

Signal Transduction Therapy for Cancer – Whither Now?

Shoshana Klein and Alexander Levitzki*

Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

Abstract: Signal transduction therapy for cancer targets pathways that are over-active in cancer cells and upon which the cancer cells depend for their survival. Protein kinases are prime targets for signal transduction therapy. A major breakthrough was the introduction of the Bcr-Abl inhibitor imatinib/Gleevec into the clinic for the treatment of chronic myelogenous leukemia (CML). Nevertheless, even for this clonal disease, which has a well-characterized principle survival factor, signal transduction therapy faces two major problems: the emergence of drug-resistant clones and the persistence of a small population of cancer stem cells that re-establish the leukemia if treatment is stopped. Most cancers are far more heterogeneous than CML, so choosing the appropriate molecular targets is a major challenge. Signal transduction therapy can potentially reduce tumor mass and control cancer as a chronic disease. Complete cures will require ways of combating cancer stem cells and preventing metastasis, such as harnessing bystander effects and the immune system during treatment.

Key Words: Signal transduction therapy, kinase inhibitor, cancer.

INTRODUCTION

It is sixty years since the introduction of chemotherapy for the treatment of cancer. Traditional chemotherapeutic agents take advantage of the increased sensitivity of many cancer cells to DNA or microtubule damage, due to their increased rates of proliferation. Although many cancers respond initially, most eventually become refractory to chemotherapy, especially at the metastatic stage. Thus, in the last two decades, the approach has been to search for specific molecular targets for selective treatment of cancer cells. In this review, we shall cite a few pertinent examples to examine the rationale and prospects for targeted molecular therapy for cancer, with an emphasis on signal transduction therapy. For a more complete catalog of tyrosine kinase inhibitors and their chemical development, see Reference [1].

Cancer can be described as a disease of miscommunication. In healthy cells, a complex network of interacting, partially overlapping signaling pathways provides robustness. In cancer cells, impaired signaling leads to blocked differentiation, excessive proliferation and defective death induction. In the course of its evolution, a cancer cell accumulates numerous chromosomal aberrations and mutations. Many signaling pathways are ablated, but a few become hyper-activated, due to mutations in oncogenes and tumor suppressor genes. Thus the cancer cell loses the robustness conferred by redundant signaling pathways and becomes dependent upon a few, highly activated pathways [2]. The rationale for signal transduction therapy is as follows: It should be possible to cause the demise of cancer cells by targeting those pathways upon which the cancer cells depend for their survival; healthy cells, on the other hand, should be less sensitive to inhibition of one or a few

signaling pathways, because alternative pathways provide protection [3] (Fig. (1)).

The term cancer actually refers to a hodgepodge of molecularly heterogeneous diseases. The list of putative oncogenes and tumor suppressor genes grows constantly, and currently numbers in the hundreds [4]. These genes encode a number of biochemically distinct families, all of which function in signal transduction. Oncogenes may code for transcription factors, nuclear hormone receptors, protein kinases, small GTP-binding proteins, growth factors or growth factor receptors. A major thrust of signal transduction therapy has been to target protein kinases (PKs). The challenge is to identify oncoproteins that are essential survival factors for a given cancer. The inherent genetic instability of cancer cells means that even a single tumor is usually composed of a heterogeneous population of cells. Furthermore, genomic instability is likely to generate a continuous shift in the list of potential targets for therapy. It is therefore becoming clear that combination therapy will be necessary for most tumors.

Twenty years after the advent of the first selective tyrosine kinase inhibitors [5, 6], kinase inhibitors are now being used successfully to treat certain cancers in the clinic. Nonetheless, it is becoming clear that their effectiveness in treating advanced cancers and preventing metastatic spread is limited. In this review we assess the promise - and the problems - of signal transduction therapy for cancer.

PROTEIN KINASES AND CANCER

The human genome encodes on the order of 500 protein kinases (PKs), which catalyze transfer of phosphate from ATP to serine, threonine or tyrosine residues. There are about 400 serine/threonine kinases and 100 tyrosine kinases (PTKs) [7]. PKs play key roles in metabolism, cellular growth and proliferation, cell death, differentiation, embryonic development, angiogenesis and the immune system. Mutation and dysregulation of PKs is associated with a variety of proliferative conditions, including psoriasis, restenosis, and of course cancer.

*Address correspondence to this author at the Unit of Cellular Signaling, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904 Israel; Tel: 972 2 6585404; Fax: 972 2 6512958; E-mail: Levitzki@vms.huji.ac.il

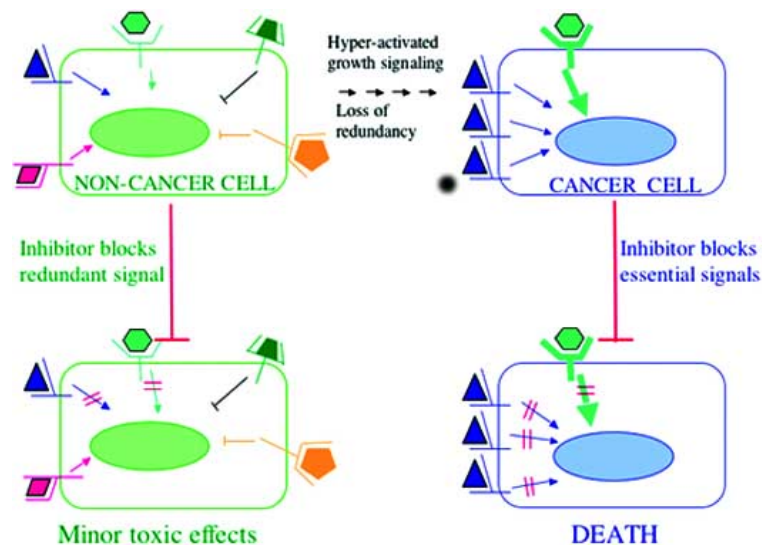


Fig. (1). Loss of redundancy – the Achilles' heel of the cancer cell.

