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ABSTRACT

Epidermal growth factor receptor (EGF-R) tyrosine kinase is known to be overexpressed in several malignancies and is an important target for anticancer drug design. We constructed a homology model to represent the structure of EGF-R and propose that this model can be used to design potent inhibitors of EGF-R. We used our EGF-R model and a docking procedure to rationally design compounds predicted to bind favorably to EGF-R. This approach led to the successful design of a leflunomide metabolite analogue, which was found to have an IC50 value of 1.7 μM in EGF-R inhibition assays and killed >99% of human breast cancer cells in vitro by triggering apoptosis. The reported studies may provide the basis for the development of a new class of potent and clinically useful anti-breast cancer agents.

INTRODUCTION

Breast cancer is the most common form of malignancy in women, representing 32% of all new cancer cases and causing 18% of the cancer-related deaths among women in the United States (1). Although the majority of patients with metastatic breast cancer will experience an initial response, survival is only modestly improved with contemporary chemotherapy programs (2–8). Front-line cytotoxic chemotherapy of metastatic breast cancer with the most effective regimens offers a median duration of response of only 8 months, and once patients progress after the front-line therapy, the response rate is only 20–35% for second-line combination chemotherapy (6, 9, 10). Thus, presently, the major challenge in the treatment of breast cancer is to cure patients who have metastatic disease (11). Consequently, the development of new potent anti-breast cancer drugs has emerged as an exceptional focal point for translational research in the treatment of breast cancer.

Human EGF2 is a 53-amino acid, single-chain polypeptide (M, 6216), which exerts pleiotropic biological effects by binding to a specific M, 170,000 cell membrane EGF-R (EGF-R/ErB-B-1; Refs. 12–15). In breast cancer, expression of the EGF-R is a significant and independent indicator for recurrence and poor relapse-free survival (16, 17). Our recent studies provided evidence that the EGF-R PTK serves as an endogenous negative regulator of apoptosis in breast cancer cells (18). Consequently, the development of PTK inhibitors that abrogate the enzymatic function of the EGF-R tyrosine kinase has become a focal point in translational research aimed at designing more effective treatment strategies for metastatic breast cancer (13, 14). LFM, the primary metabolite of the isoxazole leflunomide [N-(4-trifluoromethylphenyl)-5-methyloxazol-4-carboxamide], an anti-inflammatory agent with pleiotropic effects (19–22), was recently discovered to be capable of inhibiting the EGF-R kinase at micromolar concentrations (23).

We have constructed a homology model for the EGF-R kinase domain and used molecular modeling to evaluate structure activity relationships that affect the ability of LFM to inhibit the EGF-R tyrosine kinase and to design new LFM analogues with more potent inhibitory activity against the EGF-R tyrosine kinase. Specifically, we have used the homology model of EGF-R and its binding pocket, together with a computer docking procedure and a structure-based semiempirical score function, as a guide to predict the energetically favorable position of LFM analogues bound to EGF-R. Our modeling studies prompted the hypothesis that meta and para substitutions with groups such as CF3 and OCF3 on the aromatic ring would be more potent inhibitors of EGF-R, whereas ortho substitutions would be more likely to be inactive compounds. Our lead compound, cyano-β-hydroxy-β-methyl-N-[4-(trifluoromethoxy)phenyl]propanamide (LFM-A12), was more effective than LFM in inhibiting EGF-R kinase from breast cancer.

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2 The abbreviations used are: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; PTK, protein tyrosine kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; LFM, leflunomide metabolite; HCK, hematopoietic cell putative protein tyrosine kinase; FGFR, fibroblast growth factor receptor; IRK, insulin receptor kinase; NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet; m, multiplet; br, broad; ATCC, American Type Culture Collection; PI, propidium iodide; FACS, fluorescence-activated cell sorter.
MATERIALS AND METHODS

Construction of a Homology Model for the EGF-R Kinase Domain. Because the X-ray crystal structure of the EGF-R was not yet available at the time, a homology model of the kinase domain of EGF-R was constructed using the X-ray structures of the kinase domains of homologous kinase proteins HCK (24), FGFR (25), IRK (26), and cAMP-dependent protein kinase (27). The homology modeling of EGF-R was carried out in two steps by first determining the most reasonable sequence alignment between the EGF-R kinase and a coordinate template and then assigning new coordinates to the EGF-R kinase residues according to the template coordinates (based on the sequence alignment), followed by the determination of loop coordinates and an energy minimization of the entire structure. More specifically, in step 1, the Ca coordinates of the kinase domains of HCK, FGFR, IRK, and cAMP-dependent protein kinase were superimposed using the InsightII program (Molecular Simulations, Inc., San Diego, CA) to provide the best overall structural comparison. All four sequences were then aligned based on the superimposition of their structures (amino acid sequences were aligned together if their Ca positions were spatially related to each other). The sequence alignment accommodated such features as loops in a protein that differed from the other protein sequences. The structural superposition was performed using the Homology module of the InsightII program and a Silicon Graphics INDIGO2 computer (Silicon Graphics, Mountain View, CA). The sequence alignment was manually adjusted based on the considerations mentioned previously and produced a sequence variation profile for each superimposed Ca position. The sequence variation profile served as a basis for the next procedure, sequence alignment of the EGF-R kinase with all four proteins. In this procedure, the sequence of EGF-R kinase was read into the program and manually aligned with the four known kinase proteins based on the sequence variation profile described previously. In step 2, a set of three-dimensional coordinates was assigned to the EGF-R kinase sequence using the three-dimensional coordinates of HCK as a template, using the Homology module within the InsightII program (Molecular Simulations, Inc.). The coordinates for a loop region where a sequence insertion occurs (relative to HCK without the loop) were chosen from a limited number of possibilities automatically generated by the computer program and manually adjusted to a more ideal geometry using the program CHAIN (28). Finally, the constructed model of the EGF-R was subjected to energy minimization using the X-plor program (A. T. Brunger, X-PLOR, New Haven, CT) so that any steric strain introduced during the model-building process could be relieved. The model was screened for unfavorable steric contacts and if necessary such side chains were remodeled either by using a rotamer library database or by manually rotating the respective side chains. The constructed EGF-R kinase domain had the expected protein kinase fold with the central ATP binding region (catalytic site) dividing the kinase domain into two lobes (Fig. 1A). The constructed kinase domain is composed of a smaller N-terminal lobe connected by a flexible hinge to a larger COOH-terminal lobe (Fig. 1A). The final homology model of the EGF-R kinase domain had an rms deviation of 0.006 Å from ideal bond lengths and 2.0° from ideal bond angles after energy minimization. The homology model of EGF-R was then used, in conjunction with small molecule crystal structures of the leflunomide analogues, for our modeling studies of the EGF-R/LFM complexes.

