Supporting Information

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SI Materials and Methods

Materials. PIT-1, DM-PIT-1, PIT-1i-1, and PIT-1i-2 were purchased from Chembridge. PIT-2 was purchased from Ryan Scientific. Synthesis of other PIT-1 analogs is described below. The purity of all compounds was >90%. Tetramethylrhodamine (TMR)-labeled phosphoinositides were purchased from Echelon Biosciences. Akt inhibitor VIII and X and LY294002 were purchased from Calbiochem, zVAD.fmk and TRAIL (KillerTRAIL) were from Axxora. Propidium iodide (PI) was purchased from Roche. Mouse anti–β-tubulin antibody was purchased from Stressgene, rabbit anti-LC3 antibody from MBL, and all of the other phosphoric antibodies including rabbit anti-Akt (Thr308 or Ser473), anti-p70S6K (Thr389), anti-S6 (Ser235/236), anti–GSK-3-β (Ser9), anti–4E-BP1 (Ser65), anti-Akt substrate (Ser/Thr), anti-AMPK (Thr172), anti-ACC (Ser79), and nonphosphospecific antibodies including anti–caspase-3, anti-PARP antibodies and the secondary HRP-conjugated antibodies were purchased from Cell Signaling Technology. IGF1, PDGF, and all other reagents and chemicals were from Sigma. Btk PH domain-GFP vector was purchased from Signagen Laboratories. PLC-δ PH domain-GFP vector was a generous gift of Or Gozani, Stanford University, Stanford, CA. Akt PH domain-GFP vector was a generous gift of Joanne Brugge (Harvard Medical School, Boston). GRP1 PH domain-GFP vector was a generous gift of Tamas Balla, National Institutes of Health, Bethesda, MD. TAPP1 and TAPP2 PH domain-GFP vectors were generous gifts of Aaron J. Marshall, University of Manitoba, Winnipeg, MB, Canada. Human Akt PH domain a.a. 1–123 and human PDK1 PH domain a.a. 411–557 were cloned by PCR amplification into BamHI/EcoRI sites of pGEX-6P-1 vector (GE Healthcare). His6-Akt PH123 was prepared by cloning a corresponding PCR fragment between NdeI and EcoRI sites of pET-28 vector (Novagen). His6-GRP1 PH domain was created by cloning a.a. 245–399 into the pET-30a vector. His6-TAPP1 PH domain was created by cloning a.a. 169–329 into the pET-30a vector. His6-TAPP2 PH domain was created by cloning a.a. 179–311 into the pET-30a vector. His6-Btk PH domain was created by cloning a.a. 1–177 into the pET-30a vector. All recombinant PH domains were expressed in BL21(DE3) *Escherichia coli*. Expression was induced by addition of 100 μM IPTG for 16 h at 25 °C. Proteins were purified by using glutathione-Sepharose (GE Healthcare) or Ni-NTA beads (Qiagen) according to manufacturer's recommendations and dialyzed overnight against 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, and 5 mM β-mercaptoethanol (b-ME) buffer.

Cells and Cell Culture. Human and mouse cancer cell lines (U87MG, SUM159, A549, MDA-MB-231, 4T1 and 293T) were purchased from the American Type Culture Collection. Mouse adult lung fibroblasts (Akt-deficient and Akt1-expressing) were generous gifts of Philip Tsichlis (Tufts Medical Center, Boston) (1), Bax/Bak double knockout mouse embryonic fibroblasts (2) and matched wild-type cells were generous gifts of Stanley Korsmeyer (Dana–Farber Cancer Center, Boston). U87MG, 4T1, A549, MDA-MB-231, 293T, and fibroblast cells were cultured in DMEM with 10% FBS; SUM159 was maintained in Ham's F-12 medium with 5% FBS, 5 μg/mL insulin, and 1 μg/mL hydrocortisone. All media were supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Fluorescence Polarization (FP) Assay. FP assay was used to identify compounds that disrupt the interaction between PIP3 and PH domains. Briefly, 5 μM Akt PH domain or 100 nM PDK1 PH domain or 100 nM GRP1 PH domain or 100 nM Btk PH domain were incubated with 15 nM TMR-labeled PIP3 in the buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, and 5 mM b-ME at room temperature in the dark. PLC-δ PH domain (100 nM) or 100 nM TAPP1 or TAPP2 PH domain were incubated with 15 nM TMR-labeled PI-4,5-P2 and PI-3,4-P2, respectively. FP values were determined by using Wallac Victor 3 plate reader. For the primary screen, ≈50,000 diverse small molecules from Harvard Institute of Chemistry and Cell Biology were screened (iccb.med.harvard.edu). Incubations were carried out for 1 h. NBD-labeled PIP3 (1 μM) was used in the screen. For secondary assays, PIT compounds were added at various concentrations (6.25, 12.5, 25, 50, 100, 150, and 200 μM), followed by a 30-min incubation. The inhibition rates were calculated as: inhibition = [1 – (compounds treated group/control group)] × 100%, and IC_{50} value was determined by using Origin6 software (MicroCal).

Protein/Lipid Overlay Assay. PIP3 or PIP2 lipid was reconstituted in chloroform:methanol:water (1:2:0.8) and spotted onto HyBond C extra nitrocellulose membranes (Amersham Biosciences) at 100 pmol per spot. Membranes were air-dried and blocked with 3% (wt/vol) fat-free BSA in TBST [10 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 0.1% (vol/vol) Tween 20] for 1 h at room temperature. Blocked membranes were incubated with 0.5 μg/mL GST-fusion proteins (PH domains) in TBST containing 3% (wt/vol) fatty acid-free BSA overnight at 4 °C in the presence or absence of PIT-1 at different concentrations. The membranes were extensively washed in TBST and subjected to Western blot analysis by using anti–GST-HRP antibody (GE Healthcare) to detect the bound GST fusion protein.

Surface Plasmon Resonance (SPR) Assay. All of the interaction analyses were done with a Biacore T100 instrument, and the data were analyzed by Biacore T100 Evalulation Software 1.1.1. Akt PH domain (100 μg/mL) was immobilized on a Series S CM4 sensor chip (Biacore BR-1000-12) by using Biacore's Amine Coupling Kit (Biacore BR-1000-50) to a level of 2,400 response units with standard protocol. The concentration of PIT-1 ranges from 12.35 to 51.45 μM and PIT-6 from 12.35 to 150 μM. The DMSO concentration in all samples and running buffer was 3% (vol/vol). The PITs were injected at 25 μL/min for 60 s and dissociated for 30 s. The running buffer is HBS-EP+ (Biacore) supplemented with 0.2% (vol/vol) Surfactant P20 (Biacore). Equilibrium dissociation constants (K_d) were calculated by Biacore T100 Evalulation Software 1.1.1 using the ratio of k_d/k_a.

NMR Spectroscopy. The ^{1}H-^{15}N HSQC spectra were recorded by adding different amounts of PIT-1 into 300 μM ^{15}N-labeled Akt PH/His. The 2D HSQC titration experiments between PIT-1 and PH domain were performed as described (3).

Molecular Modeling. High resolution of crystal structure of PH domain of Akt bound to inositol-1,3,4,5-tetrakiphosphate, the head group of PIP3, was used as a receptor for modeling studies (4). Three-dimentional conformers of PIT-1 generated with OMEGA 2 (OpenEye Scientific Software) were docked to the receptor by using FRED 2 program (OpenEye Scientific Software). The models were subsequently analyzed and visualized in PyMOL.

Cell Viability and Cell Death Assays. Cancer cells were seeded into 96-well plates (white plates for ATP assay; black plates for fluorescent assay; clear plates for MTS assay) at the density of

$5–10 \times 10^3$ cells per well in 100 μL of the appropriate media. Compounds were added at a series of concentrations and incubated for different time. For the ATP assay, a luminescence-based commercial kit (CellTiter-Glo; Promega) was used. Thirty microliters of the cell lysis/ATP detection reagent was added to each well, mixed for 10 min at room temperature, and the luminescence was measured by using a Wallac Victor 3 plate reader (Perkin-Elmer). For the MTS assay, a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega) was used. Twenty microliters of the combined MTS/PMS solution was added into each well, incubated for 3 h at 37 °C, and the absorbance was measured at 490 nm by using a Wallac Victor 3 plate reader. For the Sytox cell death assay, measuring loss of plasma membrane integrity, cells were incubated with 1 μM Sytox Green reagent (Molecular Probe) for 30 min at 37 °C, and the fluorescent intensity was measured by using Wallac Victor 3 plate reader with excitation at 485 nm and emission at 545 nm. Next, 5 μL of 20% (vol/vol) Triton X-100 solution was added to each well and incubated for 1 h at 37 °C, and the signal was measured again. The ratio of values (percentage of dead cells in each well) before and after Triton X-100 treatment was calculated and normalized to the relevant controls not subjected to the cytotoxic stimuli. IC_{50} values were determined by using Origin6 software (MicroCal).

