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**The dual specificity PI3K/mTOR inhibitor PKI-587 displays efficacy against T-cell
acute lymphoblastic leukemia (T-ALL)**

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Abstract

Although significant improvements have been made in the treatment of acute lymphoblastic leukemia (ALL), there is a substantial subset of high-risk T-cell ALL (T-ALL) patients with relatively poor prognosis. Like in other leukemia types, alterations of the PI3K/mTOR pathway are predominant in ALL which is also responsible for treatment failure and relapse. In this study, we show that relapsed T-ALL patients display an enrichment of the PI3K/mTOR pathway. Using a panel of inhibitors targeting multiple components of the PI3K/mTOR pathway, we observed that the dual-specific PI3K/mTOR inhibitor PKI-587 was the most selective inhibitor for T-ALL cells dependent on the PI3K/mTOR pathway. Furthermore, we observed that PKI-587 blocked proliferation and colony formation of T-ALL cell lines. Additionally, PKI-587 selectively abrogated PI3K/mTOR signaling without affecting MAPK signaling both in *in vitro* and *in vivo*. Inhibition of the PI3K/mTOR pathway using PKI-587 delayed tumor progression, reduced tumor load and enhanced the survival rate in immune-deficient mouse xenograft models without inducing weight loss in the inhibitor treated mice. This preclinical study shows beneficial effects of PKI-587 on T-ALL that warrants further investigation in the clinical setting.

HIGHLIGHTS

- Pediatric relapsed T-ALL patients display upregulation of PI3K/mTOR pathway.
- The dual specificity PI3K/mTOR inhibitor PKI-587 is a potent inhibitor of T-ALL cells growth.
- The dual specificity PI3K/mTOR inhibitor PKI-587 inhibited *in vitro* colony formation.
- PKI-587 delayed tumor formation and extended survival in a T-ALL xenograft model.
- PKI-587 is a selective inhibitor of the PI3K/mTOR pathway both *in vitro* and *in vivo*.

Keywords: Leukemia; PI3K/mTOR; Gedatolisib; PF 05212384; T-ALL

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a neoplasm of immature lymphoid progenitors. ALL is the most common form of childhood leukemia that accounts for almost 30% of all childhood cancers [1]. T-cell acute lymphoblastic leukemia (T-ALL) is a subset of ALL affecting lymphoblast of the T-cell lineage. T-ALL represents for 15% of pediatric and 25% of adult ALL cases [2]. With risk-oriented chemotherapy and supportive care, 70-80% of children and 40% of adult ALL patients can reach long-term remission [3]. Despite significant improvements in cure rates, the survival of T-ALL patients with chemoresistant or relapsed leukemia is still very low [4]. This is mainly due to the insufficient understanding of T-ALL biology which may impede the identification of suitable prognostic factors for proper delivery of treatment.

Inhibitors of PI3K/mTOR pathway components have shown promising results in different cancers including hematological malignancies. Initially, rapamycin and its analogs were used for ALL treatment. Activation of PI3K/AKT pathway has been detected in 85% of T-ALL patients [5]. Since mTORC1 is a signaling molecule downstream of AKT, the initial treatment of T-ALL with rapamycin was logical. However, several feedback mechanisms are involved in the PI3K/mTOR signaling pathway [6]. Therefore, inhibitors targeting a single component of the pathway might not block oncogenic survival signaling. For example, activation of p70S6K by mTORC1 induces insulin receptor substrate-1 (IRS-1) phosphorylation and, thereby, dissociation of IRS-1 from its receptor. mTORC1 also phosphorylates GRB10 which leads to suppression of insulin signaling [7]. Furthermore, assembly of the mTORC2 complex is prevented by p70S6K-mediated phosphorylation of SIN1. Therefore, inhibition of mTORC1 by rapamycin (mTORC2 is insensitive to transient rapamycin treatment) can potentiate PI3K/AKT activation by removing a negative feedback control. Inhibition of both complexes through mTOR-specific inhibitors potently reduces AKT Ser⁴⁷³ phosphorylation

but, due to the feedback loop, Thr³⁰⁸ phosphorylation mediated by PDK1 is enhanced. Thus, an inhibitor with dual specificity against PI3K and mTOR provides better inhibitory capacity compared to targeting a single component of this pathway [8].

Advances in the structural and biochemical understanding of PI3K enzymes have over the last few decades enabled the development of various targeted inhibitors of the PI3K/AKT/mTOR pathway [9]. The Wyeth PI3K inhibitor discovery project identified an outstanding potent, selective, ATP-competitive, and reversible PI3K/mTOR inhibitor, PKI-587, for clinical development [10]. Preclinical data suggest its utility in the treatment of cancers with elevated PI3K/mTOR signaling and it has already exhibited effective antitumor activities against several cancers including breast, colon, glioma, and non-small cell lung cancer in *in vivo* xenograft models, which places it among the most potent dual specificity PI3K/mTOR inhibitors reported to date [11].

In this study, we analyzed the preclinical efficacy and therapeutic potential of PKI-587 on T-ALL cell line *in vitro* and on an *in vivo* mouse model. We observed strong cytotoxic activity against T-ALL cell lines and inhibition of PI3K/mTOR delayed tumor progression, reduced tumor volume and prolonged survival in a T-ALL xenograft mouse model.