Cells undergo a profound change in their signaling network during transformation. Cancer cells are dependent on a smaller ensemble of signaling pathways, and the intensity of these pathways is enhanced. This situation is the outcome of genomic instability, a hallmark of the cancer cell. Although the more intense pathways give the cancer cell its selective advantage, they also constitute the cancer's potential Achilles' heel. Blockade of these pathways, on which the cancer cells depend, causes the demise of the cancer cells, but spares normal cells, which possess redundant pathways.

Protein tyrosine kinases (PTKs) appear in evolution in multicellular organisms, where they function in communication between and within cells. Receptor tyrosine kinases (RPTKs) are transmembrane glycoproteins that transmit signals from outside the cell. Upon interaction of the extracellular domain with growth factor, the intracellular catalytic domain is activated to transmit the signal downstream. There are about sixty RPTKs, many of which have been implicated in cancer, including the epidermal growth factor receptor (EGFR) family, platelet-derived growth factor receptor (PDGFR) family, and vascular endothelial growth factor receptor (VEGFR) family. Non-receptor (cellular) PTKs have no extracellular domain, and are activated by upstream signaling molecules, such as G-protein coupled receptors, RPTKs and immune system receptors. Cellular PTKs include the Src, Abl and Jak families.

The discovery in the early 1980s that the protooncogene Src was in fact a tyrosine kinase fueled research into the relationship between PKs and cellular transformation [8]. It turned out that most PTKs are oncogenes. A number of serine/threonine PKs are oncogenes or tumor suppressors; examples include the cyclin-dependent kinases (CDKs), RAF, ERK and Akt/PKB. PKs function as master switches in the cellular communication networks. The balance between cell cycle progression, cell cycle arrest and cell death is controlled by kinase signaling pathways and can be perturbed by mutations at various stages. Oncogenic mutations that cause excessive growth factor production, growth factor independent receptor activation, constitutive activation of downstream transducers of the growth signal, and activation of the cell-cycle effectors all result in uncontrolled cellular proliferation. Mutations that block pro-apoptotic, tumor suppressive pathways are also associated with cancer.

Cancer cells are genetically unstable. They are highly aneuploid, with numerous chromosomal abnormalities, such as duplications, deletions and translocations. A number of leukemias and lymphomas are associated with specific chromosomal translocations that result in oncogene activation. It is currently a matter of controversy whether the aneuploidy is a cause or a consequence of the cancer phenotype [9], but it is clear that aneuploidy is a self-perpetuating mechanism, resulting in yet more chromosomal instability. As the cancer cell evolves, it appears to become dependent on its abnormal signaling network. The cancer cell is dependent on fewer but enhanced signaling pathways, and lacks many of the regulatory mechanisms that act as checks and balances on the proliferation of normal cells. Paradoxically, the cancer cell, because it lacks the robustness conferred by redundant pathways, is more susceptible than a healthy cell to inhibition of the specific pathways upon which it depends. In the mid-1980s, with the understanding that many oncogenes are in fact PTKs, our laboratory began a search for low molecular weight PTK inhibitors.

TYROSINE PHOSPHORYLATION INHIBITORS (TYRPHOSTINS)

The catalytic kinase domain is highly conserved. The ATP pocket is a deep cleft between the smaller N sub-domain, composed largely of β sheets and a highly conserved α helix, and the α -helical C sub-domain. A glycine-rich P-loop in the N sub-domain stabilizes the interaction between ATP and its pocket. The activation loop, in the C sub-domain, controls access to substrate. The activation loop is itself controlled commonly by phosphorylation, although, as in the case of the cyclin dependent kinases (CDKs), activation may be controlled by binding of small proteins.

