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REGULAR PAPER

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A natural product phillygenin suppresses osteosarcoma growth and metastasis by regulating the SHP-1/JAK2/STAT3 signaling

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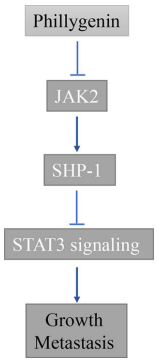
ABSTRACT

Osteosarcoma represents one of the most devastating cancers due to its high metastatic potency and fatality. Osteosarcoma is insensitive to traditional chemotherapy. Identification of a small molecule that blocks osteosarcoma progression has been a challenge in drug development. Phillygenin, a plant-derived tetrahydrofurofuran lignin, has shown to suppress cancer cell growth and inflammatory response. However, how phillygenin plays functional roles in osteosarcoma has remained unveiled. In this study, we showed that phillygenin inhibited osteosarcoma cell growth and motility *in vitro*. Further mechanistic studies indicated that phillygenin blocked STAT3 signaling pathway. Phillygenin led to significant downregulation of Janus kinase 2 and upregulation of Src homology region 2 domain-containing phosphatase 1. Gene products of STAT3 regulating cell survival and invasion were also inhibited by phillygenin. Therefore, our studies provided the first evidence that phillygenin repressed osteosarcoma progression by interfering STAT3 signaling pathway. Phillygenin is a potential candidate in osteosarcoma therapy.

Graphical Abstract



Phillygenin regulates SHP-1/JAK2/STAT3 signaling to suppress osteosarcoma growth and metastasis.

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Keywords: phillygenin, osteosarcoma, growth and metastasis, SHP-1/JAK2/STAT3 signaling

Osteosarcoma, which arises primarily in children and adolescents, represents the most common primary malignancy of bone (Isakoff *et al.* 2015). Surgery remains the vital modality for curative osteosarcoma treatment (Sluga *et al.* 1999). However, most osteosarcoma patients still develop distant metastasis and the survival of these patients is relative low, even after receiving radical surgery (Weeden *et al.* 2001). Unfortunately, conventional osteosarcoma is relatively resistant to standard chemotherapy. Current osteosarcoma therapy combines surgical removal of the primary tumor and chemotherapy (doxorubicin and cisplatin with or without methotrexate) (Ferrari *et al.* 2005). However, over the past few decades, the survival rate for patients with osteosarcoma has not improved. New therapies are time intensive.

Signal transducer and activator of transcription (STAT) proteins participate in a variety of biological process including growth, differentiation, immune response, and so on (Benekli *et al.* 2003). STAT3 is among the most well studied member of the STAT family (Jing and Tweardy 2005). Like other STAT family proteins, STAT3 can be activated in response to cytokines, growth factors, and other related ligands through the intrinsic tyrosine kinases such as Janus-like kinases (JAKs) and c-Src kinase (Yu *et al.* 1995). The JAK/STAT3 pathway is a canonical signaling system that STAT3 participates in. Once the upstream JAK kinase is activated, STAT3 is recruited to JAKs, whereupon it is phosphorylated by JAKs on the tyrosine 705 (Yu *et al.* 1995). STAT3 is then activated through tyrosine phosphorylation, leading to the SH2 domain-dependent dimerization, nuclear translocation, and further activation of the transcription of its target genes (Zhang *et al.* 2010). Persistent activation of STAT3 has been detected in the clinical tumor samples, suggesting its involvement in tumorigenesis. Preclinical studies have also demonstrated that STAT3 inhibition led to tumor regression *in vitro* and *in vivo* (Lee *et al.* 2014). These results imply that interfering STAT3 signaling is a promising approach for new targeted therapy.