MATERIALS AND METHODS

Reagents, antibodies and inhibitors: Horseradish peroxidase (HRP)-coupled secondary anti-mouse and anti-rabbit antibodies were from Thermo Fisher Scientific (Waltham, MA). Rabbit anti-phospho-AKT (pSer473), rabbit anti-phospho-ERK1/2 (pThr202/pThr204), goat anti-AKT, rabbit anti-ERK2, anti-rabbit secondary and anti-goat secondary antibodies were from Santa Cruz Biotechnology (Dallas, TX). Mouse anti-phospho-p38 and anti-p38 antibodies were from BD Transduction Laboratories (Franklin Lakes, NJ). All inhibitors were from Selleck Chemicals (Houston, TX).

108 **Drug preparation:** For *in vitro* studies, PKI-587 was dissolved in DMSO at 5 mM
109 concentration and stored at -80°C. Before starting the experiments, aliquots were thawed and
110 diluted to the desired concentrations in the corresponding medium. In the case of *in vivo*
111 studies, PKI-587 was dissolved in vehicle (5% dextrose in water and 0.3% lactic acid, pH
112 3.5). Freshly prepared inhibitor solutions were always used in both the *in vitro* and *in vivo*
113 experiments.

114 **Cell culture:** All cell lines were obtained from Deutsche Sammlung von Mikroorganismen
115 und Zellkulturen (DSMZ, Braunschweig, Germany). CCRF-CEM, MOLT3, and Jurkat cells
116 were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100
117 units/ml penicillin and 100µg/ml streptomycin.

118 **Cell lysis and western blotting:** CCRF-CEM and MOLT3 cells were washed with cold PBS
119 and then treated with increased concentrations of PKI-587 for 4 hours in RPMI-1640. Cells
120 were then washed and lysed in 1% Triton X-100 lysis buffer. The Western blotting and
121 immunodetection procedures were described earlier [12].

122 **Cell viability:** CCRF-CEM and MOLT3 cells grown in RPMI 1640 supplemented with 10%
123 FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were then seeded in 96-well
124 plates (13,000 cells per well). Two days after seeding, cell viability was counted by
125 PrestoBlue (Molecular Probes, Eugene, OR) assay.

126 **Apoptosis:** CCRF-CEM and MOLT-3 cells were washed and seeded in 12-well plates
127 (100,000 cells per well) with increasing concentrations of PKI-587. Two days after seeding,
128 apoptosis was measured by flow cytometry using Annexin V and 7-aminoactinomycin D (7-
129 AAD) apoptosis kit (BD Biosciences, Franklin Lakes, NJ).

130 **Peptide-based kinase profiling:** PamGene technology (PamGene, 's-Hertogenbosch, the
131 Netherlands) was used to measure kinase activity of T-ALL cells. Cells were serum-starved
132 overnight before lysis. Five micrograms of total protein were used for tyrosine kinase

profiling and 0.5 µg of total protein was used for serine/threonine kinase profiling using standard protocol provided by PamGene.

Colony formation assay: CCRF-CEM and MOLT3 cells were washed with IMDM and 600 cells were mixed with 500 µl of 80% methylcellulose medium and 20% IMDM. Cells were seeded in 24-well plates with increasing concentrations (0-50 nM) of PKI-587 and incubated for 7 days. After seven-day colonies were visible to the eye and counted by two individual researchers. Picture of each well was taken by using a light microscope.

Xenograft studies: Ten female NOD/SCID nude mice each weighing approximately 20g, (housed by the Laboratory Animal Facilities at Medicon Village, Lund University) were injected subcutaneously with 2 million cells. Mice were then divided into two groups that were either treated with PKI-587 or vehicle. One week after injection of cells, mice were treated twice weekly by intravenous injection of 12 mg/kg PKI-587 or vehicle for additional 20 days. Drug efficacy was monitored by checking the tumor growth in both groups and by measuring the body weight of the mice regularly. After the size of the tumors had reached about 1 cm³, mice were sacrificed. For analysis of signaling proteins, tumors excised and rapidly transferred to liquid nitrogen. Another group of female NOD/SCID nude mice were injected intravenously through the tail vein with 1 million cells and divided into two groups that were either treated with PKI-587 or vehicle. Ten days after injection of cells, treatment of mice was commenced by intravenous injection of 12 mg/kg of PKI-587 or vehicle twice weekly. Engraftment was checked by measuring the number of circulating CD45⁺ cells. Mice were euthanized when any signs of suffering such as difficulty in breathing, lack of movement, or paralysis observed. All animal experiments were performed under an ethical permit from the Swedish Animal Welfare Authority.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 5.0. One way ANOVA was used where needed.

RESULTS

Pediatric relapsed acute lymphoblastic leukemia patients display an enrichment of the PI3K/mTOR pathway:

To determine the dependency of the signaling pathways in T-ALL cells, we analyzed two different gene expression datasets (GSE13576 and GSE14618) from T-ALL patients. We compared gene set enrichment using GSEA between one group of patients that relapsed after treatment and one that got complete remission [13-15]. We observed that patients who had relapsed during disease progression displayed an enrichment of the PI3K/mTOR pathway (Fig. 1A and 1B). To follow up on the GSEA results, we used the patient-derived T-ALL cell lines Jurkat, MOLT3 and CCRF-CEM. All three cell lines displayed strong activation of the PI3K/mTOR pathway as we observed constitutive phosphorylation of AKT-Ser-473, AKT-Thr-308 and S6K-Thr-389 (Fig. 1C). Additionally, Jurkat cells show strong activation of the ERK pathway (Fig. 1D). Thus, it is likely that while MOLT3 and CCRF-CEM cells are dependent on the PI3K/mTOR pathway, Jurkat cells utilize additional survival signaling pathways. Furthermore, peptide-based kinase profiling assays suggested that while S6K was highly activated in MOLT3 cells, many tyrosine kinases were active in Jurkat cells (Fig. 2).

