Supplementary Information for

MMPP Attenuates Non-Small Cell Lung Cancer Growth by Inhibiting the STAT3 DNA-Binding Activity *via* Direct Binding to the STAT3 DNA-Binding Domain

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Supplementary Methods

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Synthesis of BHPB analogs

Compound **1**: Pd(OAc)2 (208 mg, 0.93 mmol) and tributyl phosphine (685 µL, 2.78 mmol) in t-butanol was degassed under argon for 10 min or until the solution became pale yellow. 4-(oxiran-2-yl) phenyl acetate (5.5 g, 30.87 mmol) was then added and the reaction mixture was stirred at 85 °C for 12 h. After completion of the reaction, the solvent was removed in *vacuo* followed by column purification (3:1 hexanes: ethyl acetate) to obtain a pale yellow liquid (2 g, 45%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.66 (s, 1H), 7.29 (d, 2H, *J* = 14.5Hz), 7.2 (dd, 4H, *J* = 3.5, 14.5 Hz), 7.07 (d, 2H, *J* = 14.5 Hz), 6.88 (t, 1H, *J* = 12.5 Hz), 3.72 (d, 1H, *J* = 12.5 Hz), 2.34 (s, 3H), 2.32 (s, 3H). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 338.1154, found 339.1183.

Compound **2**: Potassium carbonate (51 mg, 0.368 mmol) was added to a solution of compound **1** (50 mg, 0.147 mmol) in methanol (6 mL) and stirred at room temperature for 15 min. After completion of the reaction, the solvent was removed in *vacuo,* and DCM (50 mL) was added. Subsequently, the organic layer was washed with water (2×20 mL) and dried over anhydrous $MgSO_4$. Solvents were evaporated and product was purified by column chromatography (1:1 hexane: ethyl acetate), which yielded a yellow liquid (26 mg, 70%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.09 (d, 2H, *J* = 8.5 Hz), 6.94 (d, 2H, *J* = 8.5 Hz), 6.8 (d, 2H, *J* = 8.5 Hz), 6.68 (d, 2H, *J* = 8.5 Hz), 5.8 (t, 1H, *J* = 7.5 Hz), 4.23 (s, 2H), 3.23 (d, 2H, *J* = 7.5 Hz). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 256.1099, found 257.1145.

Compound **3**: To a solution of compound **1** (50 mg, 0.148 mmol) in ethanol (10 mL), NaBH4 (7 mg, 0.185 mmol) was added at 0 °C and stirred at room temperature for 1 h. After completion of the reaction, the solvent was removed in *vacuo.* The compound was then diluted with water (10 mL) and extracted with DCM (2×15 mL). The organic layer was separated and dried over anhydrous $MgSO_4$. Solvent was

evaporated followed by column purification (2:1 hexane: ethyl acetate), which yielded a colorless liquid (37 mg, 78%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (d, 2H, *J* = 14 Hz), 7.18-7.12 (m, 4H), 7.02 (d, 2H, *J* = 14 Hz), 5.93 (t, 1H, *J* = 12.5 Hz), 4.36 (s, 2H), 3.37 (d, 2H, *J* = 12.5 Hz), 2.34 (s, 3H), 2.31 (s, 3H). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 340.13107, found 341.1343.

Compound **4**: To a solution of compound **2** (40 mg, 0.156 mmol) in DMF, potassium carbonate (54 mg, 0.39 mmol) and methyl iodide (29 μL, 0.468 mmol) were added and stirred at room temperature for 12 h. The reaction mixture was diluted with water (15 mL) and extracted with DCM (2×30 mL). The organic layer was separated and dried over anhydrous $MgSO_4$. Solvent was evaporated followed by column purification (2:1 hexane: ethyl acetate), which yielded a colorless liquid (37 mg, 86%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (d, 2H, *J* = 8.5 Hz), 7.06 (d, 2H, *J* = 8.5 Hz), 6.93 (d, 2H, *J* = 8.5 Hz), 6.83 (d, 2H, *J* = 8.5 Hz), 5.86 (t, 1H, *J* = 7.5 Hz), 4.35 (s, 2H), 3.83 (s, 3H), 3.78 (s, 3H), 3.32 (d, 2H, *J* = 7.5 Hz). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 284.14124, found 285.1465.

Compound **5**: To a solution of compound **2** (40 mg, 0.156 mmol) in DMF, 50% NaH (30 mg, 0.624 mmol) was added at 0 °C and stirred for 10 min. Subsequently, methyl iodide (48 μL, 0.78 mmol) was added and the reaction was stirred at room temperature for 1 h. After completion, monitored by TLC, the reaction was quenched with water (10 mL) and extracted with DCM (2×30 mL). The organic layer was separated and dried over anhydrous $MgSO_4$. Solvent was evaporated followed by column purification (3:1 hexane: ethyl acetate), which yielded a pale yellow liquid (40 mg, 70%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (d, 2H, *J* = 15 Hz), 7.10 (d, 2H, *J* = 15 Hz), 6.94 (d, 2H, *J* = 15 Hz), 6.85 (d, 2H, *J* = 15 Hz), 5.90 (t, 1H, *J* = 12.5 Hz), 4.17 (s, 2H), 3.85 (s, 3H), 3.82 (s, 3H), 3.34 (d, 2H, *J* = 12.5 Hz), 3.37 (s, 3H). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 298.15689, found 299.1601.

Compound **6**: To a solution of compound **2** (40 mg, 0.156 mmol) in DMF, 50% NaH (14 mg, 0.296 mmol) was added at 0 °C and stirred for 10 min. Subsequently, benzyl chloride (34 μL, 0.296 mmol) was added and the reaction mixture was stirred at room temperature for 1 h. After completion, monitored by TLC, the reaction was quenched with water (10 mL) and extracted with DCM (2×30 mL). The organic

layer was separated and dried over anhydrous $MgSO_4$. This was followed by solvent evaporation and column purification (3:1 hexane: ethyl acetate), which yielded a colorless liquid (40 mg, 66%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49-7.34, 7.23 (d, 2H, *J* = 14.5 Hz), 7.09 (d, 2H, *J* = 14.5 Hz), 7.03 (d, 2H, *J* = 14.5 Hz), 6.93 (d, 2H, *J* = 14.5 Hz), 5.89 (t, 1H, *J* = 12.5 Hz), 5.1 (s, 2H), 5.07 (s, 2H), 4.37 (s, 2H), 3.35 (d, 2H, *J* = 12.5 Hz). HRMS (ESI) *m/z* $[M+NH4]^+$ cacld. 436.54152, found 454.2336.

Compound **7**: To a solution of compound **2** (40 mg, 0.156 mmol) in DMF, 50% NaH (30 mg, 0.624 mmol) was added at 0 °C and stirred for 10 min. Subsequently, benzyl chloride (63 µL, 0.546 mmol) was added and the reaction mixture was stirred at room temperature for 1 h. After completion, monitored by TLC, the reaction was quenched with water (10 mL) and extracted with DCM (2×30 mL). The organic layer was separated and dried over anhydrous $MgSO_4$. This was followed by solvent evaporation and column purification (3:1 hexane: ethyl acetate), which yielded a colorless liquid (70 mg, 85%).^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47-7.26 (m, 10H), 7.23 (d, 2H, *J* = 8.5 Hz), 7.09 (d, 2H, *J* = 8.5 Hz), 7.03 (d, 2H, *J* = 8.5 Hz), 6.93 (d, 2H, *J* = 8.5 Hz), 5.91 (t, 1H, *J* = 7.5 Hz), 5.09 (s, 2H), 5.05 (s, 2H), 4.55 (s, 2H), 4.24 (s, 2H), 3.37 (d, 2H, *J* = 7.5 Hz). HRMS (ESI) *m/z* $[M+NH4]^+$ cacld. 526.25079, found 544.2787.

Compound **8**: To a solution of compound **1** (50 mg, 0.148 mmol) in ethyl acetate (15 mL), 10% Pd/C was added and stirred at room temperature for 4 h. After completion of reaction, the catalyst was removed by filtration through a celite pad. This was followed by solvent evaporation and column purification (2:1 hexane: ethyl acetate), which yielded a colorless liquid (40 mg, 80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.68 (s, 1H), 7.24 (d, 2H, *J* = 14.5 Hz), 7.17 (dd, 4H, *J* = 10, 14.5 Hz), 7.02 (d, 2H, *J* = 14.5 Hz), 3.55 (t, 1H, *J* = 11 Hz), 2.66-2.53, (m, 2H), 2.49-2.37 (m, 1H), 2.34 (s, 3H), 2.32 (s, 3H), 2.11-1.99 (m, 1H). HRMS (ESI) *m/z* $[M+Na]^+$ cacld. 340.13107, found 363.1249.

