Specificity of α-Cyano-β-Hydroxy-β-Methyl-N-[4-(Trifluoromethoxy) Phenyl]-Propenamide as an Inhibitor of the Epidermal Growth Factor Receptor Tyrosine Kinase

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ABSTRACT

The epidermal growth factor receptor (EGFR) tyrosine kinase has an essential function for the survival of human breast cancer cells. In a systematic effort to design potent and specific inhibitors of this receptor family protein tyrosine kinase (PTK) as antibrust cancer agents, we recently reported the construction of a three-dimensional homology model of the EGFR kinase domain. In this model, the catalytic site is defined by two β-sheets that form an interface at the cleft between the NH2-terminal and COOH-terminal lobes of the kinase domain. Our modeling studies revealed a distinct, remarkably planar triangular binding pocket within the kinase domain with approximate dimensions of 15 Å × 12 Å × 12 Å, and the thickness of the binding pocket is ~7 Å with an estimated volume of ~600 Å3 available for inhibitor binding. Molecular docking studies had identified α-cyano-β-hydroxy-β-methyl-N-[4-(trifluoromethoxy)phenyl]-propenamide (LFM-A12) as our lead inhibitor, with an estimated binding constant of 13 μM, which subsequently inhibited EGFR kinase in vitro with an IC50 value of 1.7 μM. LFM-A12 was also discovered to be a highly specific inhibitor of the EGFR. Even at very high concentrations ranging from 175–350 μM, this inhibitor did not affect the enzymatic activity of other PTKs, including the Janus kinases JAK1 and JAK3, the Src family kinase HCK, the Tec family member Bruton’s tyrosine kinase, SYK kinase, and the receptor family PTK insulin receptor kinase. This observation is in contrast to the activity of a quinazoline inhibitor tested as a control, 4-(3-bromo, 4-hydroxyanilino)-6,7-dimethoxyquinazoline, which was shown to inhibit EGFR and other tyrosine kinases such as HCK, JAK3, and SYK.

INTRODUCTION

EGF is a 53-amino acid, single-chain polypeptide (M, 6,216,000) that exerts biological effects by binding to a specific cell membrane EGFR/ErbB-1 (1–4). EGF is a 170-kDa protein, composed of a cysteine-rich, glycosylated extracellular ligand binding domain, a short transmembrane domain, and an intracellular domain, which has tyrosine kinase activity (5). Binding of EGF to the EGFR/ErbB-1 results in receptor dimerization with itself or other members of the Erb-B (subtype I) transmembrane PTK family (e.g., Erb-B2, Erb-B3), resulting in activation and autophosphorylation of the PTK domain (6, 7). Many types of cancer cells display enhanced EGFR expression on their cell surface membranes (3). Enhanced expression of the EGFR on cancer cells has been associated with excessive proliferation and metastasis (4). Examples include breast cancer, prostate cancer, lung cancer, head and neck cancer, bladder cancer, melanoma, and brain tumors (3). In breast cancer, expression of the EGFR is a significant and independent indicator for recurrence and poor relapse-free survival (8–10). More recently, we and others have shown that the EGFR has an essential function for the survival of human breast cancer (11, 12). Therefore, the development of PTK inhibitors that abrogate the enzymatic function of the EGFR tyrosine kinase has become a focal point in drug discovery research programs aimed at designing more effective treatment strategies for metastatic breast cancer (2, 3, 13, 14).

In a systematic effort to design potent inhibitors of EGFR as antibrust cancer agents, we constructed a three-dimensional homology model of the EGFR kinase domain (Fig. 1A) and reported (15) a potent inhibitor of the EGFR tyrosine kinase, LFM-A12, targeting the catalytic site of the EGFR kinase (Fig. 1B). Here, we report that LFM-A12 is a highly specific inhibitor of the EGFR that does not affect the enzymatic activity of other PTKs, including the receptor family PTK IR kinase, the Src family kinase HCK, SYK kinase, the Janus kinases JAK1 and JAK3, and the Tec family kinase BTK, even at concentrations as high as 350 μM. This novel LFM analogue inhibited the proliferation of EGFR-positive human breast cancer cells at micromolar concentrations (15). This observation is in contrast to the activity of a quinazoline inhibitor tested as a control [4-(3-bromo, 4-hydroxyanilino)-6,7-dimethoxyquinazoline], which was shown to inhibit EGFR as well as other tyrosine kinases such as HCK, JAK3, and SYK. The identification of LFM analogues as EGFR inhibitors with antibrust cancer activity may

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The abbreviations used are: EGF, epidermal growth factor; EGFR, EGFR receptor; PTK, protein tyrosine kinase; LFM-A12, α-cyano-β-hydroxy-β-methyl-N-[4-(trifluoromethoxy)phenyl]-propenamide; IR, insulin receptor; WHI-P154, 4-(3-bromo, 4-hydroxyanilino)-6,7-dimethoxyquinazoline; FGFR, fibroblast growth factor receptor.
provide the basis for more effective cancer treatment modalities for breast cancer patients with metastatic disease.

