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ORIGINAL ARTICLE

AMP-dependent kinase/mammalian target of rapamycin complex 1 signaling in T-cell acute lymphoblastic leukemia: therapeutic implications

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The mammalian target of rapamycin (mTOR) serine/threonine kinase is the catalytic subunit of two multi-protein complexes, referred to as mTORC1 and mTORC2. Signaling downstream of mTORC1 has a critical role in leukemic cell biology by controlling mRNA translation of genes involved in both cell survival and proliferation. mTORC1 activity can be down-modulated by upregulating the liver kinase B1/AMP-activated protein kinase (LKB1/AMPK) pathway. Here, we have explored the therapeutic potential of the anti-diabetic drug, metformin (an LKB1/AMPK activator), against both T-cell acute lymphoblastic leukemia (T-ALL) cell lines and primary samples from T-ALL patients displaying mTORC1 activation. Metformin affected T-ALL cell viability by inducing autophagy and apoptosis. However, it was much less toxic against proliferating $CD4^+$ T-lymphocytes from healthy donors. Western blot analysis demonstrated dephosphorylation of mTORC1 downstream targets. Unlike rapamycin, we found a marked inhibition of mRNA translation in T-ALL cells treated with metformin. Remarkably, metformin targeted the side population of T-ALL cell lines as well as a putative leukemia-initiating cell subpopulation ($CD34^+/CD7^-/CD4^-$) in patient samples. In conclusion, metformin displayed a remarkable anti-leukemic activity, which emphasizes future development of LKB1/AMPK activators as clinical candidates for therapy in T-ALL.
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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic cancer caused by malignant transformation of developing T cells in the thymus. T-ALL accounts for 10–15% and 25% of pediatric and adult ALL, respectively.[1] After the introduction of intensified polychemotherapy schemes, a remarkable improvement has been observed in the prognosis of T-ALL patients, especially in children and adolescents. Nevertheless, the outcome of relapsed and chemoresistant T-ALL remains dismally disappointing.[2] Therefore, novel and less toxic treatment strategies are needed that may target aberrantly activated signaling pathways influencing proliferation, survival and drug resistance of T-ALL cells.[3–6] About 85% of T-ALL patients display increased phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) activation at diagnosis, and this has a negative prognostic impact.[7–9] mTOR exists in two complexes that comprise distinct sets of protein-binding partners. mTORC1, defined by the interaction between raptor (regulatory-associated protein of mTOR) and mTOR, is sensitive to rapamycin/rapalogues and is thought to mediate many of its downstream effects through p70S6 kinase (p70S6K), S6 ribosomal protein (S6RP) and initiation factor 4E-binding protein 1 (4E-BP1).[10] mTORC2, defined by the interaction between mTOR and Rictor (rapamycin-insensitive companion of mTOR), is considered to be rapamycin resistant and phosphorylates Akt at Ser 473, one of two phosphorylated residues required for full Akt activation.[10] Upregulated mTORC1 activity within cancer cells increases the synthesis of many oncogenic proteins controlled at the translation initiation level, through the phosphorylation of the physiologic translation repressor 4E-BP1 at multiple residues.[11,12] An important regulator of the mTORC1-dependent translation process is the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) axis. LKB1 is a serine/threonine protein kinase encoded by the *STK11* gene, originally identified as the gene mutated in the rare autosomal dominant human genetic disorder, Peutz–Jeghers syndrome.[13] Peutz–Jeghers syndrome patients develop numerous benign tumors (classed as hamartomas) in the gastrointestinal tract and have a 20-fold increased risk of developing malignant tumors at other sites, whereas mutations in the *LKB1* gene are also seen in some sporadic cancers, especially lung adenocarcinoma.[14] Therefore, LKB1 is a classical tumor suppressor. AMPK is a direct LKB1 substrate.[15] A consequence of AMPK activation by LKB1 is the inhibition of the mTORC1 pathway through phosphorylation of tuberous sclerosis 2 or hamartin and raptor.[16,17] Metformin (*N*,*N*-dimethylbiguanide) belongs to the biguanide class of oral hypoglycemic agents and has been widely used in treatment of Type 2 diabetes for many years. Intriguingly, there is a growing body of evidence that metformin reduces cancer risk and that it does so through activation of AMPK and repression of mTORC1.[18] In light of these findings, metformin is now being tested in several clinical trials in patients with solid tumors, especially breast cancer.[19] A recent paper has highlighted the cytotoxicity of metformin in preclinical models of acute myelogenous leukemia (AML),[20] emphasizing the effects of metformin on the translation of

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oncogenic proteins. We have recently demonstrated that inhibition of mTORC1-mediated oncogenic protein translation occurs also in T-ALL cells treated with mTORC1/mTORC2 ATP-competitive inhibitors, but not with rapamycin.[12] Here, we show that in T-ALL cell lines and patient primary cells, the LKB1/AMPK tumor-suppressor pathway is functional and can be activated by metformin treatment. Accordingly, metformin displayed a strong cytotoxic activity against both T-ALL cell lines and lymphoblasts from the T-ALL patients but barely affected the proliferation of peripheral blood $CD4^+$ T-lymphocytes from healthy donors. Moreover, metformin blocked the catalytic activity of mTORC1 and markedly repressed mRNA translation, resulting in decreased expression of highly oncogenic proteins. Treatment of T-ALL cells with metformin induced both autophagy and apoptosis. Remarkably, metformin targeted T-ALL subpopulations, which might correspond to leukemia-initiating cells (LIC). Taken together, our findings suggest that targeting AMPK-mTORC1 signaling may be an interesting option for treating T-ALL cases that display an LKB1/AMPK inducible pathway and depend on aberrant upregulation of mTORC1 for their proliferation and survival.

