Supporting Information

Small-molecule STAT3 signaling pathway modulators from

*Polygonum cuspidatum*

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Materials and Methods

General experimental procedures

The $^1$H and $^{13}$C spectra were recorded in CDCl$_3$ on an Avance 400 Bruker spectrometer operating at 400 and 100 MHz, respectively, or on an Avance 500 Bruker (500 MHz: $^1$H and 125 MHz: $^{13}$C). The chemical shifts were expressed in ppm as $\delta$ values relative to tetramethylsilane (TMS) as an internal standard. Mass spectra (ESIMS) were recorded on a DSQ ESI-mass spectrometer (Thermo). TLC was performed on silica gel 60, and detection was provided by UV at 254 and 366 nm, spraying with 10% H$_2$SO$_4$-EtOH followed by heating at 150°C. Column chromatography was on silica gel (48-74 µm, Qindao Marine Chemical). Analytical HPLC was performed on a Agilent 1200 HPLC system equipped with analytical C$_{18}$ columns (250 x 4.5 mm i.d. stainless steel, 10 µm; Waters); preparative HPLC was
performed on an Elite P270 preparative HPLC system equipped with preparative C$_{18}$ columns (150 x 30 mm i.d. stainless steel, 10 µm; Waters), the gradient mobile phase was MeOH-H$_2$O, and the elution was monitored with a UV-vis diode array detector (190-600 nm). Sepacore flash chromatography (BUCHI) was carried out on silica gel chromatography (40-60 µm, 4.1 × 23.5 cm, 120 g; Agela Technologies).

**Plant material**

The roots of *Polygonum cuspidatum* (Polygonaceae) were purchased from Guangzhou Zhixin Medicine Health Co. Ltd. in 2009. The identity of the plant samples was verified by Mr R.-T. Zhan. A voucher specimen (PC091101) of these materials was deposited for reference in the Research Center of Medicinal Plants Resource Science and Engineering, Guangzhou University of Chinese Medicine. The samples were stored in the shade at room temperature and pulverized before use.

**Extraction and bioassay-guided isolation**

Dried powdered roots of *Polygonum cuspidatum* (392 g) were extracted with EtOAc (2000 mL x 3) and MeOH (2000 mL x 3) at 45°C for 30 min using a 40 kHz ultrasonic bath (Kun Shan Ultrasonic Instruments). The filtrates were evaporated under vacuum to give the EtOAc (15.3 g) and MeOH (37.2 g) extracts. The extracts were evaluated in a STAT3-dependant luciferase reporter gene assay in HepG2 cells. The EtOAc extract was found to exhibit 100% inhibition activity at 40 µg/mL, whereas the MeOH extract was inactive. A 120 mg aliquot of the crude EtOAC
extract was dissolved in a 1:1 mixture of EtOAc-MeOH and filtered on a polyamide cartridge. The filtered extract was then fractionated on a semi-preparative C$_{18}$ column [(250 x 7.8 mm i.d. stainless steel, 10 µm, Waters); mobile phase: MeOH-H$_2$O (20:80 → 100:0); flow rate: 4 mL/min] to give six microfractions. Microfraction 5 (f-5) was shown to inhibit 100% STAT3 activity at 20 µg/mL and used as a positive reference.

