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Acta Pharmacologica Sinica

ARTICLE

3-Deoxy-2β,16-dihydroxynagilactone E, a natural compound from *Podocarpus nagi*, preferentially inhibits JAK2/STAT3 signaling by allosterically interacting with the regulatory domain of JAK2 and induces apoptosis of cancer cells

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The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways, especially the JAK2/STAT3 pathway, play vital roles in the development of many malignancies. Overactivation of STAT3 promotes cancer cell survival and proliferation. Therefore, the JAK2/STAT3-signaling pathway has been considered a promising target for cancer therapy. In this study, we identified a natural compound 3-deoxy-2β,16-dihydroxynagilactone E (B6) from the traditional Chinese medicinal plant *Podocarpus nagi* as a potent inhibitor of STAT3 signaling. B6 preferentially inhibited the phosphorylation of STAT3 by interacting with and inactivating JAK2, the main upstream kinase of STAT3. B6 dose-dependently inhibited IL-6-induced STAT3 signaling with an IC_{50} of 0.2 μM. In contrast to other JAK2 inhibitors, B6 did not interact with the catalytic domain but instead with the FERM-SH2 domain of JAK2. This interaction was JAK-specific since B6 had little effect on other tyrosine kinases. Furthermore, B6 potently inhibited the growth and induced apoptosis of MDA-MB-231 and MDA-MB-468 breast cancer cells with overactivated STAT3. Taken together, our study uncovers a novel compound and a novel mechanism for the regulation of JAK2 and offers a new therapeutic approach for the treatment of cancers with overactivated JAK2/STAT3.

Keywords: JAK/STAT; 3-deoxy-2β,16-dihydroxynagilactone E; tyrosine kinase inhibitor; allosteric inhibitor; cancer

Acta Pharmacologica Sinica (2019) 40:1578–1586; https://doi.org/10.1038/s41401-019-0254-4

INTRODUCTION

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways regulate many physiological processes, such as cell growth and differentiation, immune responses, and inflammation [1–4]. After ligand binding, the receptor-bound JAK kinases are activated through transphosphorylation and then phosphorylate their downstream mediator STATs. The phosphorylation of STATs triggers their dimerization and translocation into the nucleus, where they interact with specific promotors and induce the expression of specific genes that in turn give rise to highly specific biological responses, such as inflammation and hyperplasia [5–8]. Among the seven STATs of the STAT family, STAT3 plays an important role in cancer formation and progression. Increasing evidence suggests that overactivation of STAT3 occurs in many human solid tumors, inhibiting cell apoptosis and/or promoting cell proliferation [9–11]. Therefore, inhibiting STAT3 activation has been considered an attractive strategy for treating cancer.

JAKs, the upstream kinases of STATs, are members of a family of four nonreceptor tyrosine kinases [12]. They are the major targets for drugs that regulate the JAK/STAT pathways. JAK inhibitors have already been developed for the treatment of certain malignancies [13–15]. The majority of small-molecule JAK inhibitors are characterized as type I tyrosine kinase inhibitors that target the ATP-binding pockets of the kinases, such as ruxolitinib and AZD1480 [16, 17]. These small-molecule inhibitors, however, have poor selectivity among the JAKs as well as for the JAKS among other tyrosine kinases [18, 19]. Thus, alternative strategies to target noncatalytic domains of the JAKs are worth pursuing.

The leaves of *Podocarpus nagi*, a Chinese herbal medicine, have anti-inflammatory effects [20]. Nagilactones are major chemical constituents of *P. nagi* and have been reported to possess anticancer activities [21]. 3-Deoxy-2β,16-dihydroxynagilactone E (B6) is a biologically active nagilactone with cytotoxic activity [22]. However, the molecular mechanisms of B6 cytotoxicity are not fully understood.

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Received: 19 February 2019 Accepted: 20 May 2019
Published online: 14 June 2019

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In this study, we demonstrated that B6 acted as an inhibitor of STAT3 signaling by allosterically interacting with its upstream kinase JAK2 via its FERM-SH2 domain and thereby inducing apoptosis of cancer cells, especially those with overactivated STAT3. These data suggest the possibility of achieving the selective inhibition of JAK2 in cancer treatment.

MATERIALS AND METHODS

Cell lines and culture

HepG2/STAT3 and HepG2/STAT1 cells, which were HepG2 cells stably transfected with a STAT3-responsive or STAT1-responsive firefly luciferase reporter plasmid, were gifts from Prof. Xin-yuan Fu (National University of Singapore, Singapore). All other cell lines were purchased from the American Type Culture Collection. HepG2/STAT3 and HepG2/STAT1 cells were cultured in α-MEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). HEK293 and HeLa cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS. MDA-MB-231, MDA-MB-453, MDA-MB-468, and A549 cells were cultured in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS. All media were supplemented with 100 μg/mL ampicillin (Sangon Biotech, Shanghai, China) and 100 μg/mL streptomycin (Amresco, Solon, OH, USA). All cell lines were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Reagents

B6 (purity: ≥98%), isolated from *P. nagi*, was provided by Dr. Yang Ye (Shanghai Institute of Materia Medica, Chinese Academy of Sciences); 17-hydroxy-jolkinolide B (HJB) (purity: ≥98%) was isolated from *Euphorbia fischeriana*, as reported previously [23]; DTT (purity: ≥99%) and MTT (purity: ≥99%) were purchased from Genebase (Shanghai, China); GSH (purity: ≥99%) was obtained from Sibas Bioscience (Shanghai, China); IL-6 (#200-02), IFN-γ (#300-02), and IFN-α (human recombinant interferon-alpha 2A) (#300-02BC) were purchased from Peprotech (Saint Paul, MN, USA); and AZD1480 (#S2162, purity: ≥99%) was purchased from Selleckchem (Shanghai, China).

