Identification of 1,2,3,4,5,6-Hexabromocyclohexane as a Small Molecule Inhibitor of Jak2 Tyrosine Kinase Autophophorylation

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The commercially available Jak2 inhibitor, α-cyano-3,4-dihydroxy-*N*-benzylcinnamide (AG490), has been used extensively to study Jak2 kinase function. While α-cyano-3,4-dihydroxy-*N*-benzylcinnamide is a potent Jak2 inhibitor, it can inhibit a number of other kinase signaling pathways as well. To circumvent this problem, we sought to identify novel small molecule inhibitors of Jak2 tyrosine kinase activity. For this, we constructed a homology model of the Jak2 kinase domain and identified solvent accessible pockets on the surface of the structure. Using the DOCK program, we tested 6451 compounds of known chemical structure in silico for their ability to interact with a pocket positioned adjacent to the activation loop. We attained the top seven scoring compounds from the National Cancer Institute and tested their ability to inhibit Jak2 autophosphorylation in vitro. Using Western blot analysis, we found that one of the compounds, 1,2,3,4,5,6-hexabromocyclohexane, was able to potently, and directly, inhibit Jak2 autophosphorylation. Characterization of this compound revealed that it inhibits Jak2 tyrosine autophosphorylation in both a time- and concentration-dependent manner. It greatly reduced growth hormone-mediated Jak2 autophosphorylation but did not block autophosphorylation of the epidermal growth factor receptor. Furthermore, doses as high as 100 μM were not toxic to cells as measured by their ability to exclude propidium iodide. As such, we believe that this compound could serve as a lead compound for a new generation of Jak2 inhibitors and, perhaps, be useful in elucidating the mechanisms of Jak2 kinase function.

Introduction

Jak2 is a nonreceptor tyrosine kinase that mediates signal transduction pathways activated by members of the cytokine receptor family, seven transmembrane spanning receptor family, and by a number of cellular stressors.[1−6] This protein is important in both physiology and pathophysiology, as it plays prominent roles in embryonic development and cell signaling, as well as in cancer and heart disease.[7−12] Two impediments to better understanding Jak2 function are (1) the lack of an adult knockout animal model and (2) the lack of a Jak2-specific pharmacological inhibitor.

Jak2 knockout mice die embryonically, around E10.5, due to a lack of erythropoesis.[7,8] This work demonstrated the critical role that Jak2 plays in embryonic development and cytokine signal transduction but also raised a barrier to research on elucidating the mechanisms of Jak2 cellular function. Without an adult Jak2 knockout animal available, studying the function of Jak2 in adult physiology and pathophysiology has been difficult. Furthermore, there is no Jak2-specific pharmacological inhibitor. α-Cyano-3,4-dihydroxy-*N*-benzylcinnamide is a commercially available Jak2 inhibitor, and while it has been instrumental in elucidating some functions of Jak2 and in identifying Jak2 as a therapeutic target, it suffers from a general lack of specificity. For instance, α-cyano-3,4-dihydroxy-*N*-benzylcinnamide inhibits activation of cyclin-dependent kinases and causes growth arrest of cells in the G1 phase.[13] It inhibits calf serum-inducible cell growth and DNA synthesis and is a partial blocker of c-Src activity.[14] Most critically, α-cyano-3,4-dihydroxy-*N*-benzylcinnamide inhibits epidermal growth factor receptor autophosphorylation more potently than it inhibits Jak2 activity.[15,16] Due to these problems, there are caveats in all research relying solely on α-cyano-3,4-dihydroxy-*N*-benzylcinnamide to study Jak2 kinase function. Clearly, identification of a novel Jak2 inhibitor could greatly aid research efforts.

For these reasons, we sought to identify a potential novel Jak2 inhibitor. We first used homology modeling of the Jak2 kinase domain to identify exposed pockets on the surface of the protein. We then used a high-throughput program called DOCK to predict the ability of 6451 small molecules to interact with a solvent accessible pocket that is adjacent to the activation loop of Jak2, designated as pocket 36. The compounds were scored in silico on their potential ability to interact with pocket 36. We ordered the top seven scoring compounds and tested their ability to inhibit Jak2 tyrosine kinase function. One of these, 1,2,3,4,5,6-hexabromocyclohexane, was found to be a potent and specific inhibitor of Jak2 tyrosine kinase autophosphorylation.

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Materials and Methods

Cell Culture. BSC-40 cells were cultured exactly as described.[17,18] The creation, characterization, and maintenance of the γ2A/GHR+Jak2 cells has also been described.[19] Mouse embryonic fibroblasts were kindly provided by Dr. Hideko Kasahara (University of Florida). Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere and were maintained in DMEM containing 10% fetal bovine serum. Cell culture reagents were obtained from Invitrogen/Life Technologies, Inc. EGF and 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) were from Calbiochem. All other reagents were purchased from Sigma Chemical or Fisher Scientific.