Fluorescent Immunocytochemistry. SUM159 cells were seeded on coverslips, followed by the transient transfection as indicated. Cells were serum starved overnight and treated with different compounds at a variety of concentrations, followed by stimulation with growth factors. For translocation analysis, GFP images were obtained in live cells by using Nikon TE2000 fluorescent microscope. For detection of cell nuclei, cells were stained with Hoechst for 10 min, then the images were obtained by using Nikon TE2000 fluorescent microscope.

Western Blotting. After treatment, cells were harvested and lysed in 20 mM Hepes at pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM tetrasodium pyrophosphate, 100 mM NaF, and 17.5 mM β-glycerophosphate buffer supplemented with Complete Mini Protease Inhibitor tablet (Roche). The protein concentrations were determined by using Bio-Rad Protein Assay reagent, and equal amounts of protein were subjected to Western blotting by using the indicated antibodies. Briefly, samples separated by SDS/PAGE were transferred to nitrocellulose membranes. After being blocked in 5% BSA (wt/vol) at room temperature for 1 h, the membranes were rinsed and incubated at 4 °C overnight with a variety of primary antibodies (1:1,000 dilution). The membranes were then washed and incubated with secondary antibody (1:2,000 dilution) at room temperature for 1 h, developed with chemiluminescence ECL reagent (LumiGold; SignaGen), and exposed to Hyperfilm MP (GE Healthcare). Tumor samples were lysed in RIPA buffer (50 mM Tris·HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40, supplemented with Complete Mini protease inhibitors), and equal amounts of protein were subjected to Western blotting analysis.

EM Analysis. U87MG cells were seeded directly on Thermanox coverslips and treated with PIT-1 (100 μM) for 24 h. Cells were fixed in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and 2% paraformaldehyde for 1 h and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for another 1 h. Samples were dehydrated through a series of graded ethanol solutions from 70 to 100% and placed in 100% propylene oxide for 15 min. Propylene oxide was changed once and incubated for another 15 min. Samples were subsequently placed into propylene oxide/epoxy resin mixture (1:1) for 1 h, and transferred to embedding resin for 15 h and to a flat embedding mold with freshly prepared 100% resin for 1 h. Mold-containing samples were placed in a 60 °C oven and polymerized for 48 h. After polymerization, the appropriate blocks containing cells were cut into sections at 70–90 nm and placed on nickel mesh grids. After drying on filter paper for 1 h, the sections were stained with uranyl acetate and lead citrate for contrast. The images were acquired by using an electron microscope (Philips Tecnai 12 electron microscope).

Transient Transfection. SUM159 cells were seeded on coverslips in a 24-well plate at a density of 5×10^4 cells per well and incubated overnight. Then transient transfection was carried out by using TransIT-LT1 Transfection Reagent (Mirus Bio) according to the manufacturer's recommendations. Twenty-four hours after transfection, cells were subjected to translocation or wound healing or fluorescent immunocytochemistry analysis with or without compound treatment or growth factors stimulation.

Generation of Akt Retrovirus. PH domain-deficient activated Akt containing N-terminal myrostoylation sequence and a.a. residues 1–3 and 130–480 of human Akt was amplified by PCR and cloned into the BglII and EcoRI sites of pMSCV-puro (Clontech) viral vector. The retrovirus was generated by cotransfection of the MSCV-based vector with the plasmids encoding retroviral Gag/Pol and VSV-G proteins into human HEK 293T cells. U87MG cells were infected three times with the virus, followed by selection with puromycin to get stable populations of cells for cell viability analysis.

Flow Cytomery (FACS) Analysis. U87MG cells were seeded in six-well plates (5×10^5 cells per well), and cultured in the presence or absence of PIT-1 or PIT-2 (25, 50, and 100 μM) for 48 h. Cells were harvested, washed with PBS, fixed in 70% ethanol, and stained with propidium iodide (PI; 5 mg/liter) in the presence of RNase (1 g/liter), 1 g/liter sodium citrate and 0.5% Triton X-100 (vol/vol) in the dark for 30 min. Cells were collected for apoptosis analysis by using FACSCalibur (BD Biosciences). The percentage of hypodiploidy was analyzed by using ModFIT LT software (Verity Software).

Preparation of DM-PIT-1–Loaded Micelles. PEG-PE [1,2-disteratoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] was supplied in chloroform (10 mg/mL) and stored at −80 °C. A stock solution of DM-PIT-1 (0.5 mg/mL) was prepared by dissolving 20 mg of DM-PIT-1 in 40 mL of acetonitrile and stored at 4 °C. Two milliliters of DM-PIT-1 from stock solution was added to 2.5 mL of chloroform solution of PEG-PE. The organic solvents were removed by the rotary evaporation to form a thin film of drug/micelle mixture. This film was further dried under high vacuum overnight to remove any remaining traces of the solvent. To form micelles, the film was rehydrated in a 10 mM Hepes buffer at pH 7.4 and sonicated for 5 min. The nonincorporated, precipitated DM-PIT-1 was removed by filtration through a 0.22-μm filter (Fisher Scientific). The amount of DM-PIT-1 was determined by isocratic reverse-phase HPLC (Hitachi; Elite La Chrome) equipped with photodiode-array detector (L-2455). The chromatographic separation was performed on a C-18 column (5 μm, 4.6 × 250 mm; Hichrom). Isocratic elution was performed with a mobile phase consisted of acetonitrile and water (70:30) containing 0.1% formic acid (vol/vol). The flow rate was 1 mL/min, and the total run time was 10 min. Sample injection (10 or 20 μL) was performed with autosampler (Model L-2200, Hitachi). DM-PIT-1 was detected by the UV absorbance at 320 nm.

Drug content loading (by percent) was calculated as the weight of DM-PIT-1 in micelles divided by the PEG-PE weight that was used in micelle preparation.

The mean size of micellar preparations was measured by the dynamic light scattering (DLS) with a scattering angle of 90° at 25 °C using a N4 Plus Submicron Particle System (Coulter). The micelle suspensions were diluted with a 10 mM HBS, pH 7.4 until

a concentration providing a light scattering intensity of 5×10^4 to 1×10^6 count was achieved. The measurements were done in triplicate.

Immunohistochemistry. Tumor tissues were fixed in phosphate-buffered formalin, embed in paraffin, cut in 4-μm thickness, and applied to slides. The slides were deparaffinized in xylenes by using three changes for 5 min each and hydrated gradually through graded alcohols: 100% ethanol twice for 10 min each, 95% ethanol twice for 10 min each, and then deionized water for 1 min with stirring. For antigen unmasking, slides were placed in a container, covered with 10 mM sodium citrate buffer, pH 6.0, and heated in a convection steamer for 1 h. The slides were washed in deionized water three times for 2 min each, blocked with 5% normal goat blocking serum for 30 min, incubated with goat anti-CD31 primary antibody for 1 h, and incubated with an Alexa Fluor 488-conjugated secondary antibody for 30 min. The slides were analyzed and photographed by using a fluorescent microscope. TUNEL staining was performed by using FragEL kit according to manufacturer's instructions (Calbiochem).

Suppression of s.c. Tumor Growth in Vivo. Metastatic breast cancer cells (4T1) were first cultured in vitro in DMEM containing 10% FBS and antibiotics. After resuspension in PBS, the 4T1 cancer cells (8×10^4) were injected s.c. into axillary regions of BALB/c mice. The treatment with PIT-1 began on day 11 after inoculation of tumor cells, i.e., when tumors were palpable, but before tumors are precisely measurable (≈50 mm^3). Working solutions of all PIT-1-formulations were made in sterile 0.9% NaCl before injection to mice. Free DM-PIT-1 and micellar DM-PIT-1 were administered i.v. daily at doses of 0.4 and 1 mg/kg, respectively, for 8 d. If the tumor size reached ≈1,000 mm^3, the mice were euthanized by using carbon dioxide (CO_2) before cervical dislocation. Seven mice per group were used. The tumor volume (V) was measured every two days by microcaliper for 8 d. Tumor volume was calculated according to the formula $(a \times b^2)/2$, where "*a*" is the longest and "*b*" is the shortest diameter of tumor, respectively.

