Supplementary information

Berberine binds RXRα to suppress β-catenin signaling in colon cancer cells

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Supplementary Methods

Cell culture and treatment

The human KM12C colon cancer cell line, which was established from a Duke's B2 primary colon cancer, was kindly provided by Professor I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX, USA). Human embryonic kidney HEK293T and human colon cancer HCT-116 cells were purchased from the Institute of Cell Biology (China). KM12C cells were cultured in Eagle's minimum essential medium (Eagle's MEM) (Gibco, Grand Island, NY, USA) that had been supplemented with 1 mM sodium pyruvate (Gibco) and vitamins (Gibco). The remaining cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) that had been supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U of penicillin and 100 μg/ml of streptomycin (Life Technologies, Carlsbad, CA, USA). All cell lines were identified by STR profiling every 6 months by the source. Cells were expanded after received and subsequently stored in liquid nitrogen. The storage vials were thawed for experiments and was passaged for at most two months. All cell lines were confirmed to be mycoplasma negative. Berberine (Sigma-Aldrich, St. Louis, MO, USA) and 9-*cis*-RA (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO), which was used as the vehicle control. Prior to treatment with the various compounds, the medium was exchanged with Eagle's MEM (containing sodium pyruvate and vitamins) that did not contain serum.

Reagents and antibodies

Berberine (cat. #141433-60-5), 9-*cis*-RA (cat. #5300-03-8), MG132 (cat. #103476-89-7),

and leupeptin (cat. #103476-89-7) and mouse anti-Flag (cat. #F3165) and anti-HA (cat. #H9658) antibodies were purchased from Sigma-Aldrich, St. Louis, MO, USA. UVI3003 (cat. #sc-358586), rabbit anti-RXRα (cat. #sc-774) and anti-GAL4 (cat. #sc-577) antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Rabbit anti-cyclin D1 (cat. #2922), anti-$p21^{WAF1/CIP1}$ (cat. #2947), mouse anti-β-catenin (cat. #2677) and anti-Cdc2 (cat. #9116) antibodies were purchased from Cell Signaling Technology, Boston, MA, USA. Rabbit anti-PCNA (cat. #ab2426) was purchased from Abcam, USA. Mouse anti-c-Myc (cat. #118154150001) antibody was purchased from Roche, Nutley, NJ, USA. Rabbit anti-c-Cbl (cat. #25818-1-AP) antibody was purchased from Proteintech, Chicago, USA. PageRuler™ Prestained Protein Laddder (Marker, cat. #26616) purchased from Thermo Scientific, Waltham, USA.

Plasmids

The ligand-binding domain (LBD, residues 223-462) of human RXRα (which was purchased from Addgene, USA), human HNF4α2 and c-Cbl (purchased from Addgene, USA), β-catenin/S33Y and Wnt1 (provided by Qiao Wu, Xiamen University, Xiamen, China) were subcloned into different vectors as required, including pBIND (Promega, USA), pACT (Promega), pCMV-HA, p3×FLAG-CMV-14 and pET-14b (Invitrogen, USA). The TOPflash and FOPflash firefly luciferase plasmids were created as previously described[1]. His-hLXRα-LBD (residues 160-402) or His-hLXRβ-LBD (residues 215-461) in pET-24a plasmids were kindly provided by Yong Li (Xiamen University, Xiamen, China). hRARγ-LBD (residues 182-423) purchased from Addgene was constructed into pET-32a

(Invitrogen). The pGL3-pro-RXRE-luc, pGL3-pro-LXRE-luc, pGL3-pro-FXRE-luc, and pGL3-pro-PPRE-luc plasmids were kindly provided by Xu Shen (Shanghai Institute of Materia Medica, China) and Wen Xie (University of Pittsburgh, USA).

The RXRα or RXRα-LBD point mutations were generated using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's recommended instructions and verified using sequencing (Invitrogen).

