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Dehydrocrenatidine is a novel JAK inhibitor

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Dehydrocrenatidine is a novel JAK inhibitor

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ABBREVIATIONS:

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

JAK: Janus kinase

STAT: Signal transducers and activators of transcription

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ABSTRACT

JAK2 plays pivotal role in the tumorigenesis of STAT3 constitutively activated solid tumors. JAK2 mutations are involved in the pathogenesis of various types of hematopoietic disorders such as myeloproliferative disorders (MPDs), polycythemia vera (PV), essential thoursombocythemia (ET) and primary myelofibrosis (PMF). 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 ZINC database and 2080 natural product chemicals. We identified a novel JAK family kinase inhibitor Dehydrocrenatidine. Dehydrocrenatidine 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 IL-6, IFNα and IFNγ stimulated JAKs activity and STATs phosphorylation, suppresses STAT3 and STAT1 downstream gene expression. Dehydrocrenatidine inhibits JAKs-JH1 domain over-expression induced STAT3 and STAT1 phosphorylations. In addition, Dehydrocrenatidine inhibits JAK2-JH1 kinase activity *in vitro*. Importantly, Dehydrocrenatidine does not show significant effect on Src over-expression and EGF induced STAT3 activation. Our results indicate that Dehydrocrenatidine is a JAK specific inhibitor.

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Introduction

JAK-STAT signaling is 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 JAKs phosphorylate the downstream signal transducers and activators of transcription (STATs) family proteins. Phosphorylated STATs form homodimers or heterdimers with other STATs via reciprocal phosphotyrosine-SH2 interactions and accumulate in the nucleus. Then they bind to promoters of their target genes and initiate transcription (Darnell, 1997). Janus kinase family (JAK) consists of four family members, JAK1, JAK2, JAK3, and TYK2 (Schindler and Darnell, 1995). JAK1, JAK2, and TYK2 are ubiquitously expressed, Jak3 expression is restricted to cells of hematopoietic lineages (Johnston et al., 1994). JAK2 can be activated by cytokines such as IL-3, IL-5, erythoursopoietin (EPO), thoursombopoietin (TPO), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Valentino and Pierre, 2006).These cytokines regulate cell proliferation and differentiation in 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 contain 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 (Adelaide et al., 2006; Griesinger et al., 2005; Vainchenker et al., 2008). A single valine to phenylalanine mutation located in the pseudokinase domain of JAK2 at position 617 (JAK2 V617F), which leads to constitutively activation of JAK2, are detected in patients with myeloproliferative disorders (MPDs), polycythemia vera (PV), essential thoursombocythemia (ET) and primary myelofibrosis (PMF) (Kralovics et al., 2005; Quentmeier et al., 2006; Steensma et al., 2005).

Among STATs family, STAT3 is extensively studied for the reason that it is highly involved in oncogenesis. It was first identified to be activated by the interleukin-6 (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; Dhir et al., 2002; Garcia et al., 2001; Levy and Inghirami, 2006; Silver et al., 2004). Abnormally activated STAT3 signaling

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in tumors leads to uncontrolled cell-cycle progression and prevents cancer cells from apoptosis by dysregulation of cell cycle-associated and apoptosis-associated gene expression such as Cyclin D1, Bcl-xL, Mcl-1, c-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 are developed to improve the survival of patients with abnormal JAK2 activity. Several JAK family kinase inhibitors such as, AZD1480 (Ioannidis et al., 2011; Plimack et al., 2013) , Atiprimod (Quintas-Cardama et al., 2011), TG101348 (Pardanani et al., 2011) are under 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 existed in our lab into 1,062,608 drug-like molecules from 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, IFNα and IFNγ 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 role on EGF or Src induced STAT3 phosphorylation. Additionally, in JAK-JH1 domains over-expressed cells, Dehydrocrenatidine attenuated STAT3 and STAT1 activity. Kinase assay showed that Dehydrocrenatidine inhibited JAK2 kinase activity.

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Materials and Methods

Cell lines

JAK1-JH1 domain, JAK2-JH1 domain, TYK2-JH1 domain and c-Src over-expression HEK293T cells were constructed as previously described (Chen et al., 2013; He et al., 2012).All these transfected cells and Hela, HepG2, HEK293T, DU145 and MDA-MB-468 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). K562 and HEL cells were maintained in RMPI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml).

Antibodies and Reagents

Antibodies for phospho-STAT3(Tyr705), STAT3, JAK2, phospho-JAK2 (Tyr1007/1008), phospho-TYK2 (Tyr1054/1055), phosphor-JAK1(Tyr1022/1023), JAK1, phosphor-STAT5(Tyr694), STAT5, phosphor-p65 (Ser536) and p65, phosphor-AKT (Ser473) and AKT, PARP, c-Myc, Cyclin D1, Survivin, Bcl-xL, cleaved caspase 3 were obtained from Cell Signaling Technology; Antibody against phosphor-Tyr (PY99) and STAT2 was obtained from Santa Cruz Biotechnology Inc, anti-phospho-STAT2 was obtained from Millipore. Dehydrocrenatidine was bought from BioBioPha Co., Ltd (BBP), ^{1}H-NMR, MS and HPLC data for Dehydrocrenatidine were shown in Supplemental data (Supplemental Figure 3-5). TOP1 compound (ZINC9304906) was obtained from ASINEX, TOP2 compound (ZINC20816390) was purchased from Vitas-M. The Src/Bcr-ABL specific inhibitor Dasatinib, PI3K inhibitor LY294002, EGFR inhibitor Lapatinib and the pan-tyrosine kinase inhibitor Staurosporine were acquired from LC Lab, JAK2 inhibitor AG490, IKK and STAT3 inhibitor TPCA-1 and Flag antibody were obtained from Sigma-Aldrich. AZD1480 was purchased from Selleckchem.

