Dehydrocrenatidine Is a Novel Janus Kinase Inhibitor

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ABSTRACT

Janus kinase (JAK) 2 plays a pivotal role in the tumorigenesis of signal transducers and activators of transcription (STAT) 3 constitutively activated solid tumors. JAK2 mutations are involved in the pathogenesis of various types of hematopoietic disorders, such as myeloproliferative disorders, polycythemia vera, essential thrombocythemia, and primary myelofibrosis. Thus, small-molecular inhibitors targeting JAK2 are potent for therapy of these diseases. In this study, we screened 1,062,608 drug-like molecules from the ZINC database and 2080 natural product chemicals. We identified a novel JAK family kinase inhibitor, dehydrocrenatidine, that inhibits JAK-STAT3-dependent DU145 and MDA-MB-468 cell survival and induces cell apoptosis. Dehydrocrenatidine represses constitutively activated JAK2 and STAT3, as well as interleukin-6-, interferon-α, and interferon-γ-stimulated JAK activity, and STAT phosphorylation, and suppresses STAT3 and STAT1 downstream gene expression. Dehydrocrenatidine inhibits JAK-STAT1 domain overexpression-induced STAT3 and STAT1 phosphorylation. In addition, dehydrocrenatidine inhibits JAK2-JH1 kinase activity in vitro. Importantly, dehydrocrenatidine does not show significant effect on Src overexpression and epidermal growth factor–induced STAT3 activation. Our results indicate that dehydrocrenatidine is a JAK-specific inhibitor.

Introduction

Janus kinase (JAK)–signal transducers and activators of transcription (STAT) signaling has been well characterized for its critical role in mediating cytokine and growth factor responses (Akira et al., 1994; Darnell et al., 1994; Stark et al., 1998). Upon activation by cytokines or growth factors, receptor-associated Janus kinases phosphorylate the downstream STAT family proteins. Phosphorylated STATs form homodimers or heterodimers with other STATs via reciprocal phosphotyrosine-SH2 interactions and accumulate in the nucleus. They bind to promoters of their target genes and initiate transcription (Darnell, 1997). The JAK family consists of four members, JAK1, JAK2, JAK3, and TYK2 (Schindler and Darnell, 1995). JAK1, JAK2, and TYK2 are ubiquitously expressed, whereas JAK3 expression is restricted to cells of hematopoietic lineages (Johnston et al., 1994). JAK2 can be activated by cytokines, such as interleukin (IL)-3, IL-5, erythropoietin, thrombopoietin, and granulocyte-macrophage colony-stimulating factor (Valentino and Pierre, 2006). These cytokines regulate cell proliferation and differentiation in the hematopoietic system. Therefore, the delicate regulation of JAK family kinase activity is of great significance for the maintenance of hematopoietic system. Mutations in JAKs have been reported in many hematologic diseases. Chromosomal translocations containing fusion of JAK2 and other transcription factors result in generation of constitutively activated chimeric proteins, which are involved in the pathogenesis of various types of leukemia (Griesinger et al., 2005; Adelaïde et al., 2006; Vainchenker et al., 2008). A single valine to phenyalanine mutation located in the pseudokinase domain of JAK2 at position 617 (JAK2 V617F), which leads to constitutive activation of JAK2, are detected in patients with myeloproliferative disorders (MPDs), polycythemia vera, essential thrombocythemia and primary myelofibrosis (Kralovics et al., 2005; Steensma et al., 2005; Quentmeier et al., 2006).

Among the STATs family, STAT3 has been extensively studied because it is highly involved in oncogenesis. It was first identified as being activated by the IL-6 family of cytokines through JAKs (Akira et al., 1994). Aberrant activation of STAT3 occurs in a wide variety of human cancers, including breast, prostate, head and neck, and ovarian cancers, and other solid and hematologic tumors (Bromberg et al., 1999; Garcia et al., 2001; Dhir et al., 2002; Silver et al., 2004; Levy and Inghirami, 2005; Neal et al., 2005).

Given the critical role of JAK-STAT signaling in hematopoiesis and cancer biology, targeting JAK-STAT signaling has become a promising strategy for the treatment of hematologic cancers (Akira et al., 1994; Darnell et al., 2003; Pologe et al., 2004). Several small-molecule JAK inhibitors have been developed that show promising results in cancer cell lines and xenograft models (Barinaga, 2004; Darnell et al., 2003; Pologe et al., 2004; Xie et al., 2005). However, JAK inhibitors have been reported to show adverse effects such as myelosuppression and skin toxicity. Thus, developing selective JAK inhibitors is important.

