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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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24   **Abstract**

25   Although significant improvements have been made in the treatment of acute lymphoblastic

26   leukemia (ALL), there is a substantial subset of high-risk T-cell ALL (T-ALL) patients with

27   relatively poor prognosis. Like in other leukemia types, alterations of the PI3K/mTOR

28   pathway are predominant in ALL which is also responsible for treatment failure and relapse.

29   In this study, we show that relapsed T-ALL patients display an enrichment of the

30   PI3K/mTOR pathway. Using a panel of inhibitors targeting multiple components of the

31   PI3K/mTOR pathway, we observed that the dual-specific PI3K/mTOR inhibitor PKI-587 was

32   the most selective inhibitor for T-ALL cells dependent on the PI3K/mTOR pathway.

33   Furthermore, we observed that PKI-587 blocked proliferation and colony formation of T-ALL

34   cell lines. Additionally, PKI-587 selectively abrogated PI3K/mTOR signaling without

35   affecting MAPK signaling both in *in vitro* and *in vivo*. Inhibition of the PI3K/mTOR pathway

36   using PKI-587 delayed tumor progression, reduced tumor load and enhanced the survival rate

37   in immune-deficient mouse xenograft models without inducing weight loss in the inhibitor

38   treated mice. This preclinical study shows beneficial effects of PKI-587 on T-ALL that

39   warrants further investigation in the clinical setting.

40

41 **HIGHLIGHTS**

42 ➢ Pediatric relapsed T-ALL patients display upregulation of PI3K/mTOR pathway.

43 ➢ The dual specificity PI3K/mTOR inhibitor PKI-587 is a potent inhibitor of T-ALL

44   cells growth.

45 ➢ The dual specificity PI3K/mTOR inhibitor PKI-587 inhibited *in vitro* colony

46   formation.

47 ➢ PKI-587 delayed tumor formation and extended survival in a T-ALL xenograft model.

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

49

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

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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 Ser^{473} phosphorylation

83 but, due to the feedback loop, Thr^{308} phosphorylation mediated by PDK1 is enhanced. Thus,

84 an inhibitor with dual specificity against PI3K and mTOR provides better inhibitory capacity

85 compared to targeting a single component of this pathway [8].

86 Advances in the structural and biochemical understanding of PI3K enzymes have over the last

87 few decades enabled the development of various targeted inhibitors of the PI3K/AKT/mTOR

88 pathway [9]. The Wyeth PI3K inhibitor discovery project identified an outstanding potent,

89 selective, ATP-competitive, and reversible PI3K/mTOR inhibitor, PKI-587, for clinical

90 development [10]. Preclinical data suggest its utility in the treatment of cancers with elevated

91 PI3K/mTOR signaling and it has already exhibited effective antitumor activities against

92 several cancers including breast, colon, glioma, and non–small cell lung cancer in *in vivo*

93 xenograft models, which places it among the most potent dual specificity PI3K/mTOR

94 inhibitors reported to date [11].

95 In this study, we analyzed the preclinical efficacy and therapeutic potential of PKI-587 on T-

96 ALL cell line *in vitro* and on an *in vivo* mouse model. We observed strong cytotoxic activity

97 against T-ALL cell lines and inhibition of PI3K/mTOR delayed tumor progression, reduced

98 tumor volume and prolonged survival in a T-ALL xenograft mouse model.

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100 **MATERIALS AND METHODS**

101 **Reagents, antibodies and inhibitors:** Horseradish peroxidase (HRP)-coupled secondary

102 anti-mouse and anti-rabbit antibodies were from Thermo Fisher Scientific (Waltham, MA).

103 Rabbit anti-phospho-AKT (pSer473), rabbit anti-phospho-ERK1/2 (pThr202/pThr204), goat

104 anti-AKT, rabbit anti-ERK2, anti-rabbit secondary and anti-goat secondary antibodies were

105 from Santa Cruz Biotechnology (Dallas, TX). Mouse anti-phospho-p38 and anti-p38

106 antibodies were from BD Transduction Laboratories (Franklin Lakes, NJ). All inhibitors were

107 from Selleck Chemicals (Houston, TX).

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

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

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

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

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

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

133 profiling and 0.5 µg of total protein was used for serine/threonine kinase profiling using

134 standard protocol provided by PamGene.

135 **Colony formation assay:** CCRF-CEM and MOLT3 cells were washed with IMDM and 600

136 cells were mixed with 500 µl of 80% methylcellulose medium and 20% IMDM. Cells were

137 seeded in 24-well plates with increasing concentrations (0-50 nM) of PKI-587 and incubated

138 for 7 days. After seven-day colonies were visible to the eye and counted by two individual

139 researchers. Picture of each well was taken by using a light microscope.