Virtual screening

The initial structure of JAK2 is extracted from PDB database (PDB ID: 3RVG (Lim et al., 2011)). The small molecular database was prepared by 1,062,608 drug-like molecules from

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ZINC database (Irwin and Shoichet, 2005) and the natural product database existed in our lab were from National Compound Resource Center (China). The geometry optimizations of compounds are calculated in bulk by semi-empirical method with the Austin Model 1 (AM1) (Dewar et al., 1985) using Gaussian 09, Revision C.0.(Frisch et al., 2009) The process of virtual screening was two steps. The first screening was performed by UCSF docking based on the formed molecular database. The missing hydrogen atoms were added, and the charge method is AM1-BCC (Wang et al., 2006). The residues of JAK2 within 8 Å of crystal ligand were chosen as the docking pocket. The rigid algorithm of UCSF docking was employed for screening the prepared molecular database (Kuntz et al., 1982). Then the top 10,000 molecules were stored for further study. In the second procedure, the Autodock Vina (Trott and Olson, 2010) and MolGridCal (Bai et al., 2014) was selected for screening potential ligands from the 10,000 screened molecules. The receptor and ligand were added polar hydrogen. The 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 crystal ligand of JAK2. The efficient quasi-Newton method was used for the local optimization (Trott and Olson, 2010). Five binding modes were generated for each molecular docking. Finally, the top 5 molecules were stored for analysis. All the identified compounds did not contain any potentially reactive groups described in literature summation of Pan Assay Interference Compounds (PAINS) (Baell and Holloway, 2010). The electrostatic potentials were calculated by MGLTools (Morris et al., 2009) and APBS (Baker et al., 2001) soft package. All the method details were additionally introduced in Supplemental method 1.

Western Blotting

Cells were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 5 mM EDTA, 1 mM EGTA, 1 × protease inhibitor cocktail (Roche), 1 × Phosphatase Inhibitor Cocktail (Roche)) on ice and sonicated for 30 seconds (3 seconds on, 10 seconds off). 30-80 μg proteins were resolved in 4%–12% SDS-PAGE and transferred to PVDF membranes (Millipore). Then membranes were blocked with 5% milk in TBST for 1 hour at room temperature and incubated with indicated anti-bodies overnight at 4 °C or 1 hour at room temperature. After washing with TBST for 30 minutes (10 minutes, 3

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times), membranes were exposed to secondary antibody for one hour at room temperature. Signals were visualized with an enhanced chemiluminescence detection system (Pierce Biotechnology).

Real-Time PCR.

Total RNA was prepared by RNA Prep Pure Cell kit (TIANGEN). First-strand cDNA was synthesized from total RNA (1μg) with M-MLV reverse transcription kit (Takara). Quantitative Real-time PCR was carried out on a BIO-RAD CFX96TM Real Time system machine with SYBR Green qPCR mastermix (BIO-RAD). The PCR reaction was conducted as 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 *gapdh*. Primer sequences of tested genes are listed as follows:

gapdh forward 5'-TGGCAAATTCCATGGCAC-3',

reverse 5'-CCATGGTGGTGAAGACGC-3';

socs3 forward 5'-CCATGGTGGTGAAGACGC-3' ,

reverse5'-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 5×10^3 cells/well in DMEM plus 10% FBS, and incubated for 12 hours prior to the addition of DMSO or Dehydrocrenatidine to the culture medium. After 48 hours, 0.5 mg/ml of MTT reagent was added and incubated at 37 °C, 5% CO_2 for 4 hours. Formazan crystals in viable cells were solubilized with 200 μl DMSO. Absorbance at 490 nm was recorded using Victor^3 Plate Counter (Perkin Elmer).

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 DMEM containing 10% FBS with DMSO, or Dehydrocrenatidine as indicated. 24 hours after incubation, cells were washed

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twice with cold PBS, harvested by trypsinization and washed with cold PBS twice. Cells were resuspended in 400 μl binding buffer and stained with 5 μl Annexin-V at 4 °C in dark for 15 minutes, then 10 μl propidium (PI) was added and incubated in dark at 4 °C for 5 minutes. Samples were analyzed on a FACS calibur cytometer (BD Biosciences, Lanzhou University).

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 Signal-Chem (J02-11H-10). Approximately 150 ng hSTAT3 and 40 ng JAK2-JH1 kinase were incubated with 1 × kinase buffer, 10 × kinase buffers were obtained from New England Biolabs (NEB). 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). 15 μl of each sample was loaded for SDS-PAGE and analyzed by Western-Blotting.

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Results

Virtual screening study of drugs targeting JAK2

Based on the 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 existed in our lab which contains 2080 chemicals into 1,062,608 drug like molecules from ZINC database (Irwin and Shoichet, 2005), and then performed virtual screening to get potential candidate compounds for JAK2 inhibition. One ligand Dehydrocrenatidine, which is from the natural product database, was ranked in top 5 among the mixed molecular database (Supplemental Figure 1). A reported JAK2 inhibitor AG490 (Tyrphostin B42) (Duhe et al., 2002) was ranked in top 5 as well. We detected biological activity of top 4 ranking compounds by Western blot and found that Dehydrocrenatidine effectively inhibited JAK2 phosphorylation (Supplemental Figure 2). In addition, we selected 10 inhibitors of JAK2 from IUPHAR Database (Harmar et al., 2009) to perform 2D structure searching on ZINC database and the natural product database with 90% similarity (Supplemental Table1) (Irwin and Shoichet, 2005). The similar compound Dehydrocrenatidine is missed in the searching results. It indicates that our virtual screening protocol is an effective way to find the inhibitors of JAK2. Both AG490 and Dehydrocrenatidine locate 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.1A and 1B). Dehydrocrenatidine has a smaller planar atom group than AG490, so it can reach deeper position of JAK2 pocket more easily. The edge of JAK2 domain shows positive electrostatic potentials (Fig.1A and 1B), while deep position of JAK2 domain shows negative electrostatic potentials. Comparing the electrostatic potentials between AG490 and Dehydrocrenatidine, the part of Dehydrocrenatidine shows positive electrostatic potential in deep position of JAK2 domain, while 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 insight of molecular modeling, Dehydrocrenatidine is a more promising candidate inhibitor of JAK2 domain.

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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, in consistent with our virtual screening results, the working concentration of AG490 was much higher than Dehydrocrenatidine (Fig.2B). In order 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 non-tumorigenic human breast epithelial cell line, in which JAK-STAT signaling are 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), usually inhibits many cell processes with no cell type specificity, was used as a control. Staurosporine had no selectivity on cell growth inhibition among these cell lines (Fig. 2C).

In order 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.2D). To exclude nonspecific inhibition, phosphorylation levels of p-AKT and p-P65, key molecules of two signaling pathways which are also highly involved in tumorigenesis, were assessed. In contrast, Western blot showed that p-AKT and p-P65 phorphorylation were not changed in the presence of Dehydrocrenatidine (Fig.2D). 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

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both DU145 and MDA-MB-468 cell lines, STAT3 tyrosine 705 phosphorylation was attenuated in a dose-dependent manner (Fig.3A), with IC_{50} of 11.6 μM and 5.8 μM respectively, which confirmed its specific inhibition role on JAK-STAT3 signaling. Basal levels of JAK2 phosphorylation were inhibited in these two cell lines as well. Then we tested the kinetic 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 were undetectable under our experiment conditions (data not shown). We have demonstrated that in HEL, a human erythroleukemic cell line which contains constitutively activating mutation JAK2V617F (Levine et al., 2005), JAK2, STAT3 and STAT5 phosphorylation were inhibited in a dose and time dependent manner (Supplemental Figure 7D and 7E).