Antibodies

Antibodies against STAT3 (1:3000, #9139S), p-STAT3-Tyr705 (1:3000, #9145S), STAT1 (1:3000, #14994S), p-STAT1-Tyr701 (1:3000, #7649S), JAK2 (1:3000, #3230S), p-JAK2-Tyr1007/1008 (1:3000, #3776S), JAK1 (1:3000, #3332S), p-JAK1-Tyr1022/1023 (1:3000, #3331S), Tyk2 (1:3000, #9312S), p-Tyk2-Tyr1054/1055 (1:3000, #9321S), p-PDGFRα (1:2000, #4547S), p-PDGFRβ (1:2000, #4549S), p-EGFR (1:2000, #3777S), EGFR (1:2000, #4267T), p-IGF-1R (1:2000, #3024S), IGF-1R (1:2000, #3018S), and cleaved PARP (1:3000, #9541S) were all obtained from Cell Signaling Technology (Danvers, MA, USA); anti-PARP (1:3000, #sc-136208) and anti-α-tubulin (1:3000, #sc-5286) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA); secondary horseradish peroxidase (HRP)-conjugated antibodies were obtained from Multi Sciences Biotech (1:5000, #841403, Hangzhou, China); and Flag antibodies were purchased from Abmart (1:3000, #303875, Shanghai, China).

Luciferase assay

HepG2/STAT3 or HepG2/STAT1 cells (4000 per well) were seeded into 96-well cell culture plates and allowed to grow for 48 h. The cells were treated with B6 for 1.5 h, followed by stimulation with IL-6 (10 ng/mL) or IFN-γ (10 ng/mL) for 4 h. Luciferase activity was detected using Promega Luciferase Assay Kits according to the manufacturer's instructions (Promega, Madison, WI, USA).

MTT assay

Cells (2500 per well) were seeded into 96-well plates and allowed to grow for 24 h. The cells were treated with DMSO or B6 for 72 h. Then, 30 μL of MTT (5 mg/mL) was added to the 96-well plates. After incubation for 4 h at 37 °C, the formazan crystals were solubilized in 100 μL of "triplex solution" (10% SDS, 5% isobutanol, 12 mM HCl) for 20 h, and then the absorbance of each well was measured at 595 nm with a spectrophotometer (Tecan infinite F200, Tecan, Mannedorf, Switzerland).

Western blot analysis

Cells were lysed using 1x Laemmli sample buffer (50 mM HEPES at pH 7.5, 25 mM NaCl, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride) supplemented with cOmplete™ Mini Protease Inhibitor Cocktail (#P1860, SigmaAldrich, Saint Louis, MO, USA). Protein lysates were heated at 100 °C for 7 min. Then, the sample was separated by SDS–PAGE (8%) and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% (w/v) nonfat milk in TBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 h, followed by incubation with antibodies in 5% BSA in TBST at 4 °C overnight. The membranes were then washed three times and incubated with peroxidase-conjugated secondary antibodies (1:5000, Multi Sciences Biotech, Hangzhou, China) for 1 h at room temperature. The membranes were then washed three times. Immune complexes were detected by chemiluminescence (#WBKLS0500, Millipore, Billerica, MA, USA).

In vitro kinase assay

The JAK kinase assay was performed using the HTScan JAK2 Kinase Assay Kit (Cell Signaling Technology, Danvers, MA, USA) and streptavidin-coated 96-well plates (SigmaAldrich, Saint Louis, MO, USA). HEK293 cells were transfected with plasmids containing Flag-JAK2 or Flag-JH1 for 24 h and then harvested with lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.15% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, with complete protease inhibitor cocktail) on ice for 30 min. The cell lysates were immunoprecipitated with anti-JAK2 antibody (#sc34479, Santa Cruz Biotechnology, Saint Louis, MO, USA) overnight at 4 °C. Then, 1:10 (*v*/*v*) Protein A/G Plus Agarose (Santa Cruz Biotechnology, Saint Louis, MO, USA) was added to the lysates at 4 °C overnight. Samples were washed three times and resuspended using kinase reaction buffer (60 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 25 μM Na_3VO_4, 200 μM ATP). Samples were treated with B6, AZD1480, or DMSO for 30 min at 37 °C before incubation with biotinylated FLT3 (Cell Signaling Technology, Danvers, MA, USA) for 1 h at 37 °C. Samples were then processed following the protocol for the HTScan JAK2 Kinase Assay Kit [24].

In vitro kinase assays for other kinases

Human JAK2 JH1/JH2, ABL, EGFR, IKBKB, PDGFRα, PDGFRβ, and IGF-1R in vitro kinase assays were performed by Thermo Fisher Kinase Profiling Services (https://www.thermofisher.com/cn/en/home/industrial/pharma-biopharma/drug-discovery-development/target-and-lead-identification-and-validation/kinasebiology/kinase-proteins.html).

Plasmids and transfection

The JAK2 plasmid was a gift from Prof. David E. Levy (New York University, New York, NY, USA). Flag-JAK1 and Flag-JAK2 (JH1 domain spanning amino acids 879–1128) were generated by PCR and then cloned into the pFlag-CMV4 plasmid vector. The transfection of HEK293 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Cellular thermal shift assay

The cells were harvested and resuspended to a final concentration of 2×10^4 cells/mL and supplemented with cOmplete Protease Inhibitor Cocktail (1:1000, #P1860, SigmaAldrich, Saint Louis, MO, USA). The cells were divided into separate aliquots and treated with DMSO or 10 μM B6 for 1 h at 37 °C. Then, the samples were heated using a metal incubator bath (Major science, Saratoga, CA, USA) at various temperatures for 3 min and then freeze-thawed in liquid nitrogen and a water bath three times. The samples were clarified by centrifugation at 15000 × *g* for 20 min, and then, 40 μL of each supernatant was collected and mixed with 20 μL of 1 × Laemmli sample buffer (50 mM HEPES at pH 7.5, 25 mM NaCl, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride) supplemented with cOmpleteTM Mini Protease Inhibitor Cocktail (#P1860, SigmaAldrich, Saint Louis, MO, USA). Then, Western blotting was performed [25].