BSC-40 Cell Transfection/Infection. Jak2 autophosphorylation assays were performed in BSC-40 cells using the vaccinia virus transfection/infection protocol.[17,18] Briefly, the cells were transfected in serum-free media with 5.0 μg of a plasmid encoding the wild-type murine Jak2 cDNA under the control of the bacteriophage T7 promoter (pRC-CMV-Jak2-WT), using Lipofectin and following the manufacturer's instructions (Invitrogen). Four hours later, the cells were infected with the recombinant vaccinia virus, vTF7-3, at a multiplicity of infection (MOI) of 1.0. One hour after that, the media containing lipofectin/DNA/vTF7-3 was removed from the cells and replaced with fresh, serum-containing media. The cells were grown overnight at 37 °C to allow for high-level expression and subsequent tyrosine autophosphorylation of Jak2.

Immunoprecipitation. Cells were washed with two volumes of ice-cold PBS containing 1 mM Na_3VO_4 and lysed in 0.8 mL of ice-cold RIPA buffer containing protease inhibitors. The samples were sonicated and incubated on ice for 1 h. Samples were spun at 16 000*g* for 5 min at 4 °C and supernatants containing soluble protein lysates were normalized. Normalized lysates (approximately 400 μg/mL) were immunoprecipitated for 2−4 h at 4 °C with 2−10 μg of antibody and 20 μL of Protein A/G Plus agarose beads (Santa Cruz Biotechnology). After centrifugation, protein complexes were washed three times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) and resuspended in SDS-containing sample buffer. Bound proteins were boiled, separated by SDS−PAGE, and transferred onto nitrocellulose membranes. The immunoprecipitating anti-Jak2-pAb (HR758) was from Santa Cruz Biotechnology. The immunoprecipitating anti-Tyr(P)-mAb (clone PY20) was from BD Transduction Laboratories.

Western Blotting. Proteins were detected using enhanced chemiluminescence exactly as described.[20] Anti-Tyr(P) Western blotting was performed using a cocktail of antibodies consisting of clones 4G10 (Upstate Biotechnology), PY99 (Santa Cruz Biotecnology), and PY20 (BD Transduction Laboratories) at final dilutions of 1:1000 each. The anti-Jak2 antibody (758−776) was from Upstate Biotechnology. The anti-EGFR antibody (1005) was from Santa Cruz Biotechnology.

Jak2 In Vitro Autophosphorylation Assay. Approximately 10 ng of immunoaffinity-purified Jak2 was incubated in 20 μL of in vitro kinase reaction buffer (50 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 100 mM NaCl, 0.5 mM DTT), either in the presence or absence of 10 μM 1,2,3,4,5,6-hexabromocyclohexane. ATP was then added at a final concentration of 2.5 mM, as indicated. The samples were then incubated for 20 min at room temperature to allow for autophosphorylation. The reactions were terminated by the addition of SDS-containing sample buffer and subsequently Western blotted as described.

Propidium Iodide Staining. Cells were grown on microscope slides and stained with 1 μg/mL propidium iodide for 10 min at 37 °C. Live cells were examined using confocal microscopy. Same field images were captured under phase contrast and fluorescent conditions.

Small Molecule Inhibitors Database Preparation Using the DOCK Program. The National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) maintains a repository of approximately 139 644 samples (the plated compound set) that are nonproprietary and offered to the extramural research community for the discovery and development of new agents for the treatment of cancer, AIDS, or opportunistic infections afflicting patients with cancer or AIDS. The three-dimensional coordinates for the NCI/DTP plated compound set was obtained in the MDL SD format and converted to the mol2 format by the DOCK utility program SDF2MOL2. Partial atomic charges, solvation energies, and van der Waals parameters for the ligands were calculated using SYBDB and added to the plated compound set mol2 file.

In Silico Molecular Docking of Potential Jak2 Small Molecule Inhibitors. All docking calculations were performed with the October 15, 2002, development version of DOCK, v5.1.0. The general features of DOCK include rigid orienting of ligands to receptor spheres, AMBER energy scoring, GB/SA solvation scoring, contact scoring, internal nonbonded energy scoring, ligand flexibility, and both rigid and torsional simplex minimization. Unlike previously distributed versions, this release incorporates automated matching, internal energy (used in flexible docking), scoring function hierarchy, and new minimizer termination criteria. The coordinates for the molecular model of the Jak2 kinase domain were used in the molecular docking calculations. To prepare the site for docking, all water molecules were removed. Protonation of receptor residues was performed with Sybyl (Tripos, St. Louis, MO). The structure was explored using sets of spheres to describe potential binding pockets. The number of orientations per molecule was 100. Intermolecular AMBER energy scoring (vdw + columbic), contact scoring, and bump filtering were implemented in DOCK5.1.0. SETOR and GRASP were used to generate molecular graphic images.

Acquisition and Storage of Small Molecule Inhibitor Compounds. The top seven scoring nonproprietary compounds, designated compounds **1**−**7**, were obtained from the Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, free of charge. Upon arrival, each compound was solubilized in DMSO at a concentration of 10 mM and stored at −20 °C until needed.

