

2-Methoxystyandrone inhibits signal transducer and activator of transcription 3 and nuclear factor- κ B signaling by inhibiting Janus kinase 2 and I κ B kinase

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Key words

2-Methoxystyandrone, anticancer drug, Janus kinase 2/signal transducer and activator of transcription 3 pathway, natural compound, tumor necrosis factor- α /nuclear factor- κ B pathway

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Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that mediates cytokines and growth factors-directed transcription. An activated STAT3 by itself can cause cellular transformation and directly contributes to oncogenesis.^(1,2) Constitutive activation of STAT3 signaling is associated with enhanced cell proliferation in many different tumors, including breast, prostate, head and neck, skin and colon cancer, and melanoma.^(3–8) Small molecule inhibitors of Janus kinase 2 (JAK2)/STAT3 pathway are reported to be effective anticancer agents in both cancer cell lines and animal models.^(9–14) Therefore, targeting the JAK2/STAT3 pathway is a promising strategy for treating human tumors.

Nuclear factor- κ B (NF- κ B) and its pathway regulators modulate the expression of genes involved in inflammation, cell survival and proliferation.⁽¹⁵⁾ Constitutive activation of NF- κ B pathway is a common feature of a variety of hematological and solid tumors, demonstrating that NF- κ B plays an important role in carcinogenesis.⁽¹⁶⁾ Given the importance of the NF- κ B

Constitutive activation of the signal transducer and activator of transcription 3 (STAT3) or the nuclear factor- κ B (NF- κ B) pathway occurs frequently in cancer cells and contributes to oncogenesis. The activation of Janus kinase 2 (JAK2) and I κ B kinase (IKK) are key events in STAT3 and NF- κ B signaling, respectively. We have identified 2-methoxystyandrone (2-MS) from a traditional Chinese medicinal herb *Polygonum cuspidatum* as a novel dual inhibitor of JAK2 and IKK. 2-MS inhibits both interleukin-6-induced and constitutively-activated STAT3, as well as tumor necrosis factor- α -induced NF- κ B activation. 2-MS specifically inhibits JAK and IKK β kinase activities but has little effect on activities of other kinases tested. The inhibitory effects of 2-MS on STAT3 and NF- κ B signaling can be eliminated by DTT or glutathione and can last for 4 h after a pulse treatment. Furthermore, 2-MS inhibits growth and induces death of tumor cells, particularly those with constitutively-activated STAT3 or NF- κ B signaling. We propose that the natural compound 2-MS, as a potent dual inhibitor of STAT3 and NF- κ B pathways, is a promising anticancer drug candidate.

pathway in tumor development, many inhibitors of this pathway are under investigation for cancer therapy.⁽¹⁷⁾

The root of *Polygonum cuspidatum* Sieb. et Zucc. (Polygonaceae) is a well-known traditional Chinese medicinal herb that is used to treat various inflammatory diseases and cancer.⁽¹⁸⁾ Although several active components such as emodin and resveratrol extracted from the root of *P. cuspidatum* have been reported to exhibit anticancer activities,^(19,20) the molecular targets and modes of action of *P. cuspidatum* remain unclear.

In our previous study we reported that 2-methoxystyandrone (2-MS), isolated from the root of *P. cuspidatum*, is a potent inhibitor of interleukin-6 (IL-6)/STAT3 signaling.⁽²¹⁾ In the present study we investigate the molecular mechanisms of 2-MS and find that 2-MS inhibits both the IL-6-induced and the constitutively-activated STAT3 in human cancer cell lines through inhibition of the JAK2 kinase. In addition, 2-MS inhibits tumor necrosis factor- α (TNF- α)/NF- κ B signaling

pathway through inhibition of I κ B kinase β (IKK β). Furthermore, 2-MS inhibits growth and induces death of cancer cells. Our data strongly support the notion that 2-MS is a novel dual inhibitor of JAK2 and IKK β kinases and is a promising anticancer drug candidate.

Materials and Methods

Reagents and antibodies. 2-Methoxystyrene was isolated from the root of *P. cuspidatum* Sieb, as reported previously;⁽²¹⁾ DTT and MTT were purchased from Genebase (Shanghai, China); GSH was obtained from Shanghai Sibas Bioscience (Shanghai, China); IL-6 and IFN- α were from Peprotech (Saint Paul, MN, USA); TNF- α was from R&D Systems (Minneapolis, MN, USA); IFN- γ was obtained from Shanghai Clone (Shanghai, China); anti-phosphorylated STAT3 (Tyr705), anti-phosphorylated JAK2 (Tyr1007/1008), anti-JAK2, anti-phosphorylated JAK1 (Tyr1022/1023), anti-JAK1, anti-phosphorylated TYK2 (Tyr1054/1055), anti-TYK2, anti-phosphorylated I κ B- α (Ser32/36), anti-I κ B- α , anti-phosphorylated IKK- α (Ser176/180 for IKK α and Ser177/181 for IKK β) and anti-IKK- α antibodies were obtained from Cell Signaling Technology (Boston, MA, USA); anti-STAT3, anti-GP130 and anti- α -Tubulin antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA); anti- β -Actin antibody was from Abmart (Shanghai, China); anti-GAPDH antibody was obtained from Kang Chen Bioscience (Shanghai, China); secondary HRP-conjugated antibodies were from Multi Sciences Biotech (Hangzhou, China); and the Apoptosis Detection Kit was from Shanghai MaiYueEr Bioscience (Shanghai, China).