Materials and methods

Materials

Metformin was purchased from Sigma-Aldrich (St Louis, MO, USA). The PI3K-selective inhibitor, GDC-0941, was from Axon Medchem BV (Groningen, The Netherlands). For western blotting analysis, primary antibodies and peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). For flow cytometric analysis, AlexaFluor 488-conjugated antibodies to Ser 473 p-Akt, Thr 37/46 p-4E-BP1 and Ser 235/236 p-S6RP were obtained from Beckman Coulter (Miami, FL, USA). A blocking monoclonal antibody to the insulin-like growth factor-1 receptor (clone αIR3) was purchased from Thermo Fisher Scientific (Rockford, IL, USA) and was employed at 5 μg/ml.

Cell culture and primary samples

The T-ALL cell lines Jurkat, MOLT-4, CEM-R (drug-resistant cells overexpressing 170-kDa P-glycoprotein),[21] RPMI-8402 and BE-13 were grown in RPMI 1640, supplemented with 10% fetal bovine serum. Patient samples or peripheral blood $CD4^+$ T-lymphocytes from healthy donors were obtained with informed consent according to the Institutional guidelines and isolated using Ficoll-Paque (Amersham Biosciences, Little Chalfont, UK) for patient lymphoblasts or magnetic labeling (Miltenyi Biotec, Bergisch Gladbach, Germany) for $CD4^+$ T-lymphocytes. $CD4^+$ T-lymphocytes (10^5/well) were cultured in RPMI 1640 media containing 10% fetal bovine serum and stimulated for 48 h with a mixture of 10 μg/ml phytohemagglutinin (PHA-M) and 50 ng/ml human recombinant interleukin-2 to induce proliferation. Then, 1 μCi (0.037 MBq) [^{3}H]-thymidine was added for 16 h. The amount of radioactivity incorporated was determined by trichloracetic acid precipitation.

Cell viability analysis

MTT (3-(4,5-dimethylthythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed to assess the sensitivity of cell lines or patient samples to metformin, as previously reported.[22] Results were statistically analyzed by GraphPadPrism Software (GraphPad Software Inc., version 3.2, San Diego, CA, USA).

[^{3}H]-thymidine incorporation

T-ALL cells were incubated for 48 h in triplicate at 10^5/ml. They were then pulsed for 6 h with 1 μCi (0.037 MBq/ml) [^{3}H]-thymidine. The amount of radioactivity incorporated was determined as outlined above. For each sample, [^{3}H]-thymidine incorporation was set at 100% in the control, and was expressed relative to this value in the presence of the drugs.

Western blot analysis

This was performed as detailed elsewhere.[23] Analysis with an antibody to β-actin demonstrated equal protein loading.

Transmission electron microscope analysis

This was performed according to standard techniques, as described previously.[12]

Polysome analysis, [^{3}H]-leucine incorporation and 7methyl-GTP cap affinity assay

These were performed as reported elsewhere.[12]

Quantitative real-time PCR on mRNAs polysome fractions

The nonpolysome and polysome-containing fractions were pooled and analyzed by quantitative real-time PCR for c-Myc and Bcl-xL mRNAs. Results are expressed as fold change of polysomal-bound mRNA relative to the total mRNA amount.[24] The housekeeper gene used was human β-actin. Primers used were: c-Myc forward primer: 5′-GGAACTCTTGTGCGTA AGGA-3′; c-Myc reverse primer: 5′-CAAGACTCAGCCAAGGTT GTG-3′; Bcl-xL forward primer: 5′-GTTGAAGCGTTCCTGGC CCTTT-3′; Bcl-xL reverse primer: 5′-CAGAATGGACTGGACTG AATCGGAGAT-3′; β-actin forward primer: 5′-TCCCTGGAGAA GAGCTACGA-3′; β-actin reverse primer: 5′-AGCACTGTGTT GGCGTACAG-3′.

Flow cytometric detection of T-ALL side population cells

This was performed as reported elsewhere,[25] using a Cell Lab Quanta SC flow cytometer (Beckman Coulter) equipped with an UV lamp and a 488-nm solid-state laser.

Flow cytometric analysis of p-Akt, p-4E-BP1 and p-S6RP levels, and of putative LIC in T-ALL patient samples

These analyses were performed as detailed elsewhere,[12] using a Navios flow cytometer (Beckman Coulter), equipped with Kaluza software. At least 5000 events per sample were acquired.

Statistical evaluation

The data are presented as mean values from three separate experiments ± s.d. Data were statistically analyzed by a Dunnet's test after one-way analysis of variance at a level of significance of $P<0.05$ or $P<0.001$ vs control samples.

Results

Metformin has cytotoxic effects on T-ALL cell lines

The effects of metformin on T-ALL cells were analyzed by treating the cells with increasing concentrations of the drug and analyzing the rates of proliferation by 48 h MTT assays

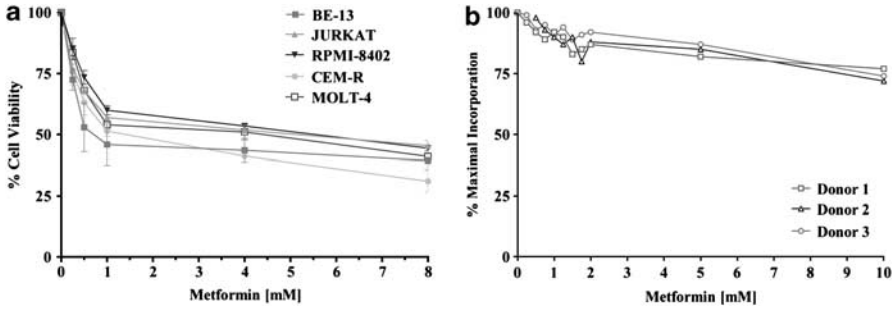

Figure 1 Metformin affects viability of T-ALL cell lines. (**a**) MTT assays of T-ALL cell lines treated with increasing concentrations of metformin for 48 h. Results are the mean of at least three different experiments ± s.d. (**b**) $CD4^+$ T-lymphocytes isolated from the peripheral blood of healthy donors and stimulated with PHA-M plus interleukin-2, were treated with increasing concentrations of metformin. One representative of three different experiments is shown. Maximal incorporation of [^{3}H]-thymidine was 73 888 c.p.m., 66 007 c.p.m. and 84 112 c.p.m. for donor 1, 2 and 3, respectively. s.d. was <15%.

