Supporting Information

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SI Discussion

Deactivation of PIP3/Akt signaling pathway suppresses the growth of tumors and the resistance of cancer cells to chemotherapeutic drugs (1–4). The signaling pathways for PIP3 production and the mechanisms of PIP3-mediated Akt activation are well established and broadly applicable to different cell types. However, how the activated Akt is deactivated in specific cellular contexts remains to be elucidated. Facilitating the deactivation of Akt would enhance the efficacy of various inhibitors of PI3K, which prevent only the activation step. Inhibiting the kinase activity of Akt will be an obvious choice. However, as elegantly demonstrated in a recent study (5), paradoxically, chemicals targeting the Akt kinase domain were shown to lead to the "inhibitor-induced Akt activation," raising concerns for the long-term clinical utility of such inhibitors (5).

Akt is known to undergo dynamic conformational changes. Controlling the structural integrity of Akt appears to serve as another regulatory mechanism. For example, it has been reported that mTorc2, apart from its activity as an S473 kinase, plays important roles in maintaining the structural integrity and maturation of Akt by phosphorylating at T450 in turn motif (6, 7). Lack of phosphorylation at this site, as a result of genetic ablation of mTorc2 components, results in the structural instability of Akt, leading to an increased susceptibility to proteasome-dependent degradation. We showed that, compared with WT, Akt1 (e17k) was unstable upon inhibition of HSP90. However, this instability did not appear to be caused by the lack of phosphorylation at T450. A recent study reported an enhanced ubiquitination of Akt1 (e17k) mutant by TRAF6 E3 ubiquitin ligase, which facilitates its membrane localization and activation (8). We also found a faster kinetics of SC66-induced ubiquitination of this mutant.

How this mutation in PH domain leads to an enhanced Akt ubiquitination is not clear. Further studies, including the identification of cellular factors involved in SC66-induced Akt ubiquitination, are needed. Ectopic expression of TRAF6 or CHIP E3 ubiquitin ligases known to be involved in Akt ubiquitination (8, 9), failed to affect the SC66-induced Akt ubiquitination. Also, as SC66 inhibits Akt phosphorylation, the drug-bound Akt is unlikely to be directly ubiquitinated by TTC E3 ubiquitin ligase, which was shown to specifically bind to and ubiquitinate the phosphorylated Akt (10).

A wide variety of PI3K inhibitors have been developed and are continuously being identified. One caveat of suppressing PIP3 signaling in cancer cells by PI3K inhibitor alone is the activation of compensatory mechanisms, as demonstrated in HeLa cells treated with LY294002 or wortmannin. Likewise, targeting the Akt activity alone can be compensated by other AGC family member kinases. Considering the heterogeneity and various genetic lesions of cancers, the effective termination of Akt signaling requires a multifaceted strategy that prevents the membrane translocation and facilitates its deactivation. The dual-function allosteric inhibitor elucidated in this study exemplifies one such new strategy.

SI Materials and Methods

Reagents and Antibodies. Plasmids encoding human Akt1 were initially obtained from Dana–Farber/Harvard Cancer Center DNA Resource Core and subcloned into the pcDNA3.1/V5-His-TOPO vector. The site-directed mutagenesis was done with the QuikChange mutagenesis kit (Stratagene). EGFP-Foxo1 was obtained from Addgene. All Akt- and phosphorylation-specific antibodies were purchased from Cell Signaling Technology; V5 antibody was from Invitrogen; ubiquitin antibody (sc-9133) was from Santa Cruz Biotechnology. All other reagents, including the HRP-conjugated secondary antibodies, for Western blot were from GE Healthcare. LY294002, Akt inhibitors, rapamycin, and wortmannin were purchased from EMD Biosciences; PI-103, PIP3-coated beads, and PIP3 ELISA kit were from Echelon.

Western Blot and Immunostaining. Preparation of cell lysates, SDS/PAGE, and Western blot, and other standard molecular biological techniques, were essentially the same as described previously (11). For immunostaining of Akt1 (1:2,000 for V5 antibody) and phospho-Akt (1:200 for pS473), cells were fixed in 3% paraformaldehyde, and followed the same procedure as previously described (12).

PIP3 ELISA and in Vitro PIP3 Binding Assay with Purified PH-EGFP Protein. The serum-starved HeLa cells (1×10^7) were pretreated with LY294002 (20 μM) or group II chemicals (4 μg/mL) for 30 min, then stimulated with IGF1 (5 ng/mL) for 20 min. Extraction of PIP3 by sequential centrifugation in methanol: chloroform:HCl buffer and measurement of the extracted PIP3 was done using the PIP3 Mass ELISA Kit (K-2500s; Echelon), according to the instructions. For purification of PH-EGFP protein, HEK cells (1×10^8) stably expressing PH-EGFP tagged with the C-terminal V5/His were suspended in PBS solution containing 0.3% CHAPS, 20 mM imidazole, and protease inhibitor mixtures. The cell suspension was frozen on dry ice for 30 min and thawed at room temperature. The lysates were cleared by centrifugation and loaded on the column packed with Ni-NTA beads (Qiagen). After washing three times in PBS solution containing 0.3% CHAPS and 50 mM imidazole, the bound fraction was eluted with 100 mM imidazole. The eluted protein was concentrated in the binding buffer (10 mM Hepes, pH 7.4, 0.25% Nonidet P-40, 150 mM NaCl, 0.5 mM β-mercaptoethanol) by centrifugation (Amicon Ultra 10K cutoff filter; Millipore). The purified PH-EGFP protein (800 ng/mL) was preincubated with group II compounds (1 μg/mL) for 20 min on ice, and incubated with 20 μL of PIP3-coated beads (Echelon) for overnight at 4 °C. After washing the beads three times with binding buffer at room temperature, the amount of bead-bound PH-EGFP protein was determined by Western blot.