MATERIALS AND METHODS

Chemical Synthesis of LFM-A12 and WHI-P154. The synthesis and characterization of LFM-A12 has been published (15), and WHI-P154 was synthesized and characterized using procedures published previously (16). The chemical structures of LFM-A12 and WHI-P154 are shown in Table 1.

Molecular Modeling Studies. The homology model for the EGFR kinase domain (Fig. 1A) was constructed as described previously (15) based on a structural alignment of the sequence of EGFR (accession #P00533, SWISS-PROT; University of Geneva, Geneva, Switzerland) obtained from GenBank (National Center for Biotechnology Information, Bethesda, MD) with the sequences of known crystal structures of other protein kinases [kinase domains of HCK (17), FGFR (18), IR (19), and cAPK (20)]. The modeling was carried out on a Silicon Graph-
Structure-based Design of a Specific EGFR Inhibitor

BTK, JAK1, or JAK3 were performed as reported previously. Inactive assays of recombinant proteins from insect cells expressing JAK3, and SYK. Inhibitors into the catalytic sites of IR, HCK, BTK, JAK1, and SYK3 [SYK protein sequence was obtained from 3 C. Mao, unpublished data.]

The above procedure was repeated to dock the compound. The procedure was also used to construct homology models for JAK1, JAK3 (21), BTK (22), and SYK3 [SYK protein sequence was obtained from Rowley et al. (23)].

Fixed docking in the Affinity program within INSIGHTII (InsightII Molecular Simulations, Inc.) was used for docking the compounds LFM-A12 and WHI-P154 to the EGFR tyrosine kinase catalytic site (Fig. 1B), as described previously (15). The modeling calculations used to study the predicted binding of inhibitors to EGFR were based on the homology model of EGFR and the crystal structure coordinates of LFM-A12 and WHI-P154. LFM-A12 and WHI-P154 were interactively docked into the catalytic site of EGFR based on the position of quercetin in the HCK/quercetin crystal structure (17). As the modeling calculations progressed, the protein residues within a defined radius from the inhibitor were allowed to shift and/or rotate to energetically favorable positions to accommodate the inhibitor. Calculations approximating hydrophobic and hydrophilic interactions were used to determine the 10 best docking positions of each inhibitor in the EGFR catalytic site. The various docked positions of each inhibitor were qualitatively evaluated using a score function in the Ludi module (24, 25) of the program INSIGHTII (InsightII Molecular Simulations, Inc.), which was then used to estimate a binding constant (Kᵢ) for each compound. The above procedure was repeated to dock the inhibitors into the catalytic sites of IR, HCK, BTK, JAK1, JAK3, and SYK.

Tyrosine Kinase Assays. Immunoprecipitation and kinase assays of recombinant proteins from insect cells expressing BTK, JAK1, or JAK3 were performed as reported previously (26, 27). Antibodies used for immunoprecipitations from insect cells were: polyclonal rabbit anti-BTK serum (28), polyclonal rabbit anti-JAK1 (HR-785, catalogue #sc-277), 0.1 mg/ml rabbit polyclonal IgG affinity purified (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-JAK3 (C-21, catalogue #sc-513), and 0.2 mg/ml rabbit polyclonal IgG affinity purified (Santa Cruz Biotechnology).

EGFR-positive human breast cancer cell line MDA-MB-231 (ATCC HTB-26) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, as reported earlier (12). For IR kinase assays, HepG2 human hepatoma cells grown to ~80% confluency were washed once with serum-free DMEM and starved for 3 h at 37°C in a CO₂ incubator. Subsequently, cells were stimulated with insulin (Eli Lilly and Co., Indianapolis, IN; catalogue #CP-410;10 units/ml/10⁶ cells) for 10 min at room temperature. Following this IR kinase activation step, cells were washed once with serum-free medium, lysed in NP40 buffer, and IR was immunoprecipitated from the lysates with an anti-IRB antibody (Santa Cruz Biotechnology; catalogue #sc-711, polyclonal IgG) and assayed for kinase activity as reported (22).

