Molecular Cancer Therapeutics

Cell-Penetrating TAT-FOXO3 Fusion Proteins Induce Apoptotic Cell Death in Leukemic Cells

Makram Essafi[1], Alice D. Baudot[1,2], Xavier Mouska[1], Jill-Patrice Cassuto[3], Michel Ticchioni[1,4], and Marcel Deckert[1,2]

Abstract

FOXO proteins are Akt-regulated transcription factors involved in the control of cell cycle, DNA repair, stress defense, apoptosis, and tumor suppression. We reported that plasmid-based overexpression of constitutively active FOXO3 in cells from chronic lymphocytic leukemia (CLL) reduced their survival, suggesting that increasing FOXO3 activity in hematologic malignancies may represent a promising therapeutic strategy. The transactivating transcription factor (TAT) protein transduction domain (PTD) derived from the HIV TAT protein was shown to efficiently deliver macromolecular cargo in various cell types. In this study, wild-type FOXO3 and FOXO3 mutated on Akt sites [FOXO3 T32A/S253A/S315A or TM (triple mutant)] were fused to the TAT-PTD. Using biochemical techniques, flow cytometry, and microscopy analysis, we found a rapid and dose-dependent cell penetration into leukemic cells of unlabeled and fluorescein isothiocyanate–labeled TAT-FOXO3 fusion proteins followed by their accumulation within nuclear and cytoplasmic compartments. Treatment with TAT-FOXO3 TM—but not wild-type TAT-FOXO3—proteins induced Jurkat and K562 leukemic cell death and affected cell viability of other hematologic malignancies including primary cells from CLL. Cell transduction with TAT-FOXO3 TM induced apoptotic cell death as shown by morphologic changes, Annexin V/7-AAD (7-amino-actinomycin D) staining, activation of effector caspases, and PARP cleavage, caspase blockade through the use of the inhibitor Z-VAD, and expression of Bim and $p27^{KIP1}$. By contrast, TAT-FOXO3 TM blocked cell proliferation of primary T cells, without affecting their viability. Together, our data show that cell penetrating TAT-FOXO3 TM fusion proteins constitute novel potential therapeutic agents in the treatment of lymphoproliferative disorders and hematologic malignancies. *Mol Cancer Ther; 10(1); 37–46. ©2011 AACR.*

Introduction

Mammalian forkhead members of the class O (FOXO) transcription factors, represented by FOXO1, FOXO3, FOXO4, and FOXO6, repress or activate a variety of genes, that are implicated in the regulation of cell death, cell cycle, DNA repair, stress response, and metabolism (reviewed in ref. 1). FOXO activity is increased by protein kinases such as JNK, MST1, and AMPK (1). By contrast, Akt, activated downstream PI3K by growth factors and cytokines, was shown to directly phosphorylate FOXO1, FOXO3, and FOXO4 at 3 residues equivalent to Thr32, Ser253, and Ser315 of FOXO3, demonstrating that Akt-dependent phosphorylation promotes nuclear exclusion of FOXO proteins and inhibition of their transcriptional activity (2–5). Consequently, the disruption of Akt phosphorylation sites on FOXO led to constitutive nuclear location and increased FOXO transcriptional activity (6). In addition to Akt, other kinases, including SGK, Iκβ, and ERK1/2, were also shown to suppress FOXO activity (1).

Genetic studies in mice, showing that disruption of all FOXO family members led to the development of thymic lymphomas and hemangiomas, demonstrated the critical role of FOXO proteins as tumor suppressor genes (7). Other studies further highlighted the importance of FOXO proteins in the maintenance of hematopoietic stem cell self-renewal capacity (8) and leukemia-initiating cells in chronic myeloid leukemia (9). Another indication that FOXO proteins are critical factors during cancer development came from the observations that FOXO overexpression in tumor cell critically affected their growth (2, 6, 10). Mutations of components within the PI3K/Akt pathway, which are frequent features of cancer cells including myeloid and lymphoid leukemia cells,