Proteasome, Deconjugation, and in Vitro Ubiquitination Assay Using Cell Lysates. HEK293 cells (1×10^6) were treated with compounds (4 μg/mL) for 1 h. After washing with PBS solution, the cell pellet was lysed on ice for 15 min in 200 μL lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1% Triton X-100). After clearing cell debris by centrifugation at 4 °C, the extract (50 μL) was subjected to proteasomal activity using Proteasome-Glo Chymotrypsin-Like Assay (G8621; Promega). The same extract was also assayed for deconjugation activity by using DUB-Glo Protease Assay (G6260; Promega). For in vitro ubiquitination assay, HEK293 expressing Akt1 tagged with V5/HIS were lysed in a buffer containing 50 mM Hepes, pH 7.4, 0.2% Nonidet P-40, 0.5 mM β-mercaptoethanol, and protease inhibitor mixtures. The lysates were frozen on dry ice for 30 min and thawed at room temperature. After centrifugation, the extract was subjected to in vitro ubiquitination reaction. Typically, 100 to 200 μg of total proteins were mixed with 1 μg of chemicals on ice for 10 min, and supplemented with MG132 (5 μM), ubiquitin aldehyde (4 μM), and ATP (5 mM) in 50 μL of ubiquitin conjugation reaction

buffer (Boston Biochem), and incubated for 1 h followed by Western blot.

Time-Lapse Live Cell Imaging Analysis for Mitotic and Apoptotic Cells. HeLa cells growing exponentially (or approximately 65%–70% confluence) in 35 mm dish were replaced with 2 mL of Leibovitz L15 medium supplemented with 10% FBS and cultured for 2 h, and then compounds were added. The time-lapse movie was taken every 15 min for 14 to 16 h. Each movie frame in the 14-h time period was analyzed for mitotic or apoptotic cells as previously described (12). The mitotic cells were identified as they underwent morphological changes from flat to round shape and cell division in later frames, and the apoptotic cells were identified as their membrane collapsed and lost adhesion to the plate.

MTT Assay. HEK293T cells (2.5×10^5) were plated in a 24-well plate in 500 μL of phenol red-free medium supplemented with 10% FBS. The next day, different amounts of each compound were added and cultured overnight (16–20 h), and 50 μL of MTT solution (5 mg/mL) were added to each well and incubated for 2 h. After directly adding 500 μL of isopropanol with 0.1 M HCl to each well to dissolve the crystals, the absorbance was measured at a wavelength of 570 nm.

Soft-Agar Colony Formation Assay. A 0.6% agar gel with 10% FBS in DMEM was prepared and added to a six-well culture dish as a base agar. HEK293T cells (3,000 per well) were plated in 0.3% agar gel with 10% FBS in DMEM supplemented with different concentrations of SC66 on top of the base agar and allowed to grow for 3 wk. Colonies were stained with Crystal violet dye. The results represent the averages from three independent experiments.

1. Fan QW, et al. (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 9:341–349.
2. Knight ZA, et al. (2006) A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* 125:733–747.
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12. Jo H, et al. (2010) Natural product Celastrol destabilizes tubulin heterodimer and facilitates mitotic cell death triggered by microtubule-targeting anti-cancer drugs. *PLoS ONE* 5:e10318.

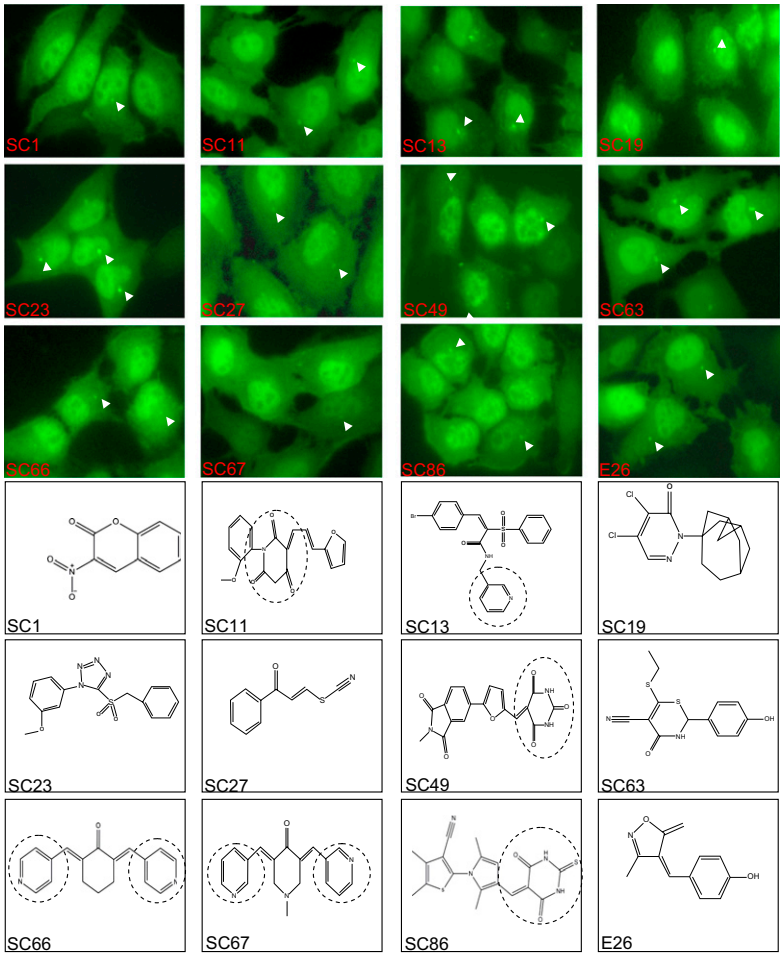


Fig. S1. Representative pictures of live imaging of HeLa-PH-EGFP cells treated with group II compounds (chemical structures, *Bottom*). Arrows indicate the pericentrosomal region. Dotted circles indicate the barbiturate-derivative and pyridine moiety.