Cell lines and culture. HEK293/NF- κ B cells, gifts from Professor Xin-Yuan Fu (National University of Singapore, Singapore), were HEK293 cells stably transfected with an NF- κ B-responsive firefly luciferase reporter plasmid, and all other cell lines were obtained from the American Type Culture Collection. HEK293/NF- κ B, HeLa, MCF-7, U87MG and SK-OV-3 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) FBS (Gibco), 100 μ g/mL ampicillin and 100 μ g/mL streptomycin. Jurkat, U937, THP-1, 786-0, Bel-7404, PC-3, BGC, A549 and H460 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 100 μ g/mL ampicillin and 100 μ g/mL streptomycin. DU-145 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μ g/mL ampicillin and 100 μ g/mL streptomycin. HepG2 cells were cultured in Minimum Essential Medium α medium (Gibco) supplemented with 10% (v/v) FBS, 100 μ g/mL ampicillin and 100 μ g/mL streptomycin. All cell lines were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Luciferase assay. HEK293/NF- κ B cells were seeded into 96-well cell culture plates and allowed to grow for 48 h and cells were then treated with 2-MS for 2 h followed by stimulation with 2 ng/mL TNF- α for 5 h. Luciferase activity was determined using the Promega luciferase kits according to the manufacturer's instruction (Promega, Madison, WI, USA). All luciferase assay experiments were repeated at least twice.

MTT assay. Cell growth rate was measured by MTT assay. Briefly, approximately 4500–12 000 cells were seeded into 96-well plates. After 24 h, cells were treated with vehicle control (DMSO) or compounds for 72 h. After treatment, 30 μ L MTT (5 mg/mL) were added to the culture medium. After incubating for 3 h at 37°C, the cells were solubilized in 100 μ L "Triplex Solution" (10% SDS-5% isobutanol-12 mM HCl) for 16 h, and then the absorbance of each well was measured at 595 nm with

a spectrophotometer (TECAN infinite F200; TECAN, Männedorf, Switzerland).

Western blot analysis. Cells were lysed with ice-cold 1 \times Laemmli buffer (Sigma-Aldrich, Saint Louis, MO, USA) and then samples were boiled for 10 min. Proteins were separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (from Millipore, Darmstadt, Hesse, Germany). Membranes were blocked with 5% nonfat dry milk in TBST (1% Tween 20) for 1 h at room temperature. Then they were subjected to primary antibodies in 5% BSA in TBST at 4°C overnight. Membranes were then washed with TBST and incubated with peroxidase-conjugated secondary antibodies at room temperature for 1 h. After washing with TBST, immune complexes on the membranes were detected by enhanced chemiluminescence (Millipore).

Janus kinase 2 and I κ B kinase β *in vitro* kinase assay. The JAK2 (IKK β) *in vitro* kinase assay was performed using JAK2 (IKK β) immunoprecipitates, an HTScan JAK2 (IKK β) Kinase Assay Kit (Cell Signaling Technology) and Streptavidin coated 96-well plates (Sigma). Briefly, HeLa cell (in IKK β assay, HEK293 cells were transfected with plasmids encoding IKK β -FLAG 24 h before lysed) growth in 10-cm dish with 90% confluence was stimulated with 30 ng/mL IL-6 (in IKK β assay, no stimulation) and then lysed with 1 mL lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 0.15% Triton X-100, 0.5 mM DTT [no DTT in IKK β assay], 2 mM sodium orthovanadate [Na₃VO₄], 2 mM sodium fluoride [NaF], 1 mM PMSF and protease inhibitor cocktail [Sigma, 1:1000]) on ice for 30 min. Cell lysates were collected and centrifuged to remove the pellet. The lysates were immunoprecipitated with 1:100 (v/v) JAK2 (Santa Cruz Biotechnology) or FLAG antibody (Abmart) at 4°C for 3 h and then 1:10 (v/v) Protein A/G Plus Agarose (Santa Cruz Biotechnology) was added to the lysates at 4°C for about 16 h. The JAK2 or IKK β immunoprecipitates were then centrifuged and washed twice with lysis buffer and once with kinase reaction buffer (60 mM HEPES [pH 7.5], 5 mM MgCl₂, 5 mM MnCl₂, 25 μ M Na₃VO₄, 200 μ M ATP and 0.5 mM DTT [no DTT in IKK β assay]). JAK2 or IKK β immunoprecipitates were incubated with DMSO or 2-MS for 5 min and then subjected to kinase reaction at 25°C for 30 min in a final volume of 50 μ L kinase reaction buffer containing 1.5 μ M FLT3 (Tyr589) or I κ B-(Ser32) biotinylated peptide substrate. Samples were then processed according to the protocol for the HTScan JAK2 (IKK β) Kinase Assay Kit (Cell Signaling Technology).

Thin layer chromatography and electrospray ionization mass spectrometry analysis. TLC was carried out on pre-coated silica gel F254 plates (Merck, Darmstadt, Germany); these plates were first run successively in methanol solvent system up to the upper edge in order to eliminate impurities. The plates then were dried, and subsequently reactivated by heating at 125°C for 60 min. 2-MS (0.3 mg) and DTT (0.7 mg) were dissolved in 100 μ L of acetone, and applied quantitatively, either as spots, or in bands (1 \times 20 cm) as with fine glass capillaries. TLC spots were developed in the CHCl₃-MeOH (4:1, v/v) solvent system and scanned under UV-366 nm using the Camag TLC Visualizer (Muttentz, Switzerland). The yellow TLC bands of Michael adducts of DTT to 2-MS were scraped from the plates and eluted in acetonitrile. The resultant solution was filtered through a 45- μ m filter for a negative-mode electrospray ionization mass spectrometry (ESI-MS) analysis. ESI-MS was performed on

a Thermo TSQ Quantum Ultra LC-MS/MS instrument (Thermo Fisher Scientific, Waltham, MA, USA).