Preparation of PIT-1 Derivatives. ***General.*** Column chromatography was carried out by using Spectrochem silica gel (60–120, 230–400 mesh). NMR spectroscopy measurements (^1H and ^{13}C) were carried out on Bruker AC 200 MHz or Bruker DRX 400 MHz, and TMS was used as internal standard. NMR chemical shifts (^1H and ^{13}C) are reported in parts per million (ppm) downfield from tetramethylsilane, and coupling constants (*J*) are reported in hertz (Hz). The following abbreviations are used to designate signal multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Mass spectroscopy (ESI; API-Q*Star* Pulsar) was carried out on a Finnigan MAT-1020 spectrometer. Elemental analysis data were obtained on a Thermo Finnigan Flash EA 1112 Series CHNS Analyzer.

Preparation of Isothiocyante. At 0 °C, a solution of 3,5-dimethylbenzoic acid (5.0 g, 33.3 mmol) in dichloromethane (30 mL) was treated with oxalyl chloride (7.0 mL, 83.2 mmol), and the contents were stirred at room temperature for 8 h. The reaction mixture was concentrated under reduced pressure and dried under high vacuum. The resulting crude acid chloride was added to a suspension of powdered ammonium thiocyanate (3.8 g, 50 mmol) and PEG-400 (100 mg) in methylene dichloride (25 mL) and stirred at room temperature for 3 h. The reaction mixture was filtered and concentrated under reduced pressure. The crude acylisothiocyanate could be stored without any decomposition for more than a week at –20 °C.

Preparation of Acylthiourea. The above isothiocyanate (≈1 mmol) was dissolved in dichloromethane or/and acetonitrile (whenever the aminophenol was not soluble in DCM) (10 mL) and treated with amino phenol (1.8 mmol). The contents were stirred at room temperature until the reaction was completed (as tracked by the TLC). The reaction mixture was washed with water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude was recrystallized from appropriate solvents to get pure acylthioureas or purified by column chromatography, when the thiourea was an oil.

PIT-3. *N*-(3-chloro-6-hydroxy-2,4-dimethylpheny lcarbamothioyl)-3,5-dimethylbenzamide. Yield 79%, yellow color solid. M. P.: 185–186 °C. IR (Nujol) $\tilde{\nu}$: 3,322, 1,681, 1,528, 1,460, 1,377, 1,296, 1,164, 1,051, 720 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz): δ 2.37 (s, 3H), 2.39 (s, 3H), 2.42 (s, 6H), 6.23 (br s, ^1H), 6.89 (s, ^1H), 7.30 (s, ^1H), 7.50 (s, 2H), 9.22 (s, ^1H), 12.32 (s, ^1H) ppm. ^{13}C NMR ($CDCl_3$, 50 MHz): δ 16.2 (q), 20.9 (q), 21.2 (q), 118.7 (d), 123.5 (s), 125.4 (d), 127.0 (s), 130.9 (s), 132.4 (s), 135.7 (d), 137.5 (s), 139.1 (s), 148.9 (s), 167.9 (s), 179.1(s) ppm. ESI-MS (*m/z*) 385.8 (35%, $[M+Na]^+$), 301.4 (100%). CHN Calcd. for $C_{18}H_{19}ClN_2O_2S$: C, 59.58; H, 5.28; N, 7.72; Found: C, 59.83; H, 5.22; N, 7.81.

PIT-4. *N*-(2-hydroxy-5-(trifluoromethyl)phenyl carbamothioyl)-3,5-dimethylbenzamide. Yield 87%, color less solid, M. P.: 208–209 °C. IR (Nujol) $\tilde{\nu}$: 3,425, 3,392, 1,740, 1,652, 1,567, 1,374, 1,233, 1,144 cm^{-1}. ^1H NMR [$CDCl_3$-DMSO-D_6 (3:1) 200 MHz]: δ 2.40 (s, 6H), 7.08 (d, $J = 8.34$ Hz, ^1H), 7.26 (s, ^1H), 7.30 (dd, $J = 2.3, 8.6$ Hz, ^1H), 7.61 (s, 2H), 9.15 (d, $J = 1.76$, Hz, ^1H), 10.47 (s, 6H) ppm. ^{13}C NMR[$CDCl_3$-DMSO-D_6 (3:1) 200 MHz]: δ 20.2 (q, 2C), 114.2 (d), 118.9 (d, $^3J_{CF} = 3.7$ Hz), 119.5 (s), 122.2 (d, $^3J_{CF} = 3.7$ Hz), 125.0 (d, 2C), 125.6 (s), 126.2 (s), 130.9 (s), 133.9 (d), 137.3 (s, 2C), 150.8 (s, $^4J_{CF} = 1.1$ Hz), 167.0 (s), 176.7 (s) ppm. ESI-MS (*m/z*): 391.3 (100%, $[M+Na]^+$), 369.4 (30%), 357.4 (10%). CHN Calcd. for $C_{17}H_{15}F_3N_2O_2S$: C, 55.43; H, 4.10; N, 7.60; Found: C, 55.38; H, 4.07; N, 7.56.

PIT-5. *N*-(2-hydroxy-4,6-*bis*(trifluoromethyl)phenylcarbamothioyl)-3,5-dimethylbenzamide. M. P.: 167–168 °C. IR (Nujol) $\tilde{\nu}$: 3,388, 2,853, 1,680, 1,604, 1,459, 1,376, 1,279, 1,214, 1,127, 761 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 2.42 (s, 6H), 6.85 (s, ^1H), 7.32 (s, ^1H), 7.52 (s, 2H), 7.61 (s, 2H), 9.32 (br s, ^1H), 12.84 (br s, ^1H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 21.1 (q, 2C), 116.1 (d, (d, $^3J_{CF} = 3.7$ Hz), 121.1 (s), 121.5 (d, $^3J_{CF} = 3.7$ Hz), 123.5 (s, $^2J_{CF} = 32.3$ Hz), 125.5 (d, 2C), 126.7 (s), 127.4 (s, $^2J_{CF} = 30.1$ Hz), 130.5 (s), 131.5 (s, $^2J_{CF} = 34.5$ Hz), 136.1 (d), 139.3 (s, 2C), 152.6 (s), 167.9 (s), 179.3 (s) ppm. ESI-MS (*m/z*): 459.4 (8%, $[M+Na]^+$), 425.4 (100%). CHN Calcd. for $C_{18}H_{14}F_6N_2O_2S$: C, 49.54; H, 3.23; N, 6.42; Found: C, 49.69; H, 3.28; N, 6.19.

PIT-6. *N*-(3-bromo-6-hydroxy-2,4-dimethylphenylcarbamothioyl)-3,5-dimethylbenzamide. Yield 81%, pale yellow solid, M. P.: 164.5–166.0 °C. IR ($CHCl_3$) $\tilde{\nu}$: 3,325, 1,679, 1,605, 1,515, 1,403, 1,211, 1,157, 1,024 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz): δ 2.41 (s, 9H), 2.44 (s, 3H), 6.92 (s, ^1H), 7.30 (s, ^1H), 7.50 (s, 2H), 9.26 (s, ^1H), 12.31 (s, ^1H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ 19.5 (q), 21.2 (q), 24.1 (q), 119.2 (d), 123.6 (s), 125.4 (d, 2C), 131.0 (s), 134.0 (s), 135.9 (d), 139.3 (s), 139.7 (s), 149.6 (s), 167.9 (s), 178.7 (s) ppm. ESI-MS (*m/z*): 431.1 (4%, $[M+Na]^+$), 395.3 (20%), 277.3 (100%). CHN Calcd. for $C_{18}H_{19}BrN_2O_2S$: C, 53.08; H, 4.70; N, 6.88; Found: C, 53.41; H, 5.00; N, 6.61.