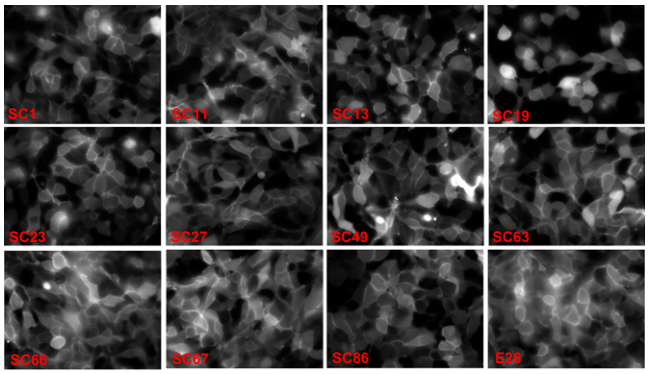


Fig. S2. The effect of group II compounds (8 μg/mL) on the PtdIns(4,5)P2-mediated membrane localization of EGFP-PLC-δ1-PH domain.

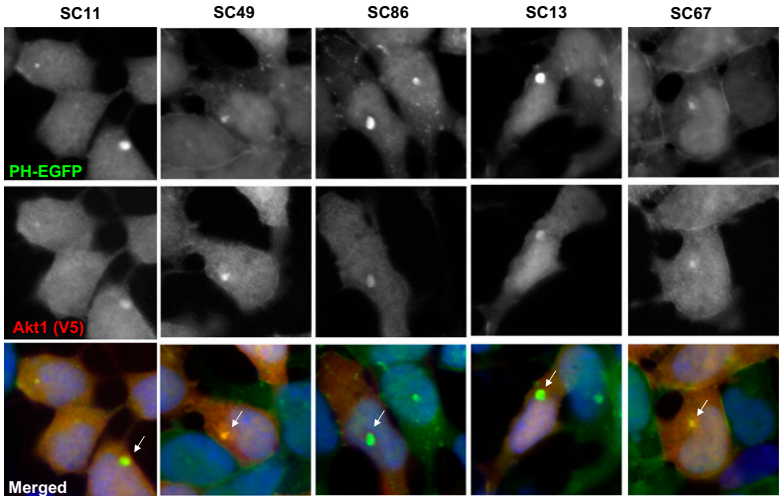


Fig. S3. HEK293 cells stably expressing PH-EGFP were transfected with the C-terminal V5/His-tagged Akt1. Following treatment with the indicated group II compounds (4 μg/mL) for 1 h, the fixed cells were stained for Akt1. Colocalization with PH-EGFP in pericentrosomal region is indicated by arrows.

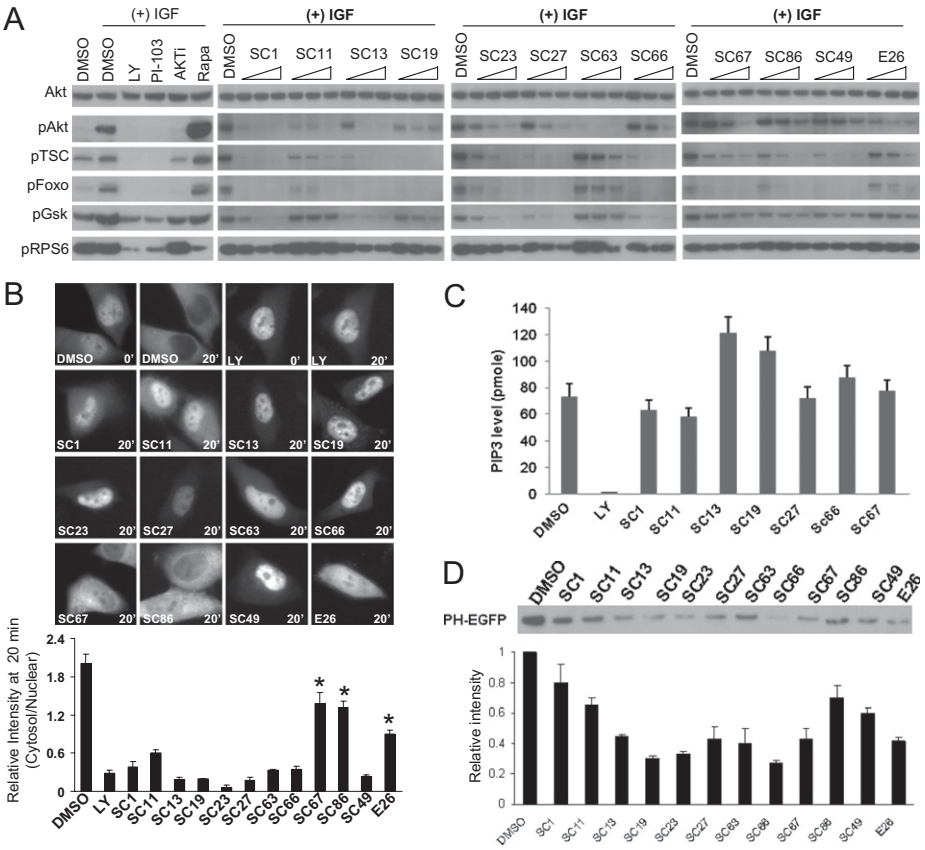


Fig. S4. (*A*) Serum-starved HeLa cells were treated with the known inhibitors of PI3K (LY294002 and PI-103), Akt (AKTi-VIII), mTorc1 (rapamycin), or three different concentrations (2, 4, or 8 μg/mL) of group II compounds for 30 min and stimulated with IGF (5 ng/mL) for an additional 30 min. The cell lysates were analyzed for the level of Akt phosphorylation at S473. The same blot was also probed for phosphorylation of the indicated target proteins. (*B*) HeLa cells transfected with EGFP-Foxo were serum-starved and treated with group II compounds for 30 min (4 μg/mL). Following addition of IGF1 (5 ng/mL), a live cell image was taken every 5 min. Representative image at 20 min IGF1 stimulation in the presence of each compound is shown. The intensity of cytoplasm and nuclear EGFP-Foxo was quantified, and the relative ratio is presented (*Bottom*; $*P < 0.05$, Student *t* test). (*C*) Serum-starved HeLa cells were treated with DMSO, LY294002 (20 mM), or the indicated group II compounds (4 μg/mL) for 30 min, followed by IGF1 (5 ng/mL) stimulation for 20 min. The amount of cellular PIP3 was measured by ELISA and calculated in reference to the standard PIP3 lipid. (*D*) The purified PH-EGFP protein (800 ng/mL) was preincubated with group II compounds (1 μg/mL) for 20 min on ice followed by incubation with the PIP3-coated beads overnight in a cold room. After washing, the bead-bound fraction was resolved on SDS/PAGE and blotted with the V5 antibody. Representative blot is shown, and the quantification is the average of three independent experiments.