Dehydrocrenatidine inhibits IL-6 and IFNs induced STAT activation

To further investigate the impact of Dehydrocrenatidine on cytokines and IFNs induced STATs activity, Hela, HepG2 and HEK293T cells were treated 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, while IFNα and IFNγ signals through type I and type II interferon receptors (Murakami et al., 1993; Platanias, 2005). Cells were pretreated with Dehydrocrenatidine and then treated by IL-6, STAT3 tyrosine 705 phosphorylation was inhibited in these three cells lines (Fig.4A). Since IL-6 activates STAT3 through JAK1 and JAK2 (Lutticken et al., 1994; Narazaki et al., 1994), we also detected JAK1 and JAK2 activity. With no surprise, we found that both JAK1 and JAK2 activity induced by IL-6 were decreased by Dehydrocrenatidine. 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 Dehydrocrenatidine. Moreover, Dehydrocrenatidine directly suppressed IFNγ activated JAK1 and JAK2 phosphorylation (Fig.4B). Additionally, we found that IFNα stimulated TYK2 and STAT3 phosphorylations were diminished by Dehydrocrenatidine (Fig.4C). Moreover, Dehydrocrenatidine inhibited IFNα induced STAT3 Phosphorylation in a dose and time dependent manner in K562 cells (Supplemental Figure 7A and 7B). IFNα also activates STAT2, and we found that this activation was inhibited by Dehydrocrenatidine. STAT1 tyrosine 701 phosphorylation level was repressed in the presence of

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Dehydrocrenatidine in a dose-dependent manner, with IC_{50} of 8.02 μM (Fig.4D). In addition, we found that Dehydrocrenatidine inhibited IFNα induced STAT1 and STAT2 phosphorylation in a concentration dependent manner in K562 cells (Supplemental Figure 7C). Since JAK-STAT3 signaling controls cell survival and proliferation by regulating their downstream genes, it is important to evaluate the effect of inhibitor on the expression of their target genes. Hela and HepG2 cells were pretreated with Dehydrocrenatidine 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 Dehydrocrenatidine (Fig.4E and 4F). Similarly, IFNα induced STAT1 regulated gene *irf1* mRNA expression was suppressed by Dehydrocrenatidine (Fig.4G).

Dehydrocrenatidine inhibits over-expression of JH1 domain induced JAK-STAT signaling

To further confirm that Dehydrocrenatidine 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 over-expressed cells to Dehydrocrenatidine resulted in decrease in total tyrosine phosphorylation. Over-expression of JH1 domains of JAK1, JAK2 or TYK2 lead to up-regulation of STAT1 and STAT3 activation, and this induction was decreased by Dehydrocrenatidine (Fig.5A). Similar results were obtained from JAK3-JH1 over-expression cells (Data not shown).These results suggested that Dehydrocrenatidine could inhibit tyrosine kinase activity of all JAK family members.

It has been reported that STAT3 can be phosphorylated by activated growth factor receptors such as EGFR, platelet-derived growth factor, and transforming growth factor and c-MET, as well as by non-receptor kinase such as Src family kinase (Aggarwal et al., 2009; Boccaccio et al., 1998; Quesnelle et al., 2007; Schindler and Darnell, 1995). We next examined influence of Dehydrocrenatidine on other tyrosine kinase. Two other tyrosine kinases which can activate STAT3, EGFR and Src were tested. STAT3 tyrosine 705 phosphorylation can be stimulated by EGF through EGFR, when we treated Hela cells with EGF, EGFR

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phosphorylation and STAT3 phosphorylation were induced. We treated Hela cell with EGF following Dehydrocrenatidine or Lapatinib, respectively. Dehydrocrenatidine had no significant effect on EGFR or STAT3 phosphorylation while Lapatinib inhibited both EGFR and STAT3 activity as previously reported (Fig.5B). In contrast to JAKs-JH1 over-expression cells, in Src over-expression 293T cells, there was no significant change of Src Y416 phosphorylation and STAT3 Y705 phosphorylation after Dehydrocrenatidine treatment (Fig.5C). Dasatinib, a BCR-ABL and Src dual inhibitor inhibited both Src and STAT3 activity. These results verified the specificity of Dehydrocrenatidine on JAK family kinase.

Dehydrocrenatidine induces apoptosis in DU145 and MDA-MB-468 cells

In order to find out whether Dehydrocrenatidine affect cell viability by inducing apoptosis, Hoechst staining was performed. We observed highly condensed chromatin stained by Hoechst in Dehydrocrenatidine treated DU145 and MDA-MB-468 cells compared to DMSO controls (Fig.6A and Fig.6B). To confirm that Dehydrocrenatidine could induce apoptosis, DU145 and MDA-MB-468 cells were incubated with 10 μM Dehydrocrenatidine for 24 hours, and double stained with annexin-V and propidium iodide, then analyzed by flow cytometry. Among DMSO treated cells, the percentage of Annexin V-positive cells was 3.74% and 6.62%, in DU145 and MDA-MB-468 respectively. Treatment with 10 μM Dehydrocrenatidine resulted in an increase to 20.46% in DU145 cells, and 31% in MDA-MB-468 cells (Fig.6C). Western blot further validated that Dehydrocrenatidine induced cell apoptosis. The presence of PARP cleavage is a marker of apoptosis. Upon treatment with Dehydrocrenatidine for 24 hours and 48 hours, cleaved PARP was evidently increased in both DU145 and MDA-MB-468 cells, while total PARP level was decreased. The executer of apoptosis, cleaved caspase3 level also elevated after Dehydrocrenatidine treatment. Moreover, Dehydrocrenatidine treatment diminished STAT3 downstream gene c-Myc, Cyclin D1, Survivin and Bcl-xL expression (Fig.6D).

Dehydrocrenatidine inhibits JAK2-JH1 kinase activity in vitro

Kinase assay was performed to confirm that Dehydrocrenatidine directly inhibited JAK2-JH1 kinase activity. In consistent with our virtual screening results, Dehydrocrenatidine inhibited JAK2-JH1 kinase catalyzed STAT3 phosphorylation in vitro, which suggested that

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Dehydrocrenatidine directly inhibited JAK2 kinase activity (Fig.7A and Fig.7B).