PIT-7. *N*-(4-hydroxybiphenyl-3-ylcarbamothioyl)-3,5-dimethylbenzamide. Yellow color gum, ^1H NMR ($CDCl_3$, 200 MHz): δ 2.42 (s, 6H), 7.17 (d, $J = 8.5$ Hz, ^1H), 7.30 (s, ^1H), 7.36–7.40 (m, ^1H), 7.43–7.48 (m, 2H), 7.50–7.60 (m, 5H), 7.69 (d, $J = 2.3$ Hz, ^1H), 9.22 (br s, ^1H), 12.76 (br s, ^1H) ppm. ESI-MS (*m/z*): 399.5 (100%, $[M+Na]^+$). CHN Calcd. for $C_{22}H_{20}N_2O_2S$: C, 70.19; H, 5.35; N, 7.44; Found: C, 70.41; H, 5.58; N, 7.21.

Statistics. Student's *t* test and analysis of variance (ANOVA) were performed by using StatView (SAS Institute). $P < 0.05$ was considered significant and $P < 0.01$ as highly significant. The data shown are representatives of at least three independent experiments with similar results, and the data points represent the mean of at least triplicate measurements with error bars corresponding to SD.

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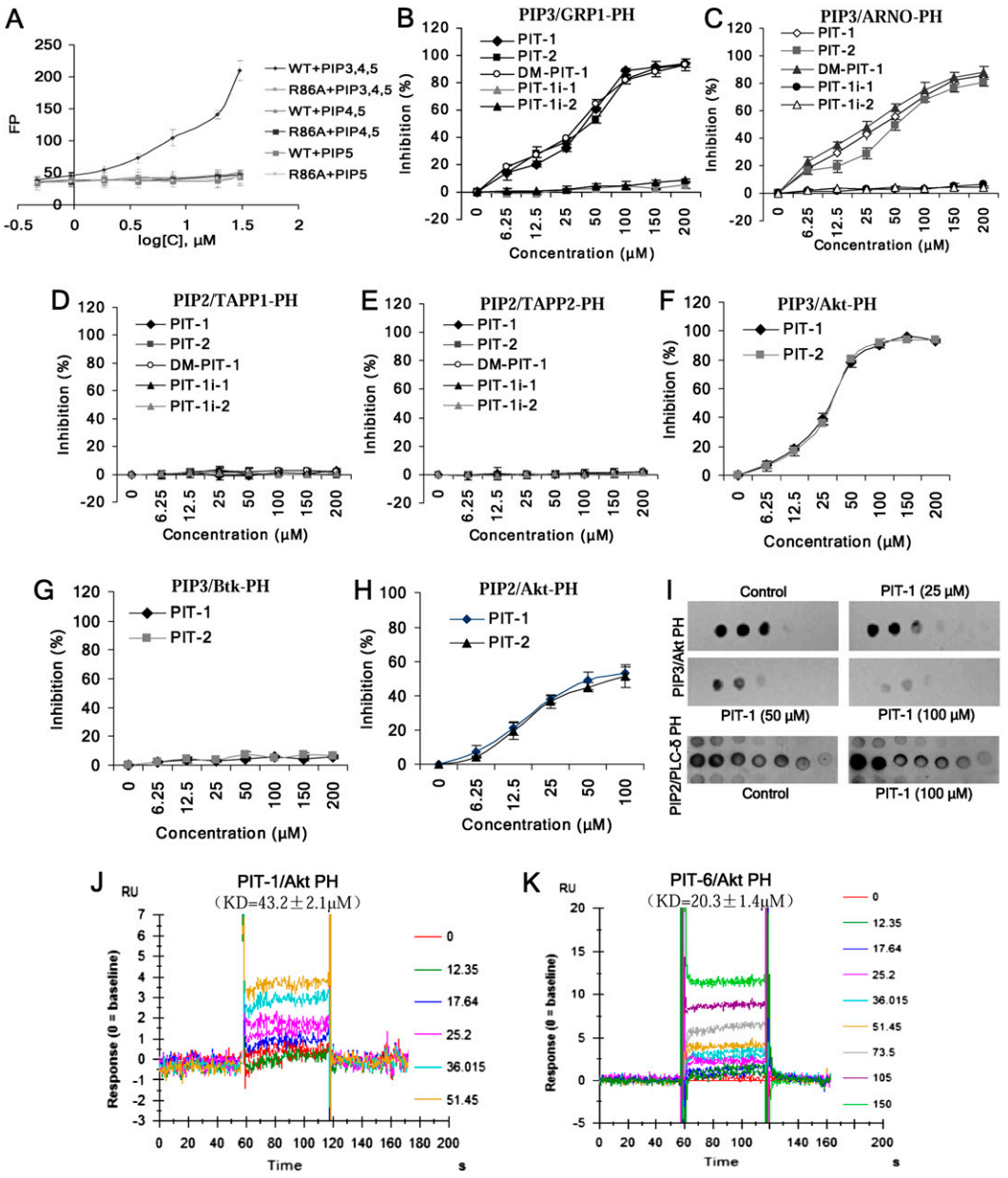


Fig. S1. Characterization of the specificity of PIT-1. (*A*) Detection of the PIP3/PH domain binding using FP assay. Various concentrations of wild-type PH123 or mutant K86A were incubated with 1 μM NBD-labeled PIP3, PIP2, or PIP5 for 15 min, followed by FP measurement. (*B–H*) Specificity analysis of phosphoinositide-PH domain inhibition by PITs. TMR-conjugated PI-3,4,5-P3 (15 nM) were incubated with 100 nM GRP1 (*B*), 100 nM ARNO (*C*), or 5 μM Btk (*G*) PH domain; 15 nM TMR-conjugated PI-3,4-P2 were incubated with 100 nM TAPP1 (*D*), 100 nM TAPP2 (*E*), or 5 μM Akt (*H*) PH domain; 60 nM TMR-conjugated PI-3,4,5-P3 were incubated with 5 μM Akt (*F*) PH domain, in the presence or absence of PITs (6.25–200 μM) for 30 min, followed by FP measurement. (*I*) GST-Akt PH domain (0.5 μg/mL) was incubated with PIP3 strip, 0.5 μg/mL GST-PLC-δ PH domain was incubated with PIP2 strip in the presence or absence of PIT-1 at indicated concentrations, and the membranes were subjected to Western blot analysis by using anti-GST antibody. (*J* and *K*) SPR analysis of direct binding of PIT-1 (*J*) and PIT-6 (*K*) to Akt PH domain. Akt PH domain was immobilized on a Series S CM4 sensor chip. PITs were passed over the sensor chip surface and the changes in mass were measured, and the sensorgrams were recorded in real time and analyzed after subtracting the control, and the K_d value was calculated.

