

MOL #47639

**Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor, A443654, in T-  
Acute Lymphoblastic Leukemia**

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MOL #47639

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**Abbreviations:** T-ALL, T-cell acute lymphoblastic leukemia; HES-1, hairy and enhancer of split 1; PI3K, phosphatidylinositol-3 kinase; mTOR, mammalian target of rapamycin; PIP3, phosphatidylinositol-3,4,5-phosphate; PIP2, phosphatidylinositol-4,5-phosphate; MMAC1, mutated multiple advanced cancers 1; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PDK1, phosphatidylinositol dependent kinase 1; GSK-3 $\alpha/\beta$ , glycogen synthetase kinase-3 $\alpha/\beta$ ; MDM2, murine double minute 2; XIAP, X-linked inhibitor of apoptosis protein; CEM-S: drug-sensitive CEM cells; CEM-R, drug-resistant CEM cells; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; CI, combination index; FITC, fluorescein isothiocyanate; PI, propidium iodide; siRNA, short interfering RNA; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; PBST, PBS containing 0.05% Tween-20; ECL, enhanced chemiluminiscence; DMSO, dimethyl sulfoxide; PIDD, p53-induced protein with a death domain; PKR, protein kinase R; PERK, PKR-like endoplasmic reticulum kinase; eIF2 $\alpha$ , eukaryotic initiation factor 2 $\alpha$ .

MOL #47639

## 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, 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 $\beta$ . 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 pre-incubation with a selective pharmacological inhibitor. Remarkably, A443654 was highly effective against the drug-resistant cell line CEM-VBL100, 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 co-administered (CI=0.39), or when pre-treated with etoposide followed by A443654 (CI=0.689). The efficacy of A443654 was confirmed using blasts from 6 patients with T-ALL, all of whom displayed low levels of PTEN and constitutive phosphorylation of Akt on S473. At 1  $\mu$ M concentration, 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. As activated AKT is seen in a large percentage of T-ALL patients, A443654, either alone or in combination with existing drugs, may be a useful therapy for primary and drug-resistant T-ALL.

MOL #47639

Treatment of adult acute T-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 greater than 50% of T-ALL patients 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, PTEN (Palomero et al., 2007). In addition, PTEN is mutated in about 20% of T-ALL patients, and virtually all T-ALL cell lines that are resistant to Notch-1 inhibition with  $\gamma$ -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 PIP2 by PI3K (Tokunaga et al., 2008). In the presence of PTEN, PIP3 is rapidly dephosphorylated to PIP2, 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, as activation of AKT by phosphorylation on T308 by PDK1 or on S473 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 following phosphorylation by AKT are involved in cell cycle arrest, apoptosis induction or homeostasis under low nutrient conditions. Targets inhibited by

MOL #47639

AKT phosphorylation include GSK-3 $\alpha/\beta$ , FoxO transcription factors, Bad, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup> (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 MDM2, XIAP, 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., 2007). Moreover, activation of 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 which overexpresses 170-kDa P-glycoprotein) 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 in RPMI 1640 supplemented with 10% FBS, 200 mM L-glutamine, and penicillin/streptomycin. A443654 was a kind gift from Abbott Pharmaceutical (Abbott Park, IL, USA). LY294002, wortmannin, etoposide, PI-103, caspase-2 inhibitor (Z-VDVAD-FMK), and caspase-3 inhibitor (Ac-DMQD-CHO) were from EMD Biosciences (La Jolla, CA, USA). 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 (Amersham Biosciences, Uppsala, Sweden) using density-gradient centrifugation. Prior to analysis, patient samples or

MOL #47639

lymphocytes from healthy donors were cultured in RPMI 1640 containing 20% FBS, 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:

$$(1) \quad CI = C_a/C_{x_a} + C_b/C_{x_b},$$

where  $C_{x_a}$  and  $C_{x_b}$  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 following 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 previously described 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, Miami, FL, USA).

**Cell Cycle Analysis.** Following their respective treatment, T-ALL cell lines and patient samples were prepared as previously described (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

MOL #47639

density of  $5 \times 10^6$  cells/0.1 ml. Cells were electroporated with 1.5  $\mu$ M SmartPool siRNA to PDK1 or control using a Nucleofector electroporator (Amaxa Biosystems, program X-05) 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 the D<sub>c</sub> Protein Assay (Bio-Rad, Hercules, CA, USA). Lysate (50  $\mu$ g) was loaded onto a SDS-PAGE gel, electrophoresed, and transferred onto nitrocellulose membranes, using a semi-dry transfer apparatus. The membranes were incubated overnight at 4<sup>0</sup>C in 5% non-fat dry milk in 1X PBST. The membranes were washed three times in 1X PBST and incubated overnight at 4<sup>0</sup>C in primary antibody diluted 1:1000 in 5% BSA in PBST. The following antibodies were from Cell Signaling Laboratories, Danvers, MA, USA: p-T308 AKT, p-S473 AKT, AKT, p-S9 GSK-3 $\beta$ , GSK-3 $\beta$ , caspase-2, caspase-3, caspase-6, caspase-8, caspase-9, PKC- $\delta$ , PDK1,  $\beta$ -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, Piscataway, NJ, USA). Band intensity was determined by densitometry using the public domain software Image J (a Java image processing program inspired by NIH Image for Macintosh), as described elsewhere (Nyakern et al., 2006).

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

**Determination of the Levels of PTEN, p-S473 AKT and Cleaved Caspase-3 in T-ALL Patient**

**Samples.** Viable lymphoblasts ( $5 \times 10^5$ ) from six T-ALL patients or peripheral blood lymphocytes from healthy donors were fixed with Reagent 1 of the Intraprep kit according to the manufacture'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<sup>0</sup>C in primary antibody [p-S473 AKT (either Alexa Fluor 488 or AlexaFluor 647 conjugate), PTEN (AlexaFluor 647 conjugate), or cleaved caspase-3 (Alexa Fluor 488 conjugated), all from Cell Signaling, diluted 1:10 in 5% BSA in PBS].

MOL #47639

Cells were then washed twice in 1X PBS and analyzed on a FC500 flow cytometer (Papa et al., 2008; Tazzari et al., 2007a; Tazzari et al., 2007b).

**Statistical evaluation.** The data are shown as mean values  $\pm$  sd. Data were statistically analyzed by a Dunnett test after one-way analysis of variance (ANOVA) at a level of significance of  $p < 0.05$  vs. 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 nM, 120 nM, and 900 nM for MOLT-4, CEM-S, and Jurkat, respectively) well below 20  $\mu$ M, 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  $\mu$ M), but this  $IC_{50}$  was still below 20  $\mu$ M (Fig. 1A).