Discussion

JAKs kinase play indispensable role 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). Due to its importance in solid tumor and hematopoietic disorders, JAKs inhibitors have become popular targets for drug development. Although there are already many JAK inhibitors available, certain defects are observed, most commonly are inhibition on other tyrosine kinase and acquired drug resistance. Physiological environment is much more complicated than *in vitro* experiment conditions; there might be unpredicted toxicity effects of a very potent inhibitor. And it has been reported that using JAK inhibitors with different mechanism could re-sensitize JAK inhibitor resistant cells in myeloproliferative neoplasm (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, which could block JAKs kinase activity through inhibiting JH1 domain. Kinase assay revealed that Dehydrocrenatidine inhibits JAK2-JH1 kinase activity *in vitro*. Besides, molecular docking results showed that Dehydrocrenatidine can bind to residues E957, F958, L959 of JAK1, residue Y931, L932 and P933of JAK2, residues E903, L905, D967 of JAK3 and E905, E979, Y980 of TYK2, respectively (Supplemental Figure 6). However, IC_{50} of *in vitro* kinase assay is higher than cell based assay. There might be two reasons: first, the kinase catalytic reaction was incubated for 2 hours, because STAT3 phosphorylation was undetectable with shorter time incubation. While, catalytic reaction with ^{32}p -labelled ATP usually requires an incubation time for only 5-15 minutes. Once incubation time was long enough when STAT3 phosphorylation could be detected, the “leaky activity” made it difficult to observe the inhibition at low concentrations. Second, in *in vivo* conditions, kinase and phosphotase are dynamically balanced, while in *in vitro* assay, the kinase catalytic reaction keeps going on before 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

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also suppressed IL-6, IFNα and IFNγ stimulated JAK activity and STAT3 phosphorylation. Nevertheless, it showed little effect on EGF 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 STA5 activity. JAK-STAT signaling controls cell proliferation through 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. A longer time treatment also diminished STAT3 downstream gene c-Myc, Cyclin D1, Survivin and Bcl-xL expression. We also detected the impact of Dehydrocrenatidine on cell survival. MTT assay showed that Dehydrocrenatidine inhibited STAT3 constitutively activated cancer cell lines DU145 and MDA-MB-468 viability in a dose-dependent manner, but showed little effect on the survival of human fibroblast cell line hTERT-BJ cells and human breast epithelial cell line MCF 10A. Hoechst staining and Flow Cytometric Analysis of apoptosis results indicated that Dehydrocrenatidine induced apoptosis. Western blot demonstrated that after treatment with Dehydrocrenatidine for 24 hours and 48 hours, the amount of cleaved PARP increased and the level of total PARP decreased. The executor of apoptosis—cleaved caspase3 level 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 to be further validated. 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 gives new insights for therapy of hyperactivated JAK2 related malignances.

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Authorship Contributions

Participated in research design: J.Zhang, Zhu, Wang and Yang.
Conducted experiments: J.Zhang, Zhu, Bai and Chen.
Contributed new reagents or analytic tools: Bai,Du,Nan,Qin,X.X. Zhang and Hou.
Performed data analysis: J.Zhang, Zhu, Bai, Du, Nan and Qin.
Wrote or contributed to the writing of the manuscript: J.Zhang, Zhu, Bai and Yang.

;

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Footnotes

Jing Zhang and Ning Zhu contributed equally to this work.

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Figure Legend

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 based on the analysis of molecular shape and electrostatic potential.

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 as indicated concentrations for 48 hours, 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.

Fig.3. Dehydrocrenatidine inhibited STAT3 phosphorylation in DU145 and MDA-MB-468 cells in a dose and time dependent manner. (A) DU145 and MDA-MB-468 cells were treated with indicated concentrations of Dehydrocrenatidine for 2 hours, and cell lysates were blotted by indicated antibodies. DMSO was used as control. p-STAT3/STAT3 relative density was measured by Image J and IC_{50} was calculated by SPSS. (B) DU145 and MDA-MB-468 cells were treated with 10 μM Dehydrocrenatidine for indicated time points, and cell extracts were analyzed by Western blot. DMSO was used as control.

Fig.4. Dehydrocrenatidine inhibited IL-6 and IFNs induced STAT3 phosphorylation and their downstream gene expression. (A) Hela, HepG2 and HEK 293T cells were cultured in DMEM with 0.2% FBS for 12 hours, serum starved cells were pretreated with DMSO or Dehydrocrenatidine (10 μM) for 1 hour and stimulated by IL-6 (250 ng/ml) for 2 hours, 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 U/ml) or

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(C) IFNα (5000 U/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 IFNα (5000 U/ml) for 2 hours, cells lysates were blotted by indicated antibodies. p-STAT1/STAT1 relative density was measured by Image J and IC_{50} was calculated by SPSS. (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, *socs3* mRNA levels were analyzed by RT-qPCR. (G) Serum starved Hela cells were pretreated with DMSO or Dehydrocrenatidine (10 μM) for 1 hour and stimulated by IFNα (5000 U/ml) for 4 hours, *irf1* mRNA levels were analyzed by RT-qPCR. Data are mean ± SD of three independent experiments. ***P< 0.001, one-way analysis of variance (ANOVA).

Fig.5. Dehydrocrenatidine inhibited over-expression of JH1 domain of JAK1, JAK2, TYK2 induced protein phosphorylation.(A) HEK293T cells transfected with JH1 domain of JAK1, JAK2, 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 (50ng/ml) for 30 minutes, cell lysates were Western blotted. (C) HEK293T cells over-expressing c-Src were incubated with Dehydrocrenatidine (15 μM) or Dasatinib (500 nM) for 4 hours. Cell lysates were analyzed by Western blot.

Fig.6. Dehydrocrenatidine induced apoptosis.(A) DU145 and (B) MDA-MB-468 cells were plated on coverslips, after 12 hours, fresh media were replaced, DMSO (a,c) or Dehydrocrenatidine (10 μM) (b,d) were added, after 16 hours treatment, cells were stained with hochest and visualized under microscope (a,b 40×magnification) (c,d 20×magnification) (C) DU145 and MDA-MB-468 cells were treated with DMSO or Dehydrocrenatidine (10 μM) for 24 hours, double stained by annexin V and propidium iodide and flow cytometry were performed. (D) DU145 and MDA-MB-468 cells were treated with Dehydrocrenatidine (10 μM) for 24 or 48 hours. Cell lysates were analyzed by Western blot. DMSO was used as control.