For HCK kinase assays, HCK-transfected COS-7 cells were used as described (22). The cloning and expression of HCK in COS-7 cells has been described previously (29). The pSV7c-HCK plasmid was transfected into 2 × 10⁶ COS-7 cells using lipofectamine (Life Technologies, Inc.), and the cells were harvested 48 h later. The cells were lysed in NP40 buffer, and HCK was immunoprecipitated from the whole cell lysates with an anti-HCK antibody (22, 29).

For EGFR immune complex kinase assays, MDA-MB-231 (breast cancer) cells were stimulated with 10 ng/ml EGF before immunoprecipitation of the EGFR with an anti-EGFR antibody reactive with the sequence Ala³⁵¹-Asp³⁶⁴ of the human EGFR (Upstate Biotechnology Inc., Lake Placid, NY; catalogue #05–104; Ref. 12). EGFR immune complexes were

### Table 1 Interaction scores, estimated Kᵢ values, and measured IC₅₀ values for the inhibition of protein tyrosine kinases by LFM-A12 and WHI-P154

<table>
<thead>
<tr>
<th>Protein</th>
<th>HBᵃ</th>
<th>Lipo score</th>
<th>Ludi score</th>
<th>Ludiᵇ Kᵢ (µM)</th>
<th>Inhibition IC₅₀ (µM)</th>
<th>HBᵇ</th>
<th>Lipo score</th>
<th>Ludi score</th>
<th>Ludiᵇ Kᵢ (µM)</th>
<th>Inhibition IC₅₀ (µM)</th>
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<tbody>
<tr>
<td>EGFR</td>
<td>1</td>
<td>510</td>
<td>489</td>
<td>13</td>
<td>1.7</td>
<td>2</td>
<td>639</td>
<td>685</td>
<td>0.14</td>
<td>5.6</td>
</tr>
<tr>
<td>BTK</td>
<td>1</td>
<td>496</td>
<td>457</td>
<td>27</td>
<td>&gt;175</td>
<td>1</td>
<td>581</td>
<td>541</td>
<td>3.8</td>
<td>&gt;300</td>
</tr>
<tr>
<td>IR</td>
<td>1</td>
<td>451</td>
<td>411</td>
<td>78</td>
<td>&gt;350</td>
<td>1</td>
<td>567</td>
<td>527</td>
<td>5.3</td>
<td>&gt;300</td>
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<tr>
<td>HCK</td>
<td>0</td>
<td>510</td>
<td>385</td>
<td>142</td>
<td>&gt;350</td>
<td>1</td>
<td>596</td>
<td>556</td>
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<td>11.6</td>
</tr>
<tr>
<td>JAK1</td>
<td>1</td>
<td>387</td>
<td>347</td>
<td>340</td>
<td>&gt;175</td>
<td>1</td>
<td>628</td>
<td>589</td>
<td>1.3</td>
<td>&gt;300</td>
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<tr>
<td>JAK3</td>
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<td>1710</td>
<td>&gt;350</td>
<td>1</td>
<td>626</td>
<td>586</td>
<td>1.4</td>
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<tr>
<td>SYK</td>
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<td>566</td>
<td>440</td>
<td>39</td>
<td>&gt;350</td>
<td>1</td>
<td>647</td>
<td>607</td>
<td>0.86</td>
<td>150</td>
</tr>
</tbody>
</table>

ᵃ HB, number of hydrogen bonds between inhibitor and protein.
ᵇ Ludi Kᵢ calculated based on the empirical score function in Ludi program (24, 25).
incubated for 1 h at room temperature with a kinase inhibitor, and tyrosine kinase assays were performed in the presence of $[\gamma-^32\text{P}]$-ATP, as previously described (12, 16, 30, 31). The kinase assay gels were analyzed both by autoradiography and using the Bio-Rad Storage Phosphor Imager System (Bio-Rad, Hercules, CA) for quantitative scanning. The IC$_{50}$ values were determined using an Inplot program (Graphpad Software, Inc., San Diego, CA).

