Rational Design and Synthesis of a Novel Anti-leukemic Agent Targeting Bruton’s Tyrosine Kinase (BTK), LFM-A13 [α-Cyano-β-Hydroxy-β-Methyl-N-(2,5-Dibromophenyl)Propenamide]*

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In a systematic effort to design potent inhibitors of the anti-apoptotic tyrosine kinase BTK (Bruton’s tyrosine kinase) as anti-leukemic agents with apoptosis-promoting and chemosensitizing properties, we have constructed a three-dimensional homology model of the BTK kinase domain. Our modeling studies revealed a distinct rectangular binding pocket near the hinge region of the BTK kinase domain with Leu$^{660}$, Tyr$^{676}$, Arg$^{625}$, and Asp$^{529}$ residues occupying the corners of the rectangle. The dimensions of this rectangle are approximately $18 \times 9 \times 9 \times 17 \, \text{Å}$, and the thickness of the pocket is approximately $7 \, \text{Å}$. Advanced docking procedures were employed for the rational design of leflunomide metabolite (LFM) analogs with a high likelihood to bind favorably to the catalytic site within the kinase domain of BTK. The lead compound LFM-A13, for which we calculated a $K_i$ value of 1.4 $\mu M$, inhibited human BTK in vitro with an $IC_{50}$ value of 17.2 $\mu M$. Similarly, LFM-A13 inhibited recombinant BTK expressed in a baculovirus expression vector system with an $IC_{50}$ value of 2.5 $\mu M$. The energetically favorable position of LFM-A13 in the binding pocket is such that its aromatic ring is close to Tyr$^{676}$, and its substituent group is sandwiched between residues Arg$^{625}$ and Asp$^{529}$. In addition, LFM-A13 is capable of favorable hydrogen bonding interactions with BTK via Asp$^{529}$ and Arg$^{625}$ residues. Besides its remarkable potency in BTK kinase assays, LFM-A13 was also discovered to be a highly specific inhibitor of BTK. Even at concentrations as high as 100 $\mu M$ ($-278 \, \text{µM}$), this novel inhibitor did not affect the enzymatic activity of other protein tyrosine kinases, including JAK1, JAK3, HCK, epidermal growth factor receptor kinase, and insulin receptor kinase. In accordance with the anti-apoptotic function of BTK, treatment of BTK+ B-lineage leukemic cells with LFM-A13 enhanced their sensitivity to ceramide- or vincristine-induced apoptosis. To our knowledge, LFM-A13 is the first BTK-specific tyrosine kinase inhibitor and the first anti-leukemic agent targeting BTK.

Apoptosis is a common mode of eukaryotic cell death that is triggered by an inducible cascade of biochemical events leading to activation of endonucleases that cleave the nuclear DNA into oligonucleosome length fragments (1–4). Several of the biochemical events that contribute to apoptotic cell death as well as both positive and negative regulators of apoptosis have recently been identified (1–4). Apoptosis plays a pivotal role in the development and maintenance of a functional immune system by ensuring the timely self-destruction of autoreactive immature and mature lymphocytes as well as any emerging target neoplastic cells by cytotoxic T cells (1–7). Inappropriate apoptosis may contribute to the development as well as chemotherapeutic resistance of human leukemias and lymphomas (5–7). Therefore, an improved understanding of the molecular basis of apoptosis and the pro-apoptotic versus anti-apoptotic regulatory signals may provide further insights into the pathogenesis of human lymphoid malignancies and have important implications for treatment of leukemias and lymphomas.

Bruton’s tyrosine kinase (BTK), a member of the BTK/Tec family of protein tyrosine kinases (PTKs), is a cytoplasmic PTK involved in signal transduction pathways regulating growth and differentiation of B-lineage lymphoid cells (8–10). BTK participates in signal transduction pathways initiated by the binding of a variety of extracellular ligands to their cell-surface receptors; following ligation of B cell antigen receptors, BTK activation by the concerted actions of the PTKs Lyn and Syk (9) is required for induction of phospholipase C-γ2-mediated calcium mobilization (9). Mutations in the human BTK gene are the cause of X-linked agammaglobulinemia, a male immune deficiency disorder characterized by a lack of mature, immunoglobulin-producing, peripheral B cells (11, 12). In mice, mutations in the BTK gene have been identified as the cause of murine X-linked immune deficiency deficiency (13).

BTK has an N-terminal region consisting of a 140-residue pleckstrin homology (PH) domain followed by an 80-residue proline-rich Tec homology (TH) domain. The PH domain is the site of activation by phosphatidylinositol phosphates and G-protein βγ subunits and inhibition by protein kinase C (14). The remaining portion of BTK contains Src homology (SH) domains SH3 (49 residues), followed by SH2 (96 residues), and a 250-residue C-terminal SH1 kinase domain. The SH2 domain mediates binding to tyrosine-phosphorylated peptide motifs on other molecules, and the SH3 domain mediates binding to proline-rich motifs. BTK is activated by transphosphorylation of Tyr$^{551}$ in the SH1 domain, followed by autophosphorylation and

† The abbreviations used are: BTK, Bruton’s tyrosine kinase; LFM, leflunomide metabolite; EGFR, EGF receptor; IRK, insulin receptor kinase; PTK, protein tyrosine kinase; PH, pleckstrin homology; TH, Tec homology; FGFR, fibroblast growth factor receptor; PIU, PhosphorImager units; DSU, densitometric scanning units; MS, mass spectrometry; SH, Src homology; PI, propidium iodide; ALL, acute lymphoblastic leukemia; GST, glutathione S-transferase; HCK, hematopoietic cell kinase.

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of Tyr223 in the SH3 domain (9). Phosphorylation of Tyr223 may function to disrupt an intramolecular TH-SH3 domain interaction, allowing BTK TH domain binding with SH3 domains in the Src family kinases FYN, LYN, and HCK, and BTK SH3 domain binding with a proline-rich region of Cbl (9, 15, 16). Mutations in the catalytic domain, SH2 domain, as well as PH domain of the BTK have been found to lead to maturational blocks at early stages of B cell ontogeny in human X-linked agammaglobulinemia (17). BTK-deficient mice generated by introducing PH domain or catalytic domain mutations in embryonic stem cells showed defective B cell development and function (18). Thus, different regions of BTK are important for its physiologic functions.