Results

2-Methoxystypane inhibited both interleukin-6-induced and constitutively-activated signal transducer and activator of transcription 3 in human tumor cells. We screened an extract library of Chinese medicinal herbs for their inhibitory effect on STAT3 signaling using a STAT3 responsive luciferase reporter gene-transfected HepG2 cell line and found 2-MS (Fig. 1), which inhibited STAT3 activity in both a luciferase assay using the HepG2 cells and a western blotting assay using MDA-MB-231 cells.⁽²¹⁾ To confirm the inhibitory effects of 2-MS on the JAK2/STAT3 signaling pathway, we examined its effects on the IL-6-induced phosphorylation/activation of STAT3. 2-MS inhibited both the IL-6-induced and the constitutive phosphorylation/activation of STAT3 in a dose-dependent manner in HeLa cells (Fig. 2a,c). The inhibition was rapid. A 15-min treatment was sufficient to block the activation (Fig. 2b), suggesting direct inhibition of the STAT3 phosphorylation. 2-MS also significantly inhibited the constitutive activation of STAT3 in MCF-7, DU-145 and HepG2 cells (Fig. 2d). Therefore, 2-MS inhibited both the IL-6 induced as well as the constitutive activation of STAT3 in human tumor cells.

2-Methoxystypane inhibited Janus kinase 2 and I κ B kinase *in vitro* kinase activity. The inhibitory effect of 2-MS on STAT3 activation suggested that 2-MS might interfere with the upstream components of the STAT3 signaling pathway.

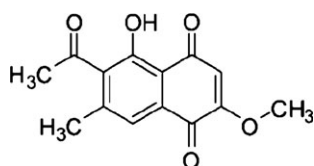


Fig. 1. Chemical structure of 2-methoxystypane.

Because JAK2 is the major kinase to activate STAT3 while GP130 is important for tyrosine phosphorylation of JAK2 in the IL-6/STAT3 pathway,⁽²²⁾ we first analyzed the effects of 2-MS on the expression and phosphorylation of JAK2 and the expression of GP130. In HeLa cells, 10 μ M 2-MS significantly inhibited phosphorylation of JAK2 (Y1007/1008), while it had no effects on the expressions of JAK2 and GP130 (Fig. 3a). In addition, 10 μ M 2-MS significantly inhibited the phosphorylation of JAK2 (Y1007/1008) in HeLa cells without IL-6 stimulation, further suggesting that JAK2 is the direct target of 2-MS (Fig. 3b).

We next analyzed whether 2-MS directly inhibit the JAK2 kinase activity *in vitro*. We immunoprecipitated the JAK2 kinase from HeLa cells and performed an *in vitro* kinase assay. We found that 10 μ M 2-MS significantly inhibited JAK2 *in vitro* kinase activity (Fig. 3c), suggesting that 2-MS is a direct JAK2 kinase inhibitor. In order to investigate the specificity of 2-MS, we tested the effects of 2-MS on eight different human tyrosine or serine/threonine kinases. 50 μ M 2-MS had almost no effect on them, except for a 25% inhibition on IKK β (Fig. 3d). 2-MS also has no effect on epidermal growth factor signaling pathway in HeLa cells (Fig. S1). We next confirmed the inhibition effects of 2-MS on IKK β kinase activity *in vitro*. We immunoprecipitated the IKK β kinase from HEK293 cells and performed an *in vitro* kinase assay. As shown in Fig. 3(e), 2-MS inhibited IKK β *in vitro* kinase activity in a dose-dependent manner. We also examined the effects of 2-MS on JAK1 and TYK2, which are two other kinases of the JAK family. As shown in Fig. S2, 10 μ M 2-MS significantly inhibited the IFN- γ -induced phosphorylation of JAK1 (Tyr1022/1023) and the IFN- α -induced phosphorylation of TYK2 (Tyr1054/1055). Thus, 2-MS is likely a pan-JAK inhibitor.

2-Methoxystypane inhibited tumor necrosis factor- α -induced NF- κ B signal transduction, phosphorylation of inhibitor of nuclear factor- κ B- α (I κ B- α) and I κ B kinase in human tumor cells. Activation of IKK in response to stimuli of various cytokines is a key event in NF- κ B signaling, which is important for tumor cell survival and growth. To fully understand the