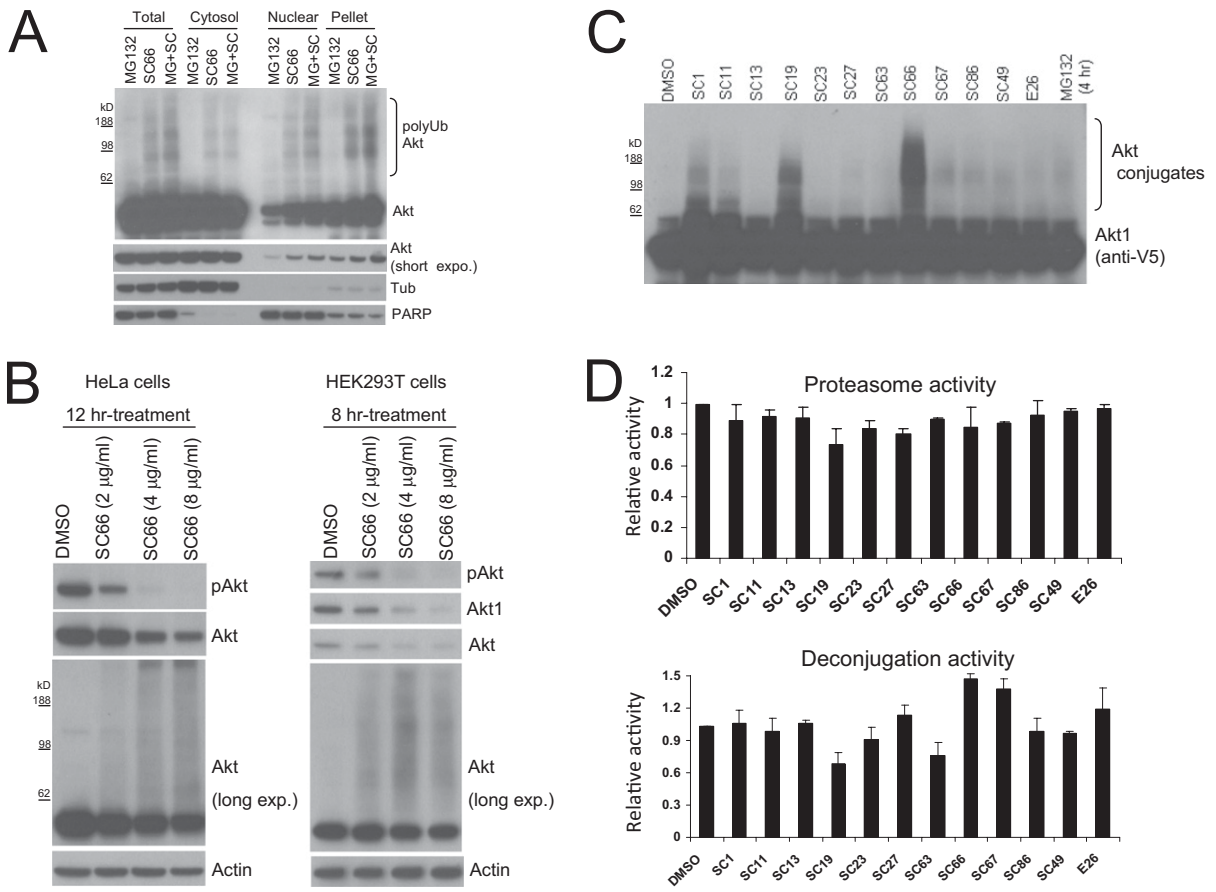


Fig. S5. (*A*) HeLa cells were pretreated with MG132 (10 μg/mL) for 2 h before addition of SC66 (4 μg/mL). Following an additional 2-h incubation, the total cell extract, cytosolic, nuclear, or pellet (insoluble) fraction were analyzed for Akt. The same fractions were simultaneously blotted for other cellular proteins: β-tubulin for cytosolic and PARP for nuclear fractions, respectively. (*B*) HeLa or HEK293T cells were treated with different amounts of SC66 for the indicated time points and the levels of pAkt, Akt, and actin were analyzed. (*C*) HEK293 cells stably expressing Akt1, HEK293-Akt1, were treated with group II compounds (4 μg/mL) for 1 h or MG132 (10 μg/mL) for 4 h, and the cell lysates were analyzed for Akt1 by Western blot with a monoclonal V5 antibody. (*D*) Proteasomal and deconjugation activity from the cytosolic cell lysates were measured. Relative activity in reference to DMSO-treated cells is presented.