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2006). Abnormally activated STAT3 signaling in tumors leads to uncontrolled cell-cycle progression and protects cancer cells from apoptosis by dysregulation of cell cycle–associated and apoptosis–associated expression of such genes as cyclin D1, Bcl-xL, Mcl-1, e-Myc, and survivin. It has been revealed that JAK2 is critical for the carcinogenesis of STAT3-activated solid tumor (Hedvat et al., 2009).

Since hyperactivated JAK2 is involved in many human diseases, small-molecular inhibitors of JAK2 continue to be developed to improve the survival of patients with abnormal JAK2 activity. Several JAK family kinase inhibitors such as AZD1480 [5-chloro-N2-[1(S)-5-fluoropyrimidin-2-yl)-ethyl]-N4-(5-methyl-1H-pyrazol-3-yl)-pyrimidine-2,4-diamine] (Ionomidis et al., 2011; Plimack et al., 2013), Atiprimod [3-(8,8-dipropyl-2-azaspiro[4,5]decan-2-yl)-N,N-diethylprop-1-amine] (Quintas-Cardama et al., 2011), and TG101348 [N-tert-butyl-3-[[5-methyl-2-([2-pyrrolidin-1-yethoxy]anilino)pyrimidin-4-yl]amino]benzenesulfonamide] (Pardaliani et al., 2011) are in clinical trials for solid tumor and hematopoietic disorders. In this study, we used virtual screening methods to find JAK2 inhibitors. We mixed the natural product chemical pool existing in our laboratory into 1,062,608 drug-like molecules from the ZINC database and screened for JAK2 inhibitors. We identified one natural product, dehydrocrenatidine, as a JAK inhibitor. It inhibited JAK-STAT3–dependent cell survival by inducing apoptosis. Dehydrocrenatidine diminished IL-6–, interferon (IFN)κ–, and interferon (IFN)γ–stimulated STAT3 phosphorylation, as well as constitutive STAT3 phosphorylation. Moreover, dehydrocrenatidine blocked IL-6– and IFNα–activated STAT3 and STAT1 downstream gene expression. However, it did not show inhibitory effect on epidermal growth factor (EGF)– or Src-induced STAT3 phosphorylation. Additionally, in cells over-expressing the Jak-JH1 domain, dehydrocrenatidine attenuated STAT3 and STAT1 activity. Kinase assay showed that dehydrocrenatidine inhibited JAK2 kinase activity.

**Materials and Methods**

**Cell Lines.** JAK1-JH1 domain, JAK2-JH1 domain, TYK2-JH1 domain, and ε-Src overexpression HEK293T cells were constructed as previously described (He et al., 2012; Chen et al., 2013). All of these transfected cells and Hela, HepG2, HEK293T, DU145, and MDA-MB-468 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS); penicillin (100 IU/ml), and streptomycin (100 mg/ml), K562 and human erythroleukemia (HEL) cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 mg/ml).