In murine B cells, BTK has been shown to act as an anti-apoptotic protein upstream of Bcl-xL in the B cell antigen receptor (but not the CD40 receptor) activation pathway (19). Our recent studies provided biochemical and genetic evidence that BTK is an inhibitor of the Fas/APO-1 death-inducing signaling complex in B-lineage lymphoid cells (20). Furthermore, we found that BTK also prevents ceramide- and vincristine-induced apoptosis (present study). The fate of leukemia/lymphoma cells may reside in the balance between the opposing proapoptotic effects of caspases activated by the death-inducing signaling complex and an upstream anti-apoptotic regulatory mechanism involving BTK and/or its substrates (20). Therefore, inhibitors of BTK are likely to enhance the chemosensitivity of leukemia/lymphoma cells.

In a systematic effort to design potent inhibitors of BTK as anti-leukemic agents with apoptosis-promoting properties, we have constructed a three-dimensional homology model of the BTK kinase domain. Advanced docking procedures were employed for the rational design of leflunomide metabolite (LFM) analogs with a high likelihood to bind favorably to the catalytic site within the kinase domain of BTK. Here, we first report the identification of the LFM analog a-cyano-β-hydroxy-β-methyl-N-(2,5-dibromomethyl)-propenamide (LFM-A13) as a potent and specific inhibitor of BTK. LFM-A13 inhibited recombinant BTK with an IC50 value of 2.5 μM, but it did not affect the enzymatic activity of other protein tyrosine kinases, including Janus kinases JAK1 and JAK2, Src family kinase HCK, and receptor family tyrosine kinases EGF-receptor kinase (EGFR) and insulin receptor kinase (IRK), at concentrations as high as 278 μM. LFM-A13 enhanced the chemosensitivity of BTK-positive B-lineage leukemia cells to vincristine and ceramide.

**EXPERIMENTAL PROCEDURES**

**Crystal Structures of Leflunomide Metabolite and Its Analogs**—The leflunomide metabolite (LFM) and two of its analogs (LFM-A12 and LFM-A13) were crystallized using various solvents by evaporation or liquid–liquid diffusion. X-ray data from single crystals were collected using a SMART CCD area detector (Bruker Analytical X-ray Systems, Madison, WI) with MoKα radiation (λ = 0.7107 Å). The space group was determined based on systematic absence and intensity statistics. A direct methods solution provided most of the non-hydrogen atoms from the electron density map. Several full-matrix least squares/difference Fourier cycles were performed to locate the remaining non-hydrogen atoms. All non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were placed in ideal positions and refined as riding atoms with relative isotropic temperature factors. The structure was refined using full matrix least squares on F2. Crystal structure calculations were performed using a Silicon Graphics INDY R4400-SC computer (Silicon Graphics Inc., Mountain View, CA) or a Pentium computer using the SHELXTL V 5.0 suite of programs (21). The structural superimposition was done using the homology module of the InsightII (26) program and a Silicon Graphics INDIGO2 computer (Silicon Graphics Inc., Mountain View, CA). The sequence alignment was manually adjusted based on the previously mentioned considerations and produced a sequence variation profile for each superimposed C-α position. The sequence variation profile served as a basis for the next procedure, which was sequence alignment of all four proteins with BTK kinase.

In this procedure, the sequence of BTK kinase was read into the program and manually aligned with the four known kinase proteins based on the sequence variation profile described previously. Next a set of three-dimensional coordinates was assigned to the BTK kinase sequence using the three-dimensional coordinates of HCK as a template, which employed the Homology module within the InsightII program (26). The coordinates for a loop region where a sequence insertion occurs (relative to HCK without the loop) was chosen from a limited number of possibilities automatically generated by the program and manually adjusted to a more ideal geometry using the program CHAIN (27). Finally, the constructed model of BTK was subjected to energy minimization using the X-plor program (28) 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 data base or by manually rotating the respective side chains. The final homology model of the BTK kinase domain had a root mean square deviation of 0.01 Å from ideal bond lengths and 2.2° from ideal bond angles after energy minimization. The homology model of BTK was then used, in conjunction with model coordinates of LFM and its analogs (which were later compared with crystal structures), for our modeling studies of the BTK-inhibitor complexes.

**Docking Procedure Using Homology Model of BTK Kinase Domain**—Modeling of the BTK-LFM analog complexes was done using the Dock module within the program INSIGHTII (26) and using the Affinity program (29) to provide energy minimization for a ligand/receptor complex. Energy-minimized coordinates for each LFM molecule were generated, and interactively docked into the ATP-binding site of BTK based on the position of quercetin in the HCK/quercetin crystal structure (22). The hydrogen atoms on the kinase domain of BTK 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 (26) program used the CVFF force field and a Monte Carlo search strategy to search for and evaluate docked structures. While the coordinates for the bulk of the receptor were 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 analog in the BTK catalytic site. The various docked positions of each LFM analog was qualitatively evaluated using Ludi (29, 30) in INSIGHTII (26) which was then used to estimate a binding constant (Kd) for each compound in order to rank their relative binding capabilities and predicted inhibition of BTK. The Kd trends for the LFM analogs were compared with the trend of the experimentally determined tyrosine kinase inhibition IC50 values for the compounds, in order to elucidate the structure-activity relationships determining the potency of the LFM analogs.
flasks at 60–90 rpm. Cell viability was maintained at 95–100% as determined by trypan blue dye exclusion.

Recombinant baculovirus containing the murine BTK gene was con-structed as described (20). In brief, the gene encoding BTK was excised from pBluescript SKII- vector (Stratagene) by digestion with BamHI, and this fragment was then ligated into pFastBac1 (Life Technologies, Inc.). The resulting vector, pFastBac1-BTK, was then used to generate the recombinant baculovirus by site-specific transposition in Esche-richtia coli DH10Bac cells (Life Technologies, Inc.) which harbor a baculovirus shuttle vector (bactm), bM0N14272. The resulting recom-binant bacmid DNA was introduced into insect cells by transfection with the standard liposome-mediated method using Cellfectin reagent (Life Technologies, Inc.). Four days later, transfection supernatants were harvested for subsequent plaque purification and analyzed as above. Kinase-dead BTK was generated as described (20) and cloned into the baculovirus expression vector as described above for wild-type BTK. Baculovirus expression vectors for JAK1 and JAK3 kinases were constructed and introduced into insect cells, as previously reported (31).

