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ORIGINAL ARTICLE

GILZ inhibits the mTORC2/AKT pathway in BCR-ABL$^+$ cells

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The malignant phenotype of chronic myeloid leukemia (CML) is due to the abnormal tyrosine kinase activity of the BCR-ABL oncoprotein, which signals several downstream cell survival pathways, including phosphoinositide 3-kinase/AKT, signal transducer and activator of transcription 5 and extracellular signal-regulated kinase 1/2. In patients with CML, tyrosine kinase inhibitors (TKIs) are used to suppress the BCR-ABL tyrosine kinase, resulting in impressive response rates. However, resistance can occur, especially in acute-phase CML, through various mechanisms. Here, we show that the glucocorticoid-induced leucine zipper protein (GILZ) modulates imatinib and dasatinib resistance and suppresses tumor growth by inactivating the mammalian target of rapamycin complex-2 (mTORC2)/AKT signaling pathway. In mouse and human models, GILZ binds to mTORC2, but not to mTORC1, inhibiting phosphorylation of AKT (at Ser473) and activating FoxO3a-mediated transcription of the pro-apoptotic protein Bim; these results demonstrate that GILZ is a key inhibitor of the mTORC2 pathway. Furthermore, CD34$^+$ stem cells isolated from relapsing CML patients underwent apoptosis and showed inhibition of mTORC2 after incubation with glucocorticoids and imatinib. Our findings provide new mechanistic insights into the role of mTORC2 in BCR-ABL$^+$ cells and indicate that regulation by GILZ may influence TKI sensitivity.
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Introduction

Chronic myeloid leukemia (CML) results from oncogenic activity of the BCR–ABL fusion protein. The downstream effectors of BCR-ABL include the JAK/signal transducer and activator of transcription, Raf/MEK/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathways, all of which affect cell viability, cell-cycle progression and leukemogenesis (Steelman *et al*., 2008). mTOR acts through its inclusion in two distinct signaling complexes: mTOR complex 1 (mTORC1/Raptor), which responds to growth signals and nutrients, and mTOR complex 2 (mTORC2/Rictor), which primarily responds to growth signals (Guertin and Sabatini, 2007; Zeng *et al*., 2007; Kharas *et al*., 2008). Inhibition of mTORC1 by rapamycin can result in increased mTORC2 activity, which results in increased AKT activity and activation of anti-apoptotic pathways. Recent data have indicated that the mTORC2 complex is activated in BCR-ABL$^+$ cells and has an important role in growth and survival (Carayol *et al*., 2010; Mancini *et al*., 2010; Vakana *et al*., 2010). Inhibition of mTORC1 by rapamycin may result in an increase in mTORC2 activity, leading to increased AKT activity and activation of anti-apoptotic pathways; only drugs that target both mTORC2 and mTORC1 have an anti-leukemic effect. Therefore, mTORC2 seems to be a key factor in BCR-ABL-induced oncogenesis, and factors that regulate mTORC2 may also limit the downstream effects of BCR-ABL signaling.

The glucocorticoid-induced leucine zipper (GILZ) protein is an essential mediator of glucocorticoid action (Ayroldi and Riccardi, 2009). GILZ has been studied extensively in T cells, and it has been shown to have both pro- and anti-apoptotic properties (D'Adamio *et al*., 1997; Asselin-Labat *et al*., 2004; Delfino *et al*., 2004). GILZ also inhibits two of the main pathways involved in oncogenesis, namely the nuclear factor-κB pathway and the Raf/Ras/ERK pathway (Ayroldi *et al*., 2001, 2002). In addition, when GILZ binds to Ras/Raf, AKT phosphorylation is reduced, suggesting that GILZ may also affect the AKT survival pathway (Ayroldi *et al*., 2007). However, the role of GILZ in BCR-ABL$^+$ cells has not been evaluated.

In this paper, we investigated the role of GILZ on AKT activation in BCR-ABL$^+$ cells. We found that some imatinib-resistant BCR-ABL$^+$ cells have reduced expression of GILZ and that increasing GILZ expression by transfection or by glucocorticoid treatment overcame imatinib resistance and suppressed BCR-ABL$^+$ tumor growth through mTORC2 inhibition. We also demonstrate that GILZ interacts with mTORC2, inhibiting P-AKT (Ser473) and activating FoxO3a-mediated transcription of the pro-apoptotic

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protein Bim. These results establish GILZ as a key inhibitor of the mTORC2 pathway in BCR-ABL$^+$ cells.

Results

GILZ expression overcomes imatinib resistance

We investigated whether the GILZ protein was expressed in different BCR-ABL$^+$ leukemia cell lines. In the DA1-3b/M0 mouse cell line (referred to as 'M0' hereafter) originally obtained after transfection of BCR-ABL into myeloid DA1 cells, BCR-ABL expression was associated with decreased GILZ expression (Figure 1a). The imatinib-resistant cell line DA1-3b/M1 (referred to as 'M1' hereafter), which was derived from M0 cells after prolonged selection with imatinib and carries an E255K BCR-ABL mutation, and the DA1-3b/d365 cell line (referred to as 'd365' hereafter), which was derived from M0 cells injected in mice and isolated after 1 year of tumor dormancy and harbors partial resistance to imatinib, also exhibited decreased GILZ expression (Figure 1a) (Saudemont *et al*., 2007; Liu *et al*., 2008). Although GILZ protein expression was decreased,

