Signal Transduction Pathway Targets for Anticancer Drug Discovery

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Abstract: There are currently over 80 agents officially approved for the treatment of cancer world-wide. However, the most common epithelial cancers, which cause greater than 75% of cancer deaths, remain incurable. Most drugs have been developed empirically by testing large numbers of chemicals on rapidly growing transplantable rodent tumors, and more recently, human tumor xenografts. This approach has identified prodeminantly DNA-active drugs that are considerably toxic and have limited efficacy. Novel molecular targets, which are selective for neoplastic cells, are needed for chemotherapeutic agents to improve cure rates of epithelial malignancies, with acceptable toxicity. In recent years, agents inhibiting signal transduction pathway molecules have entered clinical trials. These include antibodies and small molecules, which inhibit growth factor receptors and their receptor tyrosine kinases, inhibitors of cytoplasmic second messengers such as ras, raf and MEK, inhibitors of protein trafficking, and inhibitors of protein degradation.

INTRODUCTION

Cell proliferation and differentiation are regulated by a number of hormones, growth factors and cytokines. These molecules interact with cellular receptors and communicate with the nucleus of the cell through a network of intracellular signaling pathways (Fig **1**). In cancer cells, key components of these pathways may be altered by oncogenes through over-expression or mutation, leading to disregulated cell signaling and cell proliferation. The components of these abnormal signaling pathways, which are specific to neoplastic cells, represent potential selective targets for new anticancer therapies. These potential targets include ligands (typically growth factors), cellular receptors, intracellular second messengers and nuclear transcription factors. A detailed description of all these possible targets is beyond the scope of this review. The interested reader is referred to several recent, excellent reviews [1-5]. This discussion will focus exclusively on the targets for which promising anti-neoplastic agents are in clinical trials.

INHIBITION OF GROWTH FACTOR RECEPTOR BINDING

The first, obvious point of intervention in a signaling cascade is the neutralization of ligands before they can associate with their receptors. This approach has been investigated, initially through the use of antibodies, which interact with growth factors. Small cell lung cancer, and other malignancies secrete bombesin-like peptides, such as gastrin releasing peptide. A recently published phase I trial established that repeated doses of monoclonal antibody *2A11*, which binds to the bombesin-like peptide, GRP with high affinity, could be given safely to SCLC patients, and sustained plasma levels could be achieved on a 1-week schedule of antibody administration [6]. This approach is limited by the need to prospectively identify patients whose tumors express the receptor, and whose plasma contains significant amounts of circulating growth factor. Non-specific inhibition of several growth factors was explored through the use of the polysulfonated naphthylurea, suramin [7]. This agent neutralized a number of growth factors, but presented challenging dosing problems because of an extremely long half-life and significant toxicities. In spite of evidence of modest activity in prostate cancer, its development has been discontinued, because of toxicity problems.

The second approach to abrogating signaling pathways is the prevention of the binding of growth factors to their receptors. Several strategies have been under development in an attempt to block growth factor receptors. The most successful approach to date has been the development of monoclonal antibodies which bind to receptors and by so doing prevent the binding of the endogenous ligands.

