

Modifying Akt Signaling in B-Cell Chronic Lymphocytic Leukemia Cells

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Abstract

Emerging evidence suggests that the survival of B-cell chronic lymphocytic leukemia (CLL) cells is dependent on microenvironmental influences such as antigenic stimulation and support by stromal cells. Akt, also known as protein kinase B, is a central component in prosurvival signaling downstream of these events. We investigated the role of Akt and its modulation by the protooncogene T-cell leukemia 1a (Tcl1a) in the survival pathways of primary CLL samples and CLL-derived prolymphocytic cell lines MEC-1 and MEC-2. Akt activation was increased by the protective presence of human bone marrow stromal cells and B-cell receptor mimicking signals but antagonized by direct Akt blockade with the novel specific inhibitor AIX, with preferential apoptosis induction in CLL cells with an unmutated immunoglobulin status, which predicts poor clinical outcome. In addition, we found a direct interaction of Akt with Tcl1a in an endogenous coimmunoprecipitation assay. Confirming the critical role of Tcl1a in modulating Akt signaling, Akt activation was enhanced by overexpressing Tcl1a in CLL. In contrast, decreasing Tcl1a levels by small interfering RNA reduced Akt activation in the fludarabine-insensitive CLL cell line MEC-2 and sensitized the malignant cells to fludarabine treatment. In summary, our data reveal a significant role for the Akt-Tcl1a axis in CLL survival and propose a further evaluation of this interplay for targeting chemoresistance phenomena. *Cancer Res*; 70(18); 7336-44. ©2010 AACR.

Introduction

B-cell chronic lymphocytic leukemia (CLL) is the most prevalent non-Hodgkin lymphoma in the Western world and is characterized by the accumulation of CD5⁺ B lymphocytes in the blood, lymph nodes, and bone marrow (BM). The clinical course and outcome of CLL varies considerably. Some patients progress rapidly and die early despite therapy, whereas others exhibit an indolent disease course and a normal life span (1). Emerging evidence suggests that the B-cell antigen receptor (BCR) is the major determinant of this differential outcome. In particular, patients carrying mutated (MUT) variable heavy chain region genes encoding the surface immunoglobulin component of the BCR (IgVH) gen-

erally follow a more indolent course than those with unmutated (UMT) IgVH genes, who also tend to show evidence of adverse cytogenetic features, clonal evolution, and resistance to therapy (2, 3). In addition, molecular markers that are associated with the signaling capacity of the BCR in CLL, such as CD38 and the ζ -associated protein 70 (ZAP-70), serve as indicators for poor clinical prognosis (reviewed in ref. 4). In normal B lymphocytes, BCR stimulation triggers the recruitment and activation of Syk kinases, followed by the activation of effector enzymes, including protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and phospholipase C γ 2. These signaling complexes regulate key downstream pathways such as extracellular signal-regulated kinase, c-Jun NH₂-terminal kinase, p38, and Akt, the outcomes of which determine the B-cell fate (5). The serine/threonine kinase Akt, also known as protein kinase B, is a central molecule in the transmission of antigenic BCR-derived signals. Several studies suggested that Akt activation promotes CLL cell survival following BCR engagement (6, 7). It is, however, controversially discussed whether in peripheral blood CLL cells, constitutively active Akt contributes to cell survival (6, 8-11).

The protooncogene T-cell leukemia 1a (Tcl1a) is an important modulator of Akt activity. Tcl1a was initially characterized in preleukemic T cells (12) but is also expressed in subsets of naïve lymphocytes (13). Tcl1a protein is expressed in 90% of human CLL samples at variable levels correlating with ZAP-70 expression, IgVH mutational status, and cytogenetic aberrations (14). Tcl1a can form a hetero-oligomeric

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protein complex with Akt, thereby serving as a coactivator of Akt (15, 16). The significance of the Akt-Tcl1a axis is further underlined by the fact that *TCL1A*-transgenic mice expand a CD5⁺/IgM⁺ lymphocyte population, which is reminiscent of human CLL (17) and is accompanied by increased Akt activation levels (18). Intriguingly, these mice have a high-risk phenotype resembling the typical molecular features of an UMT BCR of human CLL cells.

Direct interference in Akt activation by specific Akt inhibitors or modulation of the coactivator Tcl1a has not been extensively investigated thus far. We analyzed these aspects in the context of BCR-related signaling and the tumor microenvironment—two elements centrally involved in survival and progression of CLL. We found a significant role for the Akt-Tcl1a axis in CLL cell survival. Our results warrant a further evaluation of this interplay as a therapeutic target in CLL.

Materials and Methods

Reagents and antibodies

The Akt inhibitor 10-(4'-(*N*-diethylamino)butyl)-2-chlorophenoxazine, HCl (AiX) was purchased from Calbiochem, the PI3K inhibitors LY294002 and U0126 were from Cell Signaling Technology, and 2-fluoroadenine-9- β -D-arabinofuranoside and rottlerin were from Sigma-Aldrich. Antibodies against Akt1, pan-Akt, phospho-Akt (Ser⁴⁷³), phosphoserine glycogen synthase kinase 3 α / β (GSK3 α / β), Mcl-1, Tcl1a, Bid, and Bik were purchased from Cell Signaling Technology; antibodies against Akt2, MDM2, p53, and phospho-Bcl-2 (Ser⁷⁰) were from Santa Cruz Biotechnology; Puma and tubulin were from Sigma-Aldrich; antibodies against CD5-PC7, CD19-phycoerythrin (PE), and CD19-PC7 were from Beckman-Coulter; VLA-4 (CD49d)-PE was from BD Biosciences; and CD38-PE was from Immunotech.

