ARTICLE

# Tyrosine Kinase Inhibition: An Approach to Drug Development

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Protein tyrosine kinases (PTKs) regulate cell proliferation, cell differentiation, and signaling processes in the cells of the immune system. Uncontrolled signaling from receptor tyrosine kinases and intracellular tyrosine kinases can lead to inflammatory responses and to diseases such as cancer, atherosclerosis, and psoriasis. Thus, inhibitors that block the activity of tyrosine kinases and the signaling pathways they activate may provide a useful basis for drug development. This article summarizes recent progress in the development of PTK inhibitors and demonstrates their potential use in the treatment of disease.

Phosphorylation of proteins on tyrosine constitutes less than 0.01% of the total intracellular phosphorylation. Phosphorylation on tyrosine residues occurs almost exclusively in metazoa and is absent from unicellular eukaryotes, with the exception of regulators of cell cycle kinases and members of the mitogen-activated protein (MAP) kinase family, in which simultaneous phosphorylation of tyrosine and threonine is catalyzed by a specialized kinase that differs from classical PTKs. Tyrosine phosphorylation may be the primary, or even the exclusive, indicator of signal transduction in multicellular organisms (*1*). The receptor tyrosine kinases (RTKs) participate in transmembrane signaling, whereas the intracellular tyrosine kinases take part in signal transduction within the cell, including signal transduction to the nucleus (*2*). Enhanced PTK activity resulting from tyrosine kinase overexpression can activate mutations or lead to persistent stimulation by autocrinically secreted growth factors, which in turn can lead to disease (*3*). Decreased function can also be harmful; for example, a decrease in the activity of the insulin RTK is the cause of various types of diabetes (*4*). Severe reduction of the B cell progenitor kinase leads to human X-linked agammaglobulinemia (*5*).

Enhanced activity of tyrosine kinases has been implicated in many cancers and other proliferative diseases, as well as in nonmalignant proliferative diseases such as atherosclerosis (*6*) and psoriasis (*7*) and in a large number of inflammatory responses such as septic shock (*8*, *9*). Tyrosine kinases and the signaling pathways in which they participate have therefore been identified as potential targets for drug design. In the past decade, many laboratories embarked on projects aimed at generating compounds that inhibit the activity of the signaling cascades triggered by tyrosine kinases (*10*). These signaling pathways can be intercepted at various steps, as shown in Fig. 1.

One possible approach to intercepting these pathways is to develop biological reagents that inhibit the binding of a growth factor to its receptor. This has been achieved in the case of the HER2-Neu RTK, which is overexpressed in ~30% of breast and ovary carcinomas (*11*). Antibodies to HER2 have been produced and are currently in phase II clinical trials. Another approach is to develop growth factor antagonists. This approach has not been successful, although attempts to develop low molecular weight insulin mimics or growth factor antagonists have been quite intensive. However, the drug suramin stands out as an example of the potential of this approach. This compound, initially developed as an antitrypanosome agent (*12*), was found to inhibit tumor cells with good efficacy but is quite toxic. The main mechanism of action of suramin may involve inhibition of the binding of growth factors to their receptors (*13*). Suramin has been used for treatment of renal carcinoma (*14*) and prostate carcinoma (*15*) with notable efficacy. Attempts are under way to develop suramin analogs that inhibit binding of specific growth factors to their receptors.

The action of a receptor can also be nullified with a growth factor–toxin chimera. In this case, the toxin would be internalized by receptor-mediated endocytosis of the chimera, which would destroy the receptor-harboring cell (*16*). Another possible approach is to block the interaction of an activated RTK or intracellular tyrosine

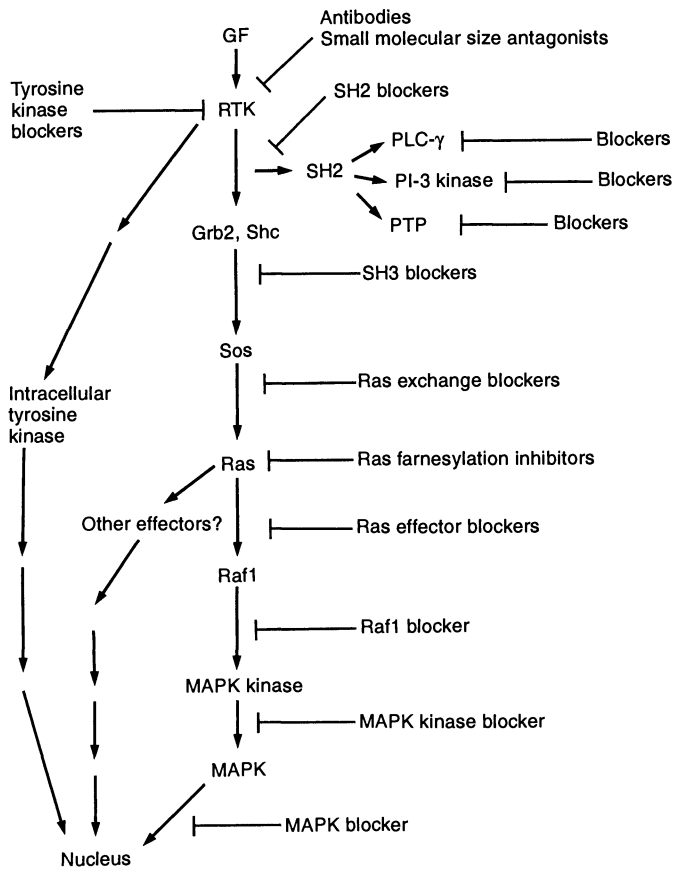


**Fig. 1.** Points of interception of PTK signaling pathways. GF, growth factor; PI, phosphatidylinositol; PTP, protein tyrosine phosphatase.