The EtOAc extract (11 g) was then submitted to Sepacore flash chromatography (BUCHI) on silica gel column (40-60 µm, 4.1 × 23.5 cm, 120 g; Agela Technologies) and eluted with a step gradient of heptane-EtOAc [100:0 (500 mL)→0:100 (500 mL)] and EtOAc-MeOH [100:0 (500 mL)→0:100 (500 mL)] to obtain twelve subfractions based on the TLC profile. The active fractions Fr.6-Fr.8 (1000 mL, 863 mg) were subjected to additional chromatography. Fr.6 (400 mL, 473 mg) was separated by a silica gel column (25-40 µm, 3.2 × 15 cm) chromatography eluting with a gradient of heptane-EtOAc as solvent [99:1 (500 mL)→80:20 (500 mL)] to give emodin (2, 119 mg, 0.03%) and physcion (4, 42 mg, 0.01%). Fr.7 (400 mL, 119 mg) was applied to a preparative C$_{18}$ column (150 x 30 mm i.d. stainless steel, 10 µm; Waters) eluting with a stepwise gradient of MeOH-H$_2$O (65:35→100:0) at 16 mL/min to yield 2-methoxystypandrone (1, 13 mg, $t_R= 9.5$ min, 0.003%). Fr.8 (200 mL, 271 mg) was chromatographed over a Sephadex LH-20 column using MeOH-H$_2$O [50:50 (720 mL)→100:0 (720 mL)] as eluting solvent to afford seventeen sub-fractions. Further separation of the sub-fractions Fr.8.3-Fr.8.9 (210 mL, 64 mg) by HPLC [using a preparative C$_{18}$ column (150 x 30 mm i.d. stainless steel, 10 µm; Waters); mobile phase: MeOH-H$_2$O (30:70→100:0); flow rate: 16 mL/min] resulted in the isolation of
Cell lines and culture

HepG2/STAT3 cells, a gift of Prof Xinyuan Fu (National University of Singapore, Singapore), were HepG2 cells stably transfected with a STAT3-responsive firefly luciferase reporter plasmid. Human breast cancer cells such as MDA-MB-231, MDA-MB-453, and MDA-MB-468 were obtained from the American Type Culture Collection. HepG2/STAT3 cells were cultured in α-MEM (Life Technologies) with 10% FCS (Life Technologies). Human breast cancer cells were cultured in DMEM (Life Technologies) with 10% FCS.

STAT3-dependent luciferase reporter assay

The inhibitory activities on IL-6/STAT3 signaling were determined by STAT3-dependent luciferase reporter assay described in our previously report [1]. HepG2/STAT3 cells (2 × 10^4 per well) were seeded into 96-well cell culture microplates (Corning) and allowed to grow for 48 h and then treated with test samples for 1 h followed by stimulation with 10 ng/mL interleukin (IL)-6 (BD Biosciences) for 5.5 h. Luciferase activity was determined using the Promega luciferase kit according to the manufacturer’s instruction. The cell number was counted at seeding and was controlled by equal seeding. All luciferase assay experiments cell cultures were repeated at least thrice to minimize the difference caused by cell number. The JAK2/STAT3 inhibitor pyridone 6 (Merck Chemical) was used as a positive control.
MTT assay

The cell growth inhibitory activities on different human breast tumor cells were determined by the MTT assay described in a previous report [1]. About 5000 cells per well were seeded into 96-well plates. Twenty-four hours later, cells were treated with vehicle control (DMSO) or compounds for 72 h. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed by adding a MTT solution (20 μL, 5 mg/mL; Sigma Chemical Co.) to each well and incubating for 3 h at 37°C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in DMSO (150 μL). The absorbance was measured by a microplate reader at a wavelength of 570 nm.

Western blot

Human breast cancer MDA-MB-231 cells were grown to 70-80% confluency and then pretreated with 2-methoxystympandrone at different concentrations for 2 h before being harvested. Cells then were lysed in Laemmli sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was blocked in TBS containing 0.1% Tween 20 (TBST) and 5% nonfat milk for 1 h at room temperature and then incubated for 2 h in TBST containing 5% bovine serum albumin and primary antibodies. Membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for
1 h, and immune complexes were detected by enhanced chemiluminescence. Primary antibodies used in Western blot were mouse anti-STAT3, mouse anti-pY-STAT3 (Tyrosine 705), and mouse anti-α-tubulin. All antibodies were purchased from Cell Signaling Technology.

**Statistic analysis**

All data were analyzed using GraphPad software (Graph-Pad Prism version 4.0 for windows) and presented as mean ± standard deviation of the mean (SD). IC$_{50}$ values (50% concentration of inhibition) were determined through non-linear regression analysis. Three independent experiments were performed.

**References**