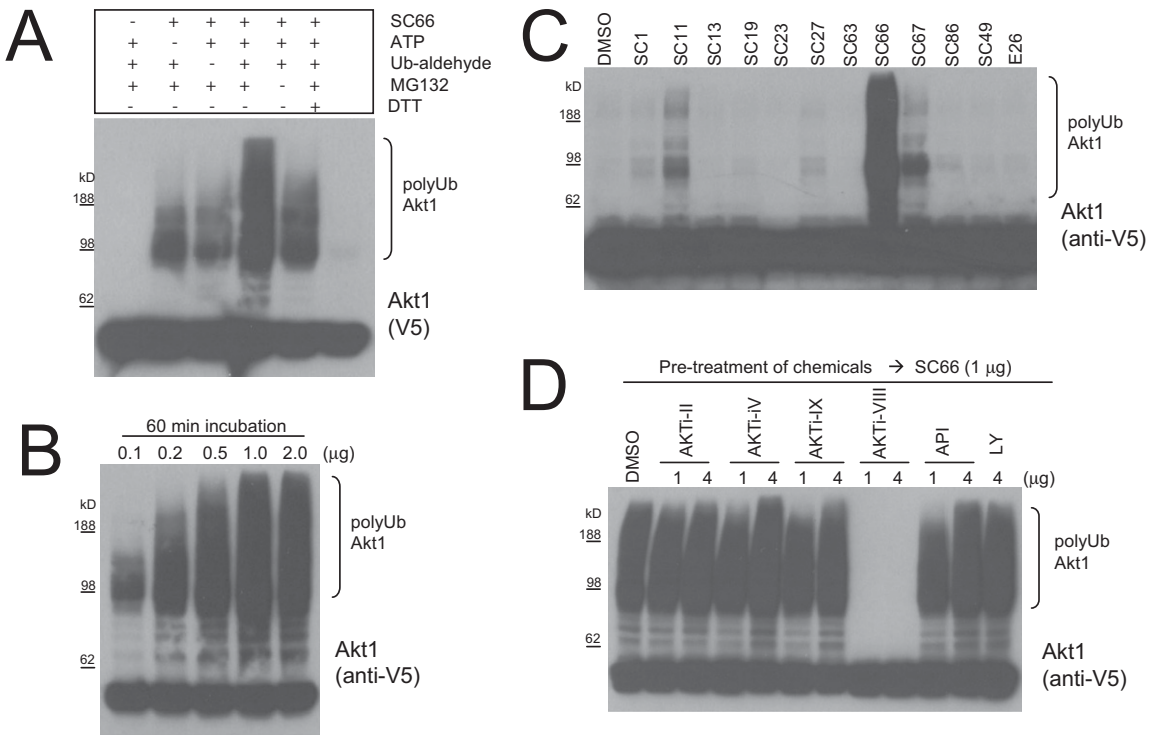


Fig. S6. (*A*) SC66-induced in vitro ubiquitination assay for Akt. HEK293-Akt1 cell lysates were incubated with the indicated combinations of ATP, ubiquitin aldehyde, MG132, DTT, and SC66 for 1 h. The absence of ubiquitin aldehyde, which inhibits deubiquitination, most significantly affected the Akt ubiquitination. The ubiquitinated Akt detected in the absence of additional ATP could be a result of residual ATP and preformed E1– and E2–ubiqutin complex present in the cell extract. In the presence of DTT, which disrupts the thioester bond between E1– and E2–ubiquitin that is required for the subsequent ubiquitination by E3 ligases, the SC66-induced Akt ubiquitination was almost completely abolished. (*B*) SC66 dose-dependent in vitro ubiquitination of Akt. (*C*) The effect of group II compounds on the in vitro ubiquitination of Akt1. (*D*) The effects of AKTi-VIII and other chemicals known to inhibit Akt pathway on in vitro ubiquitination of Akt1 by SC66. The indicated amounts of chemicals were pre- or simultaneously incubated with SC66, followed by in vitro ubiquitination reaction.

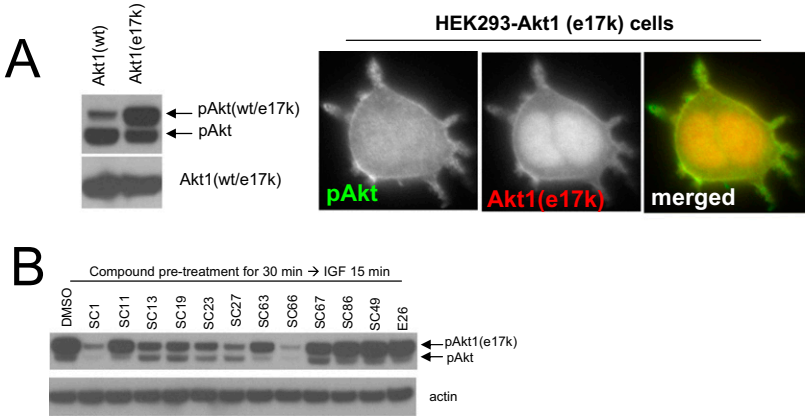


Fig. S7. (*A*) Level of phospho-Akt and cellular localization of Akt1 (e17k). (*B*) Inhibitory effects of group II compounds (8 μg/mL) on the phosphorylation of Akt1 (e17k).

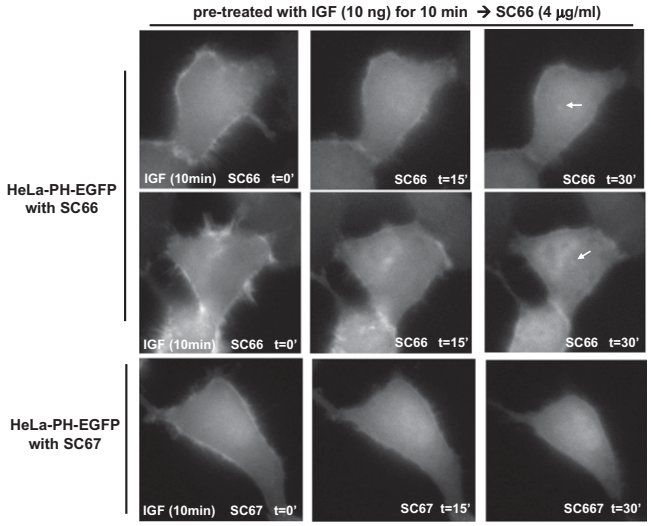


Fig. S8. Live cell imaging of the inhibitory effect of SC66 or SC67 on the membrane localization of PH-EGFP pretreated with IGF1. Arrows indicate the accumulation of PH-EGFP in the pericentrosomal region.

