

Published in final edited form as:

Mol Pharmacol. 2008 September ; 74(3): 884–895. doi:10.1124/mol.108.047639.

Proapoptotic Activity and Chemosensitizing Effect of the Novel Akt Inhibitor (2S)-1-(1H-Indol-3-yl)-3-[5-(3-methyl-2H-indazol-5-yl)pyridin-3-yl]oxypropan-2-amine (A443654) in T-Cell Acute Lymphoblastic Leukemia

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Abstract

Constitutively activated AKT kinase is a common feature of T-cell acute lymphoblastic leukemia (T-ALL). Here, we report that the novel AKT inhibitor (2S)-1-(1H-indol-3-yl)-3-[5-(3-methyl-2H-indazol-5-yl)pyridin-3-yl]oxypropan-2-amine (A443654) leads to rapid cell death of T-ALL lines and patient samples. Treatment of CEM, Jurkat, and MOLT-4 cells with nanomolar doses of the inhibitor led to AKT phosphorylation accompanied by dephosphorylation and activation of the downstream target, glycogen synthase kinase-3 β . Effects were time- and dose-dependent, resulting in apoptotic cell death. Treatment of Jurkat cells with A443654 resulted in activation of caspase-2, -3, -6, -8, and -9. Apoptotic cell death was mostly dependent on caspase-2 activation, as demonstrated by preincubation with a selective pharmacological inhibitor. It is remarkable that A443654 was highly effective against the drug-resistant cell line CEMVBL100, which expresses 170-kDa P-glycoprotein. Moreover, A443654 synergized with the DNA-damaging agent etoposide in both drug-sensitive and drug-resistant cell lines when coadministered [combination index (CI) = 0.39] or when pretreated with etoposide followed by A443654 (CI = 0.689). The efficacy of A443654 was confirmed using blasts from six patients with T-ALL, all of whom displayed low levels of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and constitutive phosphorylation of Akt on Ser473. At 1 μ M, the inhibitor was able to induce apoptotic cell death of T-ALL blast cells, as indicated by flow cytometric analysis of samples immunostained for active (cleaved) caspase-3. Because activated AKT is seen in a large percentage of patients with T-ALL, A443654, either alone or in combination with existing drugs, may be a useful therapy for primary and drug-resistant T-ALL.

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Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.

Treatment of adult T-cell acute lymphoblastic leukemia (T-ALL) has modestly improved over the last 20 years with cure rates of 15 to 40%. Novel therapies aimed at improperly activated signaling pathways in affected cells are needed to combat both the leukemia and the development of drug-resistance (Vitale et al., 2006b). Lymphoblasts from more than 50% of patients with T-ALL contain an activating mutation in the transmembrane receptor, Notch-1 (Grabher et al., 2006; Weerkamp et al., 2006). Activating mutations as well as binding with its ligands of the Delta and Serrated/Jagged family result in the cleavage of Notch-1 and release of the intracellular portion of the receptor, which translocates to the nucleus, where it interacts with transcription factors to affect gene expression. Notch-1 signaling is critical for T-cell development, proliferation, and survival (Eagar et al., 2004). Recently, it was demonstrated that activated Notch-1 leads to constitutive activation of the PI3K/AKT/mTOR pathway by HES1-mediated transcriptional suppression of the *MMAC1* gene, which encodes the dual-specificity, lipid and protein, PIP3 phosphatase known as PTEN (Palomero et al., 2007). In addition, PTEN is mutated in approximately 20% of patients with T-ALL, and virtually all T-ALL cell lines that are resistant to Notch-1 inhibition with γ -secretase inhibitors contain mutations leading to either no or low PTEN expression (Palomero et al., 2007). PTEN is a key modulator of the PI3K pathway and AKT activation through its regulation of the level of PIP3 generated from the available pool of phosphatidylinositol-4,5-phosphate by PI3K (Tokunaga et al., 2008). In the presence of PTEN, PIP3 is rapidly dephosphorylated to phosphatidylinositol-4,5-phosphate, blocking the recruitment of AKT to the membrane for activation. The loss of PTEN in T-ALL contributes to the hyperactivated state of AKT found in these cells, because activation of AKT by phosphorylation on Thr308 by PDK1 or on Ser473 by PDK2 (also referred to as the mTOR:Rictor complex) requires membrane recruitment via PIP3 (Martelli et al., 2006; Sale and Sale, 2008).

Activated AKT phosphorylates multiple targets involved in cell growth, inhibition of apoptosis and metabolism. In general, targets inhibited after phosphorylation by AKT are involved in cell cycle arrest, apoptosis induction, or homeostasis under low nutrient conditions. Targets inhibited by AKT phosphorylation include GSK-3 α/β , FoxO transcription factors, Bad, p21^{Cip1}, and p27^{Kip1} (Marone et al., 2008; Sale and Sale, 2008). Targets activated by AKT phosphorylation are involved in cell cycle progression, apoptosis inhibition, and metabolism in a high-energy environment and include murine double minute 2, X-linked inhibitor of apoptosis protein, mTOR (Marone et al., 2008; Sale and Sale, 2008). In tumors that contain low levels of, or no, PTEN, AKT activation is the lynchpin for growth and survival, as these tumors are extremely sensitive to AKT inhibition (Lopiccolo et al., 2008). Moreover, activation of the PI3K/AKT signaling pathway confers resistance to many types of cancer therapy and is a poor prognostic factor for many types of neoplastic disorders, making AKT an exciting target for innovative cancer treatment (Lindsley et al., 2008). In this study, we sought to analyze the efficacy of the novel AKT inhibitor A443654 (Luo et al., 2005) as a therapeutic agent in the treatment of T-ALL. We demonstrate that A443654 is highly cytotoxic against T-ALL cell lines (including a T-ALL drug-resistant cell line that overexpresses 170-kDa P-glyco-protein) and patient samples at doses well within the tolerated range in vivo. Moreover, it could synergize with standard therapeutic compounds to induce apoptotic cell death.

Materials and Methods

Cell Culture and Inhibitors

The T-ALL cell lines Jurkat, CEM-S, CEM-R (CEM-VBL100, drug-resistant), and MOLT-4 were cultured RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 mM L-glutamine, and penicillin/streptomycin. A443654 was a kind gift from Abbott Pharmaceutical (Abbott Park, IL). LY294002, wortmannin, etoposide, PI-103, caspase-2 inhibitor (Z-VDVAD-FMK), and caspase-3 inhibitor (*N*-acetyl-DMQD-aldehyde) were from EMD

Biosciences (La Jolla, CA). Patient samples or peripheral blood lymphocytes from healthy donors were obtained with informed consent according to institutional guidelines (World Medical Association, Declaration of Helsinki, October 2000) and isolated by Ficoll-Paque (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) using density-gradient centrifugation. Before analysis, patient samples or lymphocytes from healthy donors were cultured in RPMI 1640 medium containing 20% fetal bovine serum, 200 mM L-glutamine, and penicillin/streptomycin for a minimum of 24 h.

MTT Assay

MTT assays were performed to assess the sensitivity of the T-ALL lines to either A443654 alone or in combination with another indicated compound using the MTT assay kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol.

Combined Drug Effects Analysis

To characterize the interactions between A443654 and etoposide, the combination effect and a potential synergy were evaluated from quantitative analysis of dose-effect relationships as described previously (Nyakern et al., 2006). For each A443654/etoposide drug combination experiment, a CI number was calculated using the Biosoft CalcuSyn computer program (Cambridge, UK) and the formula $CI = C_a/Cx_a + C_b/Cx_b$, where Cx_a and Cx_b are the concentrations of compound a and b alone, respectively, needed to achieve a given effect ($x\%$) and C_a and C_b are the concentrations of A443654 and etoposide needed for the same effect ($x\%$) when the drugs are combined. This method of analysis generally defines CI values of 0.9 to 1.1 as additive, 0.3 to 0.9 as synergistic, and <0.3 as strongly synergistic, whereas values >1.1 are considered antagonistic.

Annexin V-FITC Assay

To assess the degree of apoptosis after treatment with A443654, either alone or in combination with an additional compound, the extent of Annexin V-FITC/PI staining was determined by flow cytometry as described previously using the Annexin V/PI staining kit from Bender MedSystems (Vienna, Austria) (Blalock et al., 2003). Samples were read on an Epics XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA).

Cell Cycle Analysis

After their respective treatment, T-ALL cell lines and patient samples were prepared as described previously (Shelton et al., 2004). Samples were analyzed with a FC500 flow cytometer (Beckman Coulter) equipped with CXP software.