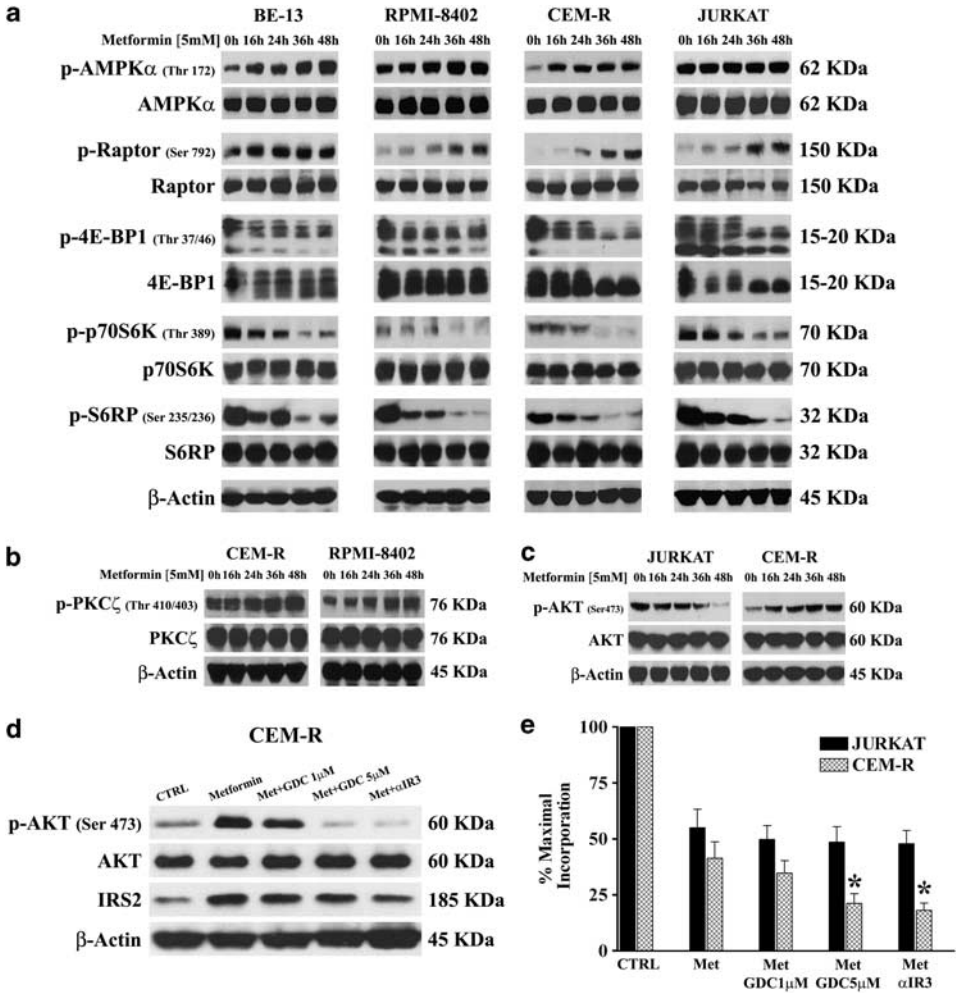

Figure 2 Effects of metformin on the phosphorylation of PKCζ/AMPKα and critical components of the mTORC1 signaling pathway. Cell lines were incubated for the indicated times in the presence of 5 mM metformin. (**a**) Western blot analysis documented increased levels of Thr 172 p-AMPKα in all cell lines, except for Jurkat, and an mTORC1 signaling inhibition in all cells was considered. (**b**) CEM-R and RPMI-8402 were analyzed by western blotting for Thr 410/403 p-PKCζ and total PKCζ. (**c**) Levels of Ser 473 p-Akt and Akt were investigated by western blot analysis in Jurkat and CEM-R cells. (**d**) Western blot analysis for Ser 473 p-Akt, Akt and IRS2 in CEM-R cells treated with metformin alone (5 mM), or with metformin plus either GDC-0941 (GDC) or αIR3 at the indicated concentrations. CTRL: untreated cells. (**e**) Jurkat and CEM-R cells were grown in the presence of metformin alone (5 mM) or with metformin plus either GDC-0941 (GDC) or αIR3 at the indicated concentrations for 48 h. They were then pulsed for 6 h with [^{3}H]-thymidine. For each sample, [^{3}H]-thymidine incorporation was set at 100% in the control (CTRL) and was expressed relative to this value in the presence of the drugs. Asterisks indicate significant differences ($P<0.01$) relative to untreated cells.

(Figure 1a). Cell lines displayed an IC_{50} (half maximal inhibitory concentration) for metformin ranging from 1.6 to 5.6 mM. The drug-resistant CEM-R cell line displayed the highest sensitivity to metformin. In contrast, $CD4^+$ T-lymphocytes isolated from the peripheral blood of healthy donors and stimulated with PHA-M plus interleukin-2, were much less sensitive to metformin concentrations as high as 10 mM as far as their proliferation (assayed by $[^3H]$-thymidine incorporation) was concerned (Figure 1b). Overall, these findings demonstrated that metformin reduced the growth of T-ALL cell lines. Moreover, they also suggested metformin could have a favorable therapeutic index, as it affected proliferation of normal $CD4^+$ T-lymphocytes to a much lower extent.

The LKB1/AMPK axis is functional and controls mTORC1 activity in T-ALL cells

Western blot analysis demonstrated a time-dependent increase in Thr 172 p-AMPKα, after treatment with 5 mM metformin in BE-13, CEM-R and RPMI-8402 cell lines. Increased p-AMPK levels were accompanied by increased phosphorylation of Ser 792 p-raptor. Although Jurkat cells did not present an evident increase in AMPKα phosphorylation, they displayed a marked increase in Ser 792 p-raptor levels (Figure 2a), an event directly mediated by AMPKα.[17] In agreement with increased Ser 792 raptor phosphorylation, we found that metformin inhibited the phosphorylation of mTORC1 downstream substrates Thr 37/46 p-4E-BP1, Thr 389 p-p70S6K and Ser 235/236 p-S6RP (Figure 2a).