140 **Xenograft studies:** Ten female NOD/SCID nude mice each weighing approximately 20g,

141 (housed by the Laboratory Animal Facilities at Medicon Village, Lund University) were

142 injected subcutaneously with 2 million cells. Mice were then divided into two groups that

143 were either treated with PKI-587 or vehicle. One week after injection of cells, mice were

144 treated twice weekly by intravenous injection of 12 mg/kg PKI-587 or vehicle for additional

145 20 days. Drug efficacy was monitored by checking the tumor growth in both groups and by

146 measuring the body weight of the mice regularly. After the size of the tumors had reached

147 about 1 cm^3, mice were sacrificed. For analysis of signaling proteins, tumors excised and

148 rapidly transferred to liquid nitrogen. Another group of female NOD/SCID nude mice were

149 injected intravenously through the tail vein with 1 million cells and divided into two groups

150 that were either treated with PKI-587 or vehicle. Ten days after injection of cells, treatment of

151 mice was commenced by intravenous injection of 12 mg/kg of PKI-587 or vehicle twice

152 weekly. Engraftment was checked by measuring the number of circulating CD45+ cells. Mice

153 were euthanized when any signs of suffering such as difficulty in breathing, lack of

154 movement, or paralysis observed. All animal experiments were performed under an ethical

155 permit from the Swedish Animal Welfare Authority.

156 **Statistical analysis:** Statistical analysis was performed using GraphPad Prism 5.0. One way

157 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

183　cell viability of Jurkat cells and thus could be used as a control. We compared cell viability

184　between Jurkat and CCRF-CEM cells in the presence of 88 different inhibitors in two

185　different concentrations, 100 nM (Fig. 3A) and 1000 nM (Fig. 3B). We observed that while

186　most of the inhibitors displayed a linear correlation between the two cell lines, PKI-587 was

187　the most selective inhibitor for CCRF-CEM cells. Additionally, PKI-587 displayed an IC50 of

188　25 nM for MOLT3 cells (Fig. 3C), 23 nM for CCRF-CEM cells (Fig. 3D) and 73 nM for

189　Jurkat cells (Fig. 3E).

190

191　**Cytokines secreted from bone marrow HS-5 cells have no effect on inhibition of cell**

192　**growth by PKI-587:**

193　Cytokines/chemokines/growth factors secreted by the bone marrow microenvironment can

194　drive the drug resistance and survival of leukemic blasts [16]. In order to investigate whether

195　PKI-587 can still efficiently block cell growth in the presence of micro-environmental factors,

196　we assessed the relative viability of MOLT3 and CCRF-CEM cells in the presence or absence

197　of conditioned medium from HS5 cell cultures with increasing concentrations of inhibitor.

198　We observed that the presence or absence of conditioned medium from HS5 cells did not

199　affect the ability of PKI-587 to induce growth inhibition in MOLT3 (Fig. 4A) and CCRF-

200　CEM (Fig. 4B) cells.

201

202　**The dual specificity PI3K/mTOR inhibitor PKI-587 reduces viability and colony**

203　**formation of T-ALL cells:**

204　We used different biological assays to evaluate the effect PKI-587 on T-ALL cells. We

205　observed a dose-dependent inhibition of cell growth of both MOLT3 (Fig. 5A) and CCRF-

206　CEM (Fig. 5B) cell lines. Colony formation assays in semi-solid medium also indicated a

207　marked decrease in the number of colonies (Fig. 5C and 5D) as well as the size of the

208   colonies (Fig. 5E and 5F) with the increasing concentration of PKI-587 on both MOLT3 and

209   CCRF-CEM cells. Additionally, treatment with PKI-587 induced apoptosis in a dose-

210   dependent manner in these cell lines (data not shown). These results demonstrate an effective

211   inhibitory action of PKI-587 on T-ALL cell lines.

212

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

214   Based on the potent inhibitory efficacy of PKI-587 on the PI3K/mTOR pathway in T-ALL

215   cell lines *in vitro*, we next assessed its therapeutic efficacy in preclinical mouse models. We

216   first developed a mouse xenograft model using immunodeficient mice. We subcutaneously

217   injected only CCRF-CEM cells since it has been reported that MOLT3 cells are unable to

218   induce tumors *in vivo* by themselves [17]. Seven days after cell injection, mice were treated

219   with 12 mg/kg body weight of PKI-587, or vehicle, for an additional 20 days. Treatment with

220   PKI-587 led to a marked inhibition of tumor growth compared to vehicle treatment (Fig. 6A),

221   as well as to a delay in tumor growth in all treated mice (Fig. 6B). This three-week treatment

222   regimen also led to a significant tumor regression in the treated group (Fig. 6C). To assess the

223   survival benefit of this drug, we further developed a mouse model where we injected CCRF-

224   CEM cells intravenously. Ten days after the cells were injected, mice were treated with 12

225   mg/kg body weight of PKI-587 or vehicle control. Since CD45+ cells in the blood circulation

226   indicate the presence of leukocytes and engraftment of the injected cells, we measured those

227   cells in blood circulation in both mice treated with PKI-587 and vehicle controls. As

228   expected, the mice treated with PKI-587 exhibited significantly lower numbers of CD45+

229   cells in the blood circulation compared to the vehicle-treated (control) group (Fig. 6D). The

230   untreated first mouse was euthanized on day 40 with a median survival of 45 days, while PKI-

231   587-treatment led to a significantly higher survival advantage (Fig. 6E). The effects of this *in*

232   *vivo* treatment were well tolerated since no deceptive decrease in body weight was noticed in

233 the treated animals (data not shown). To summarize, these *in vivo* data indicate that PKI-587

234 has a substantial preclinical efficacy in treating T-ALL and warrants further investigation in a

235 clinical setting.