Statistical analyses

The plot was generated using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA), and the statistical analyses were performed using Student's *t*-test. The WB band density values were quantified using an AzureSpot densitometer and software (Azure Biosystems, Inc., Washington, USA).

RESULTS

B6 preferentially inhibited the cytokine-induced phosphorylation of STAT3 over that of STAT1

To identify specific inhibitors of the JAK/STAT3-signaling pathway, we screened more than 2000 compounds from a traditional Chinese medicinal herb compound library using a JAK/STAT luciferase reporter assay system [26]. We identified a compound, B6 (Fig. 1a), that inhibited IL-6-induced STAT3 signaling in a concentration-dependent manner (Fig. 1b) with an IC_{50} of 0.2 μM. Interestingly, B6 hardly inhibited IFN-γ/STAT1 signaling at a concentration of 1 μM (Fig. 1c), suggesting that the inhibitory effects of B6 on STAT3 signaling were relatively specific.

We then analyzed the effects of B6 on STAT1 and STAT3 phosphorylation using Western blot analyses. We found that B6 had dose-dependent but differential inhibitory effects on the IL-6-induced STAT3 Tyr705 phosphorylation, IFN-γ-induced STAT1 Tyr701 phosphorylation (Fig. 1d, e) and IFN-α-induced

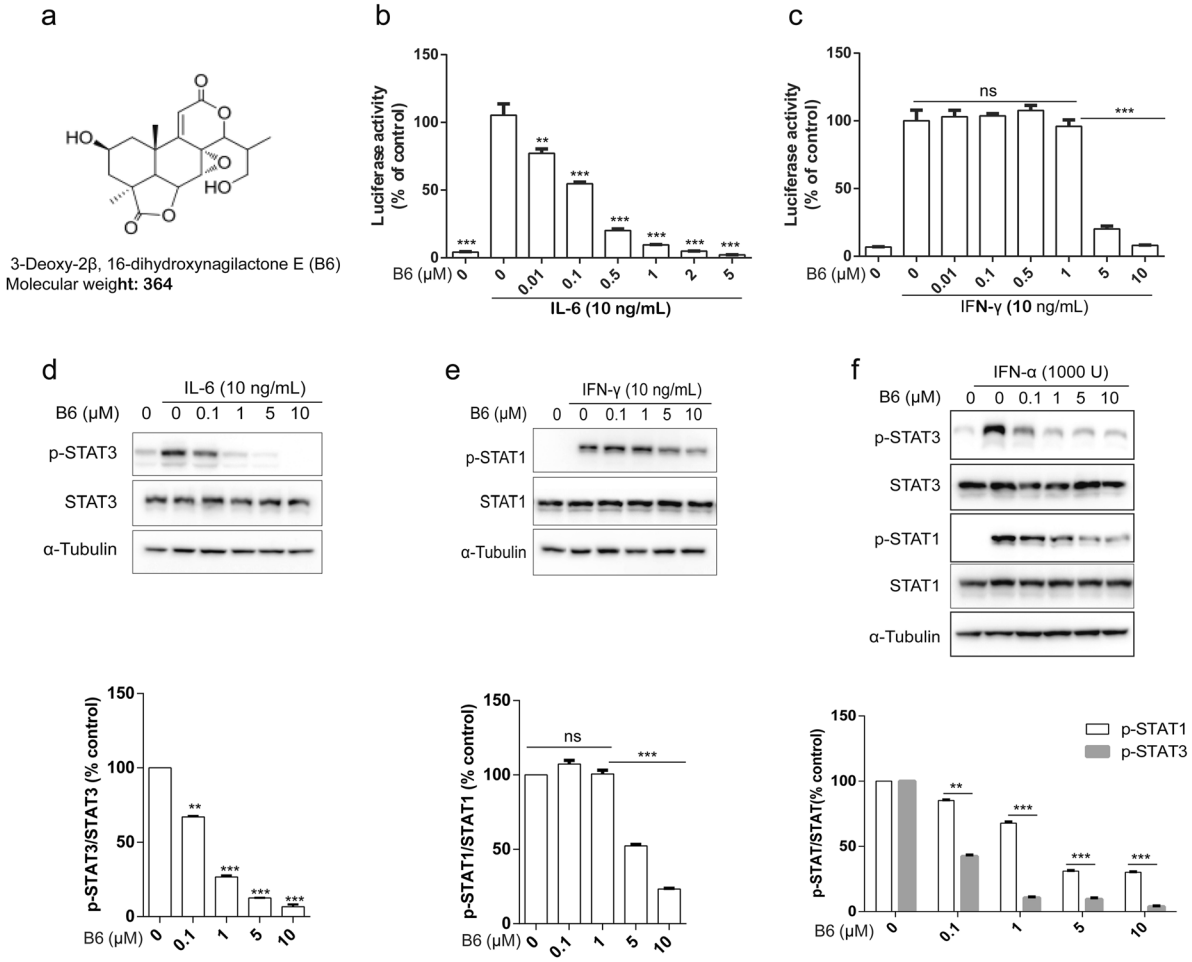

Fig. 1 B6 preferentially inhibited the cytokine-induced phosphorylation of STAT3. **a** Chemical structure of 3-deoxy-2β, 16-dihydroxynagilactone E (B6). **b**, **c** Effects of B6 on the luciferase activities of the HepG2/STAT3-luciferase or HepG2/STAT1-luciferase reporter gene-containing cells. The luciferase reporter gene-containing cells were treated with B6 at the indicated concentrations for 1 h, followed by stimulation with IL-6 (10 ng/mL) or IFN-γ (10 ng/mL) for 4 h, and then the luciferase activities were measured. These experiments were performed in triplicates. Data shown are mean ± SD ($n = 3$). ns, not significant, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the control group without B6 but with cytokine stimulation. Best-fit curve was determined using Prism software and was used to calculate the IC_{50} value. **d**–**f** Effects of B6 on the phosphorylation of STAT1 and STAT3. HeLa cells were pretreated with B6 at indicated concentrations for 1 h before stimulation with IL-6 (10 ng/mL), IFN-γ (10 ng/mL), or IFN-α (1000 U) for 15 min. The cells were then lysed and analyzed by Western blotting using antibodies as indicated. Densitometric quantification of phospho-proteins normalized to total proteins was graphed below the corresponding Western blots. WB band density was quantified three times. Data shown are mean ± SD ($n = 3$). ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$

phosphorylation of both STAT1 and STAT3 (Fig. 1f), which was consistent with the luciferase assay results. A similar pattern of inhibitory effects on STAT3 and STAT1 phosphorylation was also observed in other cell lines (Supplementary Fig. S1). Therefore, B6 preferentially inhibited STAT3 activation by inhibiting STAT3 phosphorylation.

B6 preferentially inhibited the activation and in vitro kinase activity of JAK2

The JAK family has four members, JAK1, JAK2, JAK3, and TYK2. JAK1, JAK2, and TYK2 are expressed universally, while JAK3 expression is restricted to hematopoietic cells [27, 28]. JAK1 and JAK2 are the major tyrosine kinases involved in STAT1 and STAT3 phosphorylation, respectively [29]. To understand the mechanisms by which B6 selectively inhibited STAT3 signaling, we examined the effects of B6 on JAK family proteins. As shown in Fig. 2a, b, B6 inhibited the IL-6-induced phosphorylation of JAK2 as well as the IFN-γ-induced phosphorylation of JAK1, but with different potencies, which were consistent with the inhibition of the phosphorylation of STAT3 versus STAT1, suggesting that B6 preferentially inhibits STAT3 phosphorylation by selectively inactivating JAK2. The inhibition was rapid. A 30 min treatment