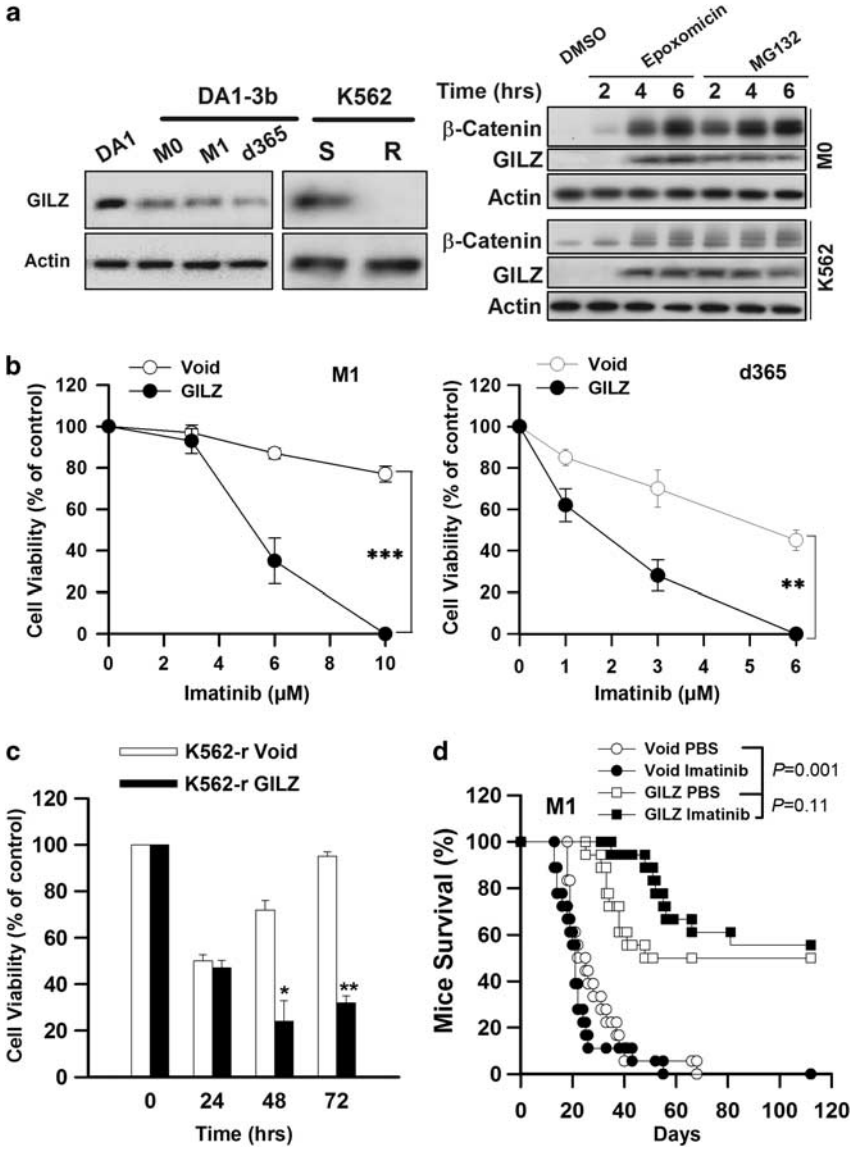

Figure 1 GILZ expression overcomes imatinib resistance. (**a**) Left panel: western blot (WB) analysis of GILZ expression in the indicated mouse (DA1-3b) and human (K562-r) cell lines. Right panel: GILZ expression in M0 and K562-r cells after addition of proteasome inhibitors (50 nM epoxomicin or 1 μM MG132). β-Catenin was used as a control for proteasome inhibition. (**b**) Cell viability measured using CMXRos retention in Void- and GILZ-M1 and d365 cells (with or without imatinib treatment). $^{**}P<0.01$, $^{***}P<0.001$, Kruskal–Wallis test. (**c**) Viability of K562-GILZ and K562-Void cells incubated with 1 μM imatinib. $^{*}P<0.05$, $^{**}P<0.01$, Kruskal–Wallis test. (**d**) Survival curve of female C3HeOuJ mice injected i.p. with 1×10^6 M1-GILZ or M1-Void cells and treated with repeated injections of PBS or imatinib. The survival of mice injected with GILZ cells was statistically different from that of mice injected with Void cells ($P=0.001$, Log-Rank test). The graphs represent mean ± s.d. of three separate experiments performed in triplicate.

GILZ mRNA levels were not, indicating that the mechanism of decreased GILZ expression occurs at the post-transcriptional level (Supplementary Figure S1a). Dexamethasone was still able to induce GILZ mRNA in M0 and M1 cells, showing that these cells remained sensitive to glucocorticoids (Supplementary Figure S1b). MG132 and epoxomicin administered for various lengths of time (Figure 1a, right panel) at various concentrations (Supplementary Figure S1c) restored GILZ expression, confirming that the proteasome has a role in regulating GILZ expression, as previously reported in thymocytes (Delfino *et al*., 2010).

We explored the effects of increasing GILZ expression by stably transfecting a vector expressing GILZ or an empty vector into cells (referred to as 'GILZ-transfected' and 'Void-transfected', respectively). Forced GILZ expression through stable plasmid transfection led to higher levels of GILZ than those observed in the cell lines (Supplementary Figure S1d). In M1 and d365 cells, imatinib induced massive apoptosis in GILZ-transfected cells but not in Void-transfected cells (Figure 1b). Similar results were also obtained with the human K562-r cell line, which was originally generated by long-term selection with imatinib; the mechanism of resistance in K562-r cells is not yet known (Pocaly *et al*., 2008; Figures 1a and c).

Staurosporine (STS), another tyrosine kinase inhibitor (TKI) that targets a different spectrum of kinases than imatinib, also induced apoptosis in GILZ-transfected but not in Void-transfected M1 cells (Supplementary Figures S2a–c) and had no effect on BCR-ABL phosphorylation (Supplementary Figure S2d), suggesting that GILZ may have a more general role in facilitating apoptosis.

These results were confirmed *in vivo* in our mouse model (Figure 1d). Compared with mice injected with Void-transfected M1 cells, fewer mice injected with GILZ-transfected M1 cells and treated with imatinib or vehicle developed leukemia. This result was confirmed by the absence of dormant tumor cells in mice killed 9 or 12 months after injection, as reported previously (Saudemont and Quesnel, 2004).

Similar results were observed using the double imatinib/dasatinib-resistant line DA1-3b/M2 (referred to as 'M2'), which carries an additional T315I mutation, which confers broad resistance to TKIs. Dexamethasone was able to induce GILZ mRNA in M2 cells (Supplementary Figure S1b). Ectopic GILZ expression did not modify resistance to dasatinib but restored imatinib and STS sensitivity (Figure 2a), and these results were confirmed *in vivo* (Figure 2b). Mice injected with GILZ-transfected M2 cells and treated with imatinib manifested delayed leukogenesis when compared with mice injected with GILZ-transfected cells treated with dasatinib or mice injected with Void-transfected M2 cells and treated with either imatinib or dasatinib.

Taken together, our results suggest that GILZ sensitizes cells to the off-target effects of imatinib, and that GILZ overexpression suppresses BCR-ABL oncogenicity, even when the tumor has become TKI resistant.

GILZ upregulates Bim expression in imatinib-resistant, BCR-ABL$^+$ cells

On the basis of CMXRos measurements, imatinib-induced cell death of GILZ-transfected M1 cells was

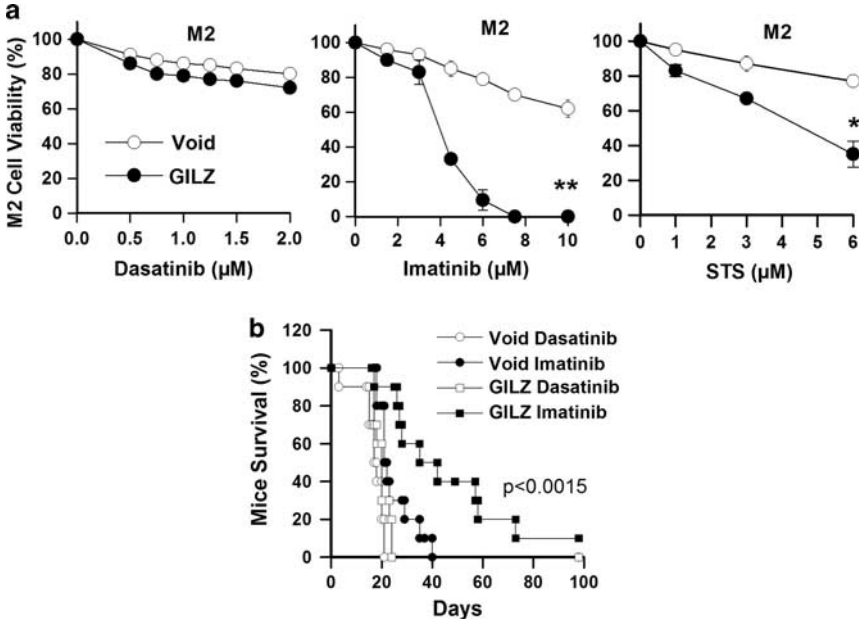

Figure 2 GILZ restores imatinib sensitivity in dasatinib-resistant M2 cells. (**a**) Cell viability of M2-GILZ and M2-Void cells exposed to dasatinib, imatinib or staurosporine (STS) for 24 h. $^{**}P<0.01$, $^{*}P<0.05$, Kruskal–Wallis test. (**b**) Survival curve of female C3HeOuJ mice injected i.p. with 1×10^6 M2-GILZ or M2-Void cells treated with daily i.p. injections of imatinib (75 mg/kg) or dasatinib (15 mg/kg). The survival of mice injected with GILZ cells and imatinib was statistically different from that of all other injected mice ($P<0.0015$, Log-Rank test).