PDK1 siRNA Knockdown

Jurkat cells were washed two times in Opti-MEM (Invitrogen, Milan, Italy) and resuspended in Nucleofector Solution V (Amaxa Biosystems, Cologne, Germany) to a density of 5×10^6 cells/0.1 ml. Cells were electroporated with 1.5 μ M SmartPool siRNA to PDK1 or control using a Nucleofector electro-porator (program X-05; Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions. Cells were then cultured in growth media for 72 h before assaying.

Protein Extraction and Western Blotting

Protein lysates were prepared as described previously (Blalock et al., 2003). The protein concentration was determined by detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA). Lysate (50 μ g) was loaded onto an SDS-polyacrylamide gel, electrophoresed, and transferred onto nitrocellulose membranes, using a semidry transfer apparatus. The

membranes were incubated overnight at 4°C in 5% nonfat dry milk in 1× PBST. The membranes were washed three times in 1× PBST and incubated overnight at 4°C in primary antibody diluted 1:1000 in 5% BSA in PBST. The following antibodies were from Cell Signaling Laboratories (Danvers, MA): p-Thr308 AKT, p-Ser473 AKT, AKT, p-Ser9 GSK-3β, GSK-3β, caspase-2, caspase-3, caspase-6, caspase-8, caspase-9, PKC-δ, PDK1, β-actin. The membranes were washed three times in 1X PBST and incubated for 2 h in the appropriate peroxidase-conjugated secondary antibody (Cell Signaling) diluted 1:2000 in 5% milk in PBST. The blots were washed three times with PBST and visualized using ECL reagent (GE Healthcare). Band intensity was determined by densitometry using the public domain software Image J (a Java image processing program inspired by NIH Image for Macintosh; <http://rsbweb.nih.gov/ij/>) as described elsewhere (Nyakern et al., 2006).

Immunoprecipitation

This was performed as described previously (Neri et al., 2003).

Determination of the Levels of PTEN, p-Ser473 AKT, and Cleaved Caspase-3 in T-ALL Patient Samples

Viable lymphoblasts (5×10^5) from six patients with T-ALL or peripheral blood lymphocytes from healthy donors were fixed with reagent 1 of the Intraprep kit according to the manufacturer's instructions (Beckman Coulter) and permeabilized with saponin-based reagent 2. Cells were blocked in 5% BSA in PBS and incubated for 12 h at 4°C in primary antibody [p-Ser473 AKT (either Alexa Fluor 488 or Alexa Fluor 647 conjugate), PTEN (Alexa Fluor 647 conjugate), or cleaved caspase-3 (Alexa Fluor 488 conjugated), all from Cell Signaling, diluted 1:10 in 5% BSA in PBS]. Cells were then washed twice in 1× PBS and analyzed on a FC500 flow cytometer (Tazzari et al., 2007a,b; Papa et al., 2008).

Statistical Evaluation

The data are shown as mean values ± S.D. Data were statistically analyzed by Dunnett's test after one-way analysis of variance at a level of significance of $p < 0.05$ versus control samples.

Results

A443654 Inhibits Proliferation and Induces Apoptosis in Drug-Sensitive and Drug-Resistant T-ALL Cell Lines

T-ALL cell lines contain constitutively elevated levels of p-AKT (Seminario et al., 2003; Uddin et al., 2004). To ascertain the effectiveness of the novel AKT inhibitor A443654 as a therapeutic agent in T-ALL, we treated the T-ALL cell lines Jurkat, MOLT-4, CEM-S, and CEM-R with serially diluted concentrations of A443654 or the vehicle (DMSO) alone (control). After 24 h, the rates of proliferation and cell viabilities were measured using MTT assays. All three parental cell lines were sensitive to nanomolar doses of A443654 ($IC_{50} = 80, 120, \text{ and } 900 \text{ nM}$ for MOLT-4, CEM-S, and Jurkat, respectively) well below 20 μM, the highest concentration reached in vivo in tumor (Fig. 1A) (Luo et al., 2005). In contrast, the drug-resistant CEM-R cell line, a cell line overexpressing the 170 kDa P-glycoprotein (Mantovani et al., 2006), showed increased resistance to A443654 ($IC_{50} = 12 \text{ μM}$), but this IC_{50} was still below 20 μM (Fig. 1A).

Because AKT is considered to be a major antiapoptotic kinase, we suspected that use of A443654 in cells that maintain active AKT would result in rapid cell cycle arrest and induction of apoptosis. To determine whether the results from the MTT assays translated into effects on cell cycle progression, cell cycle analysis was performed on Jurkat cells in the absence or presence of either 0.5 or 1.0 μM A443654 for 2, 4, 8, 16, and 24 h. No changes in cell cycle

progression were observed between treated and untreated samples after 2, 4, or 8 h of treatment (data not shown). In contrast, A443654 resulted in a G₂/M arrest of Jurkat cells after 16 h. Approximately 39% of cells treated with 0.5 μM and 45% of the cells treated with 1.0 μM were observed in G₂/M, whereas only 11% of the untreated cells were observed in G₂/M (data not shown). After 24 h of treatment with 0.5 μM A443654, 50% of the cells were observed in the sub-G₁/G₀ fraction, whereas treatment with 1.0 μM A443654 resulted in 65% of the cells in the sub-G₁/G₀ fraction. In contrast, in DMSO-treated cells, the percentage of cells in the sub-G₁/G₀ fraction was much lower (Fig. 1B). Moreover, Annexin V-FITC/PI staining indicated that A443654 induced apoptosis in Jurkat, CEM-S, and CEM-R cells in a time- and dose-dependent manner (Fig. 1C and data not shown). CEM-S and CEM-R cells were treated with 1.0 μM A443654 or DMSO (control) for 24 and 48 h. After 24 h of treatment, 70% of the CEM-S cells were negative for Annexin V-FITC/PI staining. Approximately 15% were positive for early apoptosis (Annexin V-FITC only), whereas another 15% were PI-positive only, indicating that some cells had died by necrosis or rapidly died by apoptosis, whereas others were slightly less sensitive. In contrast, no significant effects were observed in the CEM-R cells after 24 h. After 48 h of treatment at the same dose, approximately 50% of the CEM-S cells were apoptotic, with 15% in early apoptosis (Annexin V-FITC-positive only), 11% late apoptosis/necrosis (PI-positive only), and 24% in mid-late apoptosis (Annexin V-FITC/PI-positive). Again, in contrast to the CEM-S cells, only 12% of the CEM-R cells were early apoptotic (Annexin V-FITC positive only) after 48 h of treatment (Fig. 1C). The degree of apoptosis induced in Jurkat cells by A443654 was similar to that detected in CEM-S cells (data not shown).

A443654 Results in Rapid Phosphorylation of Both Thr308 and Ser473 of AKT

A443654 was reported to lead to the dephosphorylation of the AKT substrate GSK-3β and most recently to induce phosphorylation of AKT on Ser473 (Luo et al., 2005; Han et al., 2007). We examined the effects of A443654 on AKT signaling in T-ALL cells by measuring the phosphorylation status of AKT and its downstream substrate GSK-3β (Fig. 2). Treatment of Jurkat, CEM-S, and CEM-R cells with A443654 resulted in a dose-dependent phosphorylation of AKT at both Thr308 and Ser473. Treatment of Jurkat cells with 0.1 μM A443654 for 24 h resulted in a dramatic increase in AKT phosphorylated at Thr308, whereas enhancement of AKT phosphorylated on Ser473 was less marked over control cells (Fig. 2A). In addition, 0.1 μM A443654 resulted in greatly diminished levels of GSK-3β phosphorylated at S9. Phosphorylation of AKT at Ser473 as well as loss of p-S9 GSK-3β occurred rapidly (within 30 min). Treatment of CEM-S (1 μM) or CEM-R (10 μM) cells with A443654 resulted in a robust increase in the amount of AKT phosphorylated at Ser473, respectively (Fig. 2A). In contrast to Ser473, where constitutive AKT phosphorylation was observed in CEM-S and, to a greater extent, in CEM-R cells, no constitutive phosphorylation of Thr308 was detected, using a phosphospecific antibody, but phosphorylation at this site was likewise rapidly induced. Moreover, GSK-3β was rapidly dephosphorylated, although dephosphorylation of GSK-3β occurred to a lesser extent in CEM-R cells, most likely as a result of the higher constitutive levels of p-Ser473 AKT in these cells (Fig. 2A).