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generally abrogate cell dependence for survival factors and lead to inactivation of FOXO transcription factors (1). Consistently, expression of FOXO proteins mutated on Akt phosphorylation sites promotes cell-cycle arrest and apoptosis through the induction of the cell-cycle inhibitor $p27^{KIP1}$ and proapoptotic factors such as Bim (2). In leukemic cells, FOXO proteins have been shown to be essential downstream effectors of oncogenesis (11–13), growth factors (14, 15), drug resistance (16), and differentiation pathways (17). In chronic lymphocytic leukemia (CLL), we showed that both spontaneous and chemokine-induced resistance to cell death requires Akt-dependent phosphorylation and inactivation of FOXO3 and expression of a constitutively active form of FOXO3 in CLL cell–induced cell death (10). FOXO proteins are also important regulators of primary lymphocyte proliferation as documented by studies showing that activated FOXO proteins contribute to cell-cycle arrest by inducing the expression of the cell-cycle inhibitor $p27^{KIP1}$ (18–20). Interestingly, FOXO3-deficient mice displayed spontaneous lymphoproliferation and systemic inflammation associated with hyperactive NF-κB due to reduced FOXO3-dependent expression of NF-κB inhibitors, IκBβ/α (21).

Thus, targeting FOXO transcription factors in lymphoproliferative disorders and hematologic malignancies might represent an interesting novel therapeutic approach (22). The transactivating transcription factor (TAT) protein transduction domain (TAT-PTD) derived from the HIV TAT protein was shown to deliver macromolecular cargo into various cell types (23). In this study, we report the development of a protein-based method of delivery of FOXO3 proteins into leukemic cells consisting of FOXO3 mutated on all 3 Akt phosphorylation sites fused to the 11 amino acids of the TAT-PTD. We also provide evidence that recombinant TAT-FOXO3 TM (triple mutant) proteins efficiently penetrate the cell to induce apoptotic cell death of several leukemic cells, thus representing a potential novel antitumor agent.

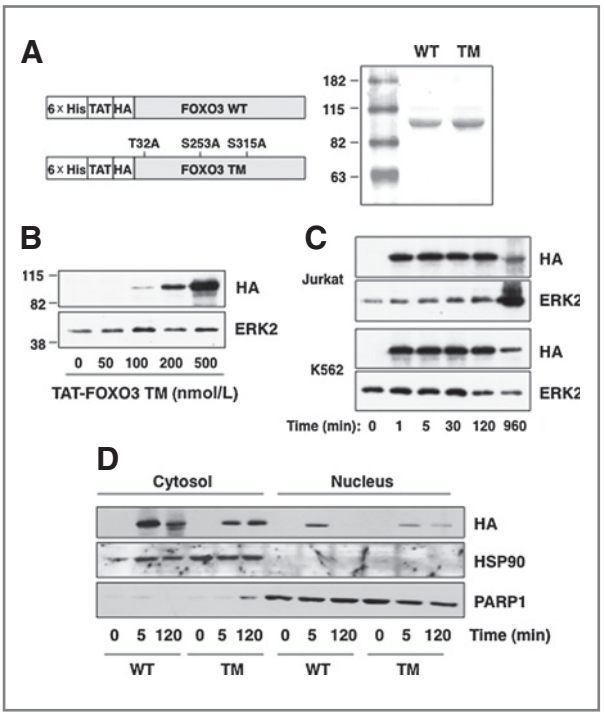

Figure 1. TAT fusion proteins are efficiently transduced into leukemic cell lines. A (left), schematic representation of the FOXO3 constructs used in this study: WT FOXO3, triple-mutated FOXO3 (TM, T32A3, S253A and S315A); 6His, 6xhistidine tag; HA, hemagglutine A epitope tag; TAT-FOXO3 wild-type (WT) and triple mutant (TM) constructs were expressed in the *Escherichia coli* BL21 strain and purified as described in the Materials and Methods section (right). Western blot analysis shows the purified recombinant forms of TAT-FOXO3 WT and TM. B, Jurkat cells were treated with the indicated concentrations of TAT-FOXO3 TM for 2 hours at 37°C. Cells were then harvested, washed 3 times in PBS, and whole-cell lysates were examined for TAT-FOXO3 TM expression by Western blotting using anti-HA antibody. Anti-ERK2 immunoblot was used as loading control. C, Jurkat and K562 cells were treated with 500 nmol/L of TAT-FOXO3 TM for the indicated times at 37°C and expression of TAT-FOXO3 TM proteins in cell lysates was analyzed as described above. D, Jurkat cells (1×10^6) were incubated for 0, 5, or 120 minutes with 500 nmol/L of WT or TM TAT-FOXO3 proteins. Nuclear and cytosolic protein extracts were prepared as described in the Materials and Methods section and analyzed by Western blot as described above. Expression of WT and TM TAT-FOXO3 proteins was examined using anti-HA antibody. HSP90 and PARP1 antibodies was used as controls for cytosolic and nuclear fractions, respectively.