Compound **9**: To a solution of compound **10** (70 mg, 0.205 mmol) in methanol (10 mL), potassium carbonate (71 mg, 0.513 mmol) was added and stirred at room temperature for 15 min. After completion of the reaction, the solvent was removed in *vacuo* and DCM (50 mL) was added. Subsequently, the organic layer was washed with water (2×10 mL) and dried over anhydrous $MgSO_4$. Solvent was

evaporated followed by column purification (1:1 hexane: ethyl acetate), which yielded a colorless liquid (40 mg, 76%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.03 (d, 2H, *J* = 8.5 Hz), 6.91 (d, 2H, *J* = 8.5 Hz), 6.76 (d, 2H, *J* = 8.5 Hz), 6.66 (d, 2H, *J* = 8.5 Hz), 3.60-3.58 (m, 2H), 2.65-2.59 (m, 1H), 2.43-2.37 (m, 1H), 2.34-2.28 (m, 2H), 2.08-2.02 (m, 1H), 1.78-1.71 (m, 1H). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 258.12559, found 259.1154.

Compound **10**: To a solution of compound **3** (30 mg, 0.088 mmol) in ethyl acetate (15 mL), 10% Pd/C was added and stirred at room temperature for 4 h. After completion of the reaction, the catalyst was removed by filtration through a celite pad. Solvent was evaporated followed by column purification (2:1 hexane: ethyl acetate), which yielded a colorless liquid (23 mg, 76%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 (d, 2H, *J* = 14.5 Hz), 7.15 (t, 4H, *J* = 13.5 Hz), 6.99 (d, 2H, *J* = 14.5 Hz), 3.76 (br s, 2H), 2.89-2.79 (m, 1H), 2.60-2.45 (m, 2H), 2.31 (s, 3H), 2.27 (s, 3H), 2.14-2.02 (m, 1H), 1.96-1.86 (m, 1H). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 342.14672, found 343.1454.

General procedure for synthesis of compounds 11-16: Heck reaction was used for the synthesis of compounds 11-16, starting from phenyl halide moieties with substituents (2.0 mmol) and allylbenzene moieties with substituents (2.0 mmol). Phenyl halide (2.0 mmol) and allylbenzene (2.0 mmol) were added to a mixture of triphenylphosphine (105 mg, 0.4 mmol), Pd(OAc)2 (44.9 mg, 0.2 mmol), and tributylamine (451 μL, 1.9 mmol) in a 25 mL round bottom flask and the reaction mixture was stirred for 2 h at 45 °C or for 2 days at ambient temperature under argon atmosphere. The product was purified by flash silica gel chromatography using hexane and ethyl acetate (3:1 mixture v/v) as the mobile phase. Compound **13** (MMPP): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 (d, 2H, *J* = 8.0 Hz), 6.88 (d, 1H, *J* = 9.0 Hz), 6.86 (d, 2H, *J* = 9.0 Hz), 6.76 (d, 1H, *J* = 8.0 Hz), 6.75 (s, 1H), 6.40 (d, 1H, *J* = 16.0 Hz), 6.21 (dt, 1H, *J* = 16.0 Hz, *J* = 6.5 Hz), 5.54 (s, 1H), 3.89 (s, 3H), 3.82 (s, 3H), 3.48 (d, 2H, 7.0 Hz). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 271.1329, found 271.1332. The structure of MMPP is shown in Figure 1a. Compound **16**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.88-6.91 (m, 4H), 6.76-6.78 (m, 2H), 6.38 (d, 1H, *J* = 16.0 Hz), 6.20 (dt,

1H, J = 16.0 Hz, J = 6.5 Hz), 3.90 (s, 3H), 3.89 (s, 3H), 3.48 (d, 2H, 6.5 Hz). HRMS (ESI) *m/z* $[M+H]^+$ cacld. 301.1440, found 301.1452.

Materials

Caspase-3 and caspase-8 antibodies were purchased from Cell Signaling Technology (Beverly, MA). CDK2, CDK4, CDK6, cyclin B1, cyclin D1, cyclin E, STAT1, phosphor-STAT1, STAT3, Tyr705 phospho-STAT3, Ser727 phospho-STAT3, Bcl-2, Bax, p21, p53, histone-H1, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture materials were obtained from Gibco/Invitrogen (Seoul, Korea), and other chemical reagents were from Sigma Aldrich (Sigma Aldrich, St. Louis, MO).

Cell culture

A549 and NCI-H460 NSCLC cell lines and LL-24 lung epithelial cell line were obtained from the American Type Culture Collection (Manassas, VA). A549 and NCI-H460 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. LL-24 lung epithelial cells were cultured in similar conditions using DMEM. Cell cultures were maintained in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C.

Cell viability assay

Cells were seeded in 96-well plates. On the next day, the cells were treated with MMPP (0-15 μg/mL) for 24 h. Cell viability was then measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions. Briefly, MTT (5 mg/mL) was added and plates were incubated at 37 °C for 4 h before dimethyl sulfoxide (100 μL) was added to each well. Finally, the absorbance in each well was read at a wavelength of 540 nm using a plate reader.

Detection of apoptosis

Cells were cultured on 8-chambered slides and then treated with MMPP (0-15 μg/mL) for 24 h. The cells were then washed twice with phosphate-buffered saline (PBS) and fixed by incubation in 4% paraformaldehyde in PBS for 1 h at room temperature. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. The total number of cells in a given area was determined using DAPI staining. The apoptotic index was determined as the number of TUNEL-positive cells divided by the total cell number × 100%.

Western blotting

Cells were treated with MMPP (0-15 μg/mL) for 24 h, homogenized with protein extraction solution (PRO-PREPTM; Intron Biotechnology), and lysed by 60 min incubation on ice. The cell lysate was centrifuged at 13,000 × g for 15 min at 4 °C. Equal amounts of protein (40 μg) were separated by 12% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (GE Water and Process technologies). The PVDF membranes were blocked for 1 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline containing Tween-20 [TBST: 10 mM Tris (pH 8.0) and 150 mM NaCl solution containing 0.05% Tween-20]. After a short wash in TBST, the membranes were immunoblotted with primary antibodies against caspase-3 and caspase-8 (1:1000 dilutions; Cell Signaling, Beverly, MA) and CDK2, CDK4, CDK6, cyclin B1, cyclin D1, cyclin E, Bax, Bcl-2, STAT1, p-STAT1, STAT3, p-STAT3 (Tyr705, Ser727), p21, p53, histone-H1, and β-actin (1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA). Reactions were detected using secondary antibodies and visualized by using a chemiluminescence detection system.

Luciferase assay

Cells were plated in 12-well plates (1×10^5 cells/well) and transiently transfected with STAT3-Luc(Stratagene, La Jolla, CA), STAT1 (pGAS)-Luc (GeneCopoeia, Rockville, MA), or STAT5 (pISRE)-Luc plasmid (Promega, Madison, WI) using Lipofectamine 3000 in OPTI-MEM according to the manufacturer's specifications (Invitrogen, Carlsbad, CA) for 24 h. Subsequently, the transfected cells were treated with 10 μg/mL of MMPP for 24 h. Luciferase activity was measured by using a luciferase assay kit (Promega, Madison, WI) and a luminometer according to the manufacturer's specifications (WinGlow, Bad Wildbad, Germany).

Flow cytometry

The cells were treated with MMPP for 24 h. Subsequently, both adherent and floating cells were harvested using 0.25% trypsin-EDTA. Harvested cells were transferred into a tube and centrifuged. Pelleted cells were washed with cold PBS and re-suspended in PBS containing propidium iodide (PI) (Sigma-Aldrich) (40 μg/mL), RNase (100 μg/mL) (Gibco), and Triton X-100 (Sigma-Aldrich) at 37 °C for 10 min. The samples were analyzed using fluorescence-activated cell sorting. The percentages of cells in G1, S, and G2/M phases were calculated using Cell Quest.

