AKT inhibitor, GSK690693, induces growth inhibition and apoptosis in acute lymphoblastic leukemia cell lines

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The PI3K/AKT signaling is activated in various hematologic malignancies. We evaluated the effect of a novel, pan-AKT kinase inhibitor, GSK690693, on the proliferation of 112 cell lines representing different hematologic neoplasia. Fifty-five percent of all cell lines tested were sensitive to AKT inhibitor (EC_{50} < 1 μM), with acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma, and Burkitt lymphoma showing 89%, 73%, and 67% sensitivity to GSK690693, respectively. The antiproliferative effect was selective for the malignant cells, as GSK690693 did not inhibit the proliferation of normal human CD4$^+$ peripheral T lymphocytes as well as mouse thymocytes. Phosphorylation of downstream substrates of AKT was reduced in both sensitive and insensitive cell lines on treatment with GSK690693, suggesting that the cause of resistance was not related to the lack of AKT kinase inhibition. Consistent with the role of AKT in cell survival, GSK690693 also induced apoptosis in sensitive ALL cell lines. Overall, our data provide direct evidence for the role of AKT signaling in various hematologic malignancies, especially ALL and some lymphomas. (Blood. 2009;113:1723-1729)

Introduction

The PI3K/AKT-signaling pathway has been shown to be associated with cancer development and disease progression.[1,2] Constitutive activation of this pathway can result from several factors, including mutation and/or amplification in certain components within this pathway (eg, EGFR, ErbB2, Met, PDGFR, c-Kit, PI3K, and AKT) as well as the down-regulation or loss of negative regulators such as the serine phosphatase, PTEN.[3,4] AKT is a serine-threonine protein kinase that transmits cellular signals regulating cell growth, survival, and metabolism. Hyperactivation of AKT promotes cell survival, cell-cycle progression, and prevention of apoptosis mediated by PTEN and proapoptotic cytotoxic agents.[5,6]

The PI3K/AKT pathway is also activated in several hematologic malignancies.[7] AKT can be constitutively activated due to a chromosomal translocation that triggers permanent activation of an upstream tyrosine kinase. Two such examples include the BCR-ABL protein, encoded by a chimeric (fusion) gene formed by a (9;22) translocation in chronic myeloid leukemia (CML)[8] and the NPM-ALK protein, encoded by a fusion gene formed by a (2;5) translocation seen in some anaplastic large cell lymphomas.[9] Deregulated expression of the T-cell leukemia/lymphoma 1 (TCL1)–family oncoproteins, which augment AKT signaling has been associated with various T-cell and B-cell malignancies.[11] AKT activation has been associated with reduced survival of cells from acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), Hodgkin and non-Hodgkin lymphoma, and mantle cell lymphoma. [10-15]

Acute lymphoblastic leukemia (ALL) is an aggressive malignancy, representing nearly one-third of all pediatric cancers as well as approximately 20% of adult leukemias. Although conventional chemotherapy has significantly improved patient outcome, a large number of patients still relapse, and the intensive treatment regimens are often associated with severe complications.[16] Constitutively active AKT has been shown to have a key role in both T-cell and B-cell ALL biology. Interleukin-7 (IL-7), a cytokine produced by thymic and bone marrow stroma, increases viability, cell proliferation, and metabolic activity of T-cell ALL (T-ALL) via activation of PI3K/AKT signaling.[16] Recent studies investigating T-ALL development describe an association between AKT phosphorylation and Notch-1 signaling.[17,18] Notch-1 is a key regulator of cell growth and metabolism during T-cell development, and activating mutations of Notch-1 have been shown to contribute to more than 50% of human T-ALL.[19,20] Loss of PTEN was associated with resistance to pharmacologic inhibition of Notch-1 signaling by γ-secretase inhibitors, suggesting a key role for PI3K/AKT signaling in Notch-1 mutant T-ALL.[17] Further, inhibition of mTOR, a downstream effector of AKT activation, leads to apoptosis of blasts from ALL patients of both B-cell and T-cell origin.[21] Taken together, these studies present the PI3K/AKT pathway as an attractive target for drug development for the treatment of ALL.

GSK690693 is a potent and selective, ATP-competitive, pan-AKT kinase inhibitor currently in clinical development for patients with various malignancies.[22] In this study, we investigated the effect of GSK690693 on the proliferation of tumor cells from various hematologic malignancies. Our results demonstrate that ALL cells from both T-cell and B-cell origin are very sensitive to AKT inhibition, and treatment with GSK690693 leads to inhibition of cell proliferation and induction of apoptosis in these cells.

Methods

Animals and thymocytes culture

All animal experiments were conducted following the guidelines and protocols approved by the GlaxoSmithKline Institutional Animal Care and Use Committee. Female C57BL6 mice obtained from Taconic Farms (Hudson, NY) from 6 to 9 weeks of age were euthanized by CO_2 inhalation.