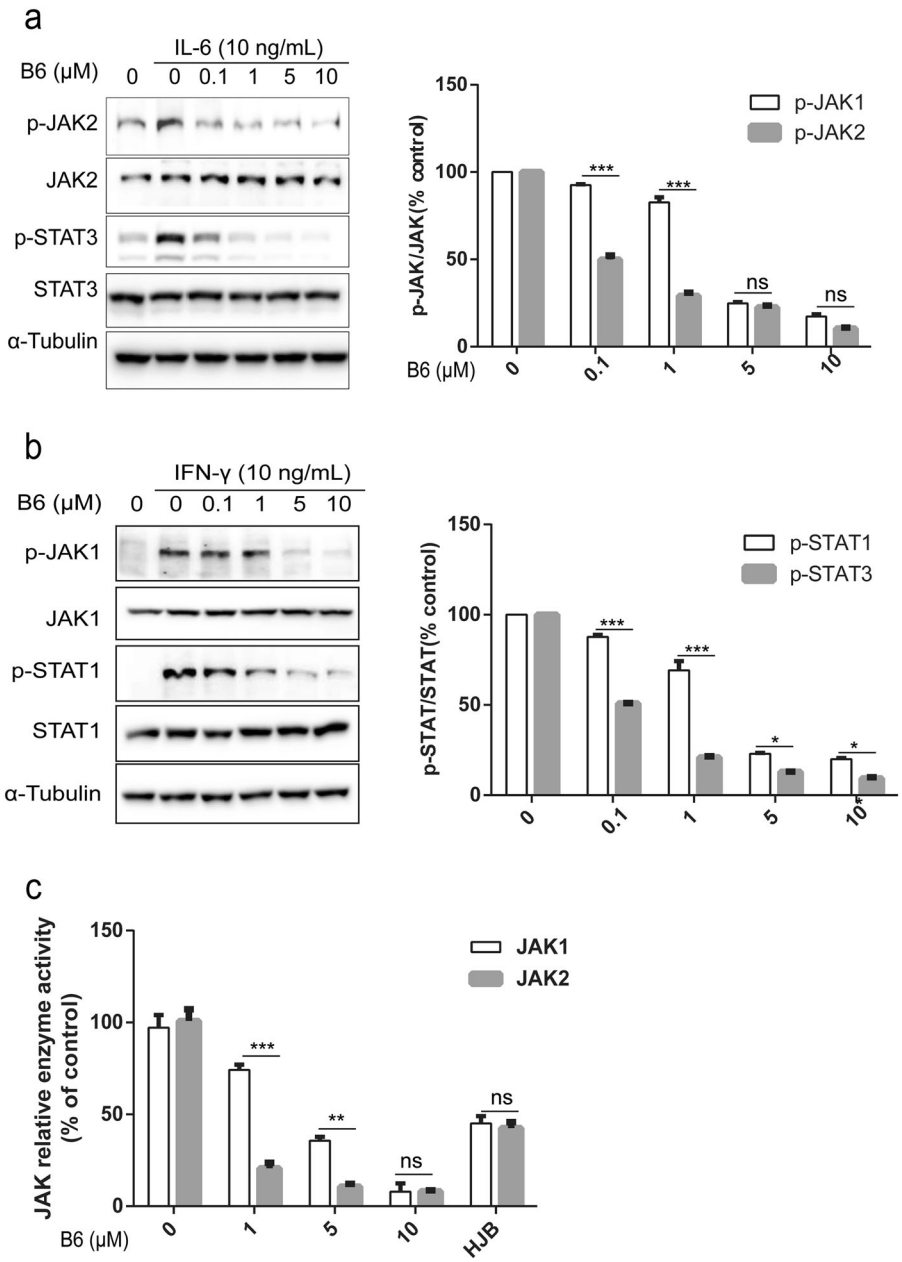

Fig. 2 B6 preferentially inhibited the activation and in vitro kinase activity of JAK2. **a**, **b** Effects of B6 on the activation/phosphorylation of STATs and JAKs. HeLa cells were pretreated with B6 at indicated concentrations for 1 h before stimulation with IL-6 (10 ng/mL) or IFN-γ (10 ng/mL) for 15 min. The cells were then lysed and analyzed by Western blotting using antibodies as indicated. Densitometric quantification of phospho-proteins normalized to total proteins was graphed right to the corresponding Western blots. WB band density was quantified three times. Data shown are mean ± SD ($n = 3$). ns, not significant, $^{*}P < 0.05$, $^{***}P < 0.001$. **c** Effects of B6 on the in vitro kinase activities of JAK1 and JAK2. The JAK proteins were immunoprecipitated from the HEK293 cells overexpressing JAK1 or JAK2, and were then subjected to in vitro kinase assays in the presence of various concentrations of B6 or the positive control compound HJB (10 μM). The experiment was performed in triplicate. Data shown are mean ± SD ($n = 3$). ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$

was sufficient to block the activation of JAKs (Supplementary Fig. S2), suggesting direct inhibition of JAK phosphorylation.

To confirm whether B6 directly interacts with the JAK kinases, we performed an in vitro kinase assay to determine the effects of B6 on the in vitro kinase activities of JAK1 or JAK2. Again, B6 preferentially inhibited the in vitro kinase activity of JAK2 over that of JAK1 (Fig. 2c). These data also suggested that B6 was a direct inhibitor of JAKs.

The inhibitory effects of B6 on JAKs were specific

To investigate whether the inhibitory effects of B6 on the JAK/STAT-signaling pathways were JAK specific, we examined the effects of B6 on other tyrosine kinases, including EGFR, IGF-IR, and PDGFR, which are important for cancer cell growth [30, 31]. Fifty micromolar B6 had little effect on the activities of the tested tyrosine kinases (Table 1). To further confirm this result, we examined the effects of B6 on the other tyrosine kinases using Western blot analyses. The results showed that B6 did not affect the phosphorylation of PDGFR, EGFR, or IGF-IR (Fig. 3a–c), suggesting that the inhibition of JAKs by B6 was specific.

B6 inhibited JAK2 activity through noncovalent interaction

The above data suggested that B6 directly inhibited the activity of JAK. B6 contains an α, β-unsaturated carbonyl group and an epoxy group that have the potential to interact covalently with cysteine thiol groups in a protein. Previous reports have shown that α,β-unsaturated carbonyl groups of several compounds inhibited JAK2 activity by covalently binding to the thiols of cysteines in JAK [32]. To investigate whether B6 also inhibited the activity of JAK2 by covalently binding to the thiols of cysteines, we examined the effects of DTT or GSH on the activities of B6. As shown in Fig. 4a, b, DTT or GSH failed to block the inhibitory effects of B6 on STAT3 signaling, suggesting that B6 might not be able to form covalent linkages with the JAKs.