related to the loss of mitochondrial transmembrane potential (Figure 1b). We confirmed apoptosis based on sub-G1 DNA content and presence of cleaved caspase 3 (Supplementary Figures S3a and b). Furthermore, inhibition of apoptosis by the pan-caspase inhibitor Z-VAD-fmk indicated that caspases were involved (Supplementary Figure S3c). In M1 cells, analysis of Bcl-2 family members indicated that GILZ overexpression resulted in downregulation of Bcl-2 protein and upregulation of Bim mRNA and protein levels but had no effect on the parental BCR-ABL DA1 cell line (Figures 3a and b). These results were confirmed by the absence of increased cell death induced by imatinib in GILZ-transfected DA1 cells (Supplementary Figure S3d) and suggested that induction of cell death by GILZ is dependent on BCR-ABL. Similar results were observed in K562-r cells (Figure 3c).

Imatinib did not further modify Bcl-xL, Bcl-2 or Bim expression in GILZ-transfected M1 cells or in Void-transfected M1 cells (Figure 3d). In GILZ-transfected cells, imatinib downregulated Mcl-1, an anti-apoptotic factor that has been identified as a BCR-ABL-dependent target in CML (Aichberger *et al*., 2005). Therefore, by increasing Bim expression, GILZ predisposes imatinib-resistant cells to imatinib-induced apoptosis by changing the Mcl-1/Bim ratio.

GILZ inhibits the P-AKT (Ser473) pathway in BCR-ABL$^+$ cells

The above results demonstrate that transfection of GILZ alone affects the protein level of Bim. The transcriptional upregulation of Bim is mediated by FoxO3a (Essafi *et al*., 2005), and FoxO3a is inactivated by phosphoinositide 3-kinase/AKT/mTOR signaling (de Jong *et al*., 1997; Dijkers *et al*., 2002). GILZ did not directly affect the kinase activity of BCR-ABL and did not affect signal transducer and activator of transcription 5 and CRKL, the downstream effectors of the BCR-ABL pathway (Figure 4a). Using western blotting and a PathScan sandwich enzyme-linked immunosorbent assay kit, we found that in BCR-ABL$^+$ M1 cells, GILZ overexpression strongly downregulated phosphorylation of AKT (Ser473), a specific substrate of mTORC2 (Sarbassov *et al*., 2005; Figures 4b and g). Consequently, phosphorylation of the downstream effector of AKT FoxO3a (Ser253 and Thr32) was also decreased (Figure 4b; Hietakangas and Cohen, 2007). In contrast, in the parental BCR-ABL DA1 cell line, AKT was not phosphorylated on Ser473, confirming that AKT (Ser473) is a downstream target of BCR-ABL (Figure 4b; Sharma *et al*., 2006). The levels of P-FoxO3a (Ser253 and Thr32) and total AKT were not altered, and GILZ overexpression did not modify these effects. In parental DA1 cells, GILZ overexpression also reduced the phosphorylation of both the p44 and p42 forms of ERK (Figure 4b), as published previously (Ayroldi *et al*., 2007). In contrast, in BCR-ABL$^+$ M1 cells, GILZ expression did not affect ERK phosphorylation or that of its downstream substrate, Bad (Ser112) (Figures 4b and c).

AKT may also be phosphorylated on Thr308 by 3-phosphoinositide-dependent protein kinase-1 (PDK1); this step is required for Bad (Ser136) phosphorylation and

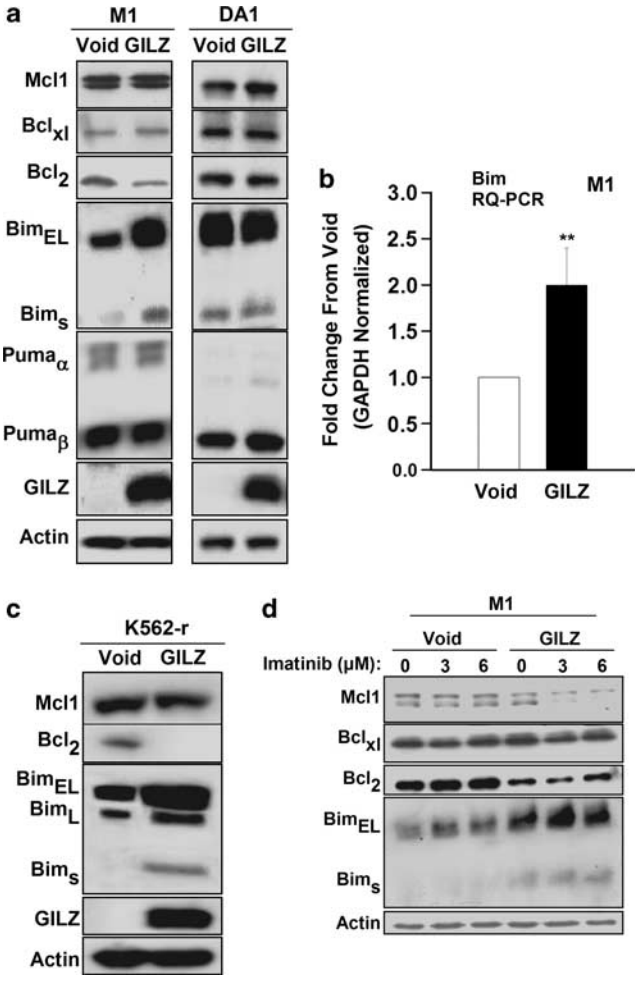

Figure 3 GILZ increases Bim expression in BCR-ABL$^+$ cells. (**a**) WB analysis of Bcl-2 family members in GILZ- and Void-M1 and Void-DA1 cells. (**b**) Real-time PCR analysis of Bim expression in M1-GILZ cells relative to M1-Void cells. $^{**}P<0.01$, Kruskal–Wallis test. (**c**) K562-r cells transfected with human GILZ were analyzed as described in panel **a**. (**d**) WB analysis of Bcl-2 family members in GILZ- and Void-M1 and Void-DA1 cells exposed for 12 h to imatinib. The graphs represent mean ± s.d. of three separate experiments performed in triplicate.

mTORC1 activity towards its downstream substrate S6 (Ly *et al*., 2003). GILZ overexpression did not affect the levels of P-Bad (Ser136), P-S6, P-Mdm2 (Figure 4c) or Mcl-1 (Figures 3a and c). GILZ overexpression also failed to affect AKT Thr308 phosphorylation in DA1 cells (Supplementary Figure S4). These results suggest that GILZ alone does not interfere with the PDK1/AKT/mTORC1 pathway or with the MEK/ERK pathway (Wang *et al*., 2005; Mills *et al*., 2008; Ikenoue *et al*., 2009). Similar results were observed in human K562-r cells (Figure 4d). mTORC1 can differentially affect S6K and 4E-BP phosphorylation, but we did not observe changes in the phosphorylation of S6K or 4E-BP in K562-r and M1 cells overexpressing GILZ (Figures 4e and f). mTORC2 has also been reported to phosphorylate protein kinase C-alpha (PKCα) and serum- and glucocorticoid-inducible kinase (SGK). GILZ-overexpressing K562-r and M1 cells showed reduced protein kinase C-alpha (PKCα) and SGK phosphorylation,

