17-Hydroxy-jolkinolide B Inhibits Signal Transducers and Activators of Transcription 3 Signaling by Covalently Cross-Linking Janus Kinases and Induces Apoptosis of Human Cancer Cells

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Abstract

Constitutive activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway occurs frequently in cancer cells and contributes to oncogenesis. Among the members of STAT family, STAT3 plays a pivotal role in the development and progression of human tumors. The STAT3-mediated signaling pathway has been recognized as a promising anticancer target. Here, we show that 17-Hydroxy-jolkinolide B (HJB), a diterpenoid from the Chinese medicinal herb Euphorbia fischeriana Steud, strongly inhibits interleukin (IL)-6–induced as well as constitutive STAT3 activation. Furthermore, we show that HJB directly targets the JAK family kinases, JAK1, JAK2, and TYK2, by inducing dimerization of the JAKs via cross-linking. Addition of DTT or glutathione prevents the JAK cross-linking and blocks the inhibitory effects of HJB on IL-6–induced STAT3 activation, suggesting that HJB may react with cystein residues of JAKs to form covalent bonds that inactivate JAKs. Liquid chromatography/mass spectrometry analysis confirmed that each HJB reacted with two thiols. In vitro analysis confirmed that each HJB reacted with two thiols. The effect of HJB on the JAK/STAT3 pathway is specific as HJB has no effect on platelet-derived growth factor, epidermal growth factor, or insulin-like growth factor I signaling pathways. Finally, we show that HJB inhibits growth and induces apoptosis of tumor cells, particularly those tumor cells with constitutively activated STAT3. We propose that the natural compound HJB is a promising anticancer drug candidate as a potent STAT3 signaling inhibitor.

Introduction

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is responsible for signal transduction of a large number of cytokines. STATs are a family of seven proteins, named STAT1, 2, 3, 4, 5a, 5b, and 6, and JAKs represent a family of four nonreceptor tyrosine kinases, JAK1, JAK2, TYK2, and JAK3 (1–3). All JAKs (except JAK3, which is restricted to hematopoietic cells) are expressed universally. After the cytokines bind to their cognate receptors, the JAKs are activated and proceed to phosphorylate the STATs. The phosphorylated STATs then form homodimers or heterodimers via reciprocal phosphotyrosine-SH2 domain interaction and translocate into the nucleus to regulate expression of target genes (4–6). Different cytokines activate different STATs (7).

Constitutive activation of the JAK/STAT pathway, particularly those in which STAT3 is involved, is frequently associated with cancer (8–10). Persistent STAT3 phosphorylation is observed in a number of human cancers including blood malignancies (leukemia, lymphomas, and multiple myeloma) and solid tumors (melanoma, head and neck, breast, lung, and prostate cancer; refs. 8, 11–18). It has been suggested that STAT3 acts as an oncogene (4, 19). All JAK family members are in vitro kinases for STAT3 and the JAKs are also frequently found to be activated in cancers and other proliferative diseases. The TEL-JAK2 fusion protein found in acute lymphoblastic leukemias with the t(9;12) translocation causes constitutive activation of JAK2 (20). Additionally, an activating mutation in JAK2 (V617F) is found in majority of patients with polycythemia vera and with other myeloproliferative disorders such as essential thrombocythemia and idiopathic myelofibrosis (21–23). Recently, it has been reported that overexpression of the JAKs promoted in vitro cell transformation (6). Its potential role in these disorders makes the JAK/STAT3 pathway an attractive target in therapeutic settings, where inappropriate or excessive cytokine signaling occurs (24, 25). Importantly, small-molecule inhibitors of the JAK/STAT3 pathway have been reported to be effective anticancer agents in vitro and in animal models (11, 26–35).