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Fig.7. Dehydrocrenatidine inhibited JAK2-JH1 induced STAT3 phosphorylation. (A) Recombined hSTAT3 protein and purified JAK2-JH1 kinase domain protein were used for kinase assay. Dehydrocrenatidine were applied at the concentration series at 20, 40, 80, 100 or 200 μM.p-STAT3 levels were analyzed by Western blot. (B) Three independent kinase assays were performed, p-STAT3/STAT3 relative density were quantified by image J. Data indicates mean ± SD of three independent experiments.

Figure 1

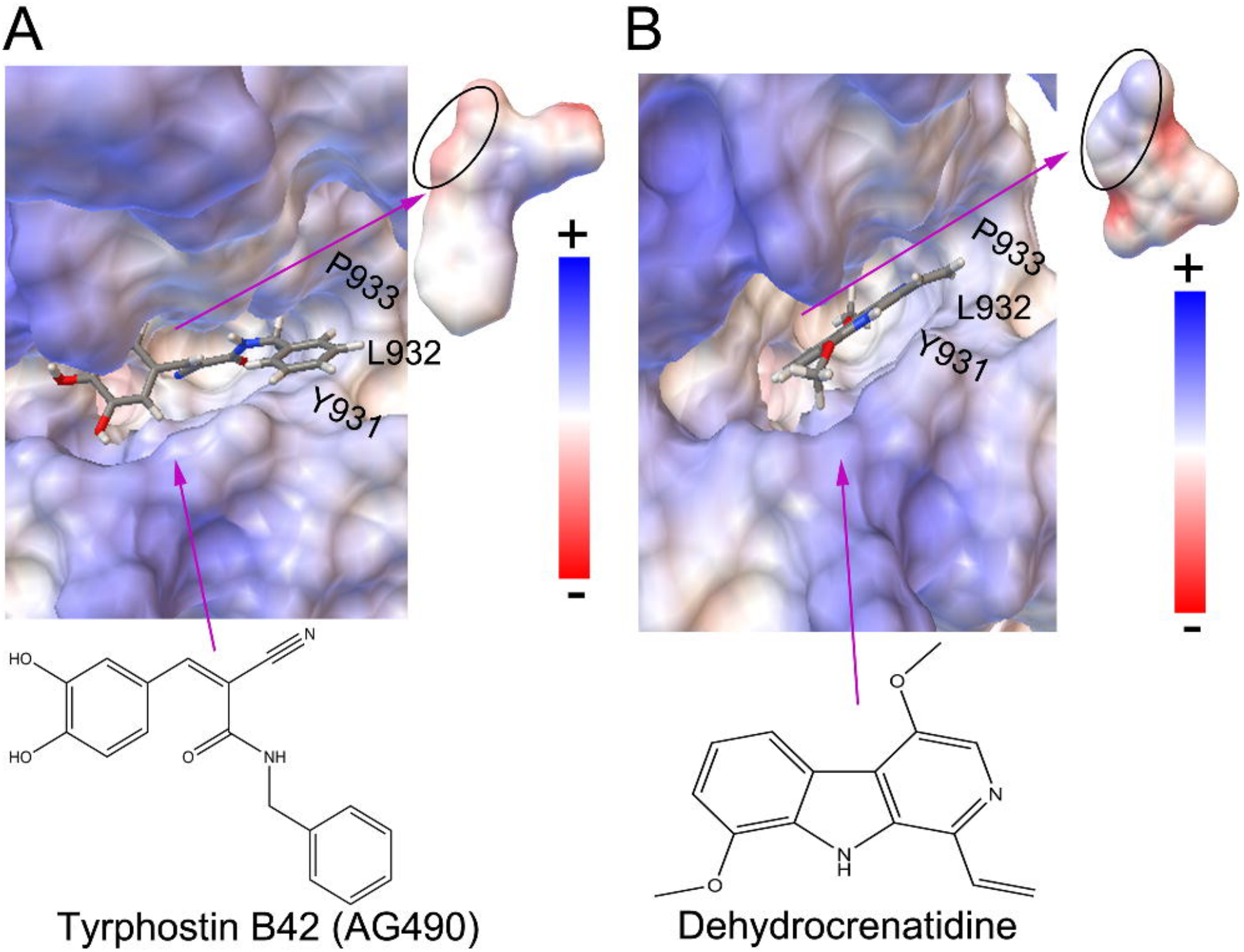

A
P933
L932
Y931
+
-
B
P933
L932
Y931
+
-
HO
HO
O
NH
N
O
N
NH
O
Tyrphostin B42 (AG490)
Dehydrocrenatidine