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kinase with its downstream targets. Because these interactions are often mediated by specific sequences at the Src homology 2 (SH2) domains on target proteins with the autophosphorylation sites on the RTK or the intracellular tyrosine kinase (*17*), it is possible to design or screen for inhibitory ligands. Certain adaptor molecules, such as Grb2, contain both SH2 and Src homology 3 (SH3) domains. The SH2 domain of Grb2 binds to an activated PTK, and its SH3 domain binds and recruits the Ras exchanger Sos to the membrane, which enables it to activate the Ras protein. Thus it is possible, in principle, to inhibit such interaction of SH3 domains and downstream effectors. Because the recognition sequences for SH2 domains (*18*) and for SH3 domains (*19*) are short, rapid progress in developing such antagonists is likely. Other targets are the interaction between Sos and Ras and between Ras and its effectors, such as Raf1. Farnesylation inhibitors that inhibit membrane localization of Ras have also been described (*20*). This review focuses on the development of inhibitors of PTK catalytic activity. This approach is productive because we know more about the mechanism of PTK activity (*21*) and the mode of inhibition of these inhibitors (*22*) than about any other step of the signaling cascade (Fig. 1).

## Structure of PTK Inhibitors

*Natural inhibitors*. The compounds quercetin (*23*), genistein (*24*), lavendustin A (*25*), erbstatin (*26*), and herbimycin A (*27*), isolated from fungal extracts, are tyrosine kinase inhibitors that exhibit rather broad specificity in the micromolar range. These compounds have served as a starting point for the development of many types of synthetic PTK inhibitors. Quercetin itself inhibits many other kinases, but derivatives that inhibit PTKs but not serine-threonine kinases have been described (*28*). Genistein and lavendustin A are competitive inhibitors of adenosine triphosphate (ATP) in the kinase reaction and are noncompetitive with the protein substrate. These compounds are broad-spectrum tyrosine kinase inhibitors, probably because the ATP binding domain is highly conserved among tyrosine kinases (*1*). Lavendustin A analogs, in which the core pharmacophore has been trimmed, are competitive with both the protein substrate and ATP, thus acting as bisubstrate inhibitors (*22*). The natural inhibitor herbimycin A irreversibly blocks the intracellular tyrosine kinases Src (*29*) and Bcr-Abl (*30*), the epidermal growth factor receptor (EGFR) (*31*), and the RTK HER2-ErbB2 (*32*). This irreversible inhibition is prevented by sulfhydryl reagents. Studies of the mode of action of herbimycin A on these proteins showed that labeling of proteins with herbimycin A targets them for degradation. The mechanism of action of herbimycin A differs from that of reversible inhibitors. It is likely that the kinases interact with the quinone moiety of herbimycin A, because simple benzoquinones have effects similar to those of herbimycin A (*33*). Erbstatin, a natural PTK inhibitor thought to be purely competitive with the protein substrate (*26*), was recently found to be competitive with both the substrate and ATP (*22*).

*Mechanistic considerations in designing PTK inhibitors*. The activation of receptors after growth factor binding is manifested by autophosphorylation (*1*), which exposes the active site to external substrates that in turn transmit the signal downstream. Autophosphorylation of receptors also creates specific phosphorylated tyrosine residues that serve as docking areas for downstream signal transducers. Thus, the efficacy of an inhibitor can be judged by its potency in inhibiting the initial autophosphorylation event or by its ability to inhibit the phosphorylation of downstream substrates (*34*). These efficacies may differ from each other; in the unphosphorylated state, the substrate binding domain may differ from its structure in the fully activated state. Indeed, some inhibitors are more effective in inhibiting autophosphorylation than they are in inhibiting the phosphorylation of exogenous substrates (*35*). In general, inhibitors would be expected to be less effective in inhibiting the autophosphorylation step, because in the autophosphorylation reaction the substrate concentration is high as a consequence of the proximity effect (*34*).

The potency of natural PTK inhibitors deserves further study with respect to PTK autophosphorylation and to the activity of the PTK toward exogenous substrates. Detailed kinetic analyses of the mode of action of tyrosine kinase inhibitors may also reveal whether the PTK inhibitor is competitive (or partially competitive) with ATP, with the protein substrate, or with both as a bisubstrate inhibitor (*22*). Preferably, compounds to be examined should be competitive with the protein substrate and should be potent in inhibiting autophosphorylation of the tyrosine kinase. A highly potent PTK inhibitor of the autophosphorylation reaction is likely to be a more effective agent, because efficacious inhibition of autophosphorylation should lead to a complete shutoff of the signaling pathway. Indeed, PTK blockers that have been found to inhibit autophosphorylation of EGFR also inhibit phosphorylation and activation of phospholipase C-γ (PLC-γ) as well as EGF-dependent cell proliferation (*34–37*). Other criteria for successful inhibitors are selectivity, cell permeability, bioavailability, appropriate pharmacokinetic properties, and nontoxicity.

