: 9351–9356, 1999.
76. Hadsell, D. L., Alexeenko, T., Klemintidis, Y., Torres, D., and Lee, A. V. Inability of overexpressed des(1–3)human insulin-like growth factor I (IGF-I) to inhibit forced mammary gland involution is associated with decreased expression of IGF signaling molecules. *Endocrinology*, *142*: 1479–1488, 2001.