Supporting Information to:

Andrographolide Inhibits Human Hepatoma-Derived Hep3B Cell Growth through the Activation of c-Jun N-Terminal Kinase

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Materials and Methods

MTT assay
Cell growth was measured using an MTT assay essentially as previously described [18]. Briefly, Hep3B cells (10⁴ per well) were pretreated with or without MAPK inhibitors for 15min, and then treated with various doses of andrographolide for 24h. 500µg/mL 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was added and incubated with cells for 4 h in a CO₂ incubator. The functional mitochondrial succinate dehydrogenases in cells can convert MTT to formazan that generates a blue color. Because the extent of this conversion is proportional to the number of surviving cells, the MTT assay is widely used to quantify viable cells. At last the formazan was dissolved in 10% SDS-5% iso-butyl alcohol-0.01 M HCl in a CO₂ incubator for 12 h. The intensity was estimated by measuring absorbance at 570/630 nm in an ELISA plate reader.

Preparation of andrographolide
Leaves of Andrographis paniculata were collected from Linquan, Anhui province, China and authenticated by Prof. Wang Zhengtao. The voucher specimen (No.2001-18) was deposited in the Herbarium of Shanghai University of Traditional Chinese Medicine (Shanghai, China). The dried leaves were powdered and macerated in 95% ethanol and kept at room temperature for 3 days. After filtration, the ethanol solution was evaporated in vacuum to remove the solvent and partitioned with ethyl acetate. The ethyl acetate soluble portion was recovered from solvent and subjected to silica gel column and eluted with petroleum-acetone in gradient. The eluates were collected and monitored by thin layer chromatography, and similar fractions were combined. Andrographolide was obtained from the petroleum eluates after recrystallization. Andrographolide was structurally elucidated based on ¹H-nuclear magnetic resonance (NMR) (Fig. 1S) and ¹³C-NMR (Fig. 2S) spectral evidences. The structure was shown in Figure 1. The purity of the compound was more than 98% as determined by high pressure liquid chromatography (HPLC) analysis.
DNA fragmentation assay

DNA fragmentation was visualized by agarose gel electrophoresis as previously described [19]. Briefly, Hep3B cells (3 × 10⁶) were cultured in 100-mm dishes and treated with andrographolide (50 μM) for indicated times, and then harvested by pipetting and centrifuging at 1,500×g. The cells were lysed with buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.4% SDS and 100 μg/mL proteinase K, and left at 37 °C overnight. The fragmented DNA in the lysate was extracted with phenol/chloroform/isopropyl alcohol (25:24:1, v/v), and then precipitated for 5 – 10 min in liquid nitrogen with chilled 100% ethanol and 3 M sodium acetate. The DNA pellet was collected by centrifuging at 17,000 × g for 15 min at 4 °C and then washed with 70% ethanol and resuspended in Tris-HCl, pH 8.0, with 100 μg/mL RNaseA at 37 °C for 1 h. The DNA fragments were separated by 2% agarose gel electrophoresis. DNA laddering in the gel was stained with ethidium bromide and photographed in UV light.

Western blot analysis

Cells were washed in ice-cold PBS, lysed in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A. Prior to Western blotting, protein concentrations were assayed and all samples were normalized to equal protein concentration. Samples containing protein were analyzed by SDS-PAGE in 10% (for phospho-p38, phospho-SAPK/JNK, phospho-ERK1/2, total p38, actin) or 15% (for caspase-3, Bcl-xL, Bcl-2) polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, and blocked with 10% non-fat dry milk in TBST [20 mM Tris (pH 8.0), 150 mM NaCl and 0.1% Tween-20]. The membranes were blotted with the primary antibodies (phospho-p38, phospho-SAPK/JNK, phospho-ERK1/2, total p38, caspase 3, Bcl-xL, Bcl-2, actin), then with horseradish peroxidase-conjugated secondary antibodies and detected by enhanced chemiluminescence according to the manufacturer’s instructions. Molecular weights of proteins were estimated by using prestained SDS-PAGE markers. For repeated immunoblotting, membranes were stripped in 62.5 mM Tris (pH 6.7), 20% SDS and 0.1 M 2-mercaptoethanol for 30 min at 50 °C.
Supplementary Fig. 1 H-NMR spectrum of andrographolide

Supplementary Fig. 2 13C-NMR spectrum of andrographolide
**Supplementary Fig 3**

Andrographolide inhibited cell growth in Hep3B and L-02 cells. Cells (10⁴ per well) were plated and incubated with or without various dose of Andro for 24 h, and then the number of viable cells was determined by MTT assay. The results (mean ± SEM, n=6) are expressed in percent of control absence of drug.

**Supplementary Fig 4**

MAPK inhibitors on Hep3B cell growth. Hep3B cells (10⁴ per well) were plated and incubated with or without SE203580 (10μM), SP600125 (2μM) or PD98059 (20μM) for 24h, and then the number of viable cells was determined by MTT assay. Data were mean ± SE of two independent experiments performed in three repeats.