Patients, cell preparation, and AiX treatment

Following informed consent, peripheral blood samples were obtained from patients fulfilling diagnostic and immunophenotypic criteria for CLL at the Salzburg University Hospital, Austria (Supplementary Table S1). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and cultured in complete RPMI 1640. To generate primary BM-derived stromal cells, adherent mononuclear cells from CLL BM aspirates were cultured for 3 weeks in RPMI 1640 supplemented with 1 μ mol/L hydrocortisone and 100 μ mol/L β -mercaptoethanol. For coculture experiments including risk stratification, BM-derived stromal cells from healthy donors were kindly provided by Reinhard Henschler (Institute for Transfusion Medicine and Immune Hematology, German Red Cross Blood Donor Service, Frankfurt, Germany).

Cell lines

HS-5 stromal cells and C7H2-CEM cells were purchased from the American Type Culture Collection, and MEC-1 and MEC-2 cells were from the German Resource Centre for Biological Material. Cell lines were cultured according to the supplier's instructions, and cell aliquots from early passages (P1–P10) were used.

Analysis of cell viability, Tcl1a expression, and Akt activation

Cells were stained with fluorochrome-labeled anti-CD5, anti-CD19, Annexin V, and 7-aminoactinomycin D (7-AAD), and at least 10,000 CLL cells were analyzed using a FC-500 flow cytometer and CXP2-2 software (Beckman-Coulter). For intracellular phospho-Akt (Ser⁴⁷³) and Tcl1a staining, cells were fixed and permeabilized using the Fix and Perm kit (AN DER GRUBB) and stained with isotype control, anti-Tcl1a, anti-phosphoserine Akt, or anti-pan-Akt (19).

Plasmid construction and transfection

TCL1A wild-type cDNA was PCR amplified and subcloned into pcDNA3.1 vectors (Clontech). For cloning, the following PCR primers with *EcoRI* and *SalI* restriction sites were used: GTGGAATTCGCCATGGCCGAGTGCCCGACACTCGGG (forward) and GTGGTCGACTCAGTCATCTGGCAGCAGCTC-GAGAAG (reverse). Plasmids were nucleofected into MEC-1 cells using the Human B Cell Nucleofector kit (Lonza Cologne AG). Transfected cells were selected by geneticin.

Small interfering RNA transfection

MEC-2 cells were transfected with 100 pmol small interfering RNA (siRNA) using Metafectene Pro (Biontex GmbH) according to the manufacturer's instructions. Assessment of transfection efficacy was monitored by transfecting an Alexa Fluor 647-conjugated nontargeting siRNA and flow cytometric analysis, and effective protein knockdown was densitometrically assessed.

Immunoblot analysis

Standard lysis buffer was supplemented with phosphatase (Roche) and protease inhibitors (Sigma-Aldrich). For coimmunoprecipitation, a buffer containing 0.3% CHAPS was used (20). The lysate protein concentration was determined using the Bio-Rad protein assay. For coimmunoprecipitation, lysates were precleared with 50 μ L protein A or G dynabeads (Invitrogen) and further incubated with the antibody overnight. Magnetically captured complexes were washed and analyzed by immunoblotting. Signals were detected by chemiluminescence using the enhanced chemiluminescence kit (Millipore).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 or SPSS 17.0. All data were tested for normal distribution, and *t* test or ANOVA and Bonferroni post test were performed for normally distributed data. For nonnormally distributed data, the Mann-Whitney test was used.

Results

Direct Akt inhibition induces CLL apoptosis that cannot be compensated by protective stimuli

We first tested the *in vitro* effect of the novel Akt inhibitor AiX on CLL survival. AiX has been previously shown to effectively inhibit Akt and Akt downstream target phosphorylation without influencing the activity of PI3K and other Akt-related

kinases (21). We treated randomly selected CLL samples with increasing concentrations of AiX for 24 hours and subsequently assessed CLL cell viability by flow cytometry following Annexin V/7-AAD staining. AiX decreased Akt phosphorylation and CLL cell viability in a dose-dependent and effective manner (Fig. 1A; data not shown) with an IC₅₀ value of 4.4 μmol/L. Comparison of apoptosis rates induced by either AiX or the PI3K inhibitor LY294002 showed similar effectivity at 5 μmol/L but dramatically higher effectivity of AiX at 10 μmol/L ($P < 0.001$; Supplementary Fig. S1). For discrimination between AiX-induced apoptosis and necrosis, we additionally monitored for caspase-dependent cleavage of the poly(ADP-ribose) polymerase (PARP) protein, internucleoso-

mal DNA fragmentation, and caspase-3 activity as commonly used indicators for apoptosis. Akt inhibition resulted in high levels of cleaved PARP and DNA ladder formation, suggesting induction of apoptosis rather than necrosis (Supplementary Fig. S2A and B). We also observed increased caspase-3 activity in CLL cells after AiX treatment, which could be mostly blocked by preincubation with the pan-caspase inhibitor Q-VD-OPH (Supplementary Fig. S2C).

CLL cell survival *ex vivo* critically depends on the presence of supportive accessory cells such as BM-derived stromal cells. Conventional *in vitro* culture conditions without these accessory cells usually result in a rapid decline in CLL cell viability. Concordantly, we observed that CLL cells cocultured