Docking Procedure Using Homology Model of EGF-R Kinase Domain. Modeling of the EGF-R/LFM analogue complexes was done using the Docking module within the program INSIGHTII (Molecular Simulations, Inc.) and using the Affinity suite of programs for automatically docking a ligand to the receptor. The coordinates of each small molecule crystal structure of the LFM analogues were docked into the homology model coordinates of the catalytic site of the EGF-R. In cases where the crystal structure of an LFM compound was not available, coordinates of an energy-minimized model of the compound was used for docking. Each leflunomide was interactively docked into the binding pocket of EGF-R based on the position of quercetin in the HCK/quercetin crystal structure. The hydrogen on the EGF-R were generated, and potentials were assigned to both receptor and ligand prior to the start of the docking procedure. The docking method in the InsightII program uses the CVFF force field and a Monte Carlo search strategy to search for and evaluate docked structures (Molecular Simulations, Inc.). Although the coordinates for the bulk of the receptor was kept fixed, a defined region of the binding site was allowed to relax, thereby allowing the protein to adjust to the binding of different inhibitors. A binding set was defined within a distance of 5 Å from the inhibitor, allowing residues within this distance to shift and/or rotate to energetically favorable positions to accommodate the ligand. An assembly was defined consisting of the receptor and inhibitor molecule, and docking was performed using the fixed docking mode. Calculations approximating hydrophobic and hydrophilic interactions were used to determine the 10 best docking positions of each LFM analogue in the EGF-R catalytic site. The various docked positions of each LFM analogue were evaluated using the Ludi module (29) in INSIGHTII (Molecular Simulations, Inc.), which was used to estimate a binding constant (Kd) for each compound to rank their relative binding capabilities and predicted inhibition of EGF-R. The Kd trends for the LFM analogues were compared with the trend of the experimentally determined tyrosine kinase inhibition as well as cytotoxicity IC50 values for the compounds to elucidate the structure-activity relationships determining the potency of LFM analogues.

Synthesis and Characterization of Active Metabolite of Leflunomide and Analogues. All chemicals were purchased from Aldrich (Milwaukee, WI) and were used without further purification. Except where noted, each reaction vessel was secured with a rubber septa, and the reaction was performed under nitrogen atmosphere. 1H and 13C NMR spectra were obtained on a Varian Mercury 300 spectrometer (Palo Alto, CA) at ambient temperature in the solvent specified. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Fourier Transform-Infrared (FT-IR) spectra were recorded on a Nicolet Protege 460 spectrometer (Madison, WI).
GC/MS was obtained on a HP 6890 GC System (Palo Alto, CA) equipped with a HP 5973 Mass Selective Detector.

Synthesis of LFM and LFM-A1-LFM-A14. Scheme 1 shows the general synthetic scheme for the preparations of LFM and LFM-A1 to LFM-A14 (30, 31). Cyanooctac acid 1 was coupled with the desired substituted-aniline 2 in the presence of disopropylcarbodiimide to form compound 3. Compound 3 was treated with NaH and then acylated with acetyl chloride to afford the final products LFM and LFM-A1-LFM-A14 (Scheme 1).

General Synthetic Procedures. 1,3-Diisopropylcarbodiimide (1.75 g; 13.9 mmol) was added to a solution of cyanooctac acid 1 (1.70 g; 20.0 mmol) and the desired substituted-aniline 2 (12.6 mmol) in tetrahydrofuran (25 ml) at 0°C (30, 31). The mixture was stirred for 12 h at room temperature. The urea precipitate (reaction side product) was removed by filtration and partitioned between ethyl acetate and 0.5 N HCl. The organic layer was sequentially washed with brine twice, dried over anhydrous Na2SO4, and concentrated by rotary evaporation. Finally, the crude solid product was recrystallized from ethanol to give pure compound 3. Sodium hydride (0.93 g; 60% in mineral oil; 23.2 mmol) was added slowly to the solution of compound 3 in tetrahydrofuran (40 ml) at 0°C. After stirring for 30 min at 0°C, acetyl chloride (1.04 g; 13.2 mmol) was added to the reaction mixture. The reaction was continued for another hour and then was quenched by the addition of acetic acid (2 ml). The mixture was poured into ice water (100 ml) containing 2.5 ml of hydrochloric acid to precipitate the crude product, which was collected by filtration and washed with water. The final pure product was obtained by recrystallization.

