Data S1

Materials and methods

Drugs, chemicals, and reagents. The drugs, chemicals and reagents included: Telmisartan (Tokyo Chemical Industry Co.), Trypan Blue (Sigma-Aldrich; Merck KGaA), RPMI-1640 medium (Gibco, Invitrogen; Thermo Fisher Scientific, Inc.), fetal bovine serum (FBS; Wako Pure Chemical Industries), penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories), Cell Cycle Phase Determination Kit (Cayman Chemical Co.), protease inhibitor cocktail (Pro-Prep, Complete Protease Inhibitor Mixture; iNtRON Biotechnology), Human Phospho-RTK Array Kits and Angiogenesis Antibody Array Kits (R&D Systems). Telmisartan was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). Stock solutions were stored at -20˚C.

Cell cycle analysis. To evaluate the mechanism by which telmisartan inhibits tumor growth, the cell cycle and apoptosis analyses were performed using a Cell Cycle Phase Determination Kit. MKN74 cells (1.0×10^6 cells in a 100 mm dish) were treated with or without 100 μM telmisartan for 24 h. The cell cycle distribution was analyzed by measuring the amount of propidium iodide (PI)-labeled DNA in ethanol-fixed cells. The fixed cells were washed with PBS and stored at -20˚C. On the day of analysis, the cells were washed with cold PBS, suspended in 100 μl of PBS plus 10 μl of RNase A (250 μg/ml) and incubated for 30 min. PI (110 μl, final concentration 100 μg/ml) was added to each tube, which was the incubated at 4˚C for at least 30 min. Flow cytometry was performed using a Cytomics FC 500 flow cytometer (Beckman Coulter). The percentage of cells in each phase of the cell cycle were analyzed using Kaluza software (Beckman Coulter).

Western blot analysis. The cells were lysed using a protease inhibitor cocktail on ice for 20 min. Suspensions of lysed cells were centrifuged at 13,000 x g at 4˚C for 5 min, and supernatants were collected and stored at -80˚C. Protein concentrations were measured using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis through 12% acrylamide gels), and the proteins were electrophoretically transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies followed by secondary antibodies. The immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (PerkinElmer Co.) with X-ray film. Primary antibodies and their dilutions were as follows: Anti-β-actin monoclonal antibody (cat. no. A5441; dilution 1:10,000), anti-cyclin D1 (cat. no. AHF0082; dilution 1:1,000), anti-cyclin E (cat. no. MA5-14336; dilution 1:1,000), anti-CDK6 (cat. no. sc-177; 1:1,000), anti-CDK4 (cat. no. sc-749; dilution 1:1,000), anti-CDK2 (cat. no. sc-163; dilution 1:2,000) and anti-phosphorylated retinoblastoma protein (pRb; cat. no. 558385; 1:1,000), anti-Rb (cat. no. sc-50; dilution 1:1,000). The secondary antibodies included horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit IgG (cat. nos. 7074 and 7076; dilution 1:2,000).

Apoptosis assay. Caspase-cleaved cytokeratin 18 (CCK18) expression was evaluated using an M30 Apoptosense ELISA Kit for detecting early during phase apoptosis (1). MKN74 cells (5×10^3 cells) were seeded in 96-well plates and cultured for 24 or 48 h following the addition of 100 μM telmisartan. The ELISAs were performed according to the manufacturer's instructions. The amounts of antigen in the control and treated samples were calculated via interpolation of a standard curve.

Antibody arrays of phosphorylated receptor tyrosine kinases (p-RTKs). Human p-RTKs were assayed using Human Phospho-RTK Array Kits according to the manufacturer's instructions. Briefly, p-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h and incubated with 2 ml of lysate prepared from the previously mentioned cells after protein amounts were normalized. After three washes for 10 min each with TBS plus 0.1% v/v Tween-20 and two washes for 10 min with TBS to remove unbound materials, the membranes were incubated with an HRP-conjugated anti-phosphotyrosine antibody for 2 h at room temperature. The unbound HRP antibody was removed with TBS plus 0.1% Tween-20. Each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co.). The density of the immunoreactive band obtained 118 on this array was analyzed using densitometric scanning (TIc 119 Scanner, Shimizu Co, Ltd., Kyoto, Japan).

Angiogenic profile analysis using an antibody array. A Human Angiogenesis Antibody Array was used according to the manufacturer's protocol. This method is a dot-based assay enabling the detection and comparison of 55 angiogenesis-specific cytokines. Each array membrane was exposed to X-ray film using a chemiluminescence detection system (PerkinElmer Co.). The immunoreactive bands were analyzed using densitometric scanning.

miRNA analyses and data analysis. Total RNA was extracted from tumor samples and cancer cell lines using an miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA samples typically exhibited $A_{260/280}$ ratios of between 1.9 and 2.1 determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). After RNA measurements were performed using an RNA 6000

Nano Kit (Agilent Technologies), the samples were labeled using a miRCURYHy3/Hy5 Power Labeling Kit (Qiagen) and were subsequently hybridized to a human miRNA Oligo chip (v. 21.0; Toray Industries, Tokyo, Japan). The chips were scanned using a 3D-Gene Scanner 3000 (Toray Industries), and the results were analyzed using 3D-Gene extraction version 1.2 software (Toray Industries). To determine the difference in miRNA levels between the telmisartan-treated and control samples, the raw data were analyzed using GeneSpring GX 10.0 software (Agilent Technologies). Quantile normalization was performed on raw data above the background values. Differentially expressed miRNAs were determined using the Mann-Whitney test. The false discovery rate was computed using the Benjamini-Hochberg method. Hierarchical clustering was performed using the farthest neighbor method using the absolute uncentered Pearson's correlation coefficient as a metric. A heat map was produced to illustrate the relative expression intensity of each miRNA, in which the base-2 logarithm of the intensity was median-centered for each row.

Reference

1. Schutte B, Henfling M, Kölgen W, Bouman M, Meex S, Leers MP, Nap M, Björklund V, Björklund P, Björklund B, *et al*: Keratin 8/18 breakdown and reorganization during apoptosis. Exp Cell Res 297: 11-26, 2004.