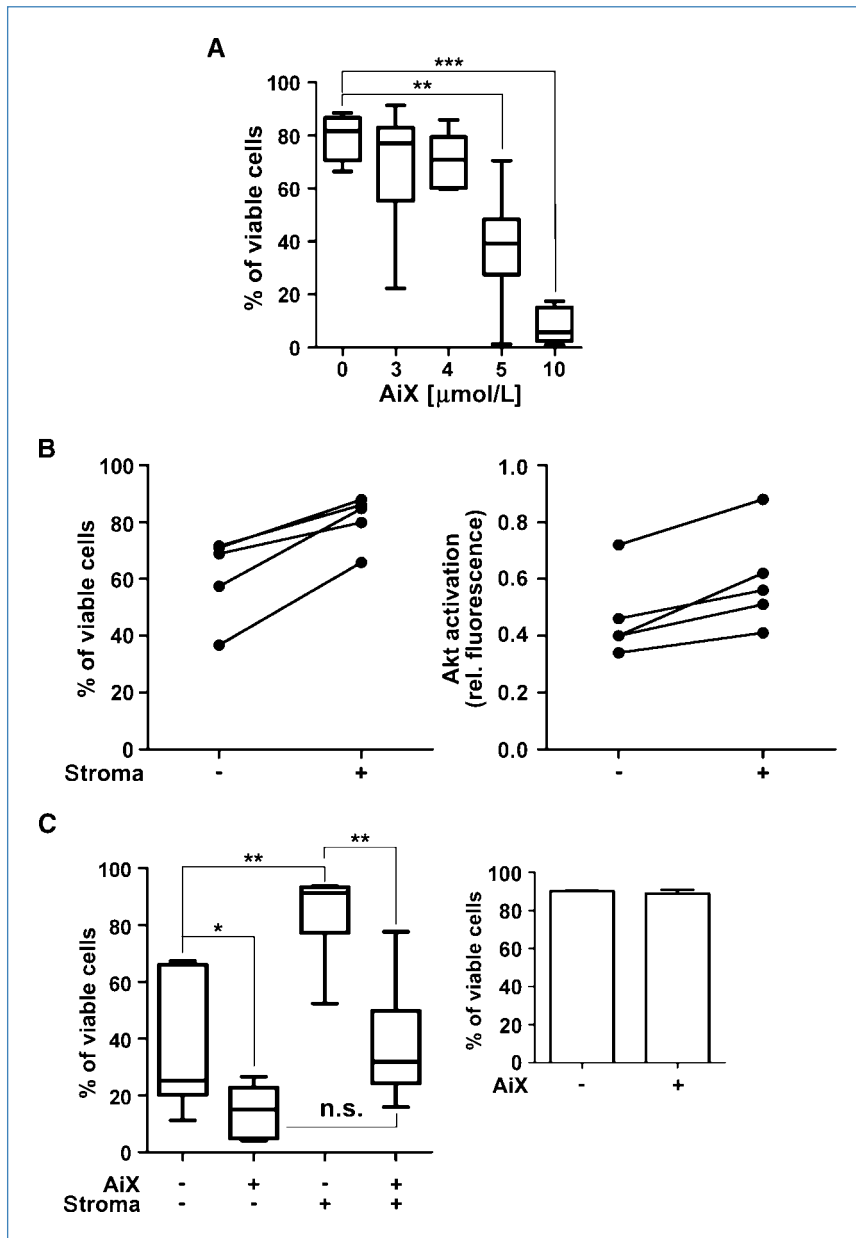
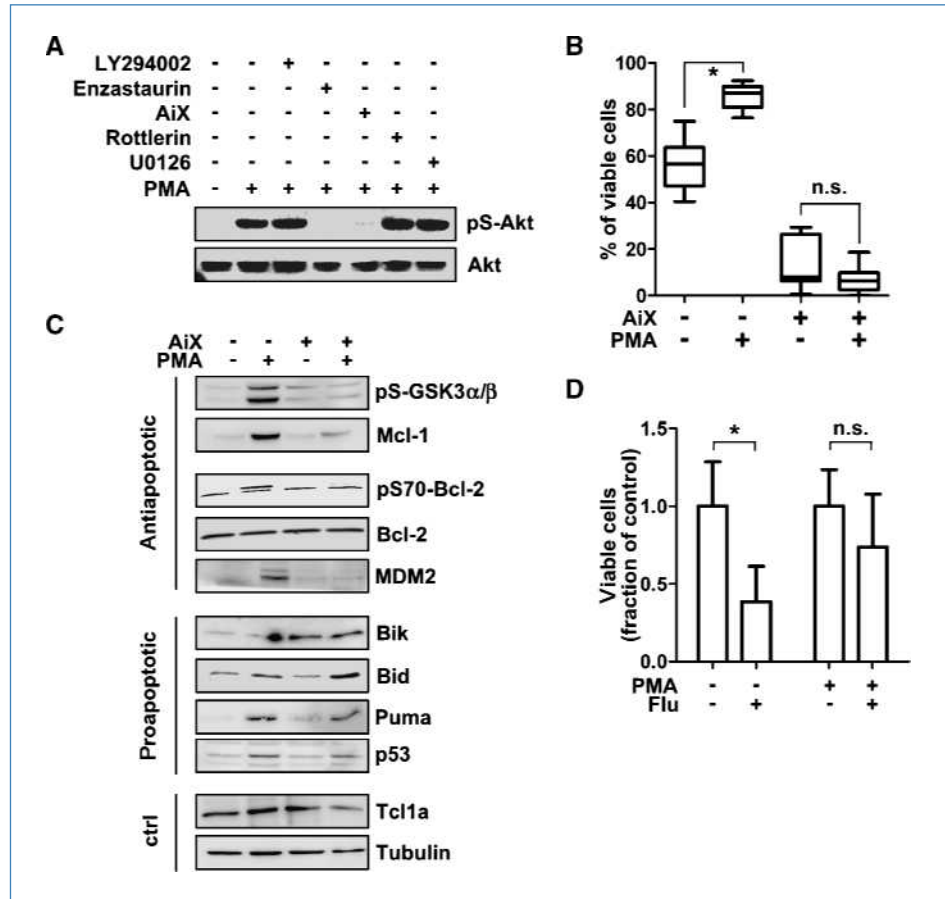