To further exclude the possibility that B6 covalently interacts with the JAKs, we examined the reversibility of the inhibitory effects of B6 on STAT3 signaling. As shown in Fig. 4c, the inhibitory effects of B6 on STAT3 signaling disappeared immediately after the compound was washed out with fresh medium. In contrast, the effects of HJB, a control compound known to inhibit STAT3 signaling through covalent cross-linking with the JAKs, were sustained after the medium change [33]. These data suggested that B6 inhibited JAK2 through noncovalent interactions.

B6 interacted with the FERM-SH2 domain of JAK2

To analyze whether B6 acted as an ATP competitive inhibitor, we performed an in vitro kinase assay with increasing amounts of ATP. As shown in Fig. 5a, the inhibitory effects of B6 on JAK2 were unaffected by increasing ATP concentrations. In contrast, the inhibitory effects of AZD1480, a known ATP-competitive JAK2 inhibitor, on JAK2 were ATP concentration dependent [34].

To identify the specific site of action of B6 on JAK2, we performed a protein thermal shift assay. We first examined the interaction of B6 with the full-length JAK2 protein. As shown in Fig. 5b, we observed a clear thermal stabilization of JAK2 upon the addition of B6 (10 μM), suggesting that B6 physically interacted with JAK2.

We next examined the interaction of B6 with a flag-tagged catalytic domain (the JH1 domain spanning amino acids 879–1128) of JAK2 [35]. As shown in Fig. 5c, B6 did not affect the thermal stability of the JH1 domain of JAK2 at different temperatures (Fig. 5d), suggesting that B6 did not interact with the catalytic domain of JAK2. In addition, we performed an in vitro kinase assay using the JH1 domain and found that B6 did not affect the activity of the JH1 kinase domain even at a concentration of 20 μM (Fig. 5e), suggesting that B6 interacted with the noncatalytic domain of JAK2.

B6 inhibited cancer cell growth by inducing apoptosis

Previous studies have shown that the constitutive activation of STAT3 frequently occurs in human cancer cells and that the inhibition of STAT3 signaling blocks cancer cell growth [10, 36]. We then examined the effects of B6 on the growth of cancer cells that had different levels of activated JAK2 and STAT3 [33]. We found that MDA-MB-231 and MDA-MB-468 breast cancer cells, which had relatively high levels of activated STAT3, were more sensitive to B6 than MDA-MB-453 cells, which had a relatively low level of phosphorylated STAT3 (Fig. 6a, b), suggesting that B6 inhibited cancer cell growth by blocking JAK2/STAT3 signaling. In addition, we analyzed the effects of B6 on IL-6-induced cell growth, which was a consequence of STAT3 activation [37], and found that B6 also inhibited the IL-6-induced growth of HeLa cells (Fig. 6c), confirming that the inhibition of cancer cell growth was caused by the inhibition of STAT3 phosphorylation/activation.