The dried plant roots of Euphorbia fischeriana Steud (Euphorbiaceae), known as "lang-du" in traditional Chinese medicine, have long been used for the treatment of cancer, edema, and ascites (36–39). Diterpenoids are major chemical constituents of the roots constituting ~0.1% of the plant’s total mass. Although it has been used in herbal medicine for thousands of years, the molecular target and mode of action of this traditional Chinese medicine remain unclear.

We identified 17-Hydroxy-jolkinolide B (HJB), an ent-ebatiane diterpene diepoxyide from Euphorbia fischeriana Steud, as a potent inhibitor of JAK/STAT3 signaling. It inactivated the JAK family kinases: JAK1, JAK2, and TYK2 by covalent cross-linking of the JAKs. The effects of HJB on the JAK/STAT3 signaling are specific. It did not affect many other growth factors signaling. Furthermore, HJB inhibited growth and induced apoptosis of tumor cells, particularly those with constitutively activated STAT3. Above all, our data explains how Euphorbia fischeriana Steud may work in cancer treatment and suggest that HJB may be a promising anti-cancer drug candidate.
Materials and Methods

Preparation of HJB. The preparation of HJB has been described previously (39), and the compound was dissolved in 100% DMSO before use.

Cell lines and culture. HepG2/STAT3 cells, gifts of Prof. Xinyuan Fu (National University of Singapore, Singapore), were HepG2 cells stably transfected with a STAT3-responsive firefly luciferase reporter plasmid. All other cell lines were obtained from the American Type Culture Collection.

Plasmids and transfection. JAK2 expression plasmid in cytomegalovirus-based vector was a gift from Prof. David E. Levy (New York University, New York, NY). Flag-JAK2 and HA-JAK2 were generated by PCR followed by cloning into pFlag-CMV4 and pcDNA3-HA, respectively. pMT2T-TYK2 expression plasmid was a gift from Prof. John Krolewski (University of California, Irvine, CA). Human full-length JAK1 cDNA was isolated from human thymus plasmid cDNA library (Clontech) using standard PCR techniques, and subsequently cloned into pFlag-CMV4. The constructs were confirmed by sequencing. pEGFP-N1 was obtained from Clontech.

Western blot analysis. Whole cell lysates were prepared in 1× Laemmli sample buffer (Sigma) to extract total proteins. Equivalent amounts of total cellular protein were electrophoresed on an 8% SDS-PAGE gel and transferred onto nitrocellulose membranes (Millipore). Membranes were blocked in 5% nonfat milk in TBS containing 0.1% Tween 20 (TBST) for 1 h at room temperature, and then incubated with primary antibodies in 5% bovine serum albumin (BSA) in TBST at 4°C overnight. Membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody in 5% BSA in TBST for 1 h at room temperature. Immune complexes were detected by enhanced chemiluminescence (Pierce).

Antibodies used in Western blotting were mouse anti-STAT3, mouse anti–pY-STAT3, rabbit anti-JAK1, rabbit anti-TYK2, rabbit anti–GFP, rabbit anti–poly ADP ribose polymerase, rabbit anti–epidermal growth factor (EGF) receptor (EGFR), rabbit anti–platelet-derived growth factor (PDGF) receptor (PDGFR), rabbit anti–phospho-insulin-like growth factor (IGF-I) receptor (IGF-IR), and rabbit anti–IGF-IR (Cell Signaling Technology); rabbit anti–phospho-EGFR and rabbit anti–phospho-PDGFR (Biosource); mouse anti–β-Actin (Sigma); and rabbit anti-JAK2 and rabbit anti-green fluorescent protein (GFP; Santa Cruz Biotechnology).