A443654 induced phosphorylation of Ser473 was recently reported to be due to the rapamycin insensitive mTOR:Rictor complex (Han et al., 2007). Likewise, we observed that part of the phosphorylation induced by treatment of Jurkat cells was PI3K-independent (Ser473) and PDK1-independent (Thr308), suggesting an additional kinase(s) responsible for the phosphorylation of AKT on Thr308 and Ser473 (Fig. 2B). Jurkat cells, which were pretreated with either DMSO (control), LY294002, wortmannin, or PI-103 (a dual PI3K/mTOR inhibitor; see Fan et al., 2006) for 1 h, were treated with 1 μM A443654 for 2 and 4 h, and the phosphorylation status of AKT was assayed. Pretreatment with wortmannin or PI-103 all but abolished the constitutive levels of p-Thr308 AKT in Jurkat cells, whereas LY294002 reduced

Thr308 phosphorylation. It is noteworthy that, in the presence of these inhibitors, A443654 was able to induce phosphorylation of AKT on Thr308 to levels similar to or above that observed with A443654 alone, suggesting that membrane recruitment and PDK1 were not responsible for A443654-induced Thr308 phosphorylation (Fig. 2B). In addition, a similar lack of inhibition of A443654-induced Thr308 phosphorylation was observed when LY294002 and wortmannin were used at 50 μ M and 300 nM, respectively (data not shown). The constitutive phosphorylation of AKT on Ser473 was not greatly affected by pretreatment with LY294002 (1.5 μ M), whereas both wortmannin (100 nM) and PI-103 (50 nM) resulted in a marked decrease in AKT Ser473 phosphorylation. Treatment with A443654 was able to restore p-Ser473 AKT levels to that of the control (Fig. 2B). Higher concentrations of LY294002 (50 μ M) and wortmannin (300 nM) abolished AKT Ser473 phosphorylation, but phosphorylation remained inducible by A443654, although not to levels of the control (data not shown). Each of the inhibitors, LY294002, wortmannin, and PI-103 (IC_{50} for PI3K, 1.4 μ M, 4 nM, and 26 nM, respectively) have been shown to also inhibit mTOR [IC_{50} , 5 μ M, 300 nM, and 20 nM (mTOR:Raptor complex) or 86 nM (mTOR:Rictor complex)] (Bain et al., 2007). At doses at or above the IC_{50} for PI3K, the inhibitors had only a partial affect on AKT Ser473 phosphorylation but, at concentrations at or above the IC_{50} for mTOR, were capable of inhibiting AKT Ser473 phosphorylation; therefore, it is likely that mTOR:Rictor is the major kinase responsible for AKT Ser473 phosphorylation. This observation is in agreement with what was recently reported by others (Han et al., 2007). To rule out PDK1 as being responsible for Thr308 phosphorylation, siRNA directed to PDK1 was used to down-regulate PDK1 levels in Jurkat cells before treatment with 1 μ M A443654. Although scrambled siRNA did not decrease PDK1 levels compared with untreated cells, as evaluated by Western blot, pretreatment with siRNA to PDK1 resulted in a marked decrease in PDK1 and in p-Thr308 AKT levels (Fig. 2C). After treatment with A443654, p-Thr308 AKT levels increased in samples pretreated with either scramble siRNA or PDK1 siRNA. By densitometric analysis of the blots, it was possible to determine that in control samples (scramble siRNA), the levels of p-Thr308 increased 3.2 times upon drug treatment, whereas in samples with down-regulated PDK1, the increase was 3.14-fold, despite a 10-fold reduction in the PDK1 levels.

Collectively, these data indicated that in Jurkat cells, part of the induced phosphorylation at Ser473 of AKT is dependent on mTOR:Rictor, whereas phosphorylation of Thr308 after treatment with A443654 is not dependent on PI3K activity, membrane recruitment by PIP3, or phosphorylation by PDK1.

It is noteworthy that, in Jurkat cells, but not in either CEM cell line, a degradation of AKT was observed after phosphorylation (Fig. 2, A and D and data not shown). As inhibition of AKT led to apoptosis, we analyzed the expression of AKT after A443654 treatment in the presence of caspase inhibitors. The loss of AKT after A443654 treatment was abolished by the inhibition of caspase-3, suggesting the loss of AKT in Jurkat cells was through a caspase-3 dependent mechanism (Fig. 2D). Moreover, a cleaved caspase-3/AKT complex was immunoprecipitated in Jurkat cells using an antibody to AKT, but not in either CEM cell line (Fig. 2E and data not shown). A similar mechanism to that of AKT may be responsible for the diminishing levels of GSK-3 β observed in Jurkat cells after A443654 treatment (Fig. 2A).

GSK-3 β Is Involved in A443654-Induced Cytotoxicity

Considering that GSK-3 β regulates the expression of cyclin D1, Myc, and a number of other key regulatory proteins that are important for cell survival (Frame and Cohen, 2001), it was investigated whether GSK-3 β played a role in A443654-dependent cytotoxicity. Jurkat cells were pretreated with LiCl (a well established inhibitor of GSK-3 β ; see Bain et al., 2007), then treated with A443654, and cell survival was assessed by MTT assay. Whereas LiCl when employed alone at 10 μ M slightly decreased cell survival, this was not statistically significant

($p > 0.05$). However, LiCl had a statistically significant ($p < 0.01$) inhibitory effect on A443654 induced cytotoxicity when employed in the range of 5 to 10 μM (i.e., concentrations at which most of GSK-3 β activity is inhibited in vitro) (Bain et al., 2007) (Fig. 3). Overall, these findings indicated that apoptotic cell death elicited by A443654 is at least partially dependent on dephosphorylation and subsequent activation of GSK-3 β .

A443654 Induces Caspase Activation

Because we had demonstrated the ability of A443654 to lead to caspase-3-dependent degradation of AKT in Jurkat cells and induce apoptosis in the tested T-ALL cell lines, we determined which caspases were responsible for the induced apoptosis. Activation of caspases, as observed by caspase cleavage, was first observed at 30 min with the cleavage of the initiator/effector caspase, caspase-2, to the p14 fragment (Fig. 4A). This was closely followed by the cleavage of two other initiator caspases, caspase-8 and -9, which were cleaved by 2 h. Finally, cleavage/activation of the effector caspase, caspase-3, was induced at 2 h with the initial observance of the cleaved product, p19 (Fig. 4A). Flow cytometric analysis of the percentage of Jurkat cells with cleaved caspase-3 was determined after 4 h of treatment with 1 μM A443654 (Fig. 4B). Approximately 56% of the cells were positive for cleaved caspase-3.

Preincubation of cells with a pharmacological inhibitor selective for caspase-2 (Z-VDVAD-FMK) almost completely prevented cleavage of effector caspases 3 and 6 (Fig. 4C). The caspase-3 downstream target PKC- δ (DeVries-Seimon et al., 2007) was cleaved after treatment with A443654, and the cleavage was strongly reduced by preincubation with Z-VDVAD-FMK (Fig. 4D). Z-VDVAD-FMK also markedly reduced apoptotic cell death induced by A443654 in Jurkat cells, as demonstrated by Annexin V-FITC/PI staining (Fig. 4E). Taken together, these findings suggested an important role played by caspase-2 activation in response to A443654 treatment.

A443654 Has a Synergistic Effect on Apoptosis Induction When Combined with Etoposide

Therapeutic protocols frequently use a drug cocktail to combat cancer. Often, the effective concentration of one or more of the cocktail components is poorly tolerated. Because A443654 showed great effectiveness at dosages within the tolerable limits, we determined whether A443654 could synergize with a chemo-therapeutic drug such as etoposide (Fig. 5). Treatment of Jurkat cells with various concentrations of A443654 combined with etoposide resulted in enhanced cell death. After the determination of the CI for the two drugs, it was observed that the best synergistic effect occurred when the drugs (100 nM A443654 and 2 μM etoposide) were administered simultaneously (CI = 0.391), whereas administration of 2 μM etoposide before 100 nM A443654 only showed a CI = 0.689. In contrast, administration of A443654 before etoposide resulted in a slight additive/antagonistic effect (CI = 1.072). Because the target of etoposide is replicating DNA and A443654 causes G₂/M arrest, etoposide may have very little cytotoxic effects on the cells if administered after A443654 treatment, an important point when administering A443654 to patients in combinational chemotherapy (Fig. 5A and data not shown).