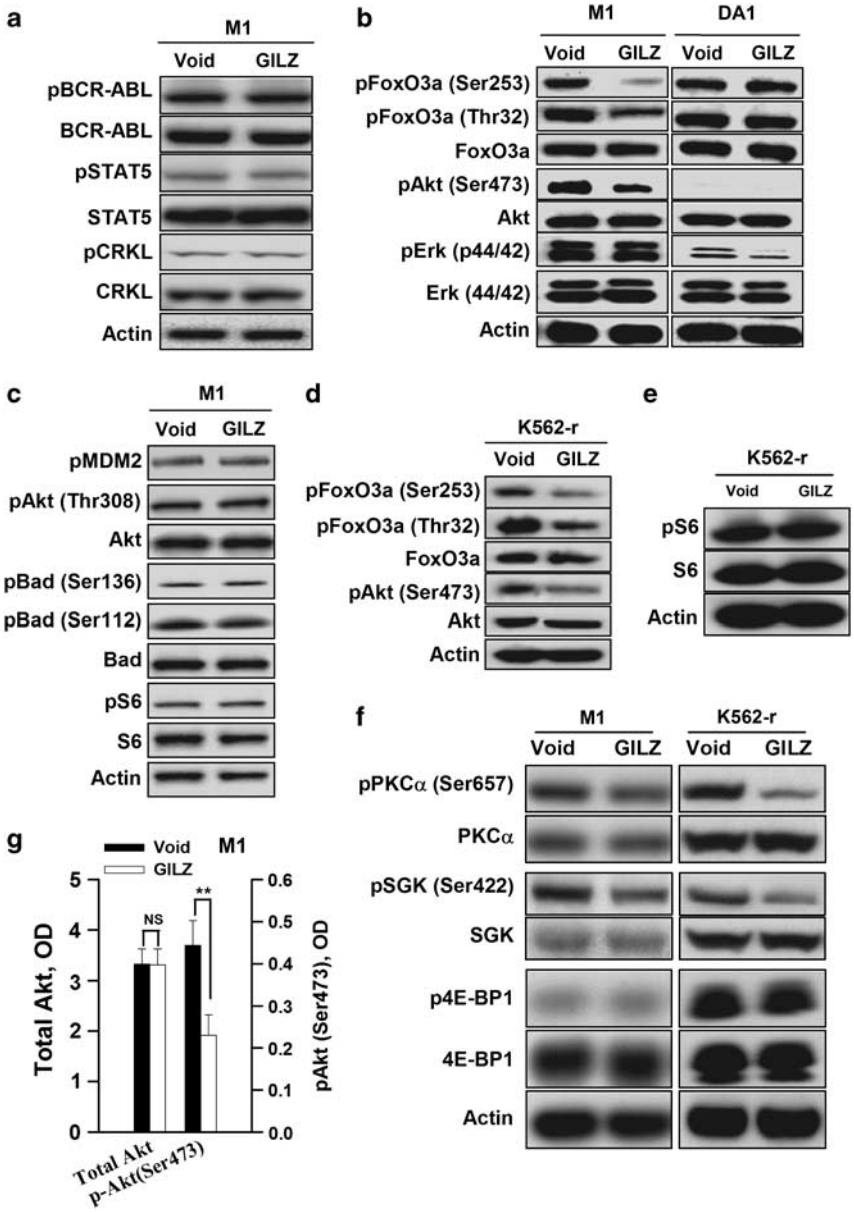

Figure 4 GILZ deregulates AKT (Ser473) phosphorylation in imatinib-resistant BCR-ABL$^+$ cells. (**a**) WB analysis of BCR-ABL, CRKL and STAT5 phosphorylation in GILZ- or Void-transfected M1 cells. (**b**) WB analysis of FoxO3a, AKT (Ser473) and Erk44/42 phosphorylation in transfected M1 and DA1 cells. (**c**) WB analysis of MDM2, AKT (Thr308), Bad and S6 phosphorylation in transfected M1 cells. (**d**) WB analysis of FoxO3a and AKT (Ser473) phosphorylation in transfected K562-r cells. (**e**) WB analysis of S6 phosphorylation in transfected K562-r cells. (**f**) WB analysis of PKCα (Ser657), SGK (Ser422) and 4E-BP1 phosphorylation in M1 (Void and GILZ) and K562-r (Void and GILZ) cells. The loading controls were actin and unphosphorylated PKCα, SGK and 4E-BP1. (**g**) ELISA analysis of P-AKT1 (Ser473) and total AKT in Void- versus GILZ-transfected M1 cells. $^{**}P<0.01$, Kruskal–Wallis test. The error bars represent mean ± s.d. of three separate experiments performed in triplicate.

confirming that GILZ-mediated inhibition of mTORC2 affected several pathways downstream of mTORC2 (Figures 4e and f). In summary, our data provide evidence that GILZ inhibits mTORC2 activity in BCR-ABL$^+$ cells. Therefore, we next investigated whether GILZ exerts its effects by directly interacting with mTORC2.

GILZ binding to mTORC2 inhibits mTORC2 activity

Using mTOR as bait for an immunoprecipitation, we found that GILZ was a component of mTORC2 (Figure 5a). GILZ co-precipitated with mTOR, Rictor and HSP70 (all of which are mTORC2 components) but not with Raptor (an mTORC1 component). Furthermore, Rictor co-precipitated with mTOR, HSP70 and GILZ but not with Raptor (Martin *et al*., 2008). We were able detect an endogenous GILZ–mTORC2 interaction in M1 cells treated with dexamethasone (Figure 5b). Similar results were observed in GILZ-transfected K562-r cells, indicating that the GILZ–mTORC2 interaction is not cell line specific (Figure 5c). Immunoprecipitation using mSin1, a component that is specific to mTORC2, as bait demonstrated that GILZ bound all mTORC2