Kinase assay. The JAK kinase assay was performed using HTScan JAK kinase assay kit (Cell Signaling Technology) with JAK immunoprecipitates. Briefly, HEK 293 cells were transfected with JAK1, JAK2, or TYK2 expression plasmid for 24 h, then lysed in 50 mmol/L HEPES (pH 7.4), 0.1% Triton X-100, 150 mmol/L NaCl, 0.5 mmol/L DTT, 2 mmol/L sodium orthovanadate, 2 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (1:1000; Sigma) on ice. Sample lysates were collected and cleared, and then 500 µL of lysates were immunoprecipitated with 400 ng antibodies specific for JAK1, JAK2, or TYK2 for 1 h at 4°C and rocked with 25 µL of protein A/G PLUS-agarose (Santa Cruz Biotechnology) for overnight at 4°C. Samples were then washed thrice with lysis buffer and once with kinase reaction buffer [60 mmol/L HEPES (pH 7.5), 5 mmol/L MgCl₂, 5 mmol/L MnCl₂, 3 µmol/L Na₃VO₄, 0.5 mmol/L DTT]. Then kinase reaction was performed with the JAK immunoprecipitates and the HTScan JAK kinase reagent.
assay kit at 30°C for 30 min in a final volume of 50 μL of reaction buffer containing 20 μmol/L ATP and 1.5 μmol/L peptide substrate. Samples were pretreated with DMSO (vehicle control) or different concentration of HJB before addition of ATP. Kinase activity was measured according to manufacturer's instruction.

**MTT assay.** About 5,000 cells per well were seeded into 96-well plates. Twenty-four hours later, cells were treated with vehicle control (DMSO) or increasing concentrations of HJB for 72 h. MTT assay was performed as described (40).

**Luciferase assay.** HepG2/STAT3 cells (1.5 × 10^5 per well) were seeded into 24-well cell culture microplates (Corning) and allowed to grow for 24 h and then treated with test samples for 2 h followed by stimulation with 10 ng/mL interleukin (IL)-6 (BD Biosciences) for 5 h. Luciferase activity was determined using the Promega luciferase kits according to the manufacturer's instruction. The cell number was counted at seeding and was controlled by equal seeding. All luciferase assay experiments were repeated at least thrice to minimize the difference caused by cell number.

**Conjugation of HJB with glutathione.** HJB (20 nmol) in DMSO was incubated with 4 eq of reduced glutathione in 20 mmol/L Tris-HCl (pH 7.5) for 1 h at 37°C. Then the sample was injected in the liquid chromatography-mass spectrometry (LC-MS) system using methanol/water (1:1) as the mobile phase at a rate of 0.2 mL/min, ESI-positive mode. High performance liquid chromatography analysis was performed using an Agilent HP 1100 series HPLC system (Agilent Technologies). Mass spectrometry was recorded using a LCQ Deca ion trap mass spectrometer (ThermoFinnigan).

**Silver staining.** BSA (2 μmol/L) in cell lysis buffer were treated with vehicle (DMSO), 10 or 100 μmol/L of HJB for 2 h at 37°C, and then boiled in 2× Laemmli sample buffer and run on 8% SDS-PAGE. Silver staining (GE Healthcare) was performed according to the manufacturer's instructions.

Figure 2. Effects of HJB on JAK family proteins. A, HepG2 cells were pretreated with HJB for 2 h before stimulation by IL-6 (50 ng/mL) for 10 min. Whole cell lysates were processed for Western blot analysis using antibodies as indicated. β-Actin served as a loading control. Protein levels were quantitated densitometrically and graphed at the right side of the corresponding blots. B, HEK293 cells were transfected with the JAK2, JAK1, or TYK2 expression plasmid for 24 h, then treated with HJB at indicated concentrations for 2 h. Cells were then harvested for Western blot analysis using antibodies specific for JAK2, JAK1, or TYK2. **, the high molecular mass form of JAK2, JAK1, or TYK2. C, the Flag-tagged JAK2 and the HA-tagged JAK2 plasmids were cotransfected into HEK293 cells for 24 h. Coimmunoprecipitation assay was then performed after cell treatment with DMSO (lane 1) or 20 μmol/L HJB for 2 h (lane 2). Cell lysates were immunoprecipitated (IP) with anti-Flag antibody and then immunoblotted (IB) with anti-Flag, anti-HA, or anti-GP130 antibodies. D, in vitro kinase assay of JAK1, JAK2, and TYK2. The JAK proteins immunoprecipitated from the HEK 293 cells overexpressing JAK1, JAK2, or TYK2 were subjected to in vitro kinase assay in the presence of various concentrations of HJB.
Results

