Tyrphostins and Other Tyrosine Kinase Inhibitors

Alexander Levitzki[1] and Eyal Mishani[2]

[1]The Silberman Institute for Life Sciences, Department of Biological Chemistry, The Hebrew University, Givat Ram Campus, Jerusalem 91904, Israel; email: levitzki@vms.huji.ac.il

[2]The Hadassah Hebrew University Hospital, Department of Medical Biophysics and Nuclear Medicine, Ein-Kerem Campus, Jerusalem 91120, Israel; email: mishani@md.huji.ac.il

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cancer therapy, signal transduction, tyrosine phosphorylation, tyrphostin

Abstract

The development of tyrosine phosphorylation inhibitors has transformed the approach to cancer therapy and is likely to affect other fields of medicine. In spite of the conservation among protein tyrosine kinases (PTKs), one can develop small molecules that block the activity of a narrow spectrum of PTKs and that exhibit much less toxicity than the currently used chemotherapeutic agents. In this review, we discuss principles for inhibiting specific PTKs. We discuss (*a*) the birth of the concept of generating targeted, nontoxic signal transduction inhibitors, (*b*) the potential of substrate-competitive versus the more common ATP-competitive PTK inhibitors, (*c*) the combination of PTK inhibitors with other signal transduction inhibitors to induce apoptosis—the best way to induce the demise of the cancer cell, and (*d*) the potential to utilize PTK inhibitors/tyrphostins to attenuate nonmalignant pathological conditions, such as immune disorders, tissue rejection, and restenosis.

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INTRODUCTION

This year marks the sixtieth anniversary of the introduction of the first chemotherapeutic agents against cancer. These agents take advantage of the enhanced cell proliferation that characterizes many cancers and target pathways related to RNA and DNA synthesis. The success of this strategy, although dramatic in some forms of cancers, has been limited. The most prevalent cancers, including lung and colon cancers, breast cancer in women, and prostate cancer in men, tend to respond initially but become refractory to chemotherapy, especially at the metastatic stage. Agents like 5-fluorouracil (5FU) and cisplatin (CDDP) are reasonably effective in the treatment of many cancers, sometimes achieving remarkable results as in the case of testicular cancer. In most cases, however, treatment brings temporary remission with the eventual reemergence of the disease, usually in a more severe, chemotherapy-resistant form. Thus, by the end of the 1970s, cancer therapy had reached an impasse.

There was no real improvement in outlook for cancer patients until the late 1990s. At this time, new therapies finally emerged, resulting from the tide of molecular and biochemical data that began in the late 1970s. A set of genes that code for proteins regulating cell proliferation, cell differentiation, and cell death had been discovered. Mutations that cause aberrant expression of these proteins, their constitutive activation, or both lead to cancer. These genes include oncogenes (genes that cause cancer) and tumor suppressor genes, which encode a number of biochemically different families of proteins such as p53 and Rb. Nuclear oncoproteins derive from transcription factors or nuclear hormone receptors. Cytoplasmic oncoproteins are signal transducers, such as protein kinases and small GTP-binding proteins. Transmembrane proteins are growth factor receptors. Last but not least, many oncoproteins are derivatives of growth factors. Although biochemically distinct, the common denominator of all these proteins is that they function in signal transduction.

The initial interaction of a growth factor receptor with its growth factor leads to activation of the inner, cytoplasmic portion of the receptor. This portion of the receptor can be itself an enzyme or can be linked to an enzyme. Activation of the enzyme portion in turn activates a signal transducer, which is another enzyme, and so the cascade begins to roll. Each of these steps is tightly controlled, and there is not only a mechanism to

activate each step but also to quench its activity. In normal transduction, every signaling event is transient. However, if there is a mutation that leads to the appearance of many copies of the receptor or to the production of both the receptor and its growth factor by the same cell, the primary receptor signal becomes amplified and persistent. This leads to abnormally high activity of the whole signaling pathway. Persistent, excess activity of the pathway can also result from a mutation in a downstream element of the signaling pathway that rids the cell of its dependence on the growth factor for activation of the pathway. Another property of cancer cells is genetic instability, leading to chromosome loss, rearrangements, and, consequently, to a reduction in the number of signaling pathways. This loss of redundancy renders the cancer cell highly dependent on those signaling pathways that have become overactive and that give the cell its growth advantage. This very property also makes the cell more susceptible to agents that target these signaling pathways. The recognition of this principle led us to the concept of "signal transduction therapy" (1).