As AKT is considered to be a major anti-apoptotic 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 if 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  $\mu$ M or 1.0  $\mu$ M A443654 for 2, 4, 8, 16, and 24 h. No changes in cell cycle progression were observed between treated and untreated samples following 2, 4 or 8 h of treatment (data not shown). In contrast,

MOL #47639

A443654 resulted in a G<sub>2</sub>/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 G<sub>2</sub>/M whereas only 11% of the untreated cells were observed in G<sub>2</sub>/M (data not shown). Following 24 h of treatment with 0.5 μM A443654, 50% of the cells were observed in the sub-G<sub>1</sub>/G<sub>0</sub> fraction, while treatment with 1.0 μM A443654 resulted in 65% of the cells in the sub-G<sub>1</sub>/G<sub>0</sub> fraction. In contrast, in DMSO-treated cells the percentage of cells in the sub-G<sub>1</sub>/G<sub>0</sub> 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) while another 15% were PI positive only, indicating that some cells had died by necrosis or rapidly died by apoptosis while others were slightly less sensitive. In contrast, no significant effects were observed in the CEM-R cells after 24 h. Following 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% 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) following 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 T308 and S473 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 S473 (Han et al., 2007; Luo et al., 2005). 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 T308 and S473. Treatment

MOL #47639

of Jurkat cells with 0.1  $\mu$ M A443654 for 24 h resulted in a dramatic increase in AKT phosphorylated at T308, while enhancement of AKT phosphorylated on S473 was less marked over control cells (Fig. 2A). In addition, 0.1  $\mu$ M A443654 resulted in greatly diminished levels of GSK-3 $\beta$  phosphorylated at S9. Phosphorylation of AKT at S473 as well as loss of p-S9 GSK-3 $\beta$  occurred rapidly (within 30 min). Treatment of CEM-S (1  $\mu$ M) or CEM-R (10  $\mu$ M) cells with A443654 resulted in a robust increase in the amount of AKT phosphorylated at S473, respectively (Fig. 2A). In contrast to S473 where constitutive AKT phosphorylation was observed in CEM-S and to a greater extent in CEM-R cells, no constitutive phosphorylation of T308 was detected, using a phosphospecific antibody, but phosphorylation at this site was likewise rapidly induced. Moreover, GSK-3 $\beta$  was rapidly dephosphorylated, although dephosphorylation of GSK-3 $\beta$  occurred to a lesser extent in CEM-R cells, most likely as a result of the higher constitutive levels of p-S473 AKT in these cells (Fig. 2A).

A443654 induced phosphorylation of S473 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 (S473) and PDK1-independent (T308), suggesting an additional kinase(s) responsible for the phosphorylation of AKT on T308 and S473 (Fig. 2B). Jurkat cells, which were either pre-treated with DMSO (control), LY294002, wortmannin or PI-103 [a dual PI3K/mTOR inhibitor, see (Fan et al., 2006)] for 1 h, were treated with 1  $\mu$ M A443654 for 2 and 4 h, and the phosphorylation status of AKT was assayed. Pre-treatment with wortmannin or PI-103 all but abolished the constitutive levels of p-T308 AKT in Jurkat cells while LY294002 reduced T308 phosphorylation. Interestingly, in the presence of these inhibitors, A443654 was able to induce phosphorylation of AKT on T308 to levels similar to or above that observed with A443654 alone, suggesting membrane recruitment and PDK1 were not responsible for A443654 induced T308 phosphorylation (Fig. 2B). In addition a similar lack of inhibition of A443654 induced T308 phosphorylation was observed when

MOL #47639

LY294002 and wortmannin were used at 50  $\mu$ M and 300 nM, respectively (data not shown). The constitutive phosphorylation of AKT on S473 was not greatly affected by pre-treatment with LY294002 (1.5  $\mu$ M) while both wortmannin (100 nM) and PI-103 (50 nM) resulted in a marked decrease in AKT S473 phosphorylation. Treatment with A443654 was able to restore p-S473 AKT levels to that of the control (Fig.2B). Higher concentrations of LY294002 (50  $\mu$ M) and wortmannin (300 nM) abolished AKT S473 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  $\mu$ M, 4 nM, and 26 nM, respectively) have been shown to also inhibit mTOR [ $IC_{50}$ = 5  $\mu$ M, 300 nM, and 20 nM (mTOR:Raptor complex) or 86 nM (mTOR:Rictor complex), respectively] (Bain et al., 2007). As at doses at or above the  $IC_{50}$  for PI3K, the inhibitors had only a partial affect on AKT S473 phosphorylation but, at concentrations at or above the  $IC_{50}$  for mTOR were capable of inhibiting AKT S473 phosphorylation, it is likely mTOR:Rictor is the major kinase responsible for AKT S473 phosphorylation. This observation is in agreement to what was recently reported by others (Han et al., 2007). To rule-out PDK1 as being responsible for T308 phosphorylation, siRNA directed to PDK1 was used to downregulate PDK1 levels in Jurkat cells prior to treatment with 1  $\mu$ M A443654. While scramble siRNA did not decrease PDK1 levels when compared with untreated cells, as evaluated by western blot, pre-treatment with siRNA to PDK1 resulted in a marked decrease in PDK1 and in p-T308 AKT levels (Fig. 2C). Following treatment with A443654, p-T308 AKT levels increased in samples pre-treated 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-T308 increased 3.2 times upon drug treatment, whereas in samples with downregulated PDK1 the increase was 3.14 fold, despite a ten-fold reduction in the PDK1 levels.

Collectively, these data indicated that in Jurkat cells, part of the induced phosphorylation at S473 of AKT is dependent on mTOR:Rictor, while phosphorylation of T308 following treatment with

MOL #47639

A443654 is not dependent on PI3K activity, membrane recruitment by PIP3, or phosphorylation by PDK1.

Interestingly, in Jurkat cells, but not in either CEM cell line, a degradation of AKT was observed following phosphorylation (Fig. 2A and D and data not shown). As inhibition of AKT led to apoptosis, we analyzed the expression of AKT following A443654 treatment in the presence of caspase inhibitors. The loss of AKT following 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 $\beta$  observed in Jurkat cells following A443654 treatment (Fig. 2A).

### **GSK-3 $\beta$ is involved in A443654 induced cytotoxicity**

Considering that GSK-3 $\beta$  regulates the expression of cyclin D1, Myc, and a number of other key regulatory proteins which are important for cell survival (Frame and Cohen, 2001), it was investigated whether GSK-3 $\beta$  played a role in A443654-dependent cytotoxicity. Jurkat cells were pre-treated with LiCl (a well established inhibitor of GSK-3 $\beta$ , see (Bain et al., 2007)), then treated with A443654, and cell survival was assessed by MTT assay. Whereas LiCl when employed alone at 10  $\mu$ 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 A-443654 induced cytotoxicity when employed in the range of 5-10 mM, i.e. concentrations at which most of GSK-3 $\beta$  activity is inhibited in vitro (Bain et al., 2007) (Figure 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 $\beta$ .