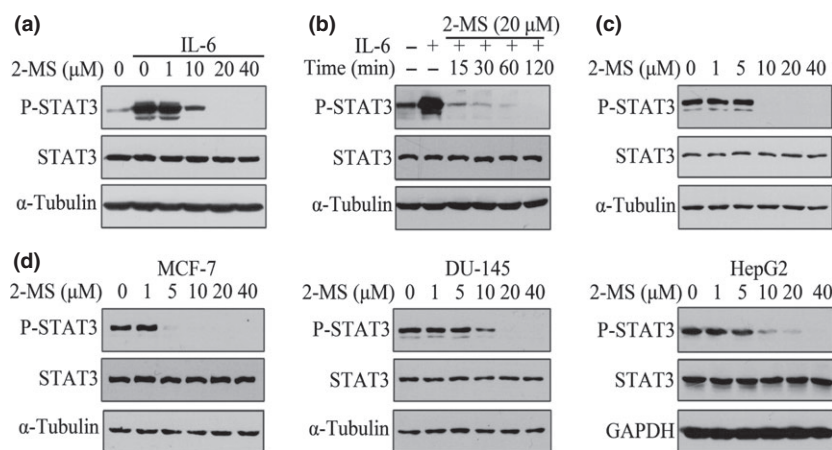


Fig. 2. 2-Methoxystypane (2-MS) inhibited both interleukin-6 (IL-6)-induced and constitutive activation of signal transducer and activator of transcription 3 (STAT3) in human tumor cell lines. (a) HeLa cells were incubated with indicated concentrations of 2-MS for 2 h before stimulation with IL-6 (30 ng/mL) for 15 min. Whole cell lysates were processed for western blot analysis and probed with anti-P-STAT3 (Y705) antibodies. Anti-STAT3 and anti- α -Tubulin antibodies were used as loading controls. (b) HeLa cells were pretreated with 20 μ M 2-MS for indicated time before stimulation with IL-6 (30 ng/mL) for 15 min. Whole cell lysates were subjected to western blot analysis. (c) HeLa cells were incubated with indicated concentrations of 2-MS for 2 h. Whole cell lysates were processed for western blot analysis. (d) MCF-7, DU-145 and HepG2 cells were incubated with indicated concentrations of 2-MS for 2 h and whole cell lysates were subjected to western blot analysis.

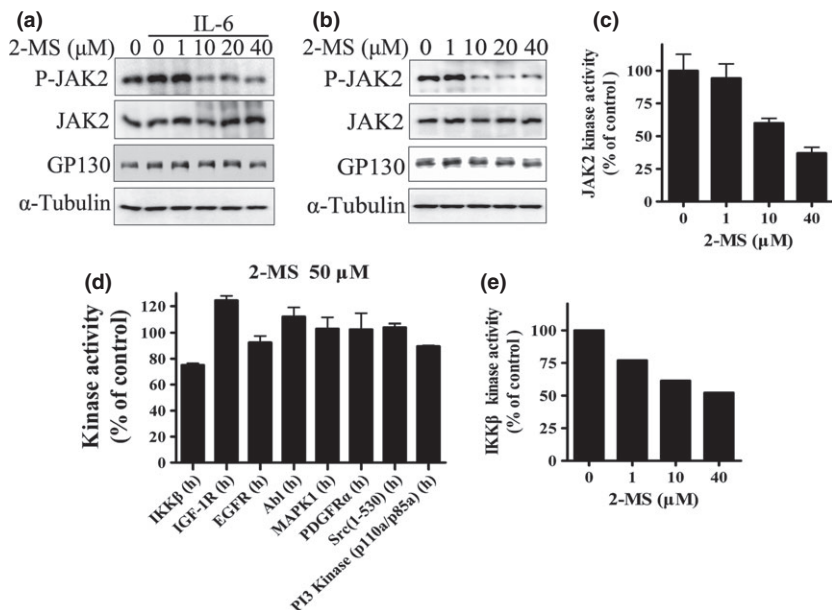


Fig. 3. Effects of 2-methoxystyrene (2-MS) on Janus kinase 2 (JAK2) and activities of other human kinases. (a) HeLa cells were incubated with indicated concentrations of 2-MS for 2 h before stimulation with interleukin-6 (IL-6) (30 ng/mL) for 15 min. Whole cell lysates were processed for western blot analysis and probed with anti-P-JAK2 (Y1007/1008), anti-JAK2 or anti-GP130 antibodies. Anti-α-Tubulin antibody was used as a loading control. (b) HeLa cells were incubated with indicated concentrations of 2-MS for 2 h before being processed for western blot analysis. (c) *In vitro* kinase assay of JAK2. JAK2 protein immunoprecipitated from HeLa cells was subjected to *in vitro* kinase assay in the presence of indicated concentrations of 2-MS. (d) Effects of 2-MS on activities of human kinases. *In vitro* kinase assays were processed by Millipore Kinase Services. (e) *In vitro* kinase assay of IKKβ. IKKβ protein immunoprecipitated from HEK293 cells was subjected to *in vitro* kinase assay in the presence of indicated concentrations of 2-MS.

effects of 2-MS on tumor cells, we next investigated the effects of 2-MS on NF-κB signaling pathway.

We examined the effects of 2-MS on the NF-κB signaling pathway using an NF-κB responsive luciferase reporter gene-transfected HEK293 cell line. We found that 2-MS inhibited TNF-α-induced NF-κB responsive reporter gene activity in a dose-dependent manner, with a half-maximal inhibition dose of 3 μM (Fig. 4a). Parallel to inhibition of

NF-κB signaling, 2-MS only slightly decreased the cell number of HEK293 cells (Fig. 4b), suggesting that the inhibition effect of 2-MS on NF-κB signaling was not caused by reduced cell number.

Activation of NF-κB signaling requires phosphorylation of IκB-α. Hence, we examined the effects of 2-MS on the phosphorylation of IκB-α. As shown in Figure 4(c), 2-MS significantly inhibited the TNF-α-induced phosphorylation of IκB-α