**Antibodies and Reagents.** Antibodies for phospho-STAT3(Tyr705), STAT3, JAK2, phospho-JAK2 (Tyr1007/1008), phospho-JAK2 (Tyr1054/1055), phospho-JAK1 (Tyr1022/1023), JAK1, phospho-STAT5(Tyr694), STAT5, phospho-p65 (Ser536) and p65, phospho-ARK (Ser473) and AKT, poly(A)DP-ribose) polymerase (PARP), e-Myc, cyclin D1, survivin, Bcl-xL, and cleaved caspase-3 were obtained from Cell Signaling Technology (Danvers, MA); antibody against phospho-Syk (PY99), and STAT2 was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX); anti–phospho-STAT2 was obtained from EMD Millipore (Billerica, MA). Dehydrocrenatidine was bought from BioBiaPha Co., Ltd. (Kunning, China). Hydrogen–1 nuclear magnetic resonance, mass spectrometry, and high-performance liquid chromatography data for dehydrocrenatidine are shown in Supplemental Data (Supplemental Figs. 3–5). TOP1 compound (ZINC9304906; 2-imino-8-methyl-1-(3-morpholin-4-yl-propyl)-10-oxo-1,10-dihydro-2H-1,9,10-triaza-anthracene-3-carboxylic acid cyclohexylamine) was obtained from ASINEX (Winston-Salem, NC) and TOP2 compound (ZINC20816390; 4,7,8-trimethyl-6-[2-(4-2-phenylethyl)piperezin-1-yl]ethylpurinol(7,8-αlimidazole-1,3-dione) was purchased from Vitas-M Laboratory Ltd. (Narva, Estonia). The Src/Brk-ABL–specific inhibitor dasatinib, PTK3 inhibitor LY294002 [2-morpholin-4-yl-6-phenylchromen-4-one], EGFR receptor (EGFR) inhibitor lapatinib, and the pan–tyrosine kinase inhibitor Staurorosporine were acquired from LC Laboratories (Woburn, MA). JAK2 inhibitor AG490 [(E)-N-benzyl-2-cyano-3-(3,4-dihydroxyphenyl)prop-2-enamide], IKK and STAT3 inhibitor TPCA-1 [2-(carbamoylamino)-5-(4-fluorophenyl)thiophene-3-carboxamide], and Flag antibody were obtained from Sigma-Aldrich (St. Louis, MO). AZD1480 was purchased from Selleckchem (Copenhagen, Germany).

**Virtual Screening.** The initial structure of JAK2 was extracted from the Protein Data Bank (PDB ID 3RVC; Lim et al., 2011). A working small-molecular database was formed from 1,062,608 drug-like molecules from the ZINC database (University of California, San Francisco; Irwin and Shoichet, 2005) and the natural product database existing in our laboratory (from the National Compound Resource Center, Shanghai, China). The geometry optimizations of compounds were calculated in bulk by semi-empirical method with the Austin Model 1 (Dewar et al., 1993) using Gaussian 09, Revision C01 (Frisch et al., 2009). The process of virtual screening had two steps. First, screening was performed by UCSF DOCK (Lang et al., 2009) with the formed molecular database as input. The missing hydrogen atoms were added, and the charge method used was Austin Model 1–bond charge correction (Wang et al., 2006). The residues of JAK2 within 8 Å of a crystal ligand were chosen as the docking pockets. The rigid algorithm of UCSF DOCK was employed for screening the prepared molecular database (Kuntz et al., 1992). Then the top 10,000 molecules were stored for further study. Second, AutoDock Vina (Scripps Research Institute; La Jolla, CA; Trott and Olson, 2010) and MolGridCal (Bai et al., 2014) were used to screen potential ligands from the 10,000 molecules. The receptor and ligand were added to the polar hydrogen. Gasteiger charges were used to prepare the ligand and receptor. The grid box was set to 24 Å × 24 Å × 24 Å around the position of the crystal ligand of JAK2. The efficient Quasi-Newton method was used for local optimization (Trott and Olson, 2010). Five binding modes were generated for each molecular docking. Finally, the top five molecules were stored for analysis. None of the identified compounds contained any potentially reactive groups, as described in a literature summation as pan assay interference compounds (Baell and Holloway, 2010). The electrostatic potentials were calculated by Molecular Graphics Tools (Scripps Research Institute; Morris et al., 2009) and Adaptive Poisson-Boltzmann Solver (Baker et al., 2001) software packages. Details for all methods are described in Supplemental Method 1.

**Western Blotting.** Cells were lysed with RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 5 mM EDTA, 1 mM EGTA, 1× protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 1× Phosphatase Inhibitor Cocktail (Roche) on ice and sonicated for 30 seconds (3 seconds on, 10 seconds off). Proteins (30–80 μg) were resolved in 4–12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore). Membranes were then blocked with 5% milk in Tris-buffered saline/ Tween 20 (TBST) for 1 hour at room temperature and incubated with the indicated antibodies overnight at 4°C or 1 hour at room temperature. After washing with TBST for 30 minutes (10 minutes, 3 times), membranes were exposed to secondary antibody for 1 hour at room temperature. Signals were visualized with an enhanced chemiluminescence detection system (Pierce Biotechnology, Rockford, IL).