An additional complication of cancer therapy is drug resistance. Because we determined that coadministration of A443654 with etoposide resulted in synergistic killing of Jurkat cells, we examined whether sublethal doses of A443654 could enhance the effectiveness of etoposide against drug-resistant cells (Fig. 5B). CEM-S and CEM-R cells were cotreated for 48 h in the presence of 25 nM (CEM-S) or 3 μM A443654 (CEM-R) and serially diluted dosages of etoposide. Although enhanced cell death was observed in CEM-S cells when treated with both A443654 and etoposide, the enhancement was not significant. In contrast, a significant enhancement of etoposide induced cell killing was observed when CEM-R cells were cotreated with sublethal A443654. At etoposide concentrations between 25 to 100 μM , A443654 was

able to enhance the extent of cell killing from 25% to 45% (Fig. 5B). By increasing the effectiveness of etoposide, a drug that is a substrate for 170-kDa P-glycoprotein, these results highlight the potential therapeutic applications A443654 has on drug-resistant cancers.

T-ALL Blasts Display Elevated p-AKT and Are Sensitive to A443654

To determine the effectiveness of A443654 as a therapeutic agent in T-ALL, we examined T-ALL patient samples (age, 19-59 years; sex, 5 male, 1 female) isolated from bone marrow or peripheral blood, for the presence of PTEN and p-Ser473 AKT and their sensitivity to A443654, using flow cytometry. All patient samples (6/6) had levels of p-Ser473 AKT higher than those observed in peripheral blood lymphocytes from healthy donors (Fig. 6, A and B). In addition, although we did not determine whether these patient samples harbored activating mutations in Notch-1, as it was beyond the scope of this study, we did determine that all six samples had decreased levels of PTEN compared with peripheral blood lymphocytes (Fig. 6, A and B). Jurkat cells served as an additional control, in that they have elevated p-Ser473 AKT and they do not express PTEN. To determine the susceptibility of these cells to inhibition of AKT, the patient samples were treated with 1 μ M A443654 for 72 h, and the percentage of sub-G₁/G₀ cells was analyzed by flow cytometry. Each of the patient samples displayed at least 50% of the cells in sub-G₁/G₀ after treatment (Fig. 6C). In addition, increased levels of cleaved caspase-3 were detected in patient samples treated with A443654 (Fig. 6D). Flow cytometric analysis of samples double-stained for p-Ser473 AKT and cleaved caspase-3 demonstrated that cells with increased levels of Akt phosphorylation as a result of drug treatment actually underwent apoptotic cell death (Fig. 6E).

Discussion

T-ALLs are rare and often difficult to treat. A small fraction results from fusion proteins such as ETV6-ABL1 and ETV6-JAK2, but the majority of T-ALL cells possess mutations in molecules such as PTEN or Notch-1 that affect PI3K/AKT/mTOR signaling (Seminario et al., 2003; Grabher et al., 2006; Chan et al., 2007; Palomero et al., 2007). Because AKT seems to play a central role in T-ALL pathology, we sought to determine whether the novel AKT inhibitor, A443654, might be a useful therapy in treating T-ALL. We found that A443654 was able to inhibit cellular proliferation in the T-ALL cell lines tested at nanomolar concentrations. Preclinical studies performed in vivo in a mouse model have shown that A443654 could be well tolerated up to a maximum concentration of approximately 20 μ M in tumor (Luo et al., 2005). It is remarkable that in the drug-resistant T-ALL cell line CEM-R, A443654 had an IC₅₀ of approximately 12 μ M; this is still well within the maximum tolerated dose, suggesting that this particular AKT inhibitor may be extremely useful in treating a majority of T-ALLs as well as drug-resistant T-ALL. It is worth emphasizing here that 170 kDa P-glycoprotein is detected in approximately 24% of patients with T-ALL and negatively correlates with the achievement of complete remission (Tafuri et al., 2002; Vitale et al., 2006a). A443654 also induced a significant amount of apoptosis in all six T-ALL patient samples.

A443654 induced cell cycle arrest in the G₂/M phase followed by subsequent apoptosis in T-ALL cell lines. It is interesting that cells were arrested in G₂/M, in that AKT is often thought of as a kinase responsible for entering into S-phase, but AKT has been demonstrated to have additional targets that control entry into G₂ and subsequent entry into the M-phase of the cell cycle (Okumura et al., 2002; Katayama et al., 2005). The observed G₂/M arrest is intriguing in that the CI of A443654 and etoposide in Jurkat cells showed synergy when the two drugs were either added simultaneously or etoposide was added before A443654. Addition of A443654 before etoposide had an additive/antagonistic effect. The fact that A443654 arrests pretreated cells in G₂/M may preclude any response to etoposide, a drug that requires DNA to be replicating to have its cytotoxic effects (Markovits et al., 1987; D'Arpa et al., 1990).

Moreover, because caspase-2 was the first caspase observed to be cleaved after treatment with A443654, the G₂/M arrest may be indicative of mitotic catastrophe, an event that results in the formation of the PIDDosome and activation of caspase-2 followed by caspase-8 and caspase-9 activation (Tinel and Tschopp, 2004). The use of a selective caspase-2 inhibitor allowed us to establish that activation of this apical caspase was very important for the activation of effector caspases 3 and 6, and for PKC- δ cleavage. It is noteworthy that, besides caspase-3, caspase-2 has been reported to possibly directly cleave PKC- δ (Zhivotovsky and Orrenius, 2005). In contrast, our unpublished data showed that inhibition of apical caspase-8 and -9 only slightly diminished caspase-3 and -6 cleavage (data not shown).

It was previously reported that A443654 induces rapid phosphorylation of AKT on Ser473 and induces a rapid dephosphorylation of the AKT substrate, GSK-3 β (Luo et al., 2005; Han et al., 2007). The phosphorylation of Ser473 was reportedly elicited through the rapamycin insensitive mTOR: Rictor complex. We found that, in addition to dephosphorylation of GSK-3 β and phosphorylation of AKT at position Ser473, Thr308 phosphorylation was rapidly induced by A443654. A443654 induced phosphorylation of Thr308 was not significantly inhibited by the PI3K/mTOR inhibitors, wortmannin, LY294002, and PI-103, or down-regulation of PDK1 through siRNA, indicating that membrane recruitment by PIP3 and subsequent phosphorylation by PDK1 were not a requirement for A443654-mediated Thr308 phosphorylation. In contrast, phosphorylation of Ser473 was partially inhibited by LY294002, wortmannin, or PI-103 when used at or above the IC₅₀ for PI3K but was greatly inhibited when these compounds were used at or above the IC₅₀ or mTOR, suggesting that, in Jurkat cells, some Ser473 phosphorylation may not be completely dependent on mTOR: Rictor, but a majority is. The phosphorylation of AKT after addition of A443654 may imply that AKT functions as a stress/ATP indicator. A443654 functions as an AKT ATPbinding site analog (Luo et al., 2005; Han et al., 2007), and the inability of AKT to phosphorylate downstream substrates is similar to a low ATP state in the cell. Such a state could occur during poor growth conditions (low O₂ or a sugar source) or after mitochondrial damage resulting in AKT phosphorylation. Such stress-induced AKT phosphorylation has been reported recently to be dependent on eukaryotic initiation factor 2 α phosphorylation after activation of protein kinase R or protein kinase R-like endoplasmic reticulum kinase (Kazemi et al., 2007). Further analysis is needed to identify the kinase(s) responsible for stress-induced AKT phosphorylation.

A443654-induced GSK-3 β dephosphorylation, despite concomitant hyperphosphorylation of AKT, could occur because A443654-bound AKT is locked into a conformation that is not amenable to dephosphorylation, and thus the phosphorylated forms of AKT rapidly accumulate. If indeed AKT relocation is necessary for its subsequent dephosphorylation, A443654 might lead to an accumulation of inactive but highly phosphorylated AKT by simply preventing its release from sites of activation (Han et al., 2007). In this connection, unpublished results of F. Falà have revealed that immunoprecipitated AKT from A443654 cells actually increased phosphorylation (and hence inhibition) of recombinant GSK-3 β in an in vitro assay. Thus, this finding could support the hypothesis of Han et al. (2007), in that immunoprecipitated AKT would be presumably freed from interactions with A443654. The occurrence of in vivo inhibition of GSK-3 β by A443654 was demonstrated by the effects of concomitant incubation with LiCl, which resulted in a mitigation of the drug-induced cytotoxicity.

