OPEN

Citation: Blood Cancer Journal (2014) 4, e228; doi:10.1038/bcj.2014.49

www.nature.com/bcj

LETTER TO THE EDITOR

Effect of CAL-101, a PI3Kδ inhibitor, on ribosomal rna synthesis and cell proliferation in acute myeloid leukemia cells

Blood Cancer Journal (2014) **4,** e228; doi:10.1038/bcj.2014.49; published online 11 July 2014

Acute myeloid leukemia (AML) accounts for ~80% of all adult leukemias and the majority of patients will relapse and die from this disease, indicating an unmet need for new therapies.[1,2] The PI3K/Akt pathway, which is frequently constitutively activated in leukemic blasts from AML patients,coordinates the synthesis of ribosomal proteins and rRNA that is required for cellular proliferation.[3–5] The overall survival of AML patients whose blasts demonstrated Akt activation was significantly shorter than that of patients without Akt activation, suggesting that this pathway could represent an important therapeutic target.[3,6]

Activation of Akt is mediated by PI3K Class I p110 isoforms. The PI3K p110δ catalytic subunit is consistently expressed at high levels in AML blasts.[7] CAL-101 is an orally bioavailable and selective inhibitor of the p110δ isoform that is currently under clinical evaluation in B-cell malignancies.[8–11] Although recent studies demonstrated that CAL-101, which is 400-fold more selective for class I PI3K than for related kinases, exhibits far greater effects against B-ALL and CLL cells as compared with AML and myeloproliferative neoplasm cells,[11] we have recently shown that Akt directly regulates rRNA synthesis activity in AML, resulting in enhancement of cell proliferation.[12] We have therefore asked whether CAL-101 could suppress rRNA synthesis by reducing Akt phosphorylation in AML cells with the goal of determining whether this approach might be therapeutically useful in this group of diseases.

We first examined the expression of the PI3Kδ isoform in 14 AML patient samples and 5 leukemic cell lines by western blot. The properties of the patient samples are shown in Supplementary Table 1. Figure 1a shows that PI3Kδ is variably expressed in patient samples and cell lines. We then examined the effects of CAL-101 on Akt phosphorylation. Treatment with CAL-101 suppresses Akt phosphorylation in K562 cells and in combined lysates from 10 primary AML cells in a dose-dependent manner (Figure 1b and Supplementary Figures 1a and b), as does the Akt inhibitor AZD8055. The molecular biomarkers of P70S6K and GSK3 phosphorylation are commonly used as indicators of PI3K pathway activity. The decrease in Akt phosphorylation induced by CAL-101 occurs concomitantly with a decrease in p-P70S6K and p-GSK3 (Figure 1b and Supplementary Figures 1a and b), further suggesting that CAL-101 suppresses PI3K/Akt downstream signaling pathways in AML cells.

To determine whether the suppression of Akt signaling by CAL-101 impairs cell proliferation, we treated K562 cells with CAL-101 or AZD8055 and examined cell proliferation over time. Treatment with CAL-101 reduced cell growth (Figure 1c, left) and inhibited PCNA expression (Figure 1c, right) in a dose-dependent manner. CAL-101 also inhibited K562 cell proliferation as measured by multiparametric cell viability (MTT) and colony-forming assays (Figure 1d) and reduced cell survival and PCNA expression in primary AML cells (Figure 1e). As the expression of PI3Kδ is variable among AML samples (Figure 1a), we asked whether the expression level of PI3Kδ is related to the response of AML cells to CAL-101. We divided AML samples into high and low PI3Kδ expression groups based on the densitometry results of western blots in Figure 1a and treated the two groups of cells with CAL-101 at pharmacologically relevant concentrations (100 nM). Figure 1f demonstrates that there is no difference in the levels of p-Akt between the two groups (right, second panel), suggesting that Akt phosphorylation in AML cells is regulated by factors in addition to PI3Kδ isoform expression, as suggested previously.[13] CAL-101 inhibited proliferation as measured by MTT assay in both groups; however, a greater reduction of p-Akt, PCNA levels and cell survival was observed in the high PI3Kδ expression group (Figure 1f and Supplementary Figures 1c–e).