Figure 1. AiX-induced CLL apoptosis cannot be antagonized by the presence of protective stromal cells. **A**, PBMCs from CLL patients were cultured in the presence of increasing concentrations of AiX for 24 h and cell viability was cytometrically measured after Annexin V/7-AAD staining. CLL cells were hereby defined by gating on CD19⁺/CD5⁺ expression. The median with min/max of eight CLL samples is shown, and statistically significant differences are indicated. **, $P < 0.01$; ***, $P < 0.001$. **B**, left, PBMCs from CLL patients were cultured in the presence of HS-5 stromal cells for 5 d. CLL cell viability was defined as described above ($n = 5$). Right, Akt activation in CLL cells gained by the presence of HS-5 cells was cytometrically measured as the ratio of phospho-Akt (Ser⁴⁷³)/Akt in an intracellular stain. **C**, left, PBMCs from eight patients were cultured in the presence or absence of CLL BM-derived stromal cells and treated with 5 μmol/L AiX for 72 h. Right, stromal cells were treated with 5 μmol/L AiX for 72 h. CLL and stroma cell viability was measured as described (median with min/max). *, $P < 0.05$; **, $P < 0.01$. n.s., not significant.

Figure 2. PKC β -dependent Akt activation enhances CLL cell viability and fludarabine resistance. A, cells were stimulated with 10 nmol/L PMA for 20 min in the presence or absence of the indicated inhibitors. The activation status of Akt was monitored by immunoblotting the lysates for phosphoserine Akt (pS-Akt) and total (pan-) Akt. Note the very short exposure time. B, PBMCs from CLL patients were cultured for 48 h with 10 nmol/L PMA, 10 μ mol/L AiX, or both ($n = 7$). Cell viability was measured as described (median with min/max). *, $P < 0.05$. C, CLL cells were treated with 10 nmol/L PMA and 10 μ mol/L AiX for 6 h. Cell lysates were separated by SDS-PAGE, and expression status of Mcl-1, phosphoserine GSK3 α/β , Tcl1a, MDM2, p53, Puma, phospho-Bcl-2 (Ser⁷⁰), total Bcl-2, Bid, and Bik was monitored by immunoblotting. Tubulin expression is shown as equal protein loading control. D, PBMCs from CLL patients were activated with 10 nmol/L PMA and treated with 5 μ mol/L fludarabine. After 48 h, cell viability was assessed as described. The median with min/max of five independent experiments is shown. *, $P < 0.05$.



with the human stromal cell line HS-5 displayed significant increased survival (Fig. 1B, left). Notably, this stromal-induced prosurvival effect correlated with increased Akt phosphorylation in the same CLL cells ($n = 5$, correlation coefficient $r^2 = 0.81$, $P = 0.032$; Fig. 1B, right) and could be completely countered by treatment with AiX (data not shown). As we wished to determine if Akt inhibition of the CLL cells or rather the reduced viability of the stromal cells accounted for the decline in stromal-mediated protective effects, we assessed the viability of the HS-5 cells on AiX treatment. We observed a slight reduction in HS-5 viability in response to $\geq 5 \mu\text{mol/L}$ AiX (data not shown). Therefore, we repeated these experiments using primary patient-derived BM stromal cells, which were resistant to AiX treatment. Strikingly, coculture of CLL cells with primary stromal cells almost completely protected CLL cells against spontaneous apoptosis during the whole culture period of 5 days (Fig. 1C, left). The median viability of CLL cells after 5 days of *in vitro* culture in the absence of stromal cells was 25.3% compared with 91.4% in the presence of the CLL BM-derived stromal cells. However, despite this strong protective effect, Akt inhibition was highly efficient in inducing CLL apoptosis without affecting stromal cell viability (Fig. 1C, right).

Besides the stromal microenvironment, antigenic input is one of the most relevant factors in CLL survival (22). We recently showed a critical role of PKC β , a molecule centrally involved in BCR signaling, in CLL development (23). We now stimulated CLL cells with phorbol 12-myristate 13-acetate (PMA), thereby mimicking a PKC β signal (24). This stimulation resulted in strong Akt phosphorylation at Ser⁴⁷³, a major and for full activation indispensable phosphorylation site (25), which was entirely reversible by AiX treatment (Fig. 2A). We confirmed the PKC β mimicking nature of PMA stimulation by use of enzastaurin. This PKC β -specific inhibitor, but not the PI3K inhibitor LY294002 or the PKC θ inhibitor rottlerin, antagonized PMA-induced Akt activation (Fig. 2A), as previously observed (23). PMA stimulation led to a significant increase in CLL cell viability but failed to inhibit AiX-induced apoptosis (Fig. 2B). Determining the molecular basis of this observation, we found a dramatic activation of the Akt signaling pathway on PMA stimulation, such as increased levels of phosphoserine GSK3 α/β and strong upregulation of the antiapoptotic protein Mcl-1, which was mostly antagonized by AiX treatment. Expression of the direct Akt modulator Tcl1a was not visibly affected by PMA stimulation or direct Akt inhibition (Fig. 2C). Importantly, Akt inhibition was efficient in antagonizing PMA-induced Bcl-2 Ser⁷⁰

phosphorylation, recently reported to be crucial for CLL survival (26). In addition, proapoptotic BH3-only proteins Bid and Bik were upregulated in the presence of Akt inhibition (Fig. 2C).