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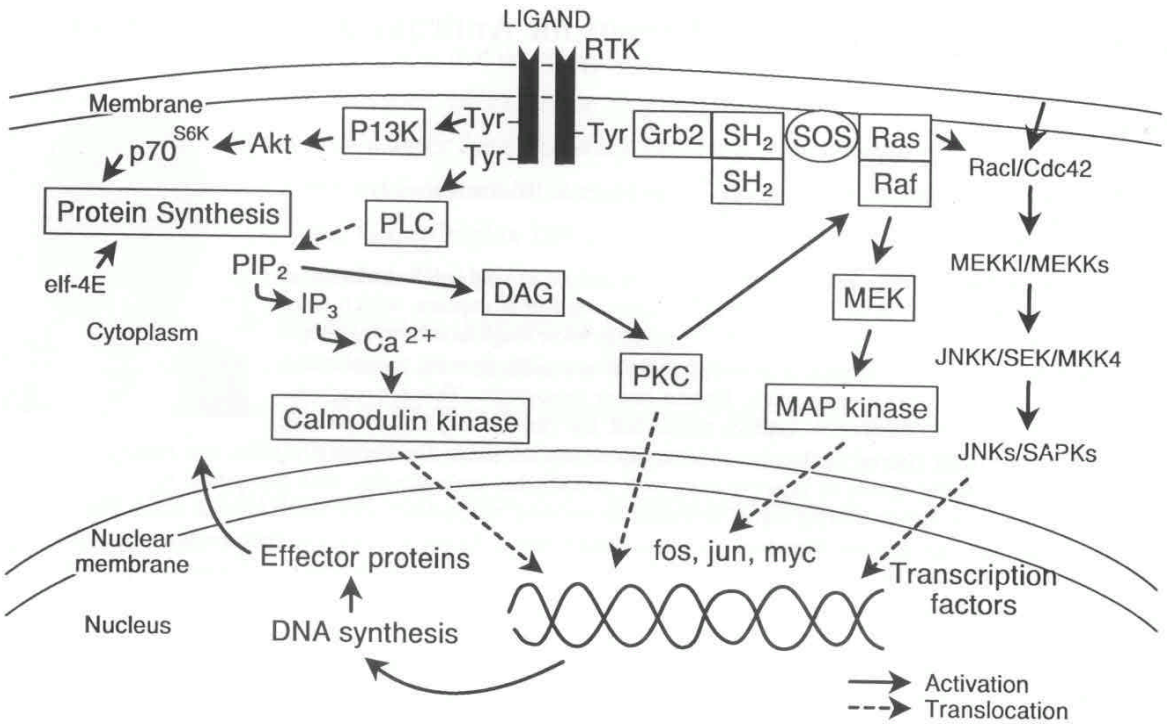

Fig. (1). Schematic representation of some important signal transduction pathways in cancer cell proliferation. DAG, diacylglecerol; IP3, inositide triphosphate; PI3K, phosphoinositide-3-kinase; PLC, phospholipase C; PIP2, phosphoinositide diphosphate; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; MAP kinase, mitogen-activated protein kinase.

The Epidermal Growth Factor Receptor (EGFR) Family

The epidermal growth factor (EGF) receptor was cloned in 1984 by Ullrich *et al.* [8]. This receptor has two cysteine-rich regions in the extracellular domain and a single kinase domain. Three other members of this family, HER-2, HER-3 and HER-4 (referred to also as *erb*B2, *erb*B3 and *erb*B4) are known. The designation HER-2 stands for **H**uman **E**pidermal growth factor-like **R**eceptor type **2**. Members of the EGF receptor family and their ligands are overexpressed or expressed as an autocrine loop in a number of tumor types, including pancreatic, lung, ovarian, renal cell,gastric, hepatocellular and breast cancers [10-12].

Anti-EGFR Antibodies

Because over-expression of EGFR has been associated with a more aggressive disease and a poor prognosis, the blockade of EGFR activation has been proposed as a target for anticancer therapy. The most promising agent is the human-mouse chimeric monoclonal antibody 225 (C225), which inhibits activation of the EGFR receptor tyrosine kinase. This inhibition of EGFR activation causes cell cycle arrest in G1. The mechanism of growth inhibition has been shown to involve an elevation in the levels of $p27^{KIP1}$ and inhibition of cyclin-dependent kinase-2 activity [13]. Preclinically, C225 in combination with several chemotherapy agents, including cisplatin, doxorubicin and paclitaxel exhibited synergistic antitumor activity, with successful eradication of well-established tumor xenografts that were resistant to treatment with either C225 or drug alone [14]. Phase I clinical trials have established the safety of repeated administration of single-agent C225 at concentrations that maintain receptor-saturating blood levels for up to 3 months [15]. Phase I trials exploring C225 treatment in combination with the chemotherapy agents mentioned above, are ongoing [16], and single-agent phase II trials are in progress. Preliminary data indicate that antitumor activity is significantly augmented when the antibody is utilized in combination with cytotoxic chemotherapy.