Ribosomal RNA synthesis is essential for cellular proliferation and Akt directly regulates rRNA synthesis in AML cells.[12] The effects of CAL-101 in inhibiting Akt activation and cell proliferation in AML cells (Figure 1) led us to hypothesize that CAL-101 might also repress rRNA synthesis. Treatment of K562 and primary AML cells with CAL-101 suppressed both the 5′external transcribed spacer pre-rRNA abundance and the extent of Pol I recruitment to rDNA (Figures 2a–d). Given the high rate of rRNA turnover in these cells, the levels of pre-rRNA transcript abundance are a valid approximation of the overall rate of rRNA transcription.[14,15] The incorporation of ^{32}P into newly synthesized RNA in AML was also decreased by CAL-101 (Figures 2a–c, right).

We have recently found that inhibition of mTORC1 with Rapamycin does not completely ablate rRNA synthesis.[12] We therefore compared the effects of Rapamycin and CAL-101 on rRNA synthesis and cell survival in AML cells. Based on previous *in vitro* data demonstrating that p-P70S6K is inhibited at a dose of 100 nM Rapamycin,[16,17] we treated AML cells at this final concentration. Treatment of AML cells with CAL-101 decreased Akt signaling (Figure 2e), pre-rRNA synthesis (Figure 2f and Supplementary Figure 2a) and cell survival (Figure 2g) to a significantly greater extent than did Rapamycin. Similar results were obtained with K562 cells (Supplementary Figures 1b–d). These results demonstrate that the effects of CAL-101 on AML cells are independent of the mTOR pathway. Finally, although CAL-101 treatment decreased pre-rRNA synthesis in both high and low PI3Kδ expression groups, the effect was stronger in cells expressing higher levels of PI3Kδ (Figure 2h). Our results demonstrate that CAL-101 inhibits rRNA synthesis and cell proliferation in AML cells through inhibition of Akt activation