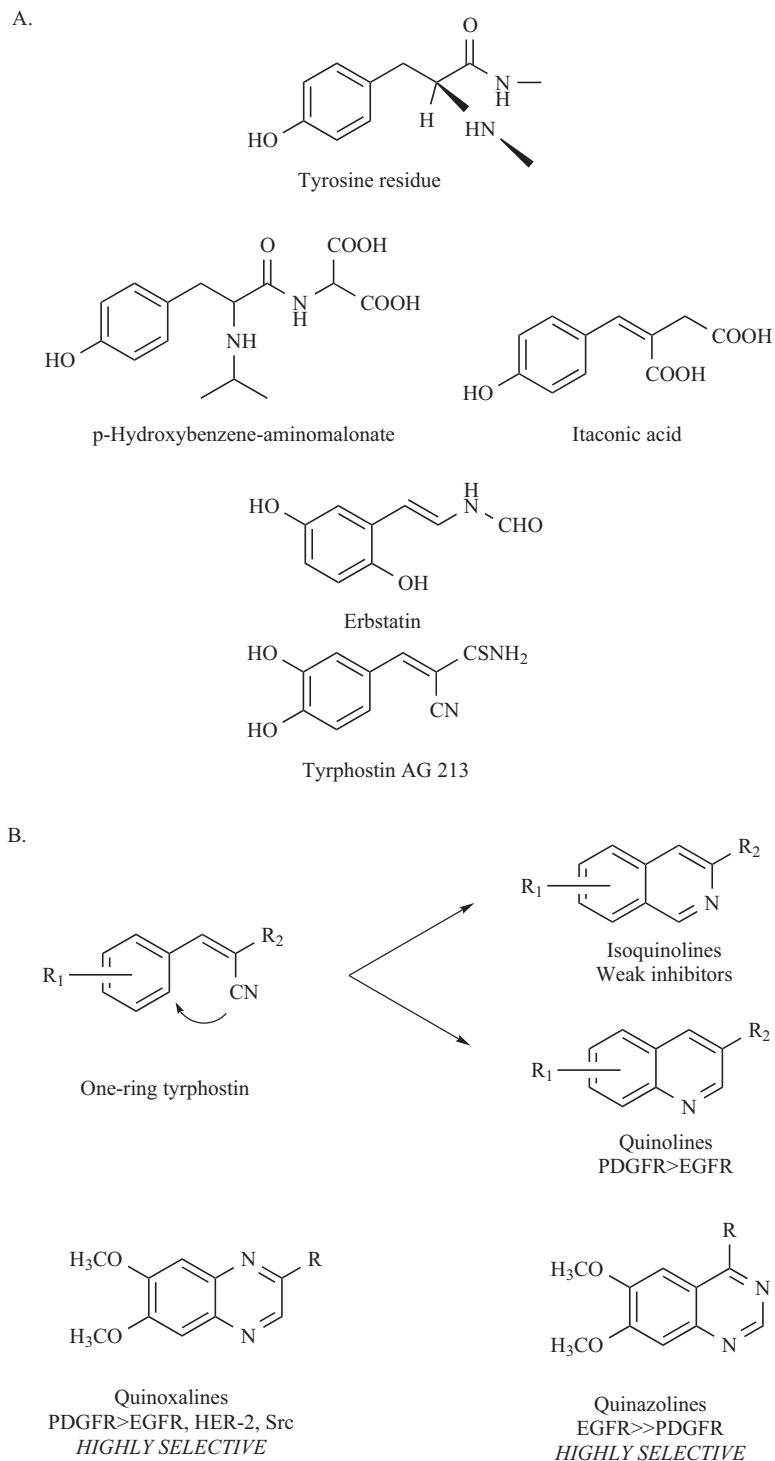


Fig. (2). The evolution of protein tyrosine kinase inhibitors (tyrphostins)

A. Tyrosine mimics as inhibitors of protein tyrosine kinases

The first small molecules that exhibited selectivity towards protein tyrosine kinases vis-à-vis serine/threonine kinases: itaconic acid, tBoc-p-hydroxy-benzene malonic acid and erbstatin.

B. The emergence of tyrphostins

Initially compounds that closely mimic tyrosine were synthesized, mostly from the benzene malono nitrile family. These compounds led to quinazolines and quinoxalines. In contrast to the early tyrphostins, which are substrate competitive, the bicyclic compounds are predominantly ATP competitive.

Our search for specific PTK inhibitors was initially met with skepticism. It was believed that the strong degree of conservation between kinase domains [10] would make it impossible to find selective inhibitors. Indeed, several naturally occurring phosphorylation inhibitors were isolated in the early 1980s, and these displayed poor selectivity [11-14]. Nonetheless, our group pursued the search for PTK inhibitors, by synthesizing a series of hydroxyphenyl containing molecules as tyrosine mimics. We built a series of molecules, based on the structure of itaconic acid, which we found inhibits the insulin receptor with no effect on Ser/Thr kinases [15], and erbstatin, a natural compound that inhibits the EGFR and pp60^{Src} [14], (Fig. (2)). In 1988, we succeeded in obtaining the first potent, selective tyrosine phosphorylation inhibitors, which we dubbed tyrphostins [5, 6] (Fig. (2)). The compounds we obtained inhibited EGF-dependent autophosphorylation of the EGFR, EGF-stimulated cancer cell proliferation *in vitro*, and also tumor growth in nude mice [16]. These studies provided support for our

contention that small molecular weight kinase inhibitors would be effective in cancer therapy.

PROOF OF PRINCIPLE: CML, BCR-ABL AND GLEEVEC

The demonstration that inhibition of kinase activity can repress cancer cell growth selectively, *in vitro* and *in vivo*, provided the impetus for Novartis to develop an inhibitor of the Bcr-Abl kinase. This PTK, which results from a translocation between chromosomes 9 and 22 ("Philadelphia chromosome"), causes chronic myelogenous leukemia (CML). Because virtually all cases of early CML are associated with the presence of the *BCR-ABL* oncogene, this leukemia provided a good test case for signal transduction therapy.

The first selective Bcr-Abl kinase inhibitors were described in 1992 and 1993 [17, 18] (Fig. (3)). A derivative of AG 957, Adaphostin, is being developed for use in the clinic. In 1996, Druker *et al.* reported on their successful