SPR analysis

BIAcore 2000 and a CM5 sensor chip were both supplied by BIAcore (Uppsala, Sweden). HEPES-buffered saline (10 mM HEPES, 1 mM EDTA, 0.001% Tween-20, and 0.15 M NaCl, pH 7.4) was used as the constant flow buffer unless otherwise stated. All buffers and solutions used during BIAcore analysis were prepared using ultrapure water, degassed, and filter-sterilized. Activated CM-dextran matrix was prepared by mixing ethyl-N-(dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide and was used to coat a sensor chip. Recombinant STAT3 protein or MMPP was immobilized onto the CM-dextran sensor chip using 1 M ethanolamine, pH 8.5. Serial dilutions of MMPP and STAT3 antibodies were prepared using HEPES-buffered saline, and then flowed sequentially at increasing concentrations. The regeneration of protein interaction was tested with solutions at different pH; optimum interaction was

observed at pH 12. The BIAcore 2000 system was used to continuously monitor the change in mass at the sensor surface, and the kinetics of protein interaction were analyzed with BIAevaluation 3.0 software (BIAcore).

Docking experiment

A docking study of MMPP with STAT3 or STAT1 was performed using Autodock VINA [1]. Only one monomer of the homodimeric STAT3 (PDB code: 3CWG) or STAT1 (PDB code: 1YVL) crystal structures was used in the docking experiments and conditioned using AutodockTools by adding all polar hydrogen atoms. Initially, the grid box was centered on the STAT3 or STAT1 monomer and its size was adjusted to include the whole monomer. After initial search for the binding site, docking experiments were performed again using a smaller grid size (30 × 30 × 30). Docking experiments were performed at various exhaustiveness values of the default: 16, 24, 32, 40, and 60. Molecular graphics for the best binding model were generated using Discovery Studio Visualizer 2.0.

DNA-binding activity assays

EMSA was used for testing *in vitro* DNA-binding activity of STAT3, STAT1, or STAT5 according to the manufacturer's recommendations (Promega). Briefly, cells were cultured in 60-mm culture dishes. After treatment with MMPP for 6 h, the cells were washed twice with PBS and scraped into a cold eppendorf tube in 1 mL PBS. The cells were lysed in ice-cold buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.1% protease inhibitor, 0.1% phosphatase inhibitor, and 0.5% NP-40) for 30 min and centrifuged for 6 min at 6,000 rpm. The pellet was resuspended in buffer C (10 mM HEPES, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 0.1% protease inhibitor, 0.1% phosphatase inhibitor, 420 mM NaCl, 0.2 mM EDTA, and 20% glycerol). After incubation at 4 °C for 1 h, the lysate was centrifuged for 15 min at 13,000 × g and nuclear extracts were prepared and processed for EMSA. The relative densities of the DNA-protein bands were determined by densitometry using MyImage (SLB), and quantified by

Labworks 4.0 software (UVP, Upland, CA).

Pull-down assay

MMPP (1 mg) was dissolved in 1 mL of coupling buffer (0.1 M $NaHCO_3$, pH 11.0 containing 0.5 M NaCl) and conjugated with epoxy-activated Sepharose 6B. The epoxy-activated Sepharose 6B was swelled and washed in distilled water on a sintered-glass filter and then washed with the coupling buffer. The epoxy-activated Sepharose 6B beads were added to the MMPP-containing coupling buffer and rotated at 4 °C overnight. After washing, unoccupied binding sites were blocked with 0.1 M Tris-HCl (pH 8.0) for 2 h at room temperature. The MMPP-conjugated Sepharose 6B was washed with three cycles of alternating pH wash buffers (buffer 1: 0.1 M acetate and 0.5 M NaCl, pH 4.0; buffer 2: 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0). The control unconjugated epoxy-activated Sepharose 6B beads were prepared as described above in the absence of MMPP. The cell lysate was mixed with MMPP-conjugated Sepharose 6B or with Sepharose 6B at 4 °C overnight. The beads were then washed three times with TBST. The bound proteins were eluted with SDS loading buffer. The proteins were resolved by SDS-PAGE followed by immunoblotting with an antibody against STAT3 (1:1000 dilutions, Santa Cruz Biotechnology).

Immunoprecipitation assay

Cells were harvested in NET-NL lysis buffer containing 50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, 0.2 mM PMSF, and a mixture of protease inhibitors (Roche, Mannheim, Germany). Cell lysates were clarified by centrifugation and then incubated at 4 °C overnight with a monoclonal antibody against STAT3 (Santa Cruz). An aliquot of 50 μL pre-washed protein G-agarose was added and the incubation was continued for another 2 h at 4 °C. Immunoprecipitates were recovered by centrifugation, washed three times in NET-NW buffer, and resolved by western blotting.

Plasmid construction

The coding region of *Mus musculus* STAT3 was amplified by PCR using full-length *M. musculus* STAT3 cDNA as a template. Purified PCR products were double-digested with *EcoR*I and *Xho*I and then subcloned into the pcDNA3.1 vector containing a cytomegalovirus promoter, pUC origin, and ampicillin-resistance gene. Mutated STAT3 (T456A or T456F) was generated by Cosmo Genetech (Seoul, Korea) and the mutants were checked by sequencing.

GST pull-down assay

For expression of the STAT3 deletion mutant, plasmids harboring full-length wild-type (pcDNA-FLAG-full-length STAT3) and mutant (T456A or T456F) STAT3 were transfected into A549 cells using the TNT Quick coupled transcription/translation system (Promega). GST fusion proteins were collected on glutathione-Sepharose beads (Amersham Pharmacia Biotech), incubated with 200 μg cell lysate at 4 °C for 4 h or boiled with 2.5× sample buffer for 3 min, and centrifuged. The supernatant fraction was resolved on 12% SDS-PAGE. The binding was detected by autoradiography or western blotting.

Xenograft mouse models

Seven-week-old male BALB/c nude mice were purchased from Orient-Bio (Gyunggi-do, Korea). The mice were maintained in accordance with the Korea Ministry of Food and Drug Safety Guidelines as well as the Guide for the Care and Use of Laboratory Animals of Chungbuk National University (Cheongju, Chungbuk, Korea). All protocols involving mice in this study were reviewed and approved by the Chungbuk National University Institutional Animal Care and Use Committee and complied with the Korean National Institute of Health Guide of the Care and Use of Laboratory Animals (CBNU-929-16-01). NCI-H460 cells (1×10^7 cells in 0.1 mL PBS/animal) were subcutaneously injected with a 27-gauge needle into the right lower flanks of mice (n = 10 each group). After 14 days, when the tumors had reached an average volume of 100-150 mm^3, the tumor-bearing nude mice were i.p. administered MMPP (2.5 mg/kg and 5 mg/kg dissolved in 0.01% DMSO) twice per week for 3 weeks or administered MMPP orally three times per week for 1 month. The tumor-bearing nude mice were also i.p. administered cisplatin (5 mg/kg dissolved in 0.01% DMSO)

and docetaxel (5 mg/kg dissolved in 0.01% DMSO). The group treated with 0.01% DMSO was designated as the control. The weights and tumor volumes of the animals were monitored twice per week. Tumor volume was measured with a Vernier caliper and calculated using the following formula: $(A \times B^2)/2$, where A is the larger and B is the smaller of the two dimensions. At the end of the experiment, the animals were sacrificed. The tumors were separated from the surrounding muscles and dermis, excised, and weighed.

Freshly isolated human NSCLC surgical samples

All human surgical specimens were obtained and studied under informed consent in accordance with the Declaration of Helsinki and the specimens were acquired from The Catholic University of Korea Daejeon St. Mary's Hospital after approval from the Institutional Review Boards (No. DC16TISI0028). Surgical specimens were obtained from 6 patients with NSCLC who underwent surgical removal of the primary tumors. The specimens were processed for paraffin and frozen sectioning, and were classified as NSCLC based on the World Health Organization (WHO) criteria by licensed pathologist after examination. Tumor tissue accounted for at least 80% of the samples. Fresh surgical specimens were divided into 1-2 mm^3-sized fragments in antibiotic-containing RPMI medium for implantation into immune-deficient mice.