236

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

238 It is a common phenomenon for oncogenic signaling network to exhibit crosstalk between

239 multiple cellular signaling pathways. This has sometimes led to wrong interpretation and

240 overestimation of the role of a single protein in response to drug treatment [18]. PKI-587 has

241 already been shown to be a highly selective inhibitor of the PI3K/mTOR pathway in several

242 cancer types [10] and has been studied in different cell models, animal models and in clinical

243 trials [19, 20]. On the basis of the previous reports and our preliminary data, we further

244 evaluated the effect of PKI-587 on other signaling pathways. While treating the cells with a

245 higher concentration of PKI-587, we observed decreased phosphorylation of AKT and S6K

246 but no effect on ERK1/2 and p38 phosphorylation *in vitro* (Fig. 7A). Similar to the *in vitro*

247 findings, PKI-587 selectively inhibited AKT and S6K phosphorylation *in vivo* (Fig. 7B).

248 These results strongly suggest that PKI-587 is a selective inhibitor of the PI3K/mTOR

249 pathway.

250

251 **DISCUSSION**

252 Although significant improvement has been observed in overall cure rates of ALL, the

253 outcome of relapsed T-ALL patients still remains a major clinical challenge. This can be

254 attributed to several factors, but it is mainly due to the insufficient understanding of T-ALL

255 biology which impedes the identification of suitable prognostic factors to deliver proper

256 treatment. With the improved cure rate in ALL, current research strategies are more focused

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257 on subsets of patients such as T-ALL with drug resistance or relapsed leukemia in order to

258 identify treatments with the effective outcome.

259 The PI3K/mTOR pathway plays a critical role in T-ALL pathogenesis since the most

260 common mutations in T-ALL, such as gain-of-function mutations of NOTCH1 or loss-of-

261 function mutation of PTEN, often cause constitutive activation of this pathway [21]. In this

262 study, two different gene expression datasets of relapsed patients also displayed a

263 predominant enrichment of the PI3K/mTOR pathway. Using a panel of 88 different inhibitors

264 which target several different components of this pathway, PKI-587 was found to be the most

265 selective drug that induces apoptosis of T-ALL cell lines that are dependent on the activity of

266 the PI3K/mTOR pathway. An important observation was made, that the inhibitor PKI-587

267 was effective even in the presence of micro-environmental factors. The use of conditioned

268 medium from the bone marrow HS5 cells had very little or no effect on PKI-587-induced

269 inhibition of cell growth. This is interesting since it has been suggested that the bone marrow

270 microenvironment, presumably through cytokine production, helps to protect leukemic cells

271 from drug-induced death and thereby contributes to therapy resistance. Our findings provide

272 a basis for the use of the dual specificity inhibitor PKI-587 as a promising drug for the

273 treatment of T-ALL. Our biological assays also suggest a strong ability of PKI-578 to block

274 cell proliferation, colony formation and to induce apoptosis of T-ALL cell lines.

275

276 Since PKI-587 is a potent ATP-competitive, and reversible PI3K/mTOR inhibitor, it can

277 selectively abrogate the PI3K/mTOR pathway, without affecting MAPK signaling, by

278 inhibiting the activity of PI3K and mTOR along with their downstream effectors AKT and

279 S6K both *in vitro* as well as *in vivo*. Our results are in line with previous findings, in a slightly

280 different context, on the signaling effects of this dual inhibitor on therapy-resistant AML

281 patient with FLT3-ITD mutation [22]. In xenograft models of the T-ALL cell, inhibition of

282 the PI3K/mTOR pathway led to delayed tumor progression, induction of tumor regression and

283 markedly increased the survival rate. No gross toxicity was observed as no deceptive

284 decrease of body weight was noticed in tumor-bearing or engrafted mice. Our conclusion is

285 that this dose is effective and well tolerated, suggesting an encouraging therapeutic window

286 for the treatment of relapsed T-ALL patients.

287 In conclusion, we have investigated the beneficial effects of the dual PI3K/mTOR inhibitor

288 PKI-587, both *in vitro* and *in vivo,* for the treatment of relapsed and therapy-resistant T-ALL.

289 Our study has dominantly revealed a preclinical rationale to explore this novel drug to

290 effectively inhibit PI3K/mTOR activated signaling pathway. However, observations of the

291 pathway enrichment and the method to block the signaling pathway with PKI-587 may be a

292 possible therapeutic way to treat relapsed T-ALL patients and thereby minimizing the diverse

293 effects. Since this drug is already in clinical trials for the treatment of other cancers, we

294 suggest that further pre-clinical trials and early phase clinical trial should be undertaken to

295 evaluate the effect of PKI-587 on patients with relapsed T-ALL.