Anti-HER-2 Antibodies

The proto-oncogene HER-2/neu is localized to chromosome 17q, and encodes a 185kDA

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transmembrane glycoprotein receptor with intrinsic tyrosine kinase activity. No endogenous ligands for the HER-2/neu protein are known. When HER-2/neu protein is activated, it can interact with many different cellular proteins such as shc, PLC, GAP and the ras MAP kinase pathway [17]. The HER-2 receptor can also form heterodimers with other members of the EGFR family. Amplification of the HER-2/neu gene or over-expression of the HER-2/neu protein has been identified in 10-34% of breast cancers. Possible techniques for evaluating HER-2/neu status in breast cancer cells include gene-based assays such as polymerase chain reaction methods and in-situ hybridization utilizing both fluorescent (FISH) and non-fluorescent approaches. Qualitative protein measurements are the most common techniques used. Immunohistochemistry, typically on archival tissues is utilized. A significant discordance between HER-2/neu detection methods has been reported [18]. The discordance has been between immunohistochemistry methods and between immunohistochemistry and gene-based assays [19]. A number of clinical studies involving over 10,000 women have examined the relationship of HER-2/neu gene and/or protein abnormalities and breast cancer outcome. Results of these studies have not been uniform. Several studies including the original study published by Slamon *et al.* [20] found that HER-2/neu over-expression independently predicted poor overall survival and disease-free survival. Some immunohistochemical studies have found significant correlation between HER-2/neu protein immunoreactivity and disease outcome in univariate, but no independent predictive status in multivariate analysis [21]. A few studies have found no correlation whatsoever with disease outcome [22.23]. Compared to the prognostic information outlined above, there are fewer studies correlating the expression of HER-2/neu protein with response to therapy. Several studies have found HER-2/neu over-expressing tumors to be resistant to tamoxifen therapy [24, 25]. One large study in 200 patients, however, failed to show resistance to tamoxifen in HER-2/neu over-expressing tumors [26]. While HER-2/neu over-expression has been associated with enhanced response to chemotherapy regimens containing doxorubicin in clinical samples, a poor response to CMF (cyclophosphamide, methotrexate, 5-fluorouracil) therapy has been found in the same population of patients [27]. In cultured breast cancer cell lines, HER-2/neu expression is associated with resistance to paclitaxel [28]. However, another study indicated a 3-fold increased response to paclitaxel in the same population [29]. The preponderance of evidence would suggest a poorer prognosis and poor response to some therapeutic agents in patients with HER-2/neu expressing tumors. The conflict in the data may be explained in part, by the different methods used to document HER-2/neu expression.

Trastuzumab (rhuMAb HER-2, Herceptin®)

Trastuzumab is a humanized monoclonal antibody that targets the HER-2 receptor with demonstrated activity in metastatic breast cancer. This is the first monoclonal antibody to be approved for the treatment of a solid tumor. In cultured cells that express high levels of HER2, trastuzumab causes growth arrest in the G0/G1 phase of the cell cycle [30]. The growth inhibitory effects have been explained by a marked induction of the cyclin dependent kinase-2 kinase inhibitor, p27, as well as the retinoblastoma-related protein p130 [30]. These data suggest that treatment of HER-2 overexpressing cells is antiproliferative, and that cytostasis may result from an inhibition of cell cycle progression.

In early preclinical studies, Slamon *et al.* demonstrated interactions between trastuzumab and several anticancer agents [31], using *in vitro* clonogenic studies. Synergistic interactions at clinically relevant drug concentrations were observed for trastuzumab in combination with cisplatin, thiotepa and etoposide. Additive cytotoxic effects were observed with trastuzumab plus doxorubicin, paclitaxel, methotrexate and vinblastine. 5-fluorouracil, was found to be less than additive with trastuzumab. Studies were further conducted with drug/trastuzumab combinations in HER-2/neu-transfected, MCF-7 human breast cancer xenografts in athymic mice. Combinations of trastuzumab and cyclophosphamide, doxorubicin , paclitaxel, methotrexate, etoposide, and vinblastine *in vivo* resulted in a significant reduction in xenograft volume compared to chemotherapy alone. Combinations of trastuzumab and 5-fluorouracil yielded equivalent results to those achieved by 5-fluorouracil alone. The 5-FU results were consistent with the sub-additive effects observed with this combination *in vitro*. The synergistic interaction of trastuzumab with alkylating agents, platinum analogs and topoisomerase II inhibitors, as well as the additive interaction with taxanes, anthracyclines and some antimetabolites in HER-2/neu over-expressing breast cancer cells guided the choice of combination studies in clinical trials. Currently, data from six breast cancer trials have