**Real-Time Polymerase Chain Reaction.** Total RNA was prepared by RNAprep Pure Kit (TIANGEN, Beijing, China). First-strand cDNA was synthesized from total RNA (1 μg) with M-MLV reverse transcription kit (TaKaRa Biotechnology, Dalian, China). Quantitative real-time polymerase chain reaction (PCR) was carried out on a CFX96™ Real-Time system with SYBR Green qPCR Master Mix (Bio-Rad, Hercules, CA). The PCR reaction was conducted with the following protocol: initial activation at 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds, and at 60°C for 1 minute. Expressions of respective genes were normalized to glyc. Primer sequences of tested genes are listed as follows:
gapdh forward 5'-TGGCAAATTCATGGCAC-3', reverse 5'-CCATGGTGGTGAAGACGC-3';
socs3 forward 5'-CCATGGTGGTGAAGACGC-3', reverse 5'-CCTGTCCAGCCCAATACCTGA-3' (He et al., 2012).
irf1 forward 5'-CGATACAAAGCAGGGGAAAA-3', reverse 5'-TAGCTGCTGTGGTCATCAGG-3' (Liu et al., 2010).

The results represent the averages from three independent experiments.

Cell Viability Assay. Cells were plated in 96-well plates at a density of 5 x 10^3 cells/well in Dulbecco's modified Eagle's medium plus 10% FBS, and incubated for 12 hours prior to the addition of dimethylsulfoxide (DMSO) or dehydrocrenatidine to the culture medium. After 48 hours, 0.5 mg/ml of MTT [3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium]

![Fig. 1. The electrostatic potentials of receptor and ligands. (A) The electrostatic potentials of AG490 and the pocket of JAK2. (B) The electrostatic potentials of dehydrocrenatidine and the pocket of JAK2. Dehydrocrenatidine can insert into the deeper position of JAK2 domain according to an analysis of molecular shape and electrostatic potential.](image)

![Fig. 2. Dehydrocrenatidine inhibited STAT3-hyperactivated cancer cell survival and STAT3 phosphorylation without affecting P65 and Akt phosphorylation. hTERT-BJ, DU145, MDA-MB-468, and MCF 10A cells were treated with (A) dehydrocrenatidine, (B) AG490, (C) AZD1480, and (D) staurosporine at indicated concentrations for 48 hours, and cell viability was measured by MTT assay. (E) DU145 and MDA-MB-468 cells were treated with DMSO, dehydrocrenatidine (10 μM), Ly294002 (50 mM), and TPCA-1 (1 μM) for 2 hours and Western blotted.](image)
reagent was added and incubated at 37°C, 5% CO2 for 4 hours. Formazan crystals in viable cells were solubilized with 200 μl DMSO. Absorbance at 490 nm was recorded using Victor Multilabel Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

**Flow Cytometric Analysis of Apoptosis.** DU145 and MDA-MB-468 cells were seeded into 60-mm dishes and allowed to attach overnight. After attachment, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% FBS with DMSO, or dehydrocrenatidine as indicated. Twenty-four hours after incubation, cells were washed twice with cold phosphate-buffered saline, harvested by trypsinization, and washed with cold phosphate-buffered saline twice. Cells were resuspended in 400 μl of binding buffer and stained with 5 μl annexin V at 4°C in dark for 15 minutes; 10 μl of propidium iodide was then added and incubated in dark at 4°C for 5 minutes. Samples were analyzed on a FACSCalibur cytometer (BD Biosciences, San Jose, CA).

**Kinase Assay.** C-terminal His-tagged hSTAT3 recombinant protein was purified as previously described (Chen et al., 2013). Active recombinant human JAK2 (804-end) was purchased from SignalChem (Richmond, BC, Canada). Approximately 150 ng of hSTAT3 and 40 ng of JAK2-JH1 kinase were incubated with 1× kinase buffer (diluted from 10× kinase buffer). 10× kinase buffer was obtained from New England Biolabs (Ipswich, MA). DMSO or dehydrocrenatidine were added as indicated concentrations. ATP was supplied at the concentration of 200 mM to the finally volume of 25 μl. Reactions were performed at 37°C for 2 hours, and stopped by 5× protein sample loading buffer (95°C, 5 minutes). Each sample (15 μl) was loaded for SDS-PAGE and analyzed by Western blotting.