NSCLC Patient-derived xenografts model

Fresh human tumor fragments were directly implanted into the subcutaneous spaces of pockets on each side of the lower back (n = 6 for each tumor sample) of 6-week-old BALB/c nude mice (OrientBio, Korea).

Immunohistochemistry

Animals were sacrificed by CO_2 gas inhalation and tumor tissues were collected. Briefly, tumor tissues were fixed in 4% formalin and paraffin-embedded tissues were cut into 5-μm serial sections using a freezing microtome (Thermo Scientific, Germany). Sections were then dehydrated in a series of graded alcohols, cleared in xylene, and mounted using Permount (Fisher Scientific, Suwanee, GA). The sections were stained with hematoxylin/eosin (H&E) for pathological examination. Immunohistochemical staining

was carried out using antibodies against PCNA, p-STAT3, cyclin D1, and active caspase-3 (1:500, Abcam, Cambridge, UK). After rinsing in PBS, the sections were incubated with a biotinylated secondary antibody. The tissue sections were then incubated with avidin-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA) for 1 h. Subsequently, the sections were washed in PBS and the immunocomplexes were visualized using a 3,3´-diaminobenzidine solution (2 mg/10 mL) containing 0.08% hydrogen peroxide in PBS.

Prediction of drug-likeness

In silico toxicology and ADME of MMPP were evaluated using computational ADME QSAR models, preADMET (http://preadmet.bmdrc.org) and StarDrop (http://www.optibrium.com/stardrop/stardrop-features.php), to predict the major toxicities and ADME of MMPP as compared with that of (E)-2,4-bis(p-hydroxyphenyl)-2-butenal.

Statistical analysis

Statistical analysis was carried out with SPSS version 18.0. All error bars reported are the standard error of the mean (SEM) unless otherwise indicated. Pairwise comparisons were made using one-way Student's *t*-test. Multiple comparisons were made using one-way analysis of variance followed by Tukey's tests. Differences between groups were considered significant at *P*-values below 0.05.

Abbreviations: STAT3: signal transducer and activator of transcription-3; DBD: DNA-binding domain; MMPP: (E)-2-methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol; NSCLC: non-small cell lung cancer; JAKs: Janus kinases; BHPB: (E)-2,4-bis(p-hydroxyphenyl)-2-butenal; SPR: surface plasmon resonance; EMSA: electrophoretic mobility shift assay; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; FACS: fluorescence-activated cell sorting; PDX: patient-derived xenograft; PCNA: proliferating cell nuclear antigen; ADME: absorption, distribution, metabolism, and excretion;

PBS: phosphate-buffered saline; PVDF: polyvinylidene fluoride; TBST: Tris-buffered saline containing Tween-20; H & E: hematoxylin/eosin; SEM: standard error of the mean.

Supplementary Results

Scheme S1. Isomerization of compounds 11, 12, 14, and 15.

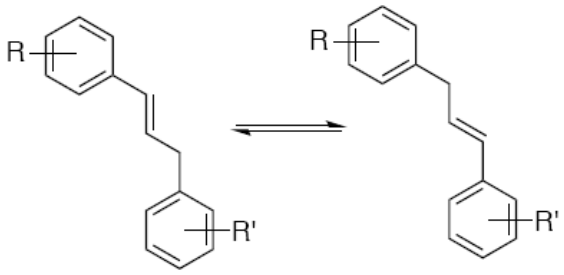
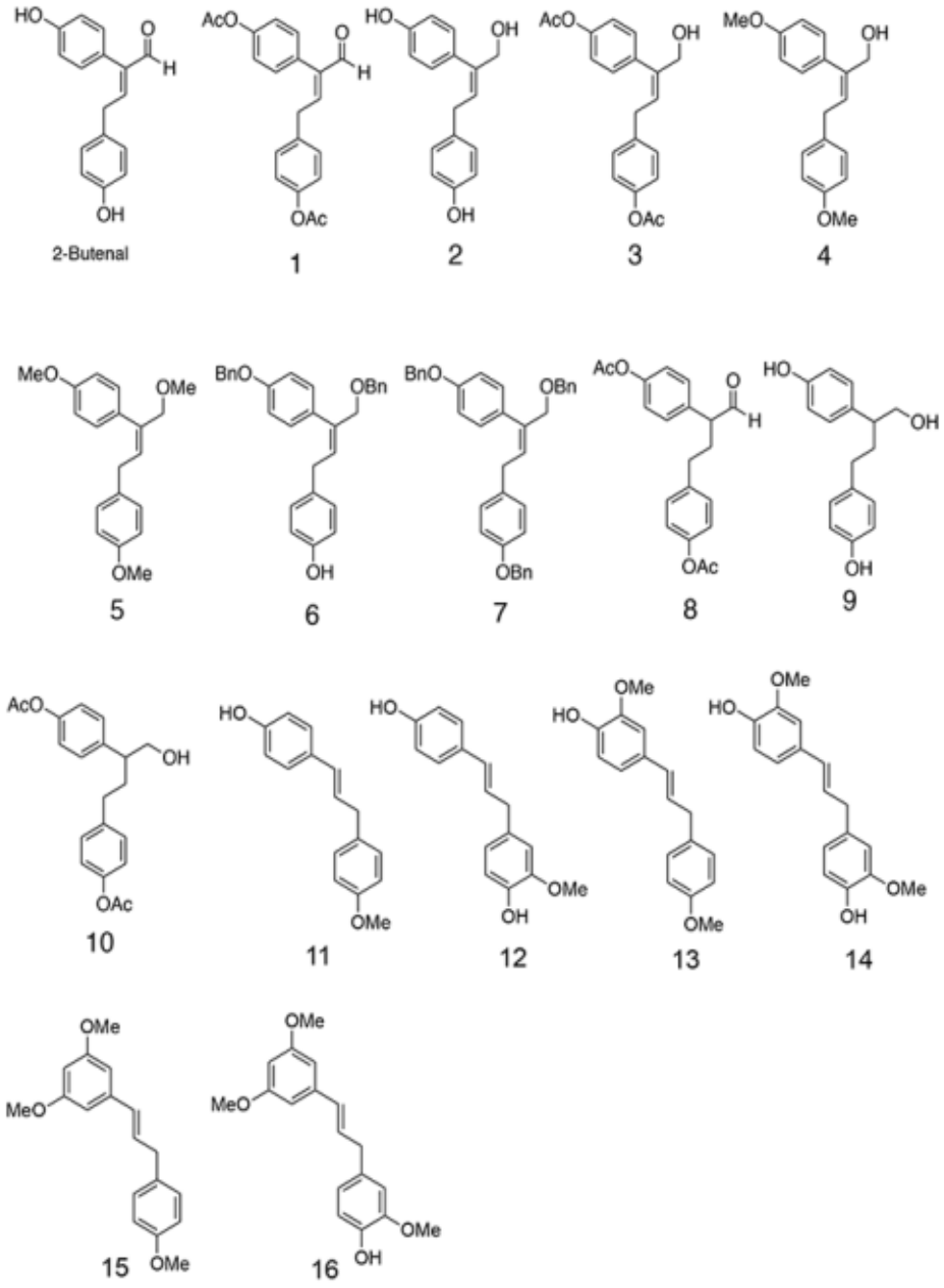


Figure S1. Chemical structure of BHPB analogs.



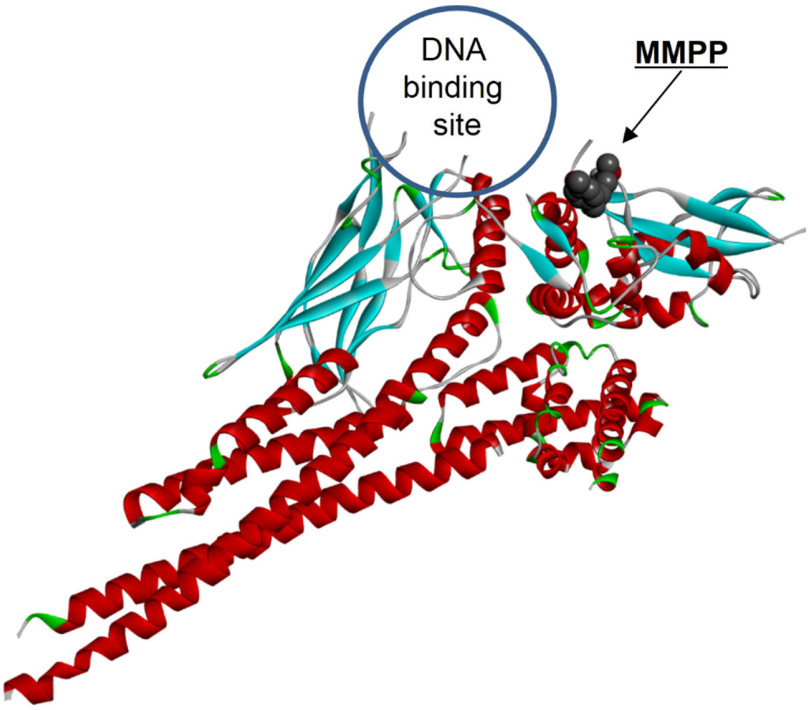


Figure S2. MMPP does not interact with STAT1. Docking model of MMPP with STAT1 is shown.