Discovery of novel drugs from plant-derived natural products have attracted much more attention as many plant chemicals exhibit potent activities that target human diseases including cancer. Natural agents such as butein (Pandey *et al.* 2009), caffeic acid (Jung *et al.* 2007), and curcumin (Yang *et al.* 2012) have shown significant activity in impairing tumor progression via interfering STAT3 signaling, indicating that natural products are suitable candidates for the discovery of STAT3 inhibitors. Phillygenin (PHI) is an active metabolite of phillyrin from the fruits of genus forsythia sp. from oleaceae family (Du *et al.* 2019). Previous studies showed PHI exerted antioxidant and antiinflammatory activity (Du *et al.* 2019). PHI was also found to inhibit esophageal cancer growth *in vitro* and *in vivo* (He *et al.* 2019). However, the role of PHI in STAT3 signaling and osteosarcoma progression has remained elusive. In this study, we demonstrated PHI can be considered as a STAT3 inhibitor and exhibits antiosteosarcoma efficacy.

Materials and methods

Reagents and antibodies

Purified PHI (MF: $C_{21}H_{24}O_6$, MW: 372.41, purity >98%) was purchased from MedChemExpress (HY-N0483, NJ, USA). All the cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Antibodies against p-STAT3 (Y705), STAT3, JAK2, Phospho-JAK2 (Tyr1007/1008), Bcl-2, survivin, matrix metalloproteinase-2 (MMP-2), and Src homology region 2 domain-containing phosphatase 1 (SHP-1) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Vanadate and antibody against β-actin was purchased from Sigma–Aldrich (Sigma–Aldrich, Inc., Shanghai, China). The DMSO was used to dissolve phillygenin, and the final concentration of DMSO in cell culture media is less than 0.1%.

Cell cultures

The osteosarcoma cell lines 143B, HOS, and SJSA were obtained from ATCC (Manassas, VA, USA), and were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C under a humidified 5% CO_2 incubator. Normal osteoblasts (hFOB1.19) were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. hFOB1.19 cells were cultured in DMEM/F12 supplemented with 10% FBS and G418 (0.3 mg/mL) and maintained in a 5% CO_2 incubator at 34 °C.

Cell viability assay

Cell viability assay was performed according to the manufacture's guidelines (Promega, Madison, WI, USA). In brief, osteosarcoma cells (5000 cells) were seeded in 96-well plate. 24 h after seeding, different concentrations of PHI were added, followed by 48 h incubation. The CellTiter 96® AQueous One Solution was then added and the OD value at 490 nm was obtained by a VERSA max microplate reader.

Western blot assay

Osteosarcoma cells were lysed with modified radioimmunoprecipitation buffer supplemented with protease and phosphatase inhibitors. Equal amount of the protein samples were subjected to the SDS-PAGE.

Colony formation assay

Osteosarcoma cells (500 cells) were seeded in 6-well plate and treated with the indicated concentrations of PHI. The culture medium was replaced every 2 days. On day 7, cells were fixed with 4% PFA and stained with 0.1% crystal violet. Representative images were acquired with a microscope (Leica). Colony numbers were counted manually.

Cell cycle and apoptosis analysis

For cell-cycle assay, 143B cells were treated with PHI for 24 h and then fixed with 70% ethanol at −20 °C overnight, and stained with propidium iodide (PI). For cell apoptosis analysis, 143B cells were stained with the PE Annexin V apoptosis detection kit I (BD Biosciences) according to the manufacturer's explanations and analyzed by flow cytometry after PHI treatment 48 h.

Wound-healing assay

A wound-healing assay was performed as previously described (Yi *et al.* 2008). Briefly, the cells were seeded into 6-well plate, and created a confluent monolayer. Then create a wound by manually scraping the cell monolayer with a 200 μL pipette tip.

Boyden chamber invasion assay

The Boyden chamber assay was performed using a Transwell filter (Millipore). The Boyden chamber was precoated with 10% Matrigel. 5×10^4 cells pretreated with PHI were resuspended in 100 μL of serum-free medium, and were added into the upper chamber of the transwell insert. 600 μL of cell culture medium was added in the lower chamber. After 12 h, the noninvaded cells on the inner side of the insert were removed by a cotton swab. Cells on the underside of the transwell filter were fixed with 4% PFA and stained with 0.1% crystal violet. Photographs of 3 random fields were acquired.