HJB inhibited IL-6–induced and constitutive STAT3 activation. In an attempt to discover novel anticancer drugs from natural products targeting the JAK/STAT3 signaling pathway, traditional Chinese medicinal herb extracts, which have been historically used to treat cancer and inflammation, were screened by using a IL-6–induced STAT3-responsive luciferase reporter assay. HJB, a diterpene diepoxide from the Euphorbiaceous extracts, was found to strongly inhibit the IL-6–induced STAT3-responsive luciferase activity in a dose-dependent manner (Fig. 1A). The half-maximal inhibition dose was ~3.34 μmol/L. To map the action site of HJB along the JAK/STAT3 signaling pathway, the effect of HJB on IL-6–induced STAT3 tyrosine phosphorylation, which is a prerequisite for STAT3 biological activity, was examined. HJB inhibited the IL-6–induced STAT3 tyrosine phosphorylation in a dose-dependent fashion (Fig. 1B) and, at 10 μmol/L, completely inhibited the IL-6–induced STAT3 tyrosine phosphorylation (Fig. 1B). The inhibition was rapid; treatment of HepG2 cells with 5 μmol/L HJB for as few as 15 min was effective (Fig. 1C). HJB showed similar inhibitory effects on constitutive STAT3 tyrosine phosphorylation in HepG2, MDA-MB-231, and MDA-MB-468 cells (Fig. 1D). The latter two breast tumor cell lines have been reported to express persistently activated STAT3, and the continued growth and survival of these cells seem to rely on STAT3 signaling (30, 32). Therefore, HJB inhibited both IL-6–induced and constitutive activation of STAT3.

HJB covalently cross-linked JAKs and inhibited their kinase activities. The ability of HJB to suppress IL-6–induced STAT3 tyrosine phosphorylation suggests that this agent might interfere with the upstream components of STAT3 signaling pathway, prompting closer analysis of the effects of HJB on GP130 and JAK family members JAK2, JAK1, and TYK2. It was found that the protein levels of the JAKs were dramatically reduced in a dose-dependent manner upon HJB treatment, whereas the protein level of the upstream receptor GP130 was not affected (Fig. 2A). To understand the mechanism through which HJB affects the JAK protein levels, JAK2, JAK1, and TYK2 were overexpressed in HEK293 cells. After HJB treatment, the proteins were analyzed. Although a similar reduction of protein level of the 130-kDa JAK2 was observed upon HJB treatment, and immunoblots showed the appearance of a high molecular mass (~260 kDa, twice the molecular mass of JAK2) protein, which was recognized by the anti-JAK2 antibody upon HJB treatment (Fig. 2B). Similar effects were observed on JAK1 and TYK2 (Fig. 2B), suggesting that HJB may covalently cross-link two JAKs into a dimer, and the JAKs may be the direct targets of HJB.

Because the effects of HJB on the JAKs were similar, the major STAT3 kinase JAK2 was chosen to further study how HJB works. To understand the nature of the JAK2 containing high molecular mass complex induced by HJB, tandem mass spectrometry (LC-MS/MS) was used to analyze the purified complex. Only JAK2 protein...
Figure 4. Inhibition of JAK/STAT3 signaling pathway by HJB is thiol dependent. A, lysates of the JAK1, JAK2, or TYK2-transfected HEK 293 cells were treated with DTT, GSH, HJB, or their mixture as indicated (HJB with DTT or HJB with GSH were premixed for 1 h at 37°C) for 2 h at 37°C, then analyzed by Western blot analysis using antibodies specific for JAK1, JAK2, or TYK2. β-Actin served as a loading control. **, the high molecular mass form of JAK1, JAK2, or TYK2.