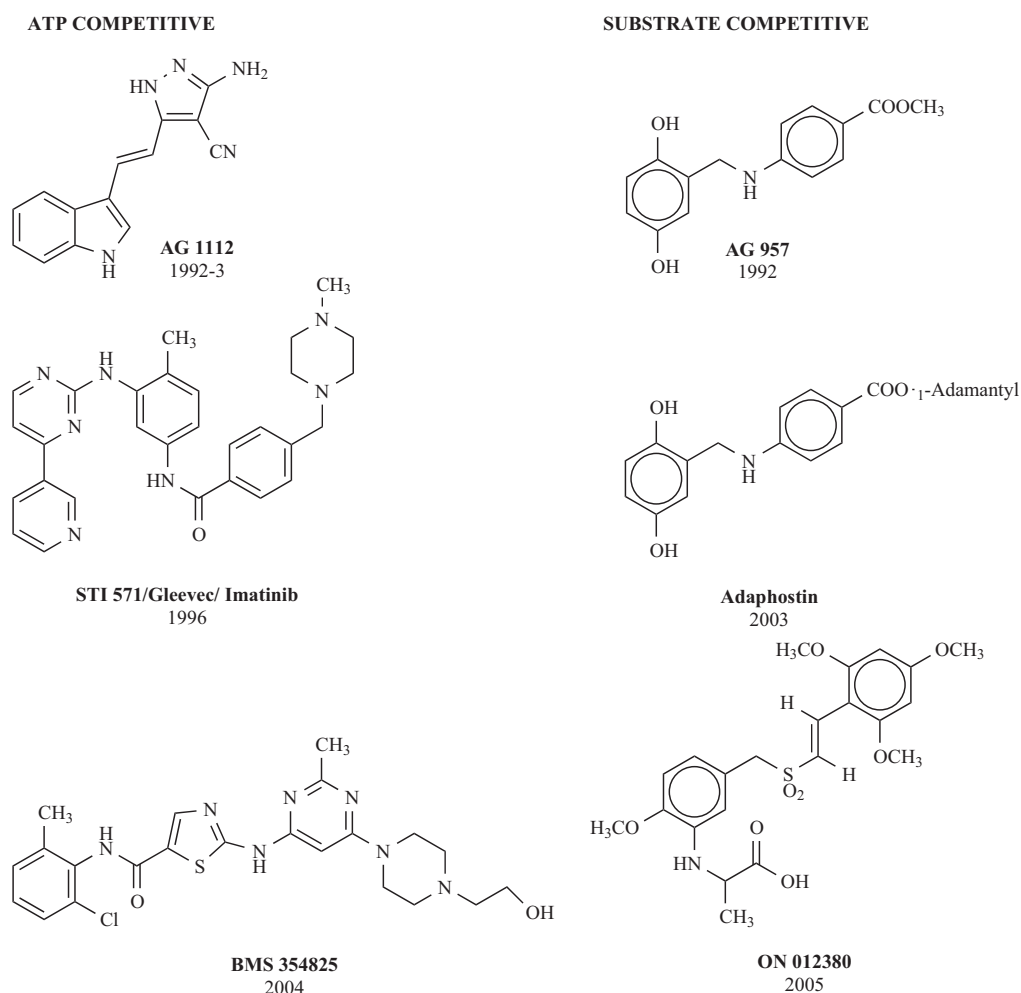


Fig. (3). Bcr-Abl kinase inhibitors

Initially two types of Bcr-Abl inhibitors were found: a substrate competitive inhibitor AG 957 and an ATP competitive inhibitor AG 1112. Gleevec, the first clinically successful inhibitor, is an ATP-mimic, binding to the inactive conformation of Bcr-Abl. BMS 534825, also an ATP competitive inhibitor, targets the active conformation of Bcr-Abl and also inhibits Src, which emerges as an important signaling element in Gleevec resistant cases. ON012380 and Adaphostin, currently under development, are substrate competitive and potently inhibit Bcr-Abl mutants that are resistant to Gleevec.

Bcr-Abl inhibitor, imatinib mesylate (Gleevec/ Glivec/ STI571, Fig. (3)) [19].

Bcr-Abl appears to be the major survival factor for CML cells. Bcr-Abl kinase activates the Ras-MAPK and JAK-STAT pathways, leading to growth factor independent proliferation. It also represses apoptosis by activating the PI3K-Akt pathway and increasing Bcl2 expression [20]. Inhibiting Bcr-Abl restores apoptosis and considerably reduces tumor load. The introduction of imatinib into the clinic several years ago led to spectacular rates of hematologic remission (over 95%), and frequent cytologic remission (75%), for early stage CML [21]. The euphoria was short-lived, as it soon became clear that imatinib does not actually cure CML. *BCR-ABL* continues to be detectable by sensitive PCR-based techniques, and eventually the disease fails to respond to imatinib. Nevertheless, imatinib has become a key tool in the control of CML, extending survival of patients for years, provided that treatment is not terminated. Imatinib has thus provided the proof of principle for the clinical application of signal transduction therapy.

TARGETS FOR SIGNAL TRANSDUCTION THERAPY

The best candidates for drug development are clearly those that are broadly applicable. Although each cancer has its own spectrum of mutations, certain mutations occur in a large number of cancers, and these are preferential targets for signal transduction therapy. Signal-transduction inhibitors in current development affect various molecular pathways [22].

Many PTK inhibitors arrest the cell cycle. Other ways of targeting the cell cycle include the use of antibodies to deplete the tumor of growth factor or to inhibit the interaction between a growth factor and its receptor (Fig. (4)). The EGFR, which is involved in many common solid tumors, including lung, breast, prostate, colon, ovary, and

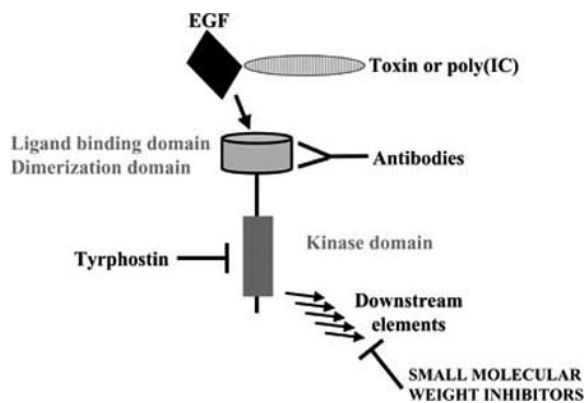


Fig. (4). Strategies to target EGFR

Antibodies targeting the extracellular domain may inhibit signaling by competing with the ligand or blocking receptor dimerization. The antibody can also serve as a Trojan horse if tethered with a toxin. Another option to target the ligand binding domain is to use EGF tethered to a bacterial toxin or to a non-viral vector carrying synthetic dsRNA such as poly(IC). In both cases ligand induced receptor mediated internalization introduces into the cell a molecule that induces cell killing. Other options include blocking the catalytic activity of the receptor or downstream signaling elements that are activated by the EGFR.

head and neck cancers, is a popular target, and we shall discuss what we have learnt from EGFR inhibitors below. Some "classical" chemotherapeutic agents cause cell growth arrest or apoptosis by inhibiting DNA synthesis.

Inducing cancer cell death by the inhibition of anti-apoptotic pathways is another attractive approach [23]. We and others are developing inhibitors of the serine/threonine kinase, PKB/Akt. PKB is overexpressed in many cancers, and in others it is persistently activated due to knockout of PTEN [24, 25]. Another popular target is p53, which is mutated in over 50% of tumors. Several groups are attempting to reactivate p53 in tumors in which it is mutant, or to block wild type p53 degradation by inhibition of MDM2 [26].