We additionally checked the involvement of the p53 signaling pathway because p53 dysfunction is a primary determinant of CLL chemoresistance (27, 28). Furthermore, protein expression of MDM2, the main counter player of p53, is regulated by Akt signaling (29, 30). MDM2 expression was increased on PMA stimulation but decreased with additional AiX treatment, coinciding with apoptosis. Expression levels of p53 and its downstream target Puma were also increased on PMA. The observation that we could modulate p53 pathway members by PMA or AiX treatment led us to investigate the influence of the PKC β /Akt axis on the fludarabine responsiveness of CLL cells. Whereas fludarabine treatment resulted in a significant decrease in cell viability of unstimulated cells, the same concentration did not affect the viability of PMA-stimulated cells, further supporting the involvement of the PKC β /Akt/Mcl-1 axis in CLL survival and chemoresistance (Fig. 2D). Concordantly, when we tested the sensitivity of fludarabine-resistant CLL samples to Akt inhibition,

we observed clear apoptosis induction by AiX. All samples displayed a 17p13 deletion in 40% to 90% of the cells, resulting in p53 dysfunction. Importantly, AiX treatment was similarly effective in these fludarabine-resistant cells ($n = 4$) and normal samples ($n = 7$, $P = 0.9972$; Supplementary Fig. S3).

High-risk CLL cells are more sensitive toward Akt inhibition than low-risk cells

We further analyzed AiX-induced apoptosis in different CLL subgroups. Stratification of patients' samples according to their molecular risk profile (marked in Supplementary Table S1) revealed that in solo culture, CLL cells with an UMT IgVH status were significantly more sensitive to treatment with AiX than those with a MUT status (Fig. 3A, left). Furthermore, AiX also preferentially killed CLL cells with high CD38 and VLA-4 expression (Fig. 3B and C, left), consistent with the association of these molecules with poor clinical outcome (2, 31, 32). Spontaneous apoptosis during *in vitro* culture did not differ between CLL subgroups. In the presence of stromal cells, Akt inhibition was very effective with a trend of preferential targeting UMT but not VLA-4-high cases (Fig. 3C, right). Due to this observation, we

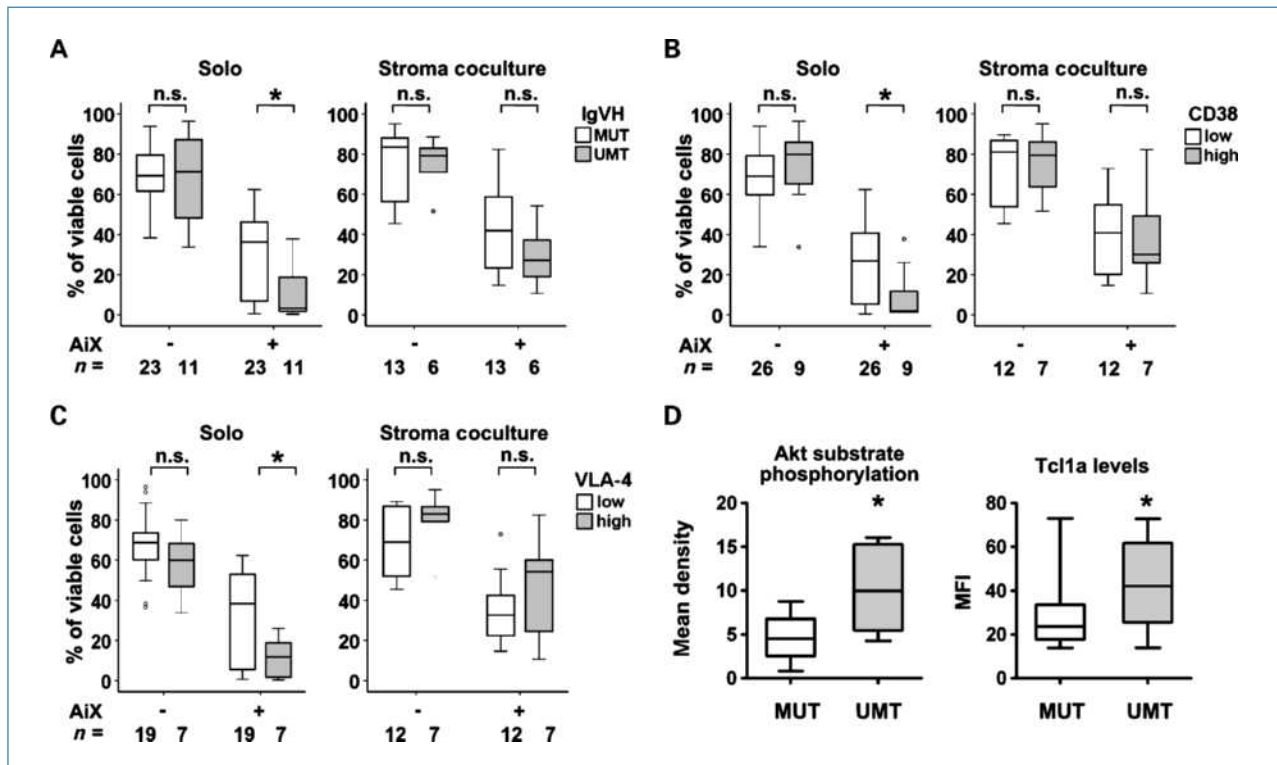


Figure 3. CLL cells with UMT IgVH genes and high CD38 or VLA-4 expression are highly sensitive to Akt inhibition. Cells were treated with AiX either in solo culture (5 μ mol/L AiX, 24 h; left) or in the presence of stromal cells (10 μ mol/L AiX, 48 h; right), and CLL cell viability was determined as described. Patient samples were stratified according to the mutational status of the IgVH genes (A), CD38 expression (B), or VLA-4 expression (C). Significance levels and analyzed case numbers are indicated in the diagrams. *, $P < 0.05$, one-way ANOVA. D, Akt substrate phosphorylation was monitored in protein lysates from eight MUT and nine UMT cases. Mean densities of Akt substrate phosphorylation were normalized to the housekeeping protein tubulin. Tcl1a expression was analyzed by intracellular staining and fluorescence-activated cell sorting analysis in 16 UMT CLL and 14 MUT CLL cases.

determined basal Akt substrate phosphorylation and Tcl1a levels in UMT versus MUT CLL subgroups. As expected, UMT CLL cells significantly displayed higher Akt activity and Tcl1a levels than MUT CLL cells (Fig. 3D).