It has been reported that metformin-mediated inhibition of complex I of the mitochondrial respiratory chain results in the generation of reactive nitrogen species that activate protein kinase C (PKC) ζ, which, in turn, phosphorylates and upregulates LKB1.[26] As PKCζ requires phosphorylation on Thr 410/403 for its activation, we examined the levels of this phosphorylated amino-acid residue after metformin treatment. As shown in Figure 2b, metformin treatment resulted in increased phosphorylation of these threonine residues of PKCζ, a result that attests to the stimulation of the PKCζ/LKB1/AMPKα pathway.

As it is known that inhibition of p70S6K phosphorylation in AML cells could relieve an inhibitory loop on Akt, which involves the IGF-1R and the insulin receptor substrate (IRS) 2,[27] the levels of Ser 473 p-Akt were analyzed in response to metformin. As shown in Figure 2c, in Jurkat cells metformin actually decreased phosphorylation of Ser 473 p-Akt. However, in CEM-R cells, the phosphorylation of this amino-acid residue markedly increased in response to metformin. This phenomenon was dependent on both PI3K and IGF-R1, as either a PI3K-selective inhibitor, GDC-0941, or a blocking antibody to the IGF-1R (αIR3) blunted the metformin-induced hyperphosphorylation of Akt. Moreover, metformin resulted in increased levels of IRS2 protein expression in CEM-R cells (Figure 2d). Thus, we investigated whether the combination of metformin plus either GDC-0941 or αIR3 was more effective than metformin alone in reducing the proliferation rate of CEM-R cells, as assessed by $[^3H]$-thymidine incorporation. As expected, the combined treatments were more effective in reducing proliferation of CEM-R, but not of Jurkat, cells (Figure 2e). Overall, our findings documented that metformin was effective in downregulating mTORC1 signaling in T-ALL cells, most notably resulting in dephosphorylation of the translation repressor 4E-BP1. However, they also demonstrated that in CEM-R cells metformin could hyperactivate Akt, which could then decrease its anti-proliferative efficacy.

Metformin induces autophagy and apoptosis in T-ALL cell lines

As mTORC1 is an inhibitor of autophagy, it was investigated whether metformin could indeed induce autophagy in T-ALL

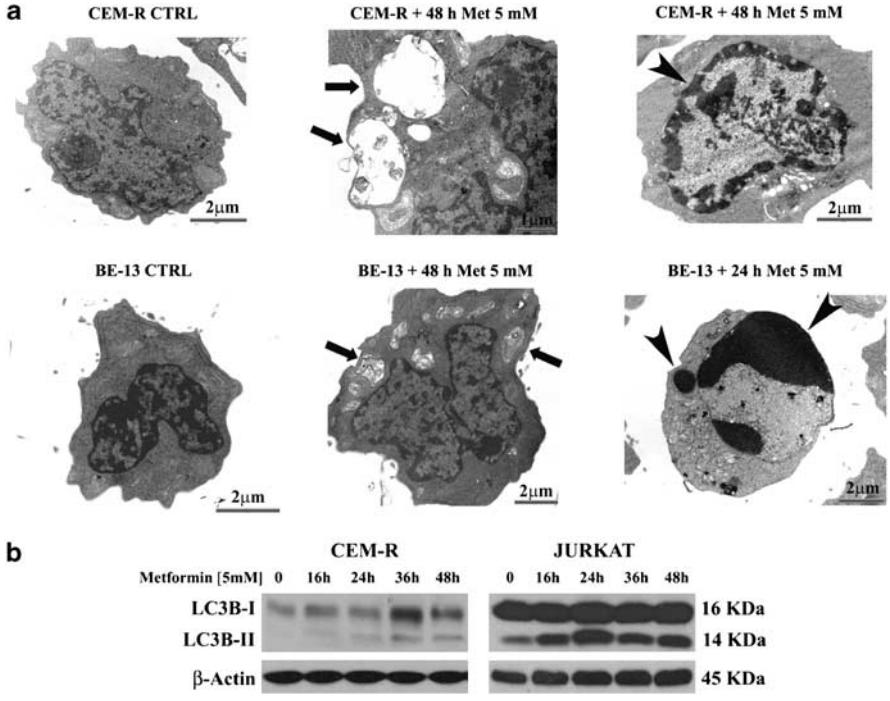

Figure 3 Metformin induces autophagy and apoptosis in T-ALL cell lines. (**a**) CEM-R and BE-13 cell lines were treated for the indicated times with 5 mM metformin, then processed for transmission electron microscope analysis that documented autophagy and apoptosis induction. Arrows indicate cytoplasmic vacuoles containing various degraded organelles, whereas arrowheads highlight condensed apoptotic chromatin. CTRL: untreated cells. (**b**) Western blot analysis was carried out for LC3B I/II in T-ALL cells treated with 5 mM metformin for the indicated times.

cells. Induction of autophagy was confirmed by transmission electron microscope analysis, the gold standard for autophagy studies,[28] which documented the presence of autophagic vacuoles in the cytoplasm of metformin-treated CEM-R and BE-13 cells (Figure 3a). However, ultrastructural analysis after metformin treatment also revealed the presence of apoptotic cells (Figure 3a). To confirm induction of autophagy, we studied the cleavage of LC3B, which is the only known mammalian protein that stably associates with the autophagosome membranes.[29] Indeed, LC3B can be detected as LC3B-I (cytosolic) and LC3B-II (membrane bound and enriched in the autophagic vacuole fraction) forms. Metformin treatment caused an increase in LC3B-II (cleaved fragment; Figure 3b).