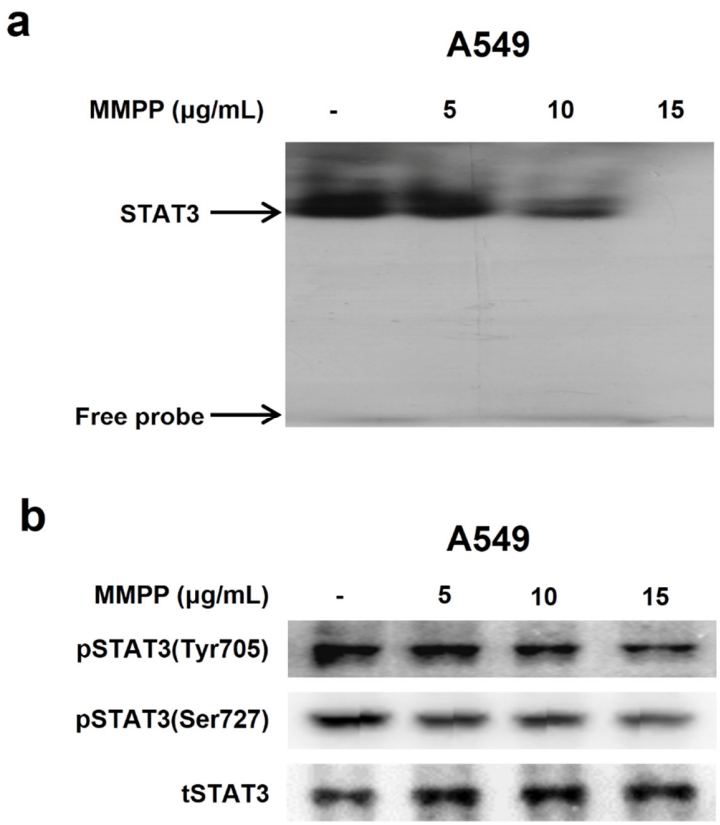


Figure S3. Effect of MMPP on the DNA-binding activity of STAT3 in A549 cells. (a-b) A549 cells were treated with MMPP (0-15 μg/mL) for 6 h and then lysed with buffers A and C. (**a**) Nuclear extracts were incubated in binding reactions of ^{32}p-end-labeled oligonucleotide containing the STAT3 sequence. EMSA was conducted to assess the effect of MMPP on DNA binding activity (**b**) MMPP-treated/or non-treated A549 cell lysates were used to determine the expression of p-STAT3 (Tyr705 and Ser727) and total-STAT3 by western blot analysis.

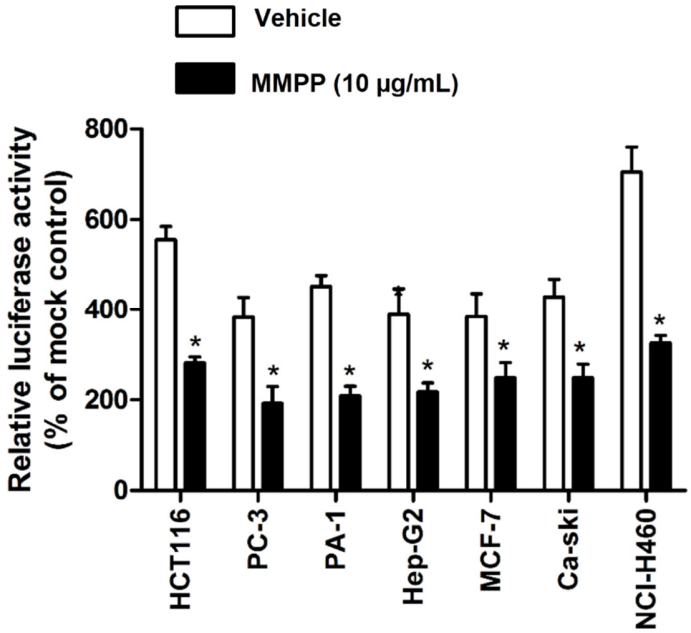


Figure S4. Inhibitory effect of MMPP on STAT3 luciferase activity in various cancer cell lines. HCT116 colon cancer cells, PC-3 prostate cancer, PA-1 ovarian cancer, Hep G2 liver cancer, MCF-7 breast cancer, Ca-ski uterine cervix carcinoma, and NCI-H460 NSCLC cells were treated with MMPP (10 μg/mL) for 24 h. Relative STAT3 luciferase activity was determined. (n = 6, data shown as mean ± SEM, *$p < 0.05$, paired t-test).

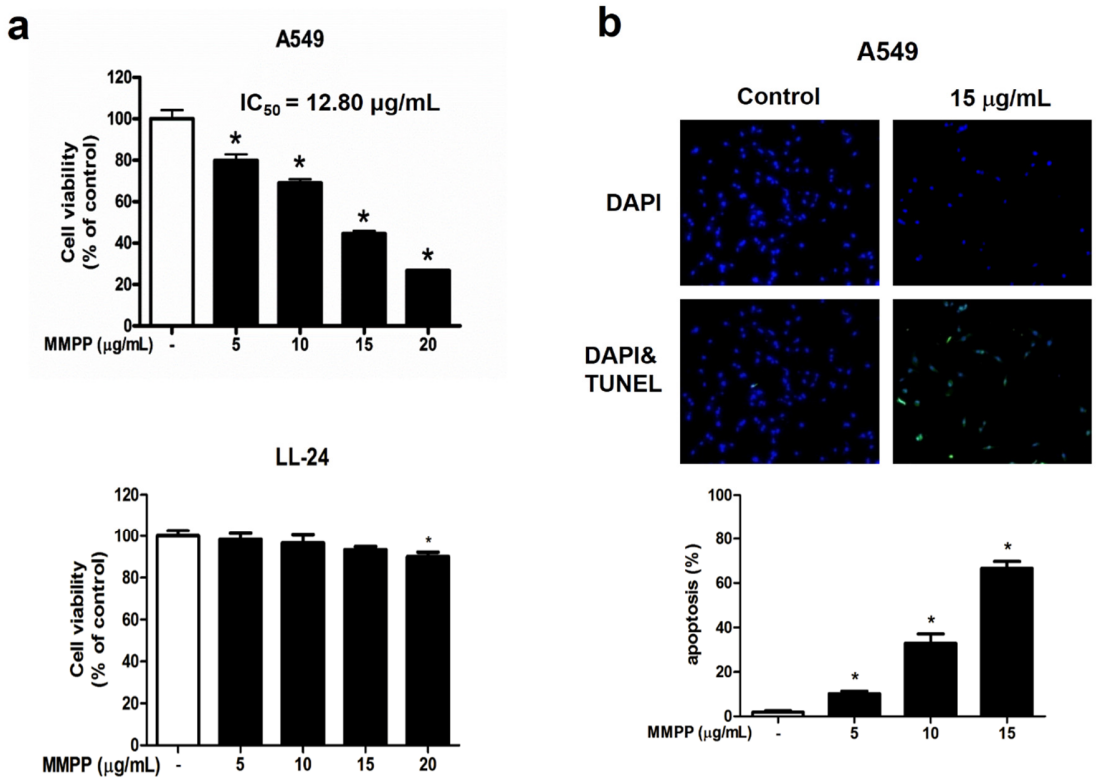


Figure S5. Inhibitory effect of MMPP on cancer cell growth in A549 cells. (**a**) A549 and LL-24 cells were treated with MMPP (0-20 μg/mL) for 24 h, and the relative cell survival rate was determined by MTT assay (n= 10, data shown as mean ± SEM, *$p < 0.05$, paired t-test). (**b**) A549 cells were cultured in a chambered glass culture slide and serum starved for 12 h. Cells were then treated with MMPP (0-15 μg/mL) for 24 h. Cell apoptosis was determined by TUNEL-assay. The apoptotic index was determined as the TUNEL-positive cell number divided by the total cell number. (n= 3, data shown as mean ± SEM, *p < 0.05, paired t-test).