3D culture assay

Three-dimensional (3D) culture assay was performed as previously described (Zhang *et al.* 2018). In brief, 100 μL Matrigel solution per well was added into 48-well plate and incubated in 37 °C for 45 min to solidify. 143B cells (1×10^4) were resuspended in 100 μL DMEM and seeded on solidified Matrigel. The top Matrigel-medium mixture was refreshed every 2 days.

Q-PCR

The total RNA was extracted with the TRIZOL reagent (Invitrogen). 1000 ng of total RNA was used for the synthesis of first-strand cDNA using the PrimeScript RT reagent kit (TaKaRa Biotechnology, Shiga, Japan). Quantitative real-time PCR (Q-PCR) was performed according to the manufacturer's instructions of the SYBR® Premix Ex Taq kit (TaKaRa) on an Mx3005P thermal cycler (Stratagene). The forward and reverse primers for Bcl-2, survivin, and MMP-2 were as follows: Bcl-2, 5′-TGCACCTGACGCCCTTCAC-3′ and 5′- AGACAGCCAGGAGAAATCAAACAG-3′, survivin, 5′-CACCGCATCTCTACATTCAA-3′ and 5′-CACTTTCTTCGCAGTTT CCT-3′ MMP-2, 5′-GGATGATGCCTTTGCTCG-3′ and 5′-ATCGGCG TTCCCATACTT-3′, SHP-1, 5′-GAGAACGCTAAGACCTACATCG-3′and 5′-CAGTATGGGACGCATTTGTT-3′.

Statistical analysis

Data are presented as mean ± SD. Comparison between 2 groups was performed using Student's *t*-test and group differences were analyzed using one-way ANOVA analysis followed by Tukey post-hoc test. *P* value of less than .05 was considered to indicate statistical significance unless otherwise indicated. All experiments were performed at least 3 times except animal model.

Results

PHI decreases osteosarcoma cell growth

To elucidate the effects of PHI (shown in Figure 1) on osteosarcoma cells, we first examined the cytotoxic efficacy of PHI. The result of cell viability assay was showed that PHI inhibited cell viability in a panel of osteosarcoma cell lines, with the half maximal inhibitory concentrations of about 50 μmol/L (Figure 2a). We also found phillygenin showed no significant inhibitory effect on hFOB1.19 cells viability (Figure S1). There is a positive correlation between cloning efficiency of the cells and the histological involvement of the tumor. Consequently, we tested whether PHI could inhibit the ability of osteosarcoma cells to form colonies. In Figure 2b, PHI markedly impaired the colony formation by 143B, HOS, and SJSA osteosarcoma cells. We also performed cell cycle and apoptosis analysis. The results demonstrated PHI dose-dependent–induced cell cycle G2/M phase arrest and cell apoptosis (Figure S2a and b). Western blot analysis further indicated addition of PHI significantly decreased the level of PCNA (Figure 2c), a proliferation marker of cancer cell. These results implied that PHI inhibited osteosarcoma cell growth *in vitro*.

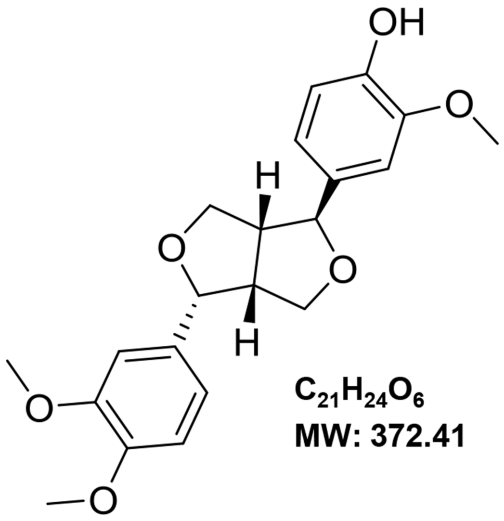

Figure 1. The chemical structure of PHI.