Results

Homology Modeling and Target Pocket Identification. We utilized a comparative structural modeling approach to generate an atomic model of murine Jak2. Such analyses find the most likely structure of a protein sequence based on its relationships to known similar structures[21] and consist of (1) generation of a multiple alignment with the sequence to be modeled,[22] (2) generation of a framework for the new sequence based on superposition of related three-dimensional structures,[23] (3) rebuilding loops and side chains,[24] (4) structural refinement by energy minimization and molecular dynamics,[25] and (5) verification of the model structure geometry.[26]

Pairwise alignments with 275 residues of murine Jak2 identified the tyrosine kinase domain of fibroblast growth factor receptor 1 as the most homologous solved structure (pdb code 1FGI). An atomic model based on this assignment was generated by ProModII.[23] The loop regions were rebuilt on the basis of a database of structural fragments derived from the Protein Data Bank, and the best fitting fragments were used as the new loop. The side chain conformations were optimized, and energy minimization was conducted. The resulting comparative model of murine Jak2 was superimposed on the most homologous structure and is shown in Figure 1A.

The target pocket for small molecule docking was selected using the program SPHGEN, which identifies potential ligand binding sites based on chemical and shape characteristics. To prepare the site for docking, all water molecules were removed. Protonation of recep-

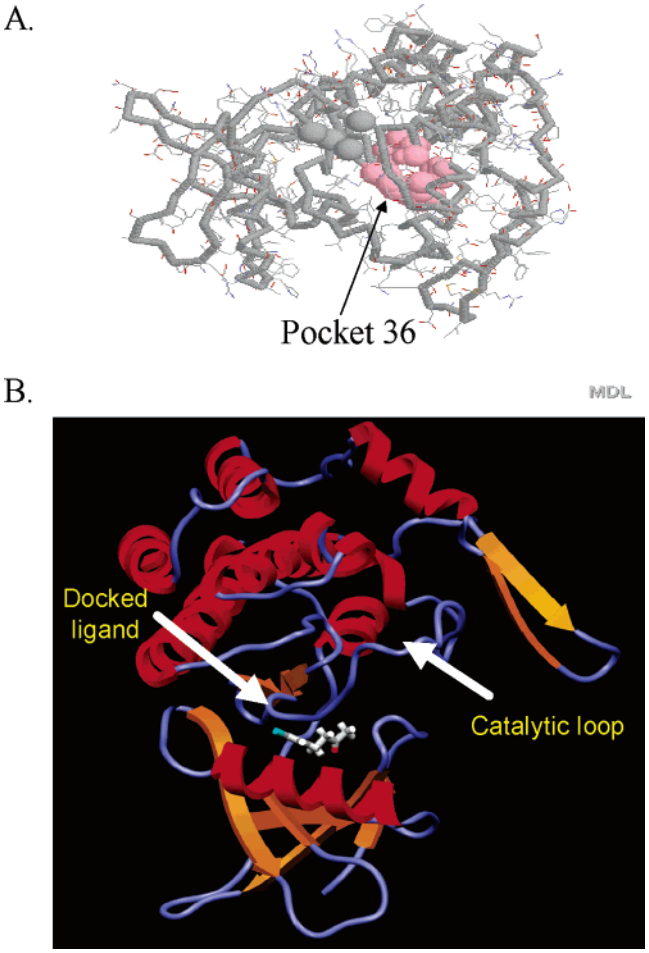

Figure 1. Atomic model of the murine Jak2 kinase domain. (A) A structural model of the Jak2 kinase domain was generated on the basis of homology with the known structure of the fibroblast growth factor receptor 1 kinase domain. Shown are two putative solvent accessible targets (colored circles) adjacent to the Jak2 tyrosine kinase activation loop. (B) The ligand shown was found to DOCK into a solvent accessible pocket of Jak2, in silico, based on contact points and energy scores.

tor residues was performed with Sybyl. The sphere set utilized for molecular docking contained 72 spheres (SPHGEN). This site was selected for molecular docking for two reasons. First, the selected site is in close proximity to the activation loop shown in Figure 1A. Second, the number of spheres in the site is in the optimum range for binding to small molecules, roughly 50−100.

Database Screening To Identify Potential Small Molecule Inhibitors of Jak2. In lieu of conducting high-throughput screening, we utilized a rapid structure-based approach combining molecular docking with functional testing. Approximately 6500 compounds with known three-dimensional structure were positioned into the structural pocket selected by SPHGEN on the homology model of mouse Jak2 in silico. This approach combined resources available through the NCI/DTP (atomic coordinates and small molecules) with improved molecular docking and scoring algorithms imposed in DOCK.

Each of the 6451 small molecule compounds was docked into the murine Jak2 structure in 100 different orientations using DOCK5.1.0. As an example, one such DOCKED ligand is shown as Figure 1B. In this case, the small molecule was found to bind into a region that is in very close proximity to the Jak2 activation loop based on contact points and energy score. The predicted binding energies of interaction between each compound and the murine Jak2 kinase domain were calculated, and those with the highest scores were obtained. Table 1 shows the seven highest scoring compounds.