The Tcl1a/Akt interaction axis is involved in fludarabine resistance of a CLL-derived prolymphocytic cell line

The expression of Tcl1a reciprocally correlates with the IgVH mutational status and is centrally involved in CLL tumorigenesis (14, 33, 34). Furthermore, Tcl1a is an important modulator of Akt activity with a central role in transmission of antigenic BCR-derived signals (35). Having observed significantly higher Tcl1a levels in UMT than MUT CLL cells, we next addressed the role of the Tcl1a/Akt interplay in CLL survival and chemosensitivity. Therefore, we used MEC-1 and MEC-2 CLL-derived prolymphocytic cells displaying different Tcl1a expression levels with higher Tcl1a expression in MEC-2 cells. Increased Tcl1a expression correlated with increased levels of phosphoserine Akt and GSK3 in MEC-2 cells (Fig. 4A). We tested the sensitivity of these cells toward Akt inhibition. AiX decreased MEC cell viability in a dose-dependent manner (Fig. 4B). After treatment with 20 μmol/L AiX, 50% of the MEC-1 cells and 65% of the MEC-2 cells underwent apoptosis (a fraction of 0.5 and 0.65 compared with untreated control cells, respectively).

To determine whether this higher constitutive Akt phosphorylation of MEC-2 is a consequence of Tcl1a/Akt interaction, we next examined for direct Tcl1a/Akt protein-protein interaction. Three isoforms of Akt have been described: Akt1, Akt2, and Akt3. We observed Akt1 and Akt2 expression in MEC-1 and MEC-2 cells but did not detect Akt3 (Fig. 4C; data not shown). Performing endogenous coimmunoprecipitation experiments, we observed direct protein-protein interaction of Tcl1a and Akt (Fig. 4C). Tcl1a coimmunoprecipitated with both Akt1 and Akt2 isoforms in lysates of the Tcl1a-high CLL cell line MEC-2. No coimmunoprecipitation of Tcl1a was observed when lysates of the Tcl1a-negative CEM cell line or anti-cytochrome *c* IgG were used in control experiments. Although Akt1 is the more predominant isoform found in MEC-2 cells, quantitatively more Tcl1a was bound to Akt2 (Fig. 4C). Next, to investigate the direct effect of Tcl1a on Akt activity, we knocked down Tcl1a expression in MEC-2 cells by siRNA. The efficacy of the transfection procedure was cytometrically assessed by nontargeting siRNA. Twenty-four hours after transfection, cell viability was not negatively affected and ~90% of MEC-2 cells were transfected (Supplementary Fig. S4). This transfection rate translated into a 70% knockdown of the Tcl1a protein (Tcl1a siRNA compared with nontargeting siRNA) and resulted in decreased Akt and GSK3 phosphorylation (Fig. 5A). Following the observation of impaired Akt signaling connected to reduced Tcl1a levels, we next investigated whether overexpressing Tcl1a would enhance Akt activation in MEC-1 cells. A plasmid encoding the human *TCL1A* cDNA was introduced into MEC-1 cells. This resulted in an activation of Akt and its downstream target GSK3α/β (Fig. 5B), further confirming the indispensable function of Tcl1a in Akt-mediated

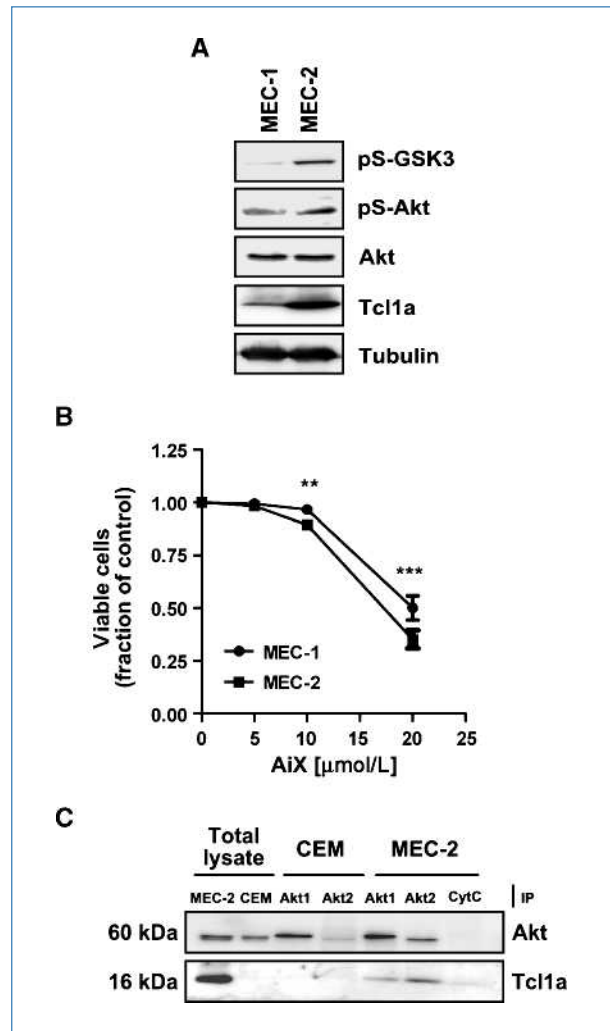


Figure 4. Enhanced Tcl1a expression corresponds to increased Akt activation. A, MEC-1 and MEC-2 cell lysates were separated by SDS-PAGE and expression levels of Tcl1a, phosphoserine Akt, and phosphoserine GSK3 were monitored by immunoblotting. One of three representative immunoblots is shown. B, MEC-1 and MEC-2 cells were treated with increasing concentrations of AiX (5–20 μmol/L) for 24 h, and cell viability was measured by an Annexin V/7-AAD staining and flow cytometry. Points, mean of five independent experiments for each cell line; bars, SD. **, *P* < 0.01; ***, *P* < 0.001. C, CEM and MEC-2 cell lysates were used to immunoprecipitate (IP) individual Akt isoforms under non-denaturing conditions. SDS-PAGE and immunoblotting were performed for Tcl1a and pan-Akt as described in Materials and Methods. One of three representative immunoblots is shown.