Another approach to treating solid tumors is to block angiogenesis, for instance by targeting VEGF or VEGFR, thus starving the developing tumor of its blood supply. An antibody to VEGF, bevacizumab (Avastin), has been approved for adjuvant treatment of colon cancer together with chemotherapy [27], and a VEGFR2 inhibitor, BAY 43-9006 (also a B-Raf inhibitor), is in clinical trials [28, 29]. A difficulty with this approach is that advanced solid tumors largely depend on glycolysis, as they are very hypoxic, a phenomenon known as the "Warburg effect" [30]. Agents that repress glycolysis, such as inhibitors of HIF1 α , are currently being tested [31, 32].

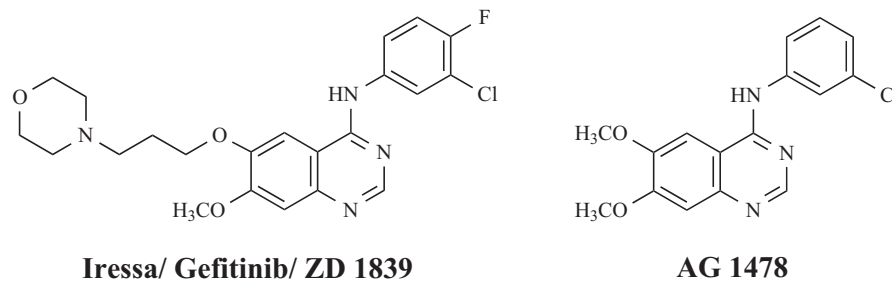
COMBINATION THERAPY

Combined Targeted Therapy

Most tumors are molecularly heterogeneous and do not have a single, principal survival factor, but rather depend on several pathways. Thus, a combination of signaling inhibitors may be required to cause significant remission of most cancers. Indeed, several of the inhibitors in current use target more than one kinase (see below). Furthermore, targeting the same molecule by more than one therapeutic moiety may be synergistic. This was first shown for inhibition of the EGFR using tyrophostins together with the monoclonal antibody mAb 108 [16]. Synergy or additivity has also been shown for the clinically approved agents Erbitux (mAb 225/ Cetuximab) and gefitinib (Iressa/ ZD1839) [33, 34] (Fig. (5)). Cooperative behavior between antibodies and small molecular inhibitors may involve inhibition of EGFR-related molecules, such as heterologous EGFR-ErbB2 dimers. Cooperativity appears to depend upon the cell type in which it is examined. This clearly complicates extrapolation to the clinical setting, and each combination will need to be tested for each cancer that is deemed likely to respond.

Targeted Therapy Plus Chemotherapy

Although early tumors often respond well to chemotherapy, advanced tumors tend to be much more refractory to treatment. At later stages of transformation, tumors are desensitized to apoptotic signals. Substantial evidence is accumulating for synergy between kinase inhibition and classical chemotherapy. On a molecular level, the inhibition of major survival factors appears to release the brakes on apoptosis, facilitating the apoptotic response to chemotherapeutic agents. The combination of an EGFR

**Fig. (5). AG 1478 and Iressa**

AG 1478 is extremely hydrophobic and therefore is used as the mesylate salt in captisol. Iressa on the other hand is more hydrophilic due to the morpholino side chain and its formulation is simpler.

kinase inhibitor with pro-apoptotic agents like cisplatin demonstrated synergistic inhibition of the intracranial growth of EGFR over-expressing glioblastoma in nude mice [35, 36]. Nonetheless, the combination of the EGFR inhibitor gefitinib (Iressa/ ZD1839) with cisplatin failed in clinical trials to treat non-small cell lung carcinoma (NSCLC), but this disappointment may have been due to poor selection of potentially responsive patients (see below) [37].

ErbB2 is over-expressed in 20-25% of breast cancers, mainly due to gene amplification. These cancers are resistant to hormonal treatment and have poor prognosis. Inhibition of the EGFR-related receptor ErbB2 (Her2/neu) is synergistic with certain forms of chemotherapy. NSCLC cell lines that over-express ErbB2 can be sensitized to chemotherapy by treatment with a small molecular weight ErbB2 inhibitor [38]. Preclinical studies showed that the humanized anti-ErbB2 monoclonal antibody, trastuzumab (Herceptin) inhibited growth of ErbB2 over-expressing cells and tumor xenografts. In addition to inhibition of the ErbB2 pathway, the effect of trastuzumab is partly due to antibody-dependent cell-mediated cytotoxicity, and may also involve inhibition of the downstream VEGF response and angiogenesis [39]. Trastuzumab appears to reduce levels of DNA repair following treatment with cisplatin or radiation [40, 41]. This provides a mechanistic understanding of the synergistic cooperation.

Even CML patients who initially respond well to imatinib may eventually cease to respond, due to the emergence of drug-resistance (Table 1). Imatinib resistance

is sometimes due to amplification of the *BCR-ABL* gene, but is more often caused by selection for mutations in the *ABL* tyrosine kinase domain that affect binding to ATP and imatinib [42, 43]. A common mutation replaces threonine with isoleucine at position 315 (T315I). The activation of pathways downstream or parallel to Bcr-Abl signaling can also lead to resistance to imatinib [44, 45]. To overcome imatinib resistance, several new inhibitors of Bcr-Abl have been synthesized (Fig. (3), see also below). There is evidence for the pre-existence of resistance mutations in a small proportion of *BCR-ABL*-positive cells, even before the administration of imatinib [46, 47]. Although "second generation" Bcr-Abl inhibitors are being developed to inhibit the resistant derivatives, combination therapy would seem to be the method of choice to prevent selection for resistant clones. The combination of imatinib treatment with cytarabine is currently being investigated [48].