The dual PI3K/mTOR inhibitor PKI-587 is a potent inhibitor of T-ALL cells growth:

Since we observed that relapsed ALL patients display upregulation of the PI3K/mTOR pathway, we decided to use a panel of inhibitors that target different components of the PI3K/mTOR pathway. We used 88 different inhibitors targeting AKT, AMPK, ATM/ATR, ATM/ATR and mTOR, GSK-3, mTOR, PDK-1, PI3K, PI3K and DNA-PK, PI3K, mTOR, PKA and S6K. As Jurkat cells display upregulation of both the PI3K/mTOR and MAPK pathways, we speculated that selective PI3K/mTOR pathway inhibitors would not block the

cell viability of Jurkat cells and thus could be used as a control. We compared cell viability between Jurkat and CCRF-CEM cells in the presence of 88 different inhibitors in two different concentrations, 100 nM (Fig. 3A) and 1000 nM (Fig. 3B). We observed that while most of the inhibitors displayed a linear correlation between the two cell lines, PKI-587 was the most selective inhibitor for CCRF-CEM cells. Additionally, PKI-587 displayed an IC₅₀ of 25 nM for MOLT3 cells (Fig. 3C), 23 nM for CCRF-CEM cells (Fig. 3D) and 73 nM for Jurkat cells (Fig. 3E).

Cytokines secreted from bone marrow HS-5 cells have no effect on inhibition of cell growth by PKI-587:

Cytokines/chemokines/growth factors secreted by the bone marrow microenvironment can drive the drug resistance and survival of leukemic blasts [16]. In order to investigate whether PKI-587 can still efficiently block cell growth in the presence of micro-environmental factors, we assessed the relative viability of MOLT3 and CCRF-CEM cells in the presence or absence of conditioned medium from HS5 cell cultures with increasing concentrations of inhibitor. We observed that the presence or absence of conditioned medium from HS5 cells did not affect the ability of PKI-587 to induce growth inhibition in MOLT3 (Fig. 4A) and CCRF-CEM (Fig. 4B) cells.

The dual specificity PI3K/mTOR inhibitor PKI-587 reduces viability and colony formation of T-ALL cells:

We used different biological assays to evaluate the effect PKI-587 on T-ALL cells. We observed a dose-dependent inhibition of cell growth of both MOLT3 (Fig. 5A) and CCRF-CEM (Fig. 5B) cell lines. Colony formation assays in semi-solid medium also indicated a marked decrease in the number of colonies (Fig. 5C and 5D) as well as the size of the

colonies (Fig. 5E and 5F) with the increasing concentration of PKI-587 on both MOLT3 and CCRF-CEM cells. Additionally, treatment with PKI-587 induced apoptosis in a dose-dependent manner in these cell lines (data not shown). These results demonstrate an effective inhibitory action of PKI-587 on T-ALL cell lines.

PKI-587 delays tumor formation and extended survival in T-ALL xenograft models:

Based on the potent inhibitory efficacy of PKI-587 on the PI3K/mTOR pathway in T-ALL cell lines *in vitro*, we next assessed its therapeutic efficacy in preclinical mouse models. We first developed a mouse xenograft model using immunodeficient mice. We subcutaneously injected only CCRF-CEM cells since it has been reported that MOLT3 cells are unable to induce tumors *in vivo* by themselves [17]. Seven days after cell injection, mice were treated with 12 mg/kg body weight of PKI-587, or vehicle, for an additional 20 days. Treatment with PKI-587 led to a marked inhibition of tumor growth compared to vehicle treatment (Fig. 6A), as well as to a delay in tumor growth in all treated mice (Fig. 6B). This three-week treatment regimen also led to a significant tumor regression in the treated group (Fig. 6C). To assess the survival benefit of this drug, we further developed a mouse model where we injected CCRF-CEM cells intravenously. Ten days after the cells were injected, mice were treated with 12 mg/kg body weight of PKI-587 or vehicle control. Since CD45⁺ cells in the blood circulation indicate the presence of leukocytes and engraftment of the injected cells, we measured those cells in blood circulation in both mice treated with PKI-587 and vehicle controls. As expected, the mice treated with PKI-587 exhibited significantly lower numbers of CD45⁺ cells in the blood circulation compared to the vehicle-treated (control) group (Fig. 6D). The untreated first mouse was euthanized on day 40 with a median survival of 45 days, while PKI-587-treatment led to a significantly higher survival advantage (Fig. 6E). The effects of this *in vivo* treatment were well tolerated since no deceptive decrease in body weight was noticed in

the treated animals (data not shown). To summarize, these *in vivo* data indicate that PKI-587 has a substantial preclinical efficacy in treating T-ALL and warrants further investigation in a clinical setting.

PKI-587 is a selective inhibitor of the PI3K/mTOR pathway both *in vitro* and *in vivo*:

It is a common phenomenon for oncogenic signaling network to exhibit crosstalk between multiple cellular signaling pathways. This has sometimes led to wrong interpretation and overestimation of the role of a single protein in response to drug treatment [18]. PKI-587 has already been shown to be a highly selective inhibitor of the PI3K/mTOR pathway in several cancer types [10] and has been studied in different cell models, animal models and in clinical trials [19, 20]. On the basis of the previous reports and our preliminary data, we further evaluated the effect of PKI-587 on other signaling pathways. While treating the cells with a higher concentration of PKI-587, we observed decreased phosphorylation of AKT and S6K but no effect on ERK1/2 and p38 phosphorylation *in vitro* (Fig. 7A). Similar to the *in vitro* findings, PKI-587 selectively inhibited AKT and S6K phosphorylation *in vivo* (Fig. 7B). These results strongly suggest that PKI-587 is a selective inhibitor of the PI3K/mTOR pathway.