B, HepG2 cells were pretreated with DTT, GSH, HJB, or their mixture for 2 h and then stimulated with IL-6 for 10 min. Whole cell lysates were processed for Western blot analysis using antibodies as indicated.

C, HepG2/STAT3 cells were pretreated with DTT, GSH, HJB, or their mixture for 2 h and then stimulated with IL-6 for 5 h. Cells were harvested for luciferase assay.

D, HJB was incubated with 4 eq of GSH for 60 min at 37°C, and the mixture was resolved by mass spectrometry. Molecular weights of the molecules are indicated. Arrows, the functional groups of HJB, which may be reactive with thiol nucleophiles.
sequences were identified in the high molecular mass complex (Supplementary Fig. S1), suggesting that HJB elicits JAK2 homotypic dimerization. To independently verify the dimerization of JAK2 by HJB, two tagged JAK2 expression plasmids, HA-JAK2 and Flag-JAK2, were cotransfected into HEK293 cells, and the two tagged JAK2 proteins were analyzed by coimmunoprecipitation assays after treatment with HJB. The results showed that HA-JAK2, as well as GP130, were coimmunoprecipitated with Flag-JAK2 under normal conditions (Fig. 2C). After HJB treatment, the three proteins could still be coimmunoprecipitated; the high molecular mass band immunoprecipitated by the anti-Flag antibody, however, contained only the two tagged JAK proteins (Fig. 2C), suggesting that a JAK2 dimer was formed. No GP130 could be detected in the high molecular mass band (Fig. 2C), ruling out the possibility that the high molecular mass band is a complex of JAK2 and GP130.

To determine whether the HJB-induced covalent cross-linking of JAKs affected their kinase activities, the effects of HJB on JAK1, JAK2, and TYK2 enzymatic activity were analyzed in vitro. The results indicated that the kinase activities of all the JAKs were efficiently inhibited by HJB in vitro (Fig. 2D), leading to the conclusion that the cross-linking of JAKs by HJB inactivated their activities.

The effects of HJB on JAK/STAT3 signaling pathway were specific. To investigate whether the modification of JAKs by HJB is
specific, the effects of HJB on two randomly chosen proteins, BSA and GPP, were analyzed in vitro and compared with that of JAK2. HJB cross-linked JAK2 in vitro in a time-dependent fashion (Fig. 3A), whereas no changes occurred to BSA or GPP after HJB treatment (Fig. 3B), suggesting that the effects of HJB on JAKs was not due to nonspecific cross-linking of proteins.

The effects of HJB on a panel of kinases by in vitro kinase assays were also analyzed. At 50 μmol/L concentration, HJB had very little inhibitory effect on all of the kinases analyzed except one, the IKK-β, confirming the specificity of HJB toward the JAK family proteins (Supplementary Table S1).

To further investigate the specificity of HJB on signaling pathways, the effects of HJB on growth factors including EGF, PDGF, and IGF-I were examined. HJB did not affect any of these growth factor signaling pathways (Fig. 3C), again suggesting that HJB acted selectively on the JAK/STAT3 pathway.

HJB interacted with thiol groups of the JAKs and inhibited STAT3 activation in a thiol-dependent manner. Because HJB contains epoxy groups and α, β-unsaturated carbonyl group, which are reactive with thiols, speculation arose regarding whether the modification of the JAKs by HJB was through reaction with the cysteines in the JAK proteins. If so, other thiol-containing reagents may compete with the cysteines of JAKs for HJB and alleviate the effects of HJB on JAKs. As shown in Fig. 4A, the induction of the high molecular mass form of JAKs by HJB was indeed abrogated in the presence of the thiol-containing reagent DTT or reduced glutathione. Accordingly, the inhibition of IL-6–induced STAT3 tyrosine phosphorylation by HJB was also abolished in the presence of DTT or GSH (Fig. 4B); so was the inhibition of the IL-6–induced STAT3-responsive luciferase reporter activity by HJB (Fig. 4C). These results showed that HJB inhibited the JAK/STAT3 signaling pathway through interacting with the cysteine thiol groups of the JAKs.