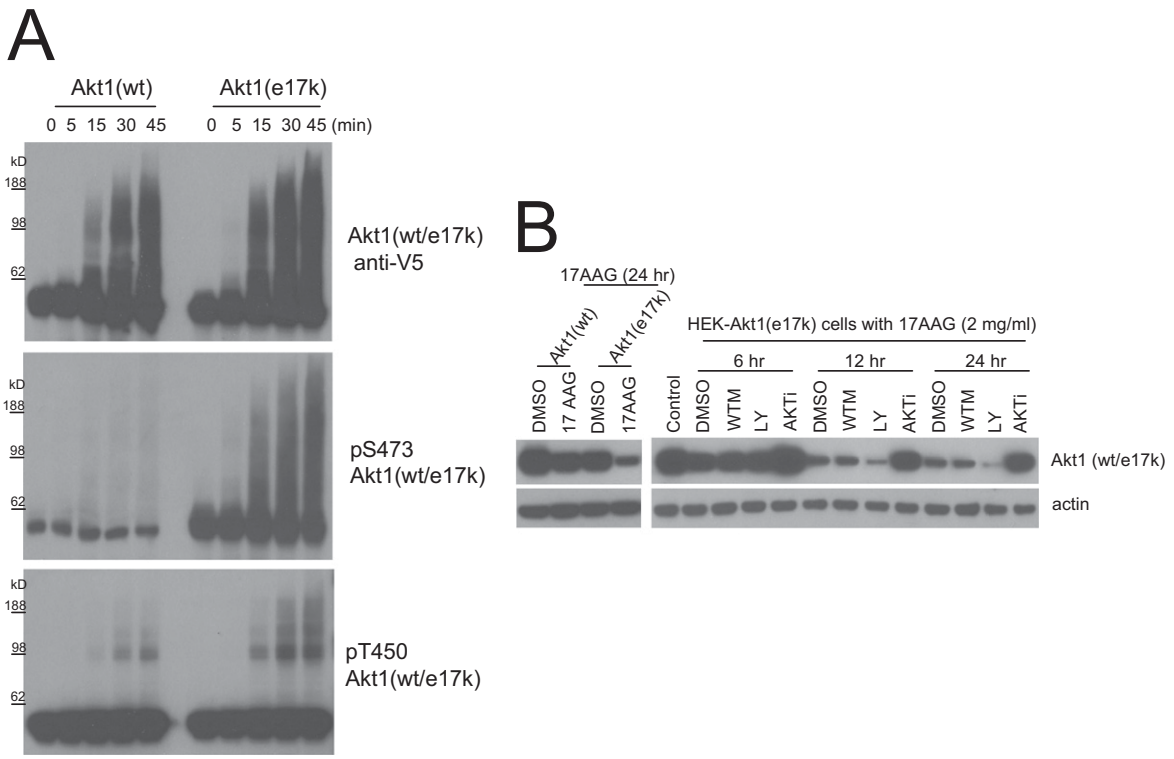


Fig. S9. (*A*) The kinetics of SC66-induced in vitro ubiquitination of WT and Akt1 (e17k). (*B*) The effect of HSP90 inhibition on the stability of WT or Akt1 (e17k) mutant. LY294002 (40 μM), wortmannin (200 nM), and AKTi-VIII (8 μM) were used.

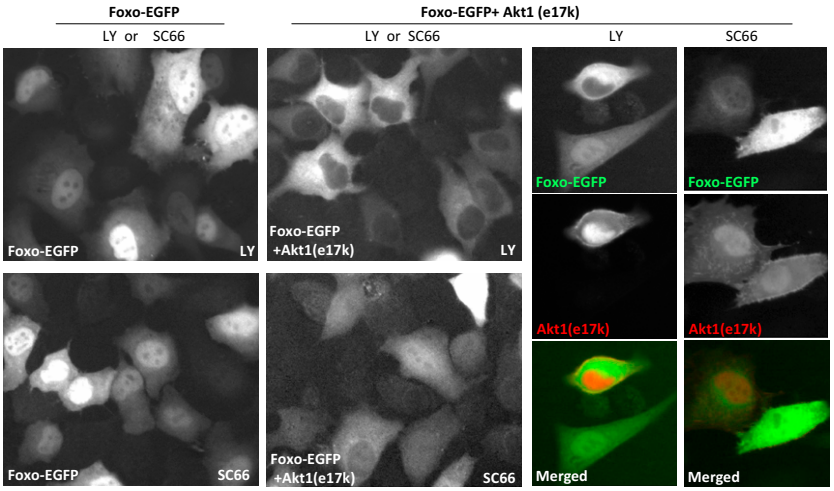


Fig. S10. Effect of LY294002 (40 μM) or SC66 (4 μg/mL) on the cellular localization of EGFP-Foxo in HeLa cells expressing Akt1 (e17k) mutant. HeLa cells were transfected with EGFP-Foxo alone or cotransfected with Akt1 (e17k) and treated with the chemicals for 1 h. Representative immunostaining shows coexpression of EGFP-Foxo and Akt1 (e17k).

