Structure-based Design of Specific Inhibitors of Janus Kinase 3 as Apoptosis-inducing Antileukemic Agents

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ABSTRACT

A novel homology model of the kinase domain of Janus kinase (JAK) 3 was used for the structure-based design of dimethoxyquinazoline compounds with potent and specific inhibitory activity against JAK3. The active site of JAK3 in this homology model measures roughly 8 Å × 11 Å × 20 Å, with a volume of ~530 Å³ available for inhibitor binding. Modeling studies indicated that 4-(phenyl)-amino-6,7-dimethoxyquinazoline (parent compound WHI-258) would likely fit into the catalytic site of JAK3 and that derivatives of this compound that contain an OH group at the 4’ position of the phenyl ring would more strongly bind to JAK3 because of added interactions with Asp-967, a key residue in the catalytic site of JAK3. These predictions were consistent with docking studies indicating that compounds containing a 4′-OH group, WHI-P131 [4-(4′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline], WHI-P154 [4-(3′-bromo-4′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline], and WHI-P97 [4-(3′,5′-dibromo-4′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline], were likely to be favorably bound to JAK3, with estimated Kᵢ ranging from 0.6 to 2.3 μM. These compounds inhibited JAK3 in immune complex kinase assays in a dose-dependent fashion. In contrast, compounds lacking the 4′-OH group, WHI-P79 [4-(3′-bromo-phenyl)-amino-6,7-dimethoxyquinazoline], WHI-P111 [4-(3′-bromo-4′-methylphenyl)-amino-6,7-dimethoxyquinazoline], WHI-P112 [4-(2′,5′- dibromophenyl)-amino-6,7-dimethoxyquinazoline], WHI-P132 [4-(2′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline], and WHI-P258 [4-(phenyl)-amino-6,7-dimethoxyquinazoline], were predicted to bind less strongly, with estimated Kᵢ ranging from 28 to 72 μM. These compounds did not show any significant JAK3 inhibition in kinase assays. Furthermore, the lead dimethoxyquinazoline compound, WHI-P131, which showed potent JAK3-inhibitory activity (IC₅₀ of 78 μM), did not inhibit JAK1 and JAK2, the ZAP/SYK family tyrosine kinase SYK, the TEC family tyrosine kinase BTK, the SRC family tyrosine kinase Lyn, or the receptor family tyrosine kinase insulin receptor kinase, even at concentrations as high as 350 μM. WHI-P131 induced apoptosis in JAK3-expressing human leukemia cell lines NALM-6 and LC1;19 but not in melanoma (M24-MET) or squamous carcinoma (SQ20B) cells. Leukemia cells were not killed by dimethoxyquinazoline compounds that were inactive against JAK3. WHI-P131 inhibited the clonogenic growth of JAK3-positive leukemia cell lines DAUDI, RAMOS, LC1;19, NALM-6, MOLT-3, and HL-60 (but not JAK3-negative BT-20 breast cancer, M24-MET melanoma, or SQ20B squamous carcinoma cell lines) in a concentration-dependent fashion. Potent and specific inhibitors of JAK3 such as WHI-P131 may provide the basis for the design of new treatment strategies against acute lymphoblastic leukemia, the most common form of childhood cancer.

INTRODUCTION

STATs³ constitute a family of DNA-binding proteins that reside in the cytoplasm until they are activated by tyrosine phosphorylation. This phosphorylation event is catalyzed by members of the Janus family of tyrosine kinases, including JAK3 (1, 2). The dual role of STATs as signaling molecules and transcription factors is reflected in their structure. All STAT proteins contain a DNA-binding domain, an SH2 domain, and a transactivation domain necessary for transcriptional induction. In unstimulated cells, latent forms of STATs are predominantly localized in the cytoplasm. Ligand binding induces STAT proteins to bind with their SH2 domains to the tyrosine-phosphorylated motifs in the intracellular domains of various transmembrane cell surface receptors (3, 4). Once STATs are bound to receptors, the receptor-associated JAKs phosphorylate STATs on a single tyrosine residue located near the SH2 domain. Two STATs then dimerize through specific reciprocal SH2-phosphotyrosine interactions. The dimerized STAT proteins can also

³ The abbreviations used are: STAT, signal transducers and activators of transcription; JAK, Janus kinase; ALL, acute lymphoblastic leukemia; IRK, insulin receptor kinase; FGFR, fibroblast growth factor receptor; ZAP, ζ associated protein; SYK, spleen tyrosine kinase; TEC, tyrosine kinase expressed in hepatocellular carcinoma; BTK, Bruton’s tyrosine kinase; SRC, cellular homologue of oncogene product from Rous avian sarcoma virus; LYN, PTK related to LCK and YES; PTK, protein tyrosine kinase; EMSA, electrophoretic mobility-shift assay; NAO, 10-nonyl-acridine orange; B-ALL, B-cell ALL; IL, interleukin; TdT, terminal deoxynucleotidyl transferase.

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form complexes with other DNA-binding proteins. The STAT dimers/complexes next translocate to the nucleus and use their DNA-binding domain to interact with DNA response elements in promoters of target genes (5). STATs then interact directly or indirectly, via their transactivation domain, with components of the RNA polymerase II complex to activate transcription of target genes. Different ligands use specific JAK and STAT family members; thus, utilization of this pathway mandates specificity in signaling cascades and contributes to a diverse array of cellular responses (3, 4). JAKs, including JAK3, are abundantly expressed in primary leukemic cells from children with ALL, the most common form of childhood cancer, and recent studies have correlated STAT activation in ALL cells with signals regulating apoptosis (5–8).