Materials and Methods

Reagents, antibodies, and plasmid constructs

Culture media, oligonucleotides, and enzymes were from Invitrogen. Chemicals were obtained from Sigma. Polyclonal antibodies were from Santa Cruz Biotechnology, except for the antihemagglutinine (HA) monoclonal antibody (mAb; clone 12CA5) that was from Roche Diagnostics. Annexin V-PE apoptosis Detection Kit, anti-CD3, and anti-CD28 mAbs were from BD Biosciences. CFSE (carboxyfluorescein diacetate succinimyl ester) was from Invitrogen. *N*-Hydroxysuccinimide (NHS)-fluorescein was purchased from Thermo Scientific. Enhanced chemiluminescence (ECL) detection solutions were from GE Healthcare Bio-Sciences. The Bim promoter reporter plasmid was obtained from Eric W-F Lam. The pTAT-HA vector encoding the 11 amino acids of TAT-PTD YGRKKRRQRRR derived from the HIV TAT protein, in frame with 6x His and HA tags, was a generous gift from S.F. Dowdy (24, 25). Cell permeant forms of FOXO3 were generated by fusing the TAT-PTD to its amino terminal using standard molecular cloning techniques (Fig. 1A). Briefly, the cDNAs encoding wild-type (WT) FOXO3a or FOXO3a mutated on all 3 Akt phosphorylation sites (2) were amplified by PCR from pcDNA3-HA-FOXO3 WT or triple mutant (TM) (26). Following digestion with *Kpn*I and *Xho*I, the PCR products were cloned into pTAT-HA vector and the obtained plasmids pTAT-HA-FOXO3 WT and pTAT-HA-FOXO3 TM were sequenced prior to bacterial transformation.

Protein purification

pTAT-HA-FOXO3 WT and pTAT-HA-FOXO3 TM plasmids were transformed into the *E. coli* BL21(DE3) pLysS bacterial strain. Bacterial cultures were grown at 37°C until an $OD_{600\ nm}$ of 0.3 to 0.4 was reached. TAT fusion protein expression was induced by isopropylthiogalactoside (IPTG) treatment (0.5 mmol/L) for 5 to 6 hours at 30°C. Bacteria were then harvested and lysed by sonication in 20 mL of denaturing lysis buffer (20 mmol/L of HEPES, pH 8.0, 8 mol/L of urea, 100 mmol/L of NaCl). The His-tagged fusion proteins were purified using Ni-NTA-agarose columns (Qiagen) that were preequilibrated with denaturing lysis buffer containing 20 mmol/L of imidazole as described before (24). Clarified lysates were applied to the columns, and after extensive washing with lysis buffer plus 20 mmol/L of imidazole, recombinant proteins were eluted with 250 mmol/L of imidazole. Eluted protein solutions were then dialyzed against 20 mmol/L of HEPES (pH 8.0) plus 150 mmol/L of NaCl at 4°C and frozen in 10% glycerol at −80°C. TAT-HA proteins were used as control.

Cell lines, primary cell preparation, and culture conditions

The human leukemia cell lines Jurkat (T lymphoblast), Raji, RPMI 8866, SKW6.4 (B lymphoblast), U937 (promonocytic), and K562 (chronic myelogenous) were from the American Type Culture Collection (ATCC). The B-CLL cell line EHEB was obtained from DSMZ. Cell lines were not tested and authenticated by the authors after receiving from ATCC and DSMZ. Primary B-CLL cells were obtained at the Nice University Hospital (Nice, France) from peripheral blood samples of CLL patients after informed consent. The diagnosis of CLL was based on clinical and immunophenotypic criteria. Peripheral mononuclear cells were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation. Leukemic B cells were isolated by magnetic selection as previously described (10, 27). For primary T cells, mononuclear cells were isolated from blood by Ficoll-Hypaque sedimentation and T cells were purified by negative depletion using a Pan T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. All cells were cultured in RPMI 1640 medium (Gibco BRL) containing 10% fetal bovine serum (Hyclone), 1 mmol/L of L-glutamine, and 1 mmol/L of pyruvate, at 37°C in 5% CO_2 atmosphere.