MOL #47639

**A443654 induces caspase activation.** As 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 following 4 h of treatment with 1  $\mu$ M of A443654 (Fig. 4B). Approximately 56% of the cells were positive for cleaved caspase-3.

Pre-incubation 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- $\delta$  (DeVries-Seimon et al., 2007) was cleaved following treatment with A443654, and the cleavage was strongly reduced by pre-incubation 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 utilize a drug cocktail to combat cancer. Often, the effective concentration of one or more of the cocktail components is poorly tolerated. As A443654 showed great effectiveness at dosages within the tolerable limits, we determined whether A443654 could synergize with a chemotherapeutic drug such as etoposide (Fig. 5). Treatment of Jurkat cells with various concentrations of A443654 combined with etoposide resulted in enhanced cell death. Following 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  $\mu$ M etoposide) were administered simultaneously (CI=0.391), while administration of 2  $\mu$ M etoposide prior to 100 nM A443654 only

MOL #47639

showed a CI=0.689. In contrast, administration of A443654 prior to etoposide resulted in a slight additive/antagonistic effect (CI=1.072). Since the target of etoposide is replicating DNA and A443654 causes G<sub>2</sub>/M arrest, etoposide may have very little cytotoxic effects on the cells if administered following 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. Since we determined that co-administration of A443654 with etoposide resulted in synergistic killing of Jurkat cells, we examined whether sub-lethal doses of A443654 could enhance the effectiveness of etoposide against drug-resistant cells (Fig. 5B). CEM-S and CEM-R cells were co-treated for 48 h in the presence of 25 nM (CEM-S) or 3  $\mu$ M 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 co-treated with sub-lethal A443654. At etoposide concentrations between 25  $\mu$ M to 100  $\mu$ M, A443654 was able to enhance the extent of cell killing from 25% to 45% (Fig. 5B). By increasing the effectiveness of etoposide, a drug which 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 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-S473 AKT and their sensitivity to A443654, using flow cytometry. All patient samples (6/6) had levels of p-S473 AKT higher than those observed in peripheral blood lymphocytes from healthy donors (Fig. 6A 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 as compared to peripheral blood lymphocytes (Fig 6A and B). Jurkat cells served as an additional control, as they

MOL #47639

have elevated p-S473 AKT and they do not express PTEN. In order to determine the susceptibility of these cells to inhibition of AKT, the patient samples were treated with 1  $\mu$ M A443654 for 72 h, and the percentage of sub-G<sub>1</sub>/G<sub>0</sub> cells was analyzed by flow cytometry. Each of the patient samples displayed at least 50% of the cells in sub-G<sub>1</sub>/G<sub>0</sub> following 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-S473 AKT and cleaved caspase-3, demonstrated that cells with increased levels of Akt phosphorylation due to 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 which affect PI3K/AKT/mTOR signaling (Chan et al., 2007; Grabher et al., 2006; Palomero et al., 2007; Seminario et al., 2003). As AKT seems to play a central role in T-ALL pathology, we sought to determine if 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. Pre-clinical studies performed *in vivo* in a mouse model, have shown that A443654 could be well tolerated up to a maximum concentration of approximately 20  $\mu$ M in tumor (Luo et al., 2005). Remarkably, in the drug-resistant T-ALL cell line CEM-R, A443654 had an IC<sub>50</sub> of approximately 12  $\mu$ M; 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 about 24% of T-ALL patients and negatively correlates with the achievement of complete remission (Tafari et al., 2002; Vitale et al., 2006a). A443654 also induced a significant amount of apoptosis in all six T-ALL patient samples.

MOL #47639

A443654 induced cell cycle arrest in the G<sub>2</sub>/M phase followed by subsequent apoptosis in T-ALL cell lines. It is interesting that cells were arrested in G<sub>2</sub>/M as AKT is often thought of as a kinase responsible for entering into S-phase, but AKT has been demonstrated to have additional targets which control entry into G<sub>2</sub> and subsequent entry into the M-phase of the cell cycle (Katayama et al., 2005; Okumura et al., 2002). The observed G<sub>2</sub>/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 prior to A443654. Addition of A443654 prior to etoposide had an additive/antagonistic effect. The fact that A443654 arrests pre-treated cells in G<sub>2</sub>/M, may preclude any response to etoposide, a drug that requires DNA to be replicating in order to have its cytotoxic effects (D'Arpa et al., 1990; Markovits et al., 1987). Moreover, as caspase-2 was the first caspase observed to be cleaved following treatment with A443654, the G<sub>2</sub>/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- $\delta$  cleavage. Interestingly, it has been reported that, besides caspase-3, also caspase-2 could directly cleave PKC- $\delta$  (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 (not shown).

It was previously reported that A443654 induces rapid phosphorylation of AKT on S473 and induces a rapid dephosphorylation of the AKT substrate, GSK-3 $\beta$  (Han et al., 2007; Luo et al., 2005). The phosphorylation of S473 was reportedly elicited through the rapamycin insensitive mTOR:Rictor complex. We found that, in addition to dephosphorylation of GSK-3 $\beta$  and phosphorylation of AKT at position S473, T308 phosphorylation was rapidly induced by A443654. A443654 induced phosphorylation of T308 was not significantly inhibited by the PI3K/mTOR inhibitors, wortmannin, LY294002, and PI-103, or downregulation of PDK1 through siRNA, indicating that membrane recruitment by PIP3 and subsequent phosphorylation by PDK1 were not a

MOL #47639

requirement for A443654-mediated T308 phosphorylation. In contrast, phosphorylation of S473 was partially inhibited by LY294002, wortmannin or PI-103 when used at or above the IC<sub>50</sub> for PI3K but was greatly inhibited when these compounds were used at or above the IC<sub>50</sub> for mTOR, suggesting that, in Jurkat cells, some S473 phosphorylation may not be completely dependent on mTOR:Rictor, but a majority is. The phosphorylation of AKT following addition of A443654 may imply AKT functions as a stress/ATP indicator. A443654 functions as an AKT ATP-binding site analogue (Han et al., 2007; Luo et al., 2005), 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 O<sub>2</sub> or a sugar source) or following mitochondrial damage resulting in AKT phosphorylation. Such stress induced AKT phosphorylation has been reported recently to be dependent on eIF2 $\alpha$  phosphorylation following PKR or PERK activation (Kazemi et al., 2007). Further analysis is needed to identify the kinase(s) responsible for stress-induced AKT phosphorylation.