Harnessing the Immune System

Immunotherapy has little impact on established tumors, but can induce long-term anti-tumor immunity once tumor volume has been reduced. Therefore, it may be possible to control certain cancers by reducing tumor load using targeted therapy, and then stimulating the immune system to generate an immune response against the remaining tumor cells. Unlike cytotoxic agents, tyrosinostins do not harm the immune system, and therefore can be used in combination with immunotherapy. Treatment with AG-490, a tyrosinostin against Jak2, was more effective when followed by interleukin-2 (IL-2) treatment [49]. Similarly, the combination of imatinib

Table 1. Mechanisms of Resistance to PTK Inhibitors

Mechanism	Examples	References
Gene amplification	<i>BCR-ABL</i> amplification leads to resistance to imatinib	[42]
Point mutation	Mutations in the Abl, Kit and PDGFR α kinase domains lead to resistance to imatinib. The Abl kinase mutation, T315I, leads to resistance to all known ATP-competitive Abl inhibitors. Analogous mutations in the EGFR lead to resistance to erlotinib and gefitinib.	[42,43] [85]
Activation of downstream or alternative signals	Activation of Src family members can replace or augment Bcr-Abl signaling, leading to imatinib insensitivity.	[44,45]
Multi-drug resistance	Although not demonstrated for PTK inhibitors, in principle drug efflux and/or inactivation should be considered.	

with interferon- α is being investigated for the treatment of CML [50]. The utilization of the immune system is particularly significant since cytokines like interferon- α used against CML lead to the demise of the cancer stem cells (see below).

PROPERTIES OF THE "IDEAL" PK INHIBITOR

ATP-Competitive or Substrate-Competitive?

Most small molecular weight kinase inhibitors are ATP mimics. The ATP cleft is strongly conserved between kinases, so ATP-competitive inhibitors tend to lack specificity and to affect groups of related kinases. Furthermore, ATP-competitive inhibitors have much higher IC_{50} values in cells or *in vivo* than *in vitro*, because they need to compete with the high intracellular concentrations of ATP. The high doses required for ATP-competitive inhibitors lead to increased toxicity.

It is therefore important to develop inhibitors that target less highly conserved domains. Some of the original typhostins were substrate-competitive [6, 15] (Fig. (2)). Recently, several interesting substrate-competitive PK inhibitors have been described. The Bcr-Abl inhibitor, ON012380 (Fig. (3)), is effective against all known imatinib-resistant mutants of Bcr-Abl and synergizes well with imatinib [51]. In addition to being more potent, combined inhibition with an ATP mimic and a substrate mimic should reduce the frequency of drug resistance. Substrate-competitive inhibitors of the IGF1-R have also been produced [52, 53]. A substrate-competitive inhibitor of PKB, PTR 6164, is more selective for PKB than ATP-mimic PKB inhibitors [Litman *et al* submitted]. PTR 6164 is highly effective in the inhibition of prostate cancer cell lines in which PKB is highly active and shows no activity against tumor cell lines or normal cells in which PKB is minimally active. This excellent toxicity profile and the stability of PTR 6164 in serum make it a true candidate for further development. PTR 6164 also synergizes with cytotoxic agents to enhance apoptosis of prostate cancer cells possessing high PKB activity [Litman *et al*. submitted]. Mixed-competitive inhibitors of PKB, which appear to target the PH or hinge domain, have recently been reported to be so selective as to distinguish between different PKB isozymes [54, 55].

Selectivity

Despite the intuitive reasoning that a more selective inhibitor should have lower toxicity and cause fewer side effects, it is not clear that strict selectivity of a targeted inhibitor is always desirable. A less selective inhibitor might target multiple pathways, thus achieving "combination therapy" with a single form of treatment. We have seen, for example, that trastuzumab (Herceptin) inhibits both ErbB2A activation and angiogenesis [39]. A less selective inhibitor might also be effective in treatment of a broader range of tumors. In fact, imatinib was originally developed as a PDGFR inhibitor [56], and only later found to inhibit the Abl and Kit kinases [19, 57, 58]. Imatinib is also effective in the treatment of gastrointestinal stromal tumors (GIST), which usually involve Kit activation [59, 60] and occasionally PDGFR α [61]. Chronic myelomonocytic leukemia (CMML)

is sometimes associated with a translocation that leads to activation of PDGFR β (*EVT6-PDGFRB*; t5;12) [62], and imatinib leads to remission in these cases [63]. It was hoped that imatinib might be effective in treating solid tumors, as Kit and PDGFR activation are fairly common, but clinical trials have not yielded success [64].

In a search for second generation Bcr-Abl inhibitors that would be effective against imatinib-resistant mutants, a number of even less specific inhibitors have been investigated. Imatinib resistance may be accompanied by increased activity of Src family kinases [44, 45] (Table 1). Molecules that inhibit both Src and Abl kinases blocked growth of cells expressing imatinib-resistant Bcr-Abl, suggesting that such inhibitors might be useful in preventing emergence of imatinib-resistant CML (Fig. (4)) [65].

Targeting the Inactive Versus the Active form of an Enzyme

The balance between selectivity and toxicity might depend on whether an inhibitor interacts with the active or inactive form of its target. The small molecule, BMS-345825 (Fig. (3)), targets the active forms of the Src and Abl kinases and is even less selective than imatinib. This agent is much more potent than imatinib, and inhibits all of the clinically-derived imatinib-resistant Bcr-Abl mutants except the T3151 mutant [66, 67]. An inhibitor that targets the active form of a kinase may be less toxic than one that binds to the inactive form. This is because the active form is the predominant form of the kinase in cancer cells, but in normal, healthy cells the inactive form of the kinase predominates, the active form being transient. Thus, in cancer cells the active kinase is largely available to the inhibitor, but it is only fleetingly available in healthy cells. Therefore an inhibitor that targets the active kinase can effectively attack cancer cells at concentrations that are sub-toxic to healthy cells [68].

Reversible or Irreversible Inhibition?

As explained above, ATP-competitive inhibitors need to prevail over the high endogenous concentrations of ATP. In the past decade, dozens of EGFR inhibitors have been produced [1]. Gefitinib (Iressa/ ZD 1839) and a similar compound, Erlotinib (Tarceva/ OSI-774) have been approved for clinical use (see below). Animal studies using positron emission tomography (PET) have shown that reversible, ATP-competitive EGFR inhibitors are rapidly cleared from EGFR-over-expressing tumors [69], and similar results have been seen with Gefitinib [70]. Thus, effective labeling or treatment using reversible inhibitors requires sustained delivery.