Determination of transduction efficiency

For determination of protein transduction efficiency, we generated fluorescein isothiocyanate (FITC)-labeled TAT fusion proteins using NHS-Fluorescein (Pierce), according to the manufacturer's protocol. Briefly, 5 μg of NHS-Fluorescein were incubated with 100 μg of TAT-proteins for 2 hours on ice in the dark. Free, unbound fluorescein was removed by dialysis against 20 mmol/L of HEPES (pH 8.0), 150 mmol/L of NaCl. Protein transduction was evaluated by adding 10 μg of FITC-labeled TAT fusion proteins to a Jurkat cell suspension (1×10^5) for the indicated times. After 2 washes, total fluorescein staining of transduced cells was examined by flow cytometry (FACScan, Becton-Dickinson). Unlabeled TAT fusion proteins were used as negative controls. Internalized fluorescein staining was analyzed by fluorescence microscopy. Cells were plated onto poly-L-lysine–coated slides for 20 minutes before fixation in 3.7% (w/v) paraformaldehyde for 15 minutes at 37°C. After 3 washes in PBS 0.1% BSA, cell plasma membrane and nucleus were stained using AlexaFluor 594-wheat germ agglutinin (WGA) and Hoechst 33342, respectively according to manufacturer's instructions (Image-iT labeling kit, Molecular probes). After 3 washes, cells were mounted with Fluoromount (Sigma-Aldrich) and analyzed using a Zeiss Axiovert 200M fluorescence microscope (Zeiss Leica Microsystems) equipped by a Hamamatsu ORCA-ER digital camera. Image analysis was performed with Volocity software (Improvision Inc).

Analysis of cell death and cell proliferation

The analysis of cell death induced by treatment with TAT fusion proteins was performed by flow cytometry as previously described (10). Briefly, cells were resuspended in RPMI 10% FCS (5×10^5/mL) and incubated with the indicated concentrations of TAT fusion proteins at 37°C. Cells were collected at different times and analyzed for apoptosis following double staining with Annexin V-PE and 7-AAD (7-amino-actinomycin D) as indicated by the manufacturer (BD Biosciences). Analysis was performed on FACScan cytometer (BD Biosciences) using CellQuest software. For the analysis of T-cell proliferation, cells (2×10^7/mL) were labeled with 2 μmol/L of CFSE (Invitrogen) for 15 minutes at 37°C. After extensive washing in culture medium, cells (1×10^5) were stimulated by a combination of immobilized anti-CD3 antibodies (5 μg/mL) plus soluble anti-CD28 antibodies (5 μg/mL; BD Biosciences) for 48 hours 37°C. CFSE-labeled T cells were then analyzed for cell proliferation by flow cytometry as described above.

Cell fractionation, immunoprecipitation, and Western blot analysis

For preparation of nuclear and cytoplasmic protein extracts, cells (1×10^6) were incubated for the indicated time with WT or TM TAT-FOXO3 proteins. Cell extracts were then prepared using ProteoExtract Fractionation kit (Merck), according to the manufacturer's instructions. For preparation of whole-cell lysates, cells were lysed in ice-cold lysis buffer (1% Triton X-100 in 150 mmol/L of NaCl, 50 mmol/L of HEPES, pH 7.4, 5 mmol/L of NaF, 5 mmol/L of sodium pyrophosphate, 1 mmol/L of sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L of phenylmethylsulfonyl fluoride, PMSF) for 15 minutes in ice. Lysates were clarified by centrifugation at 15,000 × g for 15 minutes at 4°C. Nuclear and cytoplasmic protein extracts (10 μg per lane) and whole-cell lysates (30 μg per lane) were separated by SDS-PAGE and transferred

to a nitrocellulose membrane. Immunoprecipitations were performed as described before (27) on 50 μg of whole-cell lysates using 1 μg of anti-HA antibodies immobilized on protein G-sepharose beads. Immunoprecipitates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were probed by immunoblotting with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies followed by ECL detection as described before (10). Intensity of the bands was evaluated and compared using ImageJ software.