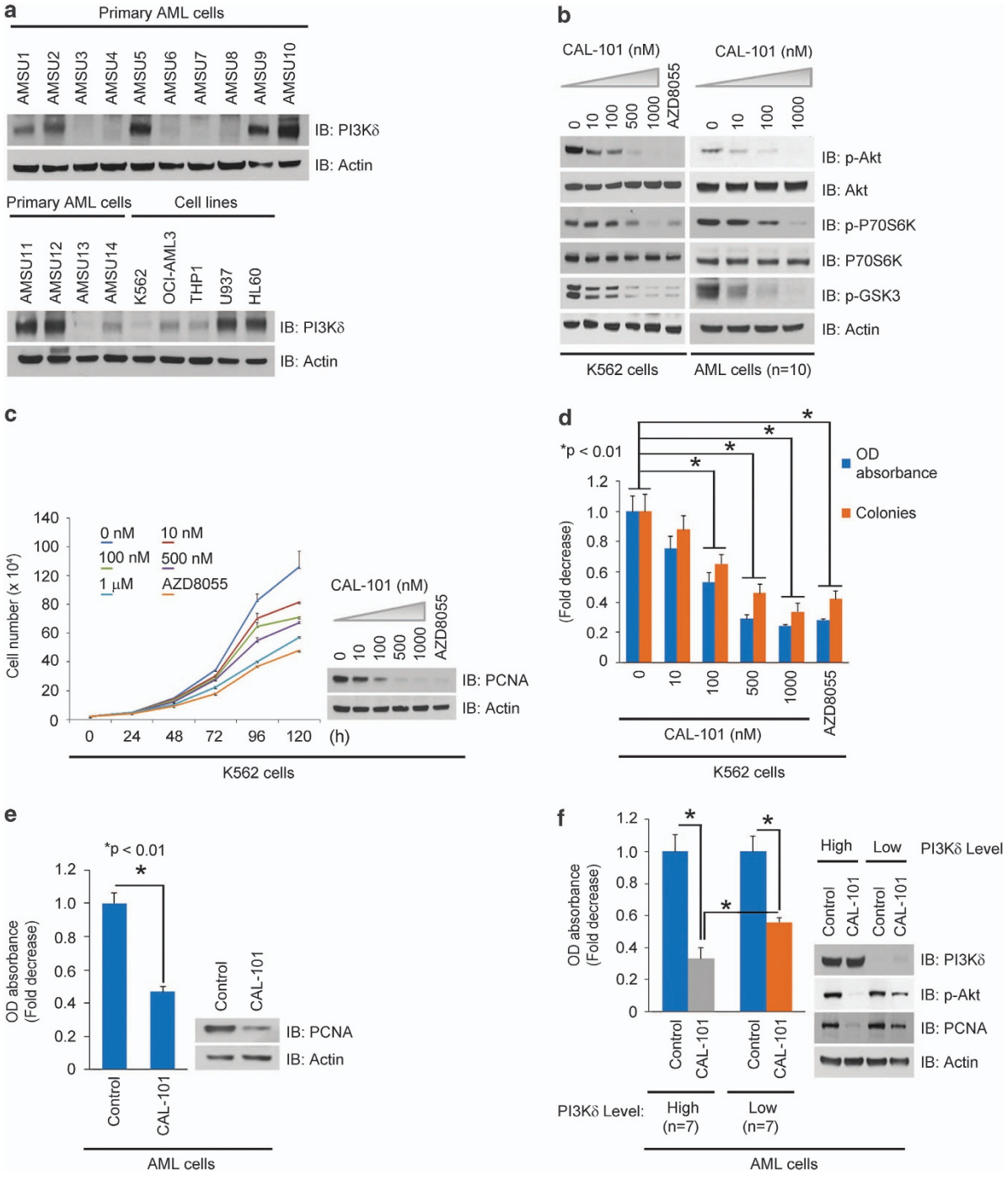

Figure 1. Inhibition of Akt activation and cell proliferation by CAL-101 in AML cells. (**a**) Expression of PI3Kδ protein in leukemic cell lines and primary AML cells. Thirty micrograms cell lysate from five leukemic cell lines and 14 AML samples were separated on SDS gels and immunoblotted with anti-PI3Kδ and anti-actin antibodies. (**b**) Effects of CAL-101 on Akt phosphorylation in AML cells. K562 (left) and a mixture of primary AML cell lysates ($n = 10$) (right) were treated with the indicated concentrations of CAL-101 or AZD8055 for 3 h. Thirty micrograms of cell lysate were separated on SDS gels and immunoblotted with the indicated antibodies. Densitometry measurements of p-Akt, p-P70S6K and p-GSK3 are shown in Supplementary Figure 1a and b. (**c**) and (**d**) Effects of CAL-101 on K562 cell proliferation. (**c**) K562 cells were plated, treated with indicated concentration of CAL-101 or AZD8055 and counted at the times indicated (left) and PCNA protein levels were determined by western blot after 72 h of culture (right). (**d**) K562 cells were treated with the indicated concentration of CAL-101 or AZD8055. MTT assays were performed after 72 h of culture and colonies were counted after 10 days of culture in soft agar. Values represent the mean ± − s.d. of triplicate determinations. (**e**) Effects of CAL-101 on proliferation in primary AML cells. A mixture of AML cells ($n = 10$) was plated and treated with 100 nM of CAL-101 for 72 h. (left) MTT assay; (right) western blot of PCNA protein. (**f**) Effects of PI3Kδ expression levels on response to CAL-101. Primary AML cells ($n = 14$) were divided into low and high PI3Kδ expression groups (each group, $n = 7$) and the level of PI3Kδ was measured based on the densitometry results from panel (**a**). Cells were plated and treated with CAL-101 or vehicle for 72 h. (left) MTT assay; (right) western blot with indicated antibodies. The effects of CAL-101 on p-Akt level in individual AML samples are shown on Supplementary Figure 1c and d. The densitometry measurement of p-Akt and PCNA in Figure 1f was shown in Supplementary Figure 1e.