**Results**

**Virtual Screening Study of Drugs Targeting JAK2.** On the basis of computational technology, virtual screening is an effective and economic way to obtain potential drugs targeting proteins of interest. In this experiment, we mixed the natural product database existing in our laboratory, which contains 2080 chemicals, into 1,062,608 drug-like molecules from the ZINC database (Irwin and Shoichet, 2005), and then performed virtual screening to obtain candidate compounds for JAK2 inhibition. One ligand, dehydrocrenatidine, which is from the natural product database, was ranked in the top five among the mixed molecular database (Supplemental Fig. 1). A reported JAK2 inhibitor, AG490 (Tyrphostin B42) (Duhe et al., 2002), was ranked in the top five as well. We detected biologic activity by Western blot from the top four ranking compounds and found that dehydrocrenatidine effectively inhibited JAK2 phosphorylation (Supplemental Fig. 2). In addition, we selected 10 inhibitors of JAK2 from the IUPHAR Database (Harmar et al., 2009) to perform two-dimensional structure searching on the ZINC and natural product database with 90% similarity (Supplemental Table 1) (Irwin and Shoichet, 2005). The similar compound, dehydrocrenatidine, was missed in the search results. This indicates that our virtual screening protocol is an effective way to find the inhibitors of JAK2. Both AG490 and dehydrocrenatidine are located near the residues P933, L932, and Y931 of JAK2, which are identified as key sites for ligand-binding. AG490 has slightly lower affinity energy than dehydrocrenatidine. However, JAK2 has a long and deep pocket for ligand-binding (Fig. 1, A and B). Dehydrocrenatidine has a smaller planar atom group than AG490, so it can reach the deeper position of the JAK2 pocket more easily. The edge of the JAK2 domain...
shows positive electrostatic potentials (Fig. 1, A and B), whereas the deep position of JAK2 domain shows negative electrostatic potentials. Comparing the electrostatic potentials between AG490 and dehydrocrenatidine, part of dehydrocrenatidine shows positive electrostatic potential in deep position of JAK2 domain, whereas AG490 shows negative electrostatic potential in the deep position of JAK2 pocket. It indicates that dehydrocrenatidine can insert into deeper position of JAK2 pocket than AG490. So from the perspective of molecular modeling, dehydrocrenatidine is a more promising inhibitor candidate of JAK2 domain.

Dehydrocrenatidine Inhibits STAT3 Phosphorylation and DU145, MDA-MB-468 Cell Viability. To evaluate the specificity and efficiency of dehydrocrenatidine, we assessed the impact of dehydrocrenatidine on JAK-STAT3 constitutively activated cancer cell survival. We choose DU145 and MDA-MB-468 cells, which have been reported to have IL-6 autocrine loop and persistently activated STAT3 (Berishaj et al., 2007; Okamoto et al., 1997). We treated these two cell lines with indicated concentrations of dehydrocrenatidine for 48 hours and determined cell viability by MTT assay. The viability of DU145 and MDA-MB-468 cells were significantly decreased upon dehydrocrenatidine treatment in a dose-dependent manner (Fig. 2A). Meanwhile, we tested the effect of AG490 and AZD1480 on those two cell lines; consistent with our virtual screening results, the working concentration of AG490 was much higher than that for dehydrocrenatidine (Fig. 2, B and C). To rule out the possibility that dehydrocrenatidine causes cell death by cytotoxicity, we examined its influence on cell viability of hTERT-BJ, a telomerase-immortalized cell line derived from a human primary foreskin fibroblast cell line, and MCF 10A, a nontumorigenic human breast epithelial cell line in which JAK-STAT signaling is