PROTEIN TYROSINE KINASES

Protein tyrosine kinases (PTKs) appear in evolution with multicellular organisms. Yeasts such as *Saccharomyces cerevisiae* or *Candida albicans* do not possess PTKs, whereas Dictostylium, nematodes, and Drosophila do. It appears from what we have learned over the past 25 years that PTKs specialize in communication between cells and within cells. PTKs are involved in embryonic development, metabolism, cell proliferation, angiogenesis, and the immune system. Because PTKs occupy key positions in the function of the multicellular organism, it is not surprising that malfunction of PTKs can lead to disease. Most diseases involve enhanced activity of PTKs owing to various mutations, either in the PTK itself or in the mechanisms of their activation, leading to enhanced, and sometimes persistent, stimulation. Some diseases, such as various forms of diabetes, involve the lack or diminished activity of the PTK [insulin receptor (InsR) in the case of diabetes]. In this review, we discuss the much more common situation—the enhanced activities of PTKs leading to proliferative diseases, including cancers, leukemias, psoriasis, restenosis, and others. PTKs comprise receptor protein tyrosine kinases (RPTKs), which receive signals from outside the cell and transmit it into the cell, and nonreceptor (cellular) PTKs, which do not possess a domain facing outside the cell. RPTKs are glycoproteins spanning the cell membrane where their extracellular domains face outward, interacting with growth factors, triggering the activation of the catalytic intracellular domains, and thereby transmitting the messages. This family of RPTKs consists of ~60 known proteins, such as EGF receptor (EGFR), platelet-derived growth factor receptor (PDGFR), InsR, vascular endothelial growth factor receptors (VEGFRs), and more. Nonreceptor (cellular) PTKs, for example, Abl, the Src family, and the Jak family are activated by upstream signaling molecules, such as receptors of the immune system, G protein–coupled receptors, and RPTKs. In our review, we do not list and discuss all the PTKs (2) but rather focus on the principles of developing inhibitors as manifested through the discussion of representative PTKs.

Signal Transduction Therapy and Protein Tyrosine Kinases

The strategy behind signal transduction therapy is inhibition of the hyperactive signaling pathways on which the cancer cell is dependent for survival and/or proliferation. In 1985, we decided to test the feasibility of this approach on PTKs. After the discovery that pp60Src was a tyrosine kinase, many RPTKs (except the InsR) and cellular (nonreceptor) PTKs, identified in the 1980s, were all found to be associated with

various forms of cancer and leukemia. We recognized the immense emerging opportunity to generate low-molecular-weight inhibitors of tyrosine phosphorylation and use them for therapy and the dissection of signal transduction pathways (3). We have since generated many hundreds of PTK inhibitors and coined the term "tyrphostins" (TYRosine PHOSphorylation INhibitors) for such compounds. Initially the work on tyrphostins was met with skepticism because the common belief was that the high degree of conservation of the kinase domain (4) would preclude the possibility of obtaining highly selective molecules. This dogma was reinforced by the observation that all of the newly identified PTK inhibitors obtained from natural sources, including quercetin (5), genistein (6), erbstatin (7), and lavendustin (8), were not selective and also inhibited Ser/Thr kinases (erbstatin inhibits PKC) and other ATP-requiring enzymes (quercetin).

Since its inception, signal transduction therapy has expanded exponentially (1, 9–12). Small molecules that modulate the activity of an aberrant signaling element are entering the clinic in ever increasing numbers. Most of the agents are inhibitors of a signaling protein whose activity is abnormally enhanced. Occasionally, as in the case of p53, an attempt is made to generate agents that activate a signaling element. In addition to small molecules, signal transduction agents include antibodies and recombinant proteins (11, 13). In this review, we focus on PTKs whose enhanced signaling is involved in many cancers as well as in other pathophysiological conditions.

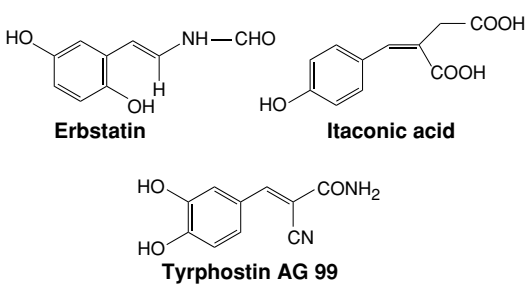

Figure 1

The origin of early tyrphostins. Itaconic acid was found to be a substrate-competitive inhibitor (14), whereas erbstatin is a mixed-type competitive inhibitor (20). AG 99, one of the early tyrphostins, was designed on the basis of these two inhibitors.

TYRPHOSTINS

The first step in the development of PTK inhibitors began soon after the recognition in the early 1980s that natural compounds, such as quercetin, erbstatin, genistein, and lavendustin A, inhibit the activities of PTKs such as $pp60^{Src}$ and EGFR. Although these natural compounds have rather poor selectivity or mediocre potency, they served as lead compounds for the design and development of synthetic, more potent, and selective PTK inhibitors (tyrphostins). In parallel with the search for natural compounds, our group prepared hydroxyphenyl-containing molecules as tyrosine mimics. We showed that these molecules are competitive inhibitors of the InsR kinase and block insulin-dependent glucose uptake and the antilipolytic activity of the hormone (14). The activity of itaconic acid observed by us (14, 15) along with the observations of Umezawa et al. (7) regarding erbstatin triggered the first systematic synthesis of numerous potential PTK inhibitors (tyrphostins) (**Figure 1**) from the family of benzene malononitriles (16, 17). The structure of itaconic acid and erbstatin served as the template for a large number of compounds, many of which demonstrated excellent PTK inhibition, with no significant inhibition of Ser/Thr kinases (16, 17). This initial class of tyrphostins showed for the first time that one can generate a series of compounds that inhibit a particular PTK with minimal toxic effects in cells and in vivo (18). These findings convinced the chief executive officer of Novartis, Daniel Vasella, to move on and develop an inhibitor for the protein tyrosine kinase Bcr-Abl (19, p. 46).