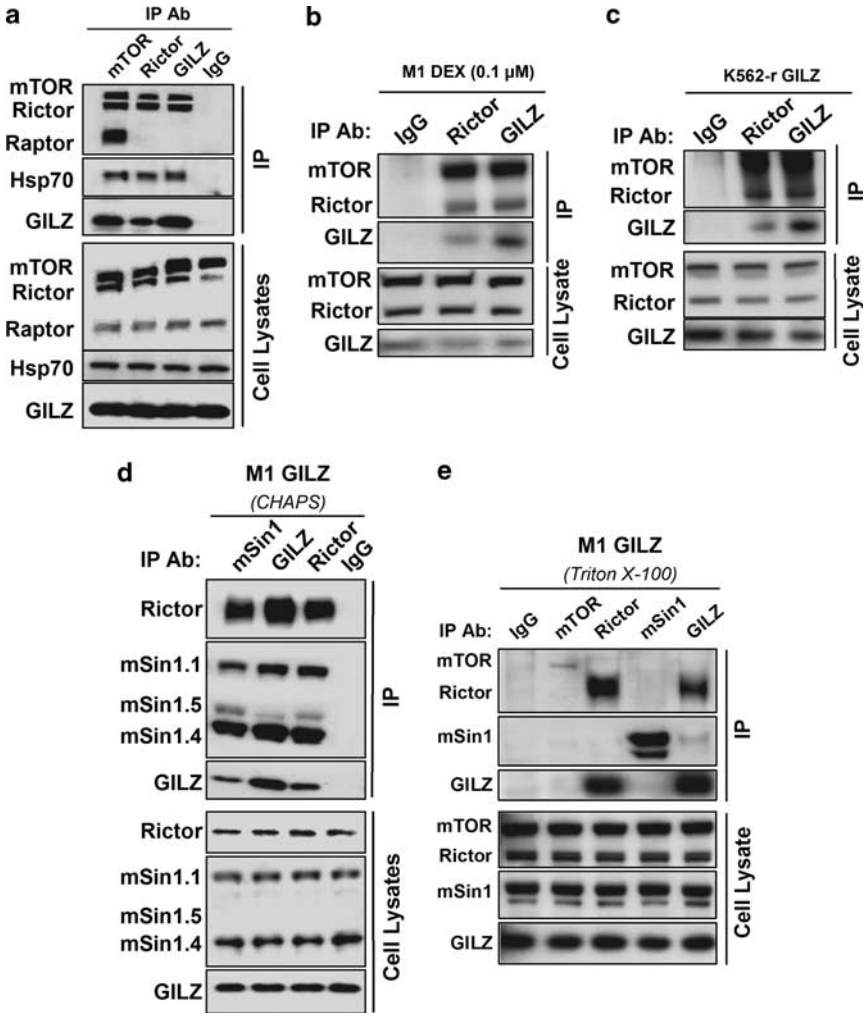

Figure 5 GILZ interferes with the mTORC2/AKT pathway. (**a**) Co-IP: M1-GILZ cells were lysed in CHAPS buffer, and immunoprecipitations (IP) were performed using anti-mTOR, anti-Rictor, anti-GILZ and control (nonspecific) antibodies. Immunoprecipitates and cell lysates were subjected to WB analysis using anti-mTOR, anti-Rictor, anti-HSP70 and anti-GILZ antibodies (**b**) As described in panel **a**, but with M1 cells pretreated with dexamethasone (DEX) for 24 h and using anti-Rictor, anti-GILZ and control IgG antibodies. (**c**) Same as in panel **b**, but using K562-r-GILZ cells. (**d**) Co-IP: M1 GILZ cells were lysed in CHAPS buffer, and immunoprecipitations were performed using anti-mSin1, anti-GILZ, anti-Rictor and control (nonspecific) antibodies. The immunoprecipitates and cell lysates were subjected to WB analysis using anti-Rictor, anti-mSin1 and anti-GILZ antibodies. (**e**) Same as in panel **d**, except that cells were lysed in a buffer that contained Triton X-100, which disrupts the interaction between mTOR and Rictor.

complexes (Figure 5d; Frias *et al*., 2006). Like the Raptor–mTOR interaction, the Rictor–mTOR interaction is sensitive to Triton X-100 but is stable in CHAPS-containing buffers (Sarbassov *et al*., 2004). The GILZ–Rictor interaction was not destabilized in the presence of Triton X-100, suggesting that GILZ bound Rictor preferentially and independently of mTOR (Figure 5e). Silencing of mSin1 or Rictor expression led to a significant reduction in the amount of GILZ associated with mTORC2, suggesting that GILZ interaction with mTORC2 requires Rictor and/or mSin1 (Figures 6a and b). As mTORC2 is known to be sensitive to prolonged rapamycin treatment, and because rapamycin enhances the effects of imatinib, we performed mTOR and Raptor immunoprecipitations in lysates from GILZ-transfected M1 cells treated with rapamycin for 24 h (Mohi *et al*., 2004). Rapamycin profoundly affected the interaction between GILZ and mTOR complexes (Figure 6c). Furthermore, incubation of purified GILZ with mTORC2 inhibited AKT (Ser473) phosphorylation in an *in vitro* kinase assay (Figure 6d). This was confirmed using myc-tagged recombinant human GILZ and recombinant, active human AKT1 (Figure 6e). Taken together, these data suggest that GILZ is a novel mTORC2 component that acts to inhibit mTOR kinase activity in BCR-ABL$^+$ cells.

Modulation of imatinib resistance by GCs in BCR-ABL$^+$ myeloid cells

As the ectopic expression of GILZ in imatinib-resistant, BCR-ABL$^+$ myeloid cells was able to induce apoptosis in combination with imatinib or STS, we investigated whether glucocorticoids (GCs), which are the main physiological inducers of GILZ expression, could also

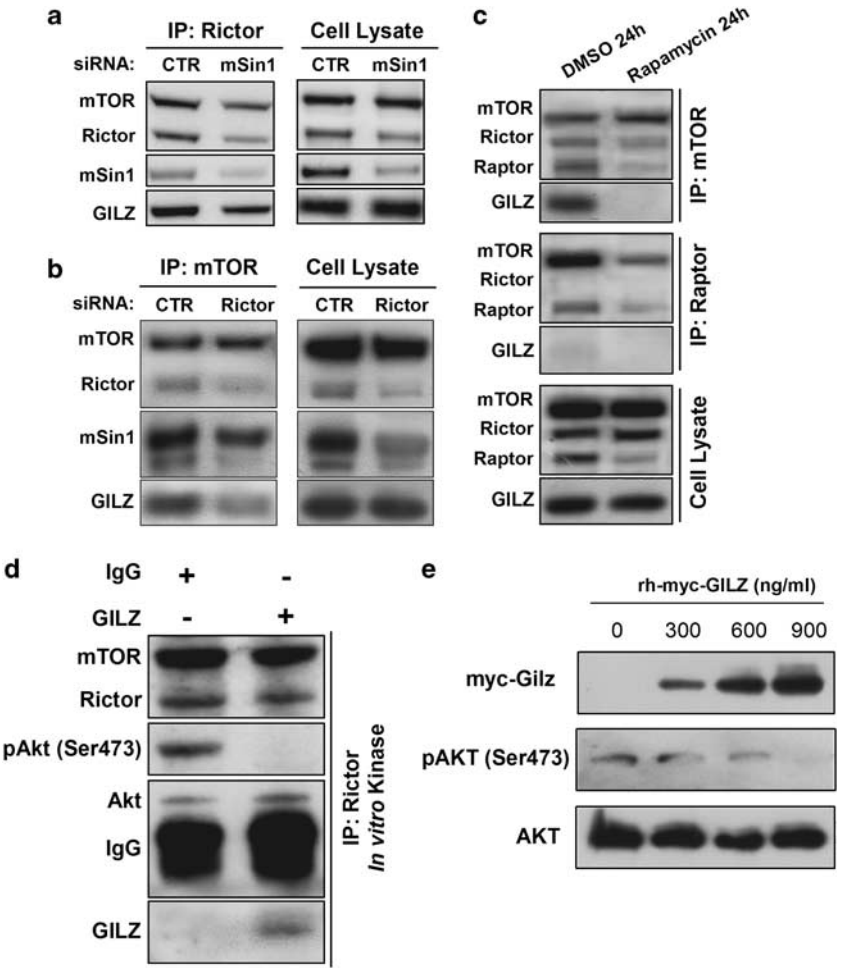

Figure 6 GILZ interacts with mTORC2. (**a**) mSin1 or scrambled (CTR) siRNA was transfected into M1 GILZ cells. One day post transfection, cells were lysed, and immunoprecipitation was performed using an anti-Rictor antibody, as described previously. (**b**) Rictor or scrambled (CTR) siRNA was transfected into M1 GILZ cells. One day post transfection, cells were lysed, and immunoprecipitation was performed using an anti-mTOR antibody, as described previously. (**c**) M1 GILZ cells were treated for 24 h with 100 nM rapamycin. Cell lysates (lower panel) and mTOR or Raptor immunoprecipitates (upper panels) obtained from cell lysates were analyzed for levels of the indicated proteins using WB. (**d**) *In vitro* kinase assay: mTORC2 was purified from M1 cells using an anti-Rictor antibody. mTORC2 kinase assays containing anti-GILZ or IgG immunoprecipitates were then performed using AKT as a substrate. (**e**) *In vitro* kinase assay: various concentrations of myc-tagged human recombinant GILZ were preincubated with immunoprecipitated Rictor, and recombinant, active human AKT1 was added.