Here, we used a novel homology model of the kinase domain of JAK3 to design compounds with potent and specific JAK3-inhibitory activity as potential antileukemic agents. The lead compound WHI-P131 \[4-(4'\text{-hydroxyphenyl})\text{-amino-6,7-dimethoxyquinazoline}\] inhibited JAK3 but not JAK1 or JAK2. Similarly, the ZAP/SYK family tyrosine kinase SYK, the TEC family tyrosine kinase BTK, the SRC family tyrosine kinase LYN, and the receptor family tyrosine kinase IRK were not inhibited by WHI-P131. WHI-P131 induced apoptosis in JAK3-expressing human leukemia cell lines but not in melanoma or squamous carcinoma cells. Leukemia cells were not killed by dimethoxyquinazoline compounds that were inactive against JAK3. Potent and specific inhibitors of JAK3, such as the dimethoxyquinazoline compound WHI-P131, may provide the basis for the design of new treatment strategies against ALL.

**MATERIALS AND METHODS**

**Constructing a Homology Model for the JAK3 Kinase Domain.** Because the three-dimensional coordinates of the JAK3 kinase domain are currently unknown, a structural model of JAK3 was required for a docking analysis of JAK3 inhibitors.
A homology model of JAK3 was constructed (Fig. 1) by using known coordinates of homologous kinase domains as references. The JAK3 homology model was built by first obtaining the protein sequence of JAK3 (Swiss-Prot database access no. P52333; University of Geneva, Geneva, Switzerland) from GenBank (National Center for Biotechnology Information, Bethesda, MD) and determining the most reasonable sequence alignment for the JAK3 kinase domain relative to some template coordinates [known kinase structures such as HCK (9), FGFR (10, 11), and IRK (12)]. This was accomplished by first superimposing the Cα coordinates of the kinase domains of HCK, FGFR, and IRK using the InsightII program (InsightII, Molecular Simulations Inc. San Diego, CA, 1996) to provide the best overall structural comparison. The sequences were then aligned based on the superimposition of their structures (amino acid sequences were aligned together if their Cα positions were spatially related to each other). The alignment accommodated features, such as loops in a protein, that differed from the other protein sequences. The structural superimposition 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 performed manually and produced a sequence variation profile for each superimposed Cα position. The sequence variation profile served as a basis for the subsequent sequence alignment of the JAK3 kinase with the other three proteins. In this procedure, the sequence of JAK3 was entered and aligned with the three known kinase proteins based on the sequence variation profiles described previously. Next, a set of three-dimensional coordinates was assigned to the JAK3 kinase sequence using the three-dimensional coordinates of HCK as a template and the Homology module within the InsightII program (InsightII, Molecular Simulations Inc. San Diego, CA, 1996). 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 (13). Finally, the constructed model of the JAK3 kinase domain was subjected to energy minimization using the X-PLOR program (14) 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 procedure for homology model construction was repeated for JAK1 (Swiss-Prot database access no. P23458) and JAK2 (GenBank database access no. AF005216) using the JAK3 model as a structural template. The energy-minimized homology models of JAK1, JAK2, and JAK3 were then used, in conjunction with energy-minimized structural models of dimethoxyquinazoline compounds, for modeling studies of JAK-dimethoxyquinazoline complexes.

Docking Procedure Using Homology Model of JAK3 Kinase Domain. Modeling of the JAK3-dimethoxyquinazoline complexes was accomplished using the Docking module within the program InsightII (InsightII, Molecular Simulations Inc. San Diego, CA, 1996) and using the Affinity suite of programs for automatically docking an inhibitor into a protein binding site [a similar procedure for epidermal growth factor receptor (15) and BTK (16) was described previously]. The various docked positions of each compound were evaluated using a Ludi (17) scoring procedure in InsightII (InsightII, Molecular Simulations Inc. San Diego, CA, 1996), which estimated a binding constant, Kᵢ, taking into account the predicted lipophilic, hydrogen bonding, and van der Waals interactions between the inhibitor and the protein. A comparison of the catalytic site residues of several different PTKs was made by manually superimposing crystal structure coordinates of the kinase domains of IRK (12) and HCK (9) and models of JAK1, JAK2, JAK3, BTK (16), and SYK (18) and then identifying features in the active site that were unique to JAK3 (Figs. 2C and 3).

Chemical Synthesis of Quinazoline Derivatives. The compounds listed in Table I were synthesized and characterized using procedures published previously (18).

Immune Complex Kinase Assays. SF21 (IPLB-SF21-AE) cells (19), derived from the ovarian tissue of the fall armyworm Spodotera frugiperda, were obtained from Invitrogen (Carlsbad, CA) and maintained at 26–28°C in Grace’s insect cell medium supplemented with 10% fetal bovine serum and 1.0% antibiotic/antimycotic (Life Technologies, Inc.). Stock cells were maintained in suspension at 0.2 × 10⁶–1.6 × 10⁶ cells/ml in a total culture volume of 600 ml in 1-liter Bellco spinner flasks at 60–90 rpm. Cell viability was maintained at 95–100%, as determined by trypan blue dye exclusion. SF21 cells were infected with a baculovirus expression vector for BTK, SYK, JAK1, JAK2, or JAK3, as reported previously (16). Cells were harvested and lysed [10 mM Tris (pH 7.6), 100 mM NaCl, 1% NP40, 10% glycerol, 50 mM NaF, 100 μM Na3VO4, 50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin], the kinases were immunoprecipitated from the lysates, and their enzymatic activity was assayed, as reported previously (19–23). The immunoprecipitates were subjected to Western blot analysis, as described previously (19, 20).