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306 **Conflict of interest:**

307 The authors declare no conflict of interest.

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REFERENCES

310 [1] R. Siegel, D. Naishadham, A. Jemal, Cancer statistics, 2013, CA Cancer J Clin, 63 (2013)
311 11-30.
312 [2] A. Lonetti, A. Cappellini, A. Bertaina, F. Locatelli, A. Pession, F. Buontempo, C.
313 Evangelisti, C. Evangelisti, E. Orsini, L. Zambonin, L.M. Neri, A.M. Martelli, F. Chiarini,
314 Improving nelarabine efficacy in T cell acute lymphoblastic leukemia by targeting aberrant
315 PI3K/AKT/mTOR signaling pathway, J Hematol Oncol, 9 (2016) 114.
316 [3] M.D. Kraszewska, M. Dawidowska, T. Szczepanski, M. Witt, T-cell acute lymphoblastic
317 leukaemia: recent molecular biology findings, Br J Haematol, 156 (2012) 303-315.
318 [4] P. Van Vlierberghe, A. Ferrando, The molecular basis of T cell acute lymphoblastic
319 leukemia, J Clin Invest, 122 (2012) 3398-3406.
320 [5] A. Silva, J.A. Yunes, B.A. Cardoso, L.R. Martins, P.Y. Jotta, M. Abecasis, A.E. Nowill,
321 N.R. Leslie, A.A. Cardoso, J.T. Barata, PTEN posttranslational inactivation and
322 hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability, J Clin
323 Invest, 118 (2008) 3762-3774.
324 [6] E. Rozengurt, H.P. Soares, J. Sinnet-Smith, Suppression of feedback loops mediated by
325 PI3K/mTOR induces multiple overactivation of compensatory pathways: an unintended
326 consequence leading to drug resistance, Mol Cancer Ther, 13 (2014) 2477-2488.
327 [7] C.C. Dibble, L.C. Cantley, Regulation of mTORC1 by PI3K signaling, Trends Cell Biol,
328 25 (2015) 545-555.
329 [8] C.P. Hall, C.P. Reynolds, M.H. Kang, Modulation of Glucocorticoid Resistance in
330 Pediatric T-cell Acute Lymphoblastic Leukemia by Increasing BIM Expression with the
331 PI3K/mTOR Inhibitor BEZ235, Clin Cancer Res, 22 (2016) 621-632.
332 [9] S.K. Tasian, D.T. Teachey, S.R. Rheingold, Targeting the PI3K/mTOR Pathway in
333 Pediatric Hematologic Malignancies, Front Oncol, 4 (2014) 108.
334 [10] A.M. Venkatesan, C.M. Dehnhardt, E. Delos Santos, Z. Chen, O. Dos Santos, S. Ayral-
335 Kaloustian, G. Khafizova, N. Brooijmans, R. Mallon, I. Hollander, L. Feldberg, J. Lucas, K.
336 Yu, J. Gibbons, R.T. Abraham, I. Chaudhary, T.S. Mansour, Bis(morpholino-1,3,5-triazine)
337 derivatives: potent adenosine 5'-triphosphate competitive phosphatidylinositol-3-
338 kinase/mammalian target of rapamycin inhibitors: discovery of compound 26 (PKI-587), a
339 highly efficacious dual inhibitor, J Med Chem, 53 (2010) 2636-2645.
340 [11] R. Mallon, L.R. Feldberg, J. Lucas, I. Chaudhary, C. Dehnhardt, E.D. Santos, Z. Chen,
341 O. dos Santos, S. Ayral-Kaloustian, A. Venkatesan, I. Hollander, Antitumor efficacy of PKI-
342 587, a highly potent dual PI3K/mTOR kinase inhibitor, Clin Cancer Res, 17 (2011) 3193-
343 3203.
344 [12] S.A. Moharram, R.A. Chougule, X. Su, T. Li, J. Sun, H. Zhao, L. Rönnstrand, J.U. Kazi,
345 Src-like adaptor protein 2 (SLAP2) binds to and inhibits FLT3 signaling, Oncotarget, (2016).
346 [13] A. Gutierrez, A. Kentsis, T. Sanda, L. Holmfeldt, S.C. Chen, J. Zhang, A. Protopopov, L.
347 Chin, S.E. Dahlberg, D.S. Neuberg, L.B. Silverman, S.S. Winter, S.P. Hunger, S.E. Sallan, S.
348 Zha, F.W. Alt, J.R. Downing, C.G. Mulligan, A.T. Look, The BCL11B tumor suppressor is
349 mutated across the major molecular subtypes of T-cell acute lymphoblastic leukemia, Blood,
350 118 (2011) 4169-4173.
351 [14] T. Sanda, X. Li, A. Gutierrez, Y. Ahn, D.S. Neuberg, J. O'Neil, P.R. Strack, C.G. Winter,
352 S.S. Winter, R.S. Larson, H. von Boehmer, A.T. Look, Interconnecting molecular pathways

in the pathogenesis and drug sensitivity of T-cell acute lymphoblastic leukemia, Blood, 115 (2010) 1735-1745.

[15] S.S. Winter, Z. Jiang, H.M. Khawaja, T. Griffin, M. Devidas, B.L. Asselin, R.S. Larson, G. Children's Oncology, Identification of genomic classifiers that distinguish induction failure in T-lineage acute lymphoblastic leukemia: a report from the Children's Oncology Group, Blood, 110 (2007) 1429-1438.