Tyrphostins could be classified into those that were competitive with the substrate and noncompetitive with ATP, ATP competitive (20, 21), bisubstrate competitive (20), and mixed competitive (22). In the mid-1990s,

when structure-activity analysis led to bicyclic tyrphostins (21–23) (**Figure 2**), most were found to be ATP competitive but also mixed competitive. Indeed, the main thrust was to develop ATP-competitive kinase inhibitors. This is understandable because the ATP-binding fold is more structurally defined, whereas the substrate-binding domain is more open and is less easy to use for the design of low-molecular-weight inhibitors. The most common chemical entities of ATP mimics are anilinoquinazolines, anilinoquinolines, and anilino-pyridopyrimidines. Although ATP-binding sites are highly conserved among tyrosine kinases, minor differences in kinase domain structures have led to the development of selective PTK inhibitors. Yet, a strong case for substrate-competitive inhibitors can be made (see below).

CHRONIC MYELOGENOUS LEUKEMIA, BCR-ABL KINASE, AND ITS INHIBITORS

The PTK Bcr-Abl kinase causes chronic myelogenous leukemia (CML). In the early chronic phase of the disease, which lasts between three and five years, CML cells depend solely on the kinase activity of Bcr-Abl for their survival. This suggested that inhibiting this enzyme might induce the elimination of the diseased cells from the patient's body. The first potent, selective inhibitors of Bcr-Abl kinase were reported in 1992 (24). One family of inhibitors, AG 957 and its derivatives, is competitive with substrate and noncompetitive with ATP (24) (**Figure 3**). AG 957 induces CML cell death by apoptosis and synergizes with the pro-apoptotic anti-Fas antibody, CH 11 (25). Adaphostin (26) (**Figure 3**), an AG 957 analog, is being developed for clinical use. Another family of Bcr-Abl selective agents, represented by AG 1112 (**Figure 4**) and AG 1318, is ATP competitive (27) and also induces massive apoptosis of K562 (CML) cells (27). In 1996, Druker and the Novartis team (28) reported on a successful Bcr-Abl inhibitor, CGP 57148, later renamed

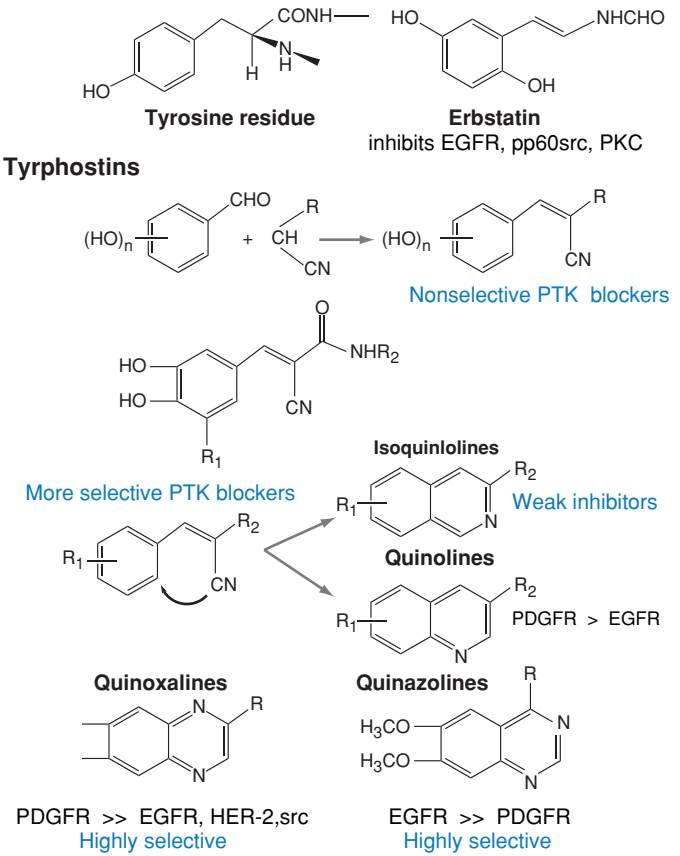

Figure 2

The evolution of tyrphostin/PTK inhibitors. Initially, small molecules tend to be substrate competitive. When more than one aromatic ring is incorporated, compounds tend to be ATP mimics, especially if they possess N atoms in the rings. Thus quinolines, quinazolines, and quinoxalines are ATP competitive or mixed competitive.

STI-571/imatinib mesylate/Gleevec/Glivec (**Figure 4**). The selective inhibition of Bcr-Abl by STI-571 is mediated via interaction between the inhibitor and the amino acids constituting the ATP-binding cleft of the PTK in the inactive state. Clinical trials have demonstrated durable responses in patients in the chronic phase, whereas those with advanced disease relapse.

The fact that STI-571 has only minor side effects and is well tolerated is of great interest and importance. This was initially surprising, given that STI-571 blocks c-Abl, PDGFR, and c-Kit. These kinases play important roles in normal cells, and knockout

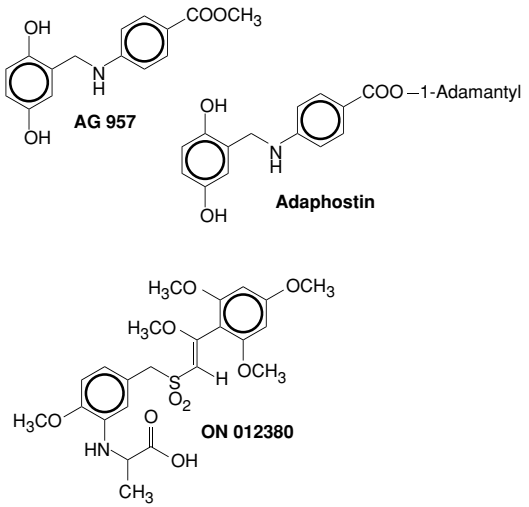

Figure 3

Bcr-Abl substrate-competitive inhibitors. AG957 is the precursor of Adaphostin, whereas ON 012380 is a completely novel structure. See Reference 38.