To further understand the HJB-thiol interaction, HJB was incubated with GSH, and the products were examined using LC-MS. The analysis detected one major component at m/z 961.2 \[HJB + H + 2GSH \]^+, indicating the addition of two molecules of GSH to one molecule of HJB (Fig. 4D). The proposed reaction sites of HJB are illustrated in Fig. 4D. Taken together, the results provided strong evidence in support of the observation that HJB reacts with thiols and induces dimerization of the JAKs.

HJB inhibited growth and induced apoptosis of tumor cells. STAT3 transmits cell survival signals and protects cells from apoptosis. Constitutive activation of STAT3 has been associated with a number of cancers. Therefore, the effects of HJB on growth and survival of a panel of human tumor cell lines from different tissues were examined. HJB inhibited growth and survival of all the tumor cells tested and the IC50 values for most of the cell lines were under 10 μmol/L (Fig. 5A). Furthermore, it was shown that HJB induced apoptosis in the human cancer cells, as indicated by the induction of PARP cleavage (data not shown; Fig. 5B).

During the examination of the HJB effects on the breast cancer cell lines, it was noticed that HJB induced significantly more death in MDA-MB-231 and MDA-MB-468 cells (which had constitutively activated STAT3) than in the MDA-MB-453 cells, which lacked constitutive STAT3 activity (Fig. 5C and D). Taken together, the data showed the potential of HJB to inhibit growth and induce apoptosis of human cancer cells, particularly those with constitutively activated STAT3.

The effects of HJB on STAT3 activation and cell viability were stronger than that of JAK2-specific inhibitor AG490. Because we observed that HJB affected JAK1, JAK2, and TYK2 proteins, it seems likely that HJB acts as a pan-JAK inhibitor. To evaluate the potential application of HJB as an anticancer agent, HJB was compared with a JAK2-specific inhibitor AG490 (41, 42) for their effects on STAT3 activation and on inducing tumor cell death. We observed that HJB exhibited much more potent inhibition on IL-6–induced STAT3 activation and cell viability than AG490 (Fig. 6). Taken together, these results suggest that HJB, as a pan-JAK inhibitor, may inhibit STAT3 activation and tumor cell growth more strongly than specific JAK inhibitors.

Discussion

Euphorbia fischeriana Steud has long been used as a folk medicine to treat cancer in China. However, little has been discovered about how it works. HJB is one of the major diterpenoids from the plant. Thus, elucidating the function and molecular mechanism of HJB in inhibiting tumor cell growth will help us to understand and to better use this traditional Chinese medicine in cancer therapy.
HJB was identified as a potent inhibitor of the JAK/STAT3 signaling pathway. It inhibited the constitutive as well as the IL-6–induced activation of STAT3, and induced apoptosis in human cancer cells. Furthermore, the direct target of HJB was discovered to be the JAK family kinases—HJB inhibited JAK kinase activities by covalently cross-linking the JAK proteins into high molecular mass, possibly dimeric, forms of the JAKs.

There are several lines of evidence to suggest that HJB induces the dimerization of JAKs. First, HJB contains two epoxy groups and α, β-unsaturated carbonyl group, both of which are thiol reactive. Either of the functional groups in HJB may form adducts with the thiols in the cysteins in the JAK proteins. The LC-MS analysis confirmed that two molecules of GSH were added to one molecule of HJB in vitro (Fig. 4D). Thus, HJB is capable of interacting with two cysteins of its target proteins. Second, the 130-kDa forms of the JAK proteins switched into double-sized 260-kDa forms in a dose-dependent manner upon HJB treatment (Fig. 2B) and the switching was thiol dependent (Fig. 4A), suggesting that covalent bonds involving cysteins were formed between two JAKs by HJB treatment. Third, using LC-MS/MS analysis and coimmunoprecipitation assays with two tagged JAK2, it was directly shown that HJB did induce the formation of a homodimer of JAK2 (Fig. 2C; Supplementary Fig. S1).