A443654-induced GSK-3 $\beta$  dephosphorylation despite concomitant hyperphosphorylation of AKT, could be due to the fact that 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 relocalization 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, our unpublished results have revealed that immunoprecipitated AKT from A443654 cells actually increased phosphorylation (and hence inhibition) of recombinant GSK-3 $\beta$  in an in vitro assay. Thus, this finding could support the hypothesis of Han and coworkers, as immunoprecipitated AKT would be presumably freed from interactions with A443654 (Han et al., 2007). The occurrence of in vivo inhibition of GSK-3 $\beta$  by A443654, was demonstrated by the effects of concomitant incubation with LiCl, which resulted in a mitigation of the drug-induced cytotoxicity.

MOL #47639

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 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 following treatment with A443654 (data not shown). In conclusion, our pre-clinical studies support the concept that inhibition of Akt signaling may have clinical application for treatment of T-ALLs. From these data it is conceivable that A443654, or other Akt inhibitors, may serve as an efficient therapeutic agents to combat T-ALLs which 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.

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MOL #47639

## FOOTNOTES

A) \*These authors contributed equally to this manuscript.

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MOL #47639

## Figure Legends

**Figure 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 3 experiments  $\pm$  sd. 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  $\mu$ M, A443654 did not decrease cell viability with respect to untreated cells (unpublished results). Therefore, cell survival values at 0.01  $\mu$ M A443654 have been set as 100%. *B*, Jurkat cells were treated with 10, 5, 1, 0.5 or 0.1  $\mu$ M A443654, or corresponding DMSO concentrations for 24 h. Cell cycle analysis was performed, and the percentage of cells in sub-G<sub>1</sub>/G<sub>0</sub> (dead cells with fragmented DNA) were determined. Bars are the averages of 3 experiments  $\pm$  sd. *C*, CEM-S and CEM-R cells were treated with DMSO (control, CTRL) or 1  $\mu$ M A443654 for 24 and 48 h. The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining. Lower Left quadrant=viable cells (Annexin V-FITC/PI negative), Lower Right quadrant=early apoptotic cells (Annexin V-FITC positive only), Upper Right=mid-late apoptotic cells (Annexin V-FITC/PI double-positive), and Upper Left=late apoptotic/necrotic (PI positive only). Percentages of cells in each quadrant are indicated with the exception of the Lower Left quadrant (viable cells). Occasionally, following treatment, some cells which were strongly PI positive/Annexin V-FITC negative would skew to the left most part of the Upper Left quadrant; the dot points for these cells are not visible but are included in the percentages indicated. One representative of three different experiments which gave similar results is shown.

**Figure 2. A443654 induces a dose-dependent and rapid phosphorylation of AKT on T308 and S473 and dephosphorylation of GSK-3 $\beta$  in T-ALL cell lines.** *A*, Jurkat cells were treated for 24 h in the presence of 0.1, 0.5 or 1  $\mu$ M A443654; CEM-S and CEM-R cells were treated with 1  $\mu$ M and 10  $\mu$ M A443654, respectively for 0.5, 2, and 4 h. Fifty  $\mu$ g of each lysate were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membrane. *B*, Jurkat cells were pre-

MOL #47639

treated for 1 h with LY294002 (1.5  $\mu$ M), wortmannin (100 nM), PI-103 (50 nM). Cells were then treated with 1  $\mu$ M A443654 for 2 or 4 h. Cells were collected and lysed, and 50  $\mu$ 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 non-specific (scramble) siRNA and cultured an additional 72 h. Cells were then treated with 1  $\mu$ M A443654 for 2 h. Cells were collected, lysed, and 50  $\mu$ g of each lysate were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membrane.  $\beta$ -actin served as loading control.

*D*, Jurkat cells, either pre-treated or not for 2 h with caspase-3 inhibitor (10  $\mu$ M), were incubated for the indicated times with 1  $\mu$ M A443654. Lysate (50  $\mu$ g) was electrophoresed on a SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with antibodies to AKT or to  $\beta$ -actin. CTRL: untreated cells. *E*, Jurkat and CEM-S cells were left untreated (0 h) or treated with 1  $\mu$ M A443654 for 2 and 4 h. Lysate (50  $\mu$ g) was immunoprecipitated with anti-AKT antibody and Protein A/G agarose. The washed immunoprecipitate was electrophoresed on a SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with anti-AKT or anti-caspase-3 antibody. The position of molecular weight markers is indicated at left. For all the blots, protein levels were quantified by using the Image J software as described in Materials and Methods. Band intensities of control was normalized to 1, and treated samples were expressed as fraction of control. Since in the blots for p-T308 AKT levels in CEM-S and CEM-R cells, there was no band in the control, quantification was not performed.

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

MOL #47639

**Figure 4. A443654 induces activation of caspases-2, -3, -6, -8, and -9 in Jurkat cells.** *A*, Jurkat cells were treated with 1  $\mu$ M A443654 for the indicated times, collected, and then lysed. Fifty  $\mu$ g of each lysate were electrophoresed on SDS-PAGE gels followed by transfer to nitrocellulose membrane. Antibody to  $\beta$ -actin served as a loading control. *B*, Jurkat cells which had been treated with 1  $\mu$ 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 AlexaFluor488 conjugate antibody. Black shaded histogram: DMSO-treated cells; grey shaded histogram: A443654-treated cells. *C*, Jurkat cells, either pre-treated or not for 2 h with caspase-2 inhibitor (10  $\mu$ M), were treated with 1  $\mu$ M A443654 for the indicated times, and activation of caspases was evaluated by western blot. Antibody to  $\beta$ -actin served as a loading control. *D*, Jurkat cells, either pre-treated or not with caspase-2 inhibitor (10  $\mu$ M) were incubated with 1  $\mu$ M A443654 for the indicated times, then lysed, and immunoblotted for PKC- $\delta$ . In *A*, *C*, and *D* the position of molecular weight markers is indicated at left. *E*, Flow cytometric analysis of Annexin V-FITC/PI- stained Jurkat cells, treated with 1  $\mu$ M A443654, with or without caspase-2 inhibitor (10  $\mu$ M) pre-incubation. CTRL: untreated cells.

**Figure 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  $\pm$  sd. Similar experiments were performed where cells were pre-treated 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 sub-lethal doses of A443654 (25 nM and 3  $\mu$ M for CEM-S and CEM-R, respectively; sub-lethal=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  $\pm$  sd.