Analytical Data. α-Cyano-β-hydroxy-β-methyl-N-[4-(trifluoromethyl)phenyl]propenamide (LFM-A1), mp: 230–233°C; IR (KBr): 3303, 2218, 1600, and 1555 cm⁻¹; 1H NMR (DMSO-d₆): δ 11.01 (s, 1 H, NH), 7.75 (d, J = 8.4 Hz, 2H, ArH), 7.64 (d, J = 8.4 Hz, 2H, ArH), 2.22 (s, 3H, CH₃); EIMS m/z [M⁺]: 270.

α-Cyano-β-hydroxy-β-methyl-N-[3-(4-fluorophenyl)]propenamide (LFM-A3), mp: 165–166°C; IR (KBr): 3298, 2218, 1610, and 1560 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.33 (s, 1H, NH), 7.80 (s, 1H, OH), 7.53 (m, 2H, ArH), 7.16 (t, J = 8.7 Hz, 2H, ArH), 2.26 (s, 3H, CH₃); EIMS m/z [M⁺]: 220.

α-Cyano-β-hydroxy-β-methyl-N-[2-[(trifluoromethyl)phenyl]propenamide (LFM-A4), mp: 61–63°C; IR (KBr): 3435, 2209, 1619, 1952, and 1548 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.99 (s, 1H, NH), 8.03 (d, J = 7.5 Hz, 1H, ArH), 7.67 (d, J = 7.5 Hz, 1H, ArH), 7.60 (d, J = 8.1 Hz, 1H, ArH), 7.29 (t, J = 7.3 Hz, 1H, ArH), 5.71 (s, br, 1H, OH), 2.20 (s, 3H, CH₃); EIMS m/z [M⁺]: 270.

α-Cyano-β-hydroxy-β-methyl-N-[2-(4-fluorophenyl)propenamide (LFM-A5), mp: 98–100°C; IR (KBr): 3351, 2214, 1609, 1585, and 1536 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.76 (s, 1H, NH), 8.06 (d, J = 7.8 Hz, 1H, ArH), 7.62 (d, J = 7.8 Hz, 1H, ArH), 7.33 (t, J = 7.5, 1H, ArH), 7.03 (t, J = 7.5 Hz, 1H, ArH), 6.60 (s, br, 1H, OH), 2.22 (s, 3H, CH₃); EIMS m/z [M⁺]: 280.

α-Cyano-β-hydroxy-β-methyl-N(1-fluorobenzyl)propenamide (LFM-A6), mp: 93–94°C; IR (KBr): 3372, 2208, 1644, 1621, and 1587 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.76 (s, 1H, NH), 8.06 (d, J = 8.4 Hz, 1H, ArH), 7.62 (d, J = 7.8 Hz, 1H, ArH), 7.33 (t, J = 7.5 Hz, 1H, ArH), 7.03 (t, J = 7.5 Hz, 1H, ArH), 2.22 (s, 3H, CH₃); EIMS m/z [M⁺]: 236.

α-Cyano-β-hydroxy-β-methyl-N(1-methoxybenzyl)propenamide (LFM-A7), mp: 118–119°C; IR (KBr): 3409, 2212, 1613, 1591 and 1532 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.70 (s, 1H, NH), 7.91 (m, 1H, ArH), 7.23 (m, 1H, ArH), 7.13 (m, 2H, ArH), 7.10 (s, br, 1H, OH), 2.22 (s, 3H, CH₃); EIMS m/z [M⁺]: 220.

α-Cyano-β-hydroxy-β-methyl-N-[3-(3-fluorophenyl)propenyl]propenamide (LFM-A8), mp: 182–184°C; IR (KBr): 3303, 2221, 1619, and 1572 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.79 (s, 1H, NH), 8.05 (s, br, 1H, OH), 8.04 (s, 1H, ArH), 7.75 (d, J = 8.4 Hz, 1H, ArH), 7.53 (t, J = 8.1 Hz, 1H, ArH), 7.34 (t, J = 7.5 Hz, 1H, ArH), 2.24 (s, 3H, CH₃); EIMS m/z [M⁺]: 270.

α-Cyano-β-hydroxy-β-methyl-N(3-fluorobenzyl)propenamide (LFM-A9), mp: 184–185°C; IR (KBr): 3303, 2228, 1610, 1595, and 1550 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.56 (s, 1H, NH), 7.89 (m, 1H, ArH), 7.47 (m, 1H, ArH), 7.28 (m, 2H, ArH), 6.37 (s, br, 1H, OH), 2.26 (s, 3H, CH₃); EIMS m/z [M⁺]: 280.

α-Cyano-β-hydroxy-β-methyl-N(3-chlorobenzyl)propenamide (LFM-A10), mp: 184–187°C; IR (KBr): 3293, 2221, 1610, 1595, and 1557 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.61 (s, 1H, NH), 7.76 (m, 1H, ArH), 7.42 (m, 1H, ArH), 7.33 (m, 1H, ArH), 7.16 (m, 1H, ArH), 2.25 (s, 3H, CH₃); EIMS m/z [M⁺]: 236.

α-Cyano-β-hydroxy-β-methyl-N(3-fluorobenzyl)propenamide (LFM-A11), mp: 136–138°C; IR (KBr): 3297, 2221, 1613, 1597, and 1567 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.54 (s, 1H, NH), 7.54 (m, 1H, ArH), 7.33 (m, 2H, ArH), 6.93 (m, 2H, ArH), 2.27 (s, 3H, CH₃); EIMS m/z [M⁺]: 220.