[16] F. Chiarini, A. Lonetti, C. Evangelisti, F. Buontempo, E. Orsini, C. Evangelisti, A. Cappellini, L.M. Neri, J.A. McCubrey, A.M. Martelli, Advances in understanding the acute lymphoblastic leukemia bone marrow microenvironment: From biology to therapeutic targeting, Biochim Biophys Acta, 1863 (2016) 449-463.

[17] S. Indraccolo, L. Stievano, S. Minuzzo, V. Tosello, G. Esposito, E. Piovan, R. Zamarchi, L. Chieco-Bianchi, A. Amadori, Interruption of tumor dormancy by a transient angiogenic burst within the tumor microenvironment, Proc Natl Acad Sci U S A, 103 (2006) 4216-4221.

[18] J.B. Fitzgerald, B. Schoeberl, U.B. Nielsen, P.K. Sorger, Systems biology and combination therapy in the quest for clinical efficacy, Nat Chem Biol, 2 (2006) 458-466.

[19] V. D'Amato, R. Rosa, C. D'Amato, L. Formisano, R. Marciano, L. Nappi, L. Raimondo, C. Di Mauro, A. Servetto, C. Fusciello, B.M. Veneziani, S. De Placido, R. Bianco, The dual PI3K/mTOR inhibitor PKI-587 enhances sensitivity to cetuximab in EGFR-resistant human head and neck cancer models, Br J Cancer, 110 (2014) 2887-2895.

[20] R. Gedaly, P. Angulo, J. Hundley, M.F. Daily, C. Chen, B.M. Evers, PKI-587 and sorafenib targeting PI3K/AKT/mTOR and Ras/Raf/MAPK pathways synergistically inhibit HCC cell proliferation, J Surg Res, 176 (2012) 542-548.

[21] T. Palomero, M.L. Sulis, M. Cortina, P.J. Real, K. Barnes, M. Ciofani, E. Caparros, J. Buteau, K. Brown, S.L. Perkins, G. Bhagat, A.M. Agarwal, G. Basso, M. Castillo, S. Nagase, C. Cordon-Cardo, R. Parsons, J.C. Zuniga-Pflucker, M. Dominguez, A.A. Ferrando, Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia, Nat Med, 13 (2007) 1203-1210.

[22] O. Lindblad, E. Cordero, A. Puissant, L. Macaulay, A. Ramos, N.N. Kabir, J. Sun, J. Vallon-Christersson, K. Haraldsson, M.T. Hemann, A. Borg, F. Levander, K. Stegmaier, K. Pietras, L. Rönnstrand, J.U. Kazi, Aberrant activation of the PI3K/mTOR pathway promotes resistance to sorafenib in AML, Oncogene, 35 (2016) 5119-5131.

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.

Figure 3: PKI-587 is a potent inhibitor of T-ALL cells growth: (A-B) A panel of inhibitors

(100 nM and 1000 nM concentrations) targeting different components of the PI3K/mTOR

pathway was used to measure cell viability using PrestoBlue assay. (C-E). Cell viability was

measured using PrestoBlue assay in the presence of increasing concentrations of PKI-587.

Figure 4: Conditioned medium from HS5 cells has no effect on PKI-587 induced growth

inhibition: (A-B) Cells were treated with increasing concentration of PKI-587 in the presence

or absence of conditioned medium from HS5 cells. PrestoBlue viability assay was used to

measure cell viability.

Figure 5: PKI-587 reduces cell viability and colony formation: (A-B) Cells were treated with

increasing concentrations of inhibitor. Cell viability was measured using PrestoBlue. (C-F)

Cells were mixed with semi-solid medium and inhibitor and seeded in 24-well plates.

Colonies were counted after 7 days. ns, not significant; ***, p< 0.001.

Figure 6: PKI-587 decreases tumor growth and increases survival: (A) Mice were treated

with PKI-587 or vehicle. Tumor volume was measured twice a week. (B-C) Tumors were

removed after sacrificing mice. The weight of individual tumors was measured, and the

picture was taken in the presence of a scale. (D) CD45+ cells in blood were counted using an

antibody against human CD45. (E) Kaplan–Meier curves showed the overall survival of mice

transplanted with CCRF-CEM cells.