responses. In addition, given the high importance of Akt-dependent signaling in CLL survival, we investigated a potential Tcl1a function in chemosensitivity of these cells. MEC-2 cells have a monoallelic deletion in 17p13 (36), resulting in fludarabine resistance based on attenuation but not complete abrogation of the p53 pathway. We confirmed residual p53 activity by showing increased expression of the p53 downstream target p21 following AiX treatment (Supplementary Fig. S5). Finally, we performed a

Tcl1a knockdown in the fludarabine-resistant MEC-2 cell line and then treated these cells with increasing concentrations of fludarabine. Tcl1a knockdown indeed sensitized the cells toward the fludarabine treatment (Fig. 5C).

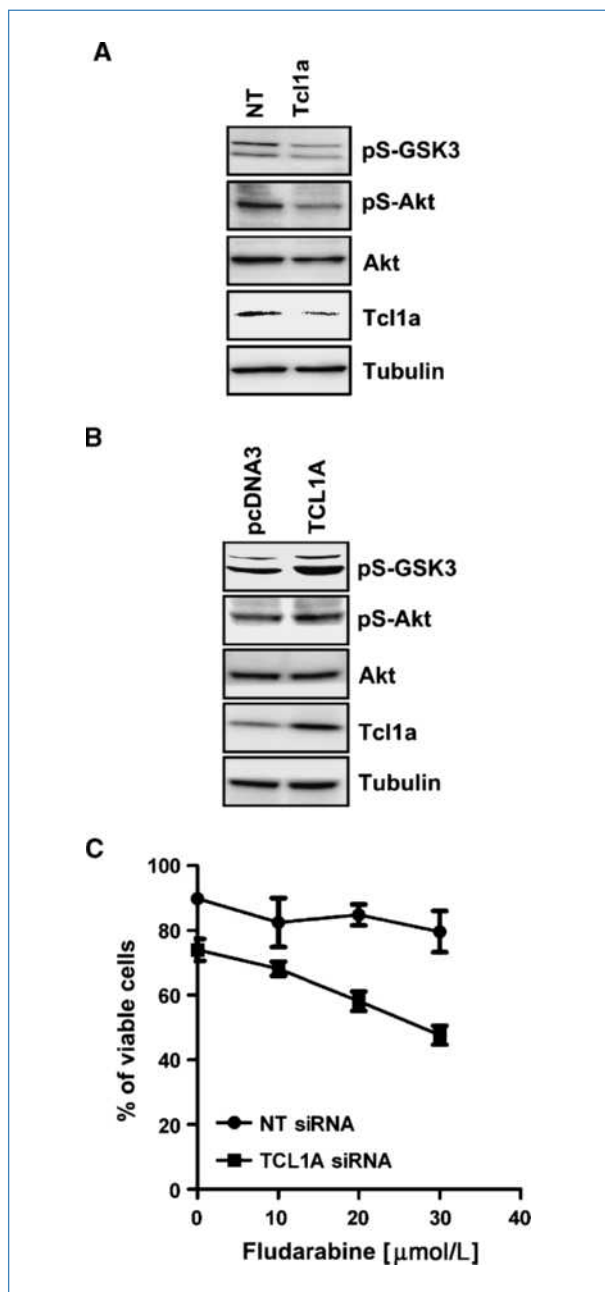


Figure 5. Tc11a expression is modulating Akt activation and fludarabine resistance of MEC cells. A, Tc11a siRNA was performed in MEC-2 cells as described in Materials and Methods and the activation status of Akt was monitored by immunoblotting. B, Tc11a was overexpressed in MEC-1 cells as described in Materials and Methods and the Akt activation status was monitored after 48 h by immunoblotting. Immunoblots of A and B are representative for three. C, MEC-2 cells were transfected with nontargeting (NT) or Tc11a-specific siRNA and cultured in the presence or absence of increasing concentrations of fludarabine for 48 h. Cell viability was measured as described. Points, mean of three independent experiments; bars, SD.

Discussion

Akt is a central protagonist in signal transduction pathways involved in cell differentiation, migration, proliferation, and survival, with innumerable regulatory circuits operating within and between these functions (37). Moreover, Akt is involved in malignant cell transformation and in the development of tumor chemoresistance (38). For these reasons, modulation of Akt activity through inhibition of phosphatidylinositol-3 kinase (PI3K), its main upstream activator, has long been deemed an attractive therapeutic approach, and a range of different PI3K inhibitors has been developed in recent years for several tumor entities. However, thus far, lack of selectivity and solubility of these inhibitors has limited their use in clinical trials (37). Constitutive PI3K activation has been shown in freshly prepared CLL cells, suggesting that the PI3K/Akt axis is relevant in CLL pathogenesis (8, 9, 39). In addition, we and others showed that Akt can also be activated by PI3K-independent pathways, such as PKC β (23, 39), recently shown to regulate the outcome of BCR signals and CLL tumorigenesis (23, 40). Likewise, stabilization of the Akt kinase activity by the oncoprotein Tc11a (41) is believed to contribute to the development of the CD5⁺ B-cell malignancy observed in the *TCL1A*-transgenic mouse model (17, 18). Furthermore, this Tc11a overexpression can partly compensate for the loss of PKC β presumably by providing the missing Akt amplification signal in the malignant cell population (23). Surprisingly, although Akt-driven pathways play an important role in CLL tumorigenesis (6, 7, 10), the effects of direct Akt targeting or modulation of its interplay with its coactivator Tc11a have not been widely addressed in CLL thus far.