Metformin inhibits mRNA translation in T-ALL cells

We performed polysome analysis using Jurkat cells treated with either rapamycin or metformin. Polysomes were separated through sucrose density gradients, and their profiles were generated by the measurement of absorbance at 254 nm. Metformin caused an inhibition of translation initiation, as documented by the shift from large to small polysomes. In contrast, rapamycin had no effects (Figure 4a). The impact of metformin on protein synthesis was further evaluated by (^{3}H)-leucine incorporation into neo-synthesized proteins of Jurkat and CEM-R cells. The drug was able to reduce proteins synthesis, whereas rapamycin did not significantly affect it (Figure 4b). Translation of mRNA is tightly regulated at the initiation level through the assembly of eIF4F complexes.[30] Using Jurkat cells, we compared the effects of rapamycin and metformin on eIF4F assembly by pull-down assays using 7methyl-GTP-Sepharose beads that mimic the cap structure of mRNA.[20] While exposure to metformin dissociated eIF4G from eIF4E and increased the amount of 4E-BP1 bound to eIF4E, indicating inhibition of eIF4F complex assembly, treatment with rapamycin did not change the amount of eIF4G or 4E-BP1 bound to eIF4E (Figure 4c). Western blot analysis

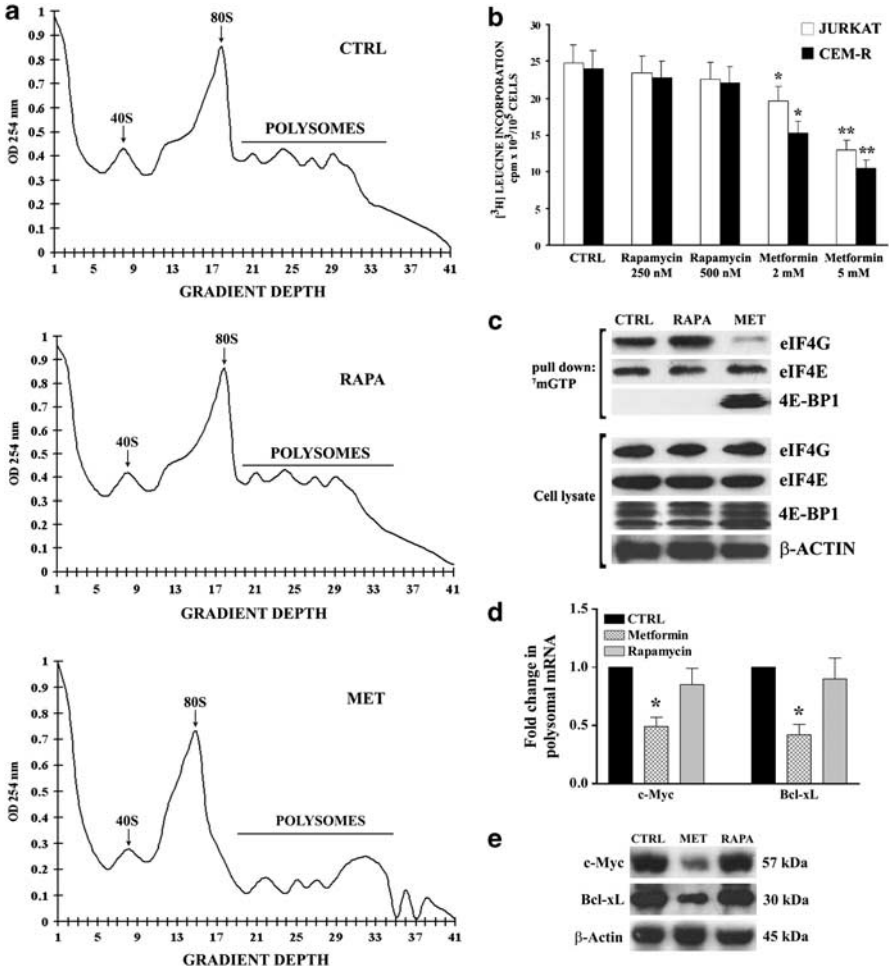

Figure 4 Metformin affects protein translation. (**a**) Jurkat cells were cultured for 48 h in the absence (CTRL) or in the presence of either rapamycin (500 nM) or metformin (5 mM). The polysome profile was generated by the measurement of optical density (OD) at 254 nm. (**b**) T-ALL cell lines were grown in the absence (CTRL) or in the presence of the drugs for 48 h, then pulse-labeled for 4 h with (^{3}H)-leucine at 2 μCi/ml (0.074 MBq/ml) in leucine-free medium. Results are the mean of three separate experiments ± s.d. Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$) relative to CTRL. (**c**) Jurkat cells were cultured for 48 h without (CTRL) or with rapamycin (RAPA, 500 nM) or metformin (MET, 5 mM). A total of 5×10^6 cells were lysed in 600 μl of solubilization buffer. Lysates were clarified by centrifugation, and supernatants were incubated with 7methyl-GTP-Sepharose beads in 400 μl binding buffer. Beads were then washed three times in binding buffer and boiled in sample buffer. Protein levels were analyzed by western blot. (**d**) The polysomal fractions of Jurkat cells were pooled and the total RNA was isolated. Quantitative real-time PCR to determine the c-Myc and Bcl-xL mRNA expression in polysomal fractions was carried out using β-actin as a control. Metformin was employed at 5 mM and rapamycin at 500 nM. Data are expressed as fold change as compared with untreated samples (CTRL) and represent means ± s.d. of three independent experiments (*$P<0.05$). (**e**) Western blot analysis for c-Myc and Bcl-xL in Jurkat cells treated with either metformin (MET, 5 mM) or rapamycin (RAPA, 500 nM) for 48 h. CTRL: untreated cells.

on whole-cell lysates demonstrated that the drugs did not significantly reduce the total amounts of eIF4G, eIF4E, or 4E-BP1 (Figure 4c). In addition, when mRNA translation for two highly oncogenic proteins (c-Myc and Bcl-xL) was directly analyzed in the polysomal fractions, we found suppression by metformin, but not rapamycin, in both Jurkat and CEM-R cells (Figure 4d). Consistently, a metformin-dependent downregulation of c-Myc and Bcl-xL protein expression was documented in Jurkat cells by western blot (Figure 4e).