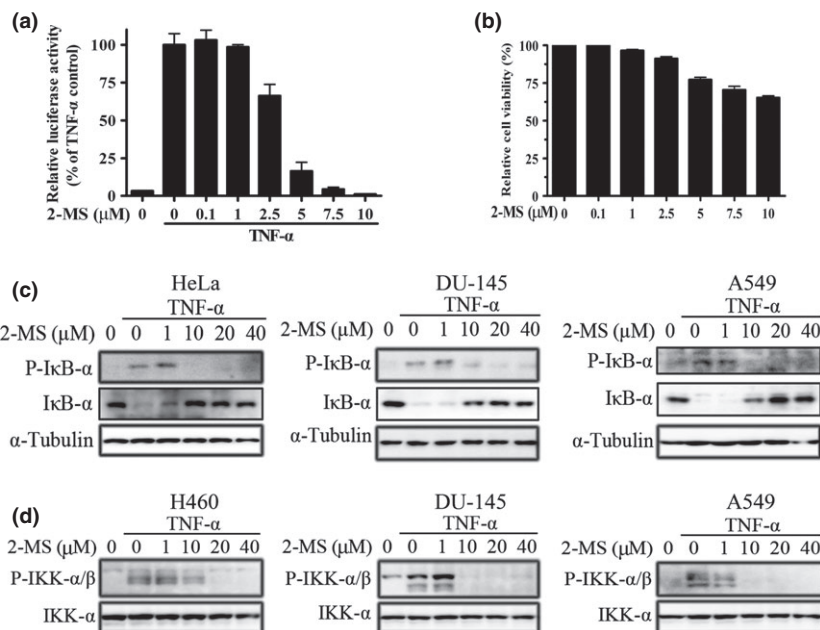


Fig. 4. 2-Methoxystyrene (2-MS) inhibited tumor necrosis factor-α (TNF-α)-induced nuclear factor-κB (NF-κB) signal transduction, phosphorylation of IκB-α and IKK in human tumor cell lines. (a) 2-MS inhibited TNF-α-induced NF-κB regulated luciferase activity. HEK293/NF-κB cells were pretreated with indicated concentrations of 2-MS for 2 h. Luciferase activity was measured following stimulation with TNF-α (2 ng/mL) for 5 h. (b) Effects of 2-MS on cell survival. HEK293/NF-κB cells were treated with indicated concentrations of 2-MS for 7 h. Cell viability was determined by MTT assay. (c) 2-MS inhibited TNF-α-induced phosphorylation of IκB-α. HeLa, DU-145 and A549 cells were incubated with indicated concentrations of 2-MS for 2 h before stimulation with TNF-α (2 ng/mL) for 15 min. Whole cell lysates were subjected to western blot analysis and probed with anti-P-IκB-α (Ser32/36) and anti-IκB-α antibodies. Anti-α-Tubulin antibodies were used as a loading control. (d) 2-MS inhibited TNF-α-induced phosphorylation of IKK. H460, DU-145 and A549 cells were incubated with indicated concentrations of 2-MS for 2 h before stimulation with TNF-α (2 ng/ml) for 15 min. Whole cell lysates were subjected to western blot analysis and probed with anti-P-IKK-α/β (Ser176/180 for IKKα and Ser177/181 for IKKβ) antibodies. Anti-IKK-α antibodies were used as a loading control.

in HeLa, DU-145 and A549 cells at a concentration of 10 μM . After phosphorylation, I κ B- α undergoes a proteasome-mediated degradation. Therefore, the changes of I κ B- α were the opposite of that of phosphorylated I κ B- α (Fig. 4c). Because IKK is the major upstream kinase of I κ B- α in TNF- α -induced NF- κ B signaling, we analyzed the effects of 2-MS on the phosphorylation of IKK. In H460, DU-145 and A549 cells, 2-MS significantly inhibited the phosphorylation of IKK α / β (Ser176/180 for IKK α and Ser177/181 for IKK β) at a concentration (10 μM) similar to that used for inhibition of the TNF- α -induced luciferase activity and the phosphorylation of I κ B- α . Thus, 2-MS, as an IKK β kinase inhibitor, inhibited the TNF- α -induced phosphorylation of IKK, I κ B- α and NF- κ B signal transduction in human tumor cells.

Effects of 2-methoxystyrene on signal transducer and activator of transcription 3 and nuclear factor- κ B signaling can be reversed by DTT or GSH and were irreversible. As shown in Fig. 1, 2-MS contains three α,β -unsaturated carbonyl groups which are reactive with thiols. The thiols of cysteines in JAK2 and IKK β are reported to be important for their kinase activities and several compounds with thiol-reactive groups have been found to covalently bind to Cysteine 179 of IKK β and inhibit its kinase activity.^(23–26) Therefore, we investigated whether the inhibitory effects of 2-MS on JAK2 or IKK β kinase activity were through interacting with their cysteine thiols. We used two thiol-containing compounds, DTT or GSH, to compete with JAK2 or IKK β on interacting with 2-MS to test whether the inhibitory effects of 2-MS on the STAT3 or NF- κ B pathway could be alleviated by the two thiol-containing compounds. As shown in Figure. 5(a) and (c), after incubation

with DTT or GSH, the inhibitory effects of 2-MS on STAT3 and NF- κ B pathways were abolished. Therefore, 2-MS may inhibit the STAT3 and NF- κ B pathways through interacting with the thiols of JAK2 and IKK β via its α,β -unsaturated carbonyl groups. To further demonstrate the 2-MS-thiol interaction, 2-MS was mixed with DTT to induce a chemical reaction of Michael addition. The proposed reaction site of 2-MS is illustrated in Fig. 6(a). The Michael adducts of DTT to 2-MS were then examined using TLC and ESI-MS analysis, which detected one product at m/z 412.97 [2-MS + DTT-H]⁻, indicating the addition of DTT to 2-MS (Fig. 6b,c). The results strongly support the possibility that 2-MS reacts with the cysteine thiols of JAK2 and IKK β .

We also found that the inhibition effects of 2-MS on the STAT3 and NF- κ B pathways lasted as long as 4 h after a pulse treatment with the compound (Fig. 5b,d), indicating that 2-MS irreversibly inhibits JAK2 and IKK β , probably through covalent modification of JAK2 and IKK β .

Taken together, these data suggest that 2-MS may covalently modify JAK2 and IKK β and inhibit their kinase activities.