PHI inhibits motility of osteosarcoma cells

Cell motility is crucial for osteosarcoma metastasis and progression. To investigate the antimotility function of PHI, the wound-healing migration assay was used. As shown in Figure 3a, PHI significantly inhibited osteosarcoma cell migration. We further examined its inhibitory function on the motility by the Boyden chamber invasion assay. Our results showed that invasive osteosarcoma cells in the PHI-treated group were dramatically less than that of the control group, suggesting a potent inhibitory effect of PHI on cell motility (Figure 3b). Cancer cells are able to form invasive stellar structures when cultured in the 3D environment (Park *et al.* 2003). To further examine whether PHI repressed the invasive stellar structure formation of osteosarcoma cells, we performed a 3D culture assay. The result indicated the invasive structure forming ability was greatly inhibited when exposure to PHI (Figure 3c). Collectively, our data demonstrated PHI suppressed osteosarcoma cell motility *in vitro*.

PHI blocks STAT3 activation in osteosarcoma cells

To find out the antiosteosarcoma molecular basis of PHI, we examined the signaling pathways mediated by PHI using western blot analysis. We found 100 μmol/L of PHI could significantly inhibit the phosphorylation of STAT3 (Tyr705). When noted, such inhibitory action of PHI on STAT3 was in parallel with a dephosphorylation of JAK2, the upstream kinase of STAT3 (Figure 4a). STAT3 activation results in target gene transcription, and we next determined whether PHI impaired STAT3