modulate imatinib resistance. In mouse and human cell lines and in $CD34^+$ cells from six relapsing CML patients (Table 1), sequential treatment with dexamethasone (a potent GC agonist) followed by imatinib modestly reduced cell viability in M1, M2 and K562-r cells and in five of six patients when compared with treatment with imatinib alone (Figures 7a–c and e). M1 and M2 cell lines were also slightly sensitive to treatment with dexamethasone alone (Figures 7a and c). This effect was associated with decreased phosphorylation of AKT (Ser473) and increased expression of Bim_{EL} and Bim_S (Figure 7d, Supplementary Figure S5). Therefore, GCs may modulate apoptosis in BCR-ABL^+ myeloid cells.

GILZ small-interfering RNA treatment only partially reduced GILZ expression and modestly inhibited the mortality caused by sequential treatment (Supplementary Figure S6). Thus, GILZ likely contributes to dexamethasone-induced mortality, but we cannot rule out the possibility that other pathways may be involved.

Table 1 Clinical features of the patients whose samples were used for *in vitro* sequential glucocorticoid/imatinib treatment

Patient no.	*Source*	*Disease phase*	*Resistance type*
1	Bone marrow	Chronic	Non-mutated
2	Bone marrow	Accelerated	Non-mutated
3	Blood	Blastic	T315I mutation
4	Blood	Blastic	T315I mutation
5	Blood	Blastic	Non-mutated
6	Bone marrow	Accelerated	E255K mutation

Discussion

The signaling pathways downstream of BCR-ABL are essential to leukemogenesis. Among these pathways, AKT has a critical role in survival and proliferation. Recent reports demonstrating that dual inhibition of the mTORC1 and mTORC2 complexes is effective in

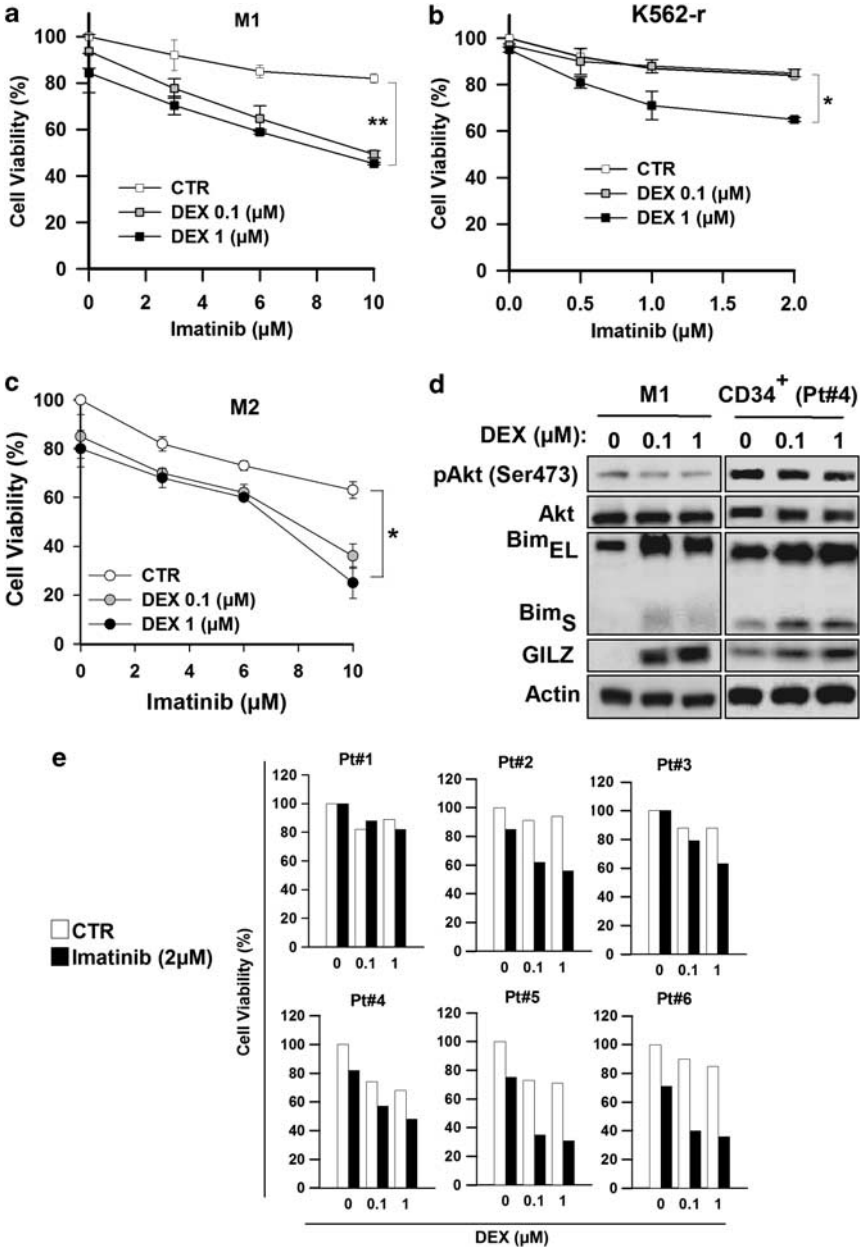

Figure 7 Sequential GC/imatinib treatment causes apoptosis in imatinib-resistant CML CD34$^+$ cells. (**a**) M1 cells were treated with dexamethasone for 24 h and then exposed to imatinib for 24 h. $^{**}P<0.001$, Kruskal–Wallis test. (**b**) Same as in panel **a**, except that K562-r cells were used ($^*P<0.05$, Kruskal–Wallis test). (**c**) Same as in panel **a**, but M2 cells were used. (**d**) WB analysis of P-AKT (Ser473), Bim and GILZ in M1 and CD34$^+$ cells (Pt no. 4) treated with the indicated concentrations of dexamethasone for 24 h. (**e**) Same as in panel **a**, except that CD34$^+$ cells from six imatinib-resistant patients were used. The graphs represent mean ± s.d. of three separate experiments performed in triplicate.

BCR-ABL$^+$ cells underscore the importance of AKT regulation (Zeng *et al*., 2007; Liu *et al*., 2008). We investigated GILZ as a possible regulator of BCR-ABL activity and found that GILZ protein expression was decreased in some BCR-ABL$^+$ cell lines (likely through proteasome-mediated degradation), and in a cell line derived from dormant BCR-ABL$^+$ cells. It was not clear whether this downregulation resulted from imatinib resistance mechanisms, because K562-r and d365 cells showed reduced expression, but M1 cells did not. However, these findings suggest that GILZ may have a role in BCR-ABL signaling. Restoration of GILZ expression re-sensitized resistant cells to imatinib, upregulated Bim expression and decreased Bcl-2 expression in human and mouse cells ectopically expressing BCR-ABL. Bim has a crucial role in imatinib-mediated cell death (van Delft *et al*., 2006). It belongs to the BH3-only family of pro-apoptotic Bcl-2 proteins and antagonizes prosurvival Bcl-2 proteins. Drugs mimicking BH3-only proteins trigger Bax/Bak-mediated apoptosis and may overcome chemoresistance by binding tightly to anti-apoptotic Bcl-2 proteins. Our data suggest that enhanced GILZ

expression sensitizes TKI-resistant cells to apoptosis through Bim. Moreover, apoptosis induced by GILZ in imatinib- and dasatinib-resistant cells indicates that imatinib may suppress unidentified kinases other than BCR-ABL, leading to its synergy with GILZ. This hypothesis was also suggested by the finding that MCL1 expression decreased only when GILZ-transfected cells were exposed to imatinib, suggesting that the same synergy between GILZ and imatinib may also induce apoptosis through modification of the Bim/MCL1 ratio. STS, another TKI that targets a different spectrum of kinases than imatinib, also induced apoptosis in GILZ-transfected cells and had no effect on BCR-ABL phosphorylation, suggesting that synergy with GILZ-induced apoptosis is not restricted to imatinib. Therefore, GILZ is a critical regulator of apoptosis in BCR-ABL$^+$ cells.

