



A Small Molecule Compound Selectively Inhibits Akt, Including AKT1-E17K, Signaling and Tumor Growth in Cancer Cells with Hyperactivated Akt

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Abstract

The serine/threonine kinase Akt/PKB is frequently hyperactivated in human cancer and functions as a cardinal nodal point for transducing extracellular and intracellular oncogenic signals, and thus presents an exciting target for molecular therapeutics. Here, we reported the identification of a small molecule Akt inhibitor, AKT-SI (Akt signaling inhibitor)-1. AKT-SI inhibited kinase activities and phosphorylation levels of the three members of Akt but had no effects on the activities of PI3K and PDK1, 2 of the upstream Akt activators. Notably, the kinase activity and phosphorylation levels of constitutively active Akt, including a naturally occurring mutant AKT1-E17K, were potently inhibited by AKT-SI. AKT-SI is highly selective for Akt and does not inhibit the activation of PKC, SGK, PKA, STAT3, Erk-1/2, or JNK. The inhibition of Akt by AKT-SI resulted in induction of cell growth arrest and apoptosis selectively in human cancer cells that harbor constitutively activated Akt. Further, AKT-SI inhibited tumor growth in nude mice of human cancer cells in which Akt is elevated but not of those cancer cells in which it is not. These data indicate that AKT-SI is an Akt signaling inhibitor with anti-tumor activity *in vitro* and *in vivo* and could be a potential anti-cancer agent for patients whose tumors express hyperactivated Akt.

Results

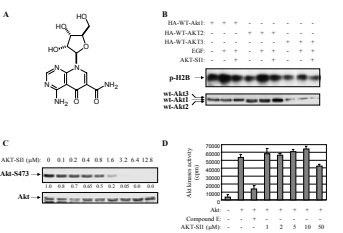


Fig. 1. Identification of AKT-SI as an Akt signaling inhibitor. (A) Chemical structure of AKT-SI. (B) AKT-SI inhibits the kinase activation of Akt1, 2 and 3. HEK293 cells were transfected with HA-Akt1, -Akt2 and -Akt3 and treated with AKT-SI (10 nM) prior to EGF stimulation; the cells were lysed and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (top). Bottom panel is a Western blot showing expression of transfected Akt1, Akt2 and Akt3 detected with anti-HA antibody. (C) AKT-SI inhibits phosphorylation levels of Akt in OVCAR3 cells, which express hyperactivated Akt. The cells were treated with AKT-SI at the indicated concentrations for 3 h and subjected to immunoblotting analysis with anti-phospho-Akt-S473 antibody (top). Bottom panel shows expression of total Akt. (D) AKT-SI does not inhibit Akt *in vitro*. *In vitro* kinase assay was performed with HEK293 cells and Histone-H2B (right). *In vivo* kinase assay was performed with recombinant constitutively active Akt protein in a kinase buffer containing the indicated concentrations of AKT-SI. Compound E, an ATP-mimic multiple kinase inhibitor, was used as positive control. The experiment was repeated three times.

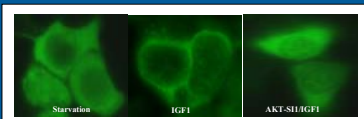


Fig. 2. AKT-SI inhibits IGF-1 induced plasma membrane translocation of Akt. HeLa cells were transfected on coverslips with Myc-AKT1, serum-starved overnight and then treated with (right) or without (middle) AKT-SI for 30 min prior to stimulation with IGF1 for 15 min. Following fixation, cells were immunostained with the anti-Myc monoclonal antibody, followed by a FITC-conjugated secondary antibody to reveal the presence of the epitope-tagged protein in the cytosol or membrane (middle). Cells without treatment with AKT-SI and IGF1 were used as control (left).

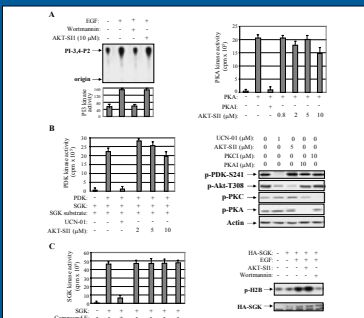


Fig. 3. AKT-SI does not inhibit PI3K, PDK1 and the closely related members of the AGC kinase family. (A) *In vitro* PI3K (left) and PKA (right) kinase assays. HEK293 cells were serum-starved and treated with AKT-SI (10 nM) or Wortmannin (1 μM) for 60 minutes prior to EGF stimulation. Immunoprecipitates were subjected to *in vitro* kinase assay using Pi-4-P as substrate (left). *In vitro* PKA kinase assay was carried out by incubation of recombinant PKA and substrate Kempeide with the indicated inhibitors. (B) Effect of AKT-SI on PDK1 activation *in vitro* (left) and on p-PKA, p-PKC and p-PDK levels in living cells (OVCAR3) (right). (C) AKT-SI does not inhibit SGK. Recombinant SGK protein was incubated with AKT-SI or compound E. Kinase assay was carried out by adding SGK substrate peptide and [³²P] ATP (left). *In vitro* kinase was performed with HA-SGK immunoprecipitated from HEK293 cells and Histone-H2B (right). (D) Effects of AKT-SI on phosphorylation of Erk, p38, JNK and Stat3 in OVCAR3 cells.