*Synthetic inhibitors*. The naturally occurring inhibitors serve as excellent models for the design of synthetic inhibitors. Selective derivatives of quercetin have been described (*28*). Erbstatin is a promising natural compound that inhibits EGFR and Src (*26*). The benzylidene moiety of erbstatin and other arylidene compounds (*38*) were incorporated into a class of PTK blockers defined as tyrphostins (to indicate tyrosine phosphorylation inhibitors) (*34, 35*). A few similar compounds were prepared independently (*39*). Compounds belonging to the dihydroxy- and dimethoxybenzylidene malononitrile (BMN) class of PTK inhibitors have good efficacy in vivo (*40*). The recently discovered natural 3,4-dihydroxybenzene PTK inhibitor was found to be active in vitro (*41*). Lavendustin A was also found to repay further investigation, and some derivatives have been found to be more selective than the parent compound (*22, 31, 42*). Some lavendustin A analogs can discriminate between the EGFR kinase and $p56^{Lck}$ in vitro (*43*). Also, AG814, an analog of lavendustin A, is competitive in the EGFR kinase reaction with both the protein substrate and ATP (*22*). In contrast, the bulky parent compound lavendustin A (*22*) is strictly an ATP competitor (*25*). Moreover, the smaller molecular size of lavendustin analogs such as AG957 (*22, 31, 42*) allows them to permeate cells, whereas lavendustin A does not. Quinazolines such as AG1478 (Fig. 2T) are highly selective for EGFR (*44*). The amount of quinazoline required to inhibit enzyme activity by 50% ($IC_{50}$) is in the nanomolar range (*44*), whereas micromolar concentrations are required for inhibition of the HER2-ErbB2 kinase and much higher concentrations are required for inhibition of the platelet-derived growth factor receptor (PDGFR) in vitro or in intact cells (*45*). The 4,5-dianilinophthalimides (Fig. 2S) selectively inhibit EGFR and HER2-ErbB2 (*46*), and quinoxalines are selective for PDGFR (*47*). Both quinazolines (*44*) and 3,4-dianilinophthalimide (*46*) are competitive with ATP, but their exact mode of competition with respect to the protein substrate is not known.

Thiazolidinediones constitute yet another family of PTK inhibitors that inhibit EGFR and Src in vitro and in intact cells in the low micromolar range (*48*). Although many of the PTK inhibitors appear to be pure competitors with the protein substrate or pure competitors with ATP, a more thorough kinetic analysis reveals that they are actually bisubstrate inhibitors that compete with both the protein substrate and ATP (*22*). It therefore appears that selectivity can be obtained by generating inhibitors

that compete in any of these three ways. Quinazolines, for example, represent a class of competitive inhibitors with respect to ATP that are highly selective for EGFR (*44*). In a number of other instances, good selectivity for protein kinase inhibitors has also been demonstrated; a detailed analysis of Bcr-Abl shows that (i) this PTK phosphorylates tyrosine-containing polymers different from those phosphorylated by the EGFR kinase, and (ii) the two kinases are sensitive to different classes of inhibitors. Moreover, c-Abl and Bcr-Abl differ from each other in their substrate specificity (*31*, *42*). Quinoxalines (Fig. 2Q) are selective for PDGFR and do not inhibit Flk1-KDR or Src (*47*).

In some cases, PTK inhibitors that are potent on isolated PTKs in vitro are ineffective in intact cells. For example, AG494 and AG825, which are, respectively, selective for EGFR and HER2-Erb2 as isolated PTKs in vitro, are ineffective in inhibiting these receptors in intact cells unless the intracellular concentration of ATP is artificially reduced (*49*). Genistein, a pure competitive inhibitor of ATP (*24*), similarly inhibits only in the 50 to 100 μM range in tissue culture systems and shows no efficacy in vivo. Kinetic analysis of many of the PTK blockers revealed that they are competitive with the substrate (*22*, *34*, *43*) and that they preferentially inhibit the EGFR kinase as compared with the insulin receptor kinase (*34*). The BMN PTK blockers inhibit not only EGFR kinase activity but also EGF-dependent cell proliferation in tissue culture (*34*, *37*, *40*). Furthermore, RG13022 and RG14620 inhibit the growth of human squamous cell carcinoma (which overexpresses EGFR) when implanted in nude mice (*40*). RG13022 and RG14620 differ from the original arylbenzenemalononitrile in the absence of hydroxyl residues on the ring (*40*). The activity of the BMN tyrphostin AG490 in inhibiting the growth of human pre-B lymphoblastic leukemia cells in vitro and in vivo is correlated with its ability to inhibit tyrosine phosphorylation by the non-Src intracellular tyrosine kinase JAK-2 (*50*). Similarly, the BMN tyrphostin AG17, which is effective in inhibiting rabbit vascular smooth muscle cell proliferation in vitro (*51*), also inhibits restenosis in the rat carotid model (*52*).