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Thymus lobes were dissected out and pooled in ice-cold RPMI media. Single-cell suspension of thymocytes was obtained by passing the thymus lobes through a nylon mesh (100-μm pore size; BD Falcon, San Jose, CA). For cell proliferation assay, the cells were immediately seeded in 96-well tissue culture plates at the density of 100 000-200 000 cells per well in medium containing 10% fetal bovine serum (FBS).

Cell culture

Tumor cell lines were obtained from ATCC (Manassas, VA) or from DSMZ, the German Resource Center for Biological Material (Braunschweig, Germany). Cells were cultured in the appropriate culture media supplemented with 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA) and either 10% or 20% FBS (JRH Biosciences, Lenexa, KS) at 37°C/5% CO_2.

Stimulation of normal human T lymphocytes

Human peripheral blood $CD4^+$ T cells were purchased from Lonza (Basel, Switzerland). Cells were cultured in RPMI 1640 media containing 10% heat-inactivated fetal calf serum and stimulated with a mixture of 10 μg/mL phytohemagglutinin-M (PHA-M) and 50 ng/mL human recombinant IL-2 (both from Sigma-Aldrich, St Louis, MO) to induce cellular proliferation.

Compound

GSK690693 and ZSTK474 were synthesized at GlaxoSmithKline and dissolved in dimethyl sulfoxide (DMSO) for treatment of cells.

Cell proliferation assay

Cell proliferation studies were assessed using the CellTiter Glo luminescent cell viability assay (Promega, Madison, WI). Cells were plated at optimal densities in 96-well plates and incubated overnight. Cells were then treated with DMSO or GSK690693 (ranging from 30 μM-1.5 nM) for 72 hours. The antiproliferative effect was determined using CellTiter Glo following the manufacturer's protocol. Data were plotted as percent of DMSO control, and the half-maximal effective concentration (EC_{50}) values were calculated using the XLFit (IDBS, Guildford, United Kingdom) curve-fitting tool from Microsoft Excel.

AKT kinase assay

In vitro AKT kinase activity was measured using the AKT kinase assay kit purchased from Cell Signaling Technology (Beverly, MA). Briefly, tumor cells were treated with DMSO or various concentrations of GSK690693 for 1 hour. With cells requiring AKT stimulation, cells were plated overnight in serum-free media, then treated with 1 μM GSK690693 for 1 hour. Cells were then treated with 5 ng/mL insulin for various times to activate AKT. Lysates were prepared, and 400 μg protein were used to immunoprecipitate AKT using immobilized anti-AKT antibody by overnight incubation at 4°C, and the kinase assay was performed according to the manufacturer's instructions using GSK-3 fusion protein as a substrate. The kinase reaction was terminated with sodium dodecyl sulfate (SDS) loading buffer (Invitrogen). The samples were run on a 4% to 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen), and the phosphorylation of GSK-3 was measured by Western blot analysis with antiphospho-GSK-3α/β (Ser21/9) antibody.

Western blot analysis

Tumor cells were treated with DMSO or various concentrations of GSK690693 for 6 hours and lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. The lysates were diluted with sample loading buffer, and 50 μg total protein were run on 4% to 12% SDS-PAGE gels. The samples were then transferred to polyvinylidene difluoride (PVDF) membranes and probed with the antibodies for phospho-GSK3β, phospho-PRAS40, phospho-p70S6K, phospho-AKT, AKT, and tubulin at 1:1000 dilution. Secondary, fluorescently labeled antibodies were used at 1:5000 dilutions. All antibodies were obtained from Cell Signaling Technology. All blots were imaged on a LiCor Odyssey instrument as directed by the manufacturer (LiCor Biosciences, Lincoln, NE).

Table 1. Antiproliferative activity of GSK690693 in cell lines derived from various hematologic malignancies

	Cell lines evaluated	Sensitive cell lines	Sensitivity, %
Total number of unique cell lines	112	62	55
ALL	35	31	89
AML	11	2	18
CLL	8	4	50
CML	5	0	0
Burkitt lymphoma	15	10	67
Non-Hodgkin lymphoma	11	8	73
Hodgkin lymphoma	4	1	25
Others	23	6	26

Antiproliferative activity of GSK690693 was determined in a 72-hour proliferation assay against various hematologic cell lines. Cell lines with an EC_{50} less than 1000 nM were classified as sensitive cells.

Cell-cycle analysis

Cells were plated in 96-well plates at a density of 5000 cells per well and incubated overnight. Cells were then treated with DMSO or various concentrations of GSK690693 for 72 hours, fixed, and stained with propidium iodide to stain nuclei based on the Vindelov method.[23] Cells were then scanned on the FACSCalibur (BD Biosciences, Franklin Lakes, NJ), and the fraction of cells in each phase of cell cycle was determined using cell-cycle analysis platform in FlowJo software (TreeStar, Ashland, OR).