DISCUSSION

Although significant improvement has been observed in overall cure rates of ALL, the outcome of relapsed T-ALL patients still remains a major clinical challenge. This can be attributed to several factors, but it is mainly due to the insufficient understanding of T-ALL biology which impedes the identification of suitable prognostic factors to deliver proper treatment. With the improved cure rate in ALL, current research strategies are more focused

on subsets of patients such as T-ALL with drug resistance or relapsed leukemia in order to identify treatments with the effective outcome.

The PI3K/mTOR pathway plays a critical role in T-ALL pathogenesis since the most common mutations in T-ALL, such as gain-of-function mutations of NOTCH1 or loss-of-function mutation of PTEN, often cause constitutive activation of this pathway [21]. In this study, two different gene expression datasets of relapsed patients also displayed a predominant enrichment of the PI3K/mTOR pathway. Using a panel of 88 different inhibitors which target several different components of this pathway, PKI-587 was found to be the most selective drug that induces apoptosis of T-ALL cell lines that are dependent on the activity of the PI3K/mTOR pathway. An important observation was made, that the inhibitor PKI-587 was effective even in the presence of micro-environmental factors. The use of conditioned medium from the bone marrow HS5 cells had very little or no effect on PKI-587-induced inhibition of cell growth. This is interesting since it has been suggested that the bone marrow microenvironment, presumably through cytokine production, helps to protect leukemic cells from drug-induced death and thereby contributes to therapy resistance. Our findings provide a basis for the use of the dual specificity inhibitor PKI-587 as a promising drug for the treatment of T-ALL. Our biological assays also suggest a strong ability of PKI-578 to block cell proliferation, colony formation and to induce apoptosis of T-ALL cell lines.

Since PKI-587 is a potent ATP-competitive, and reversible PI3K/mTOR inhibitor, it can selectively abrogate the PI3K/mTOR pathway, without affecting MAPK signaling, by inhibiting the activity of PI3K and mTOR along with their downstream effectors AKT and S6K both *in vitro* as well as *in vivo*. Our results are in line with previous findings, in a slightly different context, on the signaling effects of this dual inhibitor on therapy-resistant AML patient with FLT3-ITD mutation [22]. In xenograft models of the T-ALL cell, inhibition of

the PI3K/mTOR pathway led to delayed tumor progression, induction of tumor regression and markedly increased the survival rate. No gross toxicity was observed as no deceptive decrease of body weight was noticed in tumor-bearing or engrafted mice. Our conclusion is that this dose is effective and well tolerated, suggesting an encouraging therapeutic window for the treatment of relapsed T-ALL patients.

In conclusion, we have investigated the beneficial effects of the dual PI3K/mTOR inhibitor PKI-587, both *in vitro* and *in vivo*, for the treatment of relapsed and therapy-resistant T-ALL. Our study has dominantly revealed a preclinical rationale to explore this novel drug to effectively inhibit PI3K/mTOR activated signaling pathway. However, observations of the pathway enrichment and the method to block the signaling pathway with PKI-587 may be a possible therapeutic way to treat relapsed T-ALL patients and thereby minimizing the diverse effects. Since this drug is already in clinical trials for the treatment of other cancers, we suggest that further pre-clinical trials and early phase clinical trial should be undertaken to evaluate the effect of PKI-587 on patients with relapsed T-ALL.

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Conflict of interest:

The authors declare no conflict of interest.

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FIGURE LEGENDS:

Figure 1: T-ALL patients display upregulation of the PI3K/mTOR pathway: (A-B) Gene expression data from GSE13576 and GSE14618 were used for gene set enrichment analysis (GSEA). (C-D) Cell lysates from Jurkat, MOLT3 and CCRF-CEM were used to check phosphorylation of signaling proteins using phospho-specific antibodies.

Figure 2: Peptide-based kinase profiling shows upregulation of PI3K/mTOR pathway in MOLT3 cells. Kinome tree was generated using Kinome-render (<http://bcb.med.usherbrooke.ca/kinomerender.php>) online tool. Red: upregulated kinase activity in MOLT3 cells, Green: upregulated kinase activity in Jurkat cells.

394 **Figure 3:** PKI-587 is a potent inhibitor of T-ALL cells growth: (A-B) A panel of inhibitors
395 (100 nM and 1000 nM concentrations) targeting different components of the PI3K/mTOR
396 pathway was used to measure cell viability using PrestoBlue assay. (C-E). Cell viability was
397 measured using PrestoBlue assay in the presence of increasing concentrations of PKI-587.