of their genes is detrimental. The most likely explanation is that healthy cells, which utilize c-Abl, PDGFR, or c-Kit, can get by even when over 90% of these targets are blocked because they can utilize the alternative pathways that all normal cells possess. Conversely, CML cells are dependent on Bcr-Abl for their survival and therefore die when it is blocked. STI-571 is effective also in treating gastrointestinal stromal tumor (GIST) (29), especially in patients who harbor activating mutations in c-Kit in exon 11.

Resistance to STI-571

The most common mechanisms of relapse appear to be mutations of the amino acids involved in the binding of ATP and STI-571 (30–35). A relatively common mutation in STI-571-resistant patients is a single nucleotide change that replaces threonine with isoleucine at position 315 (T315I). Thr 315 forms critical hydrogen bonds with STI-571. Other mechanisms of STI-571 resistance include Bcr-Abl gene amplification (36) and the activation of signaling pathways downstream of or parallel to those of Bcr-Abl (30, 32, 34, 35).

In order to overcome the resistance to STI-571, a number of new inhibitors have been synthesized. The most effective ATP mimics are AMN107 (33) (**Figure 4**) and BMS-354825, which inhibit almost all STI-571-resistant forms of Bcr-Abl (32, 37) but are not effective against the T315I mutant. Interestingly, a substrate-competitive inhibitor of Bcr-Abl, ON012380 (38) (**Figure 3**), was found to bind to all Bcr-Abl kinase mutants, including the T315I mutant, with a similar affinity of ~10 nM. ON012380 synergizes with STI-571, and the combined IC50 for a 1:1 mixture is ~0.1 nM.

Altered Bcr-Abl expression and the emergence of additional signaling pathways are also involved in resistance to STI-571. For example, there is evidence of increased activity of Src family kinases in cells from STI-571-resistant patients (32, 34, 35). This prompted the evaluation of molecules that inhibit both Bcr-Abl and Src activity. Two compounds, PP1 and CGP76030, inhibited Bcr-Abl in a concentration-dependent manner by overlapping binding modes. In contrast to STI-571, PP1 and CGP76030 also blocked cell growth and survival in cells expressing STI-571-resistant Abl mutants by inhibiting the activity of Src family tyrosine kinases (39). These results suggest that the use of Src kinase inhibitors is a potential strategy to prevent or overcome clonal evolution of STI-571 resistance in Bcr-Abl-positive leukemia (39).

Another inhibitor that targets both Src and Bcr-Abl, BMS-354825, is effective in patients who have become resistant to STI-571 (32). Even though it is less specific than STI-571, it seems to be less toxic and more potent than STI-571 in patients (M. Talpaz, unpublished results). This may be because STI-571 binds to the inactive state of the Abl kinase domain, whereas BMS-354825 binds to the active state of the Abl kinase or of the Src kinase (30). In the transformed cell, the Bcr-Abl kinase is permanently in the activated state, whereas in the normal cell, the activated state of c-Abl is achieved transiently under certain biochemical conditions. Hence, the kinase domain in the tumor cell is persistently occupied by the drug. In the normal cell, the inactive kinase is not occupied by the drug and can therefore

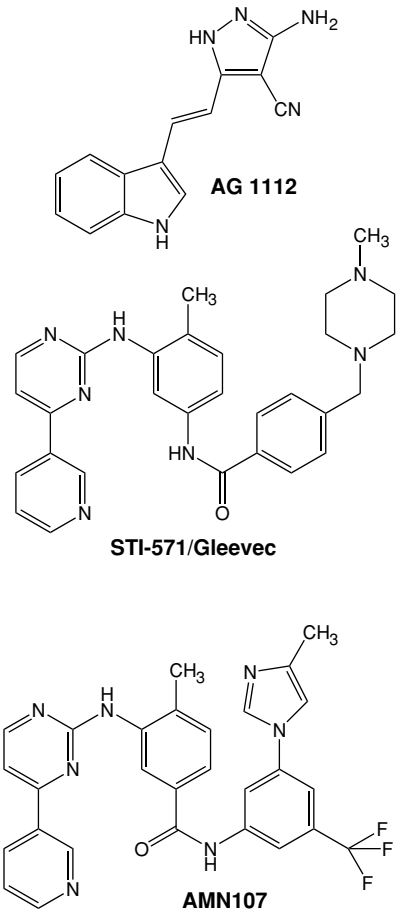

Figure 4

Bcr-Abl ATP-competitive inhibitors. AG 1112 was the first ATP-competitive inhibitor of Bcr-Abl; STI 571/Gleevec was introduced to the clinic in 1999 with great success, and AMN107 is an improved ATP mimic capable of overcoming Gleevec resistance for all Bcr-Abl mutations, except the T315I mutation. ON 012380 (**Figure 3**) overcomes all mutations, including the T315I mutation.

be activated in response to appropriate signals, at least transiently. Had the agent been bound to the inactive state too, the normal cell would have been inhibited more severely, and the toxic effect would have been more apparent. These subtle differences between the two types of compounds may explain, at least partly (40), why BMS-354825 is less toxic than STI-571.