To overcome this problem, intensive efforts are being made to produce irreversible EGFR inhibitors [1]. The current EGFR inhibitors, such as AG 1478 and Iressa/ZD 1839 (Fig. (5)), are based on anilinoquinazolines (Fig. (2)). The strategy for development of irreversible inhibitors has been to alter this structure so as to encourage covalent bonding *via* electrophilic attack of the inhibitor on a conserved cysteine residue within the ATP-binding pocket of the EGFR kinase (Cys773) [71] [72]. Three irreversible inhibitors are currently in clinical development [73-75].

PATIENT SELECTION

There is no single "magic bullet" to treat cancer. Signal transduction therapy targets the particular survival factors of a given cancer. Given the large number of potential therapeutic targets, it is vital to identify the cancers that will be susceptible to a particular treatment. Imatinib is so successful in treating early CML because this malignancy is almost always associated with *BCR-ABL*, and activated Bcr-Abl functions as an essential survival factor for CML cells. Other oncogene products, even though they may be over-expressed in a cancer cell, do not necessarily function as essential survival factors.

The EGFR, for instance, is over-expressed or persistently activated due to ligand over-expression in a large number of cancers, so it was hoped the EGFR would prove to be a broadly relevant target. Yet only 10% of NSCLC patients respond to gefitinib or erlotinib. The rate of response is higher among patients with adenocarcinomas, women, non-smokers and patients of East Asian origin. Responsiveness to gefitinib is associated with specific mutations in the EGFR kinase domain [76, 77]. These mutations activate the Akt and STAT survival pathways, and the mutant EGFR is an essential survival factor in cells from these tumors [78].

In phase 3 trials of gefitinib and erlotinib for the treatment of NSCLC, the presence of a mutation somewhat increased the likelihood of responsiveness, but did not affect overall survival [79, 80]. Intriguingly, *EGFR* and *K-ras* mutations appear to be mutually exclusive [81]. *K-ras* mutations are common in lung tumors from smokers [82]. *K-ras* mutations are associated with non-responsiveness to gefitinib or erlotinib [83], which is not surprising when one considers that Ras acts downstream from the EGFR. Gefitinib did improve survival among patients with amplification of the *EGFR* gene and strong over-expression of EGFR protein [80]. Other factors that may be associated with response to gefitinib among EGFR over-expressors are PKB activation, as indicated by phospho-PKB detection, and amplification and over-expression of the EGFR-related receptor, ErbB2 (Her2) [80, 84]. Even patients who do respond to gefitinib or erlotinib have a short reprieve. Resistance emerges rapidly, due to secondary mutations in the *EGFR* gene [85].

A similar story is emerging for gastrointestinal stromal tumors (GIST). The tumors of patients who respond to imatinib treatment have activating mutations in Kit, mostly in exon 9 or 11; for some reason, mutations in exon 11 lead to a better response to imatinib [86, 87]. Again, secondary mutations eventually lead to resistance. Analogous mutations in EGFR (M790T), Bcr-Abl (T135-I), PDGFR α (T674I) and Kit (T670I) all cause drug resistance, to gefitinib or erlotinib in the case of EGFR and to imatinib in the cases of the latter three enzymes [42, 85]. Given the evidence that these mutations are already present in a small pool of tumor cells before treatment begins [46, 47], with sensitive tests it may be possible to pinpoint those patients who are most likely to quickly develop resistance, and to use appropriate measures to forestall this.

Initial clinical trials with trastuzumab (Herceptin) to treat metastatic breast cancer, as a single agent or together with chemotherapy, indicated a slight response in a fifth of patients. Retrospective analysis of these trials revealed that patients with *ERBB2* amplification and strong over-expression of the protein were most likely to respond, and trastuzumab was approved by the FDA for treatment of such patients in 1998 [88]. Data from a later trial confirmed that the best responses were obtained in women with strong, as opposed to moderate, over-expression [89, 90]. Last spring, the results were announced of two large, randomized clinical trials testing the combination of trastuzumab and chemotherapy on women with early-stage, localized breast cancer. The criteria for inclusion in these trials included strong over-expression of ErbB2, as detected by immunohistochemistry and/or FISH. The combination of trastuzumab plus chemotherapy cut disease recurrence in these women by 50%, equating their prognoses with those of women with tumors that do not over-express ErbB2 [91, 92].

The challenge is to identify the appropriate patients for a given therapy. It is becoming clear that kinase over-expression per se is not sufficient to predict whether a given tumor will respond to a given drug: specific mutations, very strong over-expression, and/or co-expression of other markers might be better diagnostic criteria. Effective use of signal transduction inhibitors in the clinic will require a better understanding of the factors that dictate responsiveness and of methods to prevent acquired resistance.

CANCER STEM CELLS

The concept of "cancer stem cells" is changing our expectations from signal transduction therapy. Cancer stem cells were first conclusively shown for acute myeloid leukemia (AML) [93-95], and more recently for solid tumors [96-98]. These cells carry cancer-specific markers and are capable of self renewal and regeneration of the tumor. Unfortunately, they are also refractory to most, if not all, existing cytotoxic modalities. Thus, current cancer treatments that target the differentiated tumor cells can reduce tumor bulk, but do not eliminate the stem cells that appear to be responsible for self-renewal and persistence of the tumor.

A tiny pool of *BCR-ABL*-positive cells can often be detected by PCR, even after extended treatment with imatinib. This pool, which may represent cancer stem cells, expands rapidly upon cessation of treatment. Imatinib does not eliminate CML cancer stem cells [99, 100]. Patients who stop taking imatinib suffer a relapse [101, 102]. Patients with CML are now being kept on imatinib indefinitely, until the emergence of resistance.

The hunt is now on for methods to target the cancer stem cells. A number of groups are using expression profiles of cancer stem cells, in order to identify prospective therapeutic targets (for example, see [103, 104]). NF κ B is active in AML cells, including the stem cells. *In vitro*, inhibiting NF κ B activity by means of a proteasome inhibitor, in combination with anthracycline, led to apoptosis of AML stem cells, but not of healthy hematopoietic stem cells [105].