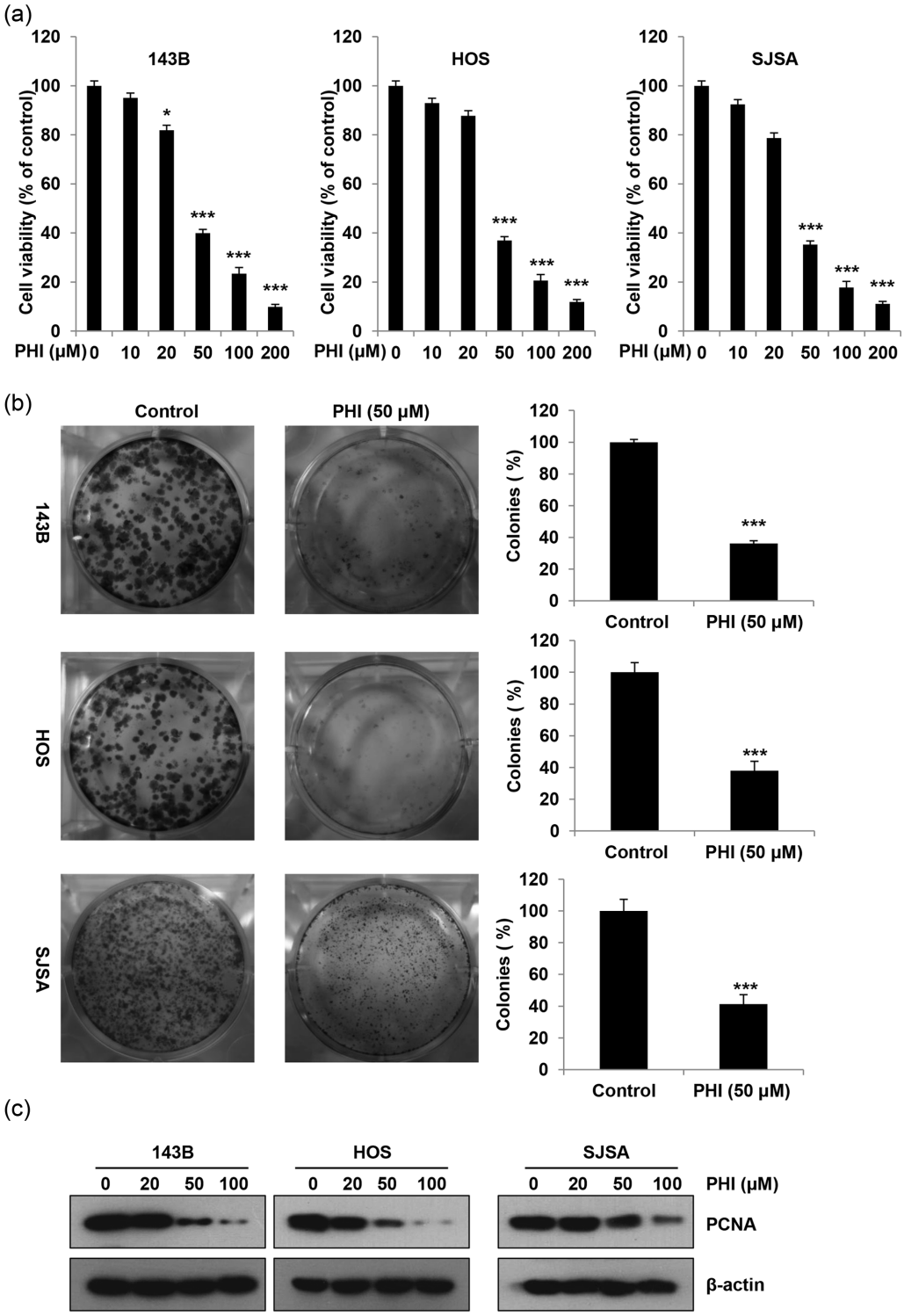

Figure 2. PHI inhibits osteosarcoma cells growth. (a) An MTS cell viability assay was performed to detect the inhibitory effect on cell viability. The osteosarcoma cells were exposed to indicated concentrations of PHI for 48 h (*$P < .05$; ***$P < .001$). (b) PHI remarkably inhibited the colony formation of osteosarcoma cells (***$P < .001$). (c) Cells were treated with PHI for 24 h. PHI dose-dependently downregulated PCNA expression in osteosarcoma cells.

target gene expression. Our results showed that addition of PHI remarkably inhibited the expression of Bcl-2, survivin, and MMP-2 (Figure 4b). In accordance with this, real-time PCR analysis indicated the mRNA level of STAT3 gene products was impaired by PHI (Figure 4c). In sum, these data implied PHI interfered STAT3 signaling pathway.

PHI induces the level of SHP-1

Several phosphatases, including SHP-1, have been reported to act as a negative mediator of JAK2/STAT3 signaling (Rhee *et al.* 2012). We set forth to explore whether PHI could upregulate the expression of SHP-1. Intriguingly, we found addition of