Purification of His-tagged fusion proteins

His-tagged fusion proteins were overexpressed in *E. coli* strain BL21(DE3) cells. The cells were grown in Luria-Bertani medium at 37℃ to an OD_{600} of 0.8-1.0, followed by 0.1 mM isopropyl-1-thio-b-D-galactopyranoside induction at 18°C for 16 hours. Cells were then harvested by centrifugation and resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.5% (v/v) Triton X-100) supplemented with PMSF (1 mM), and then subjected to sonication. After centrifugation, the cleared lysate (supernatant) were collected and incubated with 50% Ni-NTA slurry (Thermo Scientific, 200 μl per 4 ml of the cleared lysate) at 4°C for 1 h. The lysate-Ni-NTA mixture were then loaded into a column, and washed twice with 800 μl wash buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 50 mM imidazole), then the his-tagged fusion protein were eluted 4 times with 100 μl elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 250 mM imidazole). The purified proteins were further dialyzed against phosphate-buffered saline (PBS) or phosphate buffer, as needed.

Real-time PCR

Total RNA was isolated using the RNeasy Kit (QIAGEN, Valencia, CA, USA) and reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using THUNDERBIRD^{TM} SYBR$^{®}$qPCR Mix (Toyobo, Japan) with the specific primers listed in the table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were used as normalization controls.

Table 1 Primers used in quantitative PCR

Genes	Primer sequences
p21$^{WAF1/CIP1}$	F: 5'-CCACTGGAGGGTGACTTCG-3'
	R: 5'-TGCAGCAGAGCAGGTGAGG-3'
β-catenin	F: 5'-CCGCATGGAAGAAATAGTTGAAG-3'
	R: 5'-CAATTCGGTTGTGAACATCCC-3'
RXRα	F: 5'-AGACCCAACGCCAACACCT-3'
	R: 5'-CAGCGCCGGAAACGACAA-3'
c-Cbl	F: 5'-TCCCTCAAGTGCTTCTGCTC-3'
	R: 5'-GTCGGGATTCTGCTCCAACA-3'
FOXO3A	F: 5'-TCAATCAGAACTTGCTCCACCA-3'
	R: 5'-GGACTCACTCAAGCCCATGTTG-3'
APOE	F: 5'-TTGCTGGTCACATTCCTG-3'
	R: 5'-TTCAACTCCTTCATGGTCTC-3'
ABCA1	F: 5'-AACAGTTTGTGGCCCTTTTG-3'
	R: 5'-AGTTCCAGGCTGGGGTACTT-3'

COX2	F: 5'-TGCATTCTTTGCCCAGCACT-3'
	R: 5'-AAAGGCGCAGTTTACGCTGT-3'
GADPH	F: 5'-CCACTCCTCCACCTTTGAC-3'
	R: 5'-ACCCTGTTGCTGTAGCCA-3'

Immunofluorescent staining

The cells were seeded and cultured overnight on microscope coverslips (Thermo Fisher Scientific). After berberine treatment, the cells were fixed in 4% paraformaldehyde, incubated with primary antibodies as needed for 2 h, and then incubated with secondary antibodies conjugated with Cy3 and FITC (Molecular Probes, USA) for 2 h (protected from light). Nuclear localization was assessed with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Images were taken using a laser-scanning confocal microscope (Olympus, FV1000, Japan) at the same voltage level and analyzed using FV10-ASW 2.0 Viewer Software.

Co-immunoprecipitation and western blot analysis

Cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing 1 mM PMSF (Sigma-Aldrich) and a mixture of protease inhibitors (Roche), and then centrifuged at 14,000 g, 4°C for 30min. The supernatant were collected, and then incubated with protein A/G agarose and the antibody needed for 3 h. After centrifugation, the pellet was washed with pre-chilled lysis buffer for 3 times, and boiled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Then the supernatant

were collected and subjected to western blot analyses after separation using SDS-PAGE. The immunoreactive products were detected using a horseradish peroxidase (HRP)-based system (Millipore, USA) and quantified using Quantity One software (Bio-Rad).

Molecular modeling

The 3D geometry data file for the RXRα-LBD was obtained from the Protein Data Bank (http://www.pdb.org/pdb/explore/explore.do?structureId=3OAP). The 3D geometry of berberine was created and optimized using an Internal Coordinate Mechanics (ICM) Ligand Editor software package. The Molsoft ICM 3.7c program (http://www.molsoft.com/about.html) was used to investigate the binding of berberine to the RXRα-LBD. The mutation site was simulated and minimized using an ICM tool. The search was based on the Lamarckian genetic algorithm. Default values were selected for the "Docking Searching Parameter" and "Docking Run Parameter." The Pymol 1.41 program was used to generate images of berberine and the RXRα-LBD.