ATP MIMICS VERSUS SUBSTRATE MIMICS

Although ATP mimics are popular, it is advisable to consider seriously the utilization of substrate mimics. They are less likely to hit other targets because the substrate-binding domain is less conserved than the ATP fold. Secondly, a substrate-competitive inhibitor does not need to compete with the high intracellular ATP concentrations, which lead to a requirement for high doses of inhibitor for cellular and in vivo activities. The higher selectivity and the lower doses needed for in vivo activity of substrate-competitive inhibitors markedly reduce toxicity (41). In the case of Ser/Thr kinases, the situation is even more severe because there are five times more Ser/Thr kinases than PTKs. Indeed a PKB/Akt substrate-competitive inhibitor was found to be much superior to an ATP mimic (42). In the case of PTKs, only a few substrate mimics have been made (14, 16, 38, 43, 44).

EGFR KINASE INHIBITORS

In past 10 years, over 25 EGFR inhibitors have been described, many at various stages of preclinical and clinical development. Two have made it to the clinic. Here we discuss the most advanced compounds.

Reversible Inhibitors

Initially, AG1478, an anilinoquinazoline (45, 46), was identified as a potent EFGR kinase inhibitor. A more soluble anilinoquinazoline, Gefitinib, was later developed. AG1478 formulated in captisol remains in clinical development but has not yet reached the clinic (see below). Gefitinib (ZD 1839, Iressa®) (47) (**Figure 5**) is orally available and inhibits the kinase activity of EGFR. Gefitinib binds at the ATP site and is ~100 times less effective in blocking Her-2. Erlotinib [N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-anilinoquinazoline, OSI-774, Tarceva, Genentech, Inc.] is a compound

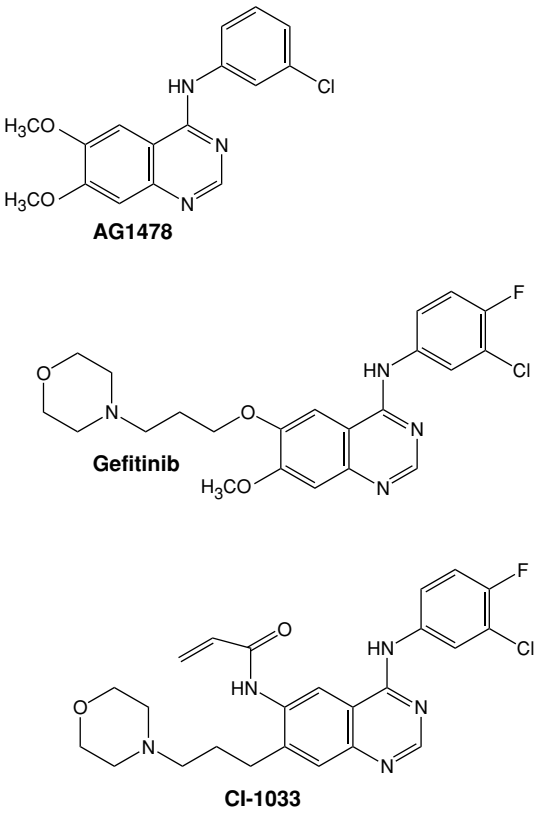

Figure 5

EGFR reversible and irreversible inhibitors.

similar to Gefitinib. Gefitinib has been in the clinic since 2002 for the treatment of non-small cell lung cancer (NSCLC) but is effective only in a small percentage of patients in which EFGR possesses activating mutations in the kinase domain (48–50). Erlotinib yielded results similar to Gefitinib. The small number of patients who respond to these agents carry specific activating mutations in the EGFR kinase domain, and tumor survival seems to depend on the activated form of the EGFR. In all other cases where EGFR overexpression is the hallmark, tumor survival does not appear to depend on EGFR activity, which accounts for inefficacy. In the absence of accurate measurements of EGFR phosphorylation in the human tumor, it is actually not possible to assess whether the failure of Gefitinib and Erlotinib is indeed due to the absence of a survival function of EGFR or to insufficient long-term occupancy of the receptor. The finding that an irreversible EGFR kinase inhibitor is more efficacious as an antitumor agent supports the latter view (51). In view of the weak performance of Gefitinib and Erlotinib, no new reversible EGFR kinase inhibitors are heading for the clinic at present. This attitude may change in view of the recent finding that 10% to 20% of EGFR-overexpressing glioblastoma multiforme patients respond to EGFR kinase inhibitor therapy. It seems that their responses correlate with the expression of PTEN and the mutant Δ(2-7)EGFR (52). This finding suggests that EGFR kinase inhibitors will find their way to the clinic in cases in which the receptor plays a survival role by itself and/or when the inhibitors can be combined with other signal transduction agents to induce apoptosis selectively in the cancer cell.

Resistance to Gefitinib and Erlotinib

NSCLC patients who initially respond to Gefitinib and Erlotinib become resistant because of secondary mutations in the EGFR (53), in addition to the primary mutations that made them responsive to these inhibitors. Interestingly, the M790T mutation in the EGFR, which confers resistance to Gefitinib and Erlotinib (53), is homologous to the mutations that make Bcr-Abl (T315I), PDGFRα (T674I), and c-Kit (T670I) resistant to STI-571. Current preclinical data show that cell lines expressing the Gefitinib-resistant mutants are inhibited by irreversible EGFR inhibitors, suggesting that these may have future clinical utility (see below). It should be stressed, however, that the primary cause of resistance to EGFR-directed agents is the activity of other signal transduction pathways, such as K-ras activating mutations in lung cancer (54) and the lack of PTEN leading to PKB/Akt hyperactivity (52).