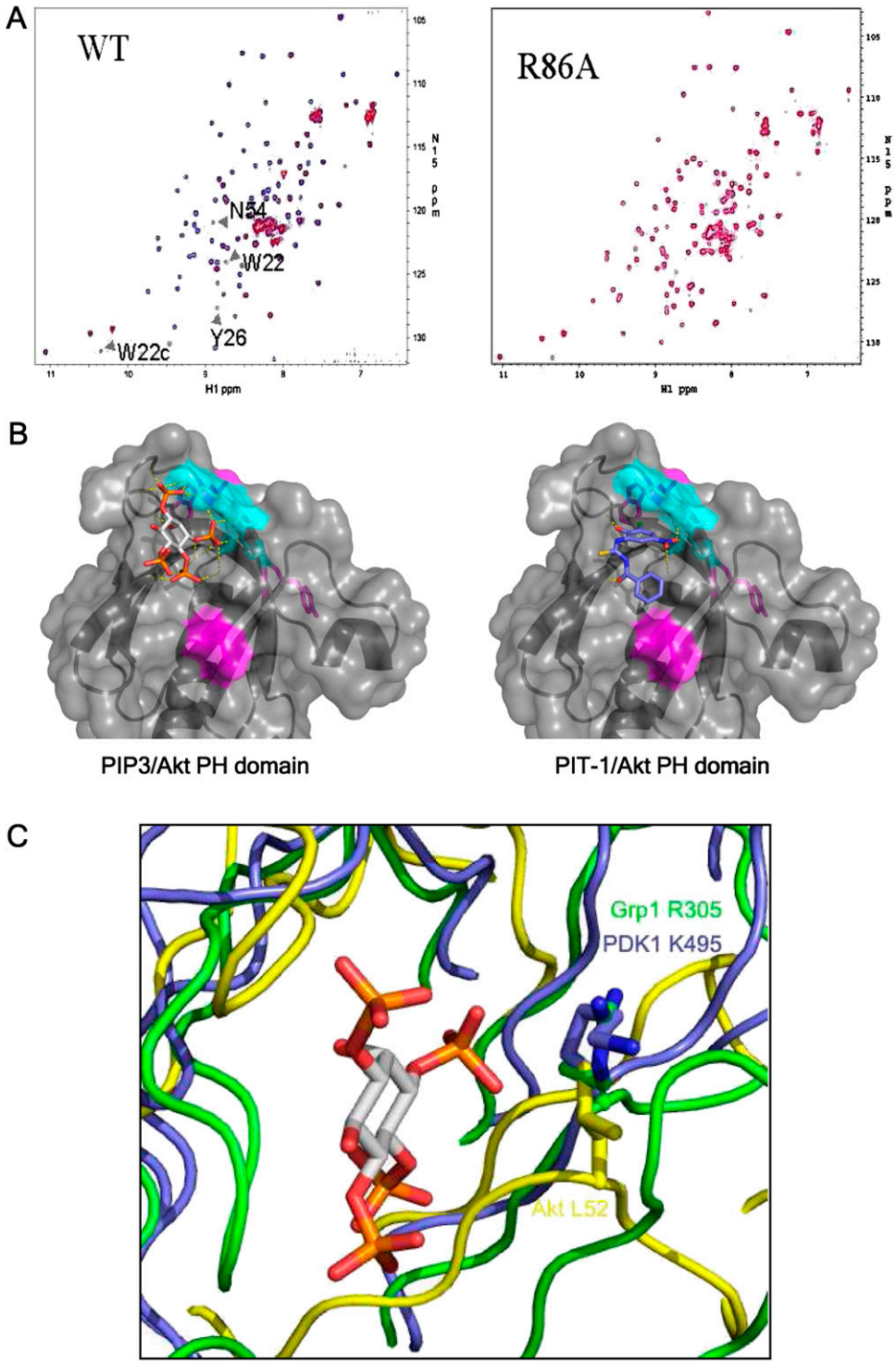


Fig. S2. NMR analysis and molecular modeling of PIT-1/Akt PH domain binding, as well as molecular modeling of overlay of distinct PH domains. (*A*) PIT-1 binding induces significant changes in the 2D HSQC ^{1}H-15N correlation spectrum of Akt PH domain measured by NMR. Red color indicates peaks observed at 2-, 1-, and 0-fold excess of PIT-1. Blue color denotes peaks not observed at 2-fold excess of PIT-1; gray color signals not observed at 1-fold excess of PIT-1. Gray crosspeaks correspond to residues preferentially affected by compound binding. In contrast, PIT-1 does not affect the spectrum of the R86A mutant of the Akt PH domain, which is deficient in PIP3 binding. (*B*) Three-dimensional structure of the PIP3/Akt PH domain and PIT-1/Akt PH domain complexes. Tryptophan 22, tyrosine 26, and asparagine 54 preferentially affected by the PIT-1 molecule in NMR experiments are shown in magenta. Arginines 23 and 25, essential for PIP3 binding, are shown in cyan. Polar contacts between ligands and Akt PH domain are illustrated by yellow dashed lines. (*C*) Inositol-(1,3,4,5)-tetrakisphosphate (shown in gray) -based overlay of structures of PH domain of Akt [Protein Data Bank (PDB) ID code 1UNQ, shown in yellow], GRP1 (PDB ID code: 1FHX, shown in green), and PDK1 (PDB ID code:1W1D, shown in blue). Isosteric sidechains of leucine 52 of Akt, arginine 305 of GRP1, and lysine 495 of PDK1 as well as adjacent inositol-(1,3,4,5)-tetrakisphosphate are shown in stick representation.

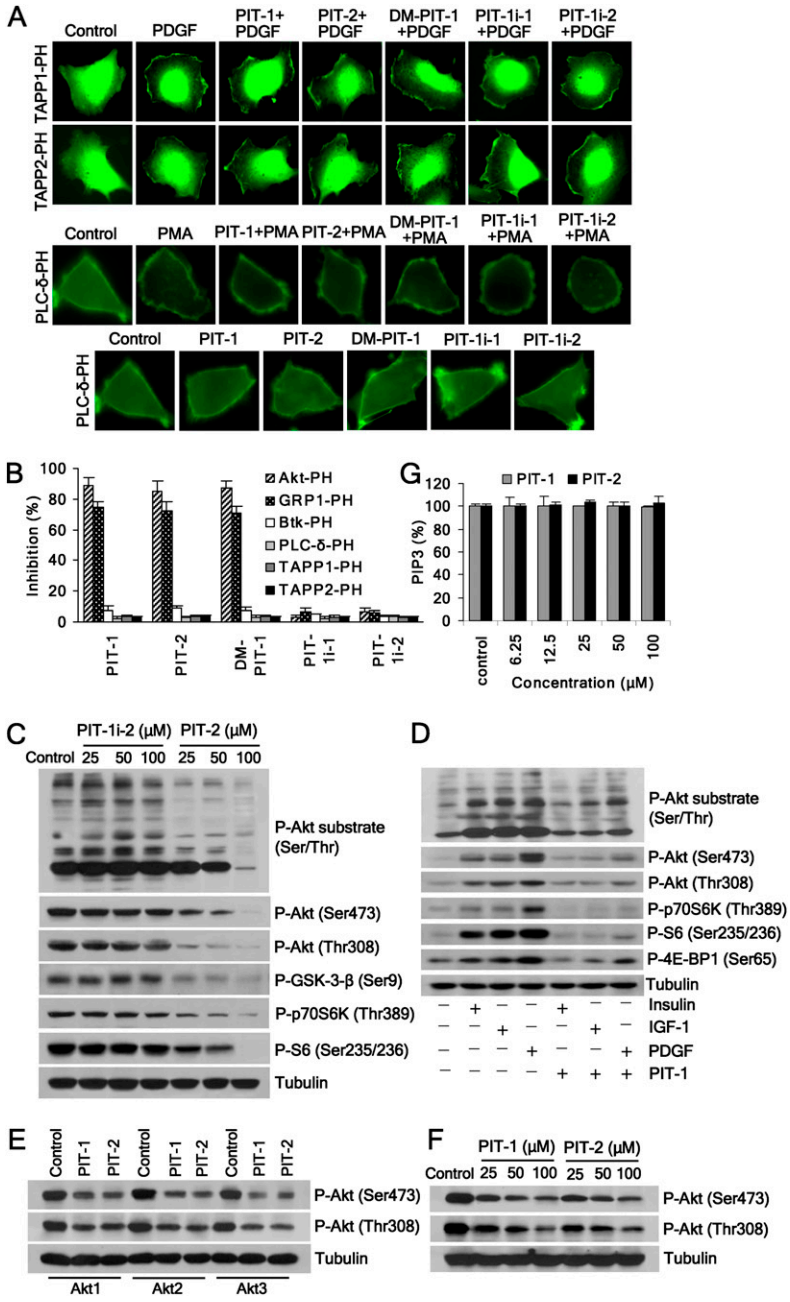


Fig. S3. PITs inhibit PI3K/PIP3/Akt signaling in cancer cells. (*A* and *B*) PITs don't inhibit growth factor or PMA-induced plasma membrane translocation of the PIP2-specific TAPP1, TAPP2, and PLC-δ PH domains. At the same time, they do not affect membrane location of PLC-δ PH domain without stimulation. SUM159 cells were transfected with the GFP-fusion vectors, serum starved overnight, and incubated with 100 μM PITs or PIT-1is for 1 h, followed by stimulation with 100 ng/mL PDGF or 250 ng/mL PMA for 5 min. All translocations were quantified by counting the number of cells with translocated PH domains in five random fields (*B*), and the representative fluorescent images are shown in *A*. (*C*) U87MG cells were treated with PIT-2 or PIT-1i-2 (25, 50, 100 μM) for 12 h, and the phosphorylation levels of the proteins in the Akt pathway were evaluated by Western blot using the indicated antibodies. (*D*) SUM159 cells were serum-starved overnight and incubated with or without 100 μM PIT-1 for 12 h, followed by stimulation with 200 nM Insulin, 100 ng/mL IGF, or 100 ng/mL PDGF for 15 min. The phosphorylation levels of the proteins in the Akt pathway were evaluated by Western blot using the indicated antibodies. (*E*) Akt1-, Akt2-, or Akt3-expressing cells were treated with 100 μM PIT-1 or PIT-2 for 12 h, and the phosphorylation levels of Akt were evaluated by Western blot using the indicated antibodies. (*F*) MCF-7 cells were treated with PIT-1 or PIT-2 (25, 50, 100 μM) for 12 h, and the phosphorylation levels of Akt were evaluated by Western blot using the indicated antibodies. (*G*) Analysis of PIP3 levels with treatments of PITs. U87MG cells were incubated with PITs (6.25–100 μM) for 12 h, and the PIP3 levels were measured by using a PIP3 ELISA (Echelon).