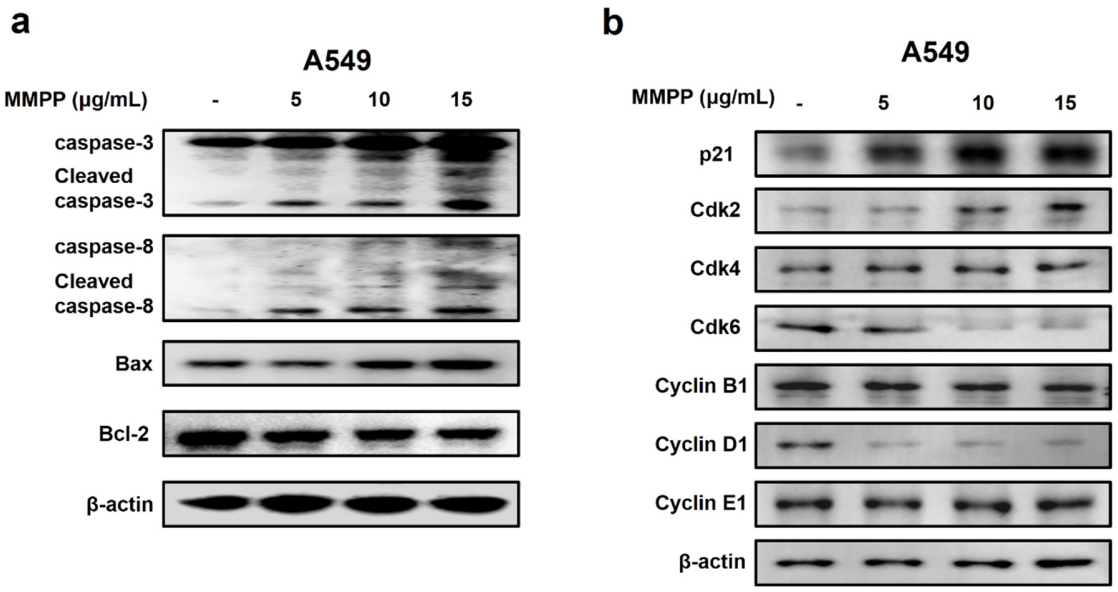


Figure S6. Effect of MMPP on apoptosis and cell cycle regulatory proteins in NSCLC cells. **(a-b)** A549 cells were treated with MMPP (0-15 μg/mL) for 24 h. Cells were then lysed and total proteins were prepared. (**a**) Expression of apoptosis regulatory proteins was determined by western blot analysis with antibodies against cleaved caspase-3, cleaved caspase-8, Bax, and Bcl-2, using β-actin as a loading control. **(b)** Expression of cell cycle regulatory proteins was determined by western blot analysis with antibodies against CDK2, CDK4, CDK6, cyclin B1, cyclin D1, and cyclin E1, using β-actin as a loading control.

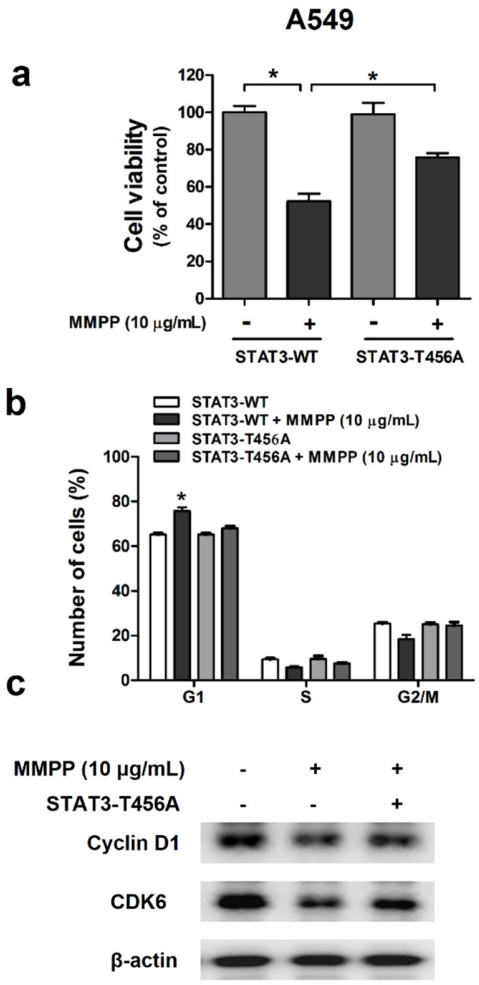


Figure S7. Effects of mutant STAT3 (T456A) on MMPP-induced cell growth inhibition, CDK6 expression, and cyclin D1/G1 cell cycle arrest, and combinatorial effect of MMPP and STAT3 inhibition on the expression of G1 cell cycle regulatory proteins in A549 cells. **(a)** A549 cells were transfected with wild-type STAT3 (STAT3-WT) or STAT3-T456A mutant plasmid for 24 h. Cells were then treated with MMPP (10 μg/mL) for another 24 h. Cell viability was determined by MTT assay (n= 10, data shown as mean ± SEM, *$p < 0.05$, paired t-test). (**b-c**) Cells were transfected with STAT3-WT or STAT3-T456A mutant plasmid. (**b**) FACS analysis (PI staining) was conducted to assess the effect of STAT3 mutation (STAT3-T456A) on the

inhibitory effect of MMPP on cell cycle arrest. **(c)** Cells were lysed and analyzed by western blotting with antibodies against cyclin D1 and CDK6, using β-actin as a loading control.