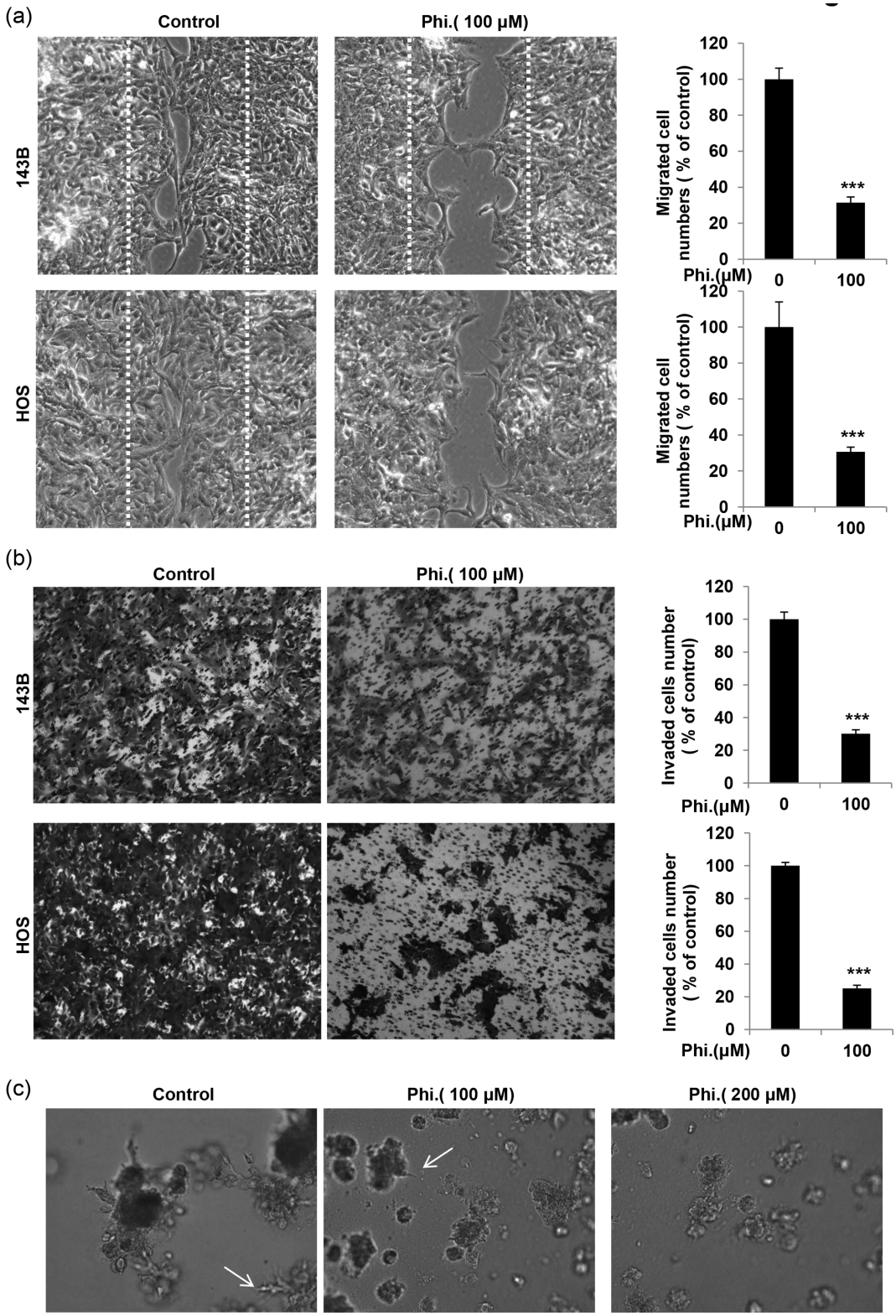

Figure 3. PHI suppresses osteosarcoma cells migration and invasion. (a) The wound-healing assay was conducted to detect the inhibitory effect of PHI on osteosarcoma cells (143B and HOS) migration (***$P < .001$). (b) PHI remarkably inhibited osteosarcoma cells (143B and HOS) invasion (***$P < .001$). (c) 143B cells were grown in 3D culture system and PHI suppressed invasive structures formation.