1,2,3,4,5,6-Hexabromocyclohexane Inhibits Jak2 Tyrosine Autophosphorylation. To test the ability of each of the seven compounds to inhibit Jak2 tyrosine kinase activity, we utilized the vaccinia virus transfection/infection protocol. Briefly, BSC-40 cells, a vaccinia virus permissive cell line, were transfected with an expression vector encoding the wild-type murine Jak2 cDNA under the control of the T7 promoter. The cells were then infected with a vaccinia virus that produces T7 RNA polymerase. This results in high-level Jak2 expression and subsequent tyrosine autophosphorylation independent of exogenous ligand addition. This system has been used by our laboratory and by others as a means of better understanding Jak2 kinase function.[17,18,27−29]

Following the initial 1 h vaccinia virus infection, the cells were switched to serum-containing media, and each compound was added at a final concentration of 100 μM and incubated overnight. Sixteen hours after the addition of the inhibitor compounds, cellular lysates were prepared and immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-phosphotyrosine antibody to detect tyrosine phosphorylated Jak2 (Figure 2A, top). The results showed that 1,2,3,4,5,6-hexabromocyclohexane was the only compound to inhibit Jak2 tyrosine autophosphorylation. The membrane was then stripped and reprobed with anti-Jak2 antibody to demonstrate equal protein expression among all samples (Figure 2A, bottom). 1,2,3,4,5,6-Hexabromocyclohexane is a single aromatic ring structure with a halide on each carbon. It has a molecular mass of 558 Da. The structure is shown as Figure 2B.

1,2,3,4,5,6-Hexabromocyclohexane Inhibits Jak2 Autophosphorylation in a Time-Dependent Manner. We next wanted to determine whether 1,2,3,4,5,6-hexabromocyclohexane could inhibit Jak2 tyrosine autophosphorylation in a time-dependent manner. For this, BSC-40 cells were transfected/infected as described. Prior to cell lysis, 50 μM 1,2,3,4,5,6-hexabromocyclohexane was applied to the cells for 0, 1, 4, or 16 h. Cellular lysates were then prepared, immunoprecipitated with anti-Jak2 antibody, and immunoblotted with anti-phosphotyrosine antibody to measure tyrosine phosphorylated Jak2 levels (Figure 3, top). The results showed that 1 or 4 h treatment with 1,2,3,4,5,6-hexabromocyclohexane was sufficient to block ~75% of the tyrosine autophosphorylation of Jak2. However, 16 h treatment with a 50 μM dose of the drug resulted in a virtual elimination of all Jak2 tyrosine autophosphorylation. The membrane was subsequently stripped and reprobed with anti-Jak2 antibody to demonstrate equal protein expression among all samples (Figure 3, bottom).

Collectively, the data demonstrate that incubation of 1,2,3,4,5,6-hexabromocyclohexane does inhibit Jak2 tyrosine autophosphorylation in a time-dependent man-

Table 1. Top Seven Scoring Compounds

compd no.	NSC no.	formula	name	mol wt
1	7785	C_3H_5NO	2-propenamide	71.0
2	7795	$C_{28}H_{22}N_2O_8S_{22}Na$	Acid Green 25	625.0
3	7828	$C_{19}H_{12}Cl_2O_5S$	Chlorphenol Red	423.0
4	7830	$C_{36}H_{25}N_5O_6S_{22}Na$	Acid Black S	734.0
5	7851	$C_4H_3Cl_2N_3$	4,6-dichloro-5-aminopyrimidine	164.0
6	7893	$C_5H_7N_3O$	Superacil	125.0
7	7908	$C_6H_6Br_6$	1,2,3,4,5,6-hexabromocyclohexane	558.0

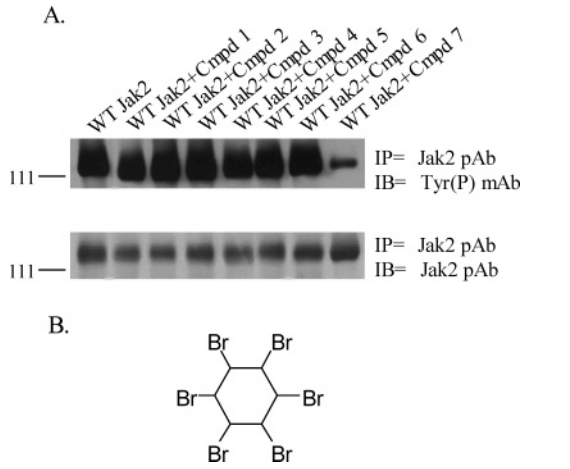

Figure 2. 1,2,3,4,5,6-Hexabromocyclohexane (compound **7**) inhibits Jak2 tyrosine autophosphorylation. (A) The seven compounds that received the highest score from the DOCK program for their ability to interact with pocket 36 within the Jak2 kinase domain were tested for their ability to inhibit Jak2 autophosphorylation. BSC-40 cells were transfected with 5 μg of wild-type Jak2 cDNA and then infected with 1 MOI of vaccinia virus for 16 h to drive high-level expression and subsequent Jak2 tyrosine autophosphorylation. The seven compounds were incubated with the cells at a concentration of 100 μM each, for 16 h. Cell lysates were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation levels (top panel). The membrane was stripped and reprobed with anti-Jak2 antibody to demonstrate equal Jak2 expression among all samples (bottom panel). Shown is one of three independent experiments. (B) Shown is the molecular structure of 1,2,3,4,5,6-hexabromocyclohexane.

ner; treatment of cells with 50 μM 1,2,3,4,5,6-hexabromocyclohexane for 1 or 4 h was sufficient to partially inhibit Jak2 tyrosine kinase autophosphorylation while treatment of cells for 16 h resulted in near total elimination of Jak2 tyrosine autophosphorylation.