We next investigated the mechanisms of B6-induced cell growth inhibition and examined the effects of B6 on the induction of apoptosis in cancer cells. A known JAK2 inhibitor and apoptosis inducer, AZD1480 [38], was used as a positive control. We found that B6 induced apoptosis of cancer cells in a dose-dependent

Table 1. Effects of B6 on non-JAK family tyrosine kinases. In vitro kinase assays were processed by Thermo Fisher Kinase Profiling Services

Kinase	ABL1 (h)	EGFR (h)	IGF-1R (h)	IKBKB (h)	JAK2 JH1JH2 (h)	PDGFRα (h)	PDGFRβ (h)
Activity (%)	95	102	101	103	101	103	100

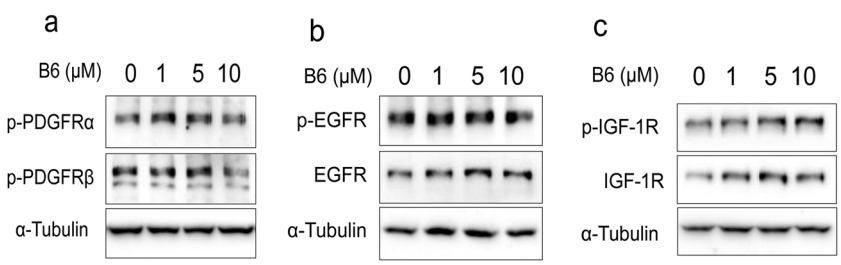

Fig. 3 The effects of B6 on non-JAK family tyrosine kinases. **a**–**c** Western blot analyses of HeLa cell lysates treated with B6 at indicated concentrations for 1 h. Separate blots were probed with the antibodies as indicated, respectively

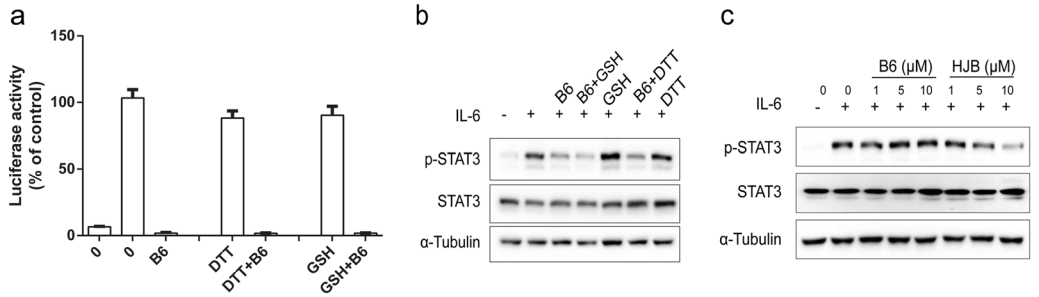

Fig. 4 B6 inhibited JAK2 activity through noncovalent interaction. **a** Effects of GSH or DTT on the inhibition of luciferase activities by B6 in the HepG2/STAT3-luciferase gene reporter cells. B6 (1 μM) was incubated with or without 1 mM DTT or GSH for 1 h at 37 °C before being added to the HepG2/STAT3-luciferase gene reporter cells for 1 h, and the luciferase activities were measured following stimulation with IL-6 (10 ng/mL) for 4 h. The experiment was performed in triplicate. Data shown are mean ± SD ($n = 3$). **b** Western blotting analyses of STAT3 phosphorylation in the HeLa cells treated with B6. B6 (1 μM) was incubated with or without 1 mM DTT or GSH at 37 °C for 1 h before being added to HeLa cells for 1 h, followed by stimulation with IL-6 (10 ng/mL) for 15 min. The cells were then lysed and analyzed by Western blotting using antibodies as indicated. **c** Reversibility of B6 treatment. HeLa cells were treated with B6 or HJB at indicated concentrations for 1 h and then were washed with fresh medium three times followed by culturing in fresh medium for 2 h. The cells were then stimulated with IL-6 (10 ng/mL) for 15 min and lysed and analyzed by Western blotting using antibodies as indicated