2-Methoxystyrene inhibited growth of human cancer cells. The STAT3 and NF- κ B signaling pathways play an important role in tumor cell survival and growth. Therefore, we analyzed the effects of 2-MS on the growth and survival of a panel of human tumor cell lines. We first examined the 2-MS's effects on HeLa cells using a real-time cell analysis system. As shown in Figure. 7(a), 2-MS exhibited inhibitory effects on HeLa cells and 10 μM 2-MS totally inhibited the cell growth. 2-MS inhibited the growth of all human tumor cell lines we examined with an IC₅₀ between 2.5 to 12.5 μM (Figure. 7b). Many STAT3 and NF- κ B pathway inhibitors inhibit cell growth by inducing apoptosis.^(9–14,24,25) Therefore, we tested the ability of 2-MS to induce apoptosis. As shown in Figure. 7(c), 2-MS mostly induced necrosis of the tumor cells, as indicated by the single PI staining. We also examined the effects of 2-MS on the PARP cleavage in the tumor cells. However, we did not detect the PARP cleavage caused by 2-MS (data not shown).

As a kinase inhibitor of JAK2 and IKK β , 2-MS effectively inhibits the STAT3 and NF- κ B signaling pathways and growth of cancer cells. We examined whether the growth inhibition effects of 2-MS on cancer cells are related to the activation status of STAT3 or NF- κ B pathway. As shown in Figure. 7(d) and (e), prostate cancer cell lines DU-145, PC-3 (which have constitutively activated NF- κ B pathway) and non-small-cell lung cancer (NSCLC) cell line A549 (which has constitutively activated STAT3 pathway) cells are significantly more sensitive than H460 cells (NSCLC cell line which lacked constitutively activated NF- κ B or STAT3 pathway) to growth inhibition of 2-MS. Taken together, these data demonstrate the potential of 2-MS to inhibit growth and induce cell death of human cancer cells, particularly those with constitutively activated STAT3 and/or NF- κ B pathway.

Discussion

The root of *P. cuspidatum* is used as a traditional Chinese medicine to treat cancer.⁽¹⁸⁾ Although many active components from the root of *P. cuspidatum* have been reported to be kinase inhibitors,⁽²⁷⁾ only a few have been found to inhibit JAK2 or IKK β .^(20,28) Here we report the identification of a new dual JAK2 and IKK β kinase inhibitor, 2-MS, from the root of *P. cuspidatum*. Because both STAT3 and NF- κ B pathways play

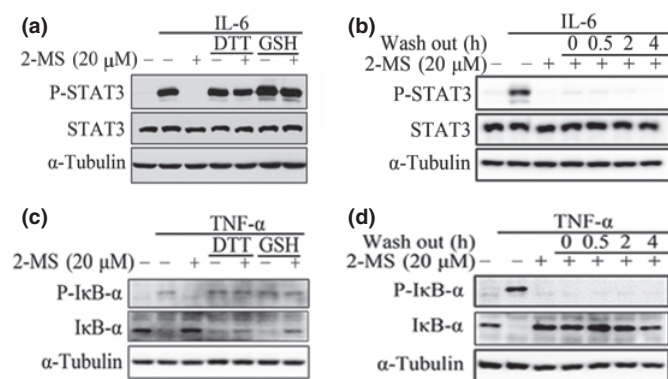


Fig. 5. Effects of 2-methoxystyrene (2-MS) on signal transducer and activator of transcription 3 (STAT3) and NF- κ B signaling can be abolished by DTT or GSH treatment and were irreversible. (a) 20 μM 2-MS were incubated with or without 500 μM DTT or GSH at 37°C for 1 h before being added to HeLa cells for 2 h, followed by stimulation with interleukin-6 (IL-6) (30 ng/mL) for 15 min. Whole cell lysates were processed for western blot analysis and probed with anti-P-STAT3 (Y705) antibodies. Anti-STAT3 and anti- α -Tubulin antibodies were used as loading controls. (b) HeLa cells were pretreated with 20 μM 2-MS for 2 h and then washed with fresh medium for three times. After the indicated time periods, the cells were stimulated with IL-6 (30 ng/mL) for 15 min. Whole cell lysates were processed for western blot analysis. (c) 20 μM 2-MS were incubated with or without 500 μM DTT or GSH at 37°C for 1 h before being added to HeLa cells for 2 h, followed by stimulation with tumor necrosis factor- α (TNF- α) (2 ng/mL) for 15 min. Whole cell lysates were subjected to western blot analysis and probed with anti-P-I κ B- α (Ser32/36) and anti-I κ B- α antibodies. Anti- α -Tubulin antibodies were used as a loading control. (d) HeLa cells were pretreated with 20 μM 2-MS for 2 h and then washed with fresh medium three times. After the indicated time periods, the cells were stimulated with TNF- α (2 ng/mL) for 15 min. Whole cell lysates were processed for western blot analysis.