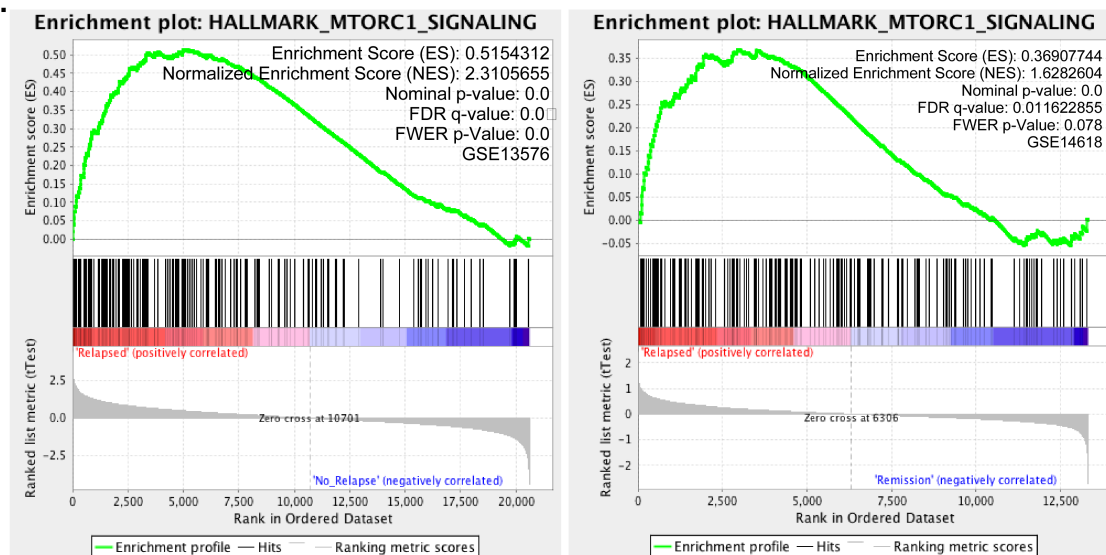
398 **Figure 4:** Conditioned medium from HS5 cells has no effect on PKI-587 induced growth
399 inhibition: (A-B) Cells were treated with increasing concentration of PKI-587 in the presence
400 or absence of conditioned medium from HS5 cells. PrestoBlue viability assay was used to
401 measure cell viability.

402 **Figure 5:** PKI-587 reduces cell viability and colony formation: (A-B) Cells were treated with
403 increasing concentrations of inhibitor. Cell viability was measured using PrestoBlue. (C-F)
404 Cells were mixed with semi-solid medium and inhibitor and seeded in 24-well plates.
405 Colonies were counted after 7 days. ns, not significant; ***, $p < 0.001$.

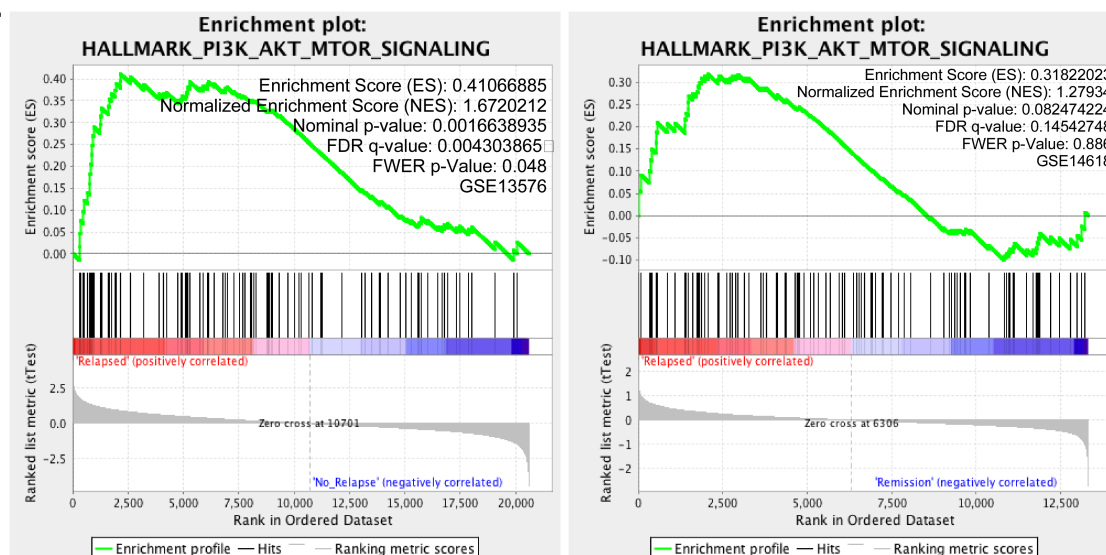
406 **Figure 6:** PKI-587 decreases tumor growth and increases survival: (A) Mice were treated
407 with PKI-587 or vehicle. Tumor volume was measured twice a week. (B-C) Tumors were
408 removed after sacrificing mice. The weight of individual tumors was measured, and the
409 picture was taken in the presence of a scale. (D) CD45+ cells in blood were counted using an
410 antibody against human CD45. (E) Kaplan–Meier curves showed the overall survival of mice
411 transplanted with CCRF-CEM cells.

412 **Figure 7:** PKI-587 selectively inhibits AKT and S6K phosphorylation: (A) Cells were treated
413 with increasing concentrations of PKI-587 before lysis. Cell lysates were used to assess
414 phosphorylation of signaling proteins using phospho-specific antibodies. (B) Tumors from
415 mice treated with either PKI-587 or vehicle were used to assess phosphorylation of signaling
416 proteins using phospho-specific antibodies.

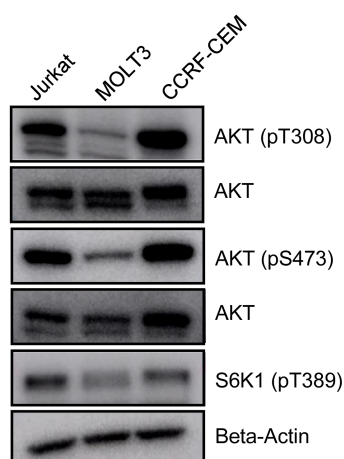
A.



B.



C.



D.