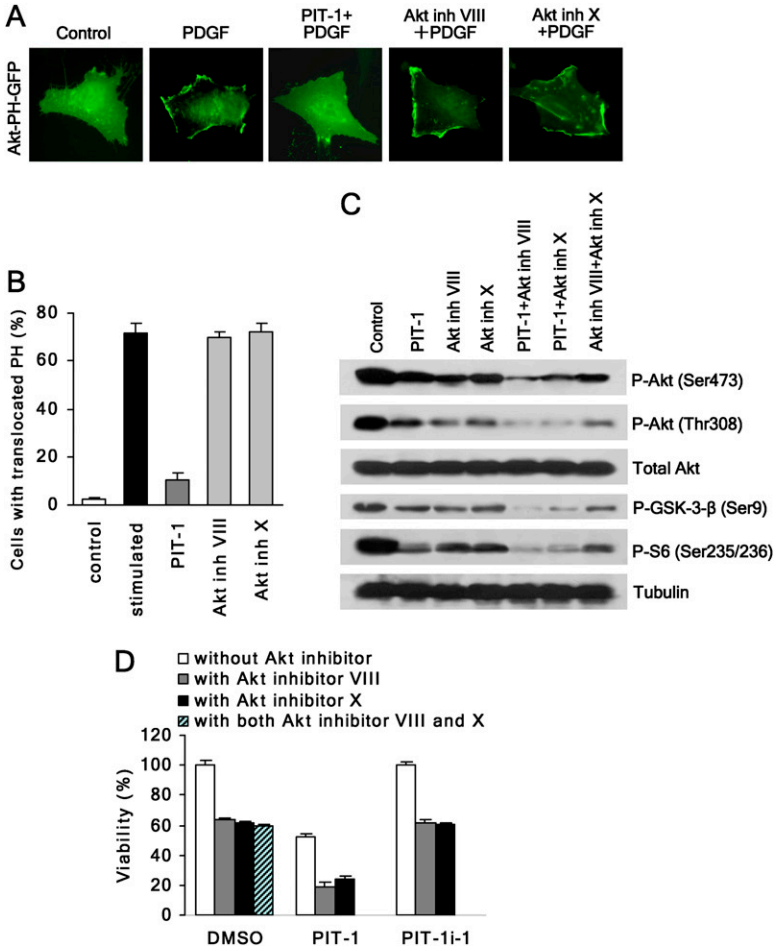


Fig. S4. PIT-1 synergizes with Akt kinase inhibitors VIII and X in attenuating Akt activation, phosphorylation of downstream targets and inhibition of cell survival. (*A* and *B*) PIT-1, but not Akt kinase inhibitors VIII and X, inhibits growth factor-induced plasma membrane translocation of Akt PH domain. SUM159 cells were transfected with Akt PH domain-GFP vector, serum-starved overnight, and incubated with 100 μM PIT-1 or 10 μM Akt inhibitor VIII or X for 1 h, followed by stimulation with 100 ng/mL PDGF for 5 min. The translocation was analyzed by using fluorescent microscope and was quantitated by counting the number of cells with translocated PH domains in five random fields (*B*). Representative fluorescent images are shown in *A*. (*C*) PIT-1 synergizes with Akt kinase inhibitors VIII and X in suppressing Akt activation and phosphorylation of downstream targets. U87MG cells were treated with 100 μM PIT-1, 10 μM Akt inhibitor VIII or X, or together for 12 h, and phosphorylation events were evaluated by Western blot. (*D*) PIT-1 synergizes with Akt kinase inhibitors VIII and X in reducing cancer cell viability. U87MG cells were incubated with 100 μM PIT-1 or PIT-1i-1, 10 μM Akt inhibitor VIII or X, or together for 24 h. Cell viability was determined by using ATP assay.