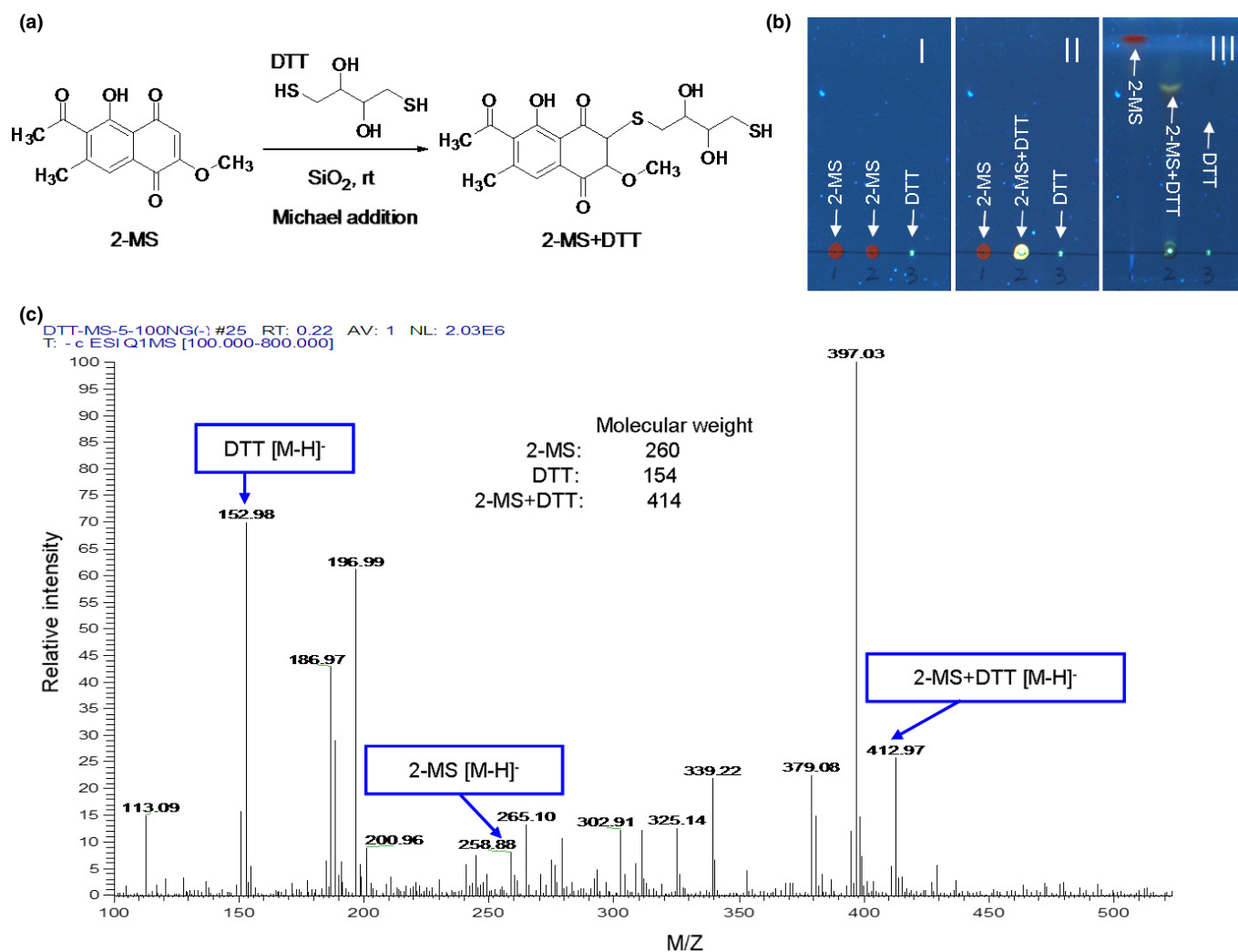


Fig. 6. The covalent interaction of 2-methoxystypane (2-MS) with DTT. (a) The Michael addition reaction of α,β -unsaturated carbonyl of 2-MS with DTT; (b) TLC photography of 2-MS, DTT and Michael adducts of DTT to 2-MS under the ultraviolet light, 366 nm. TLC was performed on silica gel F254 TLC plates and was scanned using the Camag TLC visualizer. The No. III plate was developed in CHCl₃-MeOH (4:1, v/v) solvent system, the spots in the No. III plate: 1: 2-MS (red); 2: 2-MS+DTT (yellow); 3: DTT. (c) Detection of a mixture of DTT, 2-MS and Michael adduct of DTT to 2-MS on the silica gel plate by a negative-mode electrospray ionization mass spectrometry (ESI-MS). The TLC bands of Michael adducts of DTT to 2-MS were scraped from the plates and eluted in acetonitrile. The resultant solution was filtered through a 45 μ m filter and then collected for ESI-MS analysis.

important roles in oncogenesis and tumor growth,^(1,2,16) elucidating the molecular mechanisms of 2-MS in regulating the STAT3 and NF- κ B pathways will help us to understand and better use 2-MS in cancer treatment.

2-Methoxystypane is a potent inhibitor of the STAT3 and NF- κ B pathways. It inhibited the IL-6-induced and the constitutive activation of STAT3 as well as the TNF- α -induced activation of NF- κ B. 2-MS inhibited tumor cell growth, especially those with activated STAT3 and/or NF- κ B pathway. The direct targets of 2-MS are JAK2 and IKK β kinases. 2-MS specifically inhibited JAK2 and IKK β kinase activities while had little effect on the activities of seven other kinases analyzed.

The exact mechanisms of 2-MS inhibiting the JAK2 and IKK β kinases are not clear. However, we have several lines of evidence suggesting that 2-MS may inhibit the JAK2 and IKK β kinase activities by covalently interacting with the JAK2 and IKK β proteins. First, 2-MS has three α,β -unsaturated

carbonyl groups which are thiol-reactive and formed covalent linkages with DTT. Second, the inhibitory effects of 2-MS on the STAT3 and NF- κ B pathways were abolished after incubating with DTT or GSH, suggesting that the α,β -unsaturated carbonyl groups in 2-MS are active groups that may form adducts with the thiols of the cysteines in the JAK2 and IKK β proteins. Third, the inhibition effects of 2-MS on STAT3 and NF- κ B pathways lasted for as long as 4 h after a pulse treatment with the compound, suggesting that the inhibition effects of 2-MS were irreversible. Taken together, these data suggest that 2-MS may covalently modify JAK2 and IKK β and inhibit their kinase activities.