RESULTS

Modeling Studies of LFM-A12 with the EGFR Kinase Domain. Our earlier study (15) reported the construction of a novel homology model of the EGFR kinase domain. In this model, the EGFR kinase domain (Fig. 1A) is composed of a smaller NH$_2$-terminal lobe, which is rich in $\beta$-strands, connected by a flexible hinge to a COOH-terminal lobe, which is mostly helical. The catalytic site is defined by two $\beta$-sheets that form an interface at the cleft between the two lobes. The catalytic site of the EGFR kinase domain displays a remarkably planar triangular binding pocket, which can bind the base ring portion of ATP. The sides of this triangle are $\sim 15$ Å $\times 12$ Å $\times 12$ Å, and the thickness of the binding pocket is $\sim 7$ Å, with an estimated volume of $\sim 600$ Å$^3$. Two sides of the triangle can be visualized as beginning at an apex located between Thr$^{766}$ (peach residue in Fig. 1B) and Asp$^{831}$ (lavender residue in Fig. 1B) and extending toward the solvent-accessible opening of the catalytic site. One side of the triangle extends from the apex along the hinge region of the catalytic site (blue residues in Fig. 1B), and a second side extends from the apex to Arg$^{817}$ (green residue in Fig. 1B), which is immediately adjacent to the binding subsites for the sugar and triphosphate groups of ATP. The third side of the triangle extends along the slot-shaped opening to the catalytic site. It is in this catalytic region where small molecule inhibitors can bind.

Our earlier studies (15) had also proposed a binding mode for the leflunomide metabolite analogues on the basis of the constructed three dimensional homology model of the tyrosine kinase domain of EGFR (Fig. 1A) and had identified the synthetic leflunomide analogue LFM-A12 as a potent inhibitor of the EGFR tyrosine kinase. The binding mode of LFM-A12 is such that the compound can maintain close contact with the hinge region on the edge of the inhibitor and is sandwiched between the residues Leu$^{694}$ and Val$^{705}$ from above and Leu$^{820}$ and Thr$^{830}$ from below, and the para-substituted

![Fig. 2](https://example.com/fig2.png) Selective inhibition of EGFR tyrosine kinase by LFM-A12. A, EGFR immune complexes from lysates of MDA-MB-231 human breast cancer cells were treated with LFM or LFM-A12 for 1 h and then assayed for tyrosine kinase activity, as described in “Materials and Methods.” B, lack of inhibition of IR immunoprecipitated from HepG2 hepatoma cells in immune complex kinase assays after treatment with LFM or LFM-A12. C, lack of inhibition of HCK immunoprecipitated from transfected COS7 cells in immune complex kinase assays after treatment with LFM or LFM-A12. D-F, lack of inhibition of JAK3, JAK1, and BTK immunoprecipitated from lysates of transfected insect ovary cells.
OCF₃ group is clamped between the residues Thr⁷⁶⁶ and Asp⁸³¹. The nitrile nitrogen of LFM-A12 faces the hinge region and is involved in hydrogen bonding with the amide NH of Met⁷⁶⁹. In addition, the para-substituted OCF₃ group on LFM-A12 can form close contacts between residues Thr⁷⁶⁶ and Asp⁸³¹.

Tyrosine Kinase Assays. We examined the effects of LFM-A12 on the enzymatic activity of the EGFR kinase in cell-free immune complex kinase assays. As shown in Fig. 2A, a 1-h incubation with LFM-A12 inhibited the EGFR tyrosine kinase in a concentration-dependent fashion in anti-EGFR immunoprecipitates from lysates of MDA-MB-231 human breast cancer cells. The IC₅₀ values for EGFR inhibition were 1.7 µM for LFM-A12 and 5.4 µM for the synthetic unmodified leflunomide metabolite, LFM (Fig. 2A). We also examined the effects of LFM-A12 on the enzymatic activity of the EGFR tyrosine kinase in breast cancer cells. After a 24-h exposure to LFM-A12, MDA-MB-231 cells were stimulated with EGF for 10 min, and EGFR immune complexes from whole cell lysates were subjected to Western blot analysis with a polyclonal antiphosphotyrosine antibody to measure the autophosphorylation of the EGFR. Treatment of MDA-MB-231 cells with LFM-A12 resulted in markedly decreased tyrosine phosphorylation of the EGFR after EGF stimulation (data not shown). LFM-A12 did not affect the enzymatic activity of other protein tyrosine kinases, including receptor family tyrosine kinase IR (Fig. 2B), Src family tyrosine kinase HCK (Fig. 2C), Janus kinases JAK1 and JAK3 (Fig. 2D-E), Tec family tyrosine kinase BTK (Fig. 2F), and SYK at concentrations as high as 350 µM (Table 1). In contrast to LFM-A12, the dimethoxyquinazoline compound WHI-P154 inhibited not only EGFR (IC₅₀ = 5.6 µM) but also HCK (IC₅₀ = 11.6 µM), JAK3 (IC₅₀ = 128 µM), and SYK (IC₅₀ = 150 µM; Table 1).