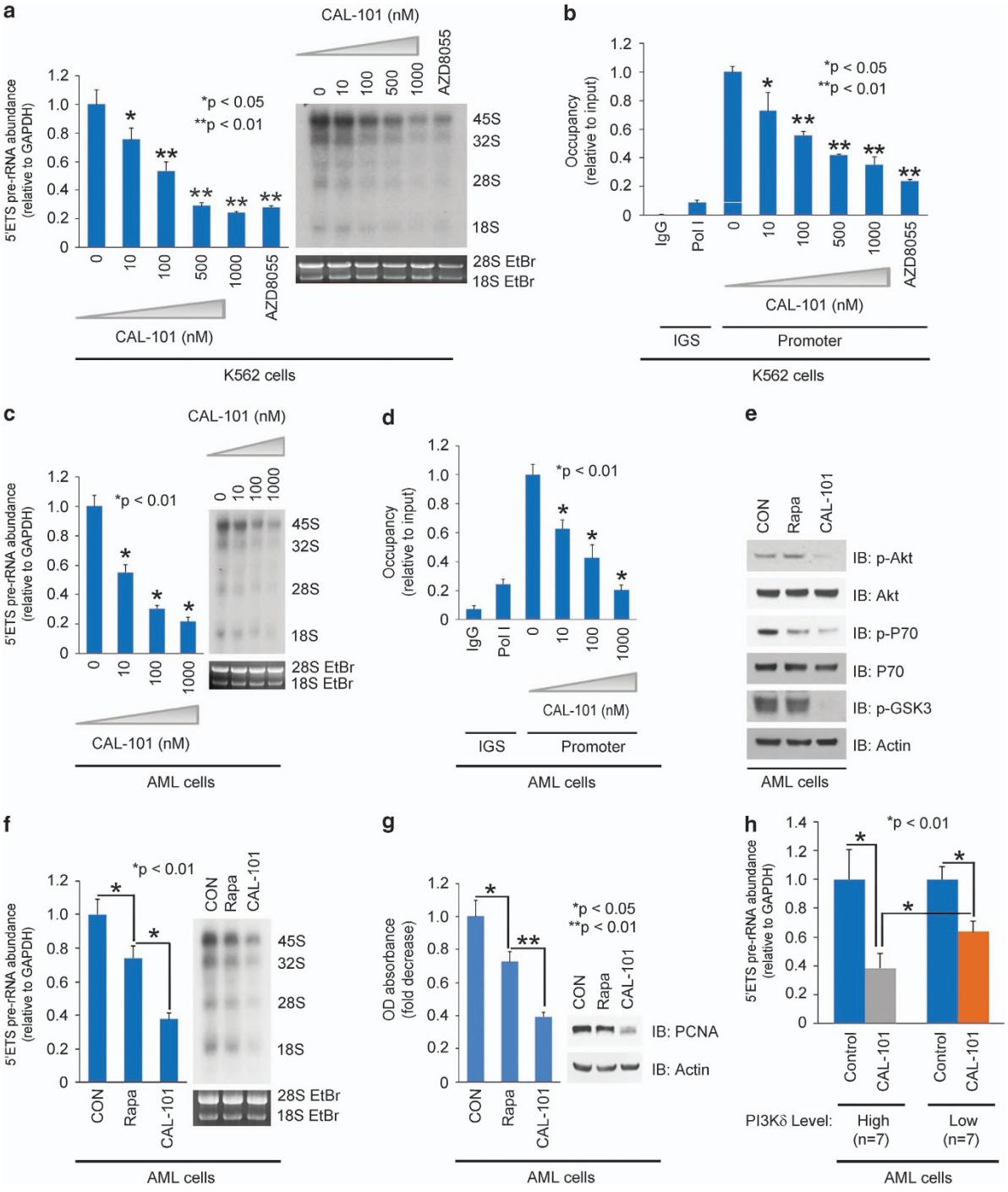

Figure 2. Repression of rRNA synthesis by CAL-101 in AML cells. (**a**) and (**b**) Effects of CAL-101 on Pol I binding to rDNA and rRNA synthesis in K562 cells. Cells were treated with the indicated concentration of CAL-101 or AZD8055 for 3 h. (**a**) Levels of pre-rRNA expression relative to GAPDH for each sample as determined by qPCR and RNA synthesis using ^{32}P labeling. (**b**) ChIP assays were performed as described in Supplementary Materials and Methods using anti-Pol I antibody. Values represent the mean ± s.d. of triplicate determinations ($n = 3$). (**c**) and (**d**) Effects of CAL-101 on rRNA synthesis in primary AML cells. A mixture of AML cells ($n = 10$) was treated with indicated concentration of CAL-101 for 3 h. (**c**) Pre-rRNA expression (left) and RNA labeling (right). (**d**) ChIP assay with Pol I antibody. (**e**) Comparison of effects of Rapamycin and CAL-101 on inhibition of Akt phosphorylation. A mixture of AML cells ($n = 10$) was treated with Rapamycin (100 nM) or CAL-101 (100 nM) for 3 h. The cell lysate was immunoblotted with the indicated antibodies. (**f**) and (**g**) Comparison of effects of Rapamycin and CAL-101 on pre-rRNA synthesis and cell survival. (**f**) AML cells were treated as (**e**). Pre-rRNA expression (left) and RNA labeling (right) was measured. (**g**) AML cells were treated with Rapamycin or CAL-101 for 72 h. MTT assay (left) and western blot (right) were performed. (**h**) Effects of PI3Kδ expression and CAL-101 treatment on pre-rRNA synthesis in AML cells. AML cells were divided as described in Figure 1f and treated with CAL-101 or vehicle for 3 h.

with more profound effects on cells expressing higher levels of PI3Kδ.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by a translational research grant and by a SCOR award from the Leukemia and Lymphoma Society.