Apoptosis assays

Apoptosis was measured using the Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN), the Caspase 3/7 Assay Kit (Promega), and the Pathscan Cleaved Caspase-3 Sandwich ELISA Kit (Cell Signaling Technology). Western blot analysis was used to measure PARP cleavage. Cell Death ELISA is a quantitative detection of histone-complexed DNA fragments in mono- and oligonucleosomes. The cleaved caspase-3 ELISA kit is a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of cleaved caspase-3. Cells were plated at 10 000 cells/well in 96-well plates and incubated overnight. DMSO or GSK690693 at the indicated concentrations was added, and cells were incubated for an additional 24 hours for the caspase 3/7 and cleaved caspase-3 ELISA, or 72 hours for the Cell Death ELISA, and assayed based on the manufacturer's protocol. For the PARP cleavage analysis, cells were plated at 5 million cells per well in 6-well dishes and treated with DMSO or the indicated concentrations of GSK690693 for 24 hours. Lysates were prepared for Western blot analysis, and samples were probed with antibodies for cleaved PARP (Cell Signaling Technology).

Results

Sensitivity of hematologic tumor cell lines to AKT inhibitor

To evaluate the role of the AKT signaling in the growth and proliferation of hematologic tumors, the effect of GSK690693 was investigated in a 3-day cell proliferation assay in a total of 112 unique leukemia and lymphoma cell lines, representing ALL, AML, CLL, CML, Burkitt lymphoma, non-Hodgkin lymphoma, Hodgkin lymphoma, as well as several cell lines from other hematologic malignancies. Sensitivity to GSK690693 was defined as growth inhibition with an inhibitor EC_{50} value less than 1 μM. Sixty-two of the 112 cell lines were classified as sensitive cells, and they represented cell lines from various tumor types (Table 1). Of all the hematologic tumors tested, the ALL cell lines displayed the highest degree of sensitivity, with 31 of the 35 ALL cell lines (89%) sensitive to the AKT inhibitor. Fifty percent or more cell lines that originated from non-Hodgkin lymphoma, Burkitt lymphoma, and

Table 2. GSK690693 inhibits proliferation of ALL cells from both B-cell and T-cell origin

Cell line	Cell type	EC_{50}, nM
KARPAS-231	B cell	41
CCRFSB	B cell	155
SUP B15	B cell	197
SD-1	B cell	320
RS4;11	B cell	654
MN-60	B cell	3602
Tanoue	B cell	4517
RCH-ACV	pre-B cell	152
SEM	pre-B cell	202
KASUMI-2	pre-B cell	225
REH	pre-B cell	288
697	pre-B cell	338
NALM-6	pre-B cell	421
MHH-CALL–3	pre-B cell	812
MHH-CALL–2	pre-B cell	2114
J.GAMMA-1	T cell	65
JR45.01	T cell	68
A3	T cell	69
I 2.1	T cell	73
MOLT-3	T cell	74
P116	T cell	78
J.Cam1.6	T cell	79
I 9.2	T cell	80
LOUCY	T cell	117
J.RT3-T3.5	T cell	123
8E5	T cell	163
Jurkat	T cell	225
MOLT-4	T cell	232
Molt-16	T cell	241
CEM/C3	T cell	257
CEM/C2	T cell	271
CCRFCEM	T cell	327
CEM/C1	T cell	382
SUPTI[VB]	T cell	619
CCRF–HSB-2	T cell	2117

Antiproliferative activity of GSK690693 was determined in a 72-hour proliferation assay against various ALL cell lines. EC_{50} was determined as described in "Cell proliferation assay." Data represent the average value from 2 or more independent experiments.

CLL were also sensitive to GSK690693, while cell lines representing CML, AML, and Hodgkin lymphoma were less sensitive (Table 1; Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). GSK690693 was effective in inhibiting proliferation of cells from both T-cell and B-/pre-B–cell origin within the ALL cell panel, with 19 of the 20 T-ALL (95%) and 12 of the 15 B-ALL (80%) found to be sensitive to GSK690693 (Table 2). Curve-fit analysis for a representative set of both T-cell and B-cell lines are shown in Figure 1A,B, highlighting the similarity of dose response curves across sensitive cell lines in the 3-day proliferation assay.

GSK690693 does not inhibit normal T-cell growth

To elucidate whether the sensitivity of ALL to GSK690693 is associated with a general antiproliferative effect of AKT inhibition on lymphoid cells, we examined the effect of GSK690693 on normal lymphocyte growth and proliferation. Human $CD4^+$ peripheral T cells stimulated with PHA and IL-2 and the isolated mouse thymocytes were treated with GSK690693 and evaluated in the 3-day proliferation assay. An A3 T-ALL cell line was used as a reference cell line, sensitive to GSK690693. Both primary $CD4^+$ peripheral T cells and mouse thymocytes were not very sensitive to GSK690693, with EC_{50} values greater than 10 and 30 μM, respectively (Figure 1C). For comparison, the PI3K inhibitor, ZSTK474,[24] was also evaluated in the proliferation assay for both human T cells and mouse thymocytes. In contrast to GSK690693, the PI3K inhibitor inhibited the growth of both $CD4^+$ peripheral T cells and mouse thymocytes with EC_{50} values less than 1μM (Figure S1). Both the GSK690693-sensitive A3 line and GSK690693-resistant line Tanoue were included as reference cell lines. Both lines were also sensitive to ZSTK474 with less than 1 μM EC_{50} values. These results illustrate that lymphoid cells respond differently to PI3K inhibition, compared with AKT inhibition.