α-Cyano-β-hydroxy-β-methyl-N(4-trifluoromethoxy)phenyl)propenamide (LFM-A12), mp: 182–183°C; IR (KBr): 3308, 2213, 1625, and 1580 cm⁻¹; 1H NMR (DMSO-d₆): δ 10.57 (s, 1H, NH), 7.90 (s, br, 1H, OH), 7.64 (d, J = 8.7 Hz, 2H, ArH), 7.32 (d, J = 8.7 Hz, 2H, ArH), 2.25 (s, 3H, CH₃); EIMS m/z [M⁺]: 286.
\( \alpha \)-Cyano-\( \beta \)-hydroxy-\( \beta \)-methyl-N-(2,5-dibromophenyl)pro-panamide (LFM-A13), \( \delta \): 148–150°C; IR (KBr): 3353, 2211, 1648, and 1590 cm\(^{-1}\); \( ^1H \) NMR (DMSO-\( d_6 \)): \( \delta \) 11.41 (s, 1H, NH), 8.57 (m, 1H, ArH), 7.55 (d, \( J = 8.7 \) Hz, 1H, ArH), 7.14 (q, \( J_1 = 6.0 \) Hz, \( J_2 = 2.4 \) Hz, 1H, ArH), 7.10 (s br, 1H, OH), 2.17 (s, 3H, CH\(_3\)); EIMS m/z \( [M]^+ \): 358.

\( \alpha \)-Cyano-\( \beta \)-hydroxy-\( \beta \)-methyl-N-(phenyl)propanamide (LFM-A14), \( \delta \): 134–135°C; IR (KBr): 3281, 2214, 1605, 1579, and 1554 cm\(^{-1}\); \( ^1H \) NMR (DMSO-\( d_6 \)): \( \delta \) 10.33 (s, 1H, NH), 7.51 (d, \( J = 8.1 \) Hz, 2H, ArH), 7.40 (s br, 1H, OH), 7.31 (t, \( J = 7.8 \) Hz, 2H, ArH), 7.11 (t, \( J = 7.2 \) Hz, 1H, ArH), 2.26 (s, 3H, CH\(_3\)); EIMS m/z \( [M]^+ \): 202.

**In Vitro Treatment of Cells with LFM Compounds.**

To determine the cytotoxic activity of LFM and its analogues against breast cancer cells, cells in \( \alpha \)-MEM supplemented with 10% FCS were treated with various concentrations of the compounds for 24 h at 37°C, washed twice in a-MEM, and then used in either MTT assays or clonogenic assays, as described hereinafter.

**Breast Cancer Cells.** MDA-MB-231 (ATCC HTB-26), MDA-MB-361 (ATCC HTB-27), and BT-20 (ATCC HTB-19) are EGF-R-positive human breast cancer cell lines (18). These cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. For subculturing, medium was removed from the flasks containing a confluent layer of cells, and fresh 0.25% trypsin was added for 1–2 min. The trypsin was removed, and cultures were incubated for 5–10 min at 37°C until cells detached. Fresh medium was then added, and cells were aspirated and dispensed into new flasks.

**Immune-Complex Kinase Assays and Anti-Phosphotyrosine Immunoblotting.** Twenty-four h after treatment with leflunomide analogues, MDA-MB-231 breast cancer cells were stimulated with 20 ng/ml EGF for 5 min and lysed in 1% NP40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala\(^{351}\)-Asp\(^{356}\) of the human EGF-R (Upstate Biotechnology, Inc.; Ref. 18). EGF-R immune complexes were examined for tyrosine phosphorylation by Western blot analysis, as described previously (18). All anti-phosphotyrosine Western blots were subjected to densitometric scanning using the automated AMBS system (Automated Microbiology System, Inc., San Diego, CA), and for each time point a percentage inhibition value was determined by comparing the density ratios of the tyrosine phosphorylated EGF-R protein bands to those of the baseline sample and using the formula:

\[
\% \text{ inhibition} = \frac{[\text{Density of tyrosine phosphorylated EGF-R band}_\text{test sample}] - [\text{Density of tyrosine phosphorylated EGF-R band}_\text{baseline control sample}]}{[\text{Density of tyrosine phosphorylated EGF-R band}_\text{baseline control sample}]} \times 100
\]

The IC\(_{50}\) values were calculated using nonlinear regression analysis using Graphpad Prism software version 2.0 (Graphpad Software, Inc.).

**Confocal Laser Scanning Microscopy.** Immunofluorescence was used to examine the morphological features of breast cancer cells treated with LFM and its analogues. Before the experiment, cells were trypsinized from rapidly growing tissue culture flasks and seeded onto sterile 22-mm\(^2\) coverslips in DMEM supplemented with 10% (v/v) FCS were treated with various concentrations of the compounds for 36 h at 37°C in a humidified 5% CO\(_2\) atmosphere. To each well, 10 \( \mu \)l of MTT (0.5 mg/ml final concentration) were added, and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at 37°C in a solution containing 10% SDS in 0.1 M HCl. The absorbance of each well was measured in a microplate reader (Labsystems) at 540 nm and a reference wavelength of 690 nm. To translate the absorbance \( A_{440} \) values into the number of live cells in each well, the \( A_{540} \) values were compared with those on standard \( A_{540} \) versus cell number curves generated for each cell line. The percentage of survival was calculated using the formula:

\[
\% \text{ survival} = \frac{\text{Live cell number}_\text{test}}{\text{Live cell number}_\text{control}} \times 100
\]

The IC\(_{50}\) values were calculated by nonlinear regression analysis using Graphpad Prism software version 2.0 (Graphpad Software, Inc.).