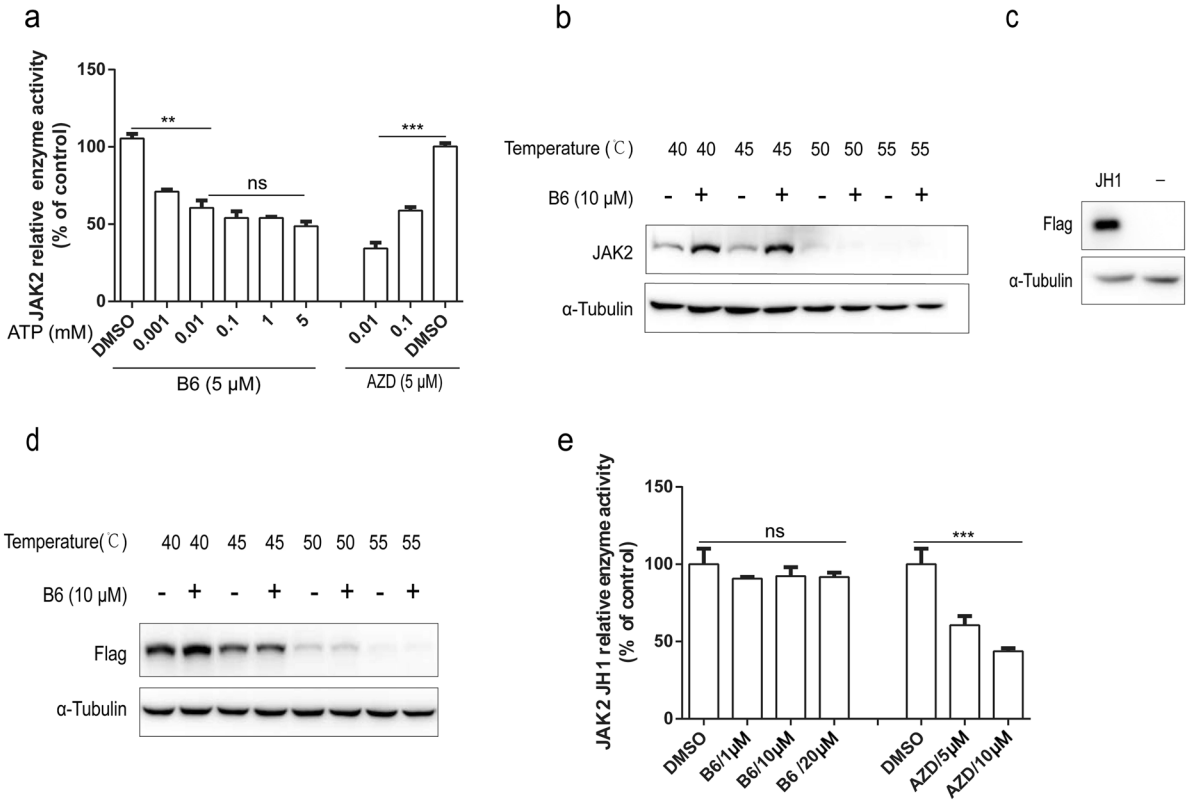

Fig. 5 B6 interacted with the FERM-SH2 domain of JAK2. **a** ATP competition assay. The JAK2 protein was immunoprecipitated from the HEK 293 cells overexpressing JAK2 and subjected to in vitro kinase assays in the presence of increasing concentrations of ATP as well as 5 μM B6. 5 μM AZD1480 was used as a positive control. The experiment was performed in triplicate. Data shown are mean ± SD ($n = 3$). ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$. **b** Cellular thermal shift assay of the interaction of JAK2 and B6. HeLa cells were incubated with or without B6 (10 μM) for 1 h, and then subjected to a cellular thermal shift assay. **c** Western blotting analysis of the expression of the flag-tagged JAK2 JH1 catalytic domain. HEK293 cells were transfected with the flag-tagged JAK2 JH1 domain plasmid for 24 h. The cell lysates were analyzed by Western blotting using the antibodies as indicated. **d** Cellular thermal shift assay of the interaction of the JAK2 JH1 domain and B6. HEK293 cells were transfected with JAK2 JH1 domain plasmid for 24 h before pretreatment with or without B6 (10 μM) for 1 h, and then subjected to the cellular thermal shift assay. **e** Effects of B6 on the enzyme activity of JAK2 JH1 domain. Immunoprecipitated JAK2 (JH1) protein was pretreated with B6 at indicated concentrations for 30 min and then assayed for their enzymatic activities. AZD1480 was used as a positive control. The experiment was performed in triplicate. Data shown are mean ± SD ($n = 3$). ns, not significant, $^{***}P < 0.001$