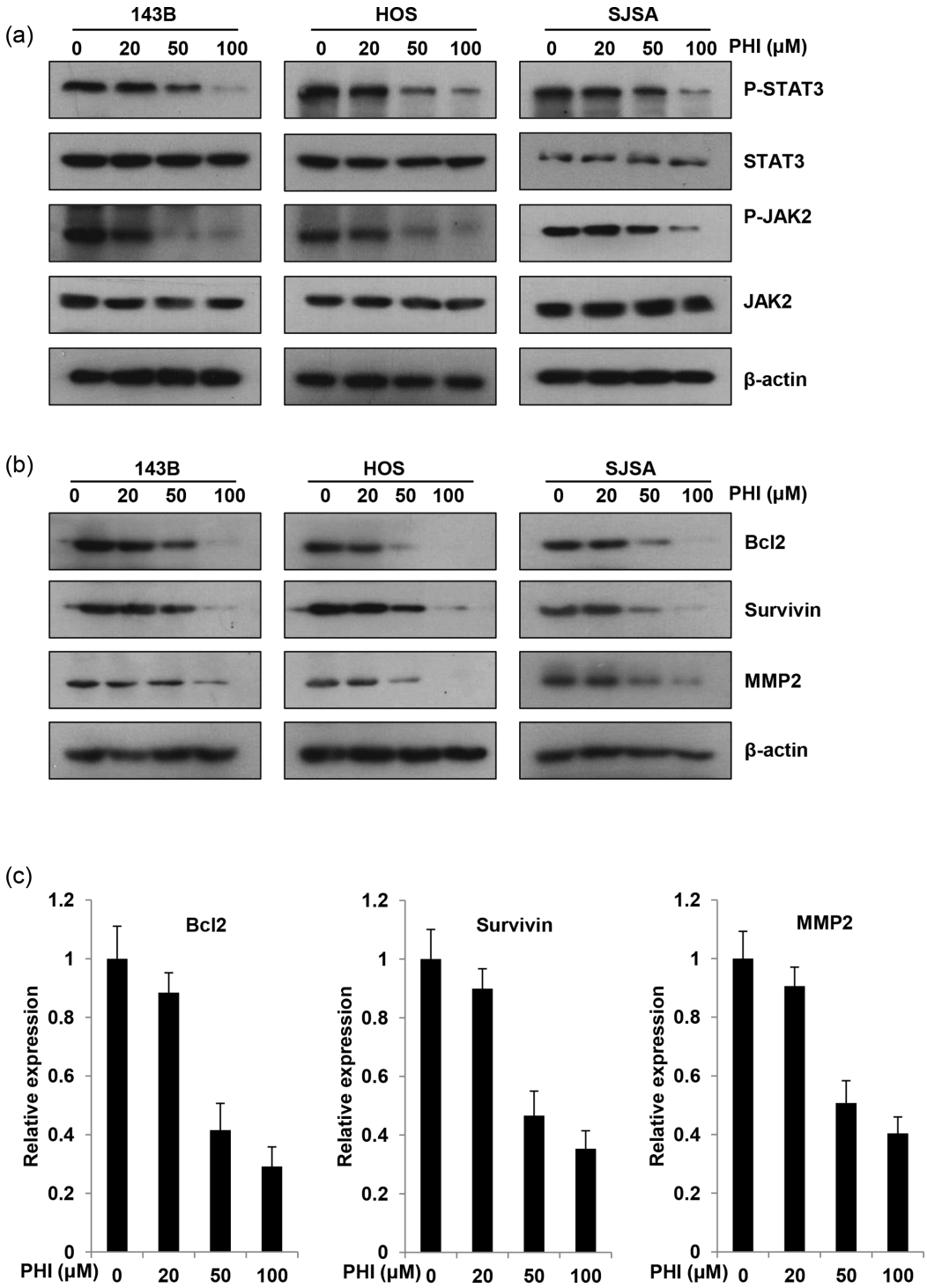

Figure 4. PHI exerts the inhibitory effect on STAT3 cascade in osteosarcoma cells. (a) Cells were treated with PHI for 24 h. PHI diminished the activation of STAT3 and JAK2 kinase in a dose-dependent manner in osteosarcoma cells. (b) The inhibitory effect of PHI on STAT3 target gene expression. (c) 143B cells were treated with PHI for 24 h. The transcription levels of Bcl-2, survivin, and MMP-2 were detected by real-time PCR.

PHI-reduced STAT3 phosphorylation; whereas addition of vanadate (100 μmol/L), a phosphatase inhibitor, in the present of PHI partly rescued the level of STAT3 (Tyr705), indicating a certain phosphatase was involved in PHI-mediated STAT3 inhibition (Figure 5a). Further western blot analysis revealed PHI treatment led to significant upregulation of SHP-1 (Figure 5b). We also found PHI induced the expression of SHP-1 at the transcriptional level in a concentration-dependent manner (Figure S3). These results indicated the PHI-induced depression of STAT3 signaling could be apparently reversed by blockade of SHP-1.