MOL #47639

**Figure 6. T-ALL patient samples have elevated levels of p-AKT and are susceptible to A443654-mediated cell death.** *A*, Jurkat cells, normal lymphocytes, and T-ALL patient samples were analyzed by flow cytometry for the levels of p-S473 AKT and PTEN. Two representative patient samples and the controls (Jurkat and healthy donor peripheral blood lymphocytes), are shown. For p-S473 AKT expression, black shaded histograms: negative control (irrelevant antibody); grey shaded histograms: p-S473 AKT positive cells. For PTEN expression in Jurkat and healthy donor lymphocytes, black shaded histogram: negative control (irrelevant antibody); grey shaded histograms: PTEN positive cells. For PTEN expression in patient samples, black shaded histogram corresponds to leukemic lymphoblasts. *B*, p-S473-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  $\mu$ M A443654 or DMSO (control, CTRL) for 72 h. Cell cycle analysis was then performed as described in Materials and Methods. The percent of cells in the sub-G<sub>1</sub>/G<sub>0</sub> fraction were plotted. Three representative patient samples are shown. *D*, Patient samples were treated with 1  $\mu$ M A443654 or DMSO for 72 h, fixed, permeabilized, immunostained for cleaved caspase-3, then analyzed by flow cytometry. Dark shaded histograms: DMSO-treated samples; grey shaded histograms: cleaved caspase-3 positive cells. *E*, Dot plot of leukemic lymphoblasts double-positive for cleaved caspase-3 and p-S473 AKT. Samples were treated with DMSO or 1  $\mu$ M A443654 for 72 hr. Lymphoblasts were fixed, permeabilized, doubly immunostained with AlexaFluor 488 conjugate anti-cleaved caspase-3 antibody and AlexaFluor 647 conjugate anti-p-S473 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.

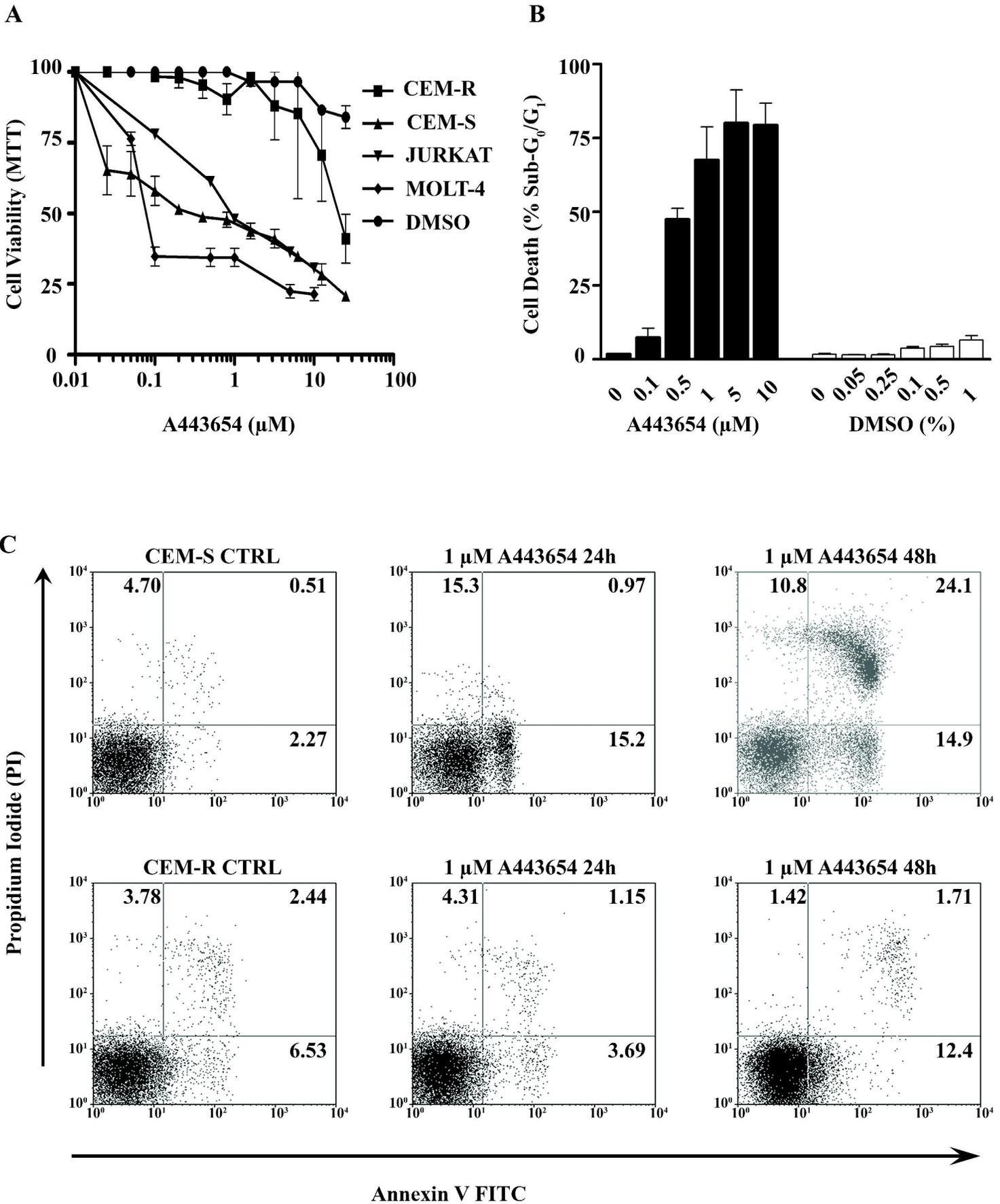
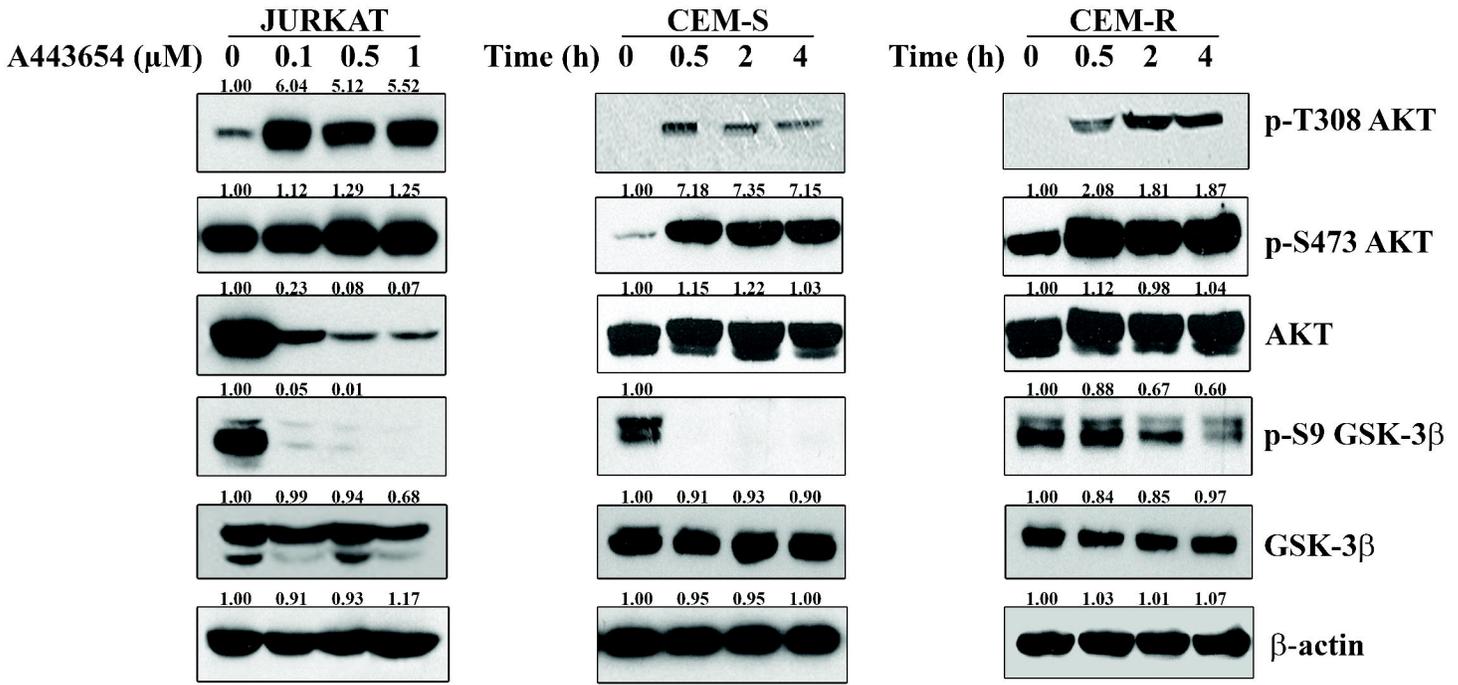
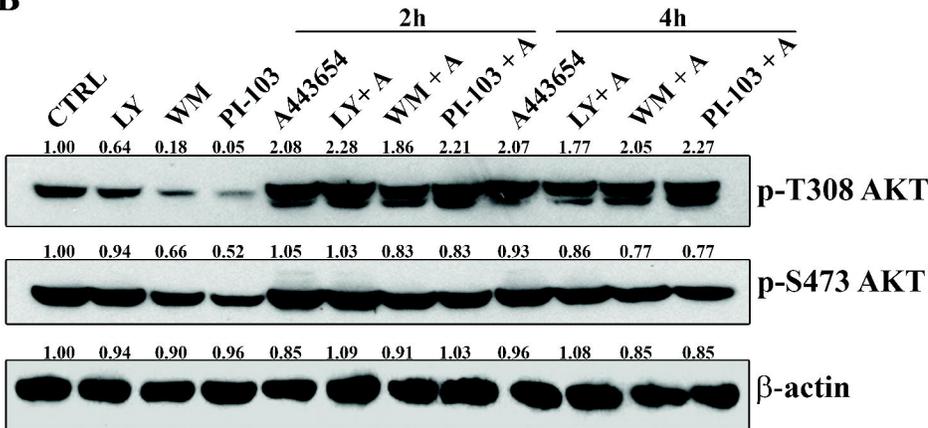


