Notoginsenoside R1 Attenuates Experimental Inflammatory Bowel Disease via Pregnane X Receptor Activation

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This file includes: Supplemental Methods, including cell lines, reagents, semi-quantitative and real-time quantitative polymerase chain reaction (qPCR), western blot analysis, PXR-mediated NF-κB repression reporter assay, gene silencing, and TR-FRET assay protocols.

Supplemental Methods

Cell Lines and Reagents. The HT-29 and LS174T human colon adenocarcinoma cell lines and the RAW264.7 mouse macrophage cell line were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Notoginsenoside R1 (3β,6α,12β)-20-(β-D-Glucopyranosyloxy)-3,12-dihydroxydammar-24-en-6-yl2-O-β-D-xylopyranosyl-β-D-glucopyranoside, $C_{47}H_{80}O_{18}$, molecular weight (MW) 933.13, high-performance liquid chromatography ≥ 98%) was kindly provided by the Shanghai R&D Center for the Standardization of Traditional Chinese Medicine, Shanghai, China. Dextran sulfate sodium (DSS) (MW 36000-50000) was acquired from MP Biochemical LLC (Solon, OH). Rifampicin (3-4-Methylpiperazinyliminomethyl, $C_{43}H_{58}N_4O_{12}$, MW 822.94), pregnenolone-16α-carbonitrile (5-pregnenolone-16a-carbonitrile, PCN, $C_{22}H_{31}NO_2$, MW 341.49), SR12813 (Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate, $C_{24}H_{42}O_7P_2$, MW 504.53), trinitrobenzene sulfonic acid (TNBS), lipopolysaccharide (LPS), donkey serum, paraformaldehyde, methylcellulose, formalin, Tween-20, ethanol and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). The NF-κB reporter vector pGL4.32[luc2P/NF-κB-RE/Hygro] and a dual-luciferase reporter assay system were purchased from Promega (Madison, WI). SYBR Premix ExTaq Mix was obtained from Takara Bio Inc. (Otsu, Japan). A LanthaScreen™ TR-FRET PXR competitive binding assay system, a SuperScript III first-strand synthesis system, Fluor 488-conjugated anti-rabbit IgG (A-21206), Triton X-100, TRIzol, and DAPI reagent were obtained from Invitrogen (Carlsbad, CA). BSA and protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany). The PXR siRNA duplex (sc-44057)，the control siRNA duplex (sc-37007) and the horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The human PXR antibody (ab85451) was obtained from Abcam (Cambridge, MA). The TNF-α reagent and mouse antibodies directed against p-IKK-α/ß (#2078), p-p65 (#3033), p-IκBα (#2859), IκBα (#4812) and ß-actin (#4970) were purchased from Cell

Signaling Technology (Danvers, MA). Mouse TNF-α and IL-6 ELISA kits were obtained from R&D systems (Minneapolis, MN). An MPO activity assay kit was purchased from CytoStore (Calgary, AB, Canada). Agarose, ethidium bromide, RIPA lysis buffer and enhanced chemiluminescence (ECL) western blot detection reagents were purchased from Thermo Scientific (Waltham, MA).

Western Blot Analysis. Cells or colon tissues in RIPA lysis buffer with fresh protease inhibitor cocktail tablets were disrupted by incubation for 30 minutes on ice or by homogenization on ice, respectively, and then collected supernatants by spinning for 10 minutes at 10,000 g, at 4°C. Equal amounts of protein (40 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Antibodies directed against PXR (1:1000), p-IKK-α/β (1:1000), p-p65 (1:1000), p-IκBα (1:1000), IκBα (1:1000 and β-actin (1:2000) were used as recommended by the manufacturers. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse) and developed using ECL detection reagents. The protein bands were quantified by the average ratios of integral optic density (IOD) following normalization to the housekeeping gene β-actin expression.