For IRK assays, HepG2 human hepatoma cells grown to ~80% confluency were washed once with serum-free DMEM and starved for 3 h at 37°C in a CO2 incubator. Subsequently, cells were stimulated with insulin (Eli Lilly and Co., Indianapolis, IN; 10 units/ml, 10 × 10⁶ cells) for 10 min at room temperature. Following this IRK activation step, cells were washed once with serum-free medium and lysed in NP40 buffer, and IRK was immunoprecipitated from the lysates with an anti-IRβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA; polyclonal IgG). Prior to performing the immune complex kinase assays, we equilibrated the beads with the kinase buffer [30 mM HEPES (pH 7.4), 30 mM NaCl, 8 mM MgCl2, and 4 mM MnCl2], LYN was immunoprecipitated from whole cell lysates of NALM-6 human leukemia cells, as reported previously (23, 24).

In JAK3 immune complex kinase assays (16, 21), KL-2 EBV-transformed human lymphoblastoid B cells (native JAK3 kinase assays) or insect ovary cells (recombinant JAK3 kinase assays) were lysed with NP40 lysis buffer [50 mM Tris (pH 8), 150 mM NaCl, 5 mM EDTA, 1% NP40, 100 μM sodium orthovanadate, 100 μM sodium molybdate, 8 μg/ml aprotinin, 5
mg/ml leupeptin, and 500 μm phenylmethylsulfonyl fluoride] and centrifuged 10 min at 13,000 × g to remove insoluble material. Samples were immunoprecipitated with antisera prepared against JAK3. The antisera were diluted and immune complexes collected by incubation with 15 μl of protein A-Sepharose. After four washes with NP40 lysis buffer, the protein A-Sepharose beads were washed once in kinase buffer [20 mM MOPS (pH 7)-10 mM MgCl₂] and resuspended in the same buffer. Reactions were initiated by the addition of 25 μCi of [γ-32P]ATP (5000 Ci/mmol) and unlabeled ATP to a final concentration of 5 μM. Reactions were terminated by boiling for 4 min in SDS sample buffer. Samples were run on 9.5% SDS polyacrylamide gels, and labeled proteins were detected by autoradiography. Following electrophoresis, kinase gels were dried onto Whatman 3M filter paper, and subjected to phosphorimaging on a Molecular Imager (Bio-Rad, Hercules, CA) as well as autoradiography on film. For each drug concentration, a kinase activity index was determined by comparing the kinase activity in phosphorimager units to that of the baseline sample. In some experiments, cold kinase assays were performed, as described previously (25).

**EMSAs.** EMSAs were performed to examine the effects of dimethoxyquinazoline compounds on cytokine-induced STAT activation in 32Dc11/IL2Rβ cells (a gift from Dr. James Ihle, St. Jude Children’s Research Hospital), as described previously (21).

**Mitochondrial Membrane Potential Assessment.** To measure the changes in mitochondria, we incubated cells with WHI-P131 at concentrations ranging from 7.4 μg/ml (25 μM) to 30 μg/ml (200 μM) for 24 or 48 h; the cells were then stained with specific fluorescent dyes and analyzed with flow cytometer. Mitochondrial membrane potential (ΔΨm) was measured using two dyes including a lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and a cyanine dye, 1,1′,3,3′,3′,3′-hexamethyldiodocarbocyanine iodide (DiIC1; Refs. 26–28) obtained from Molecular Probes (Eugene, OR). JC-1 is a monomer at 527 nm after being excited at 490 nm; with polarization of ΔΨm, J-aggregates are formed that shift emission to 590 nm (29). This can be detected on a flow cytometer by assessing the green signal (at 527 nm) and green-orange signal (at 590 nm) simultaneously, creating an index of the number of cells polarized and depolarized mitochondria. DiIC1, a cyanine dye that is amphipathic and cationic, concentrates in energized mitochondria and has been used in a variety of studies to measure the mitochondrial membrane potential (26–28). Cells were also stained with DiIC1 at 40 nM concentration for 30 min in the dark as described for JC-1.

![Fig. 2 A. model of unoccupied space in the catalytic (ATP binding) site of a JAK3 homology model. Shown in green is the binding site for ATP and the most likely binding site for dimethoxyquinazoline inhibitors. The green kinase active site region represents a total volume of ~530 Å³. Modeling studies showed that an inhibitor or a portion of an inhibitor with significant binding to this region would occupy a volume of <530 Å³ and have molecular dimensions compatible with the shape of the binding site region. Other regions near the binding site that show measurable unoccupied volume are shown in royal blue, pink, yellow, and light blue. These binding regions are either unavailable to inhibitor molecules (royal blue) or represent regions just large enough to occupy solvent molecules (pink, yellow, and light blue). A model of WHI-P131 docked into the catalytic site is shown in white, superimposed on the green region. B. model of the catalytic site of JAK3 with quinazolines WHI-P131 (multicolor), WHI-P132 (pink), and WHI-P154 (yellow). Each compound fits into the binding site but WHI-P132 (shown to be inactive against JAK3 in biological assays) lacks a OH group that is in a location to bind with Asp-967. WHI-P131 and WHI-P154, with OH groups at the C4' position of the phenyl ring, are able to form a favorable interaction with Asp-967 of JAK3, which may contribute to their enhanced inhibition activity. C. features of dimethoxyquinazoline derivatives that are predicted to aid binding to JAK3 catalytic site.](clincancerres.aacrjournals.org)
The cells were analyzed using a Vantage Becton Dickinson (San Jose, CA) cell sorter equipped with HeNe laser with excitation at 635 nm, and the fluorescence was measured at 666 nm.

**Mitochondrial Mass Determination.** Relative mitochondrial mass was measured by using Becton Dickinson Calibur flow cytometry and the fluorescent stain NAO, which binds the mitochondrial phospholipid cardiolipin, which, in turn, has been extensively used to provide an index of mitochondrial mass (30).