Figure 7: PKI-587 selectively inhibits AKT and S6K phosphorylation: (A) Cells were treated

with increasing concentrations of PKI-587 before lysis. Cell lysates were used to assess

phosphorylation of signaling proteins using phospho-specific antibodies. (B) Tumors from

mice treated with either PKI-587 or vehicle were used to assess phosphorylation of signaling
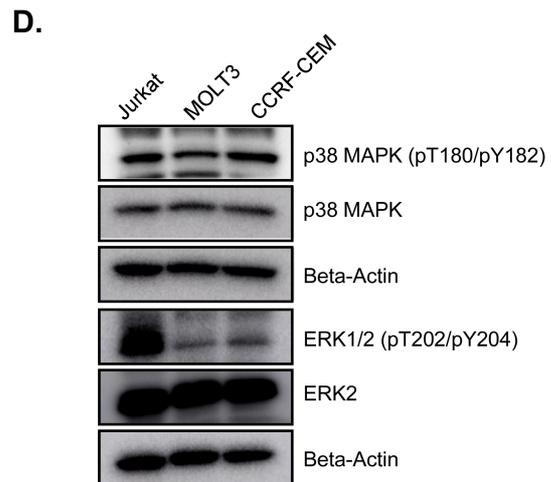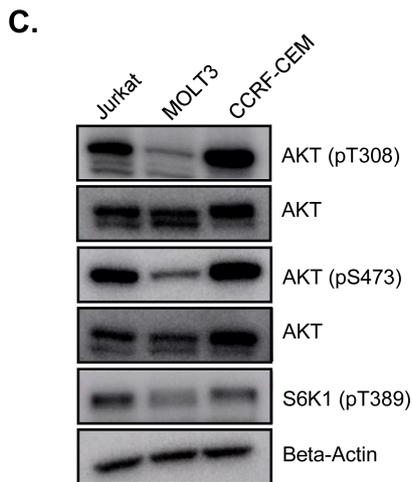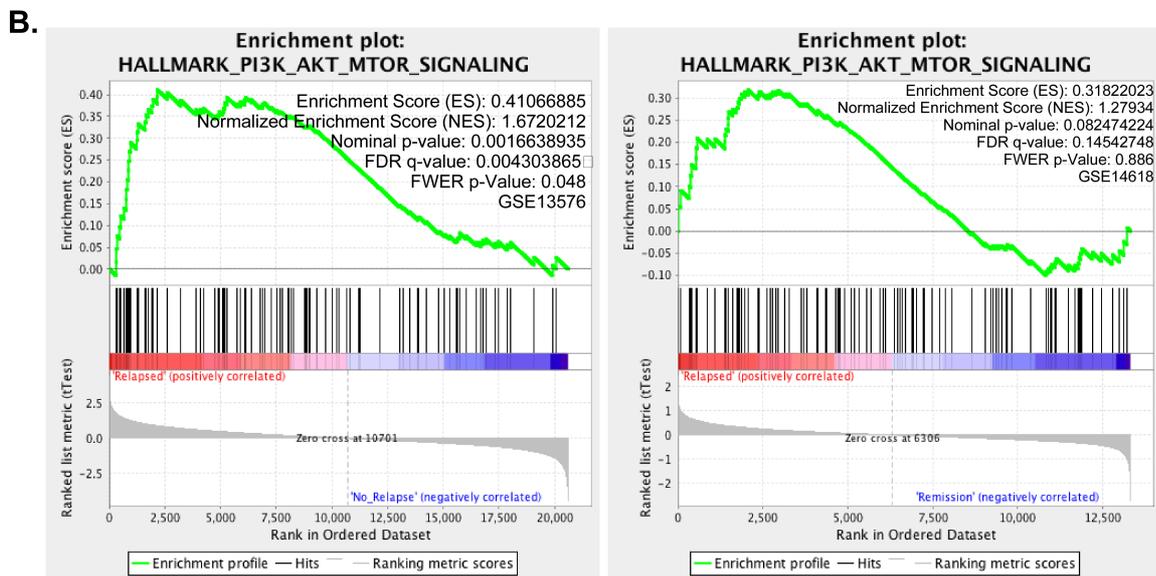
proteins using phospho-specific antibodies.

