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Tyrosine Kinase Inhibitors Against EGF Receptor-Positive Malignancies

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1. Introduction

The role of protein tyrosine kinases (PTKs) in the survival of cancer cells and their potential use in anticancer therapy has led to their selection as anticancer drug targets. Tyrosine kinases which are being studied for this purpose include epidermal growth factor receptor (EGFR) *(1–6)*, Janus kinases (JAKs) *(7–13)*, Bruton's tyrosine kinase (BTK) *(14–16)*, platelet-derived growth factor (PDGF) *(17)*, protein kinase C (PKC) *(18–24)*, Lck *(25,26)*, Trk *(27–30)*, and others. The strategies used to attenuate or disable kinases implicated in cancer include the use of antibodies, immunoconjugates, ligand-binding cytotoxic agents, and small-molecule inhibitors. Each of these strategies has shown some promise for the treatment of cancer. Herceptin *(31–35)*, for example, is an immunotherapeutic agent that binds to the extracellular domain of HER2 (also referred to as ErbB-2, a tyrosine kinase belonging to the same family as EGFR) at nanomolar levels. EGF-genistein (EGF-gen) is an EGFR-binding cytotoxic agent that also shows potency in the nanomolar range *(2,36)* and will be discussed in this chapter. The search for new small molecules that inhibit kinases has involved traditional approaches, including the testing of natural products, random screening of chemical libraries, the use of classical structure–activity relationship studies, and the incorporation of structure-based drug design approaches and combinatorial chemistry techniques. As a result, several promising small-molecule inhibitors have also been identified in recent years that may prove useful as potent new anticancer drugs.

Small-molecule inhibitors of kinases that show promise as anticancer agents include inhibitors of EGFR. EGF exerts pleiotropic biologic effects by binding

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to ErbB-1 *(37–40)*. In breast cancer, the expression of EGFR is a significant and independent indicator for recurrence and poor survival *(41–43)*. Recent studies provided evidence that EGFR serves as an endogenous negative regulator of apoptosis in breast cancer cells *(2)*. Many classes of small molecules have been reported in recent years that inhibit EGFR kinase *(1,3–6,44–47)*. Genistein (**Fig. 1**), a naturally occurring isoflavone found in soybeans, is an inhibitor of EGFR *(6)*. When genistein is linked to EGF, the potency against EGFR increases from an IC_{50} value of >10 μ*M* to a value in the nanomolar range *(2)*.

Pyrazolopyrimidines (*see* **Fig. 1**) were found to inhibit EGFR with IC_{50} values ranging around 1–8 n*M* *(3)*. Two pyrazolopyrimidines with reported IC_{50} values below 10 n*M* *(3)* also showed high selectivity towards some nonreceptor tyrosine kinases (c-*Src*, v-*Abl*, and serine/threonine kinases such as PKC-α and CDK1). The quinazoline derivative CP-358,774 *(45)* inhibits EGFR with an IC_{50} of 2 n*M* and reduces EGFR autophosphorylation in intact tumor cells with an IC_{50} of 20 n*M*. This inhibition is selective for EGFR relative to other tyrosine kinases examined as determined by assays of isolated kinases and whole cells. Despite the reported profound in vitro potency (K_i = 5 p*M*) and selectivity of the ATP-competitive brominated quinazoline derivative PD153035 (**Fig. 1**; *4,5*), the compound failed to show significant in vitro or in vivo efficacy against cancer cells. Other quinazolines reported include PD168393 and PD160678, which selectively target and irreversibly inactivate EGFR through covalent modification of a cysteine (Cys^{773}) residue present in the ATP-binding pocket *(44)*. These compounds also interact in an analogous fashion with ErbB2 (which has a conserved Cys residue at the same position) but have no activity against IR, PDGF receptor, FGFR, and PKC. The compounds have not been tested against BTK and JAK3, which also contain conserved cysteine residues at the corresponding position.

A series of new quinazoline compounds targeting EGFR have been designed more recently using structure-based methods. In this study, a three-dimensional model of the kinase domain of EGFR was constructed *(1)* using known coordinates of homologous kinase domains as reference coordinates (Hematopoietic cell putative protein tyrosine kinase [HCK; *48*], fibroblast growth factor receptor [FGFR; *49,50*], and insulin receptor kinase [IRK; *51*]). The EGFR model was used along with an inhibitor docking procedure for the rational design of compounds predicted to bind favorably to EGFR. The EGFR model indicated that inhibition may be significantly improved by increasing the size of the functional groups attached to the 4-anilinoquinazoline molecular scaffold. Chemically relevant substitutions at the 3', 4' and 5' positions on the anilino ring lead to the successful design of a dibromo quinazoline derivative, WHI-P97, with an IC_{50} value of 2.5 μ*M* in EGFR kinase inhibition assays. WHI-P97 effec-