FIGURE 1

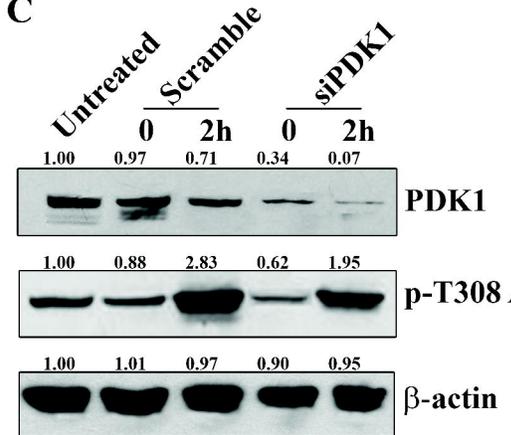
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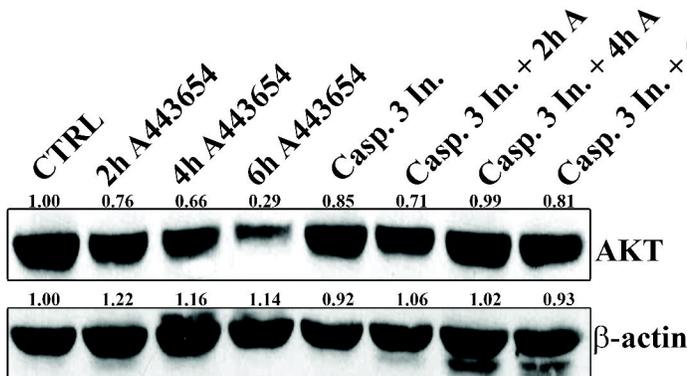
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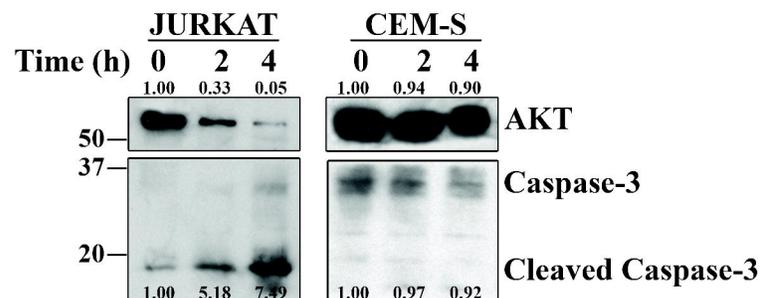