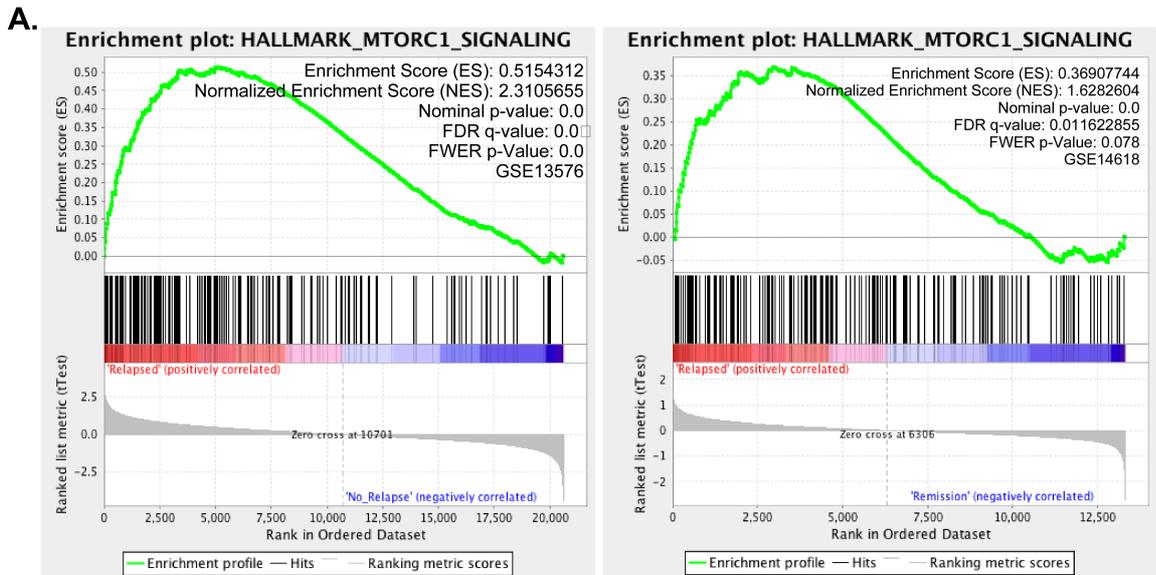Figure 1

TK

EphB2
TRKC
FGFR2 EGFR3
TRKB
TRKA
FGFR1
FGFR4
FLT1
ROR1
Ret
KDR
Axl
Tyro3/Sky
Kit
DDR2
FLT3
DDR1
PDGFR
IGF1R
EGFR
HER2
InSR
Met
HER4
ALK
LTK
Tyk2
JAK2
HER3
Fes
Src
EphA2
Lyn
BLK
HCK
Lck
ZAP70
PYK2
FAK Syk
ITK
TEC
FGR
TXK
Src
BTK
Blk
Etk/Bmx
CSK
Fes
Abl
Arg
Fer

JAK2~b
Tyk2~b

TKL

RAF1

ANPα
ANPβ

STE

MLKL

DYRK1A
DYRK1B

SgK307
SgK424
SCYL2
GCN2

NRK/ZC2
TNIK/ZC2

HIPK1
HIPK4

COT

TAO3

GCN2~b

MEK1/MAP2K1
MEK2/MAP2K2

CMGC

CK2α1
CK2α2

PINK1

CK1α

CK1

GSK3β
GSK3α
CDKL2
CDKL5
CDKL4
ERK5
ERK1
ERK2
p38γ
JNK1 JNK2
p38δ
JNK3
p38β
p38α
CDK6
PFTAIRE1
CHED
CDK3
CDC2/CDK1
CDK2

CDK7

IKKα
IKKβ
IKKε
TBK1

PLK3

BARK1/GRK2
BARK2/GRK3 RHODK/GRK1
GRK4
PDK1
RSKL2
RSK1/p90RSK
MSK1
RSK3
RSK4 RSK2
p70S6K
p70S6Kβ
Akt2/PKBβ
Akt3/PKB
Akt1/PKBα
SGK1
SGK2 SGK3
PKG2
PKG1
PRKY
NDR2
PRKX
PKCη
PKCζ
PKCι
PKCε
PKCθ
PKA
PKAη
PKCγ
PKCδ
ROCK2
PKCα
PKCβ

AGC

Nek10
CHK1

Pim1 Pim2

Nek1

Atypical Protein Kinases

ABC1
ADCK3

Alpha

Brd

PDHK

PIKK
ATR
mTOR/FRAP
DNAPK

RIO

TIF1

caMLCK

AMPKα1

CHK2

DCAMKL2
VACAMKL

MAPKAPK2
MAPKAPK3
RSK4~b
RSK1~b
MSK1~b
RSK2~b
RSK3~b

CaMK4

CAMK

Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com)

Figure 2

A. Cell viability (%) — 100 nM concentration

Jurkat vs CCRF-CEM

PKI-587

Legend:
- AKT
- AMPK
- ATM/ATR
- ATM/ATR, mTOR
- GSK-3
- mTOR
- PDK-1
- PI3K
- PI3K, DNA-PK
- PI3K, mTOR
- PKA
- S6K

B. Cell viability (%) — 1000 nM concentration

Jurkat vs CCRF-CEM

PKI-587

Legend:
- AKT
- AMPK
- ATM/ATR
- ATM/ATR, mTOR
- GSK-3
- mTOR
- PDK-1
- PI3K
- PI3K, DNA-PK
- PI3K, mTOR
- PKA
- S6K

C. MOLT3 IC$_{50}$ 25 nM

Cell viability (%) vs Concentration (Log M)