Structural Basis of Specific Inhibition of EGFR Tyrosine Kinase Activity by LFM-A12. LFM-A12 was found to be a selective inhibitor of EGFR, whereas it was a poor inhibitor of BTK, HCK, JAK1, JAK3, SYK, and IR. We next performed modeling studies using the crystal structure coordinates of HCK (17) and IR (19) and constructed homology models for the kinase domains of JAK1, JAK3 (21), BTK (22), and SYK to identify possible causes for the observed selectivity of LFM-A12 for the EGFR tyrosine kinase. LFM-A12 was docked into the kinase domains of EGFR, IR, HCK, JAK1, JAK3, SYK, and BTK. The models were then used to study the binding of LFM-A12 into the catalytic sites of these kinases, and to better understand how LFM-A12 can inhibit EGFR but not BTK, IR, JAK1, JAK3, SYK, or HCK. After energy minimization, the compound maintained favorable close contacts with the hinge region of each kinase. The orientation of LFM-A12 in the catalytic site of BTK and HCK is shown in Fig. 3A. This orientation as shown in Fig. 3A is such that the para-substituted OCF₃ group of LFM-A12 is below position B and the nitrile group is toward Asp at position C. The peach dotted line in Fig. 3A represents the hydrogen bond between the BTK residue Thr⁷⁷⁴ and the hydroxyl group of LFM-A12. There is no hydrogen bond between LFM-A12 and the HCK residues. The orientation of LFM-A12 in the catalytic site of EGFR, IR, JAK1, JAK3, and SYK is shown in Fig. 3B. This orientation is such that the para-substituted OCF₃ group of LFM-A12 is clamped between the residues at positions A and C and the nitrile group is toward the residues at position B. The white dotted line represents the hydrogen bond between the EGFR residue Met⁷⁶⁹ and the cyano nitrogen of LFM-A12. The green dotted line represents the hydrogen bond between the IR residue Asp¹⁰⁸³ and the hydroxyl group of LFM-A12. The cyan dotted line represents the hydrogen bond between the JAK1 residue Glu⁹² and the hydroxyl group of LFM-A12. There is no hydrogen bonding between LFM-A12 and the residues of SYK and JAK3. Table 1 shows the interaction scores, estimated Kᵢ values, and measured IC₅₀ data for LFM-A12 for the different kinases. Our studies indicated that the 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 the other PTKs. Likewise, unfavorable interactions with unique residues of the other PTKs that are not found in the EGFR kinase domain also contribute to this selectivity.