**Human Leukemia and Cancer Cell Lines.** The following cell lines were used in various biological assays: NALM-6 (pre-B-ALL), LC1:19 (pre-B-ALL), DAUDI (B-ALL),

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Table 1  Predicted interaction of protonated quinazolines with JAK3 kinase active site and measured inhibition values (IC₅₀) from JAK3 kinase assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>R⁵</th>
<th>R⁴</th>
<th>R³</th>
<th>R²</th>
<th>No. of H bonds</th>
<th>H bond score</th>
<th>Lipophilic score</th>
<th>Contact score</th>
<th>Total binding score</th>
<th>Estimated Ki (µM)</th>
<th>Molecular surface area (Å²)</th>
<th>Molecular volume (Å³)</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHI-P131</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>3</td>
<td>188</td>
<td>476</td>
<td>64</td>
<td>568</td>
<td>2.3</td>
<td>276</td>
<td>261</td>
<td>9.1</td>
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<tr>
<td>WHI-P97</td>
<td>Br</td>
<td>OH</td>
<td>Br</td>
<td>H</td>
<td>3</td>
<td>156</td>
<td>559</td>
<td>65</td>
<td>622</td>
<td>0.6</td>
<td>314</td>
<td>307</td>
<td>11.0</td>
</tr>
<tr>
<td>WHI-P154</td>
<td>H</td>
<td>OH</td>
<td>Br</td>
<td>H</td>
<td>3</td>
<td>171</td>
<td>512</td>
<td>64</td>
<td>587</td>
<td>1.4</td>
<td>296</td>
<td>284</td>
<td>27.9</td>
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<tr>
<td>WHI-P79</td>
<td>H</td>
<td>H</td>
<td>Br</td>
<td>H</td>
<td>1</td>
<td>9</td>
<td>531</td>
<td>63</td>
<td>444</td>
<td>36</td>
<td>278</td>
<td>272</td>
<td>&gt;300</td>
</tr>
<tr>
<td>WHI-P132</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>1</td>
<td>82</td>
<td>476</td>
<td>66</td>
<td>462</td>
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<td>269</td>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>0</td>
<td>0</td>
<td>510</td>
<td>64</td>
<td>414</td>
<td>72</td>
<td>266</td>
<td>252</td>
<td>&gt;300</td>
</tr>
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</table>
RAMOS (B-ALL), MOLT-3 (T-cell ALL), HL60 (acute myelogenous leukemia), BT-20 (breast cancer), M24-MET (melanoma), SQ20B (squamous cell carcinoma), and PC3 (prostate cancer). These cell lines were maintained in culture, as reported previously (15, 16, 19, 23, 31, 32). Cells were seeded in six-well tissue culture plates at a density of 50 × 10⁴ cells/well in a treatment medium containing various concentrations of WHI-P131 and incubated for 24–48 h at 37°C in a humidified 5% CO₂ atmosphere.

Apoptosis Assays. Cells were examined for apoptotic changes after treatment with WHI-P131 by the in situ TdT-mediated dUTP end-labeling assay using the ApopTag apoptosis detection kit (Oncor, Gaithersburg, MD) according to the manufacturer’s recommendations, as detailed in our earlier reports (32, 33).

To detect apoptotic fragmentation of DNA, we harvested cells after a 24-h exposure at 37°C to WHI-P131 or other dimethoxyquinazoline compounds at 1, 3, and/or 10 µM concentrations. DNA was prepared from Triton X-100 lysates for analysis of fragmentation (20). In brief, cells were lysed in hypotonic 10 mmol/liter Tris-HCl (pH 7.4), 1 mmol/liter EDTA, and 0.2% Triton X-100 detergent and subsequently centrifuged at 11,000 × g. To detect apoptosis-associated DNA fragmentation, we electrophoresed supernatants on a 1.2% agarose gel, and the DNA fragments were visualized by UV light after staining with ethidium bromide.

Clonogenic Assays. The antileukemic activity of WHI-P131 against clonogenic tumor cells was examined using a methylcellulose colony assay system (34, 35). In brief, cells (10⁵ P131 against clonogenic tumor cells was examined using a methylcellulose colony assay system (34, 35). In brief, cells (10⁵ cells/ml in RPMI-10% fetal bovine serum) were treated overnight at 37°C with WHI-P131 at varying concentrations. After treatment, cells were washed twice, plated at 10⁴ or 10⁵ cells/ml in RPMI, 10% fetal bovine serum, and 0.9% methylcellulose in Petri dishes and cultured for 7 days at 37°C in a humidified 5% CO₂ incubator. Subsequently, leukemic cell (or tumor cell) colonies were enumerated using an inverted phase-contrast microscope. The percentage inhibition of colony formation was calculated using the following formula:

\[
\frac{\text{Mean no. of colonies in test culture}}{\text{Mean no. of colonies in control culture}} \times 100
\]