Imatinib has replaced interferon- α as the treatment of choice for CML patients, but interferon appears to provide a longer term response. *In vitro* studies of CML cells showed that interferon was significantly more toxic to CML stem cells than imatinib, whereas imatinib was more toxic to differentiated CML cells [106]. Clinical trials of combined treatment with imatinib and interferon are currently in progress. Hopefully, this combination will give a rapid clinical improvement, due to imatinib, with a long-lasting effect, due to interferon. In this context, it is a matter of concern that clinical trials are designed to look for rapid, measureable clinical improvement – as gauged by reduction in tumor bulk—rather than long-term prognosis. It has been argued that such short-sightedness can cause premature abandonment of therapies that could actually deliver a long-term cure, so it is imperative to find new criteria for trial design [107].

UTILIZING SIGNAL TRANSDUCTION TARGETS FOR DELIVERY

The EGFR is over-expressed in about half of glioblastomas, in many cases in the truncated form (Δ 2-7EGFR) [108]. These tumors are extremely virulent, and no improvement in prognosis has been achieved. The EGFR inhibitor AG1478 in combination with temozolomide is now being evaluated in clinical trials.

Another option is to utilize the EGFR as a homing molecule, to selectively deliver an otherwise non-specific therapy (Fig. (4)). Our group is developing this idea along two avenues. One approach is to selectively activate the dsRNA-dependent kinase, PKR, in tumor cells by introducing anti-sense RNA that is complementary to a tumor-specific transcript. For instance, introducing anti-sense RNA complementary to the Δ (2-7)EGFR deletion junction activates PKR in tumor cells that express the truncated receptor [109]. PKR is a potent death-inducing molecule [110], and its selective activation resulted in apoptosis of the tumor cells, but not of healthy cells expressing the wild type EGFR receptor [109]. This approach could be widely applicable, because many cancers have identifiable translocations or deletions. The second approach is to use EGF-conjugates to deliver non-specific dsRNA to EGFR over-expressing cells [Shir *et al.*, PLoS Medicine, in press]. Again, this approach is applicable to numerous receptor-ligand combinations.

Along a similar vein, antibodies can be used to target non-specific therapies to cancer cells. Currently in clinical trials for the treatment of non-Hodgkin's lymphoma, Bexxar (^{131}I -tositumomab) and Zevalin (^{90}Y -ibritumomab tiuxetan) are anti-CD20 monoclonal antibodies bound to radioactive iodine or yttrium [111]. Encouraging interim data have been reported [112]. Another strategy is to use a recombinant immunotoxin, a chimera in which the variable portion of an antibody to a marker expressed on tumor cells is coupled to a toxin, such as *Pseudomonas* exotoxin A. Several such immunotoxins are in clinical trials [113].

BYSTANDER EFFECT

We have repeatedly stressed that most tumors, especially solid tumors, are heterogeneous. A particular target for

signal transduction therapy may be present on only some of the tumor cells. In tumors that express the truncated EGFR, co-expression of wild-type EGFR is the rule [108]. The tumors are actually mosaics of cells that over-express wild-type, over-express truncated EGFR, and do not over-express EGFR. To eradicate a tumor, it is necessary also to eliminate the tumor cells that do not express the target. One way to do this is to invoke a "bystander effect", whereby the dying targeted cells release cytokines that also kill neighboring, untargeted tumor cells [114].

CONCLUSIONS

The lessons learned so far from the successes – and failures – of signal transduction therapy should help to develop new and more effective drugs and drug cocktails. Each drug needs to be evaluated in the appropriate context: the over-expression of a kinase does not necessarily mean that a given tumor will be susceptible to inhibition of that kinase. Our ability to extrapolate from *in vitro* and in cell studies to the clinic is limited, and it is to be hoped that a better understanding of cancer development will assist in producing better models for preclinical studies. Such models ought to provide targets for cancer prevention and early detection.

It may have been naive to expect that empirically inhibiting PKs that are frequently over-expressed in common cancers would lead to discernible improvements. It is becoming increasingly clear that for "targeted molecular therapy" to succeed, it is necessary not only to identify suitable molecular targets but to target the appropriate patients. Given appropriate technological advances, cancer treatment in principle might be tailored to the individual, taking into account both the patient's genetic background and the specific proteomics profile of his tumor. Currently, signal transduction therapy involves targeting the Achilles' heel of a cancer [115], namely the signaling molecules that, on the one hand, confer unusual growth or invasive ability on the cancer, but on the other hand are essential survival factors (Fig. (1)). Approaches that might be more broadly applicable – and less expensive – involve using common cancer markers as Trojan horses for specific delivery of non-specific therapies (Fig. (4)). It is also important to implement clinical trials that test cocktails of inhibitors; although inhibiting a single kinase may not have a decisive outcome, targeting a group of kinases on key growth and survival pathways could well be more effective.

Targeted molecular therapy can play an important role in reducing tumor burden, and turning cancer into a "chronic" disease, that can be controlled over the long term by appropriate treatments. But true cures for advanced cancer will be hard to come by, unless and until cancer stem cells can be eliminated. Most cancer deaths are caused by metastases emanating from cancer stem cells, which survive the initial treatment. Bystander effects and the immune system, if harnessed during treatment, may eliminate them and induce complete tumor eradication.

ABBREVIATIONS

CML = Chronic myelogenous leukemia
CDK = Cyclin dependent kinase

EGF = Epidermal growth factor
 EGFR = EGF receptor
 GIST = Gastrointestinal stromal tumor
 PDGF = Platelet-derived growth factor
 PDGFR = PDGF receptor
 PK = Protein kinase
 PTK = Protein tyrosine kinase
 RPTK = Receptor protein tyrosine kinase
 VEGF = Vascular endothelial growth factor
 VEGFR = VEGF receptor

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