Modeling Studies of WHI-P154 with the EGFR Kinase Domain. We also studied the kinase inhibition properties of a reference compound belonging to a different class of inhibitors known as 4-anilinoquinazolines (32, 33). The binding of this compound (WHI-P154) with EGFR, JAK1, JAK3, SYK, IR, BTK, and HCK was modeled in a similar way to that of LFM-A12. The experimentally determined IC₅₀ values listed in Table 1 indicated that this compound was capable of inhibiting several kinases and did not demonstrate significant specificity for any one of them. Using the previously described homology models of EGFR, JAK1, JAK3, BTK, and SYK and the crystal structure coordinates of HCK and IR, WHI-P154 was docked into the ATP binding site of these seven kinases. LFM-A12 and WHI-P154 were predicted to bind to the same region of the catalytic site of EGFR, and a large portion of each inhibitor was aligned along the hinge region of the receptor. This position (shown in Fig. 3C for WHI-P154) favors good contact with the residues in the active site. For WHI-P154 the quinazoline moiety was aligned along the hinge region, and the anilino ring was located between the residues at positions A and C on each side for all of the seven kinases studied. The 6, 7-dimethoxy groups faced the solvent accessible region, and the 4-hydroxyl group was involved in hydrogen bonding with the conserved Asp residue at position C in all seven kinases listed in Table 1. The docked position of WHI-P154 with EGFR revealed a second hydrogen bond between the N₃ nitrogen of the quinazoline group and the backbone carbonyl of Met⁷⁶⁹. For the other six PTKs (HCK, JAK1, JAK3, IR, BTK, and SYK) modeled, these hydrogen bonds do not exist. This maybe due to both the shift of the inhibitor and the displacement of the corresponding residue in SYK, JAK1, JAK3, BTK, IR, and HCK corresponding to Met⁷⁶⁹ in EGFR due to a bulkier or longer neighboring residue at positions A and/or B. Table 1 shows the interaction scores and estimated Kᵢ values from the modeling studies of WHI-P154 with the seven different kinases. Fig. 3C shows the superimposed backbones of the ATP binding site residues of the 7 kinases with the docked position of WHI-P154 (multicolor) in EGFR (white). [WHI-P154 was predicted to bind to the catalytic site of HCK (blue), SYK (pink), JAK1 (cyan), JAK3 (red), BTK (peach), and IR (green) in an orientation similar to the EGFR result.). The dotted surface area in Fig. 3C represents the Connolly surface of WHI-P154. The white dashed lines represent...
Fig. 3  Docking of LFM-A12 and WHI-P154 into catalytic sites of kinases. A, superimposed backbones of the catalytic site residues of the kinase domain homology models of EGFR (white), BTK (peach), and crystal structure coordinates of HCK (Ref. 19; blue), with selected residues at positions A, B, and C. LFM-A12 is shown in multicolor and represents its docked position in BTK, which is also similar to its docked position in HCK. The white dotted surface area represents the Connolly surface of LFM-A12. The peach dotted line represents the hydrogen bond between the BTK residue Thr⁴⁷⁴ and the hydroxyl group of LFM-A12. There is no hydrogen bond between LFM-A12 and the HCK residues. B, superimposed backbones of the catalytic site residues of the kinase domain homology models of EGFR (white), JAK1 (cyan), JAK3 (red), SYK (pink), and crystal structure coordinates of IR (Ref. 19; green), with selected residues at positions A, B, and C. LFM-A12 is shown in multicolor and represents its docked position in EGFR, which is also similar to its docked position in IR, SYK, JAK1, and JAK3. The white dotted surface area represents the Connolly surface of LFM-A12. The white dotted line represents the hydrogen bond between the EGFR residue Met⁷⁶⁹ and the cyano nitrogen of LFM-A12. The green dotted line represents the hydrogen bond between the IR residue Asp¹⁰⁸³ and the hydroxyl group of LFM-A12. The cyan dotted line represents the hydrogen bond between the JAK1 residue Glu⁹² and the hydroxyl group of LFM-A12. C, superimposed backbones of the catalytic site residues of the kinase domain homology models of EGFR (white), JAK1 (cyan), JAK3 (red), SYK (pink), BTK (peach), and crystal structure coordinates of HCK (blue) and IR (green), with selected residues at positions A, B, and C. WHI-P154 is shown in multicolor and represents its docked position in EGFR which is also similar to its docked position in BTK, HCK, SYK, JAK1, JAK3, and IR. The white dotted surface area represents the Connolly surface of WHI-P154. The white dotted lines represent the two hydrogen bonds between the EGFR residues and WHI-P154. The conserved Asp residue at position C is involved in hydrogen bonding with the OH group of the inhibitor in all of the seven kinases.
the hydrogen bonds between the EGFR residues and WHI-P154. Our studies have, thus, identified several features that may contribute to the observed inhibition of several kinases by WHI-P154.

**DISCUSSION**

Our earlier studies (15) had proposed a binding mode for the LFM analogues on the basis of the three dimensional homology model of the tyrosine kinase domain of EGFR (Fig. 1A) and had identified the synthetic LFM analogue LFM-A12 as a potent inhibitor of the EGFR tyrosine kinase for which we estimated a Kᵢ value of 13 μM from modeling studies.

Cell-free immune complex kinase assays on the enzymatic activity of the EGFR kinase indicated that 1 h incubation with LFM-A12 inhibited the EGFR tyrosine kinase in a concentration-dependent fashion in anti-EGFR immunoprecipitates from lysates of MDA-MB-231 human breast cancer cells. The IC₅₀ values for EGFR inhibition were 1.7 μM for LFM-A12 and 5.4 μM for the synthetic unmodified leflunomide metabolite LFM (Fig. 2A). The effects of LFM-A12 on the enzymatic activity of the EGFR tyrosine kinase in MDA-MB-231 breast cancer cells resulted in markedly decreased tyrosine phosphorylation of the EGFR after EGFR stimulation (data not shown). The inhibitory effects of LFM-A12 on the EGFR tyrosine kinase were specific in that it did not affect the enzymatic activity of other protein tyrosine kinases, including receptor family tyrosine kinase IR (Fig. 2B), Src family tyrosine kinase HCK (Fig. 2C), Janus kinases JAK1 and JAK3 (Fig. 2, D and E), Tec family tyrosine kinase BTK (Fig. 2F), and SYK at concentrations as high as 350 μM (Table 1).