Immunoprecipitation of Recombinant Proteins from Insect Cells—

The insect cell line Sf21 cells were infected with a baculovirus expression vector for BTK, JAK1, or JAK3 as indicated in the figure legends. Cells were harvested and lysed (10 mM Tris, pH 7.6, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 100 µM Na3VO4, 50 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin), and the kinases were immunoprecipitated from the lysates, as reported (20). Antibodies used for immunoprecipitations from insect cells are as follows: polyclonal rabbit anti-BTK serum (15), polyclonal rabbit anti-JAK1 (HR-785), catalog number sc-277, rabbit polyclonal IgG affinity purified, 0.1 mg/ml, Santa Cruz Biotechnology, and polyclonal rabbit anti-JAK3 (C-21, catalog number sc-513), rabbit polyclonal IgG affinity purified, 0.2 mg/ml, Santa Cruz Biotechnology. Kinase assays were performed following a 1-h exposure of the immunoprecipitated tyrosine kinase kinases to the reaction compounds, as described in detail elsewhere (15, 32). The immunoprecipitates were subjected to Western blot analysis as described previously (20).

Cell Lines, Reagents, and Biochemical Assays—The establishment and characterization of DT40 lymphoma B cell line as well as BTK-deficient DT40 and its derivatives reconstituted with wild-type or muta-tant human BTK have been previously reported (32). Equal amounts of BTK protein were detected by Western blot analysis in all of the BTK-deficient DT40 clones transfected with wild-type or mutated human BTK genes, but no BTK protein was detectable in the untrans-fected BTK-deficient DT40 cells (32). All cell lines derived from the chicken B cell line DT40 were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% heat-inactivated chicken serum, 2 mM glutamine, penicillin, and streptomycin. Cells were grown at 37 °C in a 5% CO2 water-saturated atmosphere. The BTK positive human B-lineage leukemia cell lines NALM-6 and ALL-1 were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (33). COS-7 simian kidney cell line and HepG2 human hepatoma cell line were obtained from ATCC. Antibodies directed against BTK, JAK1, JAK3, and HCK have been described previously (15, 20, 31, 32). Polyclonal antibodies to BTK were generated by immunization of rabbits with glutathione S-transferase (GST) fusion proteins (Amersham Pharmacia Biotech) containing the first 150 amino acids of BTK. The monoclonal anti-Fas antibody (F22120) was obtained from the Transduction Laboratories, Inc. (Lex-ington, KY). Immunoprecipitations, immunocomplex protein kinase assays, and immunoblotting using the ECL chemiluminescence detection system (Amersham Pharmacia Biotech) were conducted as described previously (15, 20, 31, 32). Following electrophoresis, kinase gels were dried onto Whatman 3M filter paper and subjected to phosphorimagining on a Molecular Imager (Bio-Rad) as well as autoradiogra-phy on film. Similarly, all chemiluminescent BTK Western blots were subjected to three-dimensional densitometric scanning using the Molecular Imager and Imaging Densitometer using the Molecular Analyst/ Macintosh version 2.1 software following the specifications of the manufac-turer (Bio-Rad). For each drug concentration, a BTK kinase activity index was determined by comparing the ratios of the kinase activity in PhosphorImager units (PIU) and density of the protein bands in densitometric scanning units (DSU) to those of the base-line sample and using the formula: activity index = [PIU of kinase band/DSU of BTK protein band]base-line sample/[PIU of kinase band/DSU of BTK protein band]test sample. GST-IGa was sometimes used as an exogenous substrate for BTK immune complex protein kinase assays, as described (15). Horseradish peroxidase-conjugated sheep anti-mouse, donkey an-ti-rabbit secondary antibodies and ECL reagents were purchased from Amersham Pharmacia Biotech. For insulin receptor kinase (IRK) as-says, HepG2 human hepatoma cells grown to approximately 80% confluency were washed once with serum-free Dulbecco’s modified Eagle’s medium and starved for 3 h at 37 °C in a CO2 incubator. Subsequently, cells were stimulated with insulin (Lilly, catalog number CP-410; 10 units/ml/106 cells) for 10 min at room temperature. Following this IRK activation step, cells were washed once with serum-free medium and lysed in Nonidet P-40 buffer, and IRK was immunoprecipitated from the lysates with an anti-IRβ antibody (Santa Cruz Biotechnology, catalog number sc-711, polyclonal IgG). Prior to performing the immune complex kinase assays, the beads were equilibrated with the kinase buffer (30 mM Hepes, pH 7.4, 30 mM NaCl, 8 mM MgCl2, 4 mM MnCl2). For HCK kinase assays, we used HCK-transfected COS-7 cells. The cloning and expression of HCK in COS-7 cells has been described previously (34). The pS7Vt-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 Nonidet P-40 buffer, and HCK was immunoprecipitated from the whole cell lysates with an anti-HCK antibody.