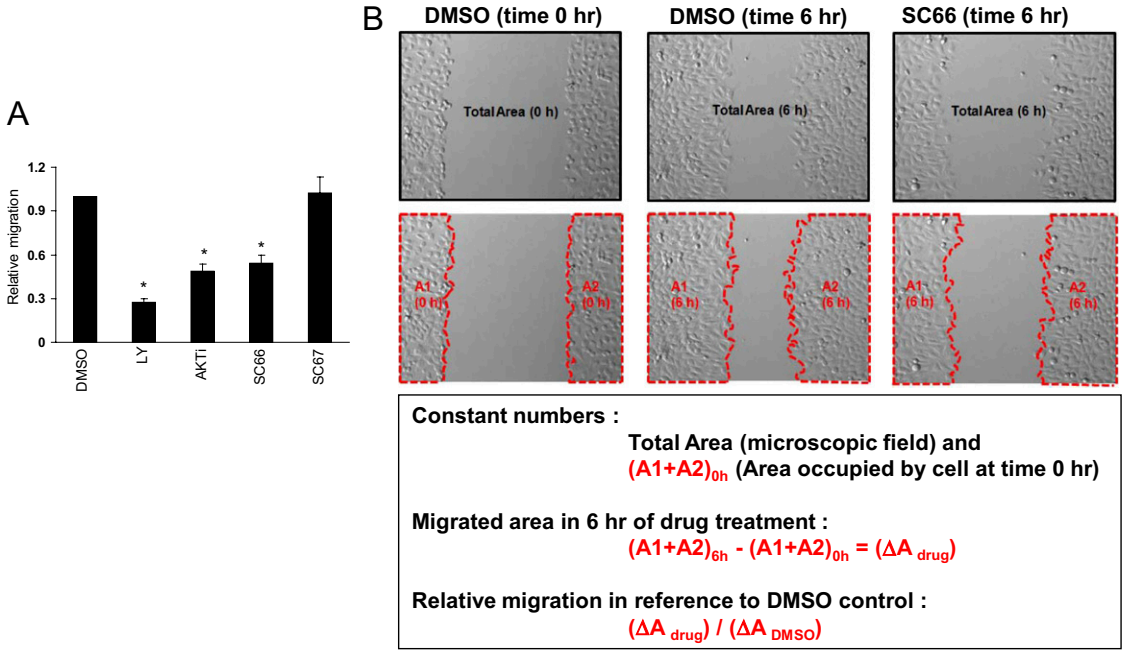


Fig. S11. (*A*) Quantification of cell migration in Fig. 3*A*. The relative migration area compared with DMSO control was presented. *$P < 0.05$ by Student t-test. (*B*) A schematic presentation of the quantification of HeLa cell migration in Fig. 3*A*.

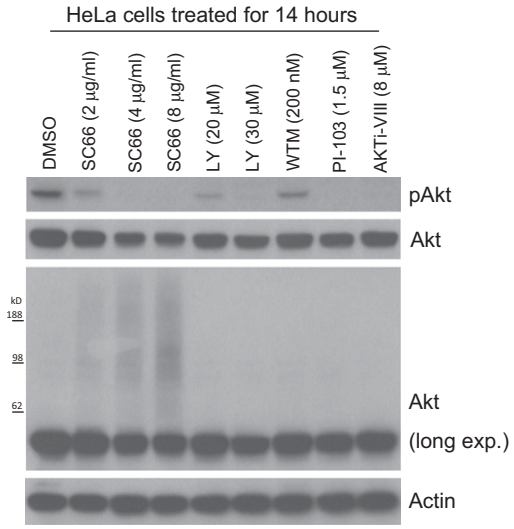


Fig. S12. Effects of long-term treatment with chemicals, as in Fig. 3*B*, on the levels of phosphorylated Akt in HeLa cells.