GSK690693 inhibits AKT signaling in ALL cells

To confirm the inhibition of AKT signaling in ALL cell lines, lysates from cells treated with DMSO or GSK690693 were analyzed by Western blot analysis for the phosphorylation of downstream substrates. Treatment with GSK690693 resulted in dose-dependent reduction in phosphorylated GSK3β (Ser9) and PRAS40 (Thr246) in both sensitive (I2.1, I9.2, and A3) and insensitive (Tanoue) ALL cell lines (Figure 2A). The same dose-dependent reduction in p70S6K (Thr421/Ser424) was also observed in the sensitive and resistant cell lines, although the effect on the Tanoue cell line was modest. An increase in AKT phosphorylation at both Ser473 and Thr308 sites was observed with GSK690693, consistent with the feedback mechanism observed previously with this and other AKT kinase inhibitors.[22,25] This feedback hyperphosphorylation appears to be very sensitive to the inhibition of AKT kinase activity and can be seen at concentrations lower than that required to see reduction in the phosphorylation of AKT substrates. These results clearly demonstrate the inhibition of AKT signaling by GSK690693 in both sensitive and insensitive ALL cell lines.

To further confirm the inhibition of AKT by GSK690693 in ALL cells, AKT-kinase activity was measured in A3 (sensitive) and Tanoue (insensitive) cells treated with DMSO or different concentrations of GSK690693 using an AKT-kinase assay kit. Treatment with GSK690693 resulted in a dose-dependent reduction in AKT-kinase activity, as measured by reduction in the phosphorylation of exogenous GSK-3 fusion protein, in both ALL cell lines (Figure 2B). To study the effect of GSK690693 on growth factor–stimulated ALL cell lines, serum-starved cells were stimulated with insulin for various times to activate AKT (Figure 2C). GSK690693 treatment resulted in the reduction of the stimulated AKT-kinase activity. This study could only be completed in the insensitive cell line, Tanoue, since AKT is constitutively activated in the A3-sensitive cell lines independent of serum. (Figure S2). Taken together with the effect of GSK690693 on the phosphorylation of various AKT substrates (Figure 2A), these results clearly demonstrate the potent inhibition of AKT activity by GSK690693 in ALL cells.

AKT inhibitor induces apoptosis in ALL cell lines

To better understand the cellular effects of AKT inhibition on ALL cell lines, cell-cycle analysis was completed in 4 cell lines (3 sensitive and 1 insensitive to GSK690693). Treatment with GSK690693 resulted in a dose-dependent increase in the population with sub-2*n* DNA, indicating cell death, in all 3 sensitive cell lines (Figure 3A,B). There was some increase in 2*n* cell population at lower drug concentrations and a decrease in cells with 4*n* DNA and cells during S-phase. These results imply that GSK690693