Irreversible Inhibitors

The current reversible EGFR kinase inhibitors are all ATP competitive. Such

reversible ATP inhibitors need to compete with high endogenous ATP concentrations within the cell for an extended period of time in order to obtain effective antitumor activity (10, 41). In addition, their rapid clearance from plasma necessitates sustained delivery. The fast washout of reversible inhibitors from the tumor area was demonstrated by positron emission tomography (PET) studies of tumor-bearing animals imaged with fluorine-18-labeled reversible EGFR inhibitors (55). Moreover, fluorine-18-labeled Gefitinib, used to image mice bearing EGFR-overexpressing tumors, did not indicate sustained or significant tumor uptake (56). In order to enhance treatment efficiency, there has been a tremendous effort to develop irreversible EGFR kinase inhibitors on the basis of 4-(phenylamino) quinazoline and quinoline core structures (57–67). The main strategy has been to attach a Michael acceptor functional group to the basic structure of the anilinoquinazoline or quinoline in order to obtain covalent bonding through electrophilic attack of the inhibitor on a cysteine residue in the kinase-binding pocket (58). This strategy was founded on the observation that residues Cys-773 in EGFR and Cys-751 in Her-2 are uniquely positioned within the ATP-binding pocket of the respective kinase. A series of irreversible EGFR and Her-2 inhibitors were synthesized with different Michael acceptor functional groups attached at the 6- and 7-positions of the quinazoline and quinoline rings, and the 6-position yielded the best results. PD168393, which contains the acrylamido functional group at the 6-position, was found to be a potent, irreversible EGFR and Her-2 inhibitor.

Three irreversible inhibitors, CI-1033 (**Figure 5**), HKI 272, and EKB-569, are currently in clinical development. Interestingly, an irreversible EGFR kinase inhibitor is effective against EGFR kinase mutants resistant to Gefitinib (51), suggesting the need for long-term occupancy of the receptor with minimal toxicity (65).

EGFR-Directed Tyrphostins in Combination with Other Agents

Because of the heterogeneity of human tumors, it is likely that a single agent will not have a significant effect over time, but when administered in combination with other agents, it may have much higher impact (9). Fifteen years ago, it was found that the combination of the EGFR-directed tyrphostin, RG 13022, with the anti-EGFR antibody, mAb108, is very effective in inhibiting squamous cell carcinoma grown in vivo (18). The action of the two agents is strongly synergistic, although they target the very same EGFR. Recently, this observation was validated with clinically approved agents. It was shown that the anti-EGFR antibody, Erbitux (mAb 225, Cetuximab), synergizes with Gefitinib to inhibit EGFR-overexpressing tumors generated from A431 cells, grown as xenografts in nude mice (68). In another study, using an EGFR-overexpressing lung cancer xenograft cell model, it was found that Erbitux and Gefitinib or Erlotinib act in an additive manner (69).

A number of reports have appeared on the synergy between Her-2-directed tyrphostins and cytotoxic agents (70) as well as between EGFR kinase inhibitors and cytotoxic agents (71, 72). These data suggest that such combinations may be suitable for the clinic. Nonetheless, Gefitinib failed in a clinical trial during which it was administered together with CDDP (73), even though in preclinical animal experiments synergism was observed.

STRUCTURAL CONSIDERATIONS

The catalytic domains of eukaryotic Ser/Thr and tyrosine kinases are highly conserved in structure and even in amino acid sequence. The kinase domain adopts the bilobate-fold characteristic. The NH_2-terminal lobe of a protein kinase (N-lobe) is formed from mostly β-strands and one conserved α-helix, where the largest COOH-terminal lobe (C-lobe) is

mostly α-helical. Although the ATP-binding cleft of GW572016/EGFR is in a relatively closed conformation, the ATP-binding cleft in Erlotinib/EGFR is in a more open one. In another crystal structure study of the kinase domain of Abl in complex with two inhibitors, STI-571 and PD173955 (74), behavior similar to that of EGFR was observed. Although the two inhibitors bind to the canonical ATP-binding site of the kinase domain, because of the differences in size of the two inhibitors their modes of binding differ. STI-571 interacts with a total of 21 amino acid residues, whereas PD173955 interacts with only 11. STI-571 captures a specific inactive conformation of the activation loop of Abl in which the loop mimics bound peptide substrate. In contrast, PD173955 binds to a conformation of Abl in which the conformation of the activation loop resembles that of the active kinase, namely the activation loop is in an extended or open conformation. Furthermore, it has been hypothesized that the reason PD173955 inhibits the Abl kinase more potently than STI-571 is because PD173955 makes fewer contacts with Abl, can bind to multiple conformations of the kinase, and is insensitive to the conformational state of the activation loop. Conversely, STI-571 requires a specific (inactive) conformation of the kinase for productive binding. Detailed knowledge of the crystal structure of Abl-STI-571 has enabled the synthesis of an improved inhibitor of Bcr-Abl, AMN107, which already has been tested and shown to possess better efficacy than STI-571 (33).