RESULTS AND DISCUSSION

Homology Model of JAK3 Kinase Domain. The three-dimensional coordinates of JAK3 used in the protein/inhibitor modeling studies were constructed based on a structural alignment with the sequences of known crystal structures of the kinase domains of three PTKs: HCK (9), FGFR (11, 16), and IRK (12, 36), as detailed in “Materials and Methods.” Fig. 1, A and B, shows the homology model of the JAK3 kinase domain, which is composed of an NH₂-terminal lobe and a COOH-terminal lobe, which are linked by a hinge region near the catalytic (ATP-binding) site. The catalytic site is a pocket located in the central region of the kinase domain, which is defined by two β-sheets at the interface between the N and C lobes. The opening to the catalytic site is solvent accessible and facilitates binding of ATP. Small molecule inhibitors can also bind to the catalytic site, which results in an attenuation of PTK activity by inhibiting ATP binding. An analysis of the JAK3 model revealed specific features of the catalytic site, which can be described as a quadrilateral-shaped pocket (Fig. 1C). The opening of the pocket is defined by residues Pro-906, Ser-907, Gly-908, Asp-912, Arg-953, Gly-829, Leu-828, and Tyr-904 (Fig. 1C, blue residues). The far wall deep inside the pocket is lined with Leu-905 (Co backbone), Glu-903, Met-902, Lys-905, and Asp-967 (Fig. 1C, pink residues), and the floor of the pocket is lined by Leu-905 (side chain), Val-884, Leu-956, and Ala-966 (Fig. 1C, yellow residues). Residues defining the roof of the pocket include Leu-828, Gly-829, Lys-830, and Gly-831 (Fig. 1C, topmost blue residues). Figs. 1C and 2A illustrate that the catalytic site of the JAK3 model has approximate dimensions of 8 Å × 11 Å × 20 Å and an available volume for binding of ~530 Å³. According to the model, the solvent exposed opening to the binding region would allow inhibitors to enter and bind if the molecule contained some planarity.

Although most of the catalytic site residues of the JAK3 kinase domain were conserved relative to other PTKs, a few specific variations were observed (Fig. 3). These differences include an alanine residue in BTK, IRK, and HCK/LYN (Fig. 3A, region A) that changes to Gln in SYK and Pro-906 in JAK3. At region B, a tyrosine residue is conserved in JAK3 (Tyr-904), BTK, and LYN but changes to Phe in HCK (which is the only apparent residue difference between HCK and LYN relevant to inhibitor binding), Met in SYK, and Leu in IRK. Region C shows a methionine residue that is conserved in BTK, IRK, and HCK/LYN but changes to Leu-905 in JAK3 and Ala in SYK. Region D shows Met-902 in JAK3, which is conserved in SYK and IRK but changes to Thr in BTK and to a much smaller residue, Ala, in LYN and HCK. This Met-902 residue in JAK3, which is located on the back wall of the pocket and protrudes in toward the center of the pocket volume, can significantly affect the shape of the binding pocket. At this location, the extended conformation of the Met-902 side chain can hinder the close contact of inhibitors with residues lining the back wall of the pocket and with the hinge region, relative to other kinases with smaller residues here such as BTK (Thr) and HCK/LYN ( Ala). Ala-966 in region E is conserved in HCK/LYN but changes to Gly in IRK and to the more hydrophilic residue Ser in BTK and SYK. Region F, which is farther away from the inhibitor location, is the least conserved region of the catalytic site and contains Asp-912 in JAK3, Asn in BTK, Lys in SYK, Ser in IRK, and Asp in HCK/LYN (Fig. 3). These residue identity differences between tyrosine kinases provide the basis for designing selective inhibitors of the JAK3 kinase domain.

Structure-based Design and Synthesis of JAK3 Inhibitors. A computer docking procedure was used to predict how well potential inhibitors could fit into and bind to the catalytic site of JAK3 and result in kinase inhibition (Fig. 2B). The dimethoxyquinazoline compound WHI-P258 [4-(phenyl)-amino-6,7-dimethoxyquinazoline] contains two methoxy groups on the quinazoline moiety but no other ring substituents. Molecular modeling studies using the homology model of JAK3 kinase domain suggested that WHI-P258 would fit into the catalytic site of JAK3 but probably would not bind very tightly due to limited hydrogen-bonding interactions. Asp-967, a key residue in the catalytic site of JAK3, can form a hydrogen bond with
molecules binding to the catalytic site, if such molecules contain a hydrogen bond donor group such as an OH group. WHI-P258, however, does not contain an OH group and, therefore, would not interact as favorably with Asp-967. We postulated that the presence of an OH group at the 4′ position of the phenyl ring of WHI-P258 would result in stronger binding to JAK3 because of added interactions with Asp-967. A series of dimethoxyquinazoline compounds were designed and synthesized to test this hypothesis.

An estimation of the molecular volume for the compounds is provided in Table 1. A summary of structural features of the designed dimethoxyquinazoline compounds that were observed to be relevant for binding to the catalytic site of JAK3 is shown in Fig. 2C. The approximate molecular volumes of the compounds in Table 1 range from 252 to 307 Å³, which are small enough to fit into the 530-Å³ binding site of JAK3 kinase. Table 1 also lists the results of molecular modeling studies, including estimated binding constants (i.e., $K_b$) for the compounds that were docked into the JAK3 catalytic site. The compounds that were evaluated in docking studies contain substitutions of similar functional groups at different positions on the phenyl ring.