Apoptosis Assays—To induce apoptosis, cells were treated with an agonistic anti-Fas/APO-1 antibody (Bender MedSystems, Lot number 04/1295) at 0.1 and 0.5 µg/ml final concentrations, vincristine (vincris-tine sulfate, USP; Amersham Pharmacia Biotech, NDC 0013-7466-86, lot number VC8019) at 10 and 100 ng/ml final concentrations, or C2-ceramide (Biologic, lot number M8107) at 10, 50, and/or 100 µM final concentrations. MC540 binding (as an early marker of apoptosis) and PI permeability (as a marker of advanced stage apoptosis) were simulta-neously measured in DT40 cells 24 h after exposure to C2-ceramide, anti-Fas, or vincristine, as described previously (32). Whole cells were analyzed using a FACStar Plus 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 660-nm short pass dichroic mirror, and a 575-nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission and a 635-nm band pass filter was used for PI emission. In order to examine the effects of the lead BTK inhibitor on ceramide-induced apoptosis in B cell antigen receptor-ABL positive human ALL cell line ALL-1, cells were treated for 4 h at 37 °C with 10 µM C2-ceramide in the presence or absence of the inhibitor (200 µM LFM-A13). Subsequently, cells were...
**FIG. 1.** The anti-apoptotic function of BTK. Wild-type and BTK-deficient (BTK\(^{-}\)) DT40 lymphoma B cells (A) as well as BTK\(^{-}\) DT40 cells reconstituted with wild-type or mutant human BTK (B) were treated with C2-ceramide (C2-CER), vincristine (VCR), or anti-Fas antibody, as described under "Experimental Procedures." BTK-deficient DT40 (BTK\(^{-}\)) cells expressing wild-type BTK, BTK\(\text{Arg}^{525} \rightarrow \text{Gln}\), BTK\(\text{Arg}^{28} \rightarrow \text{Cys}\), and BTK\(\text{Arg}^{307} \rightarrow \text{Ala}\) were designated as BTK\(^{-}\),rBTK(WT), BTK\(^{-}\),rBTK(K28), BTK\(^{-}\),rBTK(mPH) and BTK\(^{-}\),rBTK(mSH2), respectively. Vehicle (0.1% Me\(_2\)SO in phosphate-buffered saline) treated as well as drug-treated cells were maintained in culture medium for 24 h at 37 °C and 5% CO\(_2\) before harvesting. DNA from Triton X-100 lysates was analyzed for fragmentation, as described (32). bp, base pairs; WT, wild type; M, size markers.
washed and stained with PI and MC540, and the apoptotic fractions were determined by multiparameter flow cytometry, as described (32).

To detect apoptotic fragmentation of DNA, DT40 cells were harvested 24 h after exposure to anti-Fas, C2-ceramide, or vincristine. Similarly, B18.2, NALM-6, and ALL-1 cells were treated with LFM-A13 (100 μM), vincristine (VCR) (10 ng/ml), C2-ceramide (C2-CER) (10 μM), LFM-A13 (100 μM) + VCR (10 ng/ml), and LFM-A13 (100 μM) + C2-CER (10 μM) for 24 h at 37 °C. DNA was prepared from Triton X-100 lysates for analysis of fragmentation (32). In brief, cells were lysed in hypotonic 10 mmol/liter Tris-HCl, pH 7.4, 1 mmol/liter EDTA, 0.2% Triton X-100 detergent and subsequently centrifuged at 11,000 × g. To detect apoptosis-associated DNA fragmentation, supernatants were electrophoresed on a 1.2% agarose gel, and the DNA fragments were visualized by ultraviolet light after staining with ethidium bromide.

Chemical Synthesis—All chemicals were purchased from Aldrich and were used without further purification. Except where noted, each reaction vessel was secured with a rubber septum, and the reaction was performed under nitrogen atmosphere. 1H NMR spectrum was obtained on a Varian Mercury 300 instrument 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 spectra were recorded on a Nicolet Protege 460 spectrometer (Madison, WI). Gas chromatography/mass spectroscopy (MS) was obtained on a HP 6890 GC System (Palo Alto, CA) equipped with a HP 5973 Mass Selective Detector. EI and CI MS data were obtained at the University of Minnesota MS Core Facility.

Synthesis of LFM, LFM-A1-LFM-A14—Scheme 1 shows the general synthetic scheme for the preparations of LFM, and LFM-A1-LFM-A14 (35, 36). Cyanoacetic acid 1 was coupled with the desired substituted-aniline 2 in the presence of diisopropylcarbodiimide to form 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 (35, 36)—1,3-Diisopropylcarbodiimide
Chemosensitizing Novel BTK Inhibitor

Table I

| Compound | X        | M.S.a | B.S.b | Lipase score | No. of hydrogen bonds | Ludi score | Ludi c_Ki | IC50 d |
|----------|----------|-------|-------|--------------|-----------------------|------------|----------|--------|
| LFM      | p-CF3    | 240   | 168   | 491          | 1                     | 475        | 56.2     | >370   |
| LFM-A1   | p-Br     | 234   | 156   | 459          | 1                     | 446        | 36.3     | >356   |
| LFM-A2   | p-Cl     | 232   | 162   | 476          | 1                     | 461        | 24.5     | >423   |
| LFM-A3   | p-F      | 219   | 158   | 462          | 1                     | 446        | 34.7     | >454   |
| LFM-A4   | o-CF3    | 237   | 171   | 501          | 1                     | 485        | 44.7     | >370   |
| LFM-A5   | o-Br     | 226   | 162   | 474          | 1                     | 458        | 26.3     | >356   |
| LFM-A6   | o-Cl     | 229   | 165   | 483          | 1                     | 467        | 21.4     | >423   |
| LFM-A7   | o-F      | 218   | 146   | 428          | 1                     | 412        | 75.9     | >454   |
| LFM-A8   | m-CF3    | 248   | 172   | 503          | 1                     | 488        | 44.7     | >370   |
| LFM-A9   | m-Br     | 239   | 167   | 490          | 1                     | 474        | 18.2     | >356   |
| LFM-A10  | m-Cl     | 233   | 163   | 478          | 1                     | 463        | 23.4     | >423   |
| LFM-A11  | m-F      | 218   | 153   | 448          | 1                     | 432        | 47.9     | >454   |
| LFM-A12  | p-OCF3   | 257   | 170   | 497          | 1                     | 457        | 27.0     | >349   |
| LFM-A13  | 2,5-diBr | 248   | 176   | 454          | 2                     | 587        | 1.4      | 17.2 ± 0.8 |
| LFM-A14  | H        | 212   | 148   | 434          | 1                     | 419        | 64.5     | >495   |

a M.S., molecular surface area calculated using Connolly’s MS program (41). 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 molecular surface in contact with protein calculated by Ludi based on docked positions.
c Ludi K_i calculated based on the empirical score function in Ludi program (29, 30).
d Cell-free BTK inhibition assays were performed in three independent experiments on BTK immunoprecipitated from B18.2 cells and exposed to LFM and LFM analogs for 1 h prior to hot kinase assays. Except for LFM-A13, none of the compounds inhibited BTK in any of the experiments even at concentrations as high as 100 μg/ml (349–495 μM).