Semi-quantitative and Real-time Quantitative (q) Polymerase Chain Reaction (PCR). RAW 264.7 macrophages were cotreated with R1 (25 μM) and LPS (2 μg/ml) for 48 h. RNA was extracted using TRIzol reagent. 3 μg of total RNA was converted to first-strand cDNA using a SuperScript III reverse transcriptase kit. The primer sequences used in semi-quantitative PCR amplification are as follows: 5'-ACATTCAGATCCCGAAACGC-3'/5'-TTTGATGTCACGCACGATTT-3' for miNOS,
5'-TGATGGCAGCCTCTTATGTT-3'/5'-AATGAAGTCAGCGTTTCTTGG-3' for mICAM-1,
5'-AAGTTGACCCGTAAATCTGA-3'/5'-TGAAAGGGAATACCATAACA-3' for mMCP-1,
5'-GAAGTCTTTGGTCTGGTGCCT-3'/5'-CTGCTGGTTTGGAATAGTTGCT-3'

for mCOX2, 5’-GCTCTGAGACAATGAACGCTAC-3’/5’-TTTCTTCCACATCTATGCCACT-3’ for mIFNr, 5’-CTGTGAAGGGAATGGGTGTT-3’/5’-CAGGGAAGAATCTGGAAAGGTC-3’ for mTNF-α, 5’-CAGAATGGGAGGTGGTAGTGC-3’/5’-AAGAGTGGCTGGACAGAAGG-3’ for mIL-15, and 5’-TGCTGTCCCTGTATGCCTCT-3’/5’-TTTGATGTCACGCACGATTT-3’ for mß-actin. The DNA thermal cycler conditions used were 94°C for 5 min (pre-denature), and 35 cycles of 94°C for 1 min, annealing at 58°C for 30 s and extension at 72°C for 45 s, followed by a final extension of 72°C for 2 min. PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The amount of target gene was normalized with beta-actin as the internal control gene. The primer sequences used in qPCR amplification are as follows:

5’-GATGAAAGAAAGTCGCCTCG-3’/5’-GCTGGACATCAGGGTGAGTG-3’ for hCYP3A4, 5’-AATAGTTGTCCTAGCACCTGAC-3’/5’-TCTTTCACATCCTCCCTTTG-3’ for hUGT1A1, 5’-AGCCCATCCTGTTTGACTGC-3’/5’-TGTATGTTGGCCTCCTTTGC-3’ for hMDR1a, 5’-GGAAATCGTGCGTGACATTA-3’/5’-TCAGGCAGCTCGTAGCTCTT-3’ for hß-actin, 5’-TGGAGATGGAATACCTGGAT-3’/5’-GAATCATCACTGTTGACCCT-3’ for mCYP3A11, 5’-GCACGAAGTTGTGGTCATAG-3’/5’-AATCTTGATTAAAGGCAGTCC-3’ for mUGT1A1, 5’-TGTGATTGCGTTTGGAGGAC-3’/5’-CCATACCAGAATGCCAGAGC-3’ for mMDR1a, 5’-GGGAATCTTGGAGCGAGTTG-3’/5’-GTGAGGGCTTGGCTGAGTGA-3’ for

miNOS, 5’-CGCTGTGCTTTGAGAACTGT-3’/5’-AGGTCCTTGCCTACTTGCTG-3’ for mICAM-1, 5’-AAGTTGACCCGTAAATCTGA-3’/5’-TGAAAGGGAATACCATAACA-3’ for mMCP-1, 5’-GAAGTCTTTGGTCTGGTGCCT-3’/5’-GCTCCTGCTTGAGTATGTCG-3’ for mCOX2, 5’-CGTGGAACTGGCAGAAGAGG-3’/5’-AGACAGAAGAGCGTGGTGGC-3’ for mTNF-α, 5’-AGCAACAACATAAGCGTCAT-3’/5’-CCTCAAACTTGGCAATACTC-3’ for mIFNr, 5’-GTTCTGCCATTGACCATCTC-3’/5’-TGATACTGTCACCCGGCTCT-3’ for mIL-1α, 5’-GGCTGGACTGTTTCTAATGC-3’/5’-ATGGTTTCTTGTGACCCTGA-3’ for mIL-1ß, 5’-TCAGCAACTGTGGTGGACTT-3’/5’-AGTGATTAGCAAGGGTGAGA-3’ for mIL-2, 5’-ATGGCAATTCTGATTGTATG-3’/5’-GACTCTGGCTTTGTCTTTCT-3’ for mIL-6, and 5’-CAGCCTTCCTTCTTGGGTAT-3’/5’-TGGCATAGAGGTCTTTACGG-3’ for mß-actin. PCR reactions were carried out using SYBR Premix ExTaq Mix in an ABI Prism 7900 real-time PCR System (Life Technologies, Carlsbad, CA). The thermal cycler parameters were as follows: 1 cycle of 95°C for 30 s, then 40 cycles of denaturation (95°C, 5 s) and combined annealing/extension (60°C, 30 s). Gene expression changes were calculated by the comparative Ct method, and the values were normalized to the internal ß-actin control.