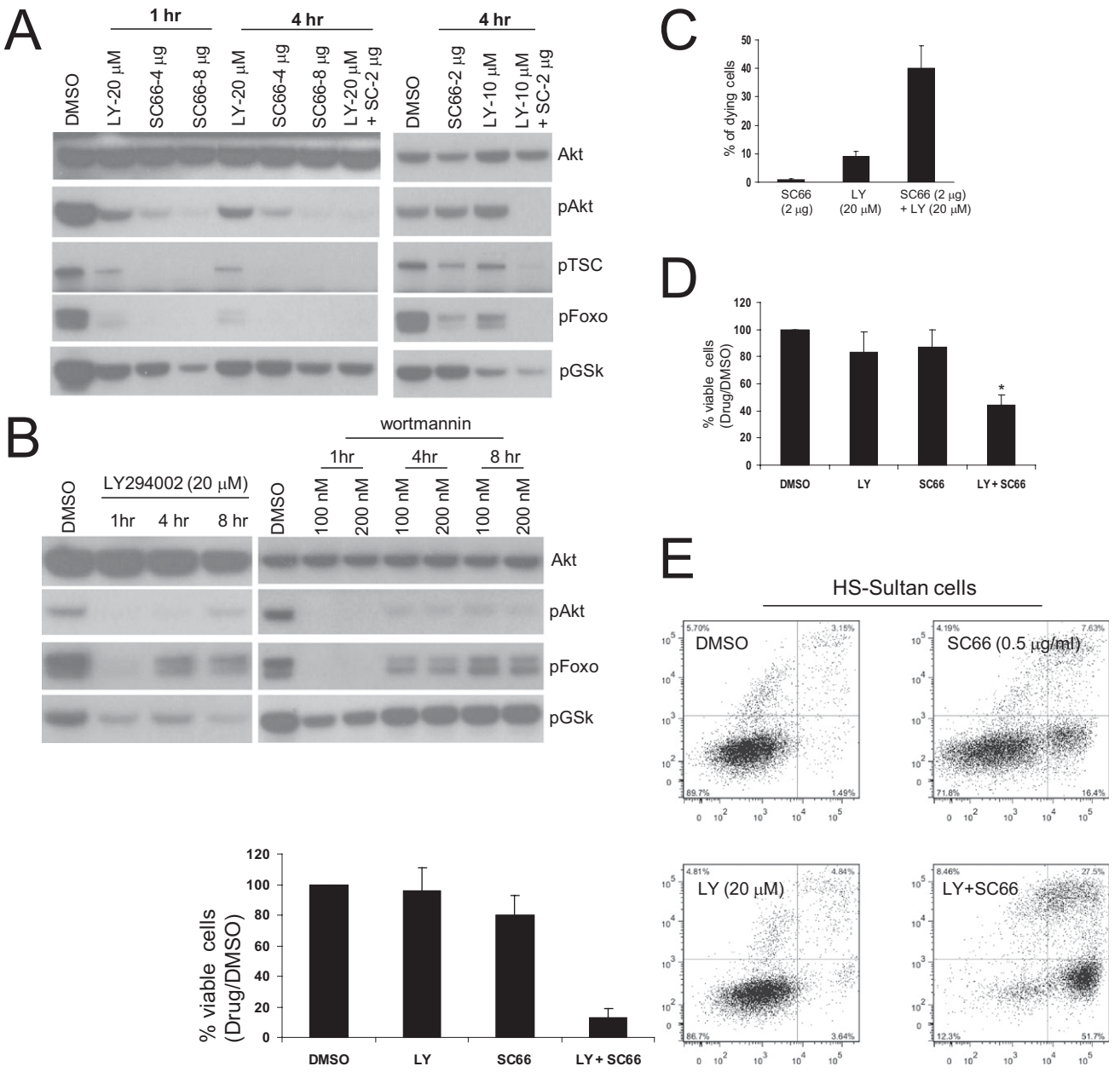


Fig. S13. (*A*) HeLa cells grown in serum-rich medium were treated with LY294002, SC66, or a combination of the two for the indicated time periods, and the cell lysates were analyzed for phosphorylation of Akt, Tsc, Foxo, and Gsk3. (*B*) HeLa cells were treated with LY294002 or wortmannin for the indicated time points, and the cell lysates were analyzed for phosphorylation of Akt and its targets. (*C*) The quantification of HeLa cell death determined by the live cell imaging in Fig. 3*D*. The percentage of dying cells in 14 h is presented. (*D*) The quantification of FACS analysis of HeLa cells treated with drugs in Fig. 3*D*. *$P < 0.05$ by Student *t* test. (*E*) The FACS analysis of HS-Sultan cells treated with LY294002, SC66, or in combination for 20 h (*$P < 0.05$, Student *t* test).

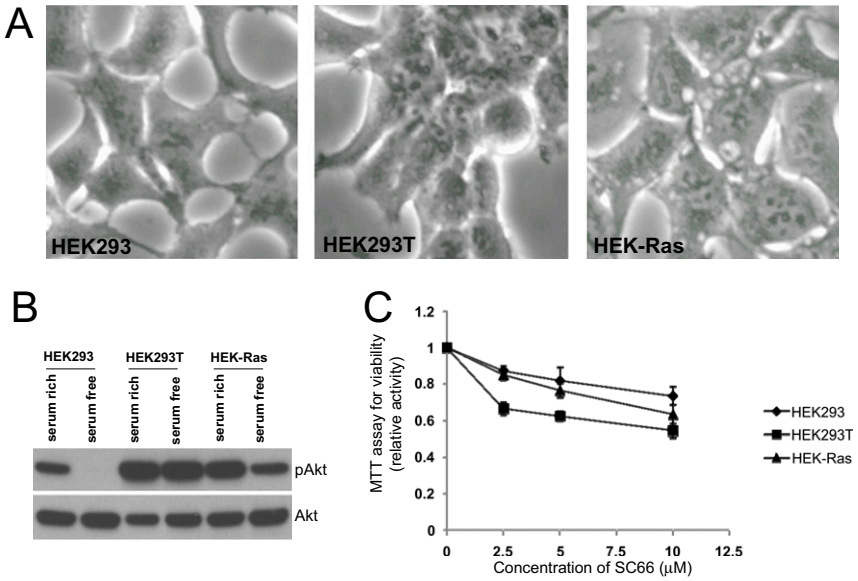
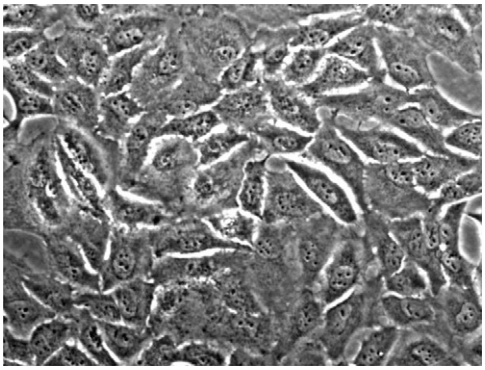
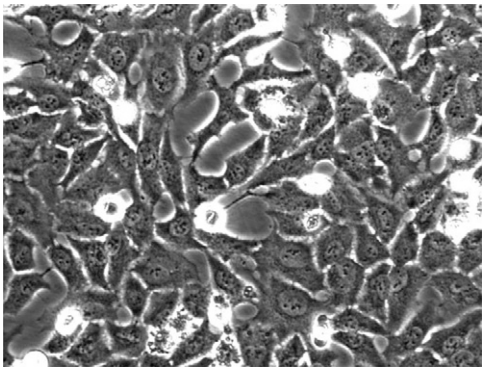


Fig. S14. (*A*) Morphology of the parental HEK293 cells and cells transformed by SV40 large T antigen (HEK293T) or H-Ras oncogene (HEK-Ras). (*B*) The level of phosphorylated Akt in cells grown in serum-rich or serum-free (for 1 h starvation) medium. (*C*) Differential growth-suppressive effects of SC66 on parental HEK cells and transformed cells. The relative viability was determined after 24 h treatment with SC66.



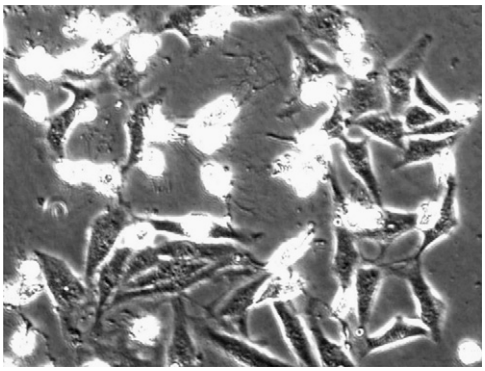
Movie S1. Live cell imaging of HeLa cells treated with SC66 (2 μg/mL). Frames were taken every 15 min for 14 h.

Movie S1



Movie S2. Live cell imaging of HeLa cells treated with LY294002 (20 μM). Frames were taken every 15 min for 14 h.

Movie S2



Movie S3. Live cell imaging of HeLa cells treated with SC66 and LY294002. Frame were taken every 15 min for 14 h.

Movie S3

Dataset S1. Characterization of group II compounds

Dataset S1 (XLS)

[a]8 μg/mL is the equivalent to 1× concentration of the initial high-throughput screening.
[b]Plus signs indicate inhibition of membrane translocation greater than 50% of control.
[c]Number of plus signs represents the relative inhibitory activity compared with DMSO control.
[d]Plus signs indicate inhibition of Akt phosphorylation (s473) greater than 50% of control.
[e]Number of plus signs represents the relative intensity of nuclear EGFP-Foxo over cytoplasm.