**Cytotoxicity Assay.** The cytotoxicity of various compounds against human breast cancer cell lines was analyzed using the MTT assay (Boehringer Mannheim Corp., Indianapolis, IN; Refs. 18 and 35). Briefly, exponentially growing breast cancer cells were seeded into a 96-well plate at a density of \( 2.5 \times 10^4 \) cells/well and incubated for 36 h at 37°C prior to drug exposure. On the day of treatment, culture medium was carefully aspirated from the wells and replaced with fresh medium containing the LFM analogues at concentrations ranging from 2 to 250 \( \mu \)g/ml. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 36 h at 37°C in a humidified 5% CO\(_2\) atmosphere. To each well, 10 \( \mu \)l of MTT (0.5 mg/ml final concentration) were added, and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at 37°C in a solution containing 10% SDS in 0.1 M HCl. The absorbance of each well was measured in a microplate reader (Labsystems) at 540 nm and a reference wavelength of 690 nm. To translate the absorbance \( A_{440} \) values into the number of live cells in each well, the \( A_{540} \) values were compared with those on standard \( A_{540} \) versus cell number curves generated for each cell line. The percentage of survival was calculated using the formula:
Fig. 1  Homology model of the EGFR kinase domain. A, molecular surface representation of the homology model of the EGFR kinase domain. B, stereoview of the catalytic site of the EGFR kinase domain. The inhibitor binding region of the EGFR is represented by a triangle-shaped grid.

prevent photobleaching, sealed with nail varnish, and stored at 4°C. Slides were examined using a Bio-Rad MRC-1024 Laser Scanning Confocal Microscope mounted on a Nikon Eclipse E800 upright microscope with high numerical aperture objectives. Digital data were processed using Lasersharp (Bio-Rad) and Adobe Photoshop softwares (Adobe Systems, Mountain View, CA) and printed on a Fuji Pictography thermal transfer printer (Fuji, Elmsford, NY).
Apoptosis Assays. Loose packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmentation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (18, 33). Plasma membrane permeability to PI (Sigma) develops at a later stage of apoptosis (18, 33). MC540 binding and PI permeability were simultaneously measured in breast cancer cells 24 h after exposure to leflunomide analogues, as described (18). Stock solutions of MC540 and PI, each at 1 mg/ml, were passed through a 0.22 μm filter and stored at 4°C in the dark. Whole cells were analyzed with a FACS Calibur or FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). All analyses were done using 488-nm excitation from an argon laser. MC540 and PI emissions were split with a 600-nm short pass dichroic mirror, and a 575-nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission. A 635-nm band pass filter was used for PI emission.

Clonogenic Assays. After treatment with LFM analogues, cells were resuspended in clonogenic medium consisting of α-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 μM 2-mercaptoethanol. Cells were plated in duplicate Petri dishes at 100,000 cells/ml/dish and cultured in a humidified 5% CO2 incubator for 7 days. Cancer cell colonies were enumerated on a grid using an inverted phase microscope of high optical resolution (18). Results were expressed as percentage of inhibition of clonogenic cells at a particular concentration of the test agent using the formula:

\[
\text{% inhibition} = 100 - \frac{\text{Mean no. of colonies [Test]}}{\text{Mean no. of colonies [Control]}} \times 100
\]

Furthermore, the dose survival curves were constructed using the percent control survival (Mean no. of colonies[Test]/Mean no. of colonies [Control] × 100) results for each drug concentration as the data points, and the IC50s were calculated. The IC50s were determined using an Prism Version II Inplot program (Graphpad Software, Inc.).

RESULTS AND DISCUSSION

Establishment of a Homology Model of the EGF-R Tyrosine Kinase. The three-dimensional coordinates of EGF-R used in the modeling studies were constructed based on a structural alignment with the sequences of known crystal structures of other protein tyrosine kinase domains, as detailed in “Materials and Methods.” The catalytic site of the EGF-R kinase domain displays a remarkably planar triangular binding region, with sides of approximately 15, 12, and 12 Å (Fig. 1). The characteristics of this triangular region, which binds the base ring portion of ATP, was elucidated using a binding sphere surface calculated by the program SPHGEN in DOCK3.5 (36). Two sides of the triangle can be visualized as beginning at an apex located between Thr76 and Asp83 (on the upper right corner of the grid lines in Fig. 1B) and extending toward the solvent-accessible opening of the catalytic site. The side of the triangle (which is to the left of the apex in Fig. 1B) is bordered by the hinge region of the catalytic site, and the side to the right of the apex is defined by Arg817, which is immediately adjacent to the binding subsites for the sugar and triphosphate groups of ATP. The hinge region of the binding site is composed of residues 764 to 773 (Fig. 2). The third side of the triangle, located opposite the apex, extends along the slot-shaped opening to the catalytic site. The crystal structures of the HCK/quercetin complex (24) and two FGFR/inhibitor complexes (25) revealed that these PTK inhibitors bind to the catalytic sites of the respective tyrosine kinases. When the catalytic sites are superimposed, all atoms of the three PTK inhibitors fall within the plane of the triangle described previously, and each molecule is in close contact with the hinge region and Asp831. Moreover, they characteristically occupy only one-half of the triangle, near the hinge region. This molecular fitting feature seems to correlate with tighter binding and may be an important determinant for inhibitor binding. Similarly, the size and planar shape of the catalytic site within the constructed EGF-R kinase domain contributes to its ability to form favorable interactions with planar molecules like the LFM analogues described in this report. This observation was in good agreement with conclusions derived from the structure-activity analyses of pyrrolo- and pyrazoloquinazolines compounds (37) and was incorporated into our modeling strategy. Although most of the catalytic site residues of the EGF-R kinase domain were conserved relative to other tyrosine