It should be stated that, in vitro, assays of A443654 inhibitory activity identified 16 kinases that were significantly affected (Bain et al., 2007). Although these results have not been repeated in vivo, it is possible that some effects of A443654 are not due directly to AKT inhibition. In our experience, however, we did not detect modifications in the activation status of the ERK kinases or JNK in T-ALL cell lines immediately after treatment with A443654 (data not shown). In conclusion, our preclinical studies support the concept that inhibition of Akt signaling may have clinical application for treatment of T-ALLs. Based on these data, it

is conceivable that A443654, or other Akt inhibitors, may serve as efficient therapeutic agents to combat T-ALLs that require elevated levels of AKT for their survival and growth. Moreover, A443654 may be an effective adjuvant to combine with current chemotherapy regimens to enhance their therapeutic efficiency, especially in drug-resistant T-ALLs.

Acknowledgements

This work was supported by grants from: Fondazione Casa di Risparmio di Bologna (to A.M.M.), Associazione Italiana Ricerca sul Cancro (to G.M. and A.T.), Italian Ministero Università e Ricerca-Progetti di Ricerca di Interesse Nazionale 2006 (to A.T.), Progetti Strategici Università di Bologna EF2006 (to A.M.M.), European LeukemiaNet (to G.M.), and a grant from the National Institutes of Health (R01-CA091025 to J.A.M.).

ABBREVIATIONS

T-ALL, T-cell acute lymphoblastic leukemia
 PI3K, phosphatidylinositol-3 kinase
 mTOR, mammalian target of rapamycin
 PTEN, phosphatase and tensin homolog deleted on chromosome 10
 PIP3, phosphatidylinositol-3,4,5-phosphate
 GSK-3 α/β , glycogen synthetase kinase-3 α/β
 A443654, (2*S*)-1-(1*H*-indol-3-yl)-3-[5-(3-methyl-2*H*-indazol-5-yl)pyridin-3-yl]oxypropan-2-amine
 LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one
 Z-VDVAD-FMK, *N*-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethyl ketone
 PI-103, 3-(4-(4-morpholinyl)pyrido[3',2',4,5]furo[3,2-*d*]pyrimidin-2-yl)phenol
 CI, combination index
 FITC, fluorescein isothiocyanate
 PI, propidium iodide
 PAGE, polyacrylamide gel electrophoresis
 PBST, PBS containing 0.05% Tween 20
 BSA, bovine serum albumin
 PKC, protein kinase C
 PDK1, phosphatidylinositol-dependent kinase 1
 PBS, phosphate-buffered saline
 CEM-S, drug-sensitive CEM cells
 CEM-R, drug-resistant CEM cells
 siRNA, short interfering RNA
 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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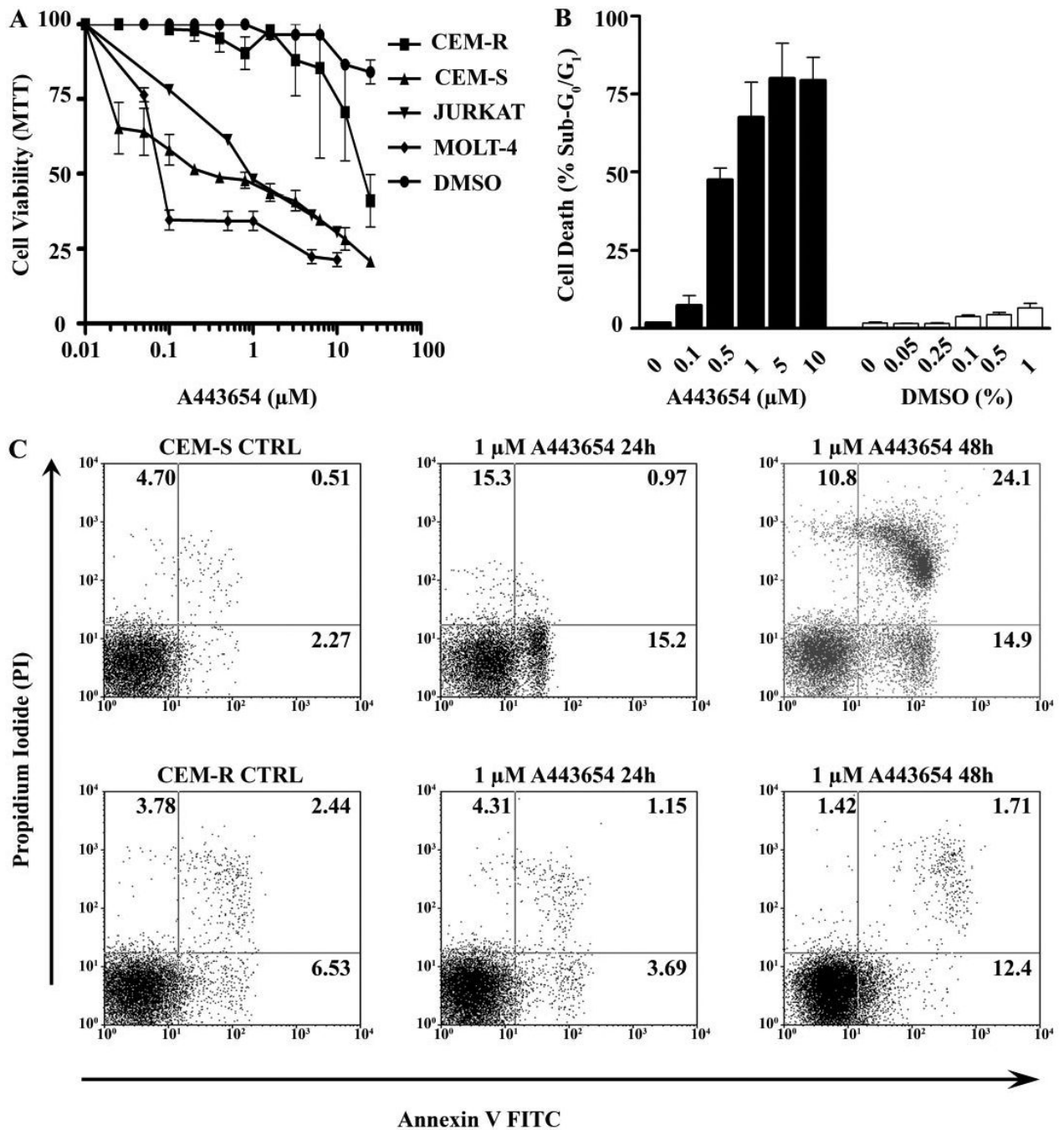


Fig. 1. A443654 inhibits proliferation and induces apoptosis in T-ALL cell lines. A, Jurkat, MOLT-4, CEM-S, and CEM-R cells were treated with serially diluted A443654 or corresponding DMSO concentrations for 24 h. MTT analysis was then performed. Points indicated are the averages of three experiments \pm S.D. The curve showing DMSO effects refer to CEM-S cells, but similar results were obtained with other cell lines (not shown). Note that at 0.01 μM A443654 did not decrease cell viability with respect to untreated cells (F. Falà, unpublished results). Therefore, cell survival values at 0.01 μM A443654 have been set as 100%. B, Jurkat cells were treated with 10, 5, 1, 0.5, or 0.1 μM A443654 or corresponding DMSO concentrations for 24 h. Cell cycle analysis was performed, and the percentage of cells in sub-G₁/G₀ (dead cells with

fragmented DNA) was determined. Bars are the averages of three experiments \pm S.D. C, CEM-S and CEM-R cells were treated with DMSO (control, CTRL) or 1 μ M A443654 for 24 and 48 h. The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining. Bottom left, viable cells (Annexin V-FITC/PI negative); bottom right, early apoptotic cells (Annexin V-FITC positive only); top right, mid-late apoptotic cells (Annexin V-FITC/PI double-positive); and top left, late apoptotic/necrotic (PI positive only). Percentages of cells in each quadrant are indicated except for the bottom left quadrant (viable cells). After treatment, some cells that were strongly PI-positive/Annexin V-FITC-negative would occasionally skew to the leftmost part of the top left; the dot points for these cells are not visible but are included in the percentages indicated. One experiment representative of three different experiments that gave similar results is shown.