Fig. 3. Docked position of the LFM-A13 molecule (multicolor) at the catalytic site (blue ribbon) of the kinase domain of BTK. Dashed lines represent hydrogen bonds between LFM-A13 and the kinase domain residues of BTK.

(1.75 g; 13.9 mmol) was added to a solution of cyanoacetic acid 1 (1.70 g; 20.0 mmol) and the desired substituted aniline 2 (12.6 mmol) in tetrahydrofuran (25 ml) at 0 °C. The mixture was stirred for 12 h at room temperature. The urea precipitate (reaction side product) was removed by filtration and the filtrate partitioned between ethyl acetate and 0.5 N HCl. The organic layer (a solution of tetrahydrofuran and ethyl acetate) was sequentially washed with brine twice, dried over anhydrous Na2SO4, and concentrated by rotary evaporation. Finally, the crude solid product was recrystallized from ethyl alcohol to give pure 3. Sodium hydride (0.93 g; 60% in mineral oil; 23.2 mmol) was added slowly to the solution of 3 in tetrahydrofuran (12 mmol in 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.

The physical data for α-cyano-β-hydroxy-β-methyl-N-(4-trifluoromethyl)phenylpropanamide (LFM) is as follows: mp, 230–233 °C; IR (KBr), 3303, 2218, 1600, and 1555 cm⁻¹; 1H NMR (Me2SO-d6), δ 11.01 (s, 1H, NH), 7.75 (d, J = 8.4 Hz, 2H, ArH), 7.64 (d, J = 8.4 Hz, 2H, ArH), 2.22 (s, 3H, CH₃); GC/MS m/z 270 (M⁺), 161, 142, 111.

The physical data for α-cyano-β-hydroxy-β-methyl-N-(4-bromophenyl)propanamide (LFM-A1) is as follows: mp, 213–214 °C; IR (KBr), 3288, 2228, 1615, 1555 cm⁻¹; 1H NMR (Me2SO-d6), δ 10.51 (s, 1H, NH), 7.49 (s, 4H, ArH), 2.25 (s, 3H, CH₃); GC/MS m/z 282 (M⁺ + 2), 280 (M⁺), 173, 171.

The physical data for α-cyano-β-hydroxy-β-methyl-N-(4-chlorophenyl)propanamide (LFM-A2) is as follows: mp, 209–211 °C; IR (KBr), 3298, 2223, 1598, and 1552 cm⁻¹; 1H NMR (Me2SO-d6), δ 10.48 (s, 1H, NH), 7.54 (d, J = 8.7 Hz, 2H, ArH), 7.45 (s br, 1H, OH), 7.36 (d, J = 8.7 Hz, 2H, ArH), 2.22 (s, 3H, CH₃); GC/MS m/z 294 (M⁺), 282 (M⁺ + 2), 280 (M⁺), 173, 171.
The physical data for 4-cyano-β-hydroxy-β-methyl-N-(2-fluorophenyl)propanamide (LFM-A3) is as follows: mp, 84–85 °C; IR (KBr), 3395, 2214, 1660, and 1587 cm⁻¹; 1H NMR (CDCl₃), δ 10.99 (s, 1H, NH), 8.03 (d, J = 6.3 Hz, 1H, ArH), 7.97 (d, J = 7.5 Hz, 1H, ArH), 7.92 (d, J = 7.5 Hz, 1H, ArH), 7.60 (d, J = 7.5 Hz, 1H, ArH), 7.59 (d, J = 7.5 Hz, 1H, ArH), 7.51 (s, 1H, OH), 2.20 (s, 3H, CH₃); GC/MS m/z 236 (M⁺). Table II

| Compound | LFM | LFM-A12 | LFM-A13 |
|----------|-----|---------|---------|
| Substitution on phenyl ring | p-OCF₃ | p-OCF₃ | 2.5-diBr |
| Empirical formula | C₁₀H₇F₃N₂O₂ | C₁₀H₇F₃N₂O₃ | C₁₀H₁₁BrN₂O₂ |
| Crystallized from | THF evaporation | THF/hexane liquid-liquid diffusion | Acetonitrile evaporation |
| Crystal system | Triclinic | Triclinic | Monoclinic |
| Space group | P-1 | P-1 | P2₁/c |
| Cell constants | a = 4.6460(1)Å | a = 4.6460(1)Å | a = 5.6134(1)Å |
| b = 11.5311(7)Å | b = 9.0781(3)Å | b = 9.9847(3)Å |
| c = 12.9013(7)Å | c = 14.688(1)Å | c = 21.5896(2)Å |
| α = 96.126(2)° | α = 94.488(2)° | β = 93.639(1)° |
| β = 105.914(1)° | β = 91.658(2)° |
| γ = 110.571(2)° | γ = 93.682(2)° |
| Wavelength, λ | 0.71073 Å | 0.71073 Å | 0.71073 Å |
| Z | 4 | 2 | 4 |
| Formula weight | 270.21 | 286.21 | 360.00 |
| Density (calculated) | 1.556 mg/m³ | 1.543 mg/m³ | 1.980 mg/m³ |
| Index range | −11 ≤ h ≤ 10 | −5 ≤ h ≤ 5 | −6 ≤ h ≤ 6 |
| | −13 ≤ k ≤ 13 | −10 ≤ k ≤ 10 | 0 ≤ k ≤ 11 |
| | 0 ≤ l ≤ 17 | 0 ≤ l ≤ 17 | 0 ≤ l ≤ 25 |
| Reflections collected | 5372 | 3856 | 5918 |
| Independent reflections | 3715 | 2059 | 2109 |
| Data/restraints/parameters | 3715/0/343 | 2057/0/181 | 2109/0/155 |
| R indices (I > 2σ(I)) | R1 = 0.090, wR2 = 0.214 | R1 = 0.0741, wR2 = 0.18 | R1 = 0.040, wR2 = 0.094 |

Chemosensitizing Novel BTK Inhibitor

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RESULTS AND DISCUSSION

BTK Is an Anti-apoptotic Enzyme—We first evaluated the anti-apoptotic activity of BTK by comparing the effects of the apoptosis-inducing agents C\textsubscript{6}-ceramide, vincristine, and anti-Fas monoclonal antibody on wild-type DT40 chicken B lymphoma cells to those on a BTK-deficient subclone of DT40 cells that was established by homologous recombination knock-out (32). Ceramide, the product of ceramide synthase and sphingomyelinase, has been shown to function as a second messenger that transmits membrane-induced apoptotic signals, including myelins, has been shown to function as a second messenger.