The effects of HJB on JAK/STAT3 signaling are specific. HJB is not a general cross-linking reagent because it did not induce cross-linking in randomly picked proteins such as BSA and GFP (Fig. 3B), nor did it cross-link many other cellular proteins analyzed during the study. HJB did not even cross-link JAKs to their upstream receptor GP130 (Fig. 2C), which is physically associated with the JAKs. The inhibition of HJB on JAKs kinase activities is also specific. HJB had very little inhibitory effects on all of the kinases that were analyzed except IKK-β (Supplementary Table S1). It was previously reported that an HJB analogue, 17-Acetyl-jolkinolide B, also inhibited IKK kinase activity by covalent thiol modification (40); however, HJB does not cross-link IKK into a dimer (data not shown), indicating that the effects of HJB on the JAKs are quite unique—it may require an appropriate tertiary protein structure of the JAK family proteins for a specific compound-protein interaction. The JAK family members have substantial amino acid similarity and homology (1, 2, 43). There are ~10 conserved cystein residues in each of the JAK family members and across the species, and we infer that these conserved cysteins are responsible for the HJB-induced cross-linking of the JAKs. Furthermore, HJB had no effects on EGFR, PDGFR, or IGF-IR signaling (Fig. 3C), indicating that HJB has the potential to be a JAK/STAT3 signaling pathway-specific inhibitor.

Specific inhibition of abnormally expressed or activated kinases is a popular strategy for the development of anticancer drugs. For example, several small molecule JAK inhibitors, such as AG490 and Pyridone 6, directly inhibit the catalytic activity of JAKs by competing with ATP binding (42, 44). Recently, a tripterpenoid CDDO-methyl ester (CDDO-Me) was reported to form adducts with both JAK1 and STAT3 as well as inhibit JAK1 kinase activity (26). CDDO-Me contains α, β-unsaturated carbonyl groups that form reversible adducts with thiol nucleophiles. Unlike HJB, CDDO-Me does not cross-link JAK1 into a large molecular mass form. Only one molecule of CDDO-Me interacts with one molecule of JAK1, therefore differentiating the mechanism by which HJB inhibits JAK kinase activity from other JAK inhibitors. Unlike most of the aforementioned JAK/STAT3 pathway inhibitors, which are more specific to inhibit a specific JAK, HJB seemed to be a pan-JAK inhibitor. Our results showed that HJB inhibited activation of STAT3 and induced death of tumor cells more potently than the JAK2-specific inhibitor AG490 (Fig. 6), suggesting that a pan-JAK inhibitor might be more likely to induce cancer cell death over specific JAK inhibitors, and therefore would be a better anticanic agent.

In summary, our study discovered HJB, a natural diterpenoid, as a potent inhibitor of JAK/STAT3 signaling pathway. HJB suppressed both IL-6–induced and constitutive STAT3 activation by direct inhibition of JAK kinase activity through covalent cross-linking of the JAKs. Moreover, HJB is a rather selective inhibitor of JAK/STAT3 signaling pathway. It does not affect growth factor signaling pathways. Constitutively activated STAT3 has been detected in a wide variety of human cancer cells (8, 45), and accumulating evidences suggest that STAT3 signaling pathway is an important target for therapeutic intervention in a variety of human cancers (35, 45–48). Here, HJB exhibited strong anticanic activity on a panel of cancer cell lines, especially those whose survival and growth are dependent on constitutively active STAT3 signaling. These data strongly support HJB to be a novel anticanic drug candidate. Future studies are geared toward evaluating the antitumor activity of HJB in animal models.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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