Cell proliferation assay

Cell proliferation rates were assessed using the MTT assay or EdU assay.

The MTT assay is based on the mitochondrial conversion of MTT (light yellow) to formazan (blue). Briefly, the cells were seeded at a density of 2×10^5 per well in 96 well culture plates. After adherence, they were treated with the indicated concentrations of berberine (Sigma-Aldrich) for 15 h (4 wells per condition). MTT (Sigma-Aldrich) was then added at a concentration of 50 μg per well. Three hours later, the medium was replaced with

150 μl of DMSO. The number of viable cells was evaluated by measuring the absorbance at an OD of 570 nm using a Model 680 microplate reader.

The EdU assay was carried out using EdU assay kit (Ribobio, China). Briefly, cells were seeded at 1×10^4 per well in 96-well plates. After drug treatment, cell medium was replaced with new medium containing 50 μM EdU and incubated for additional 4 h at 37°C. Then the cells were fixed with 4% formaldehyde for 15 min at room temperature and incubated with 2 mg/ml glycine solution for 5 min. After washed with PBS for three times, each well of the cells were reacted with 100 μl of 1×Apollo® reaction cocktail for 30 min, and then permeabilized three times with 0.5% Triton X-100 for 10 min at room temperature. After washed with PBS for three times, the nuclei were stained with Hoechst 33342 (5 μg/ml) for 30 min and visualized under a fluorescent microscope. Similar results were obtained in at least three independent experiments.

Immunohistochemistry

Xenograft tumor tissue specimens were fixed in formalin and embedded in paraffin. Five-micrometer sections were dewaxed and incubated with 0.01 Msodium citrate for antigen retrieval. Colon sections were then blocked using 5% goat serum in PBS and stained with the related antibodies overnight at 4°C, followed by an immunostaining kit (Biocare Medical, LLC). The sections were then processed using the ImmPACT™ DAB Substrate (Vector Laboratories, Inc.). Photomicrographs were obtained using an Olympus BX41 microscope, and the labeling signal was measured using an image analyzer (Image-Pro Plus 5.0).

Supplementary Figure legend

Supplementary Figure S1

(**a**) Effect of berberine (Ber) on the transcriptional activity of RXRα-involved response elements. KM12C cells were cotransfected with pRL-TK and reporter vector for FXRE, LXRE or PPRE. After 24 h of transfection, cells were treated with different doses of berberine for 15 h. The basal level of transcriptional activity in the vehicle-treated group was normalized to 1. (**b**) Synergistic effect of berberine and GW3965 on the transactivational activity of RXR/LXR heterodimer. KM12C cells cotransfected with pGL3-LXRE and pRL-TK were treated with different concentrations of berberine in the absence and presence of GW3965 for 15 h. (**c**) Berberine induced moderate decrease in RXRα expression level as detected by western blot (**Left**) and real-time PCR (**Right**) assays. (**d**) RXRα knockdown efficiency was determined by western blot (**Left**) and real-time PCR (**Right**) analyses. (**e**) Berberine elevated the transcription levels of RXRα target genes in an RXRα-dependent manner using real-time PCR analysis. (**f**) Berberine induced transformation of RXRα-LBD from tetramer to dimer as assayed by native PAGE. Purified His-RXRα-LBD proteins were incubated with an equimolar amount, 2- or 5-folds of berberine in the molar amount for 15 minutes at room temperature, and then subjected to native PAGE. All data were presented as mean ± s.e.m. of three independent experiments. * $P< 0.05$, ** $P<0.01$, and *** $P<0.001$ *vs* vehicle control (**a-c, e**) or *vs* shCtrl control (**d**). Significant differences of shRXRα *vs* shCtrl at the same dose of berberine were indicated as $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ (**e**).

Supplementary Figure S2

(**a**) Representative images of the HPLC analysis of concomitant binding of berberine and 9-*cis*-RA (9*c*RA) with RXRα-LBD proteins as described in Figure 3a. (**b**) Synergistic effect of berberine and 9-*cis*-RA on RXRα transactivational activity. KM12C cells cotransfected with pBIND-RXRα-LBD and pG5*luc* for 24h were treated with the indicated dose of berberine and/or 9-*cis*-RA for 15 h. The basal level of transcriptional activity in the vehicle-treated group was normalized to 1. All data were shown as mean ± s.e.m. of three independent experiments. Significant differences of 9-*cis*-RA *vs* vehicle control were indicated as $*P<0.05$, $**P<0.01$ and $***P<0.001$; significant differences of berberine *vs* vehicle control at the same dose of 9-*cis*-RA were indicated as $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$. (**c**) Representative images of the HPLC analysis of berberine binding to RXRα-LBD and its mutants as described in Figure 3g.