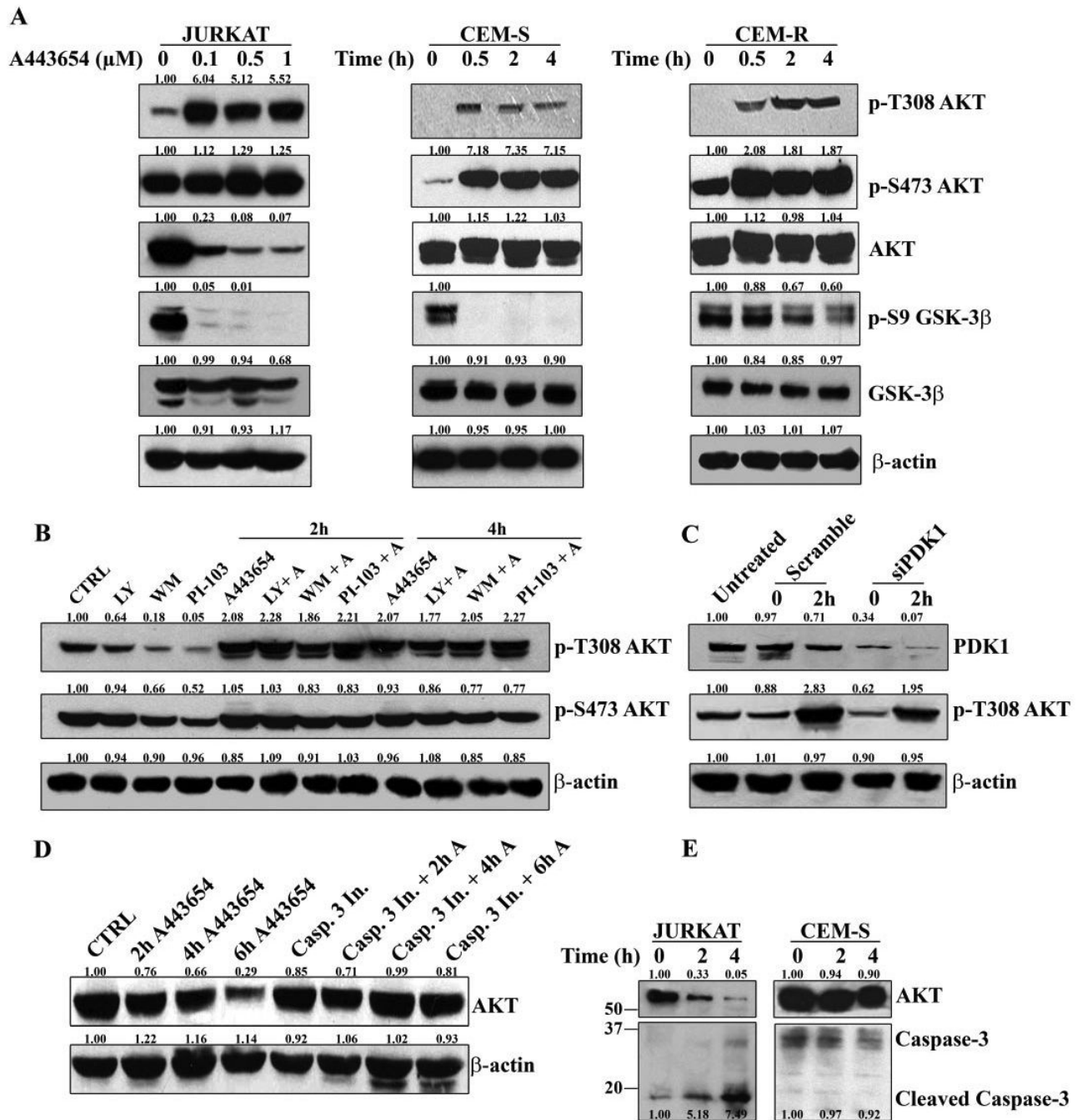


Fig. 2. A443654 induces a dose-dependent and rapid phosphorylation of AKT on Thr308 and Ser473 and dephosphorylation of GSK-3 β in T-ALL cell lines. **A**, Jurkat cells were treated for 24 h in the presence of 0.1, 0.5, or 1 μ M A443654; CEM-S and CEM-R cells were treated with 1 and 10 μ M A443654, respectively, for 0.5, 2, and 4 h. Fifty micrograms of each lysate were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membrane. **B**, Jurkat cells were pretreated for 1 h with LY294002 (1.5 μ M), wortmannin (100 nM), or PI-103 (50 nM). Cells were then treated with 1 μ M A443654 for 2 or 4 h. Cells were collected and lysed, and 50 μ g of each lysate were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membrane. CTRL, untreated cells. **C**, Jurkat cells were electroporated with

siRNA against PDK1 or nonspecific (scramble) siRNA and cultured an additional 72 h. Cells were then treated with 1 μ M A443654 for 2 h. Cells were collected and lysed, and 50 μ g of each lysate were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membrane. β -Actin served as loading control. D, Jurkat cells, either pretreated or not for 2 h with caspase-3 inhibitor (10 μ M), were incubated for the indicated times with 1 μ M A443654. Lysate (50 μ g) was electrophoresed on a SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with antibodies to AKT or to β -actin. CTRL, untreated cells. E, Jurkat and CEM-S cells were left untreated (0 h) or treated with 1 μ M A443654 for 2 and 4 h. Lysate (50 μ g) was immunoprecipitated with anti-AKT antibody and protein A/G agarose. The washed immunoprecipitate was electrophoresed on an SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with anti-AKT or anti-caspase-3 antibody. Molecular weight markers are indicated at left. For all the blots, protein levels were quantified by using Image J software as described under *Materials and Methods*. Band intensities of control was normalized to 1, and treated samples were expressed as fraction of control. Because there was no band in the control in the blots for p-Thr308 AKT levels in CEM-S and CEM-R cells, quantification was not performed.