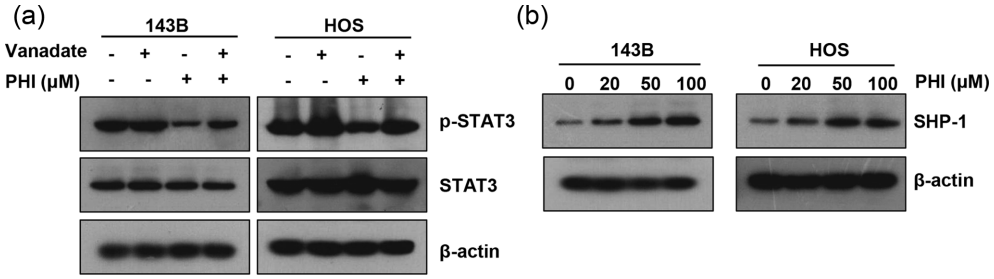

Figure 5. PHI induces the expression of SHP-1. (a) Vanadate partly reverses the inhibitory effect of PHI on STAT3 activity. Osteosarcoma cells were treated with vanadate (100 μmol/L) and PHI (50 μmol/L). Phospho-STAT3 was detected by western blotting assay. (b) PHI upregulated the total protein level of SHP-1 in a dose-dependent manner.

Discussion

Natural chemicals are considered to be an important source for anticancer drugs, and many of them are studied for efficacy *in vitro* and *in vivo*. PHI has been demonstrated to inhibit esophageal cancer growth through NF-KB signaling repression (He *et al.* 2019); however, no conclusion has been made regarding its antitumor activity in osteosarcoma. Here, we investigated whether PHI could alter osteosarcoma carcinogenesis via other molecular basis. We provide the first evidence that PHI blocks STAT3 phosphorylation and activity in osteosarcoma cells.

Our results revealed that constitutive STAT3 activation was suppressed by PHI. The activation of JAK2, the upstream kinase of STAT3, was also blocked by PHI treatment. We also observed PHI-induced STAT3 inhibition involves SHP-1. SHP-1, a nontransmembrane phosphatase, acts as a negative regulator for JAK2/STAT3 cascade (Liu *et al.* 2013). PHI treatment induces the expression of SHP-1, indicating the inhibitory effect of PHI on STAT3 signaling occurs through SHP-1 induction. However, the mechanism through which PHI induces SHP-1 expression needs further investigation. PHI may directly or indirectly affect the binding of transcription factors to the SHP-1 promoter. Epigenetic modification by PHI may be also involved (demethylation of the SHP-1 promoter for example).

One interesting finding in our study is that PHI shows remarkable efficacy in inhibiting osteosarcoma cell growth and motility *in vitro*. STAT3 participate in a variety of biological process including growth, STAT3, an important member of the STAT family, is frequently associated with tumor growth and metastasis. Previous study reported Stat3 was constitutively overexpressed in osteosarcoma tissues and p-Stat3 was overexpressed in osteosarcoma tissues (75.0%) when compared with osteoblast cell lines (Ryu *et al.* 2010). That would explain why PHI's more specific inhibition of osteosarcoma cells growth in our result. In addition, various antiapoptotic and proliferative target genes downstream of STAT3, such as cyclin D1, survivin, and Bcl-2, were significantly reduced by PHI. Matrix metalloproteinases (MMPs) are crucial downstream target genes of STAT3 and associated with invasiveness. In our study, we found PHI significantly downregulates the level of MMP-2, which could partially explain the antimetastatic efficacy of PHI.

Previous study has indicated PHI increases ROS levels and inhibits NF-KB signaling in cancer cells (He *et al.* 2019). Although we have demonstrated PHI interferes STAT3 signaling in osteosarcoma, whether PHI could regulate NF-KB signaling remains to be explored. We could not exclude the possibility that PHI inhibits osteosarcoma progression via other molecular mechanism. Further studies are needed to comprehensively reveal the antiosteosarcoma ability of PHI.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Author contribution

J.F. designed the experiment and conducted data analysis. X.D. conducted data analysis and wrote the paper. D.L. participated in the experiments. All authors read and approved this paper.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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