Figure 2

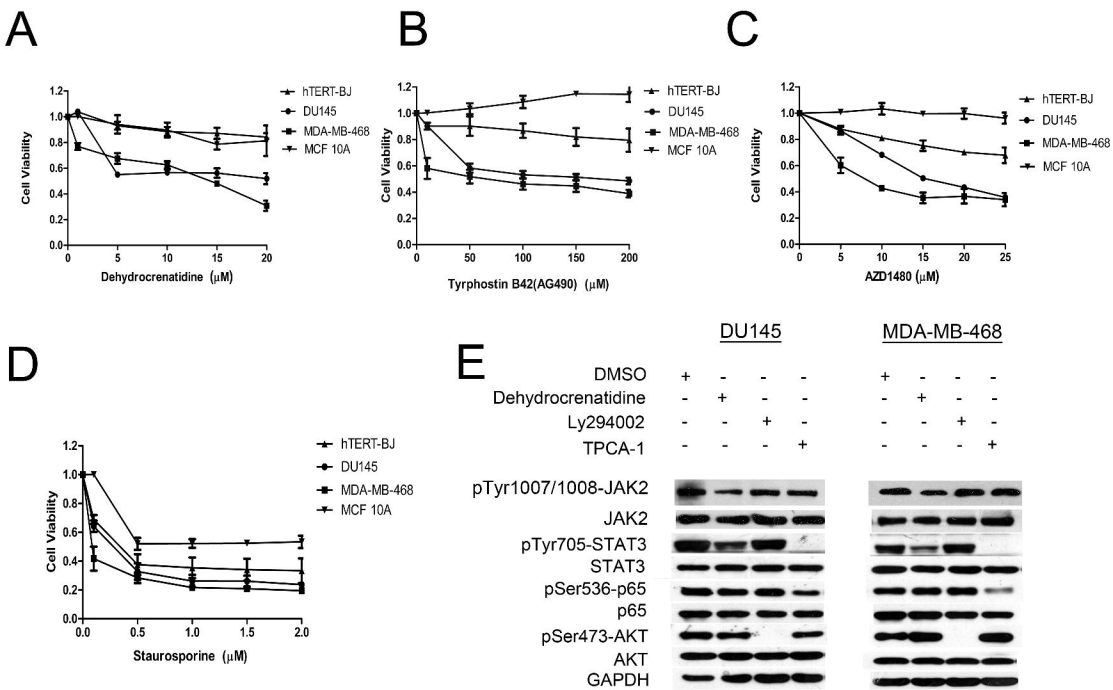
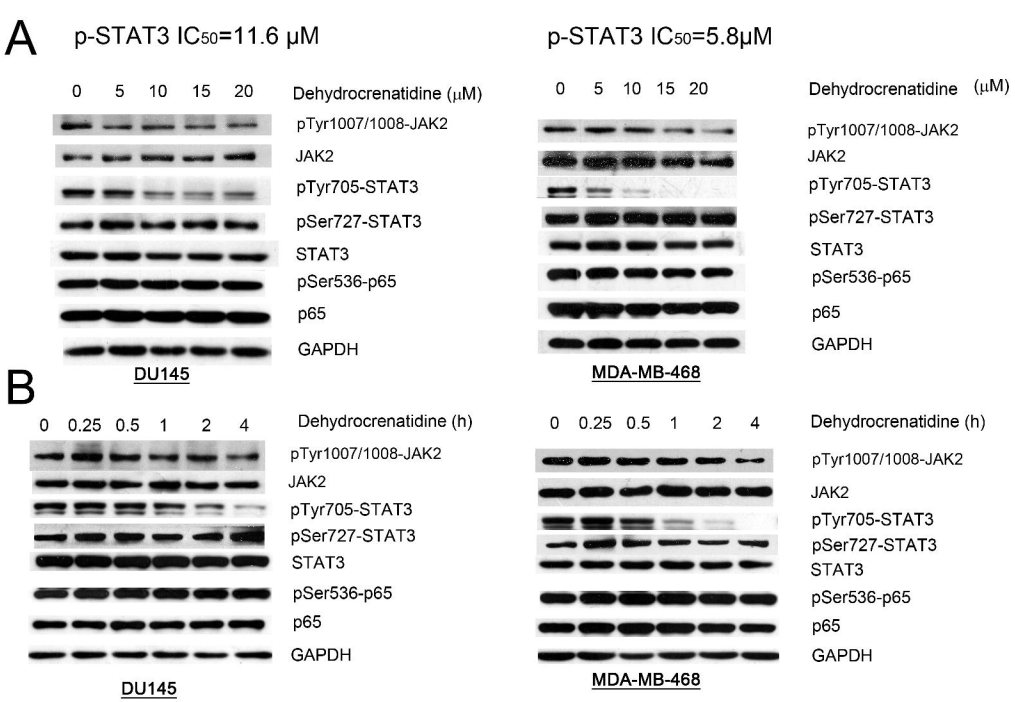

A
B
C
D
E
hTERT-BJ
DU145
MDA-MB-468
MCF 10A
Cell Viability
Dehydrocrenatidine (μM)
Tyrphostin B42(AG490) (μM)
AZD1480 (μM)
Staurosporine (μM)
DU145
MDA-MB-468
DMSO
Dehydrocrenatidine
Ly294002
TPCA-1
pTyr1007/1008-JAK2
JAK2
pTyr705-STAT3
STAT3
pSer536-p65
p65
pSer473-AKT
AKT
GAPDH

Figure 3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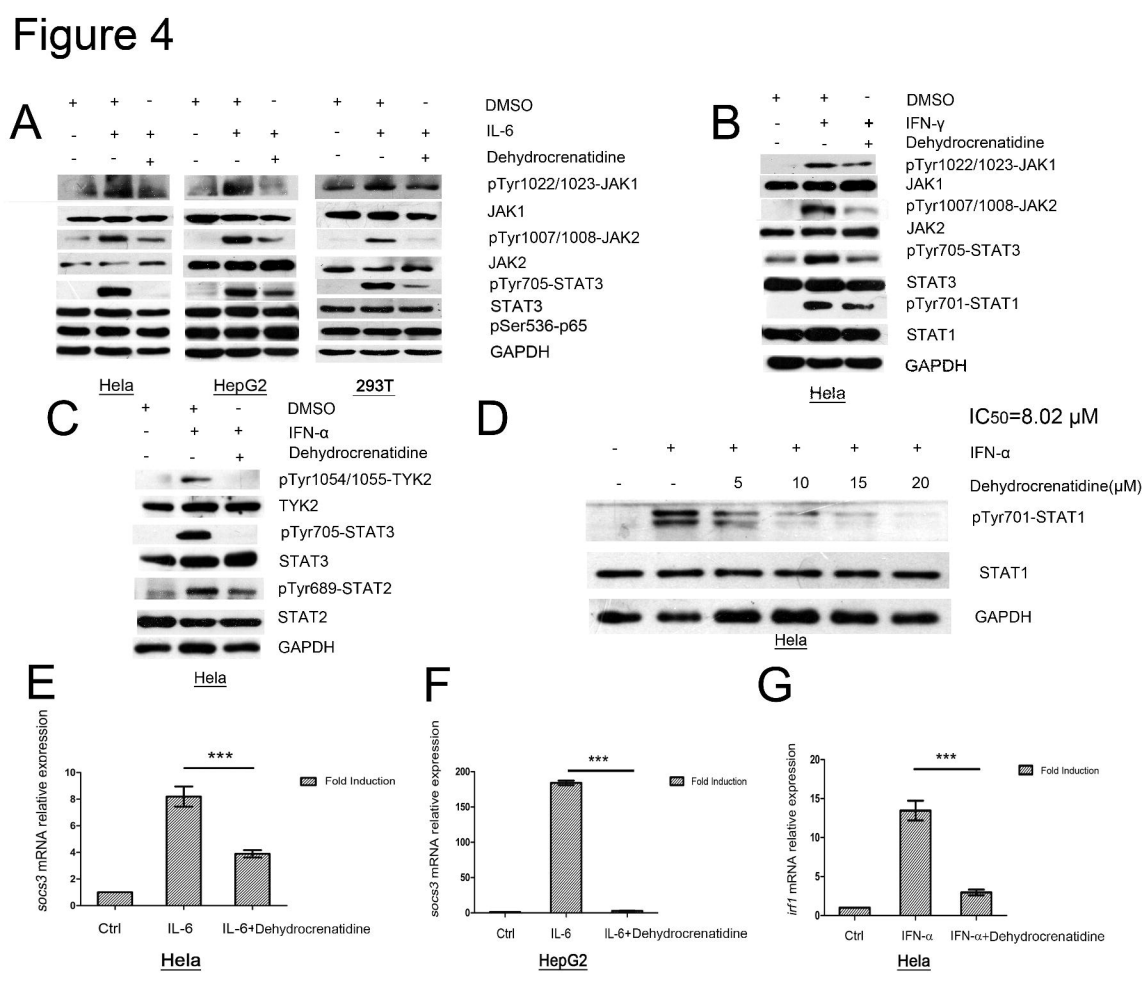
0 5 10 15 20
Dehydrocrenatidine (μM)
pTyr1007/1008-JAK2
JAK2
pTyr705-STAT3
pSer727-STAT3
STAT3
pSer536-p65
p65
GAPDH
DU145
p-STAT3 IC50=5.8μM
0 5 10 15 20
Dehydrocrenatidine (μM)
pTyr1007/1008-JAK2
JAK2
pTyr705-STAT3
pSer727-STAT3
STAT3
pSer536-p65
p65
GAPDH
MDA-MB-468
B
0 0.25 0.5 1 2 4
Dehydrocrenatidine (h)
pTyr1007/1008-JAK2
JAK2
pTyr705-STAT3
pSer727-STAT3
STAT3
pSer536-p65
p65
GAPDH
DU145
0 0.25 0.5 1 2 4
Dehydrocrenatidine (h)
pTyr1007/1008-JAK2
JAK2
pTyr705-STAT3
pSer727-STAT3
STAT3
pSer536-p65
p65
GAPDH
MDA-MB-468