LXT Nguyen, A Sesay and BS Mitchell
Departments of Medicine and Chemical and Systems Biology, Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA, USA
E-mail: bmitchell@stanford.edu

REFERENCES

1 Passegue E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci USA* 2003; **100**(Suppl 1): 11842–11849.
2 Smith M, Barnett M, Bassan R, Gatta G, Tondini C, Kern W. Adult acute myeloid leukaemia. *Crit Rev Oncol Hematol* 2004; **50**: 197–222.
3 Min YH, Eom JI, Cheong JW, Maeng HO, Kim JY, Jeung HK *et al.* Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. *Leukemia* 2003; **17**: 995–997.
4 Zhao S, Konopleva M, Cabreira-Hansen M, Xie Z, Hu W, Milella M *et al.* Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias. *Leukemia* 2004; **18**: 267–275.
5 Montanaro L, Trere D, Derenzini M. Nucleolus, ribosomes, and cancer. *Am J Pathol* 2008; **173**: 301–310.
6 Brandts CH, Sargin B, Rode M, Biermann C, Lindtner B, Schwable J *et al.* Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer Res* 2005; **65**: 9643–9650.
7 Sujobert P, Bardet V, Cornillet-Lefebvre P, Hayflick JS, Prie N, Verdier F *et al.* Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. *Blood* 2005; **106**: 1063–1066.
8 Fruman DA, Rommel C. PI3Kdelta inhibitors in cancer: rationale and serendipity merge in the clinic. *Cancer Discov* 2011; **1**: 562–572.
9 Schwamb J, Feldhaus V, Baumann M, Patz M, Brodesser S, Brinker R *et al.* B-cell receptor triggers drug sensitivity of primary CLL cells by controlling glucosylation of ceramides. *Blood* 2012; **120**: 3978–3985.
10 Berger A, Hoelbl-Kovacic A, Bourgeais J, Hoefling L, Warsch W, Grundschober E *et al.* PAK-dependent STAT5 serine phosphorylation is required for BCR-ABL-induced leukemogenesis. *Leukemia* 2014; **28**: 629–641.
11 Lannutti BJ, Meadows SA, Herman SE, Kashishian A, Steiner B, Johnson AJ *et al.* CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. *Blood* 2011; **117**: 591–594.
12 Nguyen le XT, Mitchell BS. Akt activation enhances ribosomal RNA synthesis through casein kinase II and TIF-IA. *Proc Natl Acad Sci USA* 2013; **110**: 20681–20686.
13 Park S, Chapuis N, Tamburini J, Bardet V, Cornillet-Lefebvre P, Willems L *et al.* Role of the PI3K/AKT and mTOR signaling pathways in acute myeloid leukemia. *Haematologica* 2010; **95**: 819–828.
14 Allo SN, McDermott PJ, Carl LL, Morgan HE. Phorbol ester stimulation of protein kinase C activity and ribosomal DNA transcription. Role in hypertrophic growth of cultured cardiomyocytes. *J Biol Chem* 1991; **266**: 22003–22009.
15 James MJ, Zomerdijk JC. Phosphatidylinositol 3-kinase and mTOR signaling pathways regulate RNA polymerase I transcription in response to IGF-1 and nutrients. *J Biol Chem* 2004; **279**: 8911–8918.
16 Perl AE, Kasner MT, Shank D, Luger SM, Carrol M. Single-cell pharmacodynamic monitoring of S6 ribosomal protein phosphorylation in AML blasts during a clinical trial combining the mTOR inhibitor sirolimus and intensive chemotherapy. *Clin Cancer Res* 2012; **18**: 1716–1725.
17 Ryningen A, Reikvam H, Nepstad I, Rye KP, Bruserud O. Inhibition of mammalian target of rapamycin in human acute myeloid leukemia cells has diverse effects that depend on the environment *in vitro* stress. *Bone Marrow Res* 2012; **2012**: 329061.

Supplementary Information accompanies this paper on Blood Cancer Journal website (http://www.nature.com/bcj)