1,2,3,4,5,6-Hexabromocyclohexane Inhibits Jak2 Autophosphorylation in a Dose-Dependent Manner. We next tested the ability of 1,2,3,4,5,6-hexabromocyclohexane to inhibit Jak2 autophosphorylation in a dose-dependent manner. For this, we again used the BSC-40 cell transfection/infection protocol. The cells were treated for 16 h with 1,2,3,4,5,6-hexabromocyclohexane at doses of 0, 1, 10, 50, 100, 250, or 500 μM. The following morning, soluble protein lysates were immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-phosphotyrosine antibody to measure the tyrosine phosphorylation levels of Jak2 (Figure 4, top). The results showed that 1,2,3,4,5,6-hexabromocyclohexane inhibited Jak2 tyrosine autophosphorylation in a dose-dependent manner with maximal inhibition occurring at 50 μM. The membrane was subsequently stripped and reprobed with anti-Jak2

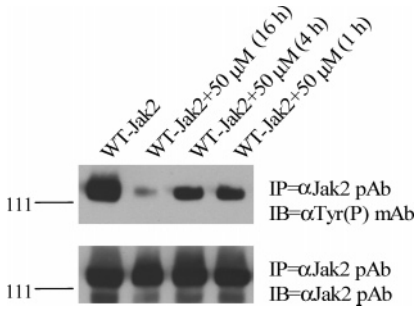

Figure 3. 1,2,3,4,5,6-Hexabromocyclohexane inhibits Jak2 tyrosine autophosphorylation in a time-dependent manner. BSC-40 cells were transfected/infected as described above. Cells were treated with 50 μM 1,2,3,4,5,6-hexabromocyclohexane for 0, 1, 4, or 16 h. Cell lysates were immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation levels (top panel). The membrane was stripped and reprobed with anti-Jak2 antibody to demonstrate equal Jak2 expression among all samples (bottom panel). Shown is one of two representative experiments.

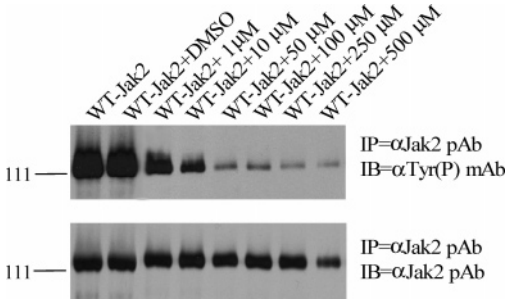

Figure 4. 1,2,3,4,5,6-Hexabromocyclohexane inhibits Jak2 tyrosine autophosphorylation in a dose-dependent manner. BSC-40 cells were transfected/infected as described. The cells were incubated for 16 h with 1,2,3,4,5,6-hexabromocyclohexane at doses 0, 1, 10, 100, 250, or 500 μM. Protein cell lysates were then immunoprecipitated with anti-Jak2 antibody and immunoblotted with anti-phosphotyrosine antibody to detect Jak2 tyrosine phosphorylation levels (top panel). The membrane was stripped and reprobed with anti-Jak2 antibody to demonstrate equal Jak2 expression among all samples (bottom panel). Shown is one of two representative experiments.

antibody to demonstrate equal protein expression among samples (Figure 4, bottom).

Collectively, the data in Figure 4 demonstrate that 1,2,3,4,5,6-hexabromocyclohexane does in fact inhibit Jak2 autophosphorylation in a dose-dependent manner. The amount of material required to inhibit 50% of the Jak2 tyrosine autophosphorylation levels in this assay (IC_{50}) was in the low micromolar range. Additionally, 50 μM 1,2,3,4,5,6-hexabromocyclohexane provided maximal inhibition.

1,2,3,4,5,6-Hexabromocyclohexane Is a Direct Inhibitor of Jak2 Tyrosine Kinase Autophospho-

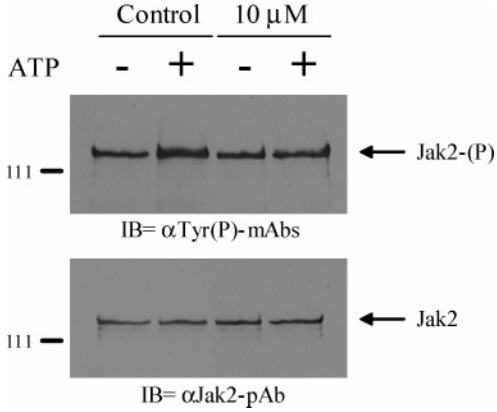

Figure 5. 1,2,3,4,5,6-Hexabromocyclohexane is a direct inhibitor of Jak2 tyrosine kinase autophosphorylation. Pure Jak2 tyrosine kinase was resuspended in kinase reaction buffer containing either vehicle control (DMSO) or 10 μM 1,2,3,4,5,6-hexabromocyclohexane. ATP was added as indicated to promote Jak2 autophosphorylation. The samples were subsequently immunoblotted with anti-phosphotyrosine antibodies to detect Jak2 tyrosine phosphorylation levels (top panel) or with anti-Jak2 antibody to demonstrate equal Jak2 content among all samples (bottom panel). Shown is one of two representative experiments.