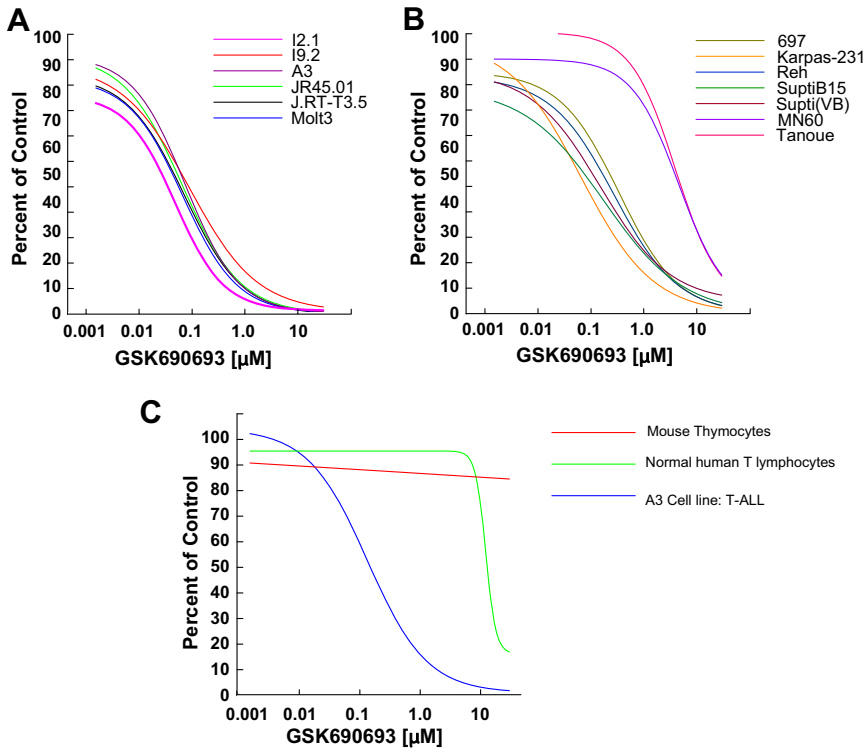

Figure 1. Inhibition of ALL cell proliferation by AKT inhibitor. ALL cell lines from both T-cell (A) and B-cell (B) origin were treated with varying concentration of GSK690693 for 72 hours. Cell proliferation was measured as described in "Methods." Graphs represent curve fit analysis of the dose response data for a subset of cell lines analyzed. Please note the relative insensitivity of MN60 and Tanoue cells to GSK690693. (C) Effect of GSK690693 on the proliferation of primary human $CD4^+$ T lymphocytes, mouse thymocytes, and an AKT inhibitor-sensitive T-ALL cell line, A3.

inhibition of the AKT signaling in ALL cell lines results in cell-cycle arrest and cell death, likely mediated by apoptosis. The results for the insensitive cell line showed no change in cell-cycle profile with less than or equal to 1 μM of treatment. There was a slight increase in the sub-2*n* population at 10 μM of treatment; however this observation is most likely due to off-target affects caused by high compound concentration.

Caspase activity, PARP cleavage, and DNA fragmentation were measured to further confirm the proapoptotic effect of GSK690693 on ALL cell lines. A dose-dependent increase in cleaved caspase-3, PARP cleavage, and caspase 3/7 activity was observed 24 hours after GSK690693 treatment in A3, I2.1, and I9.2 cells; whereas no increase in caspase activity or PARP cleavage was observed in GSK690693-insensitive, Tanoue cells

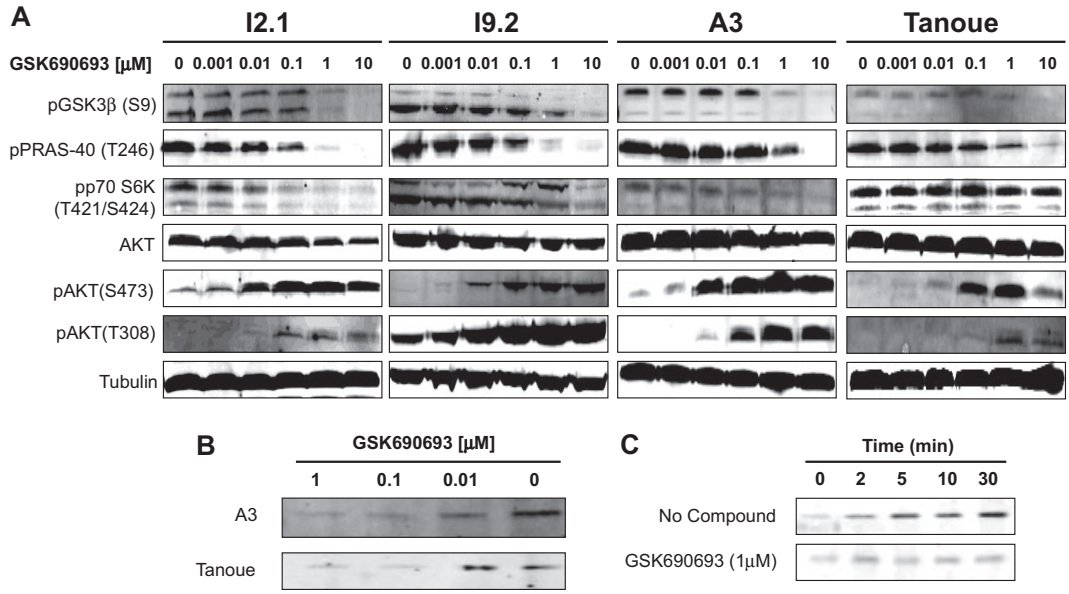

Figure 2. GSK690693 inhibits AKT signaling in ALL cells. (A) I2.1, I9.2, A3, and Tanoue cells were treated with DMSO or different concentrations of GSK690693 for 6 hours. Cell lysates were analyzed by Western blot analysis for phospho-GSK3β Ser9, phospho-PRAS40 Thr246, phospho-p70S6K Thr421/Ser424, phospho-AKT Ser473, and phospho-AKT Thr308. Total AKT and tubulin are shown as loading controls. (B) GSK690693 inhibits AKT kinase activity in ALL cells. A3 and Tanoue cell lines were treated with DMSO or various concentrations of GSK690693 for 1 hour. AKT was immunoprecipitated from cell lysates with immobilized anti-AKT antibody, and the kinase assay was performed using GSK-3 fusion protein as a substrate. Phosphorylation of GSK3 was measured by Western blot analysis, using phospho-GSK3β antibody. (C) GSK690693 inhibits stimulated AKT kinase activity in ALL cells. Tanoue cells were serum-starved overnight, then treated with DMSO or 1 μM GSK690693 for 1 hour. Cells were treated with 5 ng/mL insulin for various times to stimulate AKT activity. AKT kinase assay was performed.