Modeling studies were performed to understand how LFM-A12 could selectively inhibit EGFR tyrosine kinase, whereas it is a poor inhibitor of BTK, HCK, JAK1, JAK3, SYK, and IR. Whereas most of the catalytic site residues of the EGFR kinase domain were conserved relative to other PTKs, we noted a few specific variations. EGFR residues Leu⁶⁹⁴, Val⁷⁰², Lys⁷²¹, and Ala⁷¹⁹ are conserved in EGFR, HCK, FGFR and IR. Residues Asn⁸¹⁸ and Asp⁸³⁰ (opposite to the hinge) are conserved in EGFR, HCK, FGFR, IR, BTK, JAK1, JAK3, and SYK. Residues Cys⁷⁵¹ and Thr⁸³⁰ are specific for EGFR but vary in BTK (Val, Ser), JAK1 (Val, Gly), JAK3 (Val, Ala), IR (Val, Gly), and HCK (Val, Ala). Residues Thr⁷⁶⁶ and Leu⁷⁶⁸ in the hinge region changes to Met and Leu in IR, Met and Phe in JAK1, Met and Tyr in JAK3, to Thr and Tyr in BTK, to Thr and Phe in HCK and to Met and Met in SYK. One region of the binding pocket contains Cys⁷⁷³ in EGFR and is, therefore, considerably more hydrophobic than the corresponding residue of PDGFR (Asp), FGFR (Asn), JAK1 (Ser), HCK (Ser), and IR (Asp).

LFM-A12 was modeled into the kinase domains of IR, HCK, JAK3, JAK1, BTK, SYK, and EGFR. The models were then used to study the binding of LFM-A12 into the catalytic sites of these kinases and to better understand how LFM-A12 can inhibit EGFR but not BTK, IR, JAK1, JAK3, SYK, or HCK. Table 1 shows the interaction scores, estimated Kᵢ values, and measured IC₅₀ data for LFM-A12 with the seven different kinases. After energy minimization, the compound maintained favorable close contacts with the hinge region of each kinase, although the orientation of LFM-A12 in the catalytic site was different for BTK and HCK (as shown in Fig. 3A) than for EGFR, IR, JAK1, JAK3, and SYK (as shown in Fig. 3B). Our studies indicated that the 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 the other PTKs. Likewise, unfavorable interactions with unique residues of the other PTKs that are not found in the EGFR kinase domain also contribute to this selectivity. These residue differences are illustrated in Fig. 3, A and B, at positions A and B. Fig. 3A shows the backbone of the EGFR catalytic site, the residue differences between EGFR (white) and other kinases and the docked position of LFM-A12 (multicolor) at this site in BTK (peach), which is also similar to the docked position in HCK (blue). Fig. 3B shows the docked position of LFM-A12 (multicolor) in EGFR (white), which is also similar to the docked position in JAK1 (cyan), JAK3 (red), SYK (pink), and IR (green). We propose that the aromatic residue in BTK (Tyr) and HCK (Phe; shown at position B in Fig. 3A) is not as favorable for interactions with the p-OCF₂ group of LFM-A12. The corresponding residue in the EGFR kinase domain is leucine (shown in white at position B in Fig. 3A and 3B), which would not cause such unfavorable interactions with LFM-A12. Also, for HCK there is a loss of hydrogen bonding interaction with LFM-A12. Furthermore, JAK1, JAK3, IR, and SYK (shown in Fig. 3B) contain a methionine residue (at position A in Fig. 3B) that protrudes into the active site and could impair the close hydrophobic contact of LFM-A12 with the hinge region of the catalytic site. The longer methionine residue in these four kinases (JAK1, JAK3, IR, and SYK) does not complement the shape of LFM-A12 and may hinder its binding. As shown in Fig. 3B, the corresponding residue in the EGFR kinase domain is threonine (white); its relatively shorter side chain enables LFM-A12 (multicolor) to have a more favorable hydrophobic contact with the hinge region which may result in tighter binding to the EGFR binding site. For EGFR, the most active compound (LFM-A12) appears to be located between the residues at positions A and C. Consequently, the estimated Kᵢ value for the EGFR (13 μM) was lower than the Kᵢ values for other PTKs, which ranged from 27 μM for BTK to 1710 μM for JAK3 (Table 1).