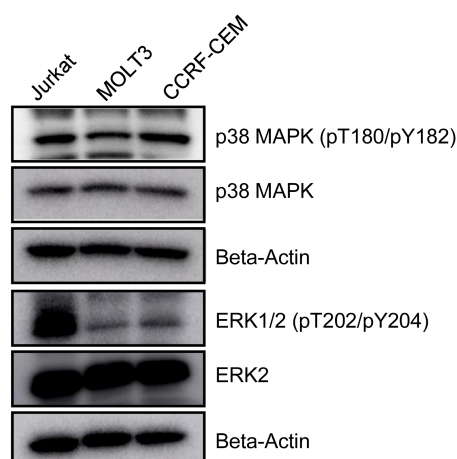


Figure 1

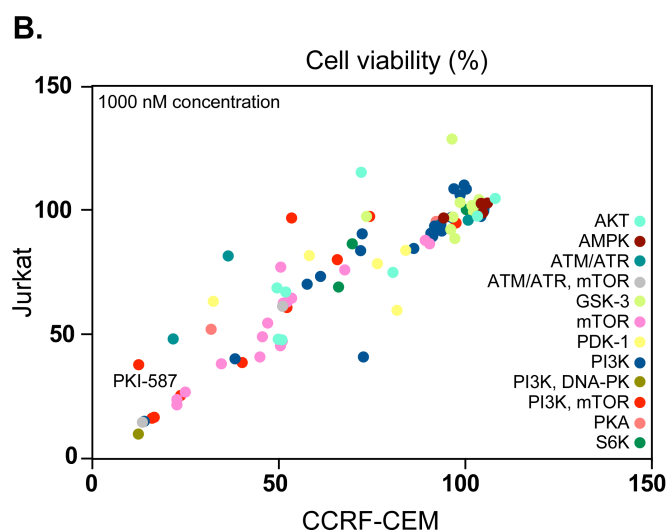
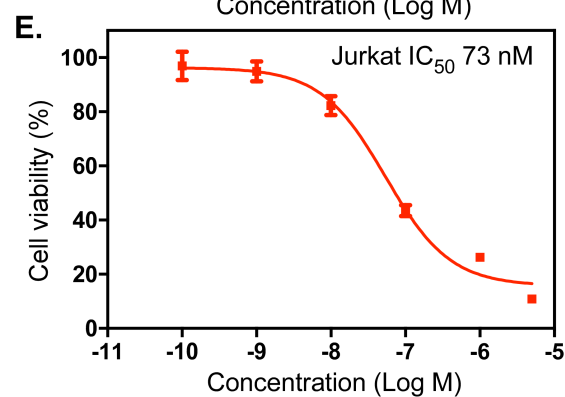
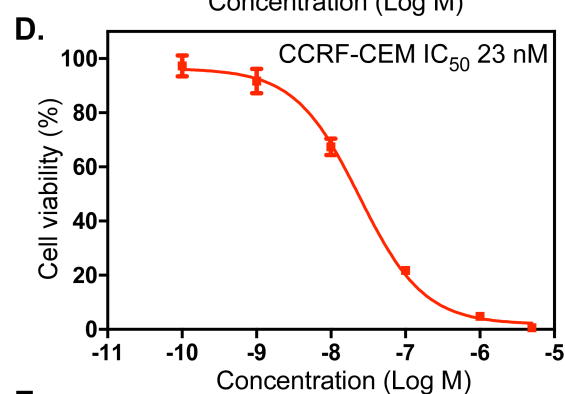
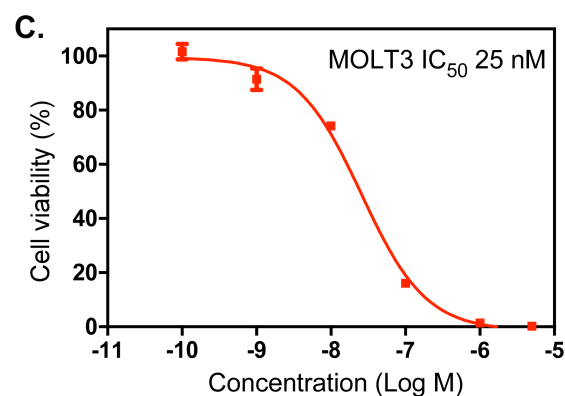
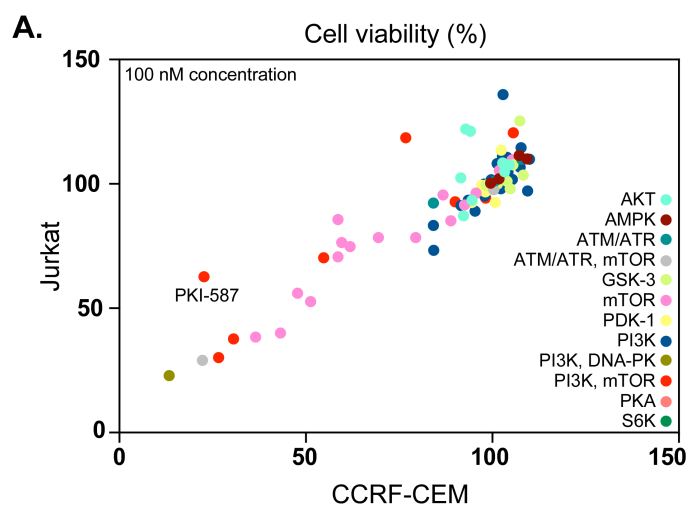