In order to examine the participation of the various domains of BTK in its anti-apoptotic function, we introduced wild-type human BTK gene as well as human BTK genes harboring mutations either in the catalytic domain (Arg\textsuperscript{307} to Ala), or PH domain (Arg\textsuperscript{286} to Cys) into the BTK-deficient DT40 cells (37). As evidenced in Fig. 1B, BTK-deficient DT40 cells reconstituted with wild-type human BTK gene (WT) did not undergo apoptosis after treatment with C\textsubscript{6}-ceramide (lanes 2–4) or vincristine (lanes 8–10), whereas DT40 subclones expressing human BTK with mutations in the kinase (K) (lanes 5–7 and 11–13), SH2 (mSH2) (lanes 15–17 and 21–23), or PH (mPH) domains (lanes 18–20 and 24–26) did. Thus, the kinase, SH2, and PH domains of BTK are all important and apparently indispensable for its anti-apoptotic function.

Homology Model of BTK Kinase Domain—The three-dimensional coordinates of BTK used in the protein/inhibitor modeling studies were constructed based on a structural alignment with the sequences of known crystal structures for four protein kinase domains (kinase domains of HCK (22), FGFR (23), IRK (24), and cAPK (25)), as detailed under “Experimental Procedures.” The modeled BTK kinase domain (Fig. 2A) has the expected protein kinase fold with the catalytic site in the center of the kinase domain. The catalytic site is defined by two \( \beta \)-sheets that form an interface at the cleft between the two lobes. It is in this catalytic region where small molecule inhibitors can bind. Our modeling studies revealed that the catalytic site of the BTK kinase domain is defined of a smaller N-terminal lobe connected by a flexible hinge to a larger C-terminal lobe. The N-terminal lobe is rich in \( \beta \)-strands, whereas the C-terminal region is mostly helical. The catalytic site is defined by two \( \beta \)-sheets that form an interface at the cleft between the two lobes. It is in this catalytic region where small molecule inhibitors can bind. Our modeling studies revealed that the catalytic site of the BTK kinase domain is composed of a distinct planar rectangular binding pocket near the hinge region. The rectangular binding region is defined by residues Leu\textsuperscript{460}, Tyr\textsuperscript{476}, Arg\textsuperscript{525}, and Asp\textsuperscript{539} which occupy the
corners of the rectangle. The dimensions of this rectangle are approximately 18 × 8 × 9 × 17 Å, and the thickness of the pocket is approximately 7 Å (Fig. 2B). The far left corner of the rectangle can be visualized as beginning close to the hinge region at Leu460 (shown in yellow, Fig. 2B) and extending 8 Å toward the upper right to Asp539 (shown in blue, Fig. 2B). This is the shortest side of the binding pocket and is located closer to the inner core of the protein. The left side of the pocket, which is the longest, extends from Leu460 and traces 18 Å along the hinge region up to Tyr476 (shown in green, Fig. 2B). The right side of the rectangular pocket, opposite to the hinge region, extends about 9 Å from Asp539 to Arg525 (shown in pink, Fig. 2B), 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 472–481. The solvent-exposed or fourth side of the rectangle extends 17 Å along the slot-shaped opening to the catalytic site from Tyr476 to Arg525. The binding pocket is wider at the solvent-accessible region, it narrows toward the innermost region of the binding site, and overall it is relatively shallow with a thickness of about 7 Å.

Although most of the catalytic site residues of the BTK kinase domain were conserved relative to other tyrosine kinases, a few specific variations were observed. Residues Asn526 and Asp539 (opposite the hinge) are conserved in EGFR, IRK, HCK, and BTK. Residue Thr474 in the hinge region changes to Met in IRK, JAK1, and JAK3 and residue Tyr476 in the hinge region changes to Leu in EGFR and IRK. Residue Ser538 changes to Gly in JAK1 and IRK, to Thr in EGFR, and to Ala in FGFR, JAK3, and HCK. One region of binding site contains Cys481 in BTK which is more hydrophobic than the corresponding residue of PDGF receptor (Asp), FGFR (Asn), and IRK (Asp). These residue identity differences may provide the basis for designing selective inhibitors of the BTK kinase domain.

Structure-based Design and Synthesis of LFM Analogs with Potent BTK-inhibitory Activity—In modeling studies aimed at identifying LFM analogs with a high likelihood to bind favorably to the catalytic site of the kinase domain of BTK, we chose to evaluate the estimated $K_i$ values that quantitate predicted binding interactions between the inhibitor and residues in the catalytic site of BTK. Each of the small molecule LFM analogs was individually modeled into the catalytic site of the BTK kinase domain using an advanced docking procedure (see “Experimental Procedures”). The position of quercetin in the HCK crystal structure (22) was used as a template to obtain a reasonable starting point for the docking procedure. The various docked positions of each LFM analog were qualitatively evaluated using a scoring procedure and consequently compared with the IC$_{50}$ values of the compounds in cell-free BTK inhibition assays. Table I shows the interaction scores, calculated $K_i$ values, and measured IC$_{50}$ values for LFM and its analogs with BTK.

The inhibitors in our modeling studies were sandwiched by two regions of mostly hydrophobic residues. The region above the docked inhibitor consisted of residues Leu408, Val416, and
TABLE III
Interaction scores, calculated $K_i$ values, and measured $IC_{50}$ values for LFM-13 with several different PTKs

| Tyrosine kinase | M.S. | B.S. | Lipo score | No. of hydrogen bonds | Ludi score | $K_i$ | $IC_{50}$ |
|----------------|------|------|------------|-----------------------|-----------|------|----------|
| JAK1           | 250  | 68   | 497        | 0                     | 396       | 110  | >278     |
| JAK3           | 246  | 67   | 484        | 0                     | 383       | 148  | >278     |
| IRK            | 248  | 64   | 466        | 1                     | 450       | 31.6 | >278     |
| EGFR           | 248  | 66   | 479        | 0                     | 378       | 166  | >278     |
| HCK            | 246  | 65   | 468        | 0                     | 367       | 214  | >278     |

* M.S., molecular surface area calculated using Connolly's MS program (41). 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.S., buried surface, percentage of molecular surface in contact with protein calculated by Ludi based on docked positions.