On the other hand, a quinazolinone inhibitor [4-(3-bromo-4-hydroxyanilino)-6,7-dimethoxyquinazolinone] tested as a control showed inhibition of EGFR tyrosine kinase, as well as other tyrosine kinases such as HCK, JAK3, and SYK. The experimentally determined IC₅₀ values listed in Table 1 indicated that this compound was capable of inhibiting several kinases and did not demonstrate significant specificity for any one of them. Using the previously described homology models of EGFR, JAK1, JAK3, BTK, and SYK and the crystal structures of HCK and IR, WHI-P154 was docked into the ATP binding site of these seven kinases. Fig. 3C shows the superimposed backbones of the ATP binding site residues of the seven kinases with the docked position of WHI-P154 (multicolor) in EGFR (white). [WHI-P154 was predicted to bind to the catalytic site of HCK (blue), SYK (pink), JAK1 (cyan), JAK3 (red), IR (green), and BTK (peach) in an orientation similar to the EGFR result]. Table 1 shows the interaction scores and estimated Kᵢ values of WHI-P154 with these kinases. Our studies have identified several features that may contribute to the observed inhibition of
specific with IC\textsubscript{50} values below 10 nM (35) and showed high activity against all kinases. An additional hydrogen bond with Met\textsuperscript{769} of EGFR can occur in this region. As seen in Fig. 3C, the inhibitor WHI-P154 maintains a close contact with the hinge region of all seven kinases. In fact, the longer methionine side chain of JAK1, JAK3, IR, and SYK at position A may serve to enhance the complementary fit of the inhibitor and increase its binding with the corresponding kinase. The same residue at position A prohibits close binding of LFM-A12 to IR, JAK1, JAK3, and SYK in this region. The aromatic residues of HCK, BTK, JAK1, and JAK3 at position B also may enhance the hydrophobic interaction with the quinazoline group of WHI-P154. This is reflected by the corresponding higher hydrophobic interaction scores of this compound for all seven kinases, relative to LFM-A12 (Table 1, Lipo Score). In addition WHI-154 forms 1 or 2 hydrogen bonds with the residues of the corresponding kinase and, like the interaction of LFM-A12 with EGFR, the anilino ring of WHI-P154 appears to be located between the residues at positions A and C for all seven kinases, thereby enhancing close contacts with each of them.

Many classes of small molecule inhibitors have been reported in the last several years for the EGFR kinase; of these, the quinazolines and the pyrazolo/pyrrolo/pyridopyrimidines seem to be the most promising in terms of specificity for inhibiting EGFR. The quinazoline derivative CP-358,774 (11) inhibits EGFR with an IC\textsubscript{50} of 2 nM and reduces EGFR autophosphorylation in intact tumor cells with an IC\textsubscript{50} of 20 nM. This inhibition is selective for EGFR tyrosine kinase relative to other tyrosine kinases examined, as determined by assays of isolated kinases and whole cells. Despite the reported profound in vitro potency (K\textsubscript{i} = 5 pm) and selectivity of the ATP-competitive brominated quinazoline derivative PD153035 (33), the compound failed to show significant in vivo efficacy. Another type of quinoline inhibitor reported, PD 168393 and PD 160678, selectively target and irreversibly inactivates EGFR through covalent modification of a cystein (Cys\textsuperscript{773}) residue present in the ATP binding pocket (34). 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, PDGFR, FGR, and PKC. However, these compounds have not been tested against BTK and JAK3, which also have a conserved cysteine residue at the corresponding position. Two pyrazolopyrimidines have been reported that inhibit EGFR specifically with IC\textsubscript{50} values below 10 nM (35) and showed high selectivity toward the tested nonreceptor tyrosine kinases (c-src, v-Abl and serine/threonine kinases (PKC \alpha, CDK1). Earlier studies have reported that the immunosuppressive activity of leflunomide is due to its metabolite A77 1726 (LFM), which is rapidly formed in vivo and functions as a pyrimidine synthesis inhibitor (36) and also inhibits the tyrosine kinase activity of EGFR (37). The compound we report here (LFM-A12) inhibits EGFR with an IC\textsubscript{50} in the low micromolar range. Our study is the first that describes the development of LFM analogues as a novel class of EGFR inhibitors which were evaluated using structure-based methods and proposes an explanation for the specificity of LFM-A12 for EGFR.

In summary, our approach led to the successful design of a leflunomide metabolite analogue LFM-A12, which showed selectivity for the EGFR tyrosine kinase. Our results suggest that the 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 the other PTKs. Likewise, unfavorable interactions with unique residues of the other PTKs that are not found in the EGFR kinase domain 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 complementary shape of the inhibitor 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, hydrogen bonding interactions with all seven kinases may enhance its binding with each of them. However, the binding volume of the EGFR catalytic site is much larger than the volume occupied by our most potent and selective compound LFM-A12. Increasing the size of the ligand by using larger ring systems might increase the contact area between the receptor and ligand and, hence, enhance binding. Interactions of the inhibitor with the nonconserved residues such as Cys\textsuperscript{771} and Thr\textsuperscript{780} in the catalytic site of EGFR may also be used for the design of more potent and selective inhibitors of EGFR.

REFERENCES


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Specificity of α-Cyano-β-Hydroxy-β-Methyl-N-[4-(Trifluoromethoxy)Phenyl]-Propenamide as an Inhibitor of the Epidermal Growth Factor Receptor Tyrosine Kinase


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