The conformations of the energy-minimized docked models of the compounds listed in Table 1 were relatively planar, with dihedral angles of ~4–18° between the phenyl ring and quinazoline ring system. This conformation allows the molecule to fit into the binding site of JAK3.
Fig. 6 WHI-P131 depolarizes mitochondrial membranes in a concentration-dependent fashion without affecting the mitochondrial mass. NALM-6 human leukemic cells were incubated with indicated concentrations of WHI-P131 for 48 h, stained with DiIC$_{1}$ to assess the mitochondrial membrane potential ($\Delta\psi_{m}$) or NAO to detect the mitochondrial mass, and then analyzed with cell sorter equipped with HeNe laser. WHI-P131 caused a progressive increase in depolarized mitochondria (as indicated by M1 in A) with increasing concentrations. At similar concentrations, no significant change in mitochondrial mass (B) was detected. C, cells were stained with JC-1 for simultaneous analysis of mitochondrial mass (green fluorescence)
to fit more easily into the catalytic site of JAK3. All of the listed compounds contain a ring nitrogen (N1), which can form a hydrogen bond with NH of Leu-905 in the hinge region of JAK3. When N1 is protonated, the NH can instead interact with the carbonyl group in Leu-905 of JAK3. The presence of a OH group at the 4-position on the phenyl ring was anticipated to be particularly important for binding to the catalytic site of JAK3. WHI-P131 (estimated $K_i = 2.3 \mu M$), WHI-P154 (estimated $K_i = 1.4 \mu M$), and WHI-P97 (estimated $K_i = 0.6 \mu M$) shown in Table 1 were predicted to have favorable binding to JAK3 and potent JAK3 inhibitory activity because they contain a 4'-OH group on the phenyl ring that can form a hydrogen bond with Asp-967 of JAK3, contributing to enhanced binding. However, the 2'-OH group of WHI-P132 is not in the right orientation to interact with Asp-967, and it would probably form an intramolecular hydrogen bond with the quinazoline ring nitrogen, which may contribute to a significantly lower affinity of WHI-P132 for the catalytic site of JAK3. The relatively large bromine substituents (WHI-P97 and WHI-P154) can increase the molecular surface area in contact with binding site residues if the molecule can fit into the binding site. Modeling of WHI-P154 and WHI-P97 showed that there is enough room to accommodate the bromine groups if the phenyl ring is tilted slightly relative to the fused ring group of the molecule. The results from the modeling studies prompted the hypothesis that WHI-P131, WHI-P154, and WHI-P97 would exhibit potent JAK3-inhibitory activity. To test this hypothesis and validate the predictive value of the described JAK3 homology model, we synthesized WHI-P131, WHI-P154, WHI-P97, and five other dimethoxyquinazoline compounds, listed in Table 1.

and mitochondrial transmembrane potential (red/orange fluorescence). Untreated NALM-6 cells (D.1) as well as NALM-6 cells treated with 50 \mu M of WHI-P131 for 24 h (D.2) were incubated with JC-1 and analyzed by confocal laser scanning microscopy. Mitochondria of control cells showed a higher membrane potential ($\Delta \psi_m$), as indicated by brighter JC-1 red fluorescence. Treatment of cells with WHI-P131 reduced mitochondrial membrane $\Delta \psi_m$ as indicated by a substantial decrease in JC-1 red fluorescence.
Inhibition of JAK3 by Rationally Designed Dimethoxyquinazoline Compounds. We first used immune complex kinase assays to compare the effects of the synthesized dimethoxyquinazoline compounds on the enzymatic activity of human JAK3 immunoprecipitated from the KL-2 EBV-transformed human lymphoblastoid B-cell line. WHI-P131, WHI-P154, and WHI-P97, which had very similar estimated $K_i$s, ranging from 0.6 to 2.3 $\mu M$ and were predicted to show significant JAK3-inhibitory activity at micromolar concentrations (which was not the case for the other compounds, which had estimated $K_i$s ranging from 25 to 72 $\mu M$), inhibited JAK3 in concentration-dependent fashion. The measured IC$_{50}$s were 9.1 $\mu M$ for WHI-P131, 11.0 $\mu M$ for WHI-P97, and 27.9 $\mu M$ for WHI-P154, but >300 $\mu M$ for all of the other dimethoxyquinazoline compounds (Table 1). WHI-P131 and WHI-P154 were also tested against recombinant murine JAK3 expressed in a
baculovirus vector expression system and inhibited JAK3 in a concentration-dependent fashion with an IC_{50} of 23.2 \mu M (78 \mu M; Fig. 4A) and 48.1 \mu M (128 \mu M; Fig. 4B), respectively. The ability of WHI-P131 and WHI-P154 to inhibit recombinant JAK3 was confirmed in four independent experiments. These kinase assay results are consistent with our modeling studies described above.

Importantly, WHI-P131 and WHI-P154 did not exhibit any detectable inhibitory activity against recombinant JAK1 or JAK2 in immune complex kinase assays (Fig. 4, C and D). EMSAs were also performed to confirm the JAK3 specificity of these dimethoxyquinazoline compounds by examining their effects on cytokine-induced STAT activation in 32Dc11/IL2R\beta cells. As shown in Fig. 4E, both WHI-P131 (10 \mu M/mL = 33.6 \mu M) and WHI-P154 (10 \mu M/mL = 26.6 \mu M) but not the control compound WHI-P132 (10 \mu M/mL = 33.6 \mu M) inhibited JAK3-dependent STAT activation after stimulation with IL-2, but they did not affect JAK1/JAK2-dependent STAT activation after stimulation with IL-3. Modeling studies suggest that this exquisite JAK3 specificity could in part be due to an alanine residue (Ala-966) that is present in the catalytic site of JAK3 but changes to glycine in JAK1 and JAK2. This alanine group, which is positioned near the phenyl ring of the bound dimethoxyquinazoline compounds, can provide greater hydrophobic contact with the phenyl group and, thus, can contribute to higher affinity relative to the smaller glycine residue in this region of the binding site in JAK1 and JAK2. However, an accurate interpretation of these remarkable differences in sensitivity of JAK3 versus JAK1 and JAK2 to WHI-131 and WHI-154 must await determination of the X-ray crystal structures of these kinases because simple amino acid discrepancies in their catalytic sites could result in pronounced structural differences.

Specificity of WHI-P131 as a Tyrosine Kinase Inhibitor. Compound WHI-P131 was selected for additional experiments designed to examine the sensitivity of non-Janus family PTKs to this novel dimethoxyquinazoline class of JAK3 inhibitors. The inhibitory activity of WHI-P131 against JAK3 was specific because it did not affect the enzymatic activity of other PTKs (Table 1; Fig. 5), including the ZAP/SYK family tyrosine kinase SYK (Fig. 5C), the TEC family tyrosine kinase BTK (Fig. 5D), the SRC family tyrosine kinase LYN (Fig. 5E), and the receptor family tyrosine kinase IRK (Fig. 5F), even at concentrations as high as 350 \mu M.