Metformin targets the side population of T-ALL cell lines

We sought to determine whether metformin could target the T-ALL side population (SP). This subpopulation, which overexpresses ABCG2 (also referred to as breast cancer resistance protein or BRCP) and other ABC plasma membrane transporters, is thought to share some properties of cancer stem cells (CSCs).[31] This has been documented in AML[32] and multiple myeloma.[33] However, candidate CSCs have been successfully identified also in the SP of a murine model of adult T-cell leukemia/lymphoma.[34] The SP of T-ALL cells was identified by the live-cell DNA-binding dye, Hoechst 33342. As a control, Hoechst 33342 staining could be inhibited after incubation with the high-specificity ABCG2 transporter inhibitor, KO143. A decrease in the amount of SP cells was evident in both MOLT-4 and BE-13 cell lines treated for 24 h with 5 mM metformin (Figure 5).

T-ALL lymphoblasts are sensitive to metformin

To better assess the efficacy of metformin as a potential therapeutic agent in T-ALL, we examined pediatric and adult T-ALL patient samples isolated from bone marrow or peripheral blood, for their sensitivity to metformin, using MTT assays, western blot and flow cytometry experiments. All of the patients displayed enhanced phosphorylation of Thr 37/46 p-4E-BP1 and Ser 235/236 p-S6RP (not shown). T-ALL lymphoblast samples were treated with increasing concentrations of metformin, and cell survival was analyzed by MTT assays. A marked reduction of cell viability at 96 h was detected. The metformin IC_{50} for patient samples ranged between 0.6 and 0.9 mM at 96 h (Figure 6a). Western blot experiments demonstrated a strong increase in Thr 172 p-AMPKα after 48 h of treatment with 1 mM metformin. At the same time, phosphorylation of 4E-BP1 and S6RP markedly decreased (Figure 6b). These observations were confirmed by flow cytometric analysis (Figure 6c). Similar to the results obtained using T-ALL cell lines, the regulation of Ser 473 p-Akt levels upon metformin treatment was variable (Figure 6b and c).

Overall, these findings demonstrated that metformin displayed cytotoxic activity also against primary cells from T-ALL patients.

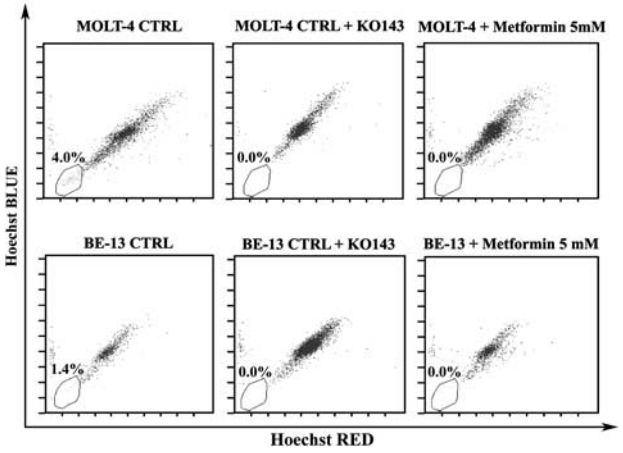

Figure 5 Metformin targets the SP in T-ALL cell lines. Cells were treated with metformin (5 mM) for 24 h, then processed for SP analysis by staining with Hoechst 33343 dye (5 μg/ml) followed by flow cytometry. To gate SP cells, untreated (CTRL) cells were stained with the Hoechst dye in the presence or in the absence of the ABCG2 blocking agent, KO-143 (0.1 μM). Cells, which disappeared in the presence of KO-143, were gated as the SP and are indicated as a percentage of the whole viable cell population.

Metformin induces apoptosis in the $CD34^+/CD7^-/CD4^-$ subset of patient lymphoblasts

Finally, we investigated whether metformin could induce apoptosis in a T-ALL patient lymphoblast subpopulation ($CD34^+/CD7^-/CD4^-$), which is enriched in putative LIC,[35] using quadruple staining and flow cytometric analysis. After electronic gating on the $CD7^-/CD4^-$ lymphoblast subset, cells were analyzed for CD34 expression and positivity to Annexin V staining. After 72 h of treatment, metformin markedly induced apoptosis in the $CD34^+/CD7^-/CD4^-$ subpopulation (Figure 6d). Intriguingly, rapamycin was much less efficacious in this respect. Also in this subpopulation, metformin was capable of decreasing phosphorylation levels of 4E-BP1, S6RP and Akt (Figure 6e).

Discussion

T-ALL is characterized by leukemic transformation of thymocytes caused by cooperative events altering proliferation, survival and differentiation of T cells. Despite major improvements in our understanding of the molecular genetics of T-ALL,[36–39] the mechanisms that lead to the abnormal proliferation and survival of T-lymphoblasts remain largely unknown. Therefore, treatment of this neoplasm remains a challenge for clinicians. Major efforts have been made to develop new compounds targeting signaling pathways implicated in T-ALL cell proliferation and survival. The AMPK1/mTORC1 axis represents a recently identified promising therapeutic target in a number of tumors, as its activity is frequently deregulated in cancer cells. Constitutive activation of mTORC1 is a common event in T-ALL,[40] which emphasizes the potential of using mTORC1-targeting drugs as therapeutic agents in this disorder. In preclinical models of T-ALL, the efficacy of the mTORC1 inhibitor, rapamycin, appears weak, at least in monotherapy settings.[41] Indeed, allosteric mTORC1 inhibitors such as rapamycin and its derivatives do not target 4E-BP1 in T-ALL cells and this limits their efficacy.[12] In this study, we tested the anti-leukemic activity of metformin on T-ALL cells, as recent data have highlighted that this drug targets 4E-BP1-dependent translation in AML.[20] Metformin demonstrated marked anti-leukemic effects *in vitro* against both T-ALL cell lines and patients lymphoblasts. The cytotoxic effects were specific to leukemic cells, as metformin only slightly affected the proliferation of $CD4^+$ T-lymphocytes from healthy donors, in agreement with a previous report, in which the AMPK activator acadesine (AICAR) was tested on healthy T-lymphocytes.[42] Moreover, metformin spared normal hematopoiesis *ex vivo*, while it affected the clonogenic activity of AML $CD34^+$ cells.[20] Taken together, these findings suggest that targeting the