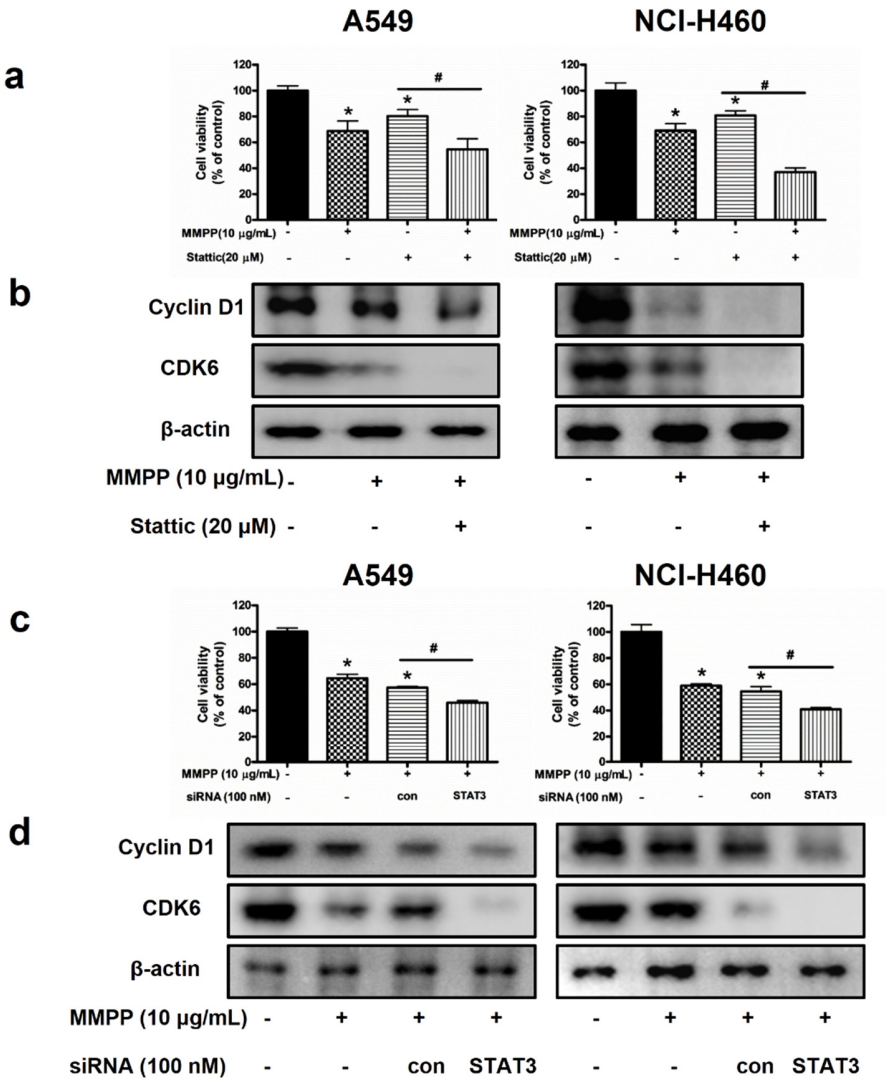


Figure S8. Inhibitory effect of MMPP on cell growth and cell cycle regulatory gene expression is abolished by treatment with STAT3 siRNA or STAT3 inhibitor. (a) A549 and NCI-H460 NSCLC cells were pretreated with STAT3 inhibitor Stattic (20 μM) for 2 h, and then treated with MMPP (10 μg/mL) for another 24 h. Relative cell survival rate was determined by

MTT assay. Data are expressed as the mean ± S.D. of three experiments. *p<0.05 indicates significant difference from the control cells. #p<0.05 indicates significant difference from the MMPP-treated cells. (b) Effect of STAT3 siRNA on the expression of G1-regulatory proteins was determined by using western blot analysis with antibodies against cyclin D1, CDK6, and β-actin (internal control). Each band is representative for three experiments. (c) A549 and NCI-H460 NSCLC cells were treated with non-targeting control siRNA (100 nM) or STAT3 siRNA (100 nM) for 24 h, or STAT3 inhibitor Stattic (20 μM) for 2 h. Cells were then were treated with MMPP (10 μg/mL) for another 24 h. Relative cell survival rate was determined by MTT assay. (d) Effect of STAT3 inhibitor (Stattic) on the expression of G1-regulatory proteins was determined by using western blot analysis with antibodies against cyclinD1, CDK6, and β-actin (internal control). Each band is representative for three experiments.

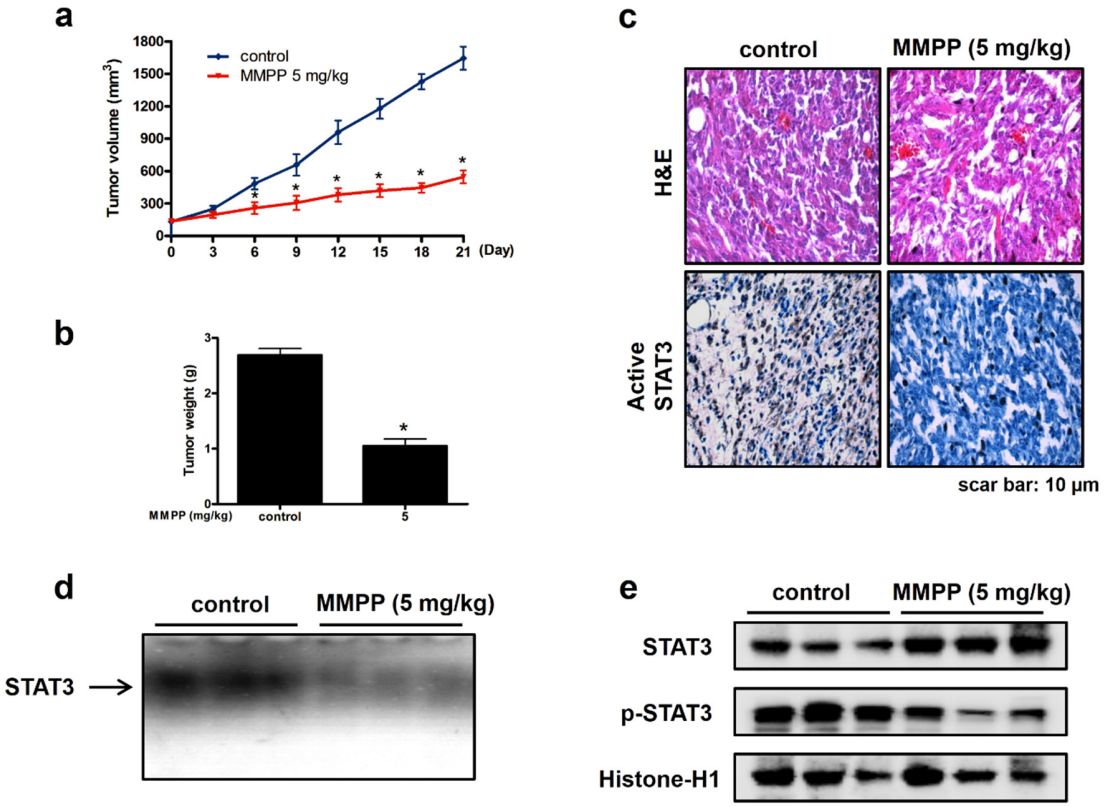


Figure S9. Antitumor activity of orally administered MMPP in NCI-H460 NSCLC xenograft model. (a) NCI-H460-tumor bearing mice (n = 5) were orally administered 0.01% DMSO or MMPP (5 mg/kg) for 1 month. Tumor volume was measured twice a week. **(b)** Tumor weight was measured after necropsy. **(c)** p-STAT3 expression in xenografts as determined by immunohistochemistry. **(d)** DNA-binding activity of STAT3 as determined by EMSA. Results are representative of three experiments. **(e)** Expression of STAT3, p-STAT3 as determined by western blot analysis. Results are representative of three experiments.