rylation. The data in Figures 2−4 suggest that 1,2,3,4,5,6-hexabromocyclohexane is an inhibitor of Jak2. However, it is possible that 1,2,3,4,5,6-hexabromocyclohexane may be acting on a tertiary protein, which indirectly, is in turn suppressing Jak2 autophosphorylation. We recently developed the means to express and purify appreciable amounts of pure Jak2.[30] Therefore, to determine whether 1,2,3,4,5,6-hexabromocyclohexane is a direct or indirect inhibitor of Jak2, we performed in vitro kinase assays using pure recombinant Jak2 protein. In the absence of 1,2,3,4,5,6-hexabromocyclohexane, ATP was able to increase the tyrosine autophosphorylation levels of Jak2, as measured by anti-Tyr(P) Western blotting (Figure 5, top). However, when 1,2,3,4,5,6-hexabromocyclohexane was present, the ability of ATP to increase Jak2 tyrosine autophosphorylation levels was virtually eliminated. The membrane was subsequently stripped and reprobed with anti-Jak2 antibody to demonstrate equal Jak2 protein content, among all conditions (Figure 5, bottom).

Collectively, the data in Figure 5 suggest that, in vitro, 1,2,3,4,5,6-hexabromocyclohexane is a direct inhibitor of Jak2 tyrosine kinase autophosphorylation.

1,2,3,4,5,6-Hexabromocyclohexane Inhibits Ligand-Dependent Jak2 Tyrosine Autophosphorylation. An important question was whether 1,2,3,4,5,6-hexabromocyclohexane could inhibit Jak2 tyrosine autophosphorylation in a ligand-dependent signaling system. For this, we utilized a γ2A-derived cell line that stably expresses the growth hormone receptor and Jak2 tyrosine kinase.[19] Here, quiescent cells were either pretreated for 16 h with either vehicle control or with 100 μM 1,2,3,4,5,6-hexabromocyclohexane. The cells were subsequently treated with 250 ng/mL growth hormone for the indicated times, to activate Jak2. To measure Jak2 tyrosine phosphorylation levels, protein lysates were first immunoprecipitated with anti-Tyr(P) antibody and then Western blotted with anti-Jak2 antibody (Figure 6A, top). We found that 1,2,3,4,5,6-hexabromocyclohexane greatly reduced growth-hormone-mediated Jak2 tyrosine autophosphorylation levels, when compared to control treated cells. To demonstrate equal levels of Jak2 protein in all the samples, equal aliquots from these same lysate samples were Western blotted with anti-Jak2 antibody to access Jak2 protein levels (Figure 6A, bottom). We found that treatment of cells with 1,2,3,4,5,6-hexabromocyclohexane did not promote Jak2 protein degradation.

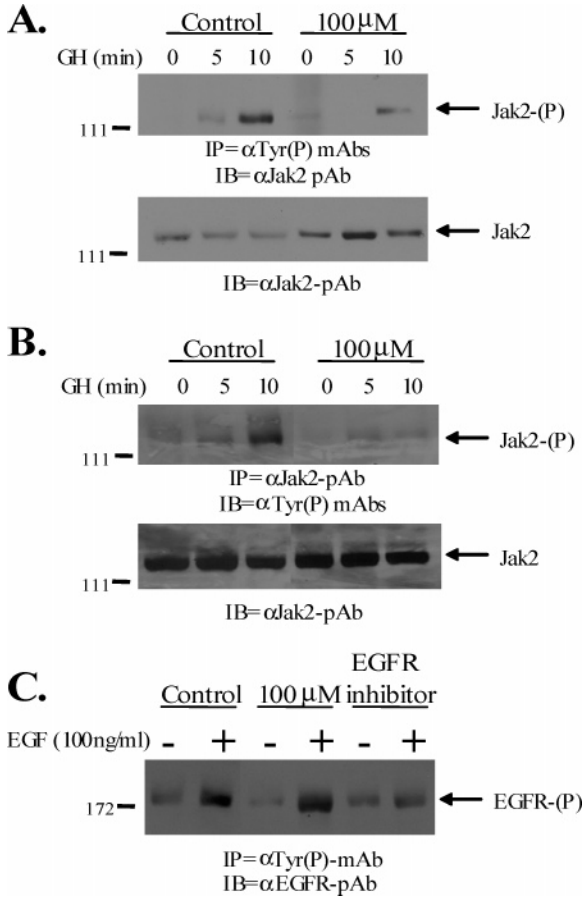

Figure 6. 1,2,3,4,5,6-Hexabromocyclohexane specifically inhibits ligand-mediated Jak2 tyrosine autophosphorylation. Cells were incubated for 16 h with either vehicle control or 100 μM 1,2,3,4,5,6-hexabromocyclohexane. (A) The cells were treated with 250 ng/mL growth hormone for the indicated times. Protein lysates were immunoprecipitated with anti-Tyr-(P) antibody and then Western blotted with anti-Jak2 antibody (top). Aliquots from these same cells were also Western blotted with anti-Jak2 antibody to access Jak2 protein levels (bottom). (B) The cells were treated with 250 ng/mL growth hormone for the indicated times. Lysates were immunoprecipitated with anti-Jak2 antibody and then Western blotted with anti-Tyr-(P) antibodies (top) or anti-Jak2 antibody (bottom). (C) Mouse fibroblasts were pretreated for 16 h with either vehicle control or 100 μM 1,2,3,4,5,6-hexabromocyclohexane. Cells were also pretreated for 1 h with 10 μM 4-(3-chloroanilino)-6,7-dimethoxyquinazoline. The cells were then either left untreated (−) or treated for 5 m with 100 ng/mL EGF (+). Protein lysates were then immunoprecipitated with anti-Tyr(P) antibody and then Western blotted with anti-EGFR antibody to access EGFR tyrosine phosphorylation levels. Shown is one of three (A and B) or two (C) representative experiments.