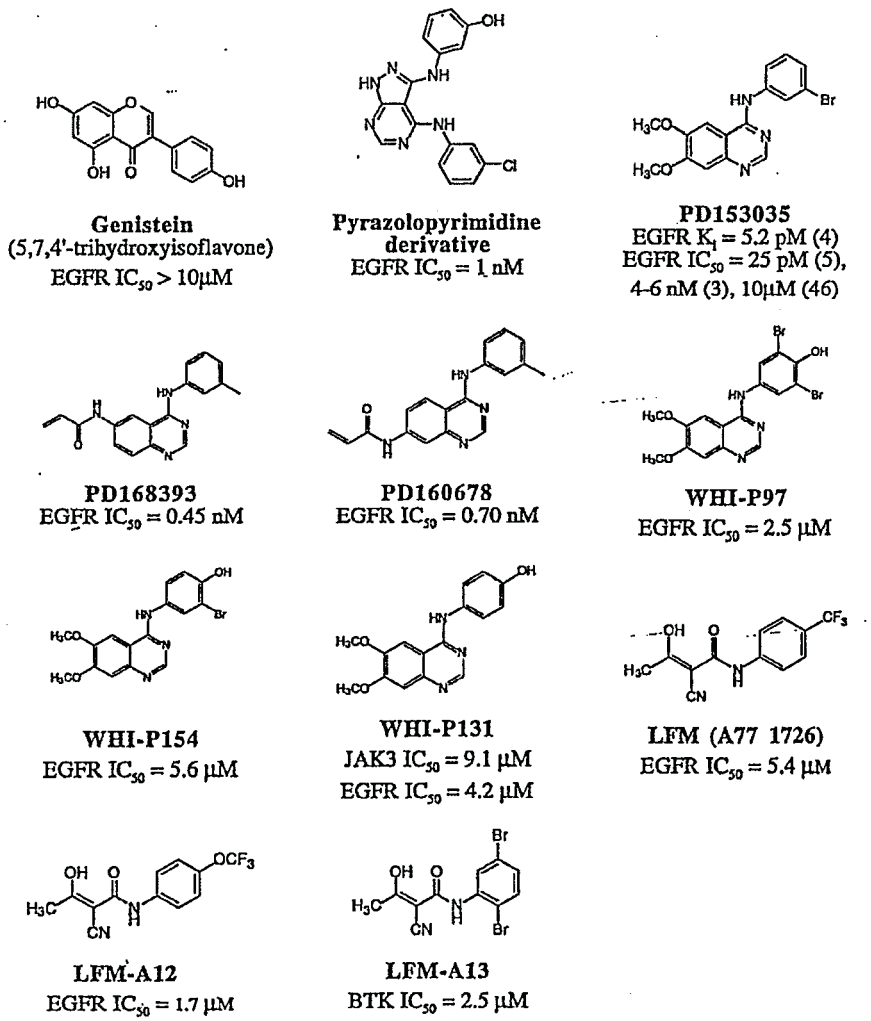

Fig. 1. Examples of tyrosine kinase inhibitors.

tively inhibited the in vitro invasiveness of EGFR-positive human cancer cells in a concentration-dependent manner. The quinazoline derivatives WHI-P97, WHI-P131, WHI-P154, WHI-P180, and WHI-P197 with 3' or 4'OH substitution on the anilino moiety were predicted to form an additional hydrogen bond with Asp^{831} in the ATP-binding region of EGFR that may enhance binding. The EGFR inhibition values for WHI-P97, WHI-P131, WHI-P154, WHI-P180, and WHI-P197 ranged from 2.5 to 5.6 μM in kinase assays. However, the

quinazolines tested were not specific for EGFR. For example, the EGFR inhibitor WHI-P154 (EGFR IC_{50} = 5.6 μ*M*) also inhibited other tyrosine kinases such as HCK (IC_{50} = 12 μ*M*), JAK3 (IC_{50} = 130 μ*M*) and spleen tyrosine kinase (SYK) (IC_{50} = 150 μ*M*) *(46)*.

In terms of selectivity for EGFR, leflunomide metabolites may show the most promise. Earlier studies reported that the immunosuppressive activity of leflunomide is due to its metabolite A77 1726 (α-cyano-β-hydroxy-β-methyl-*N*-[4–(trifluromethyl)phenyl]-propenamide, or LFM), which is rapidly formed in vivo, functions as a pyrimidine synthesis inhibitor *(52)* and also inhibits the tyrosine kinase activity of EGFR *(53)*. The leflunomide metabolite analog LFM-A12 (**Fig. 1**) showed inhibition of EGFR with an IC_{50} value of 1.7 μ*M* and killed >99% of human breast cancer cells in vitro by triggering apoptosis *(1)*. Both LFM-A12 and WHI-P97 inhibited the in vitro invasiveness of EGFR-positive human breast cancer cells at micromolar concentrations and induced apoptotic cell death. In addition, LFM-A12 inhibited the proliferation (IC_{50} = 26.3 μ*M*) and in vitro invasiveness (IC_{50} = 28.4 μ*M*) of EGFR-positive human breast-cancer cells in a concentration-dependent fashion.