D. CCRF-CEM IC$_{50}$ 23 nM

Cell viability (%) vs Concentration (Log M)
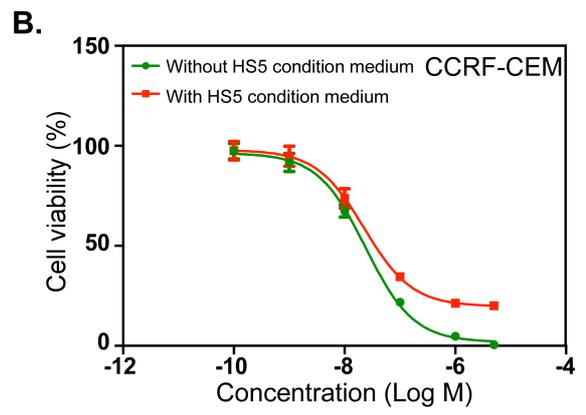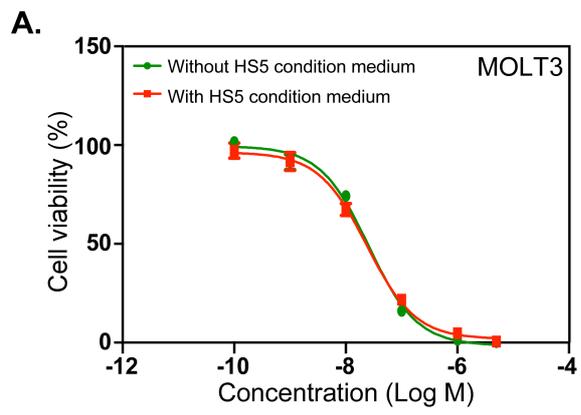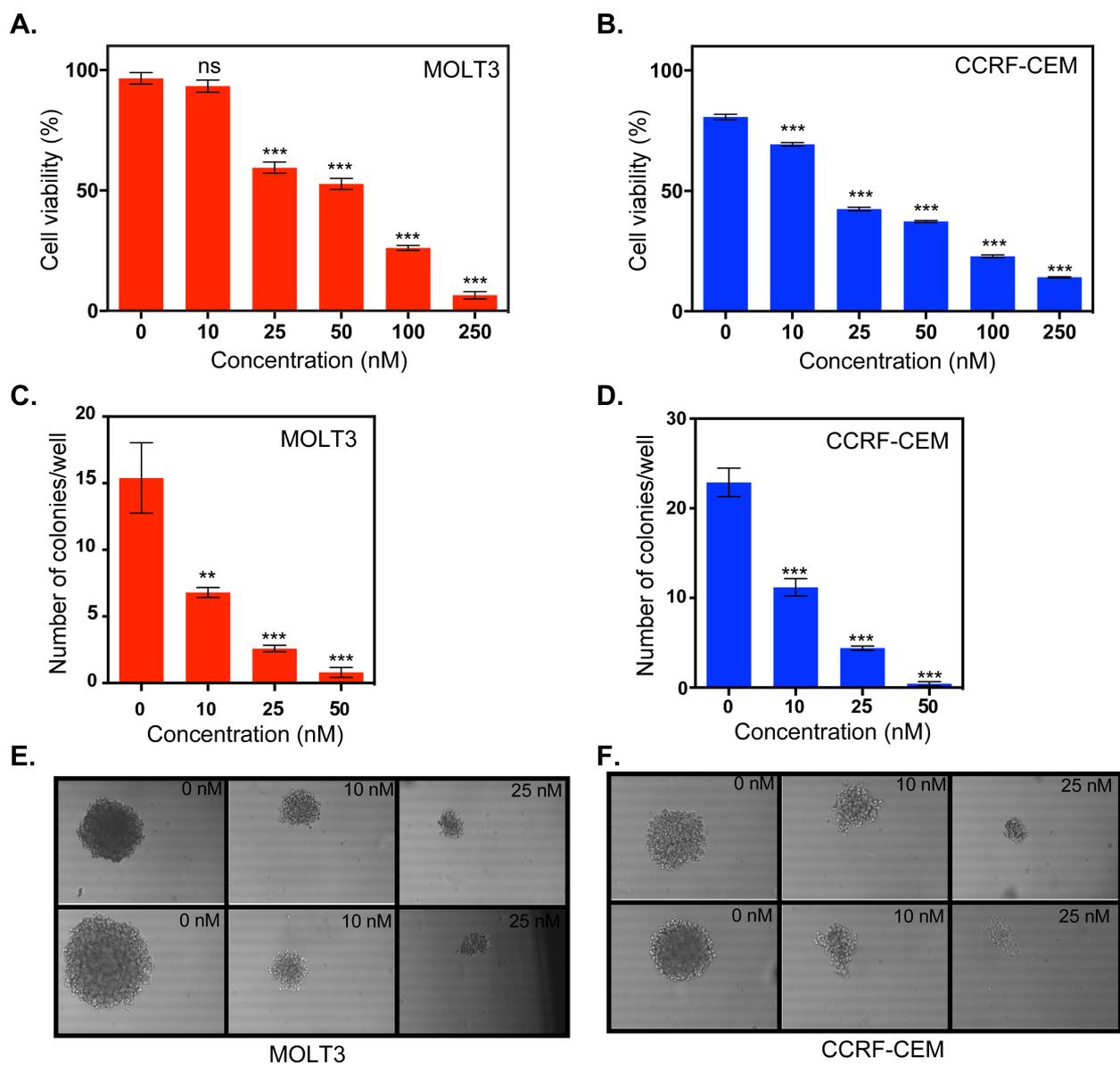
E. Jurkat IC$_{50}$ 73 nM

Cell viability (%) vs Concentration (Log M)

Figure 3

Figure 4

Figure 5

Figure 6

A.

PKI-587 (nM)

MOLT3: 0 10 100 500 1000
CCRF-CEM: 0 10 100 500 1000

pAKT
AKT
pERK1/2
ERK1/2
pS6K
S6K
pp38
p38
Tubulin

MOLT3   CCRF-CEM

B.

Control PKI-587

pAKT
AKT
pERK1/2
ERK1/2
pS6K
S6K
pp38
p38
Tubulin

Figure 7