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been published. Three studies have evaluated trastuzumab alone for the treatment of metastatic breast cancer. Two studies evaluated trastuzumab in refractory breast cancer [32]. Response rates were 11.6 and 15%, respectively [33]. The antibody was well tolerated. The third trial evaluatedtrastuzumab as first-line treatment of metastatic breast cancer. A preliminary response rate of 24% has been reported [34]. The pivotal multinational phase III study reported by Slamon *et al.* evaluated trastuzumab ± paclitaxel, or doxorubicin/cyclophosphamide in 469 first-line metastatic breast cancer patients. Overall, the median time to progression was improved from 4.6 to 7.6 months by the addition of trastuzumab, with an improvement in overall response from 32% to 48%. Toxicity was generally mild, consisting of febrile episodes and mildly increased myelossuppression. An increased incidence of symptomatic cardiac toxicity was noted when trastuzumab was added to anthracycline-based chemotherapy (19% vs. 3%). The etiology of this increased cardiotoxicity is unclear, but continues to be investigated [35]. Trastuzumab plus cisplatin was evaluated in patients with refractory breast cancer, yielding a response rate of 24% [36].

INTRACELLULAR SIGNALING PATHWAYS

The intracellular signaling pathways that are activated after growth factors and other cell-proliferation-associated ligands bind to their receptors are complex and incompletely understood. Major components of these effector systems such as the protein tyrosine kinases, protein kinase C and the ras/MAP kinase pathway have, however, been identified.

PROTEIN TYROSINE KINASES

Protein tyrosine kinases (PTKs) catalyze the phosphorylation of tyrosine residues on target proteins [37]. Two major groups of PTKs have been described to date, receptor and non-receptor tyrosine kinases. Non-receptor tyrosine kinases are cytoplasmic proteins which transduce extracellular signals to downstream intermediates in pathways that regulate cell growth, activation and differentiation. Many non-receptor tyrosine kinases are linked to transmembrane receptors including those for peptide hormones and cytokines. Unlike receptor tyrosine kinases, they lack transmembrane domains and ligand binding. They are activated by ligand binding to their associated receptors or events such as cell adhesion, calcium influx or cell cycle progression [38]. More than 30 members are classified in 10 families including src, abl, JAK, MKK2, FES [38].

Receptor tyrosine kinases (RTKs) share several structural features. They are glycoproteins possessing an extracellular ligand-binding domain, which conveys ligand specificity, and a single hydrophobic transmembrane domain, which anchors the receptor to the membrane. Intracellular sequences typically contain regulatory regions in addition to the catalytic domain. Ligand binding induces activation of the intracellular tyrosine kinase domain leading to the initiation of signaling events specific for the receptor. The RTKs have been organized into families based on sequence homology, structural characteristics and distinct motifs in the extracellular domain. There are currently 19 known families in vertebrates. The various subfamilies include receptors for epidermal growth factor (EGFR), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF). Ligand binding to a RTK induces receptor dimerization with conformational changes that result in intermolecular phosphorylation at tyrosine residues at multiple sites. Receptor heterodimerization can also occur, as reported with transforming growth factor alpha interaction with receptor heterodimers comprising HER-2 and EGFR [39]. In malignant tumors, a number of these receptors are over-expressed or mutated, leading to abnormal cell proliferation.

PLATELET DERIVED GROWTH FACTOR (PDGF)

PDGF is a major mitogen for endothelial cells, fibroblasts, smooth muscle cells and glial cells. PDGF exists as disulfide-linked homodimers and heterodimers of A and B chains, resulting in 3 isoforms (PDGF-AA, PDGF-BB, PDGF-AB). PDGF and its receptors are expressed in a wide variety of cultured neoplastic cells including breast, prostate and colon cancers. Expression has also been documented in tumor biopsies of ovarian cancer and gliomas [40].

The tyrphostins are synthetic protein tyrosine kinase inhibitors derived from erbstatin, a natural product with broad spectrum activity against protein tyrosine kinases and protein kinase C. SU101 is a tyrphostin derivative, which predominantly inhibits the PDGF receptor tyrosine kinases. In a completed phase I study, the most common toxicities were mild to moderate nausea, vomiting, and fever. Neutropenia was uncommon, and occurred only at

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the highest dose levels [41]. Phase III studies are ongoing in recurrent gliomas, and phase II studies have been completed in lung, prostate and ovarian cancers [42].