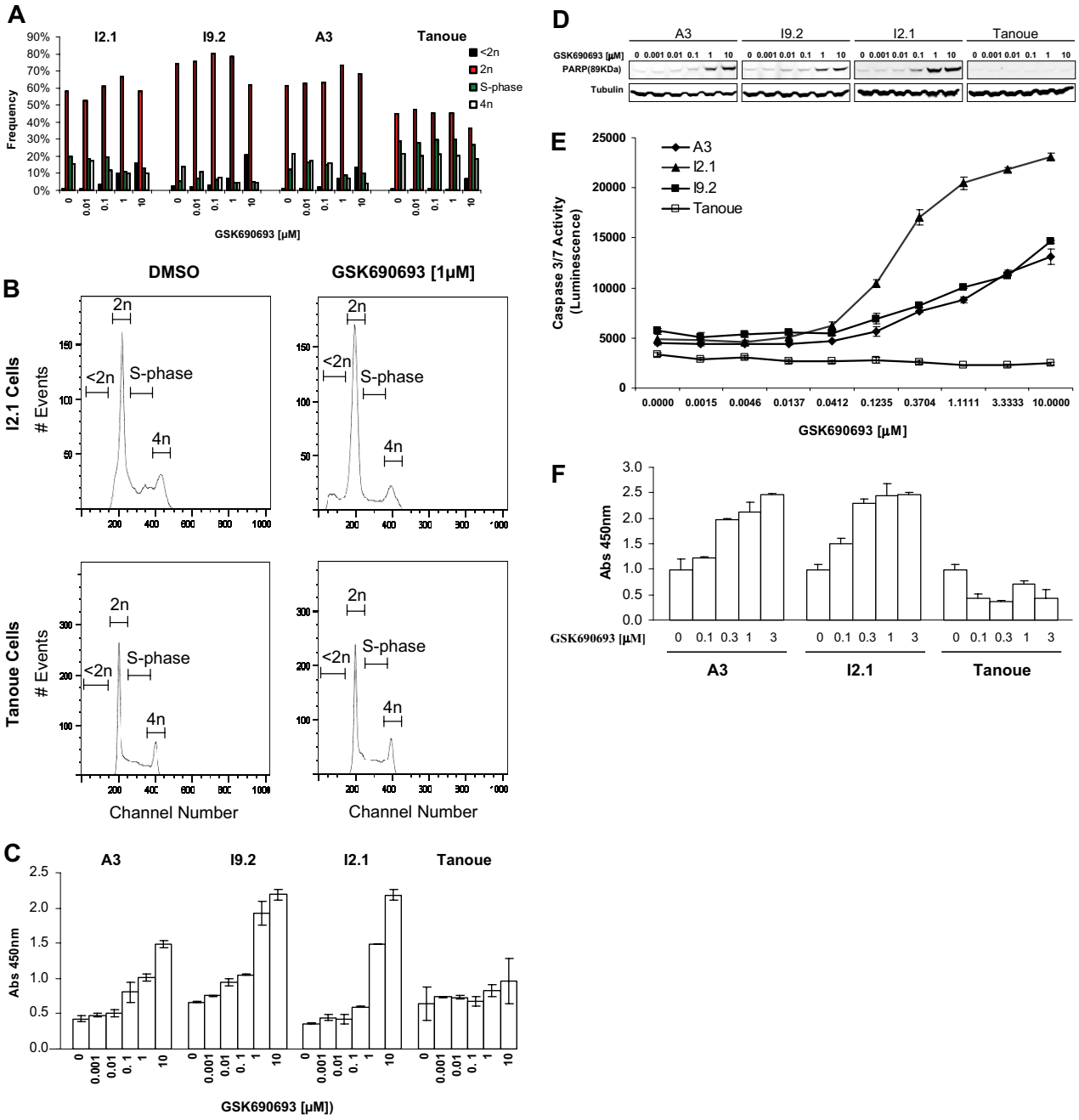

Figure 3. GSK690693 induces apoptosis in ALL cells. (A) I2.1, I9.2, A3, and Tanoue cells were treated with DMSO or varying concentrations of GSK690693 for 72 hours, and cell-cycle distribution was determined based on DNA content of propidium iodide-stained populations. (B) Representative histogram of cell-cycle analysis. Histograms illustrate cell cycle profile of DMSO- and GSK690693-treated I2.1 and Tanoue cells. (C) ALL cell lines were treated with DMSO or different concentrations of GSK690693 for 24 hours, and endogenous levels of cleaved caspase-3 were measured. (D) PARP cleavage was determined by Western blot analysis in ALL cell lines treated with DMSO or various concentrations of GSK690693 for 24 hours. (E) ALL cell lines were treated with DMSO or different concentrations of GSK690693 for 24 hours, and the caspase activity was measured using caspase 3/7 assay kit. Data represent relative luminescence signal (mean ± SD) for each cell line. (F) DNA fragmentation was measured in A3, I2.1, and Tanoue cells treated with DMSO or GSK690693 for 72 hours as described in "Methods." Data represent a comparison between GSK690693 sensitive and insensitive cell lines.