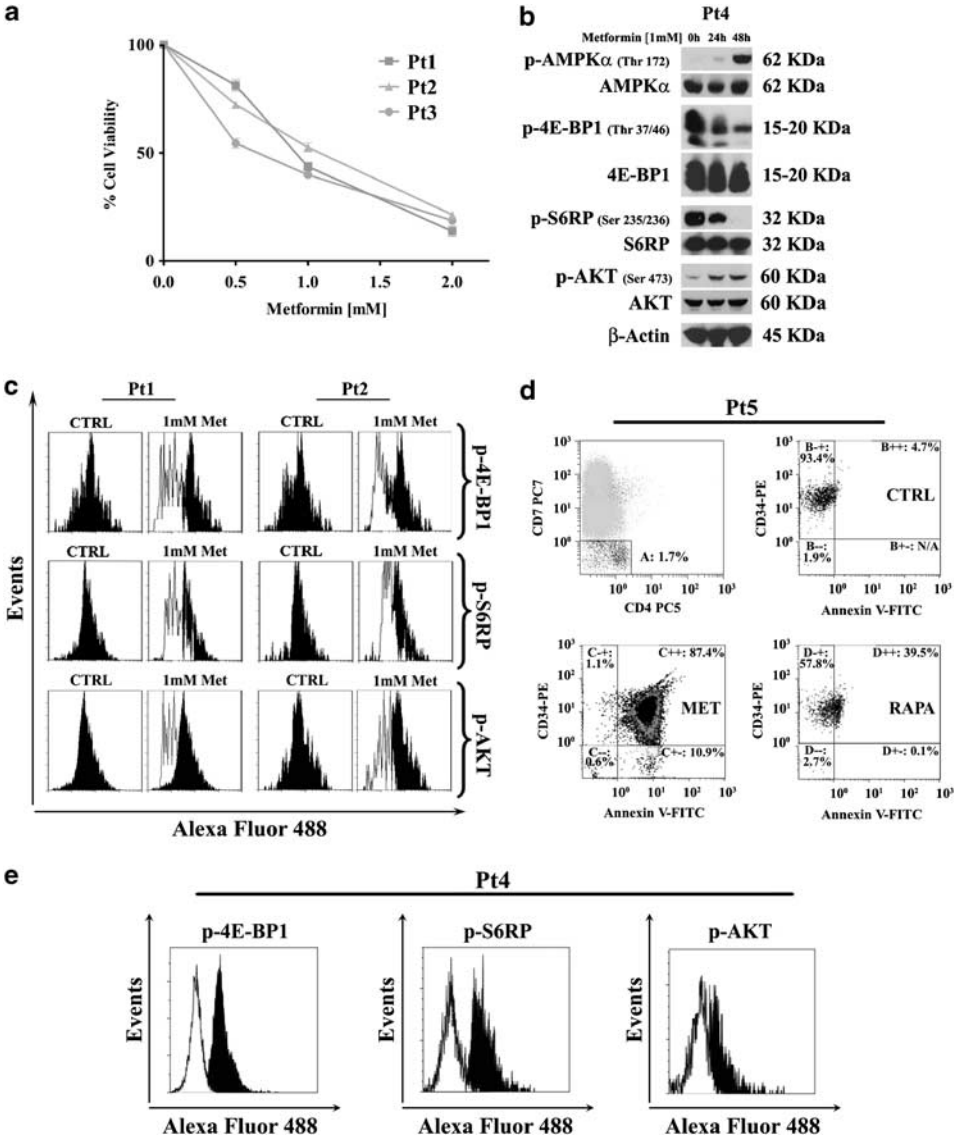

Figure 6 Metformin exerts cytotoxicity towards T-ALL primary cells displaying constitutive activation of mTORC1 signaling, and targets the $CD34^+/CD7^-/CD4^-$ lymphoblast subpopulation. (**a**) MTT assay of T-ALL lymphoblasts treated with metformin for 96 h. Results are the mean of at least two different experiments ± s.d. Three representative patients are shown. (**b**) Western blot analysis for p-AMPKα, p-4E-BP1, p-S6RP and p-Akt in T-ALL lymphoblasts. One representative patient is shown. (**c**) Samples from T-ALL patients were analyzed by flow cytometry for the levels of p-4E-BP1, p-S6RP and p-Akt in the absence (CTRL, black histograms) and presence (MET, white histograms) of 1 mM metformin for 24 h. Two representative patients are shown. (**d**) Patient samples were incubated without (CTRL) or with either metformin (MET, 1 mM) or with rapamycin (RAPA, 500 nM) for 48 h. After electronic gating on the $CD7^-/CD4^-$ lymphoblast subset, cells were analyzed for CD34 expression and positivity to Annexin V staining. One representative of three different patients is shown. (**e**) The $CD34^+/CD7^-/CD4^-$ lymphoblast sub-population was stained for p-4E-BP1, p-S6RP and p-Akt using AlexaFluor 488-conjugated antibodies. White histograms, untreated cells. Black histograms, cells treated with 1 mM metformin for 48 h. One representative patient is shown.

AMPK/mTORC1 pathway in acute leukemias could result in a favorable therapeutic window also *in vivo*.

Metformin-induced autophagy in T-ALL cells, which could be due to inhibition of ULK1 and Atg13 phosphorylation by mTORC1. Indeed, ULK1 and Atg13 are two essential components of the autophagic machinery.[43] Nevertheless, it should be considered that ceramide has emerged as a negative regulator of autophagy.[44] Considering that metformin activated AMPK and decreased ceramide levels in cardiomyocytes,[45] it would be interesting in the future to investigate the exact role of sphingolipids in metformin-elicited autophagy in T-ALL cells.