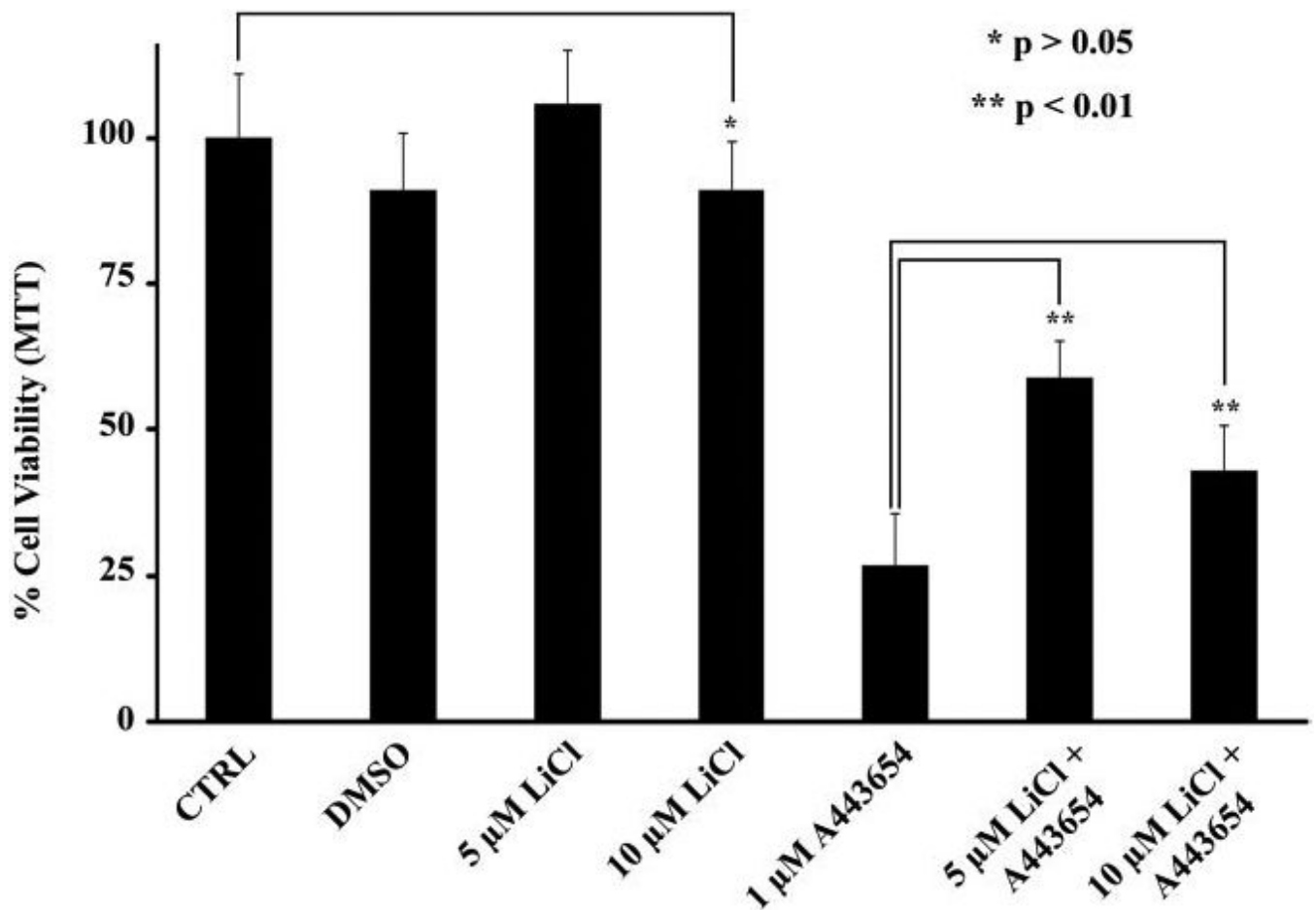
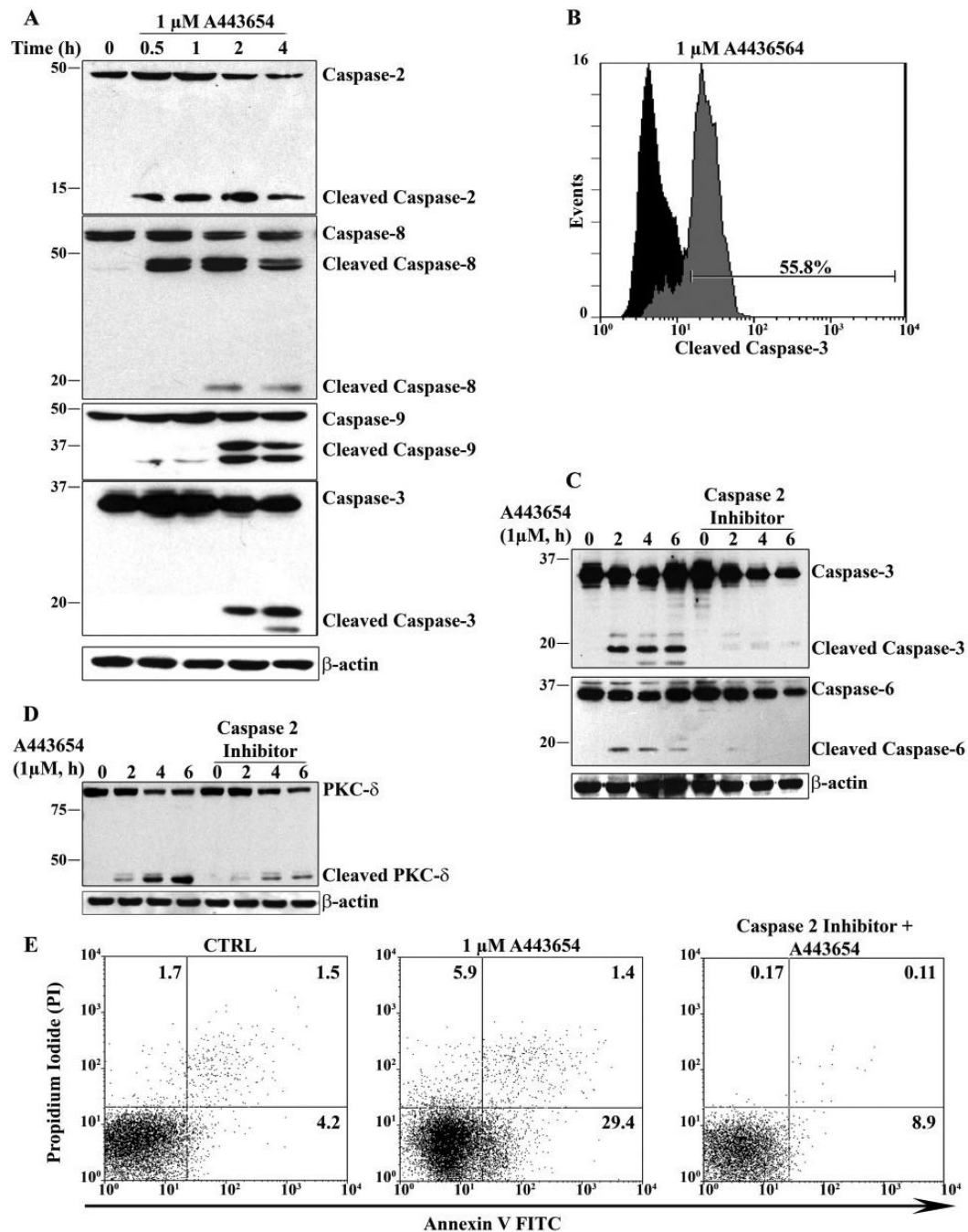


Fig. 3. A443654 induced cytotoxicity is partly dependent on GSK-3 β activity. Jurkat cells were pre-treated with LiCl for 30 min, then with A443654 (1 μ M) or DMSO for 24 h. MTT assays were then performed. CTRL: untreated cells. Points indicated are the averages of three separate experiments \pm S.D.

**Fig. 4.**

A443654 induces activation of caspase-2, -3, -6, -8, and -9 in Jurkat cells. A, Jurkat cells were treated with 1 μ M A443654 for the indicated times, collected, and then lysed. Fifty micrograms of each lysate were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membrane. Antibody to β -actin served as a loading control. B, Jurkat cells that had been treated with 1 μ M A443654 or DMSO (control) for 4 h were fixed, permeabilized, and analyzed by flow cytometry for active caspase-3 using anti-cleaved caspase-3 Alexa Fluor 488 conjugate antibody. Black histogram, DMSO-treated cells; gray histogram, A443654-treated cells. C, Jurkat cells, either pretreated or not for 2 h with caspase-2 inhibitor (10 μ M), were treated with 1 μ M A443654 for the indicated times, and activation of caspases was evaluated by Western

blot. Antibody to β -actin served as a loading control. D, Jurkat cells, either pretreated or not with caspase-2 inhibitor (10 μ M) were incubated with 1 μ M A443654 for the indicated times, then lysed, and immunoblotted for PKC- δ . In A, C, and D, molecular weight markers are indicated at left. E, flow cytometric analysis of Annexin V-FITC/PI- stained Jurkat cells, treated with 1 μ M A443654, with or without caspase-2 inhibitor (10 μ M) preincubation. CTRL, untreated cells.