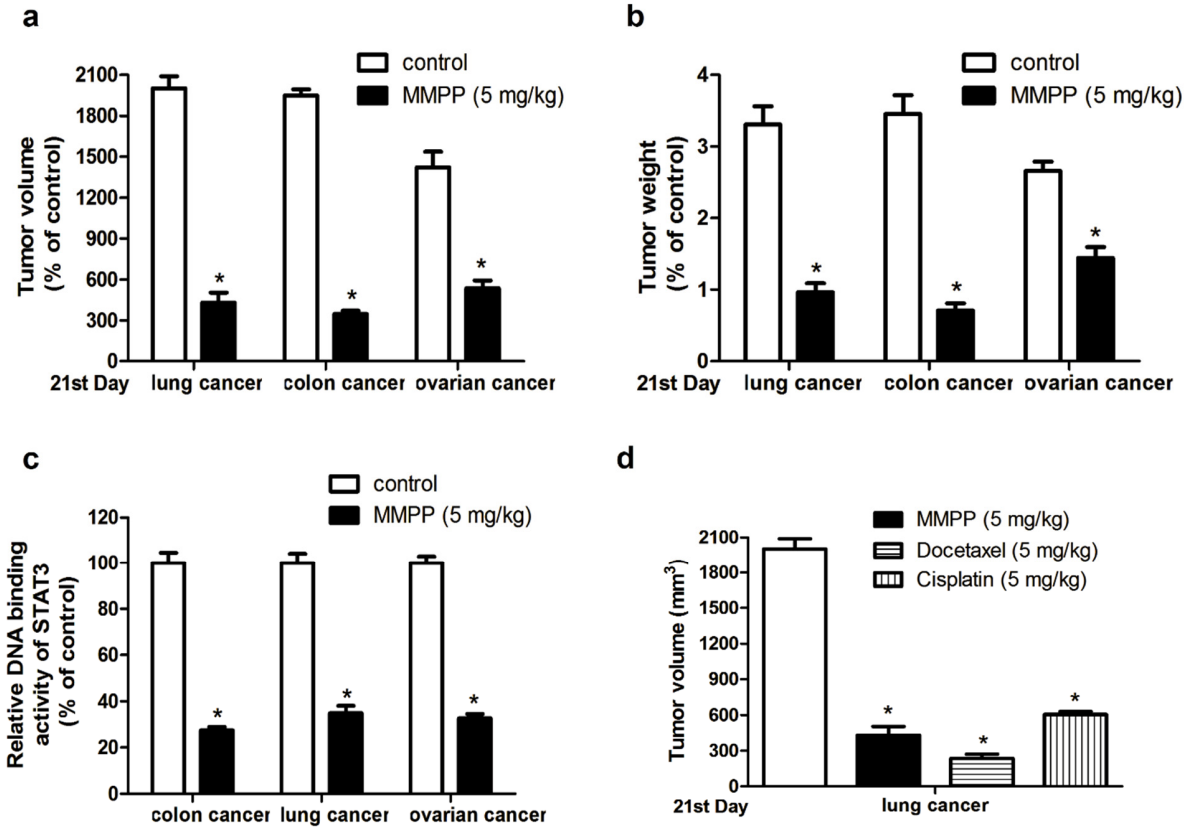


Figure S10. Inhibitory effect of MMPP on tumor growth and STAT3 activity in the lung, colon, and ovarian cancer. (a-c) A549 NSCLC, HCT116 colon cancer, and PA-1 ovarian cancer xenografts were used to assess the antitumor effect of MMPP. Mice bearing patient-derived tumor **xenografts** were intraperitoneally administered 0.01% DMSO or MMPP (5 mg/kg) for 1 month. Tumor volume (**a**) and tumor weight (**b**) were measured after necropsy (n= 10, data shown as mean ± SEM, *$p < 0.05$ as determined by paired t-test). **(c)** EMSA was conducted to identify whether the inhibitory effect of MMPP on tumor growth is correlated with STAT3 DNA binding activity. **(d)** Comparative inhibitory effect of MMPP, docetaxel, and cisplatin in NCI-H460 xenografts.

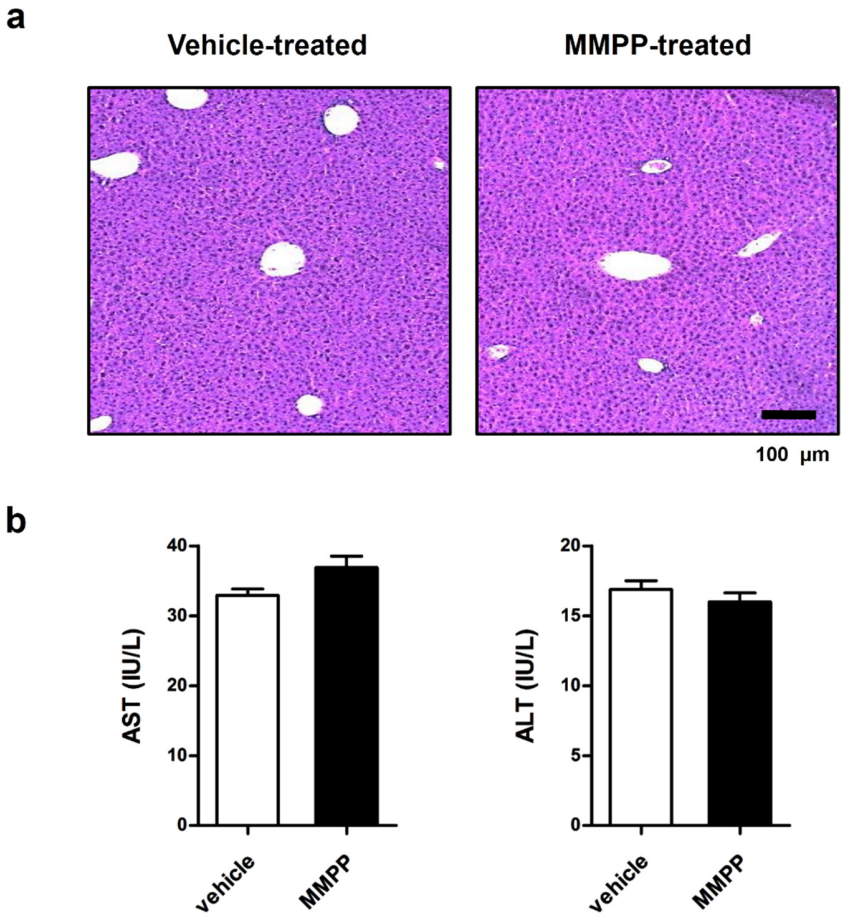


Figure S11. Administration of MMPP does not cause liver damage in C57BL/6 mice.

Supplementary Table S1. STAT3 binding affinity and inhibitory effect on STAT3-dependent luciferase reporter expression and cell viability of BHPB analogs in NCI-H460 NSCLC cells.

No.	Compound name	STAT3 binding affinity (kcal/mol)	IC_{50} value on STAT3 DNA binding activity (μg/mL)	IC_{50} value on cell viability (μg/mL)
	(E)-2,4-bis(4-hydroxyphenyl)but-2-enal	-6.4	3.11	26.92
1	(E)-2,4-bis(4-hydroxyphenyl)but-2-enal diacetate	-8.0	2.54	21.20
2	(E)-4,4'-(4-hydroxybut-2-ene-1,3-diyl) diphenol	-7.5	4.93	44.86
3	(E)-4,4'-(4-hydroxybut-2-ene-1,3-diyl)diphenol diacetate	-7.0	4.62	26.32
4	(E)-2,4-bis(4-methoxyphenyl)but-2-en-1-ol	-6.3	5.03	30.86
5	(E)-4,4'-(4-methoxybut-2-ene-1,3-diyl)bis(methoxybenzene)	-6.9	3.21	75.41
6	(E)-4-(4-(benzyloxy)-3-(4-(benzyloxy)phenyl)but-2-en-1-yl)phenol	-7.9	2.64	27.95
7	(E)-4,4'-(4-(benzyloxy)but-2-ene-1,3-diyl) bis((benzyloxy)benzene)	-7.5	4.25	48.30
8	(E)-2,4-bis(4-hydroxyphenyl)butanal diacetate	-7.5	5.91	76.55
9	4,4'-(4-hydroxybutane-1,3-diyl)diphenol	-7.4	3.34	54.96
10	4,4'-(4-hydroxybutane-1,3-diyl)diphenol diacetate	-7.3	3.57	49.51
11	(E)-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol	-7.8	2.23	14.06
12	(E)-4-(3-(4-hydroxyphenyl)allyl)-2-methoxyphenol	-7.9	2.05	19.93

13	(E)-2-methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol	-8.2	1.95	12.30
14	(E)-4,4'-(prop-1-ene-1,3-diyl)bis(2-methoxyphenol)	-7.8	2.63	17.53
15	(E)-1,3-dimethoxy-5-(3-(4-methoxyphenyl)prop-1-en-1-yl)benzene	-7.0	3.79	77.30
16	(E)-4-(3-(3,5-dimethoxyphenyl)allyl)-2-methoxyphenol	-8.0	3.64	16.10
	Oxaliplatin	-7.3	4.33	22.34

Supplementary Table S2. Predicted toxicities of BHPB and MMPP.

Test	BHPB	MMPP
Mutagenicity in vitro	negative	negative
Mutagenicity in vivo	plausible	negative
Photomutagenicity in vitro	negative	negative
Alpha-2-mu-globuline nephropathy, toxicity possibility	negative	negative
Equivocal Plausible	probable	negative
Anaphylaxis, Bladder Urothelial Hyperplasia	negative	negative
Cardiotoxicity, Cerebral oedema	negative	negative
Chloracne, Cholinesterase inhibition	negative	negative
Cyanide-type effects, High acute toxicity	negative	negative
Methaemoglobinaemia, Nephrotoxicity	negative	negative
Neurotoxicity, Oestrogenicity	negative	negative
Peroxisome proliferation, Phospholipidosis	negative	negative
Phototoxicity, Pulmonary toxicity	negative	negative
Uncoupler of oxidative phosphorylation, Lachrymation	negative	negative
HERG channel inhibition in vitro, Genotoxicity in vitro	negative	negative
Genotoxicity in vivo, Photogenotoxicity in vitro	negative	negative
Hepatotoxicity	plausible	plausible
Photogenotoxicity in vivo , Chromosome damage in vivo	negative	negative
Chromosome damage in vitro	plausible	negative
Photo-induced chromosome damage in vitro	negative	negative
Photocarcinogenicity , Carcinogenicity	negative	negative

References

1 Trott, O. & Olson, A. J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry* **31**, 455-461, doi:10.1002/jcc.21334 (2010).