Fig. 4. Dehydrocrenatidine inhibited IL-6 and IFN-induced STAT3 phosphorylation and their downstream gene expression. (A) Hela, HepG2, and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium with 0.2% FBS for 12 hours. Serum-starved cells were pretreated with DMSO or dehydrocrenatidine (10 μM) for 1 hour and stimulated with IL-6 (250 ng/ml) for 2 hours, and cell lysates were blotted by indicated antibodies. (B) Serum-starved Hela cells were incubated with DMSO or dehydrocrenatidine (10 μM) for 1 hour and treated with IFNγ (150 IU/ml) or (C) IFNα (5000 IU/ml) for 2 hours. Cells lysates were blotted by indicated antibodies. (D) Serum-starved Hela cells were incubated with DMSO or dehydrocrenatidine with indicated concentrations for 1 hour and stimulated with IFNγ (5000 IU/ml) for 2 hours. Cell lysates were blotted by indicated antibodies. p-STAT1/STAT1 relative density was measured by Image J, and IC50 was calculated by SPSS software. (E) Hela and (F) HepG2 cells were serum-starved and pretreated with DMSO or dehydrocrenatidine (10 μM) for 1 hour and stimulated by IL-6 (250 ng/ml) for 4 hours. mRNA levels of sox3 and irf1 were analyzed by real-time PCR. Data are mean ± S.D. of three independent experiments. ***P < 0.001, one-way analysis of variance. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
not constitutively activated. Like AG490 and AZD1480, dehydrocrenatidine showed little inhibition on viability of hTERT-BJ cells and MCF 10A (Fig. 2A). Staurosporine, a pan–tyrosine kinase inhibitor (Fallon, 1990) that usually inhibits many cell processes with no cell-type specificity, was used as a control. Staurosporine showed no selectivity in cell-growth inhibition among these cell lines (Fig. 2D).

To evaluate the impact of dehydrocrenatidine on STAT3 phosphorylation, DU145 and MDA-MB-468 cells were treated with 10 μM dehydrocrenatidine for 2 hours; Western blot results revealed that JAK2 and STAT3 activity were repressed in both cell lines (Fig. 2E). To exclude nonspecific inhibition, phosphorylation levels of p-AKT and p-P65, key molecules of two signaling pathways that are also highly involved in tumorigenesis, were assessed. In contrast, Western blot showed that p-AKT and p-P65 phosphorylation were not changed in the presence of dehydrocrenatidine (Fig. 2E). LY294002, a PI3K inhibitor and TPCA-1 (Nan et al., 2014), a dual inhibitor of STAT3 and NF-κB were used as controls. These data suggested that dehydrocrenatidine selectively inhibited JAK-STAT3 signaling without affecting PI3K-AKT pathway and NF-κB pathway. Moreover, in accordance with cell viability data, in both DU145 and MDA-MB-468 cell lines, STAT3 tyrosine 705 phosphorylation was attenuated in a dose-dependent manner (Fig. 3A), with IC50 of 11.6 and 5.8 μM, respectively, which confirmed its specific inhibition role on JAK-STAT3 signaling.

Dehydrocrenatidine Inhibits IL-6- and IFN-Induced STAT Activation. To further investigate the impact of dehydrocrenatidine on cytokines and interferon-induced STAT activity, we treated Hela, HepG2, and HEK293T cells with either IL-6 or IFNs. IL-6 and IFNs activate STATs through different membrane receptors: IL-6 activates STAT3 through IL-6 receptor and gp130, whereas IFNα and IFNγ signal through types I and II interferon receptors (Murakami et al., 1993; Platanias, 2005). Cells were pretreated with dehydrocrenatidine and then treated with IL-6. STAT3 tyrosine (Tyr) 705 phosphorylation was inhibited in these three cell lines (Fig. 4A). Since IL-6 activates STAT3 through JAK1 and JAK2 (Lutticken et al., 1994; Narazaki et al., 1994), we also measured JAK1 and JAK2 activity. With no surprise, we found that both JAK1 and JAK2 phosphorylation were inhibited in these two cell lines as well. We then tested the kinetic effect of dehydrocrenatidine on JAK2 and STAT3 activity inhibition and found that dehydrocrenatidine suppressed JAK2 and STAT3 activation in a time-dependent manner (Fig. 3B). We found that basal-level phosphorylation of STAT1 and STAT2 was undetectable under our experimental conditions (data not shown). We have demonstrated that in HEL, a human erythroleukemic cell line that contains constitutively activating mutation JAK2V617F (Levine et al., 2005), phosphorylation of JAK2, STAT3, and STAT5 were inhibited in a dose- and time-dependent manner (Supplemental Fig. 7, D and E).