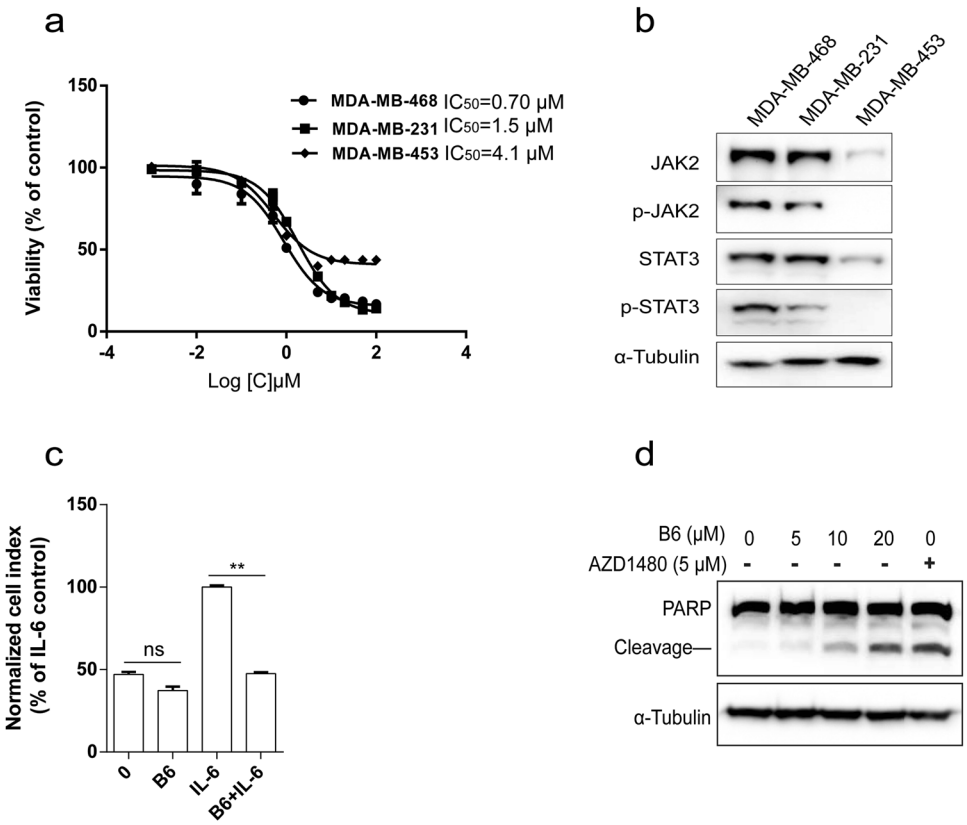

Fig. 6 B6 inhibited cancer cell growth by inducing apoptosis. **a** Effects of B6 on cancer cell growth. The indicated three human breast cancer cells were cultured with increasing concentrations of B6 for 72 h and assayed by MTT assay. The graph was calculated from one of two independent experiments performed in triplicates and the data are mean ± SD ($n = 3$). Best-fit curves were determined using Prism software and were used to calculate the IC_{50} values. **b** Expression and phosphorylation of JAK2 and STAT3 in the three breast cancer cell lines were assayed by Western blotting using the indicated antibodies. **c** Effects of B6 on the IL-6-induced cancer cell growth. HeLa cells were incubated in serum-free medium and treated with B6 (5 μM), IL-6 (100 ng/mL), or IL-6 + B6 (5 μM)) for 48 h. The cell growth was measured by MTT assay. The experiment was performed in triplicate. Data shown are mean ± SD ($n = 3$). ns, not significant, $^{**}P < 0.01$. **d** Effects of B6 on PARP cleavage/cell apoptosis in HeLa cells. The cells were treated with B6 or AZD1480 at indicated concentrations for 24 h. The cell lysates were then analyzed by Western blotting using the antibodies as indicated. AZD1480 was used as a positive control

manner (Fig. 6d), suggesting that B6 inhibited tumor cell growth by inducing apoptosis [39]. Taken together, B6 preferentially inhibited STAT3 signaling and induced apoptosis in cancer cells with constitutively activated STAT3.

DISCUSSION

Although extracts of *P nagi* have been reported to possess anticancer activities, their underlying molecular mechanisms are unclear. In this study, we found that the natural compound B6 from *P. nagi* inhibited cancer cell growth by inducing apoptosis. B6 preferentially inhibited STAT3 signaling by selectively interacting with JAK2.

We presented several lines of evidence to suggest that B6 inhibited STAT3 signaling by allosterically interacting with the FERM-SH2 domain of JAK2. First, B6 inhibited JAK2 kinase activity in in vitro kinase assays, suggesting that B6 interacted with JAK2 directly. Second, the inhibition of JAK2 by B6 was ATP concentration independent, suggesting that B6 was not an ATP-competitive kinase inhibitor. Finally, B6 did not inhibit the kinase activity of the catalytic domain of JAK2, suggesting that the inhibitory effects of B6 required the presence of the noncatalytic domain of JAK2. Taken together, these data suggested that B6 inhibited STAT3 phosphorylation by allosterically interacting with the FERM-SH2 domain of JAK2.

The N-terminal FERM and SH2 domains of the JAKs share very weak primary sequence homology. The FERM-SH2 domains of JAK1 and JAK2 have only 38% identity, which provides a molecular basis for a selective interaction of compounds with different JAKs (Supplementary Fig. S3) [40–42]. Our data suggested that B6 interacted with the FERM-SH2 domain of JAK2. Therefore, B6 was able to selectively inhibit JAK2 phosphorylation via an allosteric interaction. This mechanism of B6 is novel and different from those of many other known tyrosine kinase inhibitors, which interact with the ATP-binding pockets of JAKs. Because the ATP-binding pockets of kinases are highly conserved, it is therefore difficult to obtain compounds that specifically interact with a particular ATP-binding pocket [43, 44]. Future JAK2 inhibitors that target similar sites via allosteric mechanisms may provide alternative therapeutic strategies to inhibit specific JAK/STAT pathways.

A number of natural compounds have been reported to inhibit JAKs [33, 45]. Many of them also interact with the noncatalytic domain of JAKs. However, these compounds all contain an α, β-unsaturated carbonyl group that interacts covalently with the JAKs through a Michael addition reaction with cysteine thiols of JAKs. In contrast to B6, these compounds do not distinguish among the JAK family members [33, 46]. B6 also contains an α, β-unsaturated carbonyl group, but it inhibits JAK activity through a noncovalent interaction and preferentially interacts with JAK2, suggesting that B6 interacts with JAK2 in a different fashion from those covalent interactions.

Activated STAT3 promotes cell survival and growth and has been considered an oncogene. Our results that MDA-MB-468

and MDA-MB-231 cells, which contain overactivated STAT3, were more sensitive to B6 than MDA-MB-453 cells, which contain low levels of activated STAT3, are consistent with this conclusion. B6 preferentially inhibited the growth of cancer cells with overactivated STAT3. However, the fact that B6 still inhibited the growth of MDA-MB-453 cells at high concentrations despite the low level of phosphorylated STAT3 in the cells suggested that B6 might also target other cell growth-related proteins at high concentrations in addition to JAK2 (Fig. 6a). Further chemical modifications of B6 may help to improve its target specificity in future experiments. On the other hand, identification of the additional targets of B6 may help to better understand B6 as an anticancer agent.

In the present study, we discovered B6, a natural compound from *P. nagi*, to be a potent and selective inhibitor of JAK2/STAT3 signaling. B6 preferentially inhibited STAT3 phosphorylation/activation by allosterically interacting with the FERM-SH2 domain of JAK2. In doing so, B6 preferentially inhibited the growth of cancer cells with overactivated STAT3. In summary, our study provided a novel therapeutic approach for the treatment of cancer cells with constitutively activated STAT3 and suggested a new direction to design novel JAK inhibitors.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (grant 81673465 to QY). We thank Xin-yuan Fu (National University of Singapore) for providing HepG2/STAT3 and HepG2/STAT1 cells. We also thank Hui Wang (Shanghai Institute of Materia Medica) for help with the amino acid sequence analysis.

AUTHOR CONTRIBUTION

QY and HS conceived and designed the experiments. HS wrote the manuscript, performed experiments, and analyzed the data. SY and YY synthesized the compound 3-deoxy-2β,16-dihydroxynagilactone E (B6).

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41401-019-0254-4) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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