(Figure 3C-E). DNA fragmentation is another hallmark of apoptosis, and as such, it was measured using Cell Death ELISA in ALL cells treated with GSK690693 for 72 hours. A dose-dependent increase in cytoplasmic histone-associated DNA fragments was observed in both GSK690693-sensitive cell lines, with no significant increase in Tanoue cells at any of the GSK690693 concentration (Figure 3F). Taken together, these results provide evidence that GSK690693 treatment induces apoptosis in AKT inhibitor–sensitive ALL cell lines.

Discussion

The present study demonstrates the activity of a novel pan-AKT kinase inhibitor, GSK690693, against a large panel of cell lines derived from various hematologic tumors. Hematologic tumor cell lines were more sensitive to AKT inhibition compared with tumor cell lines of epithelial origin,[22] with 55% of all hematologic cell lines considered sensitive to GSK690693 (Table 1). Interestingly,

the frequency of sensitivity was particularly high in both T-ALL and B-ALL. T-ALL appears to be more sensitive, with only one of the 20 cell lines classified as insensitive to GSK690693, suggesting strong dependence of T-ALL on AKT signaling. Constitutive active AKT has been shown to play a key role in the biology of ALL, with multiple factors associated with AKT activation. Constitutive activation of AKT can result due to IL-7 stimulation or genetic alteration of various genes (eg, Notch-1 and PTEN). Activating mutations in Notch-1 have been observed in greater than 50% of T-ALL with concurrent loss of PTEN activity in a subset of patients.[16,17,19,20]

Due to the high sensitivity of the ALL cell lines to GSK690693-induced growth inhibition; the effect of compound treatment on normal lymphocyte proliferation was evaluated. GSK690693 did not inhibit the growth of normal human $CD4^+$ peripheral T cells or isolated mouse thymocytes in the cell proliferation assay, suggesting that the sensitivity of T-ALL cells to GSK690693 is not mediated by a general antiproliferative effect of GSK690693 in T cells.

Previous reports have shown activation of PI3K pathway in various hematologic malignancies and that pharmacologic inhibition of PI3K results in growth arrest and apoptosis of human AML cell lines in vitro.[26] Most of the AML and CML cell lines were not sensitive to GSK690693 (Table 1), suggesting a difference in the therapeutic potential of PI3K and AKT inhibitors, as these cells may have different cellular signaling. Further, few of the ALL cell lines (4 of 35 tested) were also not sensitive to GSK690693, raising the question on whether these cell lines are dependent on PI3K activity. We subsequently assessed the effect of a PI3K inhibitor, ZSTK474,[24] on the growth of GSK690693-sensitive and GSK690693-insensitive ALL cell lines, A3 and Tanoue, respectively (Figure S1). Both cell lines were sensitive to the PI3K inhibitor with similar EC_{50} (< 1 μM), suggesting that ALL cells resistant to AKT inhibition were still dependent on PI3K signaling for growth and proliferation. More importantly, primary human $CD4^+$ peripheral T cells and mouse thymocytes were also equally sensitive to the PI3K inhibitor (Figure S1), suggesting a potential difference in the therapeutic potential of PI3K and AKT inhibitors in lymphocytic malignancies.

Inhibition of AKT-signaling and kinase activity was observed in both sensitive and insensitive ALL cell lines, suggesting that lack of sensitivity in some cell lines was not due to inability to inhibit AKT signaling in those cells (Figure 2). The mechanism of resistance in these cell lines was not clear and will likely be multifactorial and differ in cells from different tumor types. Potential genetic alteration leading to the activation of Ras-Raf-MEK or JAK-STAT signaling may provide alternate growth signaling, whereby cell growth and proliferation is not driven by AKT signaling in these cells. GSK690693 inhibits cell proliferation as well as induces apoptosis in sensitive cell lines, consistent with the role of AKT signaling in cell physiology.

The PI3K/AKT signaling is critical for the development and function of normal lymphocytes, including proliferation, activation, and survival.[27,28] AKT signaling is involved in various physiologic functions of both T-lymphocytes and B-lymphocytes. Besides ALL, cell lines originating from non-Hodgkin and Burkitt lymphoma also showed high frequency of sensitivity to AKT inhibition with 73% and 67% of cell lines considered sensitive to GSK690693, respectively. Both of these malignancies are of B-cell origin, where AKT plays an important role in cellular survival.[29] Several B-cell lymphomas have overexpression of the T-cell leukemia 1 (TCL1), which enhances the activation of AKT-dependent survival pathways.[30] Constitutively active AKT expression was demonstrated in cell lines and primary human lymphomas, especially those from non-Hodgkin lymphoma.[19] The data described here validates the dependence of a large proportion of non-Hodgkin and Burkitt lymphoma cells on AKT signaling and thus provides a strong rationale for the use of AKT inhibitors in such malignancies.