Figure 3

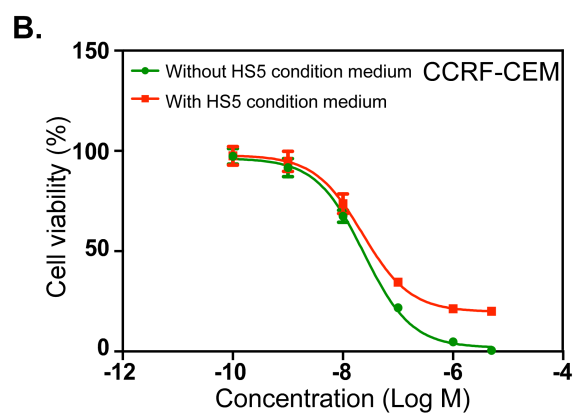
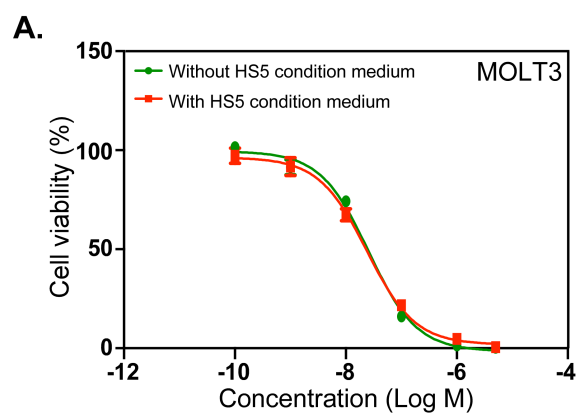