From these studies and others, we conclude that different ATP-competitive inhibitors, with a similar basic structure, yet with variations in functional groups, may recognize different conformations of the kinase and produce varied complex enzyme/inhibitor structures. Frequently, even in cases where the structure is known as it is for the Hck-PP1 (75) and Lck-PP2 (76), identifying an improved inhibitor may not be possible (77). Currently, no structural data on PTK complexes with low-molecular-weight substrate-competitive inhibitors are available. The only structure of a substrate-PTK complex involves peptide molecules. As yet, this has not been utilized to generate successful substrate-competitive inhibitors, as was done for the Ser/Thr kinase PKB/Akt (42).

VEGF RECEPTOR TYROSINE KINASE INHIBITORS

The roles of VEGFRs as important mediators in multiple processes of tumor angiogenesis, including increased vascular permeability, endothelial cell migration, proliferation and survival, have been well established (78, 79). Of these receptors, VEGFR-2 is predominantly expressed on endothelial cells and is involved in various aspects of tumor angiogenesis. Consequently, this receptor has become an attractive target for the treatment of multiple types of tumors. Starving the tumor by blocking blood supply is a key strategy to combat cancer. Indeed, many animal models and preclinical experiments have supported this idea. The first demonstration of the possible utility of angiogenesis inhibitors came with the demonstration that certain tyrphostins were able to inhibit VEGFR-2/KDR and to inhibit angiogenesis in vivo (80). Over the next few years, a number of VEGFR kinase inhibitors were synthesized, and a few entered clinical development.

On the basis of the reported crystal structure of the VGEFR-2 kinase domain, a number of small organic molecule families have been developed as potential VEGFR tyrosine kinase inhibitors (81). Among them are 4-anilinoquinazolines (82–84) and 3-substituted indolinones (85–88).

In the 3-substituted indolinone family, SU 11248, which targets VEGFR, PDGFR, Kit, and FLT3, seems to fare much better than its predecessors. This is probably because the compound is multitargeted and hits simultaneously a number of signaling RPTKs involved in tumor blood supply. In mouse xenograft models, SU 11248 exhibited broad and potent antitumor activity. The antitumor

activity is combined with antiangiogenic activity, and therefore the compound is very effective in xenograft models of prostate cancer in which PDGFR and angiogenesis play a role. Also, AML (acute myloid leukemia) patients treated with SU 11248 had reduced Flt3 activity in their blood (89, 90). The Kit kinase is a survival factor for many cases of GIST. The excellent performance of SU 11248 in STI-571-resistant GIST (91) because of its ability to block Kit, including STI-571-resistant Kit, has led to an accelerated path to clinical trials. The future of SU 11248 as an antiangiogenic agent remains to be determined.

Another promising VEGFR-2 kinase inhibitor is BAY 43-9006. Initially, BAY 43-9006 (92) was discovered as an orally available c-Raf (and B-Raf) kinase inhibitor and generated hope that it would be a good agent against metastatic melanoma in which an activating B-Raf mutation is very common. BAY 43-9006 also inhibits VEGFR-2, VEGFR-3, FLT-3, PDGFR, p38, and c-Kit (93). Very quickly it was found that the agent is not clinically effective against melanoma but is rather effective against renal cancer. The clinical utility of BAY 43-9006 appears to be due to its antiantiogenic inhibition of VEGFR-2 (94).

PDGFR AS A TARGET

PDGF receptors and their ligands are involved in a variety of diseases: cancers, atherosclerosis, balloon injury-induced restenosis, pulmonary fibrosis, and others (95–97). The role of PDGF receptors in cancer is hardly restricted to those cases in which PDGF is directly involved in the oncogenic process. PDGF receptors in the tumor stroma regulate tumor interstitial fluid pressure, tumor transvascular transport, and tumor drug uptake (95). Thus, blockade of the PDGFR in every tumor can help drug delivery to the tumor. Furthermore, the involvement of the PDGFR in recruiting blood vessels to the tumor (angiogenesis) makes it a good target for antiangiogenic therapy for many tumors. These findings have induced the research community as well as the pharmaceutical industry to develop agents that block PDGFR signaling. A large number of PDGFR kinase inhibitors with different chemical scaffolds have been synthesized and show efficacy in cells and in vivo, but only STI-571 has entered the clinic. The pyrimidinopyridines were initially developed as PDGFR kinase inhibitors (98) but found their way into the clinic as the Bcr-Abl inhibitors (see above). As a PDGFR inhibitor, STI-571 has shown efficacy in the clinic in the treatment of chronic monomyelocytic leukemia and dermatofibrosarcoma protruberans. STI-571 is also effective in the treatment of those cases of AML in which Flt-3 plays a role and in the treatment of certain cases of GIST driven by mutant c-Kit.

All of the PDGFR inhibitors also target the other members of the PDGF receptor family, c-Kit, and Flt3. The quinoxalines AG 1295, AG 1296 (22) and AGL 2033/43 (99) were found to be highly potent and selective toward the PDGFR and its family members Kit and Flt3. Because STI-571 is the only PDGFR inhibitor that also blocks Bcr-Abl kinase, it presumably has a different mode of binding to the kinase site than the rest of the PDGFR kinase inhibitors. This will be revealed once the structure of the PDGFR kinase domain in complex with the inhibitors is solved. Kinetic/mechanistic studies on the mode of action of the quinoxalines AG 1295 and AG 1296 (22) show it is competitive vis-à-vis ATP in the basal state and is mixed competitive vis-à-vis ATP subsequent to receptor activation (100). This means that AG 1296 binds differently to the kinase domain once the receptor is activated. In the activated state, AG 1296 binds outside the ATP-binding domain but still inhibits the catalytic kinase reaction. This suggests that the quinoxalines bind to unique areas of the PDGFR and may explain their high selectivity (100). In contrast, STI-571 seems to bind to common structural denominators between Bcr-Abl and PDGFR

and therefore inhibits both with similar efficacies.