We next performed a reciprocal immunoprecipitation of that shown in Figure 6A. Specifically, protein lysates were first immunoprecipitated with anti-Jak2 antibody and then Western blotted with anti-Tyr(P) antibodies (Figure 6B, top). We observed a result similar to that seen in Figure 6A, as treatment of cells with 1,2,3,4,5,6-

hexabromocyclohexane greatly reduced the ability of growth hormone to promote Jak2 tyrosine auotphosphorylation, when compared to vehicle control treated cells. The membrane was subsequently stripped and reprobed with anti-Jak2 antibody to demonstrate equal protein precipitation among all samples (Figure 6, bottom).

To determine whether 1,2,3,4,5,6-hexabromocyclohexane was specific for suppressing Jak2 autophosphorylation, mouse fibroblasts were pretreated with either vehicle control, 1,2,3,4,5,6-hexabromocyclohexane, or a EGFR specific inhibitor, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline.[31−33] The cells were subsequently either left untreated or treated for 5 min with epidermal growth factor. EGFR tyrosine autophosphorylation levels were then measured by immunoprecipitating cellular lysates with anti-Tyr(P) antibody and then Western blotting with anti-EGFR antibody (Figure 6C). The results show that treatment of cells with 1,2,3,4,5,6-hexabromocyclohexane did not inhibit EGFR tyrosine autophosphorylation. However, ligand-dependent EGFR tyrosine autophosphorylation was sensitive to the EGFR specific inhibitor 4-(3-chloroanilino)-6,7-dimethoxyquinazoline.

Collectively, the results in Figure 6 demonstrate that 1,2,3,4,5,6-hexabromocyclohexane specifically inhibited ligand-dependent, Jak2 activation.

1,2,3,4,5,6-Hexabromocyclohexane Is Noncytotoxic to Cells at Concentrations That Maximally Inhibit Jak2 Tyrosine Autophosphorylation. To determine whether 1,2,3,4,5,6-hexabromocyclohexane was cytotoxic to the cultured cells, we treated BSC-40 cells with 1,2,3,4,5,6-hexabromocyclohexane at doses of either 0, 100, or 500 μM for 16 h. The live cells were then stained with propidium iodide in order to determine whether 1,2,3,4,5,6-hexabromocyclohexane had a cytotoxic effect, which in turn reduced Jak2 tyrosine autophosphorylation levels. Propidium iodide selectively stains necrotic cells and fluoresces red, but it is excluded by the plasma membranes of healthy, intact cells. The results showed that cells treated with 100 μM 1,2,3,4,5,6-hexabromocyclohexane showed very little propidium iodide staining, akin to that of untreated cells (Figure 7). In contrast, BSC-40 cells treated with 500 μM 1,2,3,4,5,6-hexabromocyclohexane did show increased propidium iodide staining, indicating that at a dose of 500 μM 1,2,3,4,5,6-hexabromocyclohexane is cytotoxic. Since the IC_{50} of 1,2,3,4,5,6-hexabromocyclohexane is estimated to be in the low micromolar range, and 50 μM 1,2,3,4,5,6-hexabromocyclohexane maximally inhibits Jak2 tyrosine kinase autophosphorylation, we conclude that the mechanism by which 1,2,3,4,5,6-hexabromocyclohexane inhibits Jak2 tyrosine kinase autphosphorylation, at these concentrations, is independent of cellular cytotoxicity.