Figure 4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DMSO
IL-6
Dehydrocrenatidine
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JAK1
pTyr1007/1008-JAK2
JAK2
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STAT3
pSer536-p65
GAPDH
Hela
HepG2
293T
B
DMSO
IFN-γ
Dehydrocrenatidine
pTyr1022/1023-JAK1
JAK1
pTyr1007/1008-JAK2
JAK2
pTyr705-STAT3
STAT3
pTyr701-STAT1
STAT1
GAPDH
Hela
C
DMSO
IFN-α
Dehydrocrenatidine
pTyr1054/1055-TYK2
TYK2
pTyr705-STAT3
STAT3
pTyr689-STAT2
STAT2
GAPDH
Hela
D
IC50=8.02 μM
IFN-α
Dehydrocrenatidine(μM)
0
5
10
15
20
pTyr701-STAT1
STAT1
GAPDH
Hela
E
socs3 mRNA relative expression
***
Fold Induction
Ctrl
IL-6
IL-6+Dehydrocrenatidine
Hela
F
socs3 mRNA relative expression
***
Fold Induction
Ctrl
IL-6
IL-6+Dehydrocrenatidine
HepG2
G
irf1 mRNA relative expression
***
Fold Induction
Ctrl
IFN-α
IFN-α+Dehydrocrenatidine
Hela

Figure 5

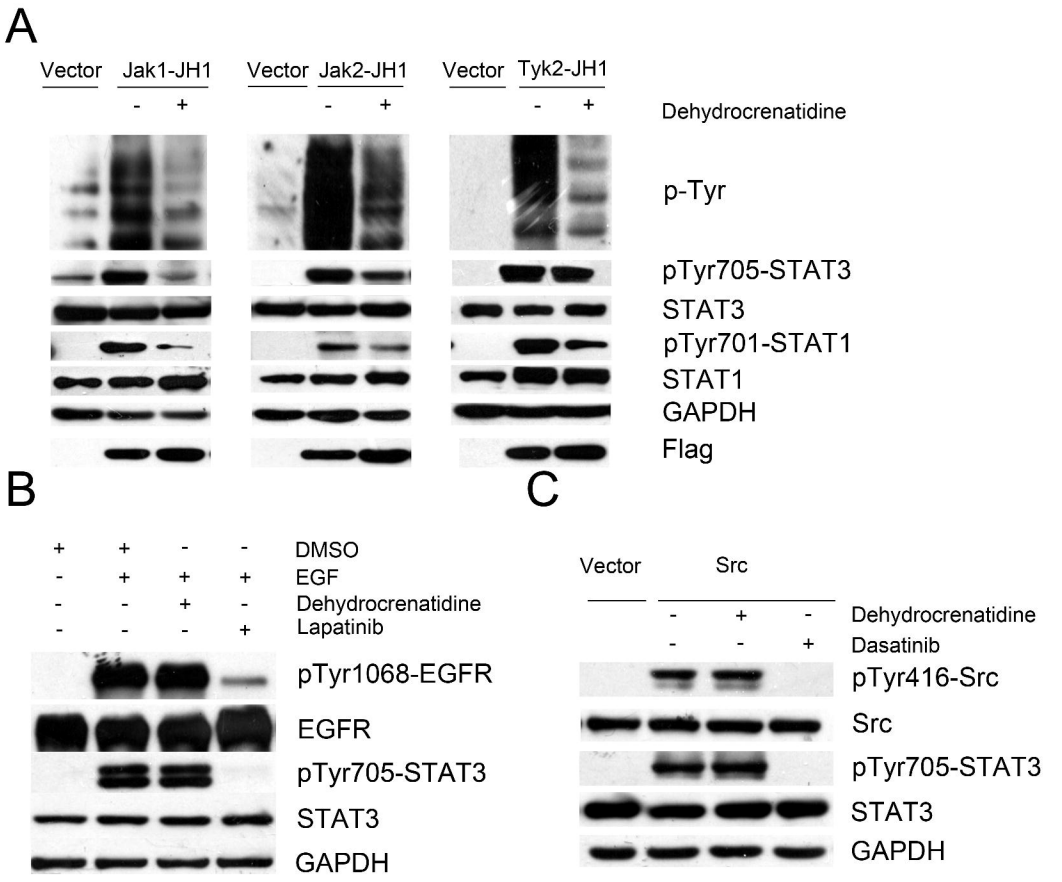

A
Vector
Jak1-JH1
Vector
Jak2-JH1
Vector
Tyk2-JH1
- + - + - +
Dehydrocrenatidine
p-Tyr
pTyr705-STAT3
STAT3
pTyr701-STAT1
STAT1
GAPDH
Flag
B
+ + - - DMSO
- + + + EGF
- - + - Dehydrocrenatidine
- - - + Lapatinib
pTyr1068-EGFR
EGFR
pTyr705-STAT3
STAT3
GAPDH
C
Vector
Src
- + - Dehydrocrenatidine
- - + Dasatinib
pTyr416-Src
Src
pTyr705-STAT3
STAT3
GAPDH