### Figures

**Fig. 5.** Dehydrocrenatidine inhibited overexpression of the JH1 domain of JAK1-, JAK2-, and TYK2-induced protein phosphorylation. (A) HEK293T cells transfected with JH1 domain of JAK1, JAK2, and TYK2 were treated with dehydrocrenatidine (15 μM) for 4 hours and blotted by indicated antibodies. (B) Serum-starved Hela cells were pretreated with DMSO, dehydrocrenatidine (15 μM), or lapatinib (10 μM) for 1 hour and stimulated with EGF (50 ng/ml) for 30 minutes, and cell lysates were Western blotted. (C) HEK293T cells overexpressing c-Src were incubated with dehydrocrenatidine (15 μM) or dasatinib (500 nM) for 4 hours. Cell lysates were analyzed by Western blot. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
JAK2 activity induced by IL-6 were decreased by dehydrocarnitidine. IFNγ activates STAT1 and STAT3 through JAK1 and JAK2 (Stark et al., 1998). Our results showed that IFNγ-induced STAT1 and STAT3 activation were blocked by dehydrocarnitidine. Moreover, dehydrocarnitidine directly suppressed IFNγ-activated JAK1 and JAK2 phosphorylation (Fig. 4B). Additionally, we found that IFNα-stimulated TYK2 and STAT3 phosphorylations were diminished by dehydrocarnitidine (Fig. 4C). Moreover, dehydrocarnitidine inhibited IFNα-induced STAT3 phosphorylation in a dose- and time-dependent manner in K562 cells (Supplemental Fig. 7, A and B). IFNα also activates STAT2, and we found that this activation was inhibited by dehydrocarnitidine. STAT1 tyrosine 701 phosphorylation level was repressed in the presence of dehydrocarnitidine in a dose-dependent manner, with IC50 of 8.02 μM (Fig. 4D). In addition, we found that dehydrocarnitidine inhibited IFNα-induced STAT1 and STAT2 phosphorylation in a concentration-dependent manner in K562 cells (Supplemental Fig. 7C). Since JAK-STAT3 signaling controls cell survival and proliferation by regulating their downstream genes, it is important to evaluate the effect of the inhibitor on expression of their target genes. Hela and HepG2 cells were pretreated with dehydrocarnitidine or DMSO, then IL-6 was added; mRNA expression of a STAT3 direct downstream gene socs3 was analyzed by real-time PCR. Results showed that IL-6 alone dramatically induced socs3 mRNA expression, and this induction was diminished by dehydrocarnitidine (Fig. 4E and F). Similarly, IFNα-induced STAT1-regulated gene irf1 mRNA expression was suppressed by dehydrocarnitidine (Fig. 4G).

**Dehydrocarnitidine Inhibits Overexpression of JH1 Domain-Induced JAK-STAT Signaling.** To further confirm that dehydrocarnitidine directly inhibits JAK kinase activity, we introduced JH1 domains of JAKs to HEK293T cells. In these cells, total tyrosine phosphorylation levels were tremendously elevated (Fig. 5A). Exposure of JAKs-JH1 overexpressed cells to dehydrocarnitidine resulted in decrease in total tyrosine phosphorylation. Overexpression of JH1 domains of JAK1, JAK2 or TYK2 lead to upregulation of STAT1 and STAT3 activation, and this induction was decreased by dehydrocarnitidine.

**Fig. 6.** Dehydrocarnitidine induced apoptosis. (A) DU145 and (B) MDA-MB-468 cells were plated on coverslips. After 12 hours, medium was replaced and DMSO (a and c) or dehydrocarnitidine (10 μM) (b and d) was added. After 16 hours of treatment, cells were stained with Hoechst and visualized under a microscope (a and b, 40× magnification; c and d, 20× magnification). (C) DU145 and MDA-MB-468 cells were treated with DMSO or dehydrocarnitidine (10 μM) for 24 hours, double stained by annexin V and propidium iodide, and flow cytometry was performed. (D) DU145 and MDA-MB-468 cells were treated with dehydrocarnitidine (10 μM) for 24 or 48 hours. Cell lysates were analyzed by Western blot. DMSO was used as control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Dehydrocrenatidine Inhibits JAK2-JH1 Kinase Activity In Vitro. Kinase assay was performed to confirm that dehydrocrenatidine directly inhibited JAK2-JH1 kinase activity. Consistent with our virtual screening results, dehydrocrenatidine inhibited JAK2-JH1 kinase--catalyzed STAT3 phosphorylation in vitro, which suggested that dehydrocrenatidine directly inhibited JAK2 kinase activity (Fig. 7, A and B).