A structural analysis of these PTKs was performed using the crystal structures of HCK (which served as a homology model for LYN; Ref. 9) and IRK (36) and constructed homology models of JAK3, BTK, and SYK. This analysis revealed some nonconserved residues located in the catalytic binding site of the different tyrosine kinases, which may contribute to the specificity of WHI-P131 (Fig. 3). One such residue, which is located closest to the docked inhibitors, is Ala-966 in JAK3 (Fig. 3, region E), which may provide the most favorable molecular surface contact with the hydrophobic phenyl ring of WHI-P131. The fact that WHI-P131 did not inhibit LYN, although LYN

### Table 2 Effects of WHI-P131 against clonogenic leukemic cells

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>WHI-P131 concentration (\mu M)</th>
<th>Mean no. of colonies/10^5 cells</th>
<th>% inhibition</th>
<th>Mean no. of colonies/10^5 cells</th>
<th>% inhibition</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NALM-6 (pre B-ALL)</td>
<td></td>
<td>BT20 (breast cancer)</td>
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<td>0</td>
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<td>3298 (2940, 3656)</td>
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</tr>
<tr>
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<td>100</td>
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<td>&gt;99.9</td>
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<td>M24-MET (melanoma)</td>
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<td>1854 (1648, 2060)</td>
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</table>

* Cells were treated with WHI-P131 and then assayed for colony formation, as described in “Materials and Methods.” NA, not applicable; ND, not determined.

T-ALL, T-cell ALL; AML, acute myelogenous leukemia.
contains the Ala residue conserved in JAK3 (Ala-966), suggests that other factors (residue differences) contribute to this selectivity. Other nonconserved residues in the catalytic site of tyrosine kinases are shown in Fig. 3 (regions A–F). All of these differences in residues, especially residues that directly contact the bound inhibitor, may play an important role in the observed specificity of WHI-P131 for JAK3.

Antileukemic Activity of WHI-P131. We hypothesized that compound WHI-P131 would exhibit significant cytotoxic activity against JAK3-expressing human leukemia cells. To test this hypothesis, we first examined leukemic cells exposed to this novel JAK3 inhibitor for apoptosis-associated changes in mitochondrial transmembrane potential (ΔΨm) and mitochondrial mass using specific fluorescent mitochondrial probes and multiparameter flow cytometry. To measure changes in ΔΨm, we used DiIC1 (which accumulates in energized mitochondria), whereas the mitochondrial mass was determined by staining the cells with NAO, a fluorescent dye that binds to the mitochondrial inner membrane independent of energetic state. Treatment of NALM-6 leukemia cells with WHI-P131 at 7.4 μg/ml (25 μM) to 60 μg/ml (200 μM) for 24 or 48 h increased the number of depolarized mitochondria in a concentration- and time-dependent manner as determined by flow cytometry using DiIC1. As shown in Fig. 6A, the fraction of DiIC1-negative cells with depolarized mitochondria increased from 1.3% in vehicle treated control cells to 81.6% in cells treated with 200 μM WHI-P131 for 48 h. The average EC50 for WHI-P131-induced depolarization of mitochondria, as measured by decreased DiIC1 staining, were 79.3 μM for a 24 h treatment and 58.4 μM for a 48 h treatment. The observed changes in ΔΨm are not due to loss in mitochondrial mass, as confirmed by a virtually identical staining intensity of NAO in the treated and untreated NALM-6 cells (Fig. 6B). To further confirm this relative change in ΔΨm, we used JC-1, a mitochondrial dye, which normally exists in solution as a monomer emitting green fluorescence and assumes a dimeric configuration emitting red fluorescence in a reaction driven by mitochondrial transmembrane potential (29). Thus, the use of JC-1 allows simultaneous analysis of mitochondrial mass (green fluorescence) and mitochondrial transmembrane potential (red/orange fluorescence). After treatment of NALM-6 cells with WHI-P131 at increasing concentrations ranging from 20 μM to 200 μM, and with increasing duration of exposure of 24 or 48 h, we observed a progressive dissociation between ΔΨm and mitochondrial mass, with decrement in JC-1 red/orange fluorescence without a significant corresponding drop in JC-1 green fluorescence (Fig. 6, C and D). As shown in Fig. 6C, the fraction of JC-1 red/orange fluorescence-positive cells decreased from 79.2% in vehicle-treated control cells to 16.9% in cells treated with 200 μM WHI-P131 for 48 h. The corresponding values for JC-1 green fluorescence were 99.3% for vehicle-treated cells and 99.8% for WHI-P131-treated (200 μM for 48 h) cells. The average EC50 for WHI-P131 induced depolarization of mitochondria, as measured by decreased JC-1 red/orange fluorescence were 94.2 μM for a 24 h treatment and 50.4 μM for a 48 h treatment. Fig. 6D compares the single color (red/orange) fluorescent confocal images of vehicle-treated and WHI-P131-treated (100 μM × 48 h) NALM-6 cells stained with JC-1. These results collectively demonstrate that WHI-P131 causes a significant decrease in mitochondrial transmembrane potential in NALM-6 human leukemia cells.