![Fig. 2 Superimposed docked positions of the para-substituted LFM analogues at the active site of the EGF-R tyrosine kinase. Selected residues in the active site of EGF-R are shown in pink. LFM, unsubstituted active metabolite of leflunomide (green); LFM-A1, para-Br substituted LFM analogue (blue); LFM-A2, para-CI-substituted LFM analogue (red); LFM-A3, para-F substituted LFM analogue (white); LFM-A12, para-OFC, substituted LFM analogue (multicolor).](image-url)
Cancer cell cytotoxicity

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<th>Compound</th>
<th>X</th>
<th>M.S. (\text{Å}^2)</th>
<th>B.S. (\text{Å}^2)</th>
<th>Lipo score</th>
<th>Ludi score</th>
<th>Ludi (K_i) (\mu\text{M})</th>
<th>EGF-R inhibition (IC_{50}) (\mu\text{M})</th>
<th>Cancer cell cytotoxicity (IC_{50}) (\mu\text{M})</th>
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<td>p-Br</td>
<td>226.5</td>
<td>176.7</td>
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<td>449</td>
<td>32</td>
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<td>LFM-A2</td>
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<td>LFM-A3</td>
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\(a\) M.S., molecular surface area calculated using Connolly’s MS program (39). Defined as boundary of volume within any probe sphere (meant to represent a water molecule) of given radius sharing no volume with hard sphere atoms that make up the molecule.

\(b\) B.S., buried surface: molecular surface (in \(\text{Å}^2\)) in contact with protein calculated by Ludi based on docked positions.

\(c\) Ludi \(K_i\) calculated based on the empirical score function in the Ludi program.

FGFR (Leu, Ala) and in IRK (Met, Gly). Residues Asn\(^{818}\) and Asp\(^{831}\) (opposite the hinge) are conserved in all four PTKs. Residue Thr\(^{766}\) in the hinge region changes to Val in FGFR and to Met in IRK. Residue Thr\(^{830}\) changes to Ala in FGFR and to Gly in IRK. The right side of the binding pocket (Fig. 1B) contains Cys\(^{773}\) in EGF-R and is therefore considerably more hydrophobic than the corresponding residue of platelet-derived growth factor receptor (Asp), FGFR (Asn), and IRK (Asn). These residue identity differences may provide the basis for designing selective inhibitors of the EGF-R tyrosine kinase. Selectivity for a particular tyrosine kinase can also be achieved by extending the binding area to the other one-half of the triangle (opposite the hinge), as has been observed in one of the FGFR inhibitor structures (38). The difference in binding affinity is provided by Phe\(^{489}\), which extends from a \(\beta\) hairpin toward the inhibitor. EGF-R has a shorter and more rigid \(\beta\) hairpin than FGFR, which would present some limitations to this strategy. The nonconserved residue Arg\(^{817}\) could also provide some binding discrimination if the inhibitor binds nearby. We concluded from our modeling studies that the catalytic site of the EGF-R has specific features that can be advantageous for the design of inhibitors. These features include a triangle-shaped region that is accessible to an inhibitor. We hypothesized that molecules fitting the triangular shape of the EGF-R catalytic site, which can also form favorable contacts with the hinge region of the binding site, will bind more strongly and hence inhibit EGF-R more effectively. To elucidate the structure activity relationships determining the ability of LFM to inhibit the EGF-R tyrosine kinase as well as test the predictive value of our homology model for the EGF-R kinase domain, we have designed and synthesized analogues of this compound by systematically replacing the p-CF3 substituent in the phenyl ring. Our modeling calculations were based on the homology model of the EGF-R kinase domain described above and
Fig. 4  Cytotoxic activity of LFM and LFM-A12 against human breast cancer cells in MTT assays. MDA-MB-231 and MDA-MB-361 cells were treated with LFM (A) or LFM-A12 (B) for 36 h in 96-well plates, and the cytotoxicity was determined by MTT assay. Data points, the mean values from three independent experiments; bars, SE.

Fig. 5 Confocal images of LFM-A12-treated breast cancer cells. MDA-MB-231 cells were treated with LFM-A3 (C) or LFM-A12 (B) at a final concentration of 100 μM for 24 h at 37°C, as described in “Materials and Methods.” After treatment, cells were processed for immunofluorescence using a monoclonal antibody to α-tubulin (green fluorescence). LFM-A12 (but not LFM-A3)-treated cells showed marked shrinkage with disruption of microtubules and lost their ability to adhere to the substratum. Blue fluorescence, nuclei stained with TOTO-3.
Fig. 6 Flow cytometric evidence of apoptosis in LFM-A12-treated breast cancer cells. FACS correlated two-parameter displays of MDA-MB-231 cells treated with PBS (CON), vehicle (DMSO), LFM (100 or 500 μM concentrations), or LFM-A12 (100 or 500 μM concentrations) for 24 h and then stained with MC540 and PI. The percentages indicate the fraction of cells at an advanced stage of apoptosis (AF, apoptotic fraction), as measured by dual MC540/PI fluorescence.

a close contact with the hinge region of the catalytic site of EGF-R, the m-CF₃ substituent of LFM-A8 is the only meta-substituted compound in the series reported that is sandwiched between residues T766 and D831; hence it is the only meta compound that gains additional hydrophobic contact via interactions with these residues. This added contact contributes to a lower calculated Kᵢ relative to other meta-substituted compounds (Kᵢ, 22 μM versus >100 μM; Table 1). The para-substituted CF₃ was more active than the m-CF₃ compound in terms of IC₅₀ inhibition values (IC₅₀, 5.4 μM versus >100 μM; Table 1), which is consistent with the modeling observation that this compound maintains closer contacts with the hinge region than the m-CF₃ compound. As shown in Fig. 3, the meta substitution is sterically less favorable for allowing a closer interaction with the hinge region of EGF-R relative to para-substituted LFM compounds, which may contribute to a loss of hydrophobic contact in this region of the binding site. This loss of hydrophobic contact would be reflected in a lower calculated Kᵢ based on docking studies.