Cells in tissue culture and in experimental animals tolerate relatively high doses of some tyrphostins, such as AG213 (RG50864), AG126, RG13022, RG14620, and AG555 (*40*, *52*, *53*). The benzene ring of the BMN compounds has been replaced by other aryl groups such as indole (*30*, *38*), quinoline (*54*), and isoquinoline (*55*). Isoquinolines, which can be viewed as cyclic BMNs (Fig. 2O), selectively inhibit EGFR rather than $p56^{Lck}$ in vitro (*55*). It is becoming apparent that although many PTK inhibitors are more selective than genistein, lavendustin A, and herbimycin A, each group inhibits several tyrosine kinases. Thus, for example, tyrphostin AG213 (RG50864), which inhibits EGFR 800 times as potently as it inhibits the insulin receptor (*34*), also inhibits several tyrosine kinases such as Bcr-Abl in vitro (*31*, *42*) and inhibits thrombin-induced activation of Src family kinases in intact platelets (*56*). RG13022, which effectively inhibits EGFR and the growth of EGFR-overexpressing tumors in vivo (*40*), also was found to inhibit the growth of breast cancer cell lines that depended on the insulin-like growth factor IGF-I (*57*). Improving the selectivity of PTK blockers remains a continuing learning process.

*Structure activity relationships of PTK inhibitors.* The core structures of most of the known PTK inhibitors are shown in Fig. 2, and data on the selectivities of some of these inhibitors are shown in Table 1. Piceatannol (Fig. 2A) is a natural product with a stilbene nucleus inhibitor (*58*), whereas nitrostyrene derivatives (Fig. 2B) are inhibitors of EGFR (*59*). The common pharmacophore for these inhibitors is the phenolic styrene (Fig. 2C), which can be viewed as a "dehydrogenated" tyrosine mimic. This pharmacophore is also part of the flavone skeleton of quercetin (Fig. 2D) and of the isoflavone skeleton of genistein (Fig. 2E). The synthesis of small molecular inhibitors was initially based on the nucleus of tyrosine itself. In designing tyrphostins (*10*, *34*), we aimed at compounds that are relatively easy to prepare and that are amenable to structure activity relationship modification. The Knoevenagel reaction (Fig.

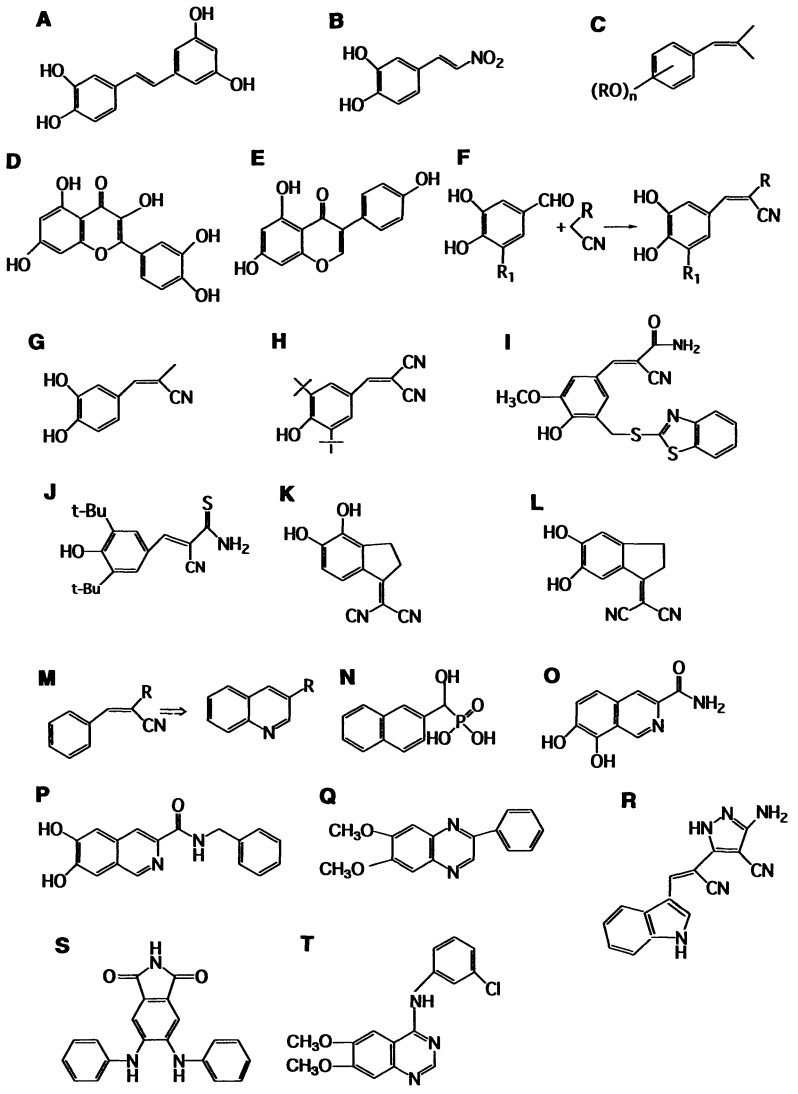
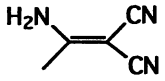



**Fig. 2.** Representative pharmacophores in tyrosine kinase inhibitors. (**A**) Piceatannol; (**B**) dihydroxynitrostyrene; (**C**) phenolic alkoxystyrene; (**D**) quercetin; (**E**) genistein; (**F**) the Knoevenagel reaction; (**G**) 3,4-dihydroxy-*cis*-cinnamonitrile; (**H**) AG17; (**I**) AG825; (**J**) AG789; (**K**) AG765; (**L**) AG308; (**M**) quinoline derivatives; (**N**) naphthalene derivatives; (**O** and **P**) isoquinoline derivatives; (**Q**) AG1296 (quinoxaline); (**R**) AG1112; (**S**) dianilinophthalimide derivatives; and (**T**) AG1478 (quinazoline).