FIGURE 2

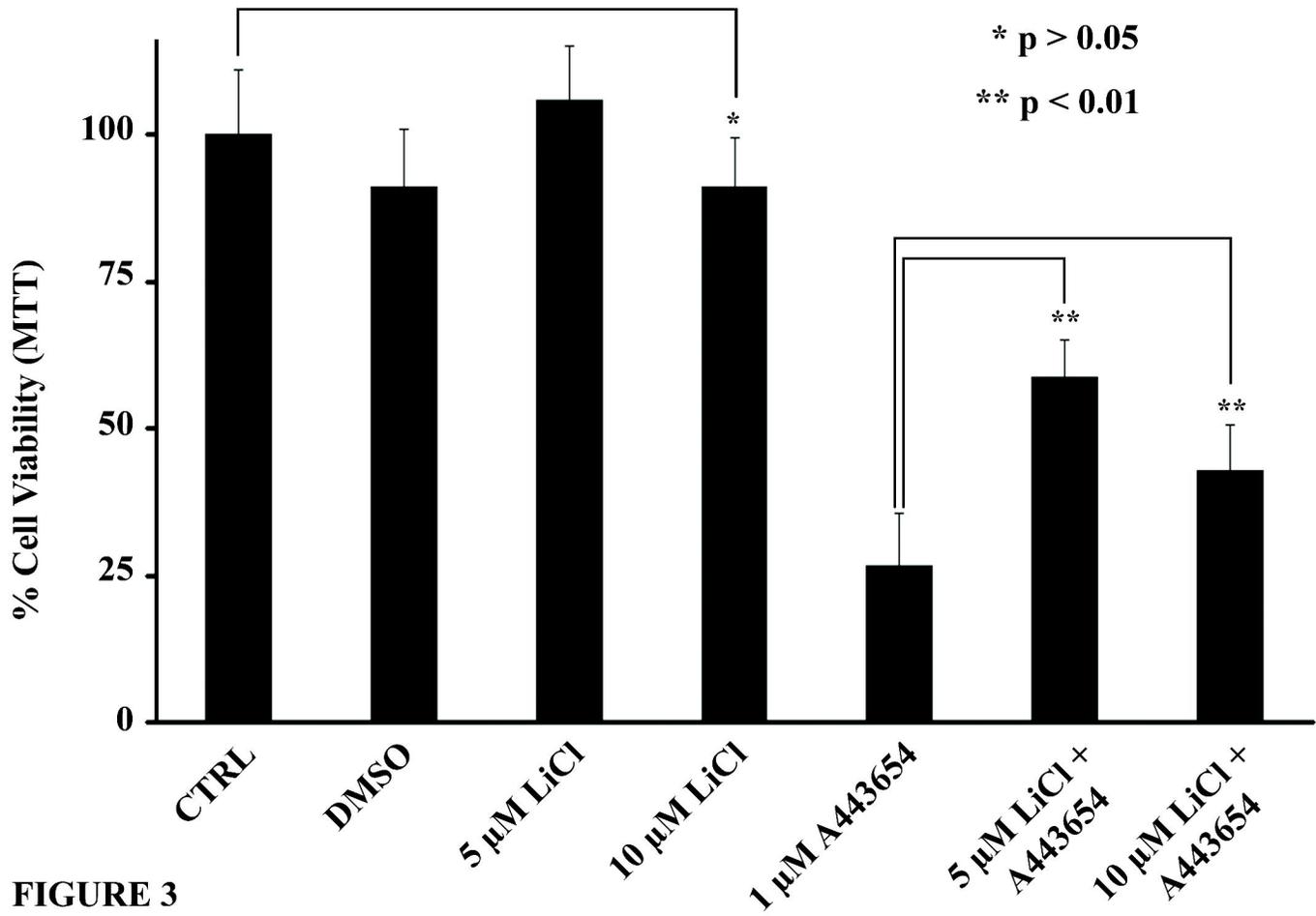
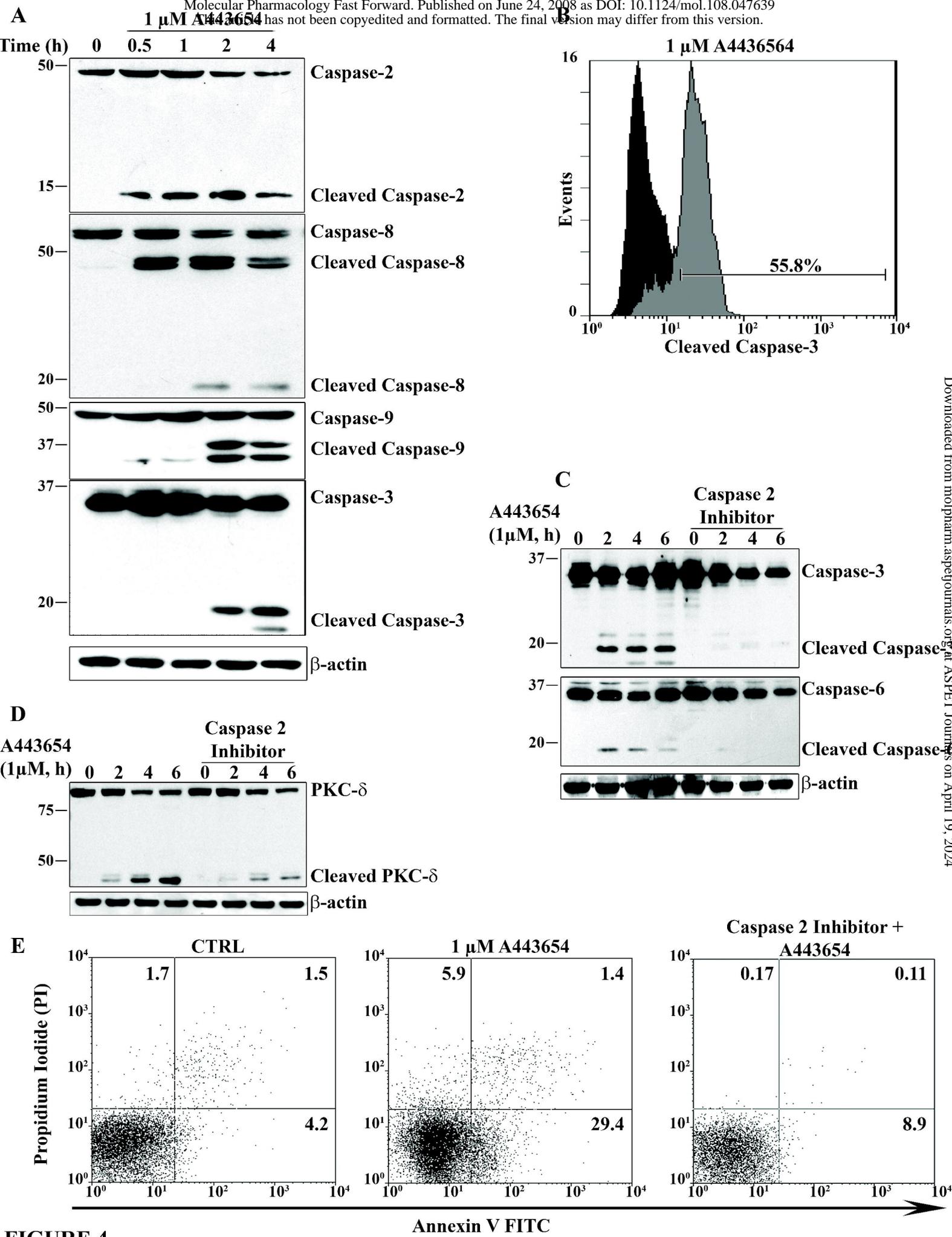
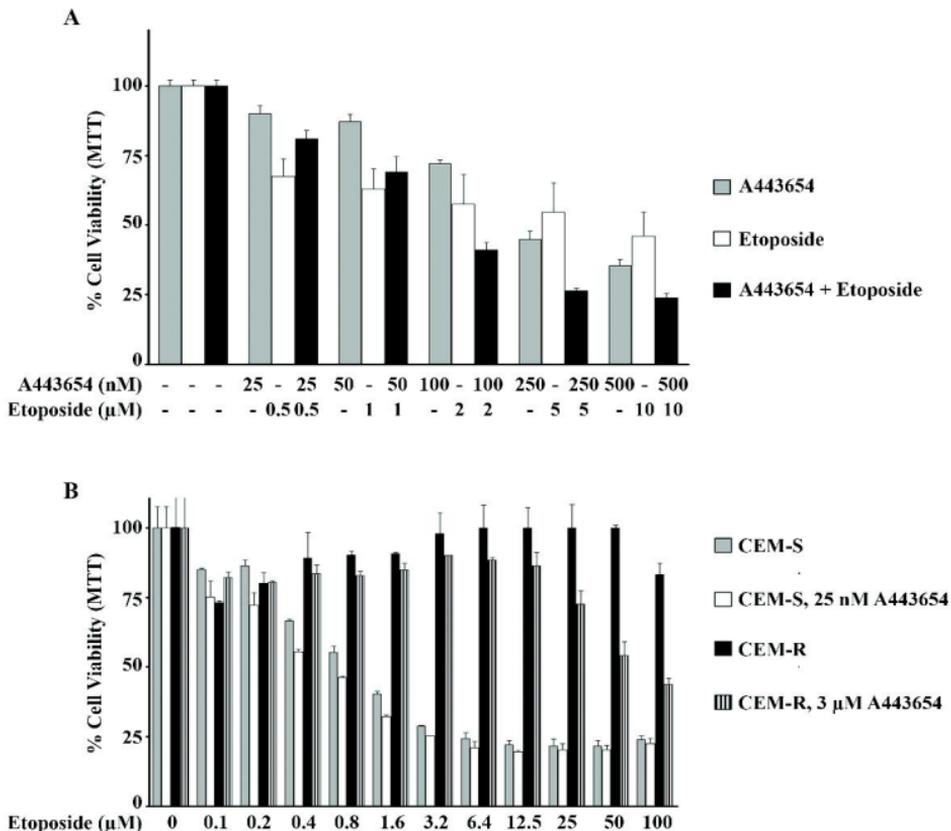