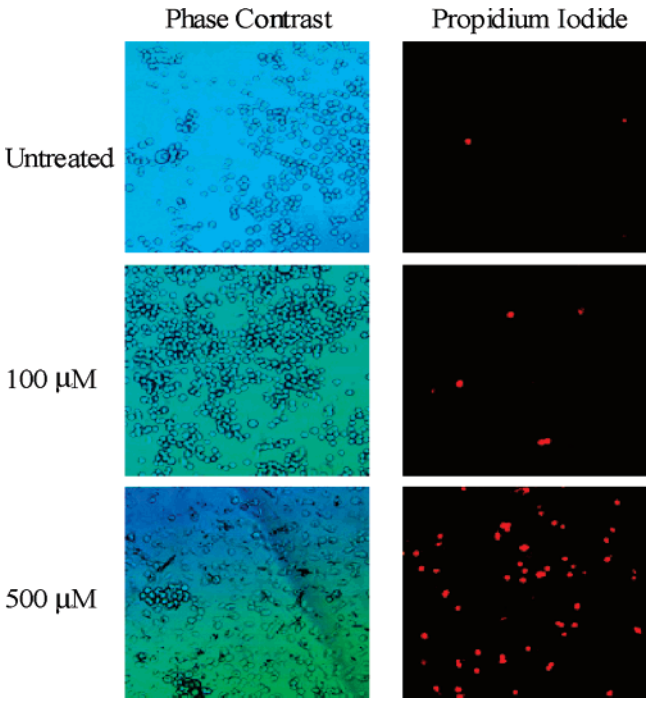

Figure 7. The concentration of 1,2,3,4,5,6-hexabromocyclohexane that inhibits Jak2 tyrosine kinase autophosphorylation is not toxic to cells. BSC-40 cells were grown on microscope slides and treated for 16 h with 0, 100, or 500 μM 1,2,3,4,5,6-hexabromocyclohexane. The live cells were then stained with 1 μg/mL propidium iodide to determine whether 1,2,3,4,5,6-hexabromocyclohexane was cytotoxic. The cells were visualized using confocal microscopy under phase contrast (left panel) and propidium iodide exclusion (right panel) conditions. Shown is one of two representative experiments.

Discussion

Since its discovery in 1992, significant progress has been made in understanding the biochemical and cellular functions of Jak2 tyrosine kinase. Studies have demonstrated essential roles for Jak2 in embryonic development, cell signaling, and the pathophysiology of heart disease and cancer.[7−12] Research on this protein, though, has been complicated by the lack of a Jak2-specific pharmacological inhibitor. α-Cyano-3,4-dihydroxy-*N*-benzylcinnamide, a commercially available Jak2 inhibitor, also inhibits several other related tyrosine kinase signaling pathways.

This work is significant for three fundamental reasons. First, we used homology modeling of the Jak2 kinase domain and high-throughput compound docking in silico to identify potential Jak2 inhibitors. We found that 1,2,3,4,5,6-hexabromocyclohexane potently inhibited Jak2 tyrosine autophosphorylation in cultured BSC-40 cells. 1,2,3,4,5,6-Hexabromocyclohexane inhibited Jak2 autophosphorylation in both a dose- and time-dependent manner. Moreover, we show that 1,2,3,4,5,6-hexabromocyclohexane is a direct inhibitor of Jak2 tyrosine kinase autophosphorylation and inhibits ligand-dependent activation of Jak2. On the basis of the autophosphorylation assays, it appears that a 16 h treatment with 1 μM 1,2,3,4,5,6-hexabromocyclohexane is sufficient to reduce Jak2 tyrosine autophosphorylation levels by about 50%, while 50 μM 1,2,3,4,5,6-hexabromocyclohexane eliminates virtually all detectable Jak2 tyrosine autophosphorylation. Furthermore, even at doses as high as 100 μM, 1,2,3,4,5,6-hexabromocyclohexane is not cytotoxic to cultured cells. As such, it inhibits Jak2 tyrosine autophosphorylation at concentrations that are well below its cytopathic threshold.

Second, the results shown here using the DOCK program demonstrate proof-of-principle for using in silico-based strategies for identifying novel Jak2 tyrosine kinase inhibitors. We showed that using the DOCK program in conjunction with homology modeling

of the Jak2 kinase domain, we could successfully identify novel small molecule inhibitors of Jak2 by database screening. We now plan to screen additional compounds for their ability to interact with multiple target pockets in the Jak2 kinase domain and test them for their ability to inhibit Jak2 tyrosine kinase autophosphorylation.

Third, α-cyano-3,4-dihydroxy-*N*-benzylcinnamide falls within the general class of tyrosine kinase inhibitors known as tyrphostins. The molecular structure of α-cyano-3,4-dihydroxy-*N*-benzylcinnamide is known.[13] It contains two aromatic ring structures linked by a spacer containing four carbons and an amide group. 1,2,3,4,5,6-Hexabromocyclohexane is noticeably different from α-cyano-3,4-dihydroxy-*N*-benzylcinnamide in that it contains only a single aromatic ring without any spacers. As such, our work here suggests that 1,2,3,4,5,6-hexabromocyclohexane, with its single aromatic ring, could serve as a potential lead compound for future synthesis reactions with the hopes of identifying specific third-generation Jak2 inhibitors.

The activation loop of a tyrosine kinase is rather adaptive in nature, as it moves either toward or away from the active site of the kinase domain. Clearly, our model places the activation loop in a single, fixed position. Thus, it must be noted that our computational model, or even any crystal structure for that matter, represents at best one of many possible tertiary conformations. Additionally, the region adjacent to the activation loop, where inhibitors might bind, is also adaptive in nature. However, once again, our model fixed this region in space in order to identify potential small molecule inhibitors via the DOCK program. As such, these are two important limitations of using computational analysis to model a tyrosine kinase domain. However, given the fact that the crystal structure of Jak2, or any other Jak family kinase for that matter, has not yet been solved, it is, to date, the best estimate of how the Jak2 kinase domain may structurally exist.

Collectively, the work shown here has identified 1,2,3,4,5,6-hexabromocyclohexane as a small molecule inhibitor of Jak2 tyrosine kinase. Because of the universal importance of Jak2 in mediating both physiological and pathophysiological actions within animals, this compound, and potential derivatives of it, may have important therapeutic value.

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