2F) of hydroxylbenzaldehydes with malononitrile or its derivatives yielded BMNs, a family of tyrphostins with the desired properties (*10*, *34*, *35*). The basic pharmacophore in tyrphostins is 3,4-dihydroxy-*cis*-cinnamonitrile (Fig. 2G). These "first-generation" tyrphostins (*34*, *35*) (Fig. 2F), in which R = CN, $CONH_2$, $CSNH_2$, or



and $R_1$ = H, OH, or $OCH_3$, inhibited EGFR in vitro in the 0.3 to 1.0 μM range, whereas the insulin receptor was inhibited only in the millimolar range (*34*, *35*). Indeed, the natural compound BE-23372M, a dihydroxybenzylidene flavone, is a potent EGFR kinase inhibitor (*41*). By preparing conformationally restricted analogs like AG765 and AG308 (Fig. 2, K and L) we established that optimal inhibition requires the 3,4-dihydroxycatechol ring and requires the nitrile to be in a cis and coplanar orientation. When the double bond is reduced, the hydroxyls are removed, or the catechol ring is substituted with a heteroaryl ring, the affinity toward EGFR is reduced (*34*, *35*). Substitution in the 5-position of the ring, or in the β-position (with R = F, Br, I, $NO_2$, or *tert*-butyl), neither improves nor diminishes activity (*35*). Substitution of the 5-position with various alkyl-aryl groups (*49*) yielded a series of potent tyrphostins. One of them, AG825, with a benzothiazole side chain (Fig. 2I and Table 1), inhibits the HER2-ErbB2 receptor 60 times as potently as it inhibits the related EGFR in vitro, despite the similarity of these tyrosine kinases (*49*).

Several analogs exhibit good inhibitory activity in cell culture. For example, AG555 and its congeners inhibit the growth of normal and psoriatic keratinocytes (*60*) (Tables 1 and 2) and of tumor cell lines that overexpress EGFR (*35*). Dimeric tyrphostins, in which two amide derivatives are linked by oligomethylene bridges of various lengths, inhibit EGFR in the range of $IC_{50}$ = 0.1 to 1.0 μM (*61*). AG17 (Fig. 2H) is a potent but nonselective inhibitor of several tyrosine kinases. A slight modification yields AG879 (Fig. 2J), which inhibits nerve growth factor (NGF)–dependent Trk tyrosine phosphorylation in PC-12 cells ($IC_{50}$ ~ 40 μM) (*62*) and inhibits HER2-ErbB2 kinase in a number of breast and ovarian cancer cell lines ($IC_{50}$ ~ 0.5 μM) (*63*). Another family of tyrphostins, derived from AG1112 (Fig. 2R and Table 1), inhibits Bcr-Abl in K562 cells and induces those cells to undergo terminal erythroid differentiation (*30*, *42*).

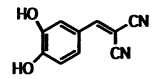
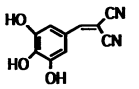
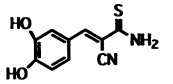
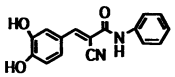
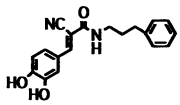
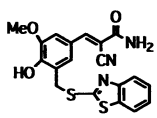
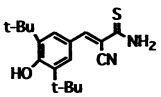
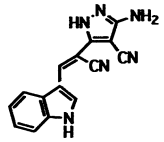
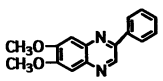
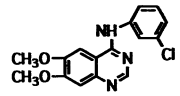
The planar bicyclic structure of flavones, and the improved potency of rigid tyrphostins such as AG308 (Fig. 2L) compared with the open-structure tyrphostins, suggest that rigid bicyclic analogs formed by incorporating the cyanostyryl radical into the ring (Fig. 2M) could yield good inhibitors. The naphthalene derivative with no nitrogen in the ring (Fig. 2N) inhibits autophosphorylation of the insulin receptor only at high concentrations (~200 μM) (*64*). Quinolines are moderate EGFR blockers with no effect on the insulin receptor (*54*). An isoquinoline derivative (Fig. 2O) inhibits its $p56^{Lck}$ autophosphorylation ($IC_{50}$ ~ 0.5 μM) and inhibits EGFR ($IC_{50}$ > 100 μM). A close analog (Fig. 2P) exhibited the opposite selectivity ($IC_{50}$ = 3.1 μM for EGFR; $IC_{50}$ > 100 μM for $p56^{Lck}$) (*55*). AG1478, a quinazoline, represents yet another class of highly selective EGFR inhibitors (Table 1) (*44*). Quinoxalines such as AG1296 (Fig. 2Q and Table 1) selectively inhibit PDGFR at 0.3 μM in intact cells (*47*) and do not inhibit EGFR, the vascular endothelial cell growth factor receptor (VEGFR), and c-Src (*47*).

## Biological Activities of Tyrphostins

Because PTKs participate in various proliferative diseases, we and others have explored the activity of PTK inhibitors as antiproliferative agents. Table 2 lists a variety of diseases in which PTKs are implicated, the mechanisms of PTK involvement for these diseases, and the PTK inhibitors that were found to inhibit the biological effects of these tyrosine kinases. The following sections discuss the potential of these PTK inhibitors in drug development.