As Bim was upregulated in GILZ-transfected cells, we investigated whether the pathways that control Bim expression are regulated by GILZ. Bim expression and activity is modulated by numerous factors, including ERK and FoxO3a. FoxO3a is a member of the FOXO subfamily of forkhead transcription factors that regulate cell survival and apoptosis. In the presence of survival factors, AKT is phosphorylated and activated and in turn phosphorylates FoxO3a, leading to its association with 14-3-3 proteins, nuclear exclusion, as well as retention and degradation in the cytoplasm. Inhibition of AKT phosphorylation leads to FoxO3a dephosphorylation, nuclear translocation and activation of Bim transcription, resulting in apoptosis. In BCR-ABL$^+$ cells transfected with GILZ, decreased phosphorylation of FoxO3a and increased mRNA and protein expression of Bim was observed. ERK-mediated phosphorylation of Bim_{EL} has been reported to promote its dissociation from MCL1, leading to proteasome-mediated degradation of Bim_{EL}. However, in our experiments, the ectopic expression of GILZ in BCR-ABL$^+$ cells did not inhibit signaling through the ERK pathway. Therefore, Bim expression is modulated through phosphorylation of FoxO3a in GILZ-expressing cells.

GILZ possesses multiple functions and can affect ERK, AP-1 and nuclear factor-κB signaling (Ayroldi and Riccardi, 2009). Here, we show that in addition to these previously reported effects, GILZ inhibits mTORC2/AKT activity in the presence of BCR-ABL. In BCR-ABL DA1 cells, GILZ decreased ERK activity, as previously reported in other BCR-ABL cell types (Ayroldi *et al*., 2007), but did not affect FoxO3a phosphorylation. However, in BCR-ABL$^+$ cells, GILZ inhibited the AKT pathway but not the ERK pathway. In BCR-ABL$^+$ cells, survival depends more on the AKT pathway than on the ERK pathway (Yu *et al*., 2002; Kawauchi *et al*., 2003). Therefore, GILZ-mediated inhibition of AKT is more likely to induce apoptosis in BCR-ABL$^+$ cells. However, the mechanism underlying the differential inhibitory effects of GILZ in BCR-ABL$^+$ and BCR-ABL cells remains unclear.

The mammalian target of rapamycin is a component of the phosphatase and tensin homolog/phosphoinositide 3-kinase/AKT signaling pathway (Essafi *et al*., 2005; Polak and Hall, 2006; Sabatini, 2006; Toker and Yoeli-Lerner, 2006; Hietakangas and Cohen, 2007; Kharas *et al*., 2008). Our study indicates that GILZ inhibits the downstream functions of mTORC2/P-AKT (Ser473), including FoxO3a, PKCα and SGK phosphorylation, but does not affect the mTORC1 pathway. We demonstrated that GILZ bound mTORC2, but not mTORC1, as illustrated by its co-immunoprecipitation with mTOR, Rictor, mSin1 and HSP70. Our results also suggest that Rictor is essential for GILZ binding to mTORC2, but we cannot rule out that other proteins in this complex may be involved. Taken together, these results establish GILZ as an inhibitor of mTORC2.

GILZ is induced by GCs, and treatment of TKI-resistant BCR-ABL$^+$ cells with GCs followed by imatinib resulted in apoptosis. GC-mediated induction of GILZ also modulated the mTORC2/AKT/FoxO3a/Bim pathway. Although their effects have been more widely studied in lymphoid cells, GCs can also induce cell death or differentiation in the myeloid–monocyte lineage and have been shown to induce apoptosis in AML cells, including BCR-ABL$^+$ cells (Miyoshi *et al*., 1997; Schmidt *et al*., 2001; Dayyani *et al*., 2003; Hicsonmez, 2006). Although several pathways may be responsible for GC-induced apoptosis in myeloid cells, our results suggest that GILZ may have a role *in vivo*, at least in BCR-ABL$^+$ cells. In addition to GCs, several factors have been reported to be inducers of GILZ expression, including interleukin-10, interleukin-15, stem cell factor (SCF) and transforming growth factor-β, suggesting that stimuli from the microenvironment may sensitize leukemia cells to apoptosis through GILZ (Berrebi *et al*., 2003; Kolbus *et al*., 2003; Perez *et al*., 2005; Cohen *et al*., 2006). GC-induced apoptosis has also been shown to be GILZ dependent in multiple myeloma, suggesting that GILZ may have broader roles (Grugan *et al*., 2008).

In summary, we show that GILZ primes leukemia cells with several types of TKI resistance for death through inhibition of the mTORC2 complex, which leads to Bim upregulation (Figure 8). Therefore, GILZ seems to be an essential regulator of BCR-ABL.

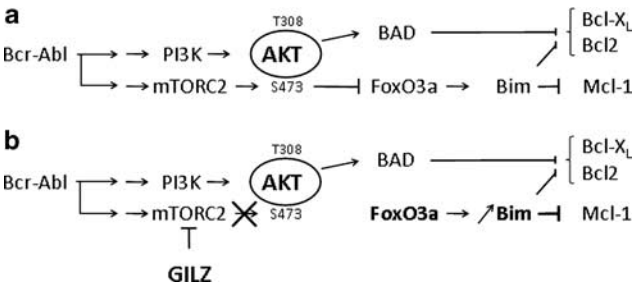

Figure 8 Summary of GILZ's regulatory effects on the AKT pathway in BCR-ABL cells. (**a**) BCR-ABL activates AKT through PI3K and mTORC2. Phosphorylation of AKT at Ser473 inhibits Bim expression and apoptosis through Foxo3A. (**b**) GILZ interacts with mTORC2 and reduces AKT (Ser473) phosphorylation, leading to Bim expression and apoptosis.

Materials and methods

Reagents and antibodies

Imatinib was purchased from Novartis (East Hanover, NJ, USA). Dasatinib was purchased from Bristol-Myers Squibb (New York, NY, USA). Dexamethasone and STS were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and stored at 20 °C as 10 mM stock solutions in ethanol. All other drugs were purchased from LC Laboratories (Woburn, MA, USA). MitoTracker Red CMXRos was purchased from Molecular Probes (Eugene, OR, USA). Z-VAD-FMK, epoxomicin and MG132 were purchased from Calbiochem (Nottingham, UK). Antibodies against Bim, BCL-2, BCL-xl and actin were purchased from Sigma-Aldrich. The GILZ (FL-134), P-SGK (Ser422), SGK and FoxO3a antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). P-FoxO3a (Thr32), P-PKCα (Ser657), PKCα and monoclonal anti-phosphotyrosine (clone 4G10) antibodies were purchased from Upstate (Lake Placid, NY, USA). The mouse MCL-1 antibody was purchased from Rockland (Philadelphia, PA, USA), and the Sin1 and Rictor antibodies were purchased from Abcam (Cambridge, MA, USA). All other antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). ECL anti-mouse IgG HRP-linked whole antibody and ECL anti-rabbit IgG HRP-linked whole antibody were purchased from Amersham Biosciences (Piscataway, NJ, USA). Mouse IgG TrueBlot and rabbit IgG Trueblot were purchased from eBioscience (San Diego, CA, USA) and used as secondary antibodies for immunoblotting of immunoprecipitated proteins.