PDGFR Kinase Inhibitors as Antirestenosis Agents

Restenosis is due to the migration of vascular smooth muscle cells (SMC) from the media into the lumen as a result of balloon- and stent-induced injuries. The SMC cross through the injured endothelium and proliferate to generate the neointima, which clogs the blood vessel.

Several growth factors and cytokines are responsible for the process of restenosis, all originating from platelets, mononuclear cells, and endothelial cells. Because PDGF and its receptor are believed to play the major role in the pathogenesis of this process (97), PDGFR kinase inhibitors, AG 1295 (22) and AGL 2043 (99, 101), were examined as antirestenosis agents by local application to the site of injury. Experiments in pigs have shown that when these agents are applied to the site of injury, either by tyrphostin-formulated nanoparticles (102) or by utilizing a tyrphostin eluting stent, balloon-induced or stent-induced stenosis is strongly inhibited (101).

JAK-2 AND JAK-3

Janus kinases (Jaks) are cytoplasmic PTKs that play a crucial role in the initial steps of cytokine signaling. The enhanced activity of Jak-2 and the constitutive activity of Jak-2 fusion proteins generated by oncogenic mutations (103) play key roles in various leukemias, lymphomas, and certain forms of metastatic cancers, such as breast cancer and prostate cancer (104). Jak-2 was therefore identified very early as a potential target for cancer therapy, but no therapeutic agent targeting this kinase has been developed for clinical use. AG 490, which was the first Jak-2 inhibitor generated, was the first to be successfully used for the eradication of a leukemia (105), lymphomas, and a myeloma (106) in animal models. Since then, AG 490 analogs with various degrees of activities in vivo have been generated (107, 108). In all the cases examined, AG 490 and its analogs inhibited the Jak-2/Stat3/5 pathway, which is a key pathway sustaining the oncogenic phenotype.

Jak-2 Inhibitors Combined with Immune Therapy

Because AG 490 does not inhibit the signaling or growth of normal T cells, B cells, and macrophages (105, 106), it was examined in combination with immunotherapy. Immunotherapy by itself has little effect on established tumors but can induce long-term antitumor immunity once tumor volume has been greatly reduced. Reduction in tumor volume can be achieved by treatment with signal transduction inhibitors. Tyrphostins, unlike cytotoxic agents, qualify for combination with immunotherapy because they do not harm the immune system. Indeed, the antitumor efficacy of AG-490 was greatly enhanced by immunotherapy when the tyrphostin was applied first to reduce tumor burden, followed by interleukin (IL)-12. In addition to inducing antitumor immune responses, IL-12 suppresses tumor angiogenesis. These findings (106) suggest that combining nontoxic signaling inhibitors of *JAK*/STAT signaling such as AG-490 with IL-12 cytokine therapy may be extended to other cancers.

Jak-3 Inhibitors

JAK3 is abundantly expressed in lymphoid cells and initiates signaling of IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15. *JAK3* is involved in T-cell activation and proliferation, and it plays a role in leukemia and autoimmune or transplant-induced inflammatory disorders. The selective targeting of *JAK3* in T cells may be clinically beneficial in T-cell-derived pathologic disorders (109), including autoimmune diabetes, allergy, and the rejection of solid organ, pancreatic islet, and bone marrow

transplants. A number of Jak-3 inhibitors have already been generated and demonstrated activity in some of these indications (for example, see Reference 110).

CONCLUSIONS

It took 20 years from the inception of the concept of signal transduction therapy until the first effective PTK inhibitors reached the clinic. Signal transduction cancer therapy rests on the finding that it is possible to specifically target and inactivate the overactive kinases upon which the cancer cell depends for its survival. Noncancerous cells are less sensitive to inhibition of a particular kinase because they rely on a larger spectrum of signal transduction pathways for their growth and survival.

The examples we have enumerated in this review highlight several principles for effective signal transduction therapy. Most of the available PTK inhibitors are ATP competitive. We believe that substrate-competitive inhibitors will be more selective because of the greater diversity in substrate-binding domains. Furthermore, ATP mimics require relatively high doses to compete with the high concentrations of intracellular ATP. Irreversibly binding ATP-competitive inhibitors may overcome this difficulty. We therefore believe that more emphasis should be placed on the development of substrate mimics and irreversible ATP mimics as PTK inhibitors. Inhibitors that target the active conformation of a kinase may be more specific for cancer cells, in which the kinase is persistently activated, and may cause fewer side effects to healthy cells, in which the activated state of the kinase is transient.

Most cancers are heterogeneous in nature, and it is therefore unlikely that a single therapeutic moiety will be effective. Even in the case of CML, which is universally associated with Bcr-Abl activity, resistance to STI-571 develops. A promising avenue for treatment of some cancers may be combination of PTK inhibition to reduce tumor load with immunotherapy to induce long-term antitumor immunity.

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