Modeling studies indicated that the LFM analogues with para substitutions show the greatest potential for inhibition in the whole series, with p-CF₃ and p-OCF₃ being the most promising (calculated Kᵢ, ~8–13 μM), followed by p-Cl and p-Br (calculated Kᵢ, ~30–40 μM), and p-F (Kᵢ >100 μM; Table 1). The best docked positions of the five para-substituted compounds showed a binding pattern similar to that of the meta-substituted compounds, except for a slight shift of the phenyl rings toward Thr⁷⁶⁶ (Fig. 2). The para-substituted compounds maintain a close contact with the hinge region of the EGF-R kinase domain and are stabilized by an additional contact area through the para substituents and the residues at the inner core of the protein. The CF₃ and OCF₃ substituents in the para position extend toward the inner core of the protein, which results in improved molecular contact. For compound LFM-A₃, the C-F bond length (1.3 Å) is shorter than the C-Cl and C-Br bond lengths in LFM-A₂ and LFM-A₁ (1.8–1.9 Å); thus, the p-F group does not approach Lys⁷²¹ as closely. This weaker contact may contribute to the poor Kᵢ for LFM-A₄. Fig. 3 shows the superimposed docked positions of the three CF₃ substituted compounds (ortho, meta, and para). Relative to para-substitu-
tuted compounds, the bulky ortho and meta substitutions prevent LFM-A4 and LFM-A8 from having closer contacts with the active site of the EGF-R kinase domain. In contrast, the para-substituted LFM maintains more favorable contact with the hinge region of the EGF-R kinase domain.

When compared with the compounds with ortho and meta substitutions in the phenyl ring, the para-substituted compounds appeared to approach Asp and force the residue to rotate away from the inhibitor, as was observed in our docking results. This action actually disrupts a hydrogen bond between the Asp83' and Asn88, which was observed in our docking results. This action actually disrupted a hydrogen bond between the Asp83' and Asn88. This action actually disrupted a hydrogen bond between the Asp83' and Asn88, which could cause a slight destabilization of the protein conformation in this region. This event becomes more likely to occur when larger para-substituted groups are involved, such as the para-Cl of LFM-Al and para-Cl of LFM-A2. Our calculations were not able to incorporate the effects of this potential increase in energy. Therefore, the true $K_{50}$ for compounds containing a larger para-substituted group, such as LFM-A1 and LFM-A2, may be higher than the estimated values shown in Table 1.

**Effects of Analogues of Leflunomide Metabolite on EGF-R Tyrosine Kinase and Survival of Human Breast Cancer Cells.** We first examined the effects of LFM analogues on the enzymatic activity of the EGF-R kinase in immune complex kinase assays. A 1-h incubation with LFM or LFM-A12 inhibited the EGF-R tyrosine kinase in the anti-EGF-R immunoprecipitates from lysates of MDA-MB-231 human breast cancer cells in a dose-dependent fashion. The $IC_{50}$s were 5.4 $\mu$M for LFM and 1.7 $\mu$M for LFM-A12 (Table 1). We next examined the effects of a 24-h exposure of MDA-MB-231 cells to LFM or LFM analogues on the enzymatic activity of the EGF-R tyrosine kinase after a 10-min stimulation with EGF. The EGF-R immune complexes were subjected to Western blot analysis with a polyclonal anti-phosphotyrosine antibody to measure the autophosphorylation of the EGF-R. Treatment of MDA-MB-231 cells with LFM-A12 and, albeit to a lesser extent, with LFM resulted in decreased tyrosine phosphorylation of the EGF-R after EGF stimulation (data not shown). In contrast, none of the other LFM analogues tested side-by-side were able to inhibit the EGF-induced tyrosine phosphorylation of the EGF-R in MDA-MB-231 cells, and most showed $IC_{50}$s of $>100$ $\mu$M in both cell-free and cellular EGF-R kinase inhibition assays (Table 1). Taken together, these experimental results are consistent with the trend of the calculated $K_{50}$ values shown in Table 1, thereby confirming the predictive value of the constructed homology model for the EGF-R kinase domain.

In MTT assays, LFM-A2 exhibited significant cytotoxicity against the MDA-MB-231 and MDA-MB-361 human breast cancer cell lines with mean $IC_{50}$s of 53.4 $\mu$M and 26.3 $\mu$M, respectively, for the composite drug dose survival curves (Fig. 4). By comparison, LFM was significantly less active against these breast cancer cell lines. The $IC_{50}$s of the LFM composite dose survival curves were 198.9 $\mu$M for MDA-MB-231 cells and 190.5 $\mu$M for MDA-MB-361 cells.