Constitutively-activated STAT3 and/or NF- κ B pathway contribute to malignant progression by preventing apoptosis.^(2,29) Many small molecule inhibitors of the STAT3 or NF- κ B pathway inhibit tumor cell growth and induce apoptosis.^(9-14,24,25) However, our data demonstrated that 2-MS induced necrosis instead of apoptosis of HeLa cells,

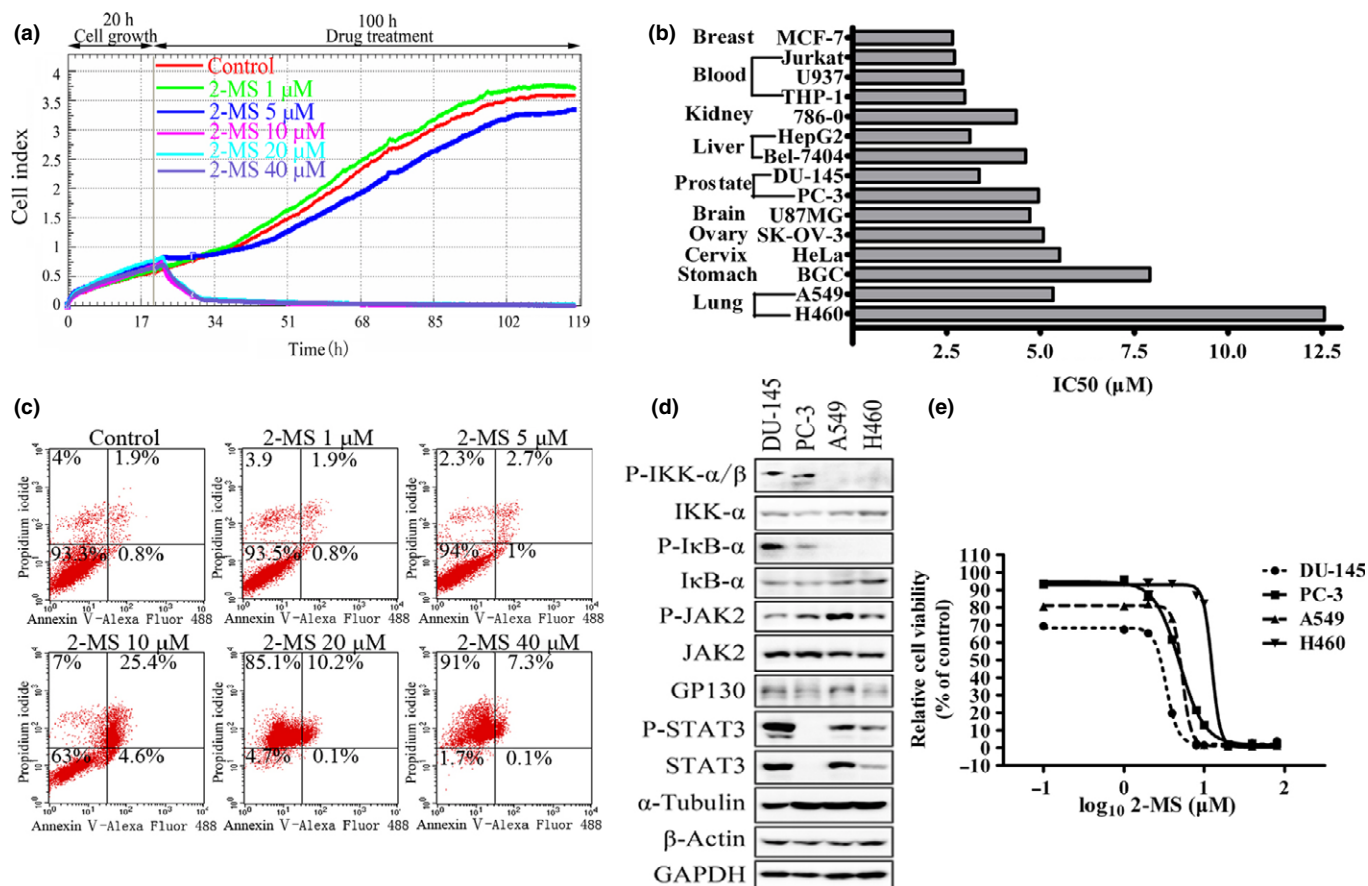


Fig. 7. 2-Methoxystypane (2-MS) inhibited growth of human cancer cells. (a) Real-time cell analysis (RTCA) system analysis of 2-MS's effects on HeLa cells. (b) IC₅₀ of 2-MS on viability of different human tumor cell lines for 72 h. (c) HeLa cells were pretreated with indicated concentrations of 2-MS for 24 h. Cells were then stained with Annexin V-Alexa Fluor 488 Apoptosis Detection Kit before analysis of apoptosis through flow cytometry. (d) Lysates from four human prostate and lung cancer cell lines DU-145, PC-3, A549 and H460 were processed for western blot analysis using specific antibodies as indicated. (e) DU-145, PC-3, A549 and H460 cells were treated with 2-MS at various concentrations for 72 h and then processed for MTT assay.

2-MS also did not cause PARP cleavage in HeLa and some other cancer cells (data not shown). It is possible that 2-MS has other targets in addition to JAK2 and IKKβ kinases and the cell death induced by 2-MS is probably a result of the combination effects of these targets.^(30–32)

In summary, our data reveals that 2-MS, a natural compound isolated from *P. cupidatum*, potently inhibits the STAT3 and NF-κB pathways through specifically inhibiting the JAK2 and IKKβ kinase activities. Furthermore, 2-MS demonstrates strong anticancer activity on a panel of cancer cell lines. These data strongly indicate that 2-MS is a new anticancer drug candidate.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Effects of 2-methoxystypandrone (2-MS) on epidermal growth factor signaling pathway in HeLa cells.

Fig. S2. 2-methoxystypandrone (2-MS) inhibited phosphorylation of JAK1 and TYK2.