Supplementary Figure S3

(**a**) Effect of berberine on cell cycle. KM12C, RKO, SW480 cells were treated with berberine at different doses as indicated for 15 h. Cell cycle analysis were then carried out. (**b**) Berberine up-regulated the expression of $p21^{WAF1/CIP1}$ in an RXRα-dependent manner. KM12C sublines, shCtrl and shRXRα, were treated with berberine for 15 h, and then subjected to real-time PCR analysis. The base expression level of $p21^{WAF1/CIP1}$ in the vehicle-treated group of each cell subline was normalized to 1. Data were shown as mean ± s.e.m. of three independent experiments. Significant differences of berberine *vs* vehicle control were indicated as $**P<0.01$ and $***P<0.001$; significant differences of shRXRα *vs* shCtrl at the same dose of berberine were indicated as $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$.

Supplementary Figure S4

(**a**) Berberine promotes RXRα interaction with β-catenin as indicated by mammalian two hybrid assay. KM12C cells were cotransfected with pBIND-β-catenin/33Y, pACT-RXRα, and pG5*luc* vector, and treated with berberine for 15 h. The basal level of transcriptional activity was normalized to 1. (**b**) Effect of berberine on mRNA expression levels of β-catenin. KM12C cells were treated with different doses of berberine for 15 h. Total RNA was then prepared, and the mRNA levels of β-catenin were analyzed by real-time PCR. The base mRNA expression level in the vehicle-treated group was normalized to 1. (**c**) Binding RXRα is essential for berberine to inhibit colon cancer cell growth. KM12C subline shRXRα were transfected with pCMV5 vector, myc-RXRα(r) or its mutants (the RXRα rescue construct RXRα(r) didn't require silence mutation as shRXRα targeted the 3′-UTR region of RXRα mRNA). 24 h after transfection, cells were treated with 25 μM berberine for 15 h, and then subjected to MTT assay. Basal proliferation rate in each vehicle-treated group was normalized to 1. (**d**) Synergistic effect of berberine and 9-*cis*-RA to promote RXRα interaction with β-catenin as indicated by mammalian two hybrid assay. KM12C cells were cotransfected with pBIND-β-catenin/33Y, pACT-RXRα and pG5*luc* vector, and treated with berberine and/or 9-*cis*-RA for 15 h. (**e**) Berberine decreases the nuclear β-catenin levels in HCT116 cells. Cells were treated with berberine (100 μM), and then subjected to immunofluorescent staining with anti-β-catenin and anti-RXRα antibodies as described in Methods. Scale bar: 5 μm. (**f**) c-Cbl knockdown efficiency was determined by real-time PCR. (**g**) Overexpression of RXRα did not affect the subcellular location of c-Cbl in KM12C cells.

Cells were transfected with GFP-c-Cbl alone or together with Myc-RXRα plasmid, and then subjected to immunofluorescent staining with anti-Myc antibodies as described in Supplementary Methods. (**h**) Overexpression of RXRα increased the expression of c-Cbl in KM12C cells as detected by western blot (**Left**) and real-time PCR (**Right**) analyses. All data were presented as mean ± s.e.m. of three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 and NS non-significant *vs* vehicle or vector control (**a, b, d, f, h**). Significant differences of RXRα mutants *vs* wide-type (WT) at the same dose of berberine were indicated as $^{\#}P$<0.05, $^{\#\#}P$<0.01, and $^{\#\#\#}P$<0.001 (**c**). Significant differences of 9-*cis*-RA *vs* vehicle control at the same dose of berberine were indicated as $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.001 (**d**).

Supplementary Figure S5

Expression levels of PCNA, Ki67, c-Myc and $p21^{WAF1/CIP1}$ in the specimens of xenograft tumors detected by immunohistochemistry experiments were quantified by densitometry. Basal protein levels in each cell subline were normalized to 1. All data represent the mean ± s.e.m. of three independent experiments.* *P*< 0.05 and NS non-significant *vs* control.

Reference

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