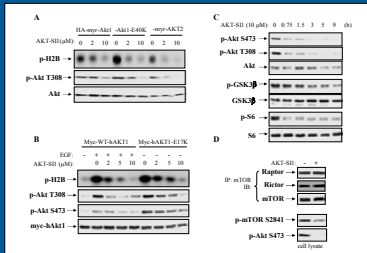


Fig. 4. AKT-SI inhibits constitutively active Akt and its downstream targets. (A) AKT-SI inhibited constitutively active Akt (AKT1-E17K, a recurrent mutation in human cancer, were inhibited by AKT-SI. (B) Kinase activity and phospho-T308 and -S473 of AKT1-E17K, a recurrent mutation in human cancer, were inhibited by AKT-SI. (C) AKT-SI inhibited phosphorylation of Akt downstream targets. (D) AKT-SI did not interfere with mTORC1 and mTORC2 complexes.

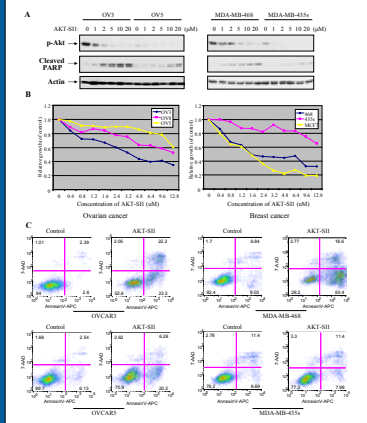


Fig. 5. AKT-SI inhibits Akt activity and cell growth and induces apoptosis in human cancer cells with hyperactivated Akt. (A) Following treatment with AKT-SI, phosphorylation levels of Akt and PARP cleavage were detected in the indicated human cancer cell lines (top and middle panels). (B) Cell proliferation after AKT-SI treatment. (C) Cells were treated with AKT-SI and stained with Annexin V for apoptosis analysis.

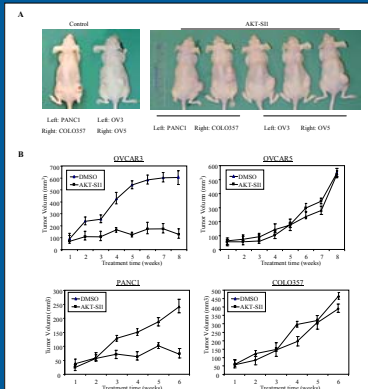


Fig. 6. AKT-SI exhibits anti-tumor activity in cancer cell lines with hyperactivated Akt in mouse xenografts. (A and B) AKT-SI inhibits tumor growth *in vivo*. Tumor cells were s.c. injected into nude mice with tumors presenting low level of p-Akt on the right flank and tumors with elevated level of p-Akt on the left flank. When the tumors reached an average size of about 100 mm³, animals were treated i.p. with either vehicle or 10 mg/kg/day AKT-SI. Representation of the mice with PANC1/OVCAR3 (left flank), which express elevated levels of p-Akt, and COLO357/OVCAR3 (right flank), which exhibit low levels of p-Akt, xenografts treated with AKT-SI or vehicle is shown in panel A. Panel B shows tumor growth curves generated with data from 10 mice/group. (C) Examples of tumor size (left) and weight (right) at the end of experiment. AKT-SI significantly reduced tumor weight in PANC1 and OVCAR3 xenografts as compared to vehicle control (*P < 0.02). (D) AKT-SI inhibits Akt phosphorylation *in vivo*. Tumor tissues from AKT-SI treated and untreated mice were lysed and immunoblotted with the indicated antibodies.

- AKT-SI is a pan-Akt signaling inhibitor.
- Constitutively activated Akts, including an active somatic mutation Akt1-E17K are effectively inhibited by AKT-SI.
- While it does not inhibit Akt *in vitro*, AKT-SI blocks Akt membrane translocation and inhibits phospho-T308 and -Ser473.
- AKT-SI induces apoptosis and cell growth arrest and inhibits the tumor growth in nude mice only in the tumor cells expressing hyperactivated Akt.

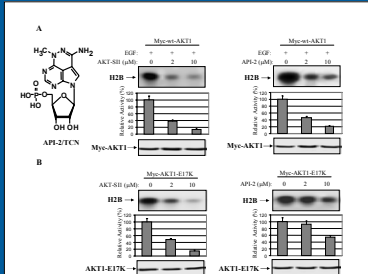


Fig. 7. AKT-SI is more potent than API-2/TCN in inhibition of Akt kinase activity, especially constitutively active Akt. While AKT-SI structure shares the ribose sugar moiety with API-2/TCN, the remaining portion of these 2 molecules have no chemical similarities. Nevertheless, we compared their capability of inhibiting Akt. HEK293 cells were transfected with wild-type-Myc-AKT1 (A) and constitutively active Myc-AKT1-E17K (B). Following 36 h incubation, cells were serum starved overnight. The wild-type Akt-transfected cells were treated with AKT-SI (left) or API-2/TCN (right) for 30 min and subsequently stimulated with EGF for 15 min. Immunoprecipitation was carried out with anti-Myc antibody and the immunoprecipitates were subjected to *in vitro* kinase assay using Histone H2B as substrate (top). Inhibition of Akt kinase activity by AKT-SI and API-2/TCN was quantified and calculated as relative activity (middle). Western blot analysis shows the immunoprecipitated AKT1 proteins (bottom panels). The experiments were repeated three times.

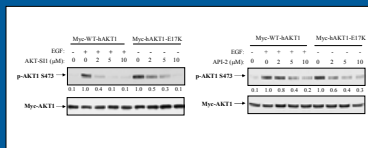


Fig. 8. AKT-SI and API-2/TCN inhibit phospho-Akt levels. Wild-type Myc-AKT1 and constitutively active Myc-AKT1-E17K transfected HEK 293 cells were treated with the indicated reagents and immunoblotted with anti-phospho-Akt-S473 (top) and -Myc (bottom) antibodies.

Conclusions