Figure 6

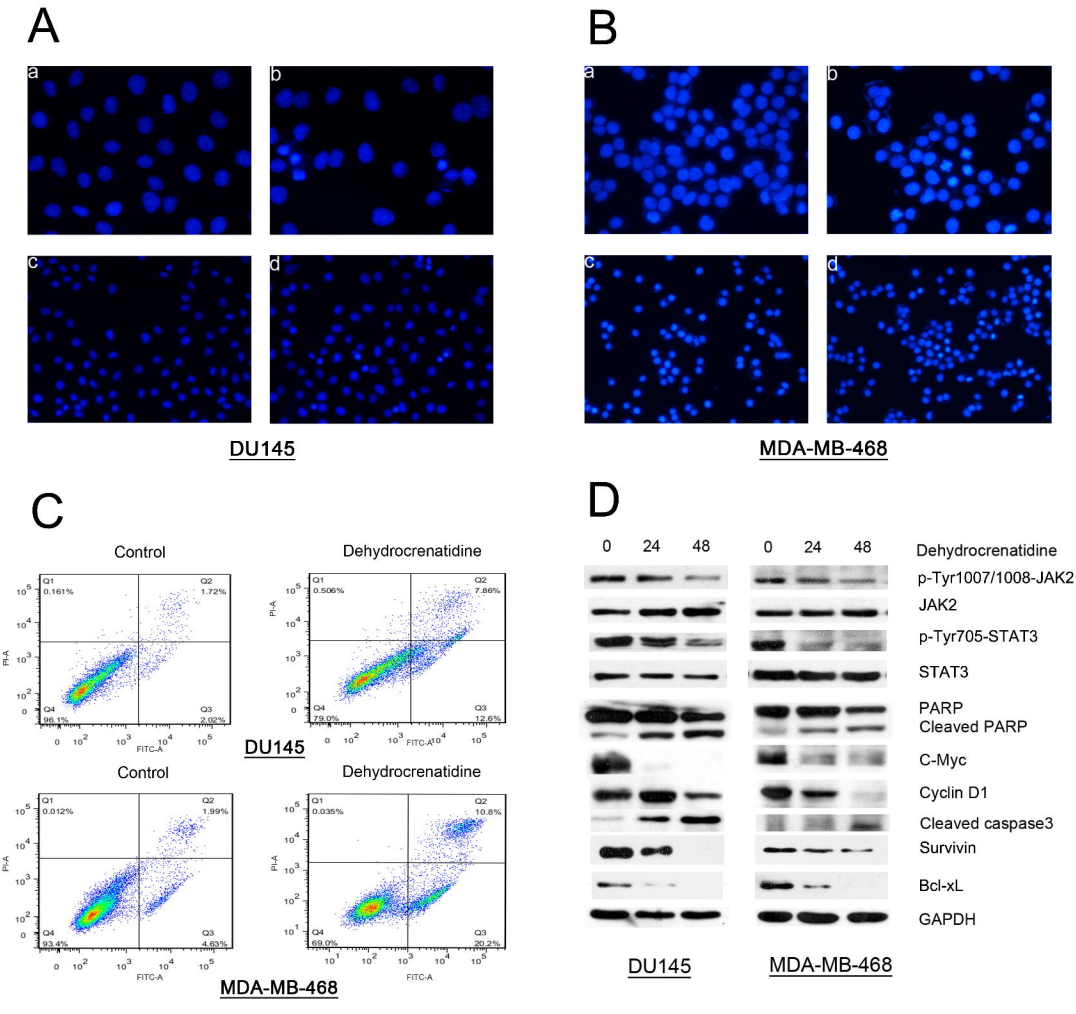
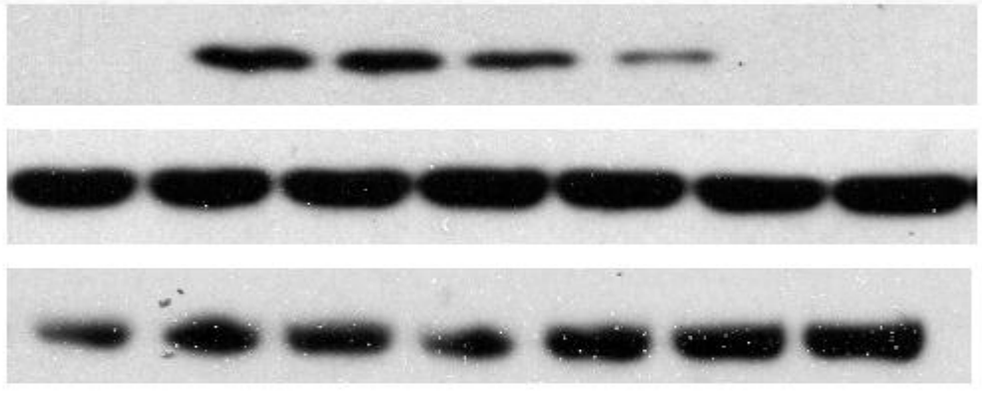

A
a
b
c
d
DU145
B
a
b
c
d
MDA-MB-468
C
Control
Dehydrocrenatidine
Q1 0.161%
Q2 1.72%
Q4 96.1%
Q3 2.02%
PI-A
FITC-A
DU145
Control
Dehydrocrenatidine
Q1 0.012%
Q2 1.99%
Q4 93.4%
Q3 4.63%
MDA-MB-468
D
0
24
48
Dehydrocrenatidine
p-Tyr1007/1008-JAK2
JAK2
p-Tyr705-STAT3
STAT3
PARP
Cleaved PARP
C-Myc
Cyclin D1
Cleaved caspase3
Survivin
Bcl-xL
GAPDH
DU145
MDA-MB-468

Figure 7

A

	DMSO	DMSO	Dehydrocrenatidine (μM)				
			20	40	80	100	200
hSTAT3	+	+	+	+	+	+	+
JAK2-JH1	+	+	+	+	+	+	+
ATP 200 μM	-	+	+	+	+	+	+



B