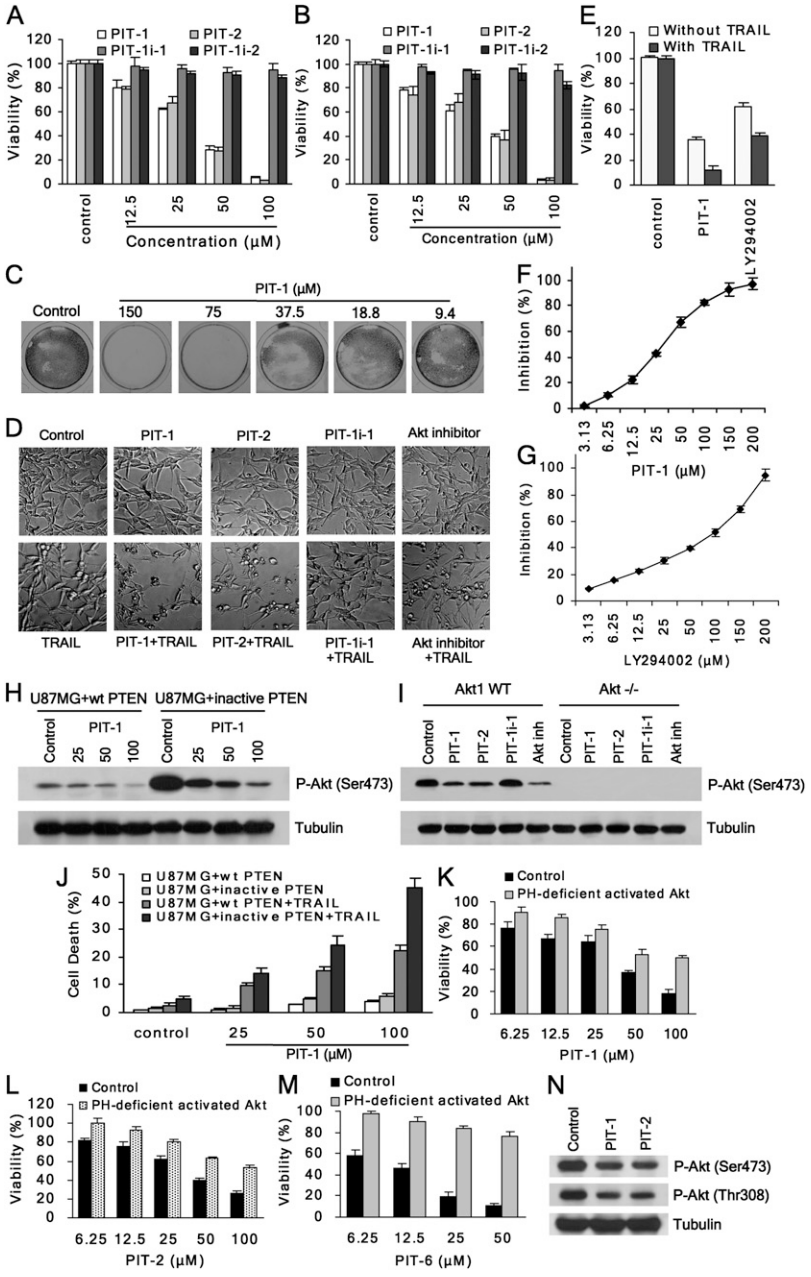


Fig. S5. PIT-1 reduces cell viability and synergizes with TRAIL more significantly in PTEN-deficient cells. (*A* and *B*) PIT-1 and PIT-2 reduce U87MG cell viability. U87MG cells were incubated with PITs or PIT-1is (12.5–100 μM) for 48 h. Cell viability was determined by using both ATP (*A*) and MTS (*B*) assays. (*C*) PIT-1 suppresses long-term colony formation by U87MG cells. U87MG cells were incubated with different concentrations of PIT-1 (9.4–150 μM) for 10 d, followed by staining with crystal violet. The representative images are shown. (*D*) PITs, as well as Akt inhibitor, sensitize U87MG cells to killing by TRAIL. U87MG cells were incubated with 100 μM PIT-1, PIT-2, PIT-1i-1, or 10 μM Akt inhibitor VIII in the presence or absence of 10 ng/mL TRAIL for 12 h, and the representative bright field images are shown. (*E*–*G*) PIT-1 exhibits a stronger activity than LY294002 in reducing cell viability. U87MG cells were incubated with 100 μM PIT-1 or LY294002 in the presence or absence of 10 ng/mL TRAIL for 48 h (*E*), or U87MG cells were incubated with 3.125–200 μM PIT-1 (*F*) or LY294002 (*G*) for 72 h, followed by cell viability analysis using ATP assay. (*H*) U87MG cells expressed with wild-type or inactive PTEN were treated with PIT-1 (25, 50, 100 μM) for 48 h, and the phosphorylation levels of Akt were evaluated by Western blot. (*I*) Akt1 expressing or triple knockout cells were incubated with 100 μM PIT-1, PIT-2, PIT-1i-1, or 10 μM Akt inhibitor VIII for 24 h, followed by analysis of Akt phosphorylation with Western blot. (*J*) The synergistic induction of cell death by PITs and TRAIL is more pronounced in U87MG cells expressing inactive PTEN compared with that in the cells expressing wild-type PTEN. Cells were treated as indicated for 24 h, followed by analysis using Sytox cell death assay. (*K*–*M*) Overexpression of PH domain-deficient activated Akt protects U87MG cells from killing by PITs. Cells stably infected with Akt retrovirus were incubated with 6.25–100 μM PIT-1 (*K*), PIT-2 (*L*), or PIT-6 (*M*) for 48 h. Cell viability was determined by using ATP assay. (*N*) Akt$^{-/-}$ cells stably infected with Akt retrovirus (Myr-Akt) were incubated with 100 μM PIT-1 or PIT-2, and the phosphorylation levels of Akt were evaluated by Western blot.

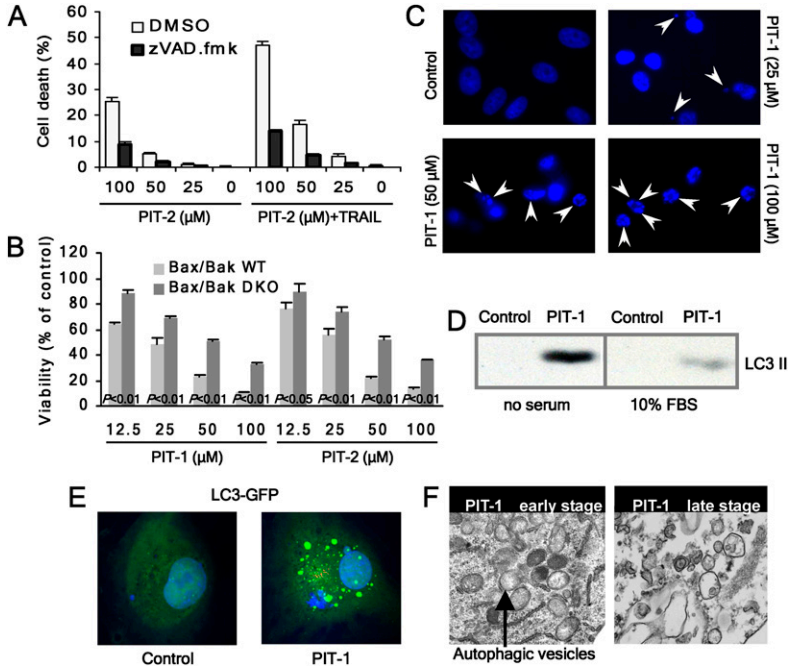


Fig. S6. PIT-1 induces metabolic stress in cancer cells. (*A*) Cell death triggered by PIT-2 alone or in combination with TRAIL is inhibited by pan-caspase inhibitor zVAD.fmk. U87MG cells were incubated with 100 μM zVAD.fmk together with PIT-2 (25, 50, 100 μM) in the presence or absence of 10 ng/mL TRAIL for 24 h. Cell death was analyzed by Sytox assay. (*B*) The loss of cell viability caused by PITs is attenuated in Bax/Bak double knockout cells compared with wild-type cells. Cells were incubated with PITs (12.5–100 μM) for 24 h, followed by viability analysis using ATP assay. (*C*) PIT-1 treatment causes nuclear condensation and fragmentation. U87MG cells were incubated with PIT-1 (25, 50, 100 μM) for 48 h, the nuclei were stained with Hoechst, and were analyzed by using a fluorescent microscope (the condensed/fragmented nuclei are indicated by arrowheads). (*D* and *E*) The treatment of U87MG cells with 100 μM PIT-1 for 48 h prominently increases the expression of LC3 II analyzed by Western blot (*D*). LC3 also displayed characteristic perinuclear punctuate signal following 100 μM PIT-1 treatment for 48 h in the cells transduced with LC3-GFP retrovirus (*E*, arrowheads). (*F*) Electron microscopy analysis shows the appearance of the characteristic double membrane-enclosed autophagic vesicles following treatment of U87MG cells with PIT-1 (100 μM) for 24 (early stage) or 48 h (late stage).

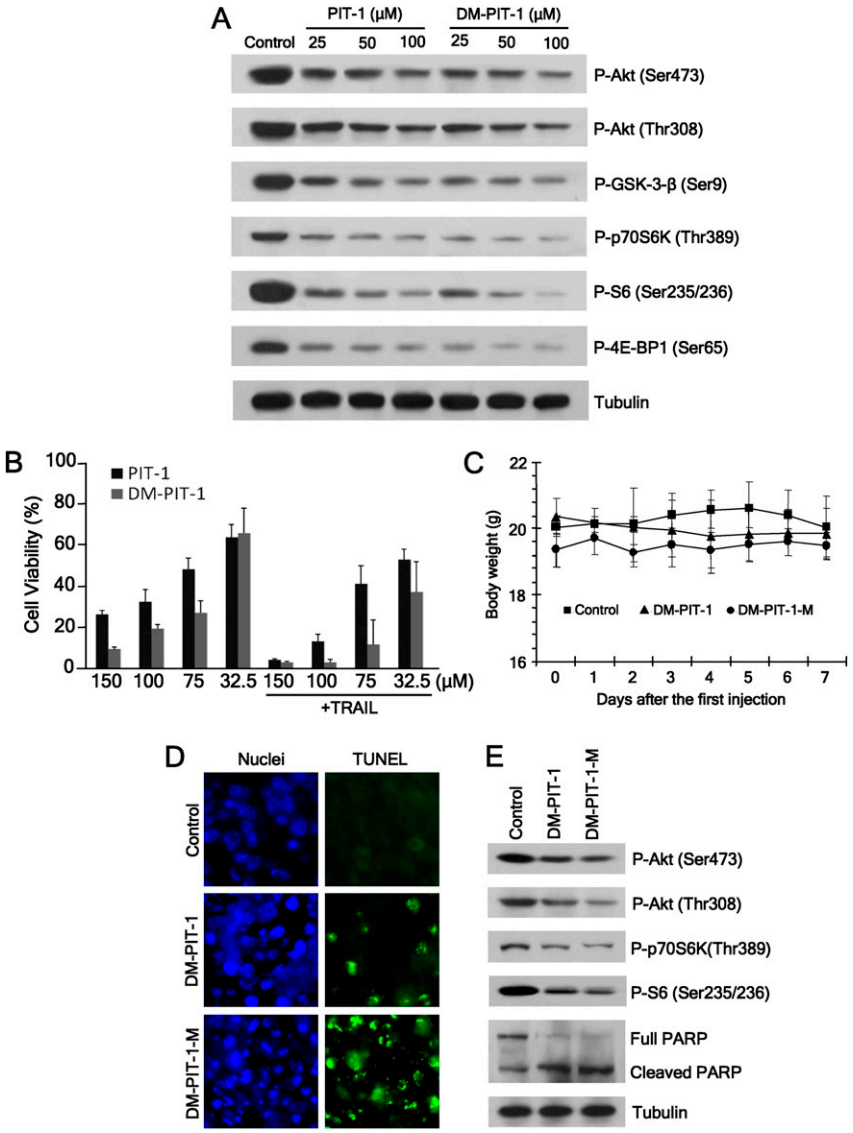


Fig. S7. DM-PIT-1 inhibits in vivo tumor growth. (*A*) DM-PIT-1 exhibits a stronger inhibition on PI3K/PIP3/Akt signaling compared with PIT-1. U87MG cells were treated with PIT-1 or DM-PIT-1 (25, 50, 100 μM) for 12 h, and the phosphorylation levels of the proteins in the Akt pathway were evaluated by Western blot using the indicated antibodies. (*B*) DM-PIT-1 is more cytotoxic toward U87MG cells than PIT-1. U87MG cells were incubated with PIT-1 or DM-PIT-1 (32.5–150 μM) with or without 10 ng/mL TRAIL for 48 h, followed by cell viability analysis using ATP assay. (*C*) No significant reduction in mouse body weight was observed during i.v. administration of DM-PIT-1 or DM-PIT-1-M in 4T1 tumor growth experiment. (*D*) DM-PIT-1 and DM-PIT-1-M administration induces apoptosis of cancer cells in vivo measured using TUNEL assay (green), and nuclear fragmentation evaluated with Hoechst staining (blue). Representative fluorescent images are shown. (*E*) DM-PIT-1 and DM-PIT-1-M administration suppresses PI3K/PIP3/Akt signaling and increases cleavage of PARP in tumor cells evaluated by using Western blot.