In conclusion, this study highlights the importance of AKT signaling in the growth and survival of ALL and other hematologic malignancies. GSK690693 is currently being evaluated in patients with various tumors, including hematologic tumors, and the results from those studies will provide the true validation of the role of AKT inhibitors in treatment of these patients.

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Authorship

Contribution: D.S.L. performed research and wrote the manuscript; J.A.K. designed and performed experiments; and R.K. designed experiments and wrote the manuscript.

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References

1. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov. 2005;4:988-1004.
2. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signaling controls tumour cell growth. Nature. 2006;441:424-430.
3. Bellacosa A, Kumar CC, DiCristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res. 2005;94:29-86.
4. Carpten JD, Faber AL, Horn C, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature. 2007;448:439-444.
5. Yang Z-Z, Tschopp O, Baudry A, Dümmler B, Hynx D, Hemmings BA. Physiological functions of protein kinase B/Akt. Biochem Soc Trans. 2004; 32:350-354.
6. DeFeo-Jones D, Barnett SF, Fu S, et al. Tumor cell sensitization to apoptotic stimuli by selective inhibition of specific /PKB family members. Mol Cancer Ther. 2005;4:271-279.
7. Witzig TE, Kaufmann SH. Inhibition of the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway in hematologic malignancies. Curr Treat Options Oncol. 2006;7:285-294.
8. Skorski T, Bellacosa A, Nieborowska-Skorska M, et al. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. EMBO J. 1997;16:6151-6161.
9. Slupianek A, Nieborowska-Skorska M, Hoser G, et al. Role of phosphatidylinositol 3-kinase-Akt-AKT pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis. Cancer Res. 2001;61:2194-2199.
10. Teitell MA. The TCL1 family of oncoproteins: co-activators of transformation. Nat Rev Cancer. 2005;5:640-648.
11. Min YH, Eom JI, Cheong JW, et al. Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. Leukemia. 2003;17:995-997.
12. Martelli AM, Nyåkern M, Tabellini G, et al. Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. Leukemia. 2006;20:911-928.
13. Munk Pedersen I, Reed J. Microenvironmental interactions and survival of CLL B-cells. Leuk Lymphoma. 2004;45:2365-2372.
14. Fillmore GC, Wang Q, Carey MJ, Kim C-H,

Elentoba-Johnson KSJ, Lim MS. Expression of Akt (protein kinase B) and its isoforms in malignant lymphomas. Leuk Lymphoma. 2005;46: 1765-1773.

15. Rudelius M, Pittaluga S, Nishizuka S, et al. Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma. Blood. 2006;108:1668-1676.
16. Barata JT, Cardoso AA, Boussiotis VA. Interleukin-7 in T-cell acute lymphoblastic leukemia: an extrinsic factor supporting leukemogenesis. Leuk Lymphoma. 2005;46:483-395.
17. Palomero T, Sulis ML, Cortina M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med. 2007;13: 1203-1210.
18. Calzavara E, Chiaramonte R, Cesana D, Basile A, Sherbet GV, Comi P. Reciprocal regulation of Notch and PI3K/AKT signaling in T-ALL cells in vitro. J Cell Biochem. 2008;103:1405-1412.
19. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306:269-271.
20. Mansour MR, Duke V, Foroni L, et al. Notch-1 mutations are secondary events in some patients with T-cell acute lymphoblastic leukemia. Clin Cancer Res. 2007;13:6964-6969.
21. Avellino R, Romano S, Parasole R, et al. Rapamycin stimulates apoptosis of childhood acute lymphoblastic leukemia cells. Blood. 2005;106: 1400-1406.
22. Rhodes N, Heerding DA, Duckett D, et al. Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity. Cancer Res. 2008;68:2366-2374.
23. Vindelov LL, Christensen IJ, Nissen NI. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry. 1982;3:323-327.
24. Yaguchi S, Fukui Y, Koshimizu I, et al. Antitumor activity of ZSTK474, a new phosphatidylinositol 3-kinase inhibitor. J Natl Cancer Inst. 2006;98: 545-556.
25. Han EK-H, Leverson JD, McGonigal T, et al. Akt inhibitor A-443654 induces rapid Akt Ser-473 phosphorylation independent on mTORC1 inhibition. Oncogene. 2007;26:5655-5661.
26. Xu Q, Simpson SE, Scialla TJ, Bagg A, Carroll M. Survival of acute myeloid leukemia cells requires PI3 kinase activation. Blood. 2003;102:972-980.
27. Marshall AJ, Niiro H, Yun TJ, Clark EA. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase $C\gamma$ pathways. Immunol Rev. 2000;176:30-46.
28. Kane LP, Weiss A. The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP_3. Immunol Rev. 2003;192:7-20.
29. Plas DR, Rathmell JC, Thompson CB. Homeostatic control of lymphocyte survival: potential origins and implications. Nat Immunol. 2002;3:515-521.
30. Gold MR. Akt is TCL-ish: implications for B-cell lymphoma. Trends Immunol. 2003;24:104-108.