PXR-mediated NF-κB Repression Reporter Assay. 2×10^6 HT-29 cells in 100 µl transfection buffer (Cell Line Nucleofector Kit V) were co-electroporated with 1 µg pGL4.32[luc2P/NF-κB-RE/Hygro] luciferase reporter vector (Promega, Madison, WI), 0.5 µg expression vector (pSG5-hPXR or pSG5 control) and 0.1 µg pRL-TK vector

using Lonza Nucleofector II instrument (program Q-009). The cells were transferred to 48-well plate following transfection. After overnight culture, the cells were treated with TNF-α (20 ng/ml) alone or in combination with R1 (0 or 25 μM) for 24 h. The cells were harvested in passive lysis buffer (Promega) and luciferase activity was detected using the dual-luciferase reporter assay system (Promega). Luminescence was detected by Turner Bio-systems Luminometer 20/20n. Normalization and calculations of fold induction were performed as previously published (Dou et al. 2013).

Gene Silencing. 2×10^6 LS174T or HT-29 cells in 100 μl transfection buffer (Cell Line Nucleofector Kit V) were electroporated with PXR siRNA targeting human PXR mRNA using program T-020 (Lonza Nucleofector II instrument). A non-targeting siRNA was used as a negative control. The cells were seeded in 6-well plates following transfection and cultured overnight. For LS174T cells, the cells were treated with or without R1 (25 μM) for 48 h and were harvested for qRT-PCR or western blot studies. For HT-29 cells, which had been previously transfected with the NF-κB-luciferase reporter and the human PXR expression plasmid (pSG5-PXR), the cells were treated with TNF-α (20 ng/ml) alone or in conjunction with R1 (25 μM) for 48 h following transfection. The luciferase activity was measured and the results were expressed as the fold induction compared with that of the control cells.

Time-resolved Fluorescence Resonance Energy Transfer (TR-FRET) Assay. The human PXR binding assay was performed using the LanthaScreen™ TR-FRET PXR competitive binding assay system (Life Technologies, NY) as described previously (Venkatesh, et al., 2011), in which a test compound competes and displaces a reference fluorescent-labeled ligand from the recombinant terbium-labeled PXR-LBD. Briefly, 10 μl of test compounds (final concentration, 0, 12.5, 25, 50 and 100 μM) was placed in quadruplicate into the wells of a black, round-bottomed 384-well assay plate. Next, 5 μl of 4× Fluormone PXR (SXR) Green was added to each well, followed by 5 μl of 4× PXR-LBD (GST)/DTT/4×Tb anti-GST antibody. The plate was gently rocked and then incubated in the dark at room temperature for 1

h. TR-FRET was measured using an EnVision® Multilabel Plate Reader (PerkinElmer, Boston, MA) at an excitation wavelength of 340 nm and at emission wavelengths of 520 and 495 nm. The TR-FRET ratio was calculated by dividing the emission signal at 520 nm by that at 495 nm. Rifampicin (final concentration, 10 μM) and SR12813 (final concentration, 1 μM) was included as positive control of PXR ligands.

References

Dou W, Zhang J, Zhang E, Sun A, Ding L, Chou G et al. (2013) Chrysin ameliorates chemically induced colitis in the mouse through modulation of a PXR/NF-κB signaling pathway. *J Pharmacol Exp Ther* **345**: 473-482.

Venkatesh M, Wang H, Cayer J, Leroux M, Salvail D, Das B *et al.* (2011). In vivo and in vitro characterization of a first-in-class novel azole analog that targets pregnane X receptor activation. *Mol Pharmacol* **80**: 124-135.