We next used the in situ TdT-mediated labeling of 3′-OH termini with digoxigenin-conjugated UTP assay method combined with confocal laser scanning microscopy to confirm that WHI-P131 can induce apoptosis in leukemia cells. At 48 h after treatment with WHI-P131 at concentrations ranging from 10 to 500 μM, NALM-6 cells were examined for digoxigenin-dUTP incorporation using FITC-conjugated antidigoxigenin (green fluorescence) and propidium iodide counterstaining (red fluorescence). The percentage of apoptotic cells increased in a concentration-dependent fashion with an average EC50 of 84.6 μM (Fig. 7A). Fig. 7, B.1 and B.2, depicts the two-color confocal microscopy images of vehicle-treated control cells and cells treated with 100 μM WHI-P131. WHI-P131-treated cells showed apoptotic yellow nuclei (yellow indicates superimposed green and red fluorescence; Fig. 7B.2). Further evidence for apoptosis was observed in DNA fragmentation assays. Because of their exquisite sensitivity in detecting DNA fragments released from a small percentage of apoptotic cells, the DNA gel assays of apoptosis are uniquely suited to examine the nonspecific toxicity of new antileukemic agents. Fig. 8A demonstrates that supernatants from NALM-6 leukemia cells, treated with 1 or 3 μM WHI-P131, contained oligonucleosome-length DNA fragments with a "ladder-like" fragmentation pattern consistent with apoptosis, whereas no DNA fragments were detected in supernatants of NALM-6 cells treated with structurally similar dimethoxyquinazoline compounds that lacked JAK3-inhibitory activity. Unlike JAK3-positive leukemia cells (NALM-6 cells in Fig. 8A and LC1;19 cells in Fig. 8B), JAK3-negative SQ20B squamous carcinoma cells and M24-MET melanoma cells did not show any evidence of apoptotic DNA fragmentation after treatment with WHI-P131 (Fig. 8B). Taken together, these results provided experimental evidence that the JAK3-specific tyrosine kinase inhibitor WHI-P131 results in depolarization of the mitochondrial membrane and triggers apoptotic death in human B-lineage ALL cells, as evidenced by the ladder-like fragmentation pattern of nuclear DNA and digoxigenin-11-UTP labeling of the exposed 3′-hydroxyl end of the fragmented nuclear DNA in the presence of TdT.

We also examined the antileukemic activity of WHI-P131 by determining its ability to inhibit the in vitro clonogenic growth of the ALL cell lines NALM-6, DAUDI, LC1;19, RAMOS, MOLT-3, and the acute myelogenous leukemia cell line HL-60. As detailed in Table 2, WHI-P131 inhibited clonogenic growth in a concentration-dependent fashion with EC50s of 24.4 μM for NALM-6 cells and 18.8 μM for DAUDI cells. At 100 μM, WHI-P131 inhibited the in vitro colony formation by these leukemia cell lines by >99%. In contrast, WHI-P131 did not inhibit the clonogenic growth of JAK3-negative M24-MET melanoma or SQ20B squamous carcinoma cell lines (Table 2).

In summary, our study demonstrates that a novel homology model of the JAK3 kinase domain can be used for structure-based design and synthesis of potent and specific inhibitors of JAK3. The reported lead compound WHI-P131 inhibited JAK3 but not other PTKs including JAK2, SYK, BTK, LYN, and IRK. ALL cells express JAK2; a JAK2 inhibitor has been reported to exhibit antileukemic activity (37). Similarly, the SRC family PTK LYN, the ZAP/SYK family PTK SYK, and the TEC...
family PTK BTK are expressed in ALL cells and affect their adhesion, proliferation, and survival (19, 23, 38, 39). IRK is the only member of the receptor PTK family that has been detected in leukemic cells, especially pre-B ALL cells with a t(1;19) translocation (40–42). Because our JAK3-specific inhibitor WHI-P131 does not inhibit these tyrosine kinases, its ability to kill ALL cells cannot be attributed to a nonspecific inhibition of JAK2, LYN, SYK, BTK, or IRK in these cells. The use of this compound in biological assays confirmed that JAK3 is a vital therapeutic target in ALL and demonstrated that WHI-P131 triggers apoptosis in leukemia cells. Potent and specific inhibitors of JAK3, such as the dimethoxyquinazoline compound WHI-P131, may, therefore, provide the basis for the design of new treatment strategies against ALL, which is the most common form of childhood cancer.

Finally, our homology model uniquely indicates that the active site of JAK3 measures approximately 8 Å × 11 Å × 20 Å with an ~530-Å³ volume available for inhibitor binding. Our modeling studies using the constructed homology model of JAK3 kinase also showed that there is significant opportunity for improvement of the quinazoline inhibitors. The JAK3 model shows that there is additional volume in the ATP-binding site which can be better used by quinazoline derivatives. The average molecular volume of our dimethoxyquinazoline compounds is 277 Å³, which is well below the estimated total volume of the binding site, 530 Å³. This leaves opportunities for the design of new inhibitors which have slightly larger functional groups at the 2’ and 3’ positions of the phenyl ring. Structural and chemical features of dimethoxyquinazoline compounds, which are proposed to facilitate their binding to the Jak3 catalytic site, include the following features, which are illustrated in Fig. 2C: (a) the presence of a 4’-OH group on the phenyl ring; (b) the presence of a hydrogen bond acceptor (N, carbonyl, OH) near the Leu-905 NH or a hydrogen bond donor (NH, OH) near the Leu-905 carbonyl; (c) a relatively planar molecular shape to allow access to the binding site; (d) the ability to fit into a 530-Å³ space defined by the residues lining the JAK3 catalytic site. These predicted binding preferences to JAK3 residues in the catalytic site can be used for the design of new and more potent inhibitors of JAK3 as antileukemic agents.

REFERENCES


Structure-based Design of Specific Inhibitors of Janus Kinase 3 as Apoptosis-inducing Antileukemic Agents

Elise A. Sudbeck, Xing-Ping Liu, Rama Krishna Narla, et al.


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