‡ Ludi $K_i$ calculated based on the empirical score function in Ludi program (29, 30).

§ Cell-free tyrosine kinase inhibition assays were performed in 2–3 independent experiments as described under “Experimental Procedures.”

Fig. 4 did not inhibit JAK1, JAK3, IRK, EGFR, or HCK in any of the experiments even at concentrations as high as 100 μg/ml (278 μM). The results from a representative experiment are depicted in Fig. 7.

Ly5430, and the residues below the docked inhibitor included BTK residues Leu528, Ser538, Gly480, and Cys481. Of all the reported compounds evaluated in our modeling studies (Table I), we predicted that compound LFM-A13 would provide the strongest binding to BTK. The positions of the critical residues in the active site of the BTK and the docked position of the lead compound LFM-A13 is shown in Fig. 3. Of all the possible orientations of this molecule bound to the catalytic site, the one shown in Fig. 3 showed the highest interaction score with BTK. This high interaction score is indicative of an energetically favored binding mode, with a correspondingly low calculated $K_i$ value of 1.4 μM. This binding position of LFM-A13 is such that the aromatic ring of the inhibitor faces the Tyr476 residue and the flexible side chain extends toward the Asp539 and Arg525 residues. The aromatic ring is also sandwiched between the hydrophobic residues Leu408 and Val418 above and Gly480 and Leu528 below. Residue Ser538 lies below the flexible side chain of the inhibitor, and the end of the side chain is located between residues Asp539 and Arg525. This position closely resembles that of the ATP analog position found in the IRK complex crystal structure (24). According to our modeling studies, the O-3 atom in the hydroxyl group of LFM-A13 would form a hydrogen bond with Asp539:O and its O-4 atom would form a hydrogen bond with Arg525:N.

Fig. 4 shows the superimposed docked positions of our lead compound LFM-A13 in the catalytic site of BTK, together with the control compounds LFM and LFM-A12. The molecules LFM and LFM-A12 are docked such that they lie along the hinge region, corresponding to the quercetin position in the HCK crystal structure. The aromatic rings of these molecules is close to Tyr476, and the end of the side chain is sandwiched between residues Asp539 and Thr474. The CF3 group in these molecules points toward the solvent-accessible region and is surrounded by Leu430 above and Gly480 below. The OH group of LFM is hydrogen-bonded to an oxygen atom of Asp539, and for LFM-A12, the same group is hydrogen-bonded to an oxygen atom of Thr474. All LFM analogs listed in Table I, except LFM-A13, lie along the hinge region like LFM or LFM-A12, and their side chains are sandwiched between Asp539 and Thr474. A comparison of the docked positions of LFM, LFM-A12, and LFM-A13 in the BTK active site shows that although the aromatic portion of the three molecules are roughly in the same region (which is also true for the other inactive LFM analogs), the side chain of LFM-A13 is tilted away from those of the others and is sandwiched between residues Asp539 and Arg525. This rotation is likely due to a more favorable orientation of the 2,5-dibromo groups of LFM-A13. This slightly tilted position and the larger bromine groups afford two advantages for the interaction of LFM-A13 with the active site residues of BTK. The first advantage is that LFM-A13 is able to form two hydrogen bonds with active site residues Asp539 and Arg525, whereas the inactive LFM analogs form only one hydrogen bond each with the Thr474 or Asp539 of BTK. The second advantage for binding is the higher contact area of LFM-A13 with active site residues of BTK, relative to the other 12 inactive LFM analogs, which leads to a greater hydrophobic interaction for LFM-A13. This feature is reflected by the correspondingly higher lipophilic score for LFM-A13 in Table I.

The results from the modeling studies prompted the hypothesis that LFM-A13 would exhibit potent BTK inhibitory activity. In order to test this hypothesis and validate the predictive value of the described BTK homology model, we synthesized LFM-A13, LFM, and 13 other LFM analogs listed in Table I. The structures of LFM, LFM-A12, and LFM-A13 were determined by single crystal x-ray diffraction (crystal data, experimental parameters, and refinement statistics for these compounds are summarized in Table II). All structures were found to have a planar conformation, and all bond lengths and angles were in the expected range. Fig. 5 shows an Oak Ridge Thermal Ellipsoid Plot representation of the lead compound LFM-A13. The crystal structure of LFM-A13 showed that its molecular conformation was very similar to the energy-minimized molecular coordinates that were generated and used for docking studies with BTK. This conformational similarity with the crystal structures indicated that the molecular models used for
**Fig. 8. Structural basis for the selectivity of LFM-A13 for BTK.** Shown in light blue is a trace of the BTK homology model with selected residues at positions A, B, and C, together with the docked position of the leflunomide metabolite analog LFM-A13 (multicolor). Shown in red is the docked position of LFM-A13 with a model of EGFR and the residue difference between EGFR and BTK at position B. Shown in yellow is the docked position of LFM-A13 with the crystal structure of HCK(22) and the residue difference between HCK and BTK at position C. Shown in pink is the docked position of LFM-A13 with models of JAK3/JAK1 and the residue difference between JAK3/JAK1 and BTK at position A. Shown in dark blue is the docked position of LFM-A13 with the crystal structure of IRK(24) and the residue differences between IRK and BTK at positions A and B.

**Fig. 9. Effects of LFM-A13 on ceramide sensitivity of human leukemia cells.** Fluorescence-activated cell sorter-correlated three-parameter (FSC, forward scatter size; fluorescence from PI, propidium iodide, and fluorescence from MC540 staining) displays of ALL-1 Ph/t(9;22) human ALL cells stained with MC540 and PI 24 h after treatment with vehicle (0.1% Me2SO in phosphate-buffered saline), C2-ceramide (C2-CER) (10 μM), LFM-A13 (200 μM), or LFM-A13 + C2-CER. The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by single MC540 fluorescence, and the fraction of cells at an advanced stage apoptosis, as measured by dual MC540/PI fluorescence.