*Cancer*. Because PTKs participate in the establishment and progression of many cancers, PTK inhibitors have potential in the

**Table 1.** Selectivity of PTK inhibitors in vitro. InsR, insulin receptor; N, not determined.

| Tyrphostin | | PTKs and $IC_{50}$ values (μM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | EGFR | HER2-Neu | PDGFR | Trk | $p210^{Bcr-Abl}$ | InsR |
| AG18 |  | 35 | N | 25 | >100 | 75 | 4000 |
| AG82 |  | 3 | N | N | >100 | 3.6 | N |
| AG213 |  | 0.8 | N | 3 | >100 | 6 | 640 |
| AG494 |  | 0.7 | 42 | 6 | N | 75 | >100 |
| AG555 |  | 0.7 | 35 | N | N | N | >100 |
| AG825 |  | 19 | 0.35 | 40 | N | 75 | >100 |
| AG879 |  | >500 | 1.0 | >100 | 10 | N | N |
| AG1112 |  | 18.5 | N | N | N | 0.8 | N |
| AG1296 |  | >100 | >100 | 0.5 | N | >50 | N |
| AG1478 |  | 0.003 | >100 | >100 | N | >50 | N |

development of antineoplastic agents. PTK inhibitors have been studied extensively in tissue culture systems of transformed cells and in vivo. Potent PTK inhibitors of the EGFR kinase arrest the growth of cells that overexpress EGFR (*34–37, 40, 46*) and inhibit tumors that overexpress EGFR. For example, tyrphostins RG13022 and RG14620 inhibit the growth of human squamous cell carcinoma implanted in nude mice (*40*). 4,5-Dianilinophthalimides inhibit the growth of cells from human tumors that overexpress EGFR or HER2-ErbB2 and exhibit good antitumor activity in mice in which these tumors are grown as xenografts. Quinazolines such as PD153035 and AG1478 (Fig. 2T) are highly selective for EGFR and inhibit the EGFR kinase in the nanomolar range (Table 1) (*44*). Quinoxalines such as AG1296 (Fig. 2Q) inhibit the kinase activity of PDGFR and reverse the transformation of Swiss 3T3 cells by the *sis* oncogene (*47*). Tyrphostins such as AG213 and AG82 reverse the transformation of chicken lens cells by v-Src as well as that of NIH 3T3 cells by the activated mutant $pp^{60Src\text{-}F527}$ (*65*). This reversal of transformation correlates with the inhibition of tyrosine phosphorylation of intracellular substrates. Tyrphostin AG17 inhibits human pancreatic cancer cell lines (*66*), and AG1112 (Table 1) inhibits the phosphorylation of Bcr-Abl and of a few intracellular protein substrates in correlation with its potency to induce erythroid differentiation of K562 cells (*30*).

These results suggest that tyrphostins might be used to purge leukemic cells in patients with chronic myelogenous leukemia (CML) and might prolong the survival of patients at the chronic stage by eliminating cells that harbor the Philadelphia chromosome. Tyrphostin AG568 induces erythroid differentiation of mouse erythroleukemic cells in correlation with its inhibition of tyrosine phosphorylation of at least one protein (*67*). The selective inhibition of the proliferation of human pre-B acute lymphoblastic leukemia (ALL) cells by AG490 is in excellent correlation with its inhibition of intracellular tyrosine kinases. AG490 also has been shown to have excellent efficacy in vivo; its use resulted in a total cure of established ALL in mice with SCID (severe combined immunodeficiency disease) (*50*).

*Restenosis.* Tyrphostins that inhibit PDGFR have potential in the development of drugs that inhibit restenosis, because PDGF and its receptor contribute to the formation of the atherosclerotic plaque (*6*). Because basic fibroblast growth factor (bFGF) and cytokines also take part in the formation of the atherosclerotic plaque, relatively broad-spectrum PTK inhibitors may be useful. Indeed, AG213 (RG50864) and AG18 inhibit the effect of the proliferative signals of both bFGF (*68*) and PDGF on rabbit vascular smooth muscle cells (*51*) and human bone marrow fibroblasts (*38*) and also inhibit thrombin-induced platelet aggregation (*56*). Thus, the therapeutic synthesis might include tyrphostins with a relatively broad spectrum that inhibit PDGFR, fibroblast growth factor receptor (FGFR), and nonreceptor PTKs that are activated during formation of the atherosclerotic plaque (*6*). Plaque formation is accompanied by an inflammatory component that is also mediated by intracellular tyrosine kinases, whose activities could be inhibited. It has been shown that tyrphostins effectively inhibit the activation of B cells (*69*), T cells (*70*), macrophages (*71*), neutrophiles, mast cells, basophiles, and monocytes (*72*). Therefore, broad-specificity tyrphostins may be especially well suited for treating restenosis. Indeed, the nonselective tyrphostin AG17 (RG50872), which is effective as an antiproliferative agent on rabbit smooth muscle vascular cells grown in vitro (*51*), seems to fit this purpose. When restenosis is induced in the rat carotid artery and AG17 is applied from an engrafted polymer mantle encompassing the artery, the process of the disease is inhibited by about 70% (*52*).