Cell lines

The leukemic DA1-3b/M0 p210$^{BCR-ABL}$ cell line (referred to here as 'M0') and the DA1-3b/C3HeOuJ mouse model have been described previously (Saudemont and Quesnel, 2004; Saudemont *et al*., 2005). We recently reported the generation of the DA1-3b/M1 cell line (referred to here as 'M1') carrying an E255K mutation and the double imatinib/dasatinib-resistant line DA1-3b/M2 (referred to here as 'M2') with an additional T315I mutation (Liu *et al*., 2008). Using the same model, we previously described a dormant cell line (DA1-3b/d365, referred to here as 'd365') that persisted for a year in mice without causing symptoms and developed resistance to imatinib that was independent of BCR-ABL mutation (Saudemont *et al*., 2007). These cell lines provide a model to examine imatinib resistance and mechanisms necessary to overcome it (Liu *et al*., 2008). Imatinib (1 μM) was added continuously to maintain selective pressure. BCR-ABL parental DA1 cells (obtained from and established by Ihle (1985)) were maintained in the same medium supplemented with 4 ng/ml mouse interleukin-3 (PeproTech, London, UK). The human imatinib-resistant K562 line (K562-r) has been described previously (Pocaly *et al*., 2008). The identity of each cell line was authenticated based on BCR-ABL expression, sequencing analyses and imatinib IC_{50} determinations before beginning the experiments.

Primary CML samples

Bone marrow or peripheral blood was obtained from imatinib-resistant patients during routine blood withdrawal. Donors provided informed consent in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board at the Tumorotheque du Centre Hospitalier et Universitaire de Lille (Centre de Biologie, Lille, France). $CD34^+$ cell separation (>95% purity) was carried out using positive-selection magnetic beads (Miltenyi Biotec, Auburn, CA, USA). Primary cells were cultured in RPMI 1640 supplemented with 1% glutamine, 1% penicillin/streptomycin, 20% fetal calf serum, 200 pg/ml stem cell factor, 10 ng/ml interleukin-6 and 200 pg/ml granulocyte colony-stimulating factor (all from PeproTech).

Plasmids and small-interfering RNA transfection

Human and mouse GILZ cDNAs were cloned into the pVITRO eGFP plasmid (InvivoGen, Toulouse, France). Stably transfected cells were termed 'Void' or 'GILZ'. The mouse Rictor expression plasmid was kindly provided by Michael N Hall (Jacinto *et al*., 2004). The mouse Rictor-, mSIN1-, GILZ- and AKT-specific small-interfering RNAs and the scrambled control small-interfering RNA were obtained from Santa Cruz Biotechnology.

Measurement of AKT phosphorylation

Total AKT1 and P-AKT1 (Ser473) were analyzed using a PathScan sandwich enzyme-linked immunosorbent assay kit (Cell Signaling Technologies) according to the manufacturer's instructions.

In vivo *studies*

Seven- to eight-week-old C3H/HeOuJ female mice (Charles River Laboratories, Lyon, France) were injected intraperitoneally with 1×10^6 Void- or GILZ-transfected M1 cells. Treatment was initiated 24 h after cell injection and consisted of an intraperitoneal injection of phosphate-buffered saline (vehicle) or imatinib (50 mg/kg) twice daily. Imatinib injections were terminated after 1 month because of the death of all mice injected with Void-transfected cells. Quantification of residual disease in surviving mice was performed as described previously (Saudemont and Quesnel, 2004). For M2 experiments, the same protocol was followed, except that treatment consisted of daily intraperitoneal injections of imatinib (75 mg/kg) or dasatinib (15 mg/kg) resuspended in dimethyl sulfoxide/polyethylene glycol 300 (vol/vol). All animal experiments were approved by the Animal Care Ethical Committee CEEA.NPDC (Agreement no. AF-03-2008).

Co-immunoprecipitation and western blot analyses

Whole cell extracts were prepared under non-denaturing conditions using CHAPS lysis buffer (0.3% CHAPS in phosphate-buffered saline). When specified, cell extracts were also prepared using a buffer containing Triton X-100 (Cell Signaling Technologies), which disrupts the interaction between mTOR and Rictor (Sarbassov *et al*., 2004).

In vitro *mTORC2 kinase assays*

The effect of GILZ on the ability of mTORC2 to phosphorylate AKT on Ser473 was determined using the following two different methods:

A previously described method (Sancak *et al*., 2007; Peterson *et al*., 2009): In brief, the mTORC2 complex was purified by lysing M1 cells in CHAPS buffer, and immunoprecipitation was performed using an anti-Rictor antibody. The GILZ protein was purified from GILZ-transfected M1 cells as described above, except for the use of TBS wash buffer containing 0.2% Tween and lysis buffer containing Triton X-100 (Cell Signaling Technologies).Purified human recombinant proteins (GILZ and AKT1): To assess the inhibitory effect of GILZ on phosphorylation of AKT by mTORC2, we preincubated immunoprecipitated human Rictor (5 μl) with various concentration of myc-tagged human recombinant GILZ (Origine; 0, 300, 600 and 900 ng) for 30 min at 30 °C.

Active recombinant human AKT1 (BPS Biosciences, San Diego, CA, USA; 150 ng) was then added, and the mixture was incubated for 20 min at 30 °C before the addition of 200 μM ATP for 20 min at 30 °C in a kinase buffer (Cell Signaling Technologies). The reaction was terminated by addition of 20 μl of 2 × SDS sample buffer to 20 μl of the reaction mixture and heating at 95 °C for 5 min.

Quantitative real-time PCR
Real-time PCR analysis of the BIM transcript was performed using SYBR Green PCR Master Mix (Applied Biosystems, Bedford, MA, USA). The mouse BIM sense primer was 5′-CACCTGCTGTGTGCTTCCTA-3′, and the antisense primer was 5′-GCTGGCCTAAAGCAGTGAAC-3′. The mouse GAPDH (glyceraldehyde 3-phosphate dehydrogenase) sense primer was 5′-CAGCTTCGGCACATATTTCA-3′, and the antisense primer was 5′-TCGTTCACTCCCATGACAAA-3′. Real-time PCR analysis of the GILZ transcript was performed using the TaqMan Gene Expression Assay (Applied Biosystems), as recommended by the manufacturer.

Cell viability and cell-cycle analysis
Apoptosis was analyzed by determining the mitochondrial transmembrane potential, which was quantified based on CMXRos retention. MitoTracker Red CMXRos was purchased from Molecular Probes. When specified, cell viability was also analyzed using the MTT assay (Promega, Madison, WI, USA). Cell-cycle analysis and sub-diploid populations were analyzed using propidium iodide staining and conventional techniques.

Statistics
All data were analyzed using Sigma Stat 3.1 software (SPSS, Chicago, IL, USA).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)