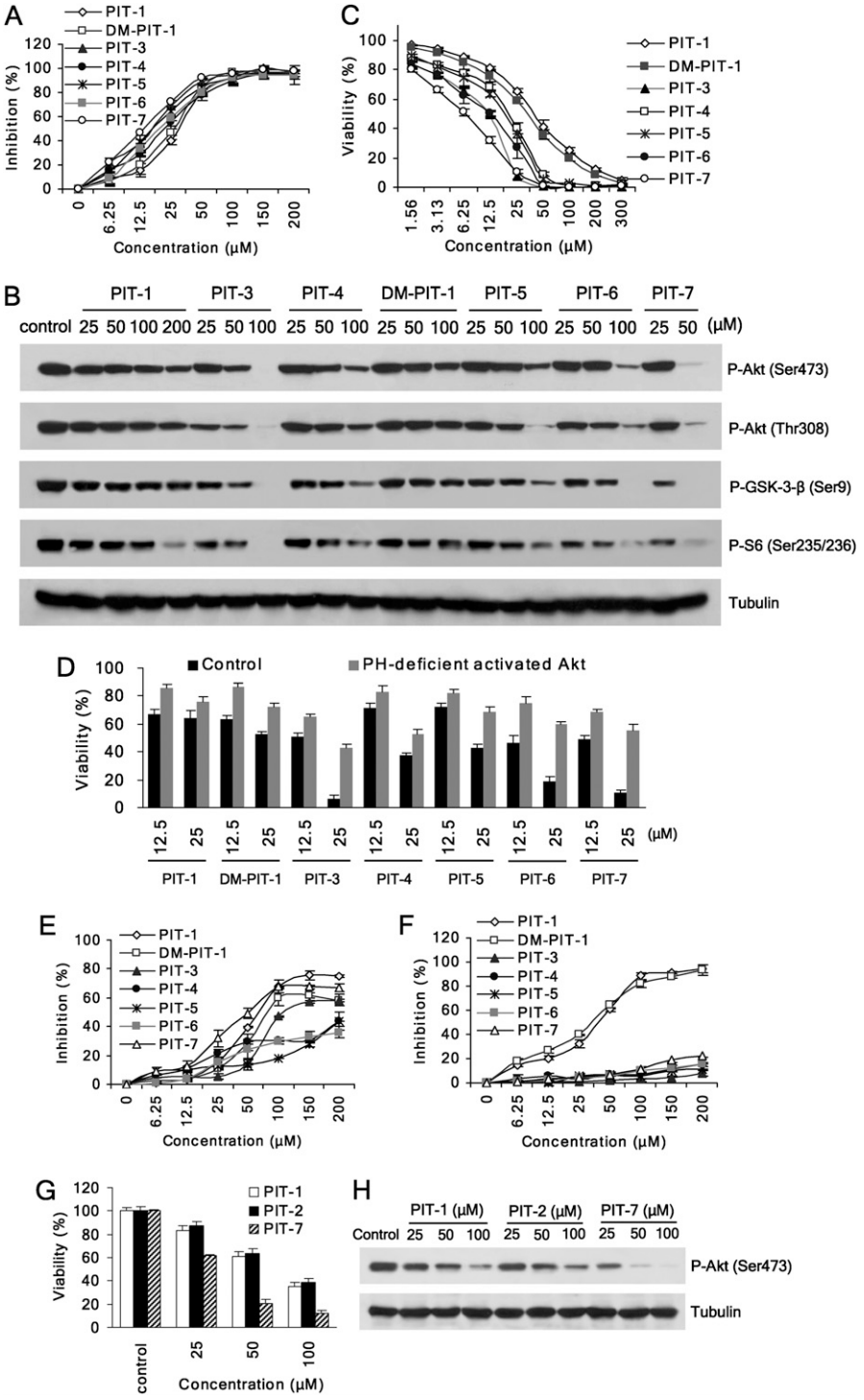
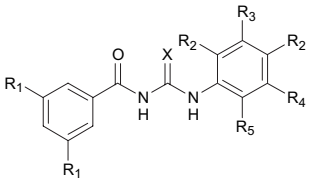


Fig. S8. Inhibition of PIP3/PH domain interactions, cell survival and Akt signaling by PIT-1 derivatives. (*A*) Inhibition of PIP3/Akt PH domain interaction by PIT-1 derivatives. TMR-conjugated PIP3 (15 nM) were incubated with 5 μM Akt PH domain in the presence or absence of PITs (6.25–200 μM) for 30 min, followed by FP measurement. (*B*) Inhibition of Akt signaling in U87MG cells by PIT-1 derivatives. U87MG cells were treated with PITs at indicated concentrations for 3 h, and the indicated phosphorylation events were evaluated by Western blot. (*C*) Induction of cell death by PIT-1 derivatives. U87MG cells were incubated with PITs (1.56–300 μM) for 48 h. Cell viability was determined by using ATP assay. (*D*) Overexpression of PH domain-deficient activated Akt protects U87MG cells from killing by PIT-1 derivatives. Cells stably infected with Akt retrovirus were incubated with 12.5 or 25 μM PITs for 48 h. Cell viability was determined by using ATP assay. (*E*) Inhibition of PIP3/PDK1 PH domain interaction by PIT-1 derivatives. TMR-conjugated PIP3 (15 nM) were incubated with 100 nM PDK1 PH domain in the presence or absence of PITs (6.25–200 μM) for 30 min, followed by FP measurement. (*F*) Inhibition of PIP3/GRP1 PH domain interaction by PIT-1 derivatives. TMR-conjugated PIP3 (15 nM) were incubated with 100 nM GRP1 PH domain in the presence or absence of PITs (6.25–200 μM) for 30 min, followed by FP measurement. (*G*) U87MG cells were incubated with PITs (25, 50, 100 μM) for 12 h. Cell viability was determined by using ATP assay. (*H*) U87MG cells were incubated with PITs (25, 50, 100 μM) for 12 h, and the phosphorylation level of Akt was evaluated by Western blot.

Table S1. Structure-activity relationship of PITs



Compounds	R_1	R_2	R_3	R_4	R_5	X	FP-Akt IC_{50}, μM	FP-PDK1 IC_{50}, μM	FP-GRP1 IC_{50}, μM	Cell viability IC_{50}, μM
PIT-1	H	H	NO_2	Cl	OH	S	31.0 ± 2.5	66.7 ± 3.5	40.3 ± 2.8	39.9 ± 3.2
PIT-1i-1	H	H	NO_2	Cl	OH	O	—	—	—	—
PIT-1i-2	H	H	NO_2	Cl	OMe	S	—	—	—	—
DM-PIT-1	Me	H	NO_2	H	OH	S	27.1 ± 3.2	80.5 ± 3.1	35.5 ± 3.8	33.9 ± 2.2
PIT-3	Me	Me	Cl	H	OH	S	17.5 ± 1.6	106.8 ± 3.9	—	12.4 ± 0.9
PIT-4	Me	H	CF_3	H	OH	S	22.6 ± 3.0	—	—	20.7 ± 3.3
PIT-5	Me	CF_3	H	H	OH	S	20.8 ± 2.5	—	—	18.8 ± 1.7
PIT-6	Me	Me	Br	H	OH	S	18.1 ± 3.1	—	—	12.6 ± 2.1
PIT-7	Me	H	Ph	H	OH	S	13.4 ± 2.1	52.3 ± 2.3	—	6.6 ± 0.3

—, >200 μM. Cell death was measured in U87MG cells. FP assay was performed by using Akt, PDK1, or GRP1 PH domains as described in Fig. 1.