*Psoriasis and other skin conditions.* Psoriatic lesions are typified by a few molecular indicators that strongly implicate the hyperactivity of PTKs. These include amplification of the gene for transforming growth factor–α (TGF-α) or for amphiregulin, or both, which leads to the persistent activation of EGFR on the psoriatic keratinocyte (*7*); the involvement of interleukin-6 (IL-6) (*73*) as the result of activation of cellular tyrosine kinases (*74*); and the involvement of IGF-I (*75*). We therefore examined the effect of tyrphostins on the growth of normal and psoriatic keratinocytes (*60*). Tyrphostins AG555 and AG18 arrest the growth of these cells without adverse cytotoxic effects. Because psoriasis affects up to 3% of the population, many people will benefit if compounds such as AG555 can be developed into effective antipsoriatic ointments. Such an approach can, in principle, be extended to other skin conditions such as Kaposi's sarcoma and papilloma, in which a number of PTKs have been implicated (*76*).

*Sepsis and other inflammatory conditions.* Lipopolysaccharide (LPS) is a Gram-nega-

**Table 2.** Selected diseases, the PTKs that are implicated in their progress, and tyrphostins (with reference numbers) that act as PTK inhibitors and have potential for drug development.

| Disease | PTK implicated | Mechanism of PTK involvement | Tyrphostin |
|---|---|---|---|
| Certain cancers (squamous cell carcinoma) | EGFR | Amplification of the gene and overexpression of EGFR | RG13022 (*40*)<br>AG1478 (*44*)<br>3,4-dianilinophthalimide (*46*) |
| Psoriasis | EGFR | Overexpression of the amphiregulin or TGF-α gene, or both, leading to persistent autocrine stimulation of EGFR | AG213<br>AG555 (*60*) |
| Mammary and ovary carcinomas | HER2-Neu | Gene amplification and overexpression of the HER2 protein | AG825 (*49*)<br>AG1377 (*61*)<br>AG879 (*61*) |
| Atherosclerosis, restenosis, pulmonary fibrosis | PDGFR | Stimulation of PDGFR by pathological release of PDGF (restenosis) | AG1295 (*47*)<br>AG1296 (*47*) |
| Gliomas, glioblastomas | PDGFR | Amplification of PDGFR; coexpression of PDGF and PDGFR in the tumor | AG17 (*51, 52*)<br>AG370 (*51*) |
| CML | $p210^{Bcr\text{-}Abl}$ and $p185^{Bcr\text{-}Abl}$ | Chromosome rearrangement that results in fusion of Bcr and p140c-Abl, leading to enhanced kinase activity | AG1112 (*30, 31*)<br>AG957 (*30, 31*) |
| ALL | JAK-2 | Enhanced tyrosine phosphorylation of intracellular proteins | AG490 (*50*) |
| Sepsis and other inflammatory conditions | LPS, TNF-α dependent phosphorylation on tyrosines | Probably enhanced activity of cytoplasmic PTKs from the Src family | AG126 (*53*)<br>AG556 (*53*) |

tive bacterial endotoxin that causes the development of sepsis (septic shock) (*77*). This condition afflicts about 400,000 patients annually in the United States alone, and of the 200,000 hospitalized, 30 to 50% die (*77*). LPS induces tyrosine phosphorylation in target cells (*8*, *78*); it also activates macrophages, inducing them to produce tumor necrosis factor α (TNF-α) and IL-1, which are important mediators of sepsis and of other inflammatory responses such as rheumatoid arthritis (*79*). TNF-α and IL-1 cause many of the symptoms of sepsis (*80*), and, like LPS, they induce tyrosine phosphorylation in target cells (*81*). Tyrphostins may therefore offer a double block by inhibiting LPS-induced TNF-α production as well as the action of TNF-α. Tyrphostins such as AG126 offer complete protection when injected into mice 2 hours before a lethal dose of LPS, and partial protection when injected at the same time as LPS (*53*). More potent tyrphostins such as AG556 offer nearly complete protection even when injected 2 hours after LPS. The protective tyrphostins inhibit LPS-induced TNF-α production, tyrosine phosphorylation of mitogen-activated protein kinase (MAPK), and production of NO in mouse macrophages (*53*). Inhibition of LPS-induced tyrosine phosphorylation and NO production also was reported for tyrphostin AG82, herbimycin A, and genistein (*71*, *82*). These successes with tyrphostins in model systems for sepsis suggest that they may be useful in the management of other inflammatory diseases, such as rheumatoid arthritis, in which TNF-α is an important mediator (*83*).

*Tyrosine kinase inhibitors in combination with other drugs*. Because tyrphostins and other PTK inhibitors exhibit different selectivities, various combinations of these compounds might be useful. In most of the conditions described above, a number of tyrosine kinases are involved. Thus, an inhibitor with broad specificity or a combination of PTK inhibitors may be most effective. Tyrphostins also synergize with antibodies to EGFR to inhibit the growth of squamous cell carcinoma in vivo (*40*) and with *cis*-platin to block the growth of human cancer cells that overexpress HER2-ErbB2 (*84*). Another opportunity to be explored is the use of the antiestrogen ICI182,780 (*85*), in combination with tyrosine kinase inhibitors and cytotoxic drugs, to treat breast and ovarian cancers.

## Conclusion

Approaches to drug design have become refocused in response to our rapidly emerging understanding of the role of signaling pathways in health and disease. Identification of receptors, enzymes, and adaptor proteins that mediate proliferative metabolic and inflammatory signals provide targets for drug design. In this review we have examined PTK inhibitors as potential drugs for a variety of disease states. The recent publication of the structure of the insulin receptor kinase domain (*86*) and its extensive homology to other PTKs will probably enhance the rational design of other PTK blockers. The reported success of tyrosine kinase inhibitors and inhibitors of other signaling molecules (*87*) suggests that such agents may be useful in the treatment of disease.