We show here that direct inhibition of Akt by AiX even under conditions that normally confer protection to these cells, such as survival signals emanating from the tumor microenvironment and signals mimicking sustained antigenic input, induced apoptosis of CLL cells. As CLL cells display an extended life span within the context of their immediate tumor microenvironment, but rapidly undergo spontaneous apoptosis *ex vivo*, increasing emphasis is being placed on these leukemic cell-microenvironment interactions (42). Concordantly, coculturing CLL cells with accessory cells (43) or activation of the BCR signaling pathway (7, 23) rescues the malignant cells from succumbing rapidly to apoptosis *ex vivo*. Importantly, these prosurvival signals may converge on the Akt signaling pathway (6). Indeed, we show that both stromal- and BCR-related prosurvival stimuli result in Akt activation and that inhibition of Akt effectively overcomes these protective signals. The data indicate that direct Akt targeting can kill not only the bulk of the tumor cells circulating in the blood but also those residing within protective BM niches. Considering the pivotal role these niches play in the development of chemoresistance and disease relapse, our results underscore the potential value of Akt inhibitors in the treatment of CLL.

Furthermore, we show that direct Akt inhibition leads to strong proapoptotic effects especially in high-risk samples and is also effective in chemoresistant cases. In the presence of primary stromal cells, Akt inhibition was very potent with

a trend of preferential targeting in those cases with an UMT IgVH status. In light of increased Tcl1a levels of these cells, we assume that their increased reliance on an activated Akt pathway downstream of the BCR (6, 7) renders them more sensitive toward Akt inhibition. These risk-specific differences in BCR signaling capacity could be overruled by PKC β activation. Indeed, PKC β stimulation with PMA resulted in high phospho-Akt levels as well as increased cell viability and chemoresistance to fludarabine regardless of the risk group. PMA-induced survival benefits were based on elevated levels of Mcl-1 and activated (phospho-)Bcl-2 (Fig. 2) with simultaneous reduction of BimEL (data not shown). CLL cells exist in a delicate balance between the proapoptotic and prosurvival members of the Bcl-2 protein family. High levels of prosurvival Bcl-2 and Mcl-1, along with high levels of proapoptotic Bcl-xL, Bax, Bim, and Bmf, have been observed in the majority of CLL cells (44, 45). Our data support the described role of Mcl-1 and Bcl-2 in CLL cell survival and drug resistance (6, 7, 26). Furthermore, chemoresistance in CLL is largely dependent on loss of p53 function, in the form of 17p13 deletion, which also represents one of the worst prognostic markers in CLL (46–48). Interestingly, we observed that activation of the BCR pathway using PMA led to increased protein levels of MDM2 and p53, as well as induction of p53 target Puma. However, simultaneous activation of Akt and Mcl-1 by PMA could effectively suppress the proapoptotic activity of these proteins (Fig. 2C). In support of this, the strong downregulation of Mcl-1 by Akt inhibition combined with simultaneous increase in proapoptotic BH3-only proteins Bid and Bik resulted in apoptosis even in PMA-stimulated CLL cells. Our observation on Bid cleavage on Akt inhibition may indicate the involvement of the extrinsic apoptotic pathway, as Bid conjoins the extrinsic and intrinsic apoptotic pathways via caspase-8. As we indeed observed caspase-8 activation after Akt inhibition (data not shown), it is likely that both apoptotic pathways are involved. Our data are in line with coincidental Bid cleavage and caspase-8 activation after inhibition of the PI3K/Akt pathway in different lymphoma entities (49, 50).

Importantly, Akt inhibition also induced apoptosis in CLL cells with p53 deletion or dysfunction (Supplementary Fig. S3), an observation in consensus with a recent report (11). Therefore, our results show that targeting Akt by specific small molecules represents a rational strategy to combat not only high-risk but also therapy-resistant CLL entities, including those with p53 dysfunction.

Besides direct Akt inhibition, disruption of the Akt interaction with its coactivator Tcl1a can represent a valuable

therapeutic approach. Expression of Tcl1a has been observed in 90% of human CLL samples, with higher levels correlating with an UMT IgVH status and ZAP-70 expression (14). Moreover, higher Tcl1a levels predict for an inferior clinical outcome likely by modulating Akt activation (33). To show the utility of a modulating approach, we made use of CLL cell-derived lines that differed intrinsically in their expression of Tcl1a. Concordantly, we found more Akt activation and higher AiX response rates with regard to cell death in high Tcl1a-expressing MEC-2 cells than in MEC-1 cells (Fig. 4). Of note, overexpression of myr-Akt1 in CLL cells increased leukemic cell size (6), which is an interesting observation, as Akt-active MEC-2 cells have a bigger cell size than MEC-1 cells. Moreover, we are the first to report the coimmunoprecipitation of endogenous Tcl1a with Akt1 and Akt2 in the CLL cell line MEC-2, as direct Tcl1a-Akt interaction has only ever been shown in cell lines engineered to overexpress Tcl1a (35). Concordant with our results, Tcl1a was shown to be corecruited with pan-Akt in BCR-activated CLL cells (33). RNA interference with Tcl1a in these cells led to reduced Akt activation and enhanced response toward cytotoxic treatment such as fludarabine. In contrast, overexpression of *TCL1A* in low Tcl1a-expressing MEC-1 cells led to enhanced phosphoserine Akt and GSK3 α/β levels (Fig. 5). Collectively, our data show the central role of the prosurvival kinase Akt and its coactivator Tcl1a in the survival of stimulated CLL cells, which otherwise would be protected from treatment with cytotoxic agents such as fludarabine, and suggest that interference of this signaling axis represents a potential therapeutic strategy to combat CLL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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