Like the quinazolines WHI-P97 and WHI-P154, the design of LFM-A12 was aided by a model of the EGFR kinase domain. In kinase assays, LFM-A12 was found to be specific for EGFR and did not inhibit other PTKs such as BTK, HCK, JAK1, JAK3, IRK, and SYK at concentrations ranging from 175 to 350 μ*M*. The observed selectivity of LFM-A12 for EGFR likely results from its molecular shape and from favorable interactions with unique EGFR residues that are not present in the kinase domains of other PTKs. Likewise, unfavorable interactions with unique residues of other PTKs that are not found in the EGFR kinase domain may also contribute to this selectivity. This observation is in contrast to the observed inhibition of several kinases (EGFR, HCK, JAK3 and SYK) by WHI-P154. The first contributing factor for the nonselectivity of WHI-P154 may be the inhibitor's complementary shape with the hinge region of the binding cavity of all seven kinases, which in turn leads to favorable hydrophobic contact between the compound and the residues in this cavity. Additionally, predicted hydrogen bonding interactions with all seven kinases may enhance its binding with each of them.

The structure-based method used to design leflunomide inhibitors of EGFR was also successful for the identification of small-molecule inhibitors of JAK3 *(7)*. JAK3 is expressed abundantly in primary leukemic cells from children with acute lymphoblastic leukemia (ALL). The construction of a three-dimensional model of JAK3 *(7)* was used to design a quinazoline inhibitor, WHI-P131 (**Fig. 1**), shown to have specificity for JAK3. WHI-P131 inhibited JAK3 (IC_{50} = 9.1 μ*M*) but not JAK1 or JAK2 and did not inhibit the ZAP/SYK-family tyrosine kinase SYK, TEC-family tyrosine kinase BTK, *Src*-family tyrosine kinase LYN, or

the receptor-family tyrosine kinase IRK, even at concentrations as high as 350 μ*M* *(7)*. WHI-P131 induced apoptosis in JAK3-expressing human leukemia cell lines but not in JAK3-negative melanoma or squamous carcinoma cells.

Another study to identify kinase inhibitors focused on inhibitors of BTK as antileukemic agents with apoptosis-promoting properties *(14)*. A three-dimensional homology model of the BTK kinase domain was constructed *(14)* and inhibitor docking procedures led to the identification of an LFM analog, LFM-A13 (**Fig. 1**), which was found to be a potent and specific inhibitor of BTK. LFM-A13 inhibited recombinant BTK with an IC_{50} value of 2.5 μ*M*, but it did not affect the enzymatic activity of other protein tyrosine kinases including JAK1 and JAK2, *Src*-family kinase HCK, and receptor-family tyrosine kinases EGFR and IRK, at concentrations as high as 278 μ*M*. LFM-A13 also enhanced the chemosensitivity of BTK-positive B-lineage leukemia cells to vincristine and ceramide.

Although several agents have been identified in recent years that inhibit tyrosine kinases such as EGFR, JAK3, and BTK, a future challenge is to ensure the specificity of inhibitors for one targeted tyrosine kinase. A successful strategy to accomplish this involves conjugating small-molecule inhibitors to ligand-binding entities; this enables the inhibitor to be delivered to a specific tyrosine kinase. An example of this strategy is to link a kinase inhibitor (soybean-derived genistein, 5,7,4'-trihydroxyisoflavone) to a protein (recombinant human EGF) that binds to a receptor kinase (EGFR). The resulting protein–inhibitor conjugate is an EGFR-directed cytotoxic agent (EGF-gen) with PTK inhibitory activity *(2,36)*, which will be described in further detail in this chapter. (A similar method was successfully applied to the targeted delivery of genistein to CD19-receptor–associated vital PTK and shows considerable promise for more effective treatment of human leukemias and lymphomas *[25,54]*)

2. Materials and Methods

2.1. Structure-Based Design of Small-Molecule Inhibitors of EGFR

The three-dimensional coordinates of the EGFR kinase domain used in protein–inhibitor modeling studies (**Fig. 2**) were constructed based on a structural alignment of the sequence of EGFR with the sequences of known crystal structures of other protein kinases (kinase domains of HCK *[48]*, FGFR *[50]*, IR *[55]*, and cAPK *[56]*) as described previously *(1)*. The procedure was also used to construct homology models for JAK1, JAK3 *(7)*, BTK *(14)*, and SYK (Mao, C., unpublished data). Molecular docking and scoring procedures were used to estimate binding of inhibitors in the catalytic site of EGFR (**Tables 1** and **2**, **Figs. 3** and **4**) *(1,47)*. Leflunomide metabolite analogs such as LFM-A12 (**Scheme 1**) and quinazoline compounds such as WHI-P97 (**Schemes 2&3**) were synthesized, and their ability to inhibit EGFR in breast cancer cells was tested as previously described *(1,57)*.

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