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RESEARCH ARTICLE

# Induction of Ectopic Eyes by Targeted Expression of the *eyeless* Gene in *Drosophila*

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The *Drosophila* gene *eyeless* (*ey*) encodes a transcription factor with both a paired domain and a homeodomain. It is homologous to the mouse *Small eye* (*Pax-6*) gene and to the *Aniridia gene* in humans. These genes share extensive sequence identity, the position of three intron splice sites is conserved, and these genes are expressed similarly in the developing nervous system and in the eye during morphogenesis. Loss-of-function mutations in both the insect and in the mammalian genes have been shown to lead to a reduction or absence of eye structures, which suggests that *ey* functions in eye morphogenesis. By targeted expression of the *ey* complementary DNA in various imaginal disc primordia of *Drosophila*, ectopic eye structures were induced on the wings, the legs, and on the antennae. The ectopic eyes appeared morphologically normal and consisted of groups of fully differentiated ommatidia with a complete set of photoreceptor cells. These results support the proposition that *ey* is the master control gene for eye morphogenesis. Because homologous genes are present in vertebrates, ascidians, insects, cephalopods, and nemerteans, *ey* may function as a master control gene throughout the metazoa.

The *eyeless* (*ey*) mutation of *Drosophila* was first described in 1915 (*1*) on the basis of its characteristic phenotype, the partial or complete absence of the compound eyes. The *ey* alleles available today are recessive hypomorphs (weak alleles) and they lead to the reduction or complete absence of the compound eyes but do not affect the ocelli (*simple eyes*) on the head of the fly. Apparent null alleles that are lethal when homozygous have also been isolated (*2*), but they have been lost, and a detailed analysis of their phenotype is not available. Cloning and sequencing of the *ey* gene (*3*) have shown that it encodes a transcription factor that contains both a paired domain and a homeodomain. The *ey* gene is homologous to *Small eye* (*Sey* = *Pax-6*) in the mouse and to *Aniridia* in humans. The proteins encoded by these genes share 94 percent sequence identity in the paired domain, and 90 percent identity in the homeodomain and they contain additional similarities in the flanking sequences. Furthermore, two out of three splice sites in the paired box and one out of two splice sites in the homeobox are conserved between the *Drosophila* and the mammalian genes, which indicates that these genes are orthologous.

Both the mouse and the *Drosophila* gene have similar expression patterns during development. In the mouse, the expression of *Sey* is observed in the spinal cord, in discrete regions of the brain, and in the developing eye. The *Sey* gene is expressed from the earliest stages until the end of eye morphogenesis: first, in the optic sulcus, and subsequently in the eye vesicle, in the lens, in the differentiating retina, and finally in the cornea (*4*). In *Drosophila*, *ey* is first expressed in the embryonic ventral nerve cord and in defined regions of the brain. Later in embryogenesis, *ey* is transcribed in the embryonic primordia of the eye as soon as these cells can be detected. In subsequent larval stages, it continues to be expressed in the developing eye imaginal discs. During the third larval stage, *ey* expression becomes largely restricted to the part of the eye disc that is anterior to the morphogenetic furrow. This region consists of undifferentiated cells whereas posterior to the furrow the differentiating ommatidia are apparent (*5*). Because mutations in the mouse and *Drosophila* genes lead to a reduction or complete absence of all eye structures, and because these genes are similar in DNA sequence and in expression pattern even at the earliest stage of eye development, it has been suggested that *ey* and *Sey* may be the master control genes involved in eye morphogenesis (*3*). Furthermore, mutations in four other *Drosophila* genes with similar phenotypes (*eyes absent*, *sine oculis*, *eye gone*, and *eyelisch*) do not affect the expression pattern of *ey*, which indicates that *ey* acts upstream of these other genes (*6*). These results are consistent with its possible role as a gene that controls eye morphogenesis, even though it may have additional functions in the developing nervous system. The cloning of the homologous genes from ascidians, cephalopods, and nemerteans (ribbon worms) suggests that this gene may be present in all metazoa (*3*).

Master control genes that act as developmental switches can be detected on the basis of their mutant phenotypes. Thus, homeotic mutations have identified master control genes that specify the body plan along the antero-posterior axis. These genes, which are characterized by a homeobox, are clustered in the Antennapedia (*Antp*) and Bithorax Complexes in *Drosophila*, and in the Hox gene clusters of the mouse (*7*). Loss- and gain-of-function mutations in these genes lead to opposite homeotic transformations. For example, in *Antp*, recessive loss-of-function mutations are lethal at the embryonic or larval stage and lead to a transformation of the second thoracic segment (T2) toward the first thoracic segment (T2→T1). Dominant gain-of-function mutations lead to a transformation in the opposite direction, that is from the anterior head and T1 segments toward T2 (H,T1→T2) (*8*). These transformations can be explained by the combinatorial interaction of several homeotic genes in order to specify a given body segment. These genes have partially overlapping expression domains in several body segments and each segment is specified by a combination of homeobox genes, that is by a Hox code (*9*). By ubiquitous (ectopic) expression of *Antp* under the control of a heat-shock promoter, we have changed the body plan of *Drosophila* and induced the formation of middle legs in place of the antennae, and

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