Discussion

JAK kinases play indispensable roles in the activation of STAT3 in STAT3 constitutively activated human cancer cell lines. Richard Jove and Michael Zinda reported that inhibition of JAK, but not Src or EGFR, activity ultimately resulted in diminished STAT3 phosphorylation. Their murine model data also demonstrated the importance of JAK2 in STAT3-dependent solid tumorigenesis (Hedvat et al., 2009). Owing to its importance in solid tumor and hematopoietic disorders, JAK inhibitors have become popular targets for drug development. Although many JAK inhibitors are already available, certain defects have been observed; most common are inhibition of other tyrosine kinases and acquired drug resistance. The physiologic environment is much more complicated than in vitro experimental conditions; there might be unpredicted toxicity effects from a very potent inhibitor. In addition, it has been reported that using JAK inhibitors with different mechanisms could re sensitize JAK inhibitor–resistant cells in myeloproliferative neoplasms (Koppikar et al., 2012). Thus, it is necessary to develop new JAK inhibitors. In this study, we used a computer-based screening method to identify small-molecular inhibitors of JAK2. We found a natural product, dehydrocrenatidine, that could block JAK activity through inhibition of the JH1 domain. Kinase assay revealed that dehydrocrenatidine inhibits JAK2-JH1 kinase activity in vitro. In addition, molecular docking results showed that dehydrocrenatidine can bind to residues E957, F958, and L959 of JAK1, residues Y931, L932, and P933 of JAK2, residues E905, E979, and D967 of JAK3, and E903, E905 of JAK1, and Y980 of TYK2 (Supplemental Fig. 6). However, the IC50 of the in vitro kinase assay was higher than that for the cell-based assay. There might be two reasons for this. First, the kinase catalytic reaction was incubated for 2 hours, because STAT3 phosphorylation was undetectable with a shorter incubation, whereas catalytic reaction with 32P labeled ATP usually requires an incubation time for only 5–15 minutes. Once incubation time
was long enough to allow STAT3 phosphorylation to be detected, the “leaky activity” made it difficult to observe the inhibition at low concentrations. Second, under in vivo conditions, kinase and phosphatase are dynamically balanced, whereas in vitro the kinase catalytic reaction keeps going until substrates are saturated. We demonstrated that dehydrocrenatidine inhibited constitutively activated JAK2 and STAT3 in DU145 and MDA-MB-468 cells in a dose-dependent and time-dependent manner, and it also suppressed IL-6–, IFNα–, and IFNγ–stimulated JAK activity and STAT3 phosphorylation. Nevertheless, dehydrocrenatidine showed little effect on EGFR and Src-induced STAT3 activity. Our results demonstrated that dehydrocrenatidine directly inhibited JAK2 and TYK2 phosphorylation but had no significant effect on EGFR and Src phosphorylation. These results indicate that dehydrocrenatidine selectively inhibits JAK family activity. Furthermore dehydrocrenatidine suppressed IFNα-induced STAT1 and STAT2 activity and JAK2V617F mutation-induced STAT5 activity. JAK-STAT signaling controls cell proliferation by regulating expression of their downstream genes, some of which are essential for cell proliferation. Our results revealed that dehydrocrenatidine could reduce both IL-6– and IFNα–induced gene expression. Longer time treatment also diminished STAT3 downstream gene c-Myc, cyclin D1, survivin, and Bcl-xL expression. We also assessed the impact of dehydrocrenatidine on cell survival. MTT assay showed that dehydrocrenatidine inhibited cancer cell survival by inducing apoptosis. However, Western blot demonstrated that after treatment with dehydrocrenatidine for 24 and 48 hours, the amount of cleaved PARP increased and the level of total PARP decreased. Analysis of apoptosis results indicated that dehydrocrenatidine could reduce both IL-6– and IFNα–induced gene activity. Moreover, Western blot demonstrated that after treatment with dehydrocrenatidine for 24 and 48 hours, the amount of cleaved PARP increased and the level of total PARP decreased. The level of cleaved caspase—3—the executor of apoptosis—was also increased. These data confirmed that dehydrocrenatidine inhibited cancer cell survival by inducing apoptosis. However, whether dehydrocrenatidine-induced apoptosis is JAK-STAT pathway–dependent needs further validation. We could not rule out the possibility that dehydrocrenatidine could induce apoptosis through JAK-STAT pathway–independent mechanisms. In conclusion, we have discovered a novel natural product inhibitor of JAKs, dehydrocrenatidine, which may stimulate new insights for therapy of hyperactivated JAK2–related malignances.

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