We also examined the effects of LFM-A12 treatment on MDA-MB-231 cells by confocal laser scanning microscopy. Slides were examined using a Bio-Rad MR-C-1024 Laser Scanning Confocal Microscope mounted on a Nikon Eclipse E800 upright microscope with high numerical aperture objectives. Digital data were processed using Lasersharp (Bio-Rad) and Adobe Photoshop software (Adobe Systems) and printed on a Pictrography printer (Fuji, Elmsford, NY; Ref. 32). As shown in Fig. 5A, vehicle (DMSO)-treated control cells were round and large with many well organized microtubules (green fluorescence secondary to tubulin staining) in the cytoplasm. Nuclei (blue fluorescence secondary to TOTO-3 staining) were also round and homogeneous. In contrast, MDA-MB-231 cells treated with 100 $\mu$M LFM-A12 for 24 h were much smaller and had an abnormal shape with large cytoplasmic vacuoles (Fig. 5B). The microtubules of LFM-A12-treated cells were fewer in number, and they appeared less organized than those of DMSO-treated controls. The nuclei (blue) of the LFM-A12 treated cells were also smaller and misshapen. Unlike LFM-A12, 100 $\mu$M LFM-A3 did not affect the morphology or microtubular organization of MDA-MB-231 cells (Fig. 5C).

The morphological features of LFM-A12-treated MDA-MB-231 cells as determined by confocal microscopy (i.e., shrinkage, nuclear condensation, and abnormal microtubular organization) suggested that these cells might be undergoing apoptosis. Therefore, we decided to formally study whether LFM-A12 could trigger apoptosis in breast cancer cells using a quantitative flow cytometric apoptosis detection assay. In this assay, loose packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmentation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (34). MC540 binding and PI permeability of MDA-MB-231 breast cancer cells were simultaneously measured before and after a 24-h treatment with 100 or 500 $\mu$M LFM-A12. Whole cells were analyzed with a FACS Calibur or FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). Whereas less than 5% of MDA-MB-231 cells showed apoptotic changes after DMSO treatment, a significant portion of cells underwent apoptosis within 24 h after LFM-A12 treatment (apoptotic fraction with MC540/PI advanced stage apoptosis: 54% at 100 $\mu$M and 85% at 500 $\mu$M).

| Table 2 | In vitro antitumor activity of LFM and LFM-A12 against clonogenic breast cancer cells |
|-----------------|-----------------|-----------------|
| Cell line and treatment | Concentration of compound | Tumor cell colonies/106 cells | % inhibition |
| MDA-MB-361 None | 1104 (924, 1284) | 0 | 0 |
| DMSO 0.1% | 1088 (872, 1304) | 1.4 | |
| LFM 0.1 $\mu$M | 803 (702, 904) | 27.3 | |
| 10 $\mu$M | 535 (386, 684) | 51.5 | |
| 100 $\mu$M | 196 (128, 264) | 82.3 | |
| LFM-A12 0.1 $\mu$M | 746 (316, 1276) | 32.4 | |
| 10 $\mu$M | 440 (276, 604) | 60.2 | |
| 100 $\mu$M | 140 (58, 222) | 87.3 | |
| MDA-MB-231 None | 1150 (1096, 1204) | 0 | 0 |
| DMSO 0.1% | 953 (888, 1018) | 17.1 | |
| LFM 0.1 $\mu$M | 964 (588, 1340) | 16.2 | |
| 10 $\mu$M | 642 (572, 712) | 44.2 | |
| 100 $\mu$M | 297 (170, 424) | 74.2 | |
| LFM-A12 0.1 $\mu$M | 667 (454, 880) | 42.0 | |
| 10 $\mu$M | 515 (420, 610) | 55.2 | |
| 100 $\mu$M | 0 | >99 | |
μM: Fig. 6). LFM, albeit to a lesser extent, also induced apoptosis in MDA-MB-231 cells.

We next tested the anti-cancer activity of LFM and LFM-A12 against MDA-MB-361 and MDA-B-231 breast cancer cells using in vitro clonogenic assays. As shown in Table 2, 24-h treatment with LFM or LFM-A12 inhibited the clonogenic growth of MDA-MB-361 cells as well as MDA-MB-231 cells in a dose-dependent fashion. At 100 μM, our lead compound LFM-A12 killed 87.3% of clonogenic MDA-MB-361 cells and >99% of clonogenic MDA-MB-231 cells.

In summary, we constructed a homology model to represent the structure of EGF-R and used this model and a docking procedure to rationally design compounds predicted to bind favorably to EGF-R. This approach led to the successful design of a LFM analogue that was found to have an IC50 of 1.7 μM in EGF-R inhibition assays and killed >99% of human breast cancer cells in vitro by triggering apoptosis. Our ongoing efforts are aimed at synthesis and evaluation of a new generation of LFM analogues as potentially much more active inhibitors of the EGF-R tyrosine kinase. It is our working hypothesis that the LFM analogues with calculated interaction scores and Ks better than those of LFM and LFM-A12 will exhibit more potent cytotoxicity against human breast cancer cells. The development of such LFM analogues with exceptional anti-breast cancer activity may provide the basis for more effective cancer treatment modalities for breast cancer patients with metastatic disease.

REFERENCES

6. Somlo, G., Doroshov, J. H., Forman, S. H., Leong, L. A., Margolin, K. A., Morgan, R. J., Jr., Raschko, J. W., Ackman, S. A., Ahn, C., and Nagasawa, S. High-dose doxorubicin, etoposide and cyclophosphamide as potentially much more active inhibitors of the EGF-R tyrosine kinase. It is our working hypothesis that the LFM analogues as potentially much more active inhibitors of the EGF-R tyrosine kinase. It is our working hypothesis that the LFM analogues as potentially much more active inhibitors of the EGF-R tyrosine kinase.