FIGURE 3



**FIGURE 4**



**FIGURE 5**

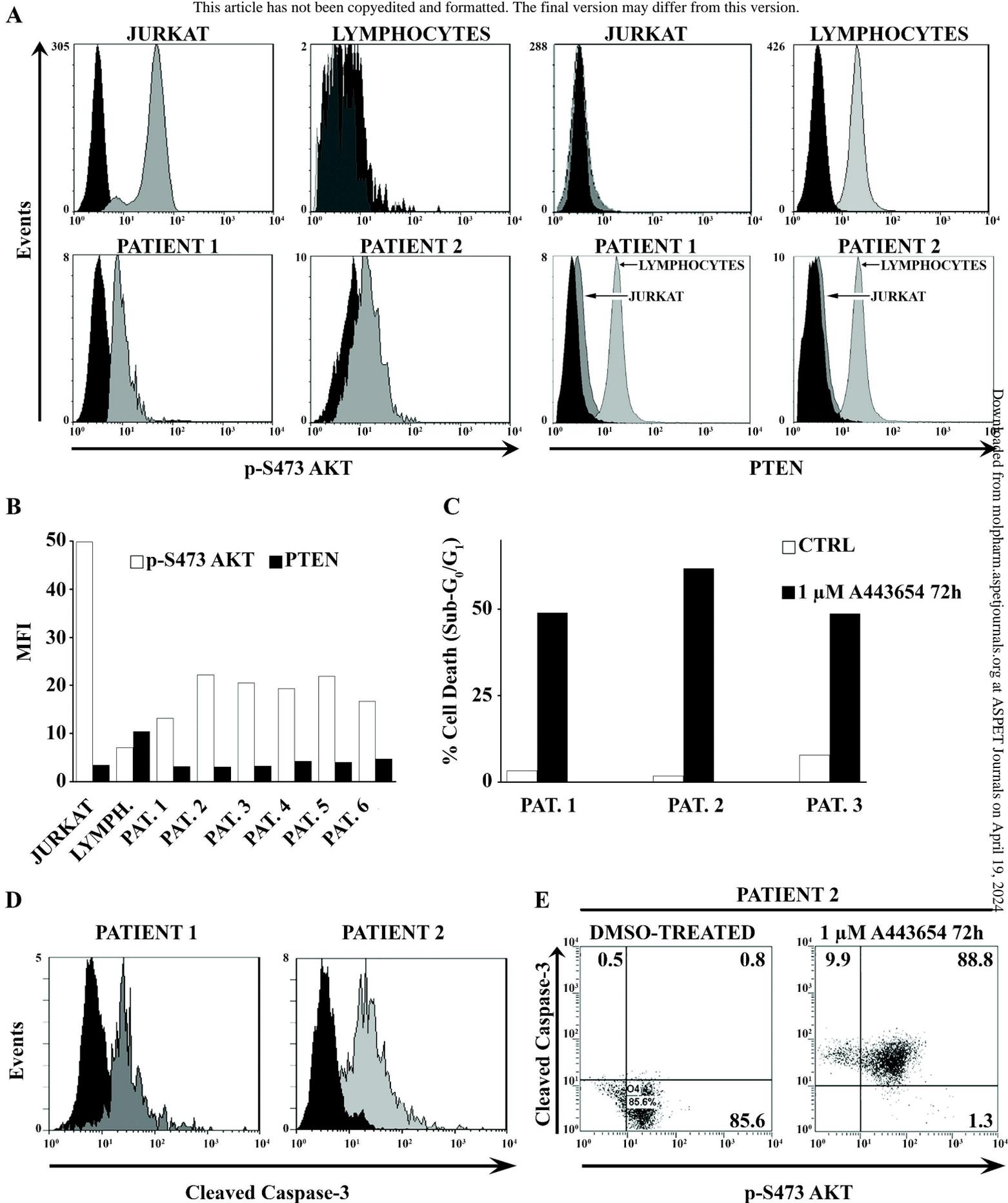


FIGURE 6