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF has 5 isoforms, which are splice variants and exist as disulfide-linked homodimers, with some structural similarities to PDGFs. These isoforms bind with high affinity to 2 receptors, *fms*-like tyrosine kinase (flt-1) and fetal liver kinase (flk-1). The biologic significance of these multiple VEGF receptor forms is not understood. VEGF stimulates the growth of endothelial cells during the process of angiogenesis, but has also been identified as a vascular permeability factor. A recombinant humanized monoclonal antibody, rhuMAb VEGF, has been developed to inhibit the effects of VEGF in the treatment of solid tumors. Phase II trials have been completed in lung and colon cancer. Results are awaited with interest A small molecule inhibitor of VEGF tyrosine kinase, SU5416 is undergoing phase I testing, and a phase I/II study in AIDS-related Kaposi's sarcoma is ongoing [43].

A second generation broad spectrum RTK inhibitor, SU6668 is currently undergoing phase I testing. This agent is a small organic molecule, that possesses anti-angiogenic and anti-proliferative properties. It inhibits the autophosphorylation of three distinct tyrosine kinases, Flk-1/KDR; PDGFR, and FGFR with IC_{50} values of 0.2 μM, 0.2 μM, and 4.1 μM, respectively. EGFR kinase activity remains uninhibited (IC_{50} > 100 μM). *In vitro* kinetic analyses demonstrate that SU6668 is a competitive inhibitor of ATP binding. This mechanism is similar to that of the bioflavonoids such as genistein and quercetin, and distinct from the tyrphostins and their derivatives, which compete for the substrate binding site of RTKs [44].

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

EGFR differs from the other receptor tyrosine kinases in that there is a single isoform, from a single 26 exon gene located across 110kb on chromosome 7p11-13. It serves as the predominant receptor for multiple distinct ligands, including EGF, TGF-α, amphiregulin and HB-EGF (Table 1). EGFR interacts with most members of the EGFR (c-*erb*B) family of RTKs. As previously mentioned, the ligand for c-erbB2 is unknown, while erbB3 and erbB4 serve as heregulin and neuregulin receptors. The major function of these other receptors appears to be as downstream effectors of each other. They cross-phosphorylate and modulate signaling from each other in specific pairs. EGFR interacts with HER-2 and HER-3 but not HER-4. HER-4 pairs with HER-2.

It is noteworthy that HER-3 lacks kinase activity, but serves as a docking protein to recruit a broader spectrum of downstream effectors after phosphorylation by EGFR or HER-2 [45]. Currently, 3 EGFR tyrosine kinase inhibitors are undergoing phase I clinical testing. One of these is ZD1839, which is an orally active, and selective inhibitor of the EGFR (HER-1) tyrosine kinase. In preclinical studies, administration of this agent daily for 4 months resulted in significant tumor growth delay in rodents bearing human xenografts. *Ex vivo* examination of xenograft tissues revealed a time and dose-dependent decrease in c-fos mRNA, a marker for EGFR signaling [46]. An ongoing phase I trial has enrolled 38 patients. A partial response has been noted in NSCLC, with minor responses in head and neck and renal cell cancers. The most common toxicity is a skin rash [47, 48]. The second agent in this class, CP-358774 is also a selective EGFR tyrosine kinase inhibitor. An acneform skin rash, diarrhea and mild hepatic transaminase elevations were the most common toxicities [48]. The third agent in this class, CI-1033 is a non-specific inhibitor of the EGFR family (HER-1, HER-2, HER-4) tyrosine kinases [49].

Table 1. Members of the Human Epidermal Growth Factor Gene Family

Gene	Ligand
HER-1 (c-erbB-1)	EGF, TGFα, Beta cellulin, Amphiregulin, Heparin binding growth factor
HER-2 (c-erbB-2)	? Heregulin
HER-3 (c-erbB-3)	Heregulin, neu differentiation factor 1+2
HER-4 (c-erbB-4)	Heregulin, neu differentiation factor 1+2

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