Figure 4

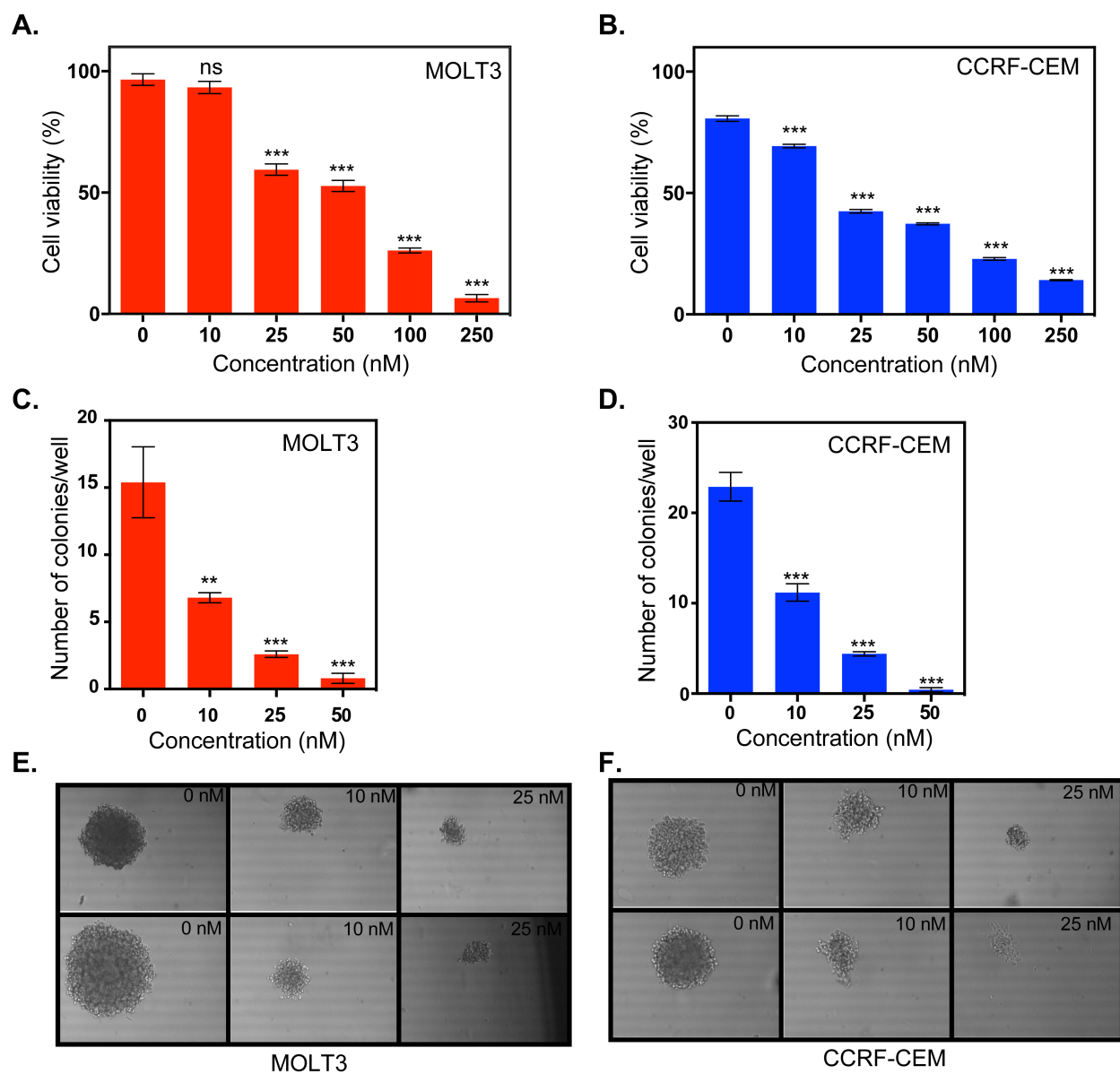


Figure 5

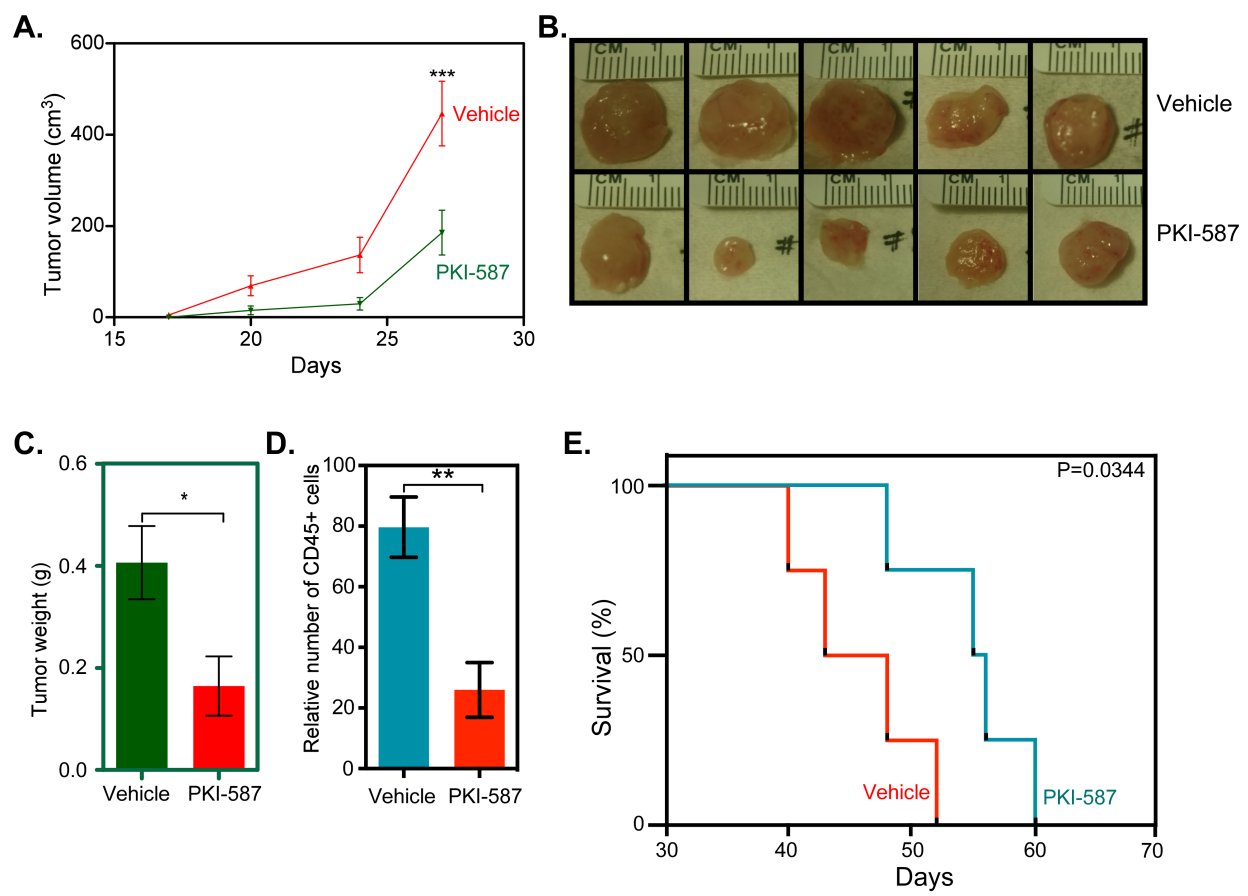


Figure 6

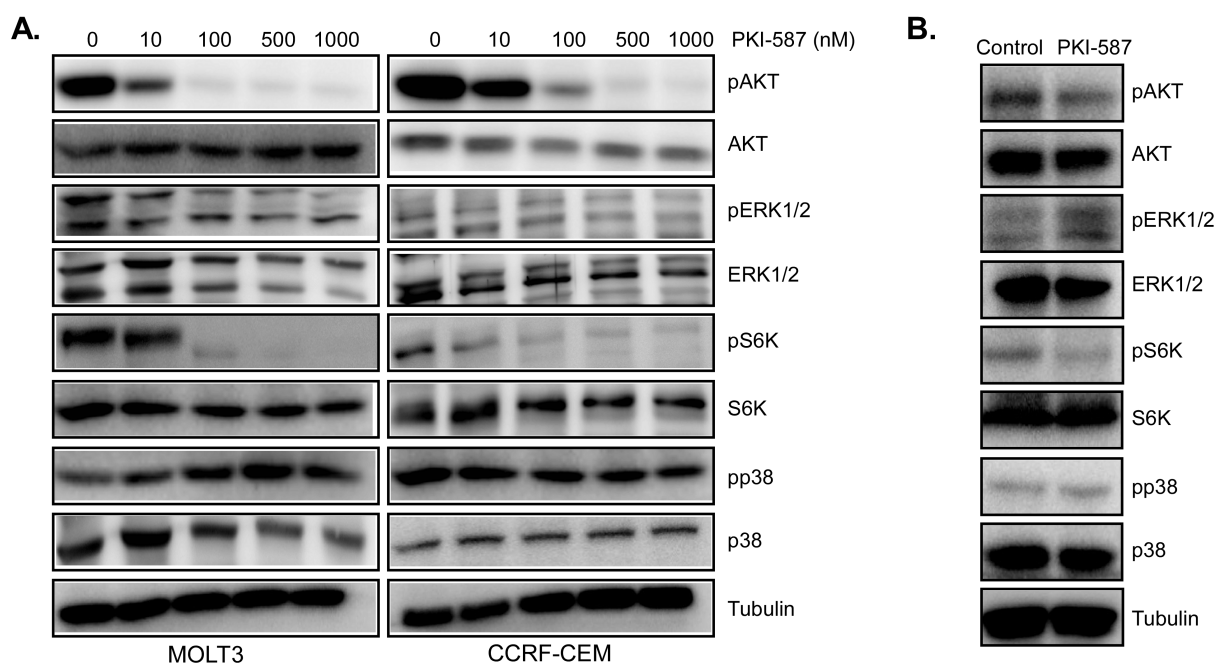


Figure 7