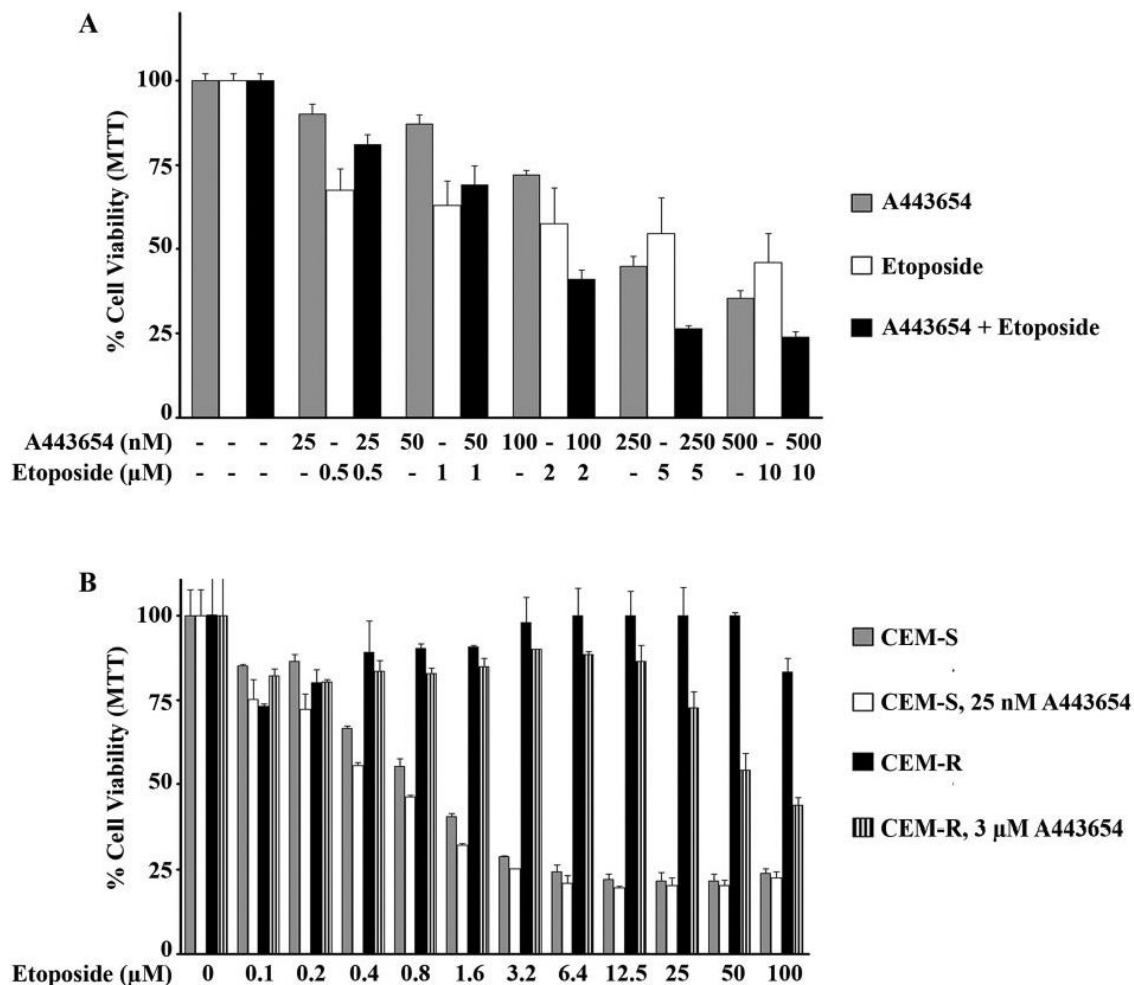


Fig. 5.

A443654 synergizes with etoposide to induce cell killing in drug-sensitive and drug-resistant T-ALL cell lines. A, Jurkat cells were treated with A443654 alone, etoposide alone, or A443654 + etoposide simultaneously at the indicated concentrations for 24 h. Viability was then determined using an MTT assay. The bars represent the average of three independent experiments \pm S.D. Similar experiments were performed in which cells were pretreated with either A443654 or etoposide before the addition of the other compound. B, CEM-S and CEM-R cells were treated with serially diluted concentrations of etoposide in the absence or presence of sublethal doses of A443654 (25 nM and 3 μ M for CEM-S and CEM-R, respectively; a sublethal dose was one that induced less than 15% inhibition in an MTT assay) for 48 h in a 96-well tissue culture plate. The effect on viability was determined by MTT assay. Bars represent the average of three independent experiments \pm S.D.

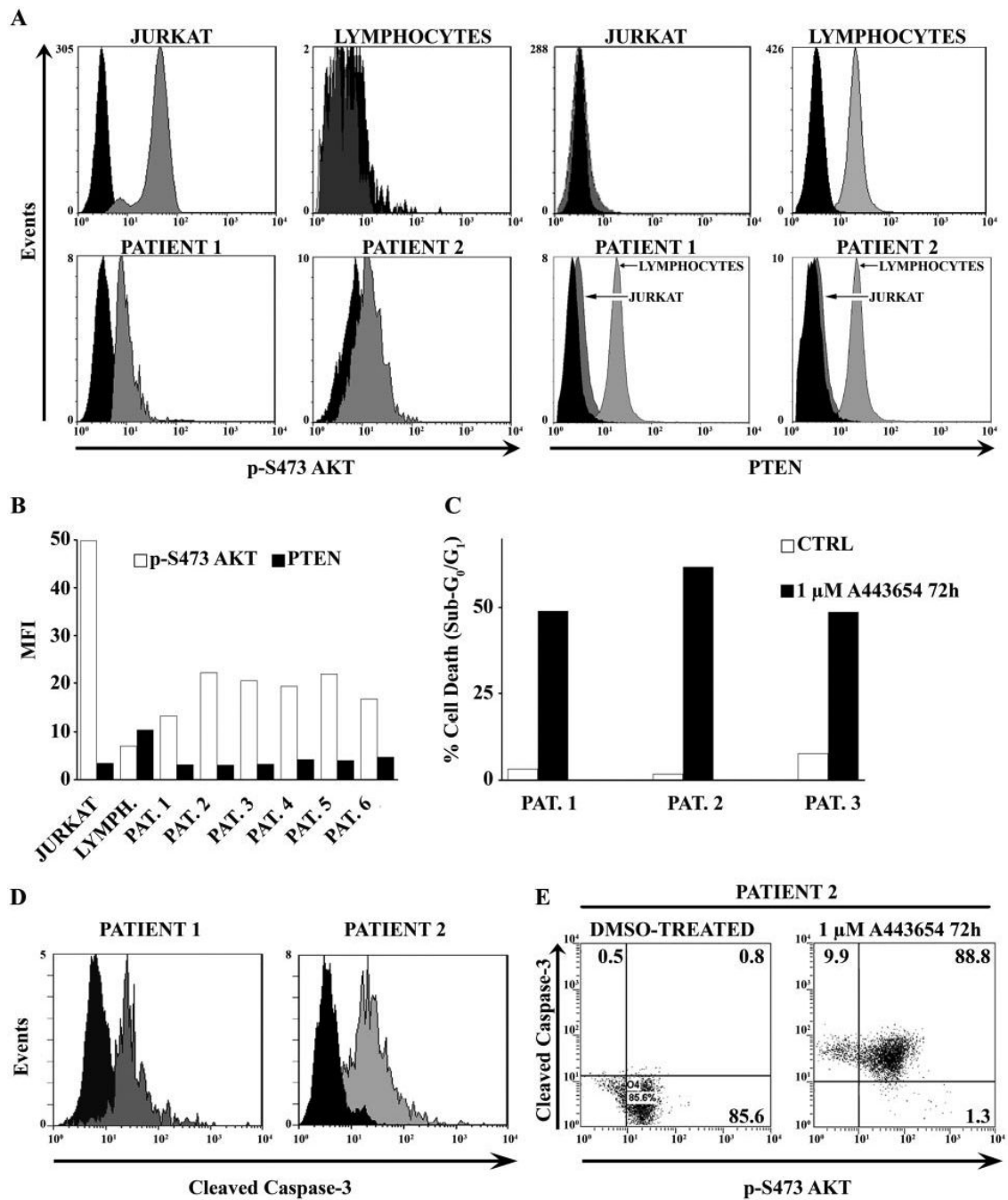


Fig. 6. Samples from T-ALL patients have elevated levels of p-AKT and are susceptible to A443654-mediated cell death. **A**, Jurkat cells, normal lymphocytes, and samples from T-ALL patients were analyzed by flow cytometry for the levels of p-Ser473 AKT and PTEN. Two representative patient samples and the controls (Jurkat and healthy donor peripheral blood lymphocytes), are shown. For p-Ser473 AKT expression: black histograms, negative control (irrelevant antibody); gray histograms, p-Ser473 AKT-positive cells. For PTEN expression in Jurkat and healthy donor lymphocytes: black histogram, negative control (irrelevant antibody); gray histograms, PTEN positive cells. For PTEN expression in patient samples: black histogram, leukemic lymphoblasts. **B**, p-Ser473 AKT and PTEN expression levels for all

patients are expressed as a bar graph. MFI, mean fluorescence intensity. C, patient samples were treated with 1 μ M A443654 or DMSO (control, CTRL) for 72 h. Cell cycle analysis was then performed as described under *Materials and Methods*. The percentage of cells in the sub-G₁/G₀ fraction were plotted. Three representative patient samples are shown. D, patient samples were treated with 1 μ M A443654 or DMSO for 72 h, fixed, permeabilized, immunostained for cleaved caspase-3, then analyzed by flow cytometry. Dark histograms, DMSO-treated samples; gray histograms, cleaved caspase-3 positive cells. E, dot plot of leukemic lymphoblasts double-positive for cleaved caspase-3 and p-Ser473 AKT. Samples were treated with DMSO or 1 μ M A443654 for 72 h. Lymphoblasts were fixed, permeabilized, doubly immunostained with Alexa Fluor 488 conjugate anti-cleaved caspase-3 antibody and Alexa Fluor 647 conjugate anti-p-Ser473 AKT antibody, then analyzed by flow cytometry. Percentages of cells in each quadrant of the dot plots are indicated with the exception of the double-negative population.