Docking were appropriate for modeling studies.

**Specific Inhibition of BTK by LFM-A13**—We first used cell-free immune complex kinase assays to compare the effects of LFM and 12 LFM analogs on the enzymatic activity of human BTK immunoprecipitated from B18–2 cells (32) (i.e. BTK-deficient DT40 chicken lymphoma B cells reconstituted with wild-type human BTK gene). As shown in Table I, only LFM-A13 exhibited significant BTK inhibitory activity with an IC50 value of 6.2 ± 0.3 μM = 17.2 ± 0.8 μM). None of the other compounds inhibited BTK even at concentrations as high as 100 μg/ml (i.e. at a range of 349 μM for LFM-A12 to 495 μM for LFM-A14). LFM-A13 was also effective against recombinant BTK expressed in a baculovirus vector expression system with an IC50 value of 0.9 μg/ml (∼2.5 μM, Fig. 6A), as well as BTK immunoprecipitated from NALM-6 human B-lineage ALL cell lysates (Fig. 6B). Furthermore, treatment of B18.2 cells (Fig. 6C) or NALM-6 cells (data not shown) with LFM-A13 resulted in a dose-dependent inhibition of cellular BTK activity. The inhibitory activity of LFM-A13 against BTK was specific since it did not affect the enzymatic activity of other protein tyrosine kinases, including Janus kinases JAK1 and JAK2, Src family kinase HCK, and receptor family tyrosine kinase IRK, at concentrations as high as 100 μg/ml (∼278 μM; Table III and Fig. 7).

**Structural Basis for the BTK Specificity of LFM-A13**—Biological assays have shown LFM-A13 to be a selective inhibitor of BTK, whereas it is a poor inhibitor of EGFR, HCK, JAK1, JAK3, and IRK. To evaluate this selectivity, we constructed a homology model of EGFR, JAK1, and JAK3 using homologous crystal structure coordinates of protein kinases IRK (24), HCK (22), and cAMP-dependent protein kinase (25) as a template. The models were then used to study the binding of small molecules such as LFM-A13 into the catalytic sites of these kinases and to understand better how LFM-A13 can inhibit BTK but not EGFR, IRK, JAK1, JAK3, or HCK. Our preliminary studies have identified three factors that may contribute to the specificity of LFM-A13 for BTK. The small molecule LFM-A13 was docked into the kinase domains of IRK, HCK, JAK3, and EGFR. Table III shows the interaction scores, calculated K and B values, and measured IC50 data for LFM-A13 with these kinases. We postulate that the selectivity of LFM-A13 for BTK results from favorable interactions with the BTK catalytic site residues that are not present in the other kinases studied. There are some residues in the BTK active site that differ from those of other PTKs. These differences are illustrated in Fig. 8 which shows the backbone of the BTK catalytic site, the residue differences between BTK and other kinases, and the docked positions of LFM-A13 in the kinase domains of BTK, HCK, JAK3, JAK1, EGFR, and IRK. We propose that the residue differences shown at positions A, B, and C in Fig. 8 may contribute to the specificity of LFM-A13 for BTK. Kinases that are not inhibited by LFM-A13, such as IRK (dark blue) and JAK1/JAK3 (pink), contain a methionine residue at position A.
which protrudes into the active site and prevents the close contact of small molecules like LFM-A13 with the hinge region of the binding site. As a result, LFM-A13 can lose favorable hydrophobic contact with the hinge region of the kinase domains of these proteins and does not bind to it tightly. Moreover, docking studies indicated that the favorable position of LFM-A13 in the BTK kinase domain is such that the side chain of the small molecule is located between residues Asp539 and Arg525 and forms hydrogen bonds with them. In addition, the aromatic residue, Tyr476, at position B of BTK increases the hydrophobic interaction between LFM-A13 and BTK, an interaction that is lost in EGFR (red, Fig. 8) and IRK (dark blue, Fig. 8). This is reflected by the lipophilic (hydrophobic interaction) scores shown in Table III. While the Lipo scores ranged between 457 and 473 for other kinases, the Lipo score for BTK was higher (more favorable) at 517. Finally, the Arg525 residue at position C of BTK can hydrogen-bond to LFM-A13. This interaction is lost in HCK (yellow), which contains an Ala at the C position. The favorable position of LFM-A13 at the HCK kinase domain (shown in yellow) is such that the small molecule is aligned along the hinge region. At this position LFM-A13 does not form hydrogen bonds with HCK, which is not the case for BTK. The longer side chain of Arg525 in BTK (position C) is involved in hydrogen bonding with LFM-A13, whereas HCK has an Ala at this position which is not able to form the same hydrogen bond.

**LFM-A13 Enhances the Sensitivity of B-lineage Acute Lymphoblastic Leukemia (ALL) Cells to Ceramide- and Vincristine-induced Apoptosis**—Patients with Philadelphia Chromosome (Ph+) ALL have a dismal outcome after intensive multimodality treatment programs. The treatment failure of these patients could be overcome if the apoptotic threshold of their leukemic cells could be decreased. We set out to determine if LFM-A13, by means of inhibiting the anti-apoptotic tyrosine kinase BTK, could alter the sensitivity of the Ph+ ALL cell line ALL-1 to C2- ceramide. As shown in Fig. 9, treatment with
LFM-A13 significantly enhanced the chemosensitivity of ALL-1 cells to ceramide-induced apoptosis, as evidenced by a greater percentage of cells treated with LFM-A13 plus C_2_ ceramide, as compared with cells treated with C_2_ ceramide alone or LFM-A13 alone, showing dual PI/MC540 fluorescence (shown in blue) consistent with advanced stage apoptosis. Furthermore, on agarose gels, DNA from Triton X-100 lysates of LFM-A13 plus ceramide or LFM-A13 plus vincristine, which was more pronounced than after treatment with LFM-A13, ceramide, or vincristine alone (Fig. 10). These results demonstrated that LFM-A13 enhances the sensitivity of B-lineage leukemia/lymphoma cells to both ceramide- and vincristine-induced apoptosis. LFM-A13 could also have clinical utility as a B-cell inhibitory agent in xenotransplantation and treatment of autoimmune diseases.

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