As expected, metformin strongly inhibited mTORC1 signaling, as evidenced by the decrease of p-p70S6K, p-S6RP and p-4E-BP1, at residues that are recognized substrates of mTORC1. However, in CEM-R cells, metformin treatment resulted in increased levels of Ser 473 p-Akt, most likely through mechanisms which involve IGF-1R, IRS2 and PI3K. Accordingly, the anti-proliferative action of metformin could be increased in CEM-R by a combination therapy consisting of either a PI3K-selective inhibitor or a blocking monoclonal antibody to IGF-1R.

In contrast, in Jurkat cells metformin actually decreased Ser 473 p-Akt. At present, we do not have an explanation for this phenomenon. Nevertheless, similar effects of metformin on Ser 473 p-Akt levels have been reported in breast cancer cells, where the drug dowregulated IRS1 at Ser 789, a site previously

reported to inhibit downstream PI3K/Akt signaling.[46] However, our unpublished data reveal that metformin did not increase Ser 789 p-IRS1 in Jurkat cells.

The critical mechanism underlying metformin action in T-ALL, however, may result from the inhibition of protein translation. It has been recently emphasized that the weak anti-leukemic activity of the rapamycin/rapalogues was mainly due to the sustained high level of 4E-BP1 phosphorylation in AML and T-ALL cells treated with these compounds.[12,47] We showed herein that, in contrast to rapamycin, metformin markedly inhibited the assembly of eIF4F-initiating complexes and reduced the recruitment of mRNA molecules to polysomes in T-ALL cells. This resulted in an inhibition of protein synthesis and a decreased expression of oncogenic proteins (c-Myc and Bcl-xL) known for being regulated at the translation initiation level.

The difficulty in eradicating tumors might result from the conventional treatments targeting the bulk of the tumor cells, but not the CSC or LIC, in case of leukemias.[48] Therefore, strategies that eliminate these cells could have significant clinical implications. On one side we were able to demonstrate that metformin targeted the SP of T-ALL cells, which could be enriched in CSCs. On the other side, metformin-induced apoptosis in a T-ALL lymphoblast subpopulation ($CD34^+/CD7^-/CD4^-$), which has been reported to be enriched in LIC in pediatric patients. Intriguingly, rapamycin was not as effective in this respect. It should be pointed out that the $CD34^+/CD7^-/CD4^-$ subpopulation is probably not the only cell subset endowed with LIC activity in T-ALL.[49] Therefore, other T-ALL subpopulations in which LIC activity has been identified, should be tested for their sensitivity to metformin.[50] In any case, it should be emphasized that in preclinical models of breast cancer, metformin displayed a sensitivity for CSCs over healthy cells.[51]

Previous investigations have highlighted the cytotoxic potential of the AMPK activator, AICAR in T-ALL and B-ALL cell lines.[52–54] However, as far as we know, this is the first report that documents the cytotoxicity of metformin against both T-ALL cell lines and patients lymphoblasts, including cell subsets that could be enriched in LIC. Nevertheless, a word of caution is necessary. In all the preclinical studies, which have been performed to explore the *in vitro* antineoplastic activity of metformin, the drug has shown efficacy in the lower millimolar rage (1–10 mM). Also in our preclinical models of T-ALL, the *in vitro* IC_{50} for metformin ranged from 1.6 to 5.6 mM in cell lines and from 0.6 to 0.9 mM in T-ALL patient samples. The plasma metformin concentration in clinical use for diabetic patients is $\sim$10 μM and normally does not exceed 50 μM.[55] Therefore, higher concentrations could be toxic to normal cells. However, it is encouraging that when metformin was combined with doxorubicin in preclinical models of breast cancer, there was a strong synergism and the drug was effective at a concentration (0.1 mM) that was not toxic to untransformed cells.[51] It is also intriguing that in this study, the combined treatment reduced tumor mass and prevented tumor relapse more effectively that either drug alone in a xenograft mice model. Mice remained tumor free for at least 2 months after the combined treatment was ended. Moreover, a recent study[56] has documented that the positive charge of metformin could promote its accumulation within the mitochondrial matrix by 1000-fold (>20 mM). It has also been demonstrated that metformin accumulates in tissues at concentrations several fold higher than those in blood,[57] indicating that AMPK-related active concentrations of metformin employed in preclinical settings might be attained also during cancer treatment in humans.

The relevance of AMPK as a potential target for innovative therapy of T-ALL is also underscored by a recent study, which has documented that in a mouse T-ALL model, overexpressed miR-19 led to downregulation of several target genes, including PTEN, PP2A subunit B56ε and Prkka1, which encodes for AMPK. This resulted in stimulation of PI3K/Akt/mTORC1 signaling through different mechanisms.[58]

Several clinical trials of metformin as a cancer therapy have been completed or are currently ongoing (http://www.clinicaltrials.gov), mainly as a neoadjuvant therapy in breast and prostate cancer. However, a clinical trial is underway in which metformin was combined with vincristine, dexamethasone, doxorubicin and PEG–asparaginase for relapsed childhood ALL (NCT01324180). These trials support the feasibility of using metformin in cancer patients, although the optimal concentrations of this drug required for achieving antitumor activity *in vivo* have yet to be determined. Overall, our findings document a potent anti-tumor role for the LKB1/AMPK/mTORC1 pathway in T-ALL, which is markedly activated by metformin and involves the repression of mTORC1-dependent oncogenic mRNA translation. Moreover, from the perspective of clinical development, metformin barely impacted upon the proliferation of $CD4^+$T-lymphocytes from healthy donors, strongly indicative of a favorable therapeutic index *in vivo*. We have thus provided a strong rationale for the development of AMPK agonists as novel therapeutic agents for T-ALL patients.

Conflict of interest

The authors declare no conflict of interest.

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