Cell transfection and luciferase reporter assays

Cell transfection and luciferase assays were performed as described elsewhere (10). Briefly, Jurkat T cells were electroporated with 5 μg of Bim-luciferase reporter plasmid using AMAXA nucleofector device (Lonza). Cells were rapidly transferred to 37°C preheated medium and incubated for 24 hours at 37°C, 5% CO_2. Cells (1×10^6) were then incubated for 16 hours with WT or TM TAT-FOXO3 proteins. Luciferase activities were assayed using Promega luciferase assay system and measured in a luminometer (EG&G Berthold; PerkinElmer Life Sciences). Normalized luciferase activities were determined in triplicate and expressed as arbitrary units.

Results

Leukemic cells are efficiently transduced with TAT-FOXO3 fusion proteins

Plasmid-based overexpression of FOXO proteins in leukemic cells critically affected their growth and induced cell death (2, 10, 28). The TAT-PTD was shown to efficiently cross biological membranes and deliver macromolecular cargo in various cell types (23, 25). We therefore evaluated whether TAT-FOXO3 fusion proteins might be a rational approach for targeting cell death pathways regulated by FOXO3 in leukemic cells. The 11 amino acids of the TAT-PTD were fused to the amino terminal sequence of full-length FOXO3, either WT or mutated on all 3 Akt phosphorylation sites (TM; Fig. 1A, left). The recombinant His-TAT-HA–tagged FOXO3 proteins were produced following bacterial expression and purification using Ni-NTA-agarose columns (Fig. 1A, right). Similar conditions were used to purify the TAT-HA control proteins (TAT-Co; data not shown). The leukemic cell line Jurkat was then incubated with increasing amounts of TAT-FOXO3 TM. Western blot analysis showed that TAT-TM protein entered Jurkat cells in a dose-dependent manner, from 50 to 500 nmol/L (Fig. 1B). For the following experiments, we selected 500 nmol/L as the working concentration. Time-course analysis of cell transduction showed that TAT-FOXO3 proteins were detectable in whole-cell lysates from Jurkat and K562 leukemic cells within 1 minute of incubation with 500 nmol/L of recombinant proteins (Fig. 1C). The expression of TAT-FOXO3 proteins remained stable for up to 2 hours, but started to decrease after 16 hours of incubation

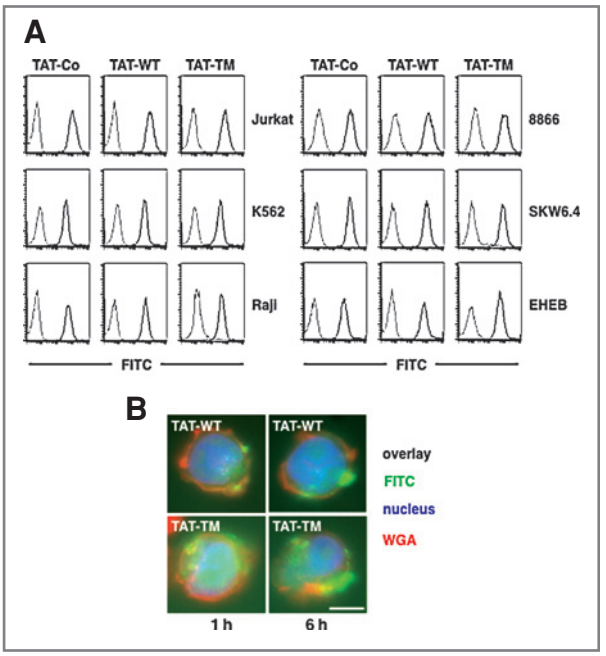

Figure 2. Analysis of cell transduction by FITC-labeled TAT fusion proteins. TAT-HA control proteins (TAT-Co), TAT-FOXO3 WT (TAT-WT), and TAT-FOXO3 TM (TAT-TM) fusion proteins were labeled with FITC as described in the Materials and Methods section. A, the indicated leukemic (Jurkat, K562, EHEB) and lymphoblastic (Raji, RPMI 8866, SKW6.4) cell lines were treated with 500 nmol/L of unlabeled (gray line) or FITC-labeled TAT-Co, TAT-WT, and TAT-TM fusion proteins (dark line) for 1 hour at 37°C before extensive washing with PBS and analysis by flow cytometry. B, Jurkat cells were treated with 500 nmol/L of FITC-labeled TAT-WT and TAT-TM fusion proteins for 1 and 6 hours at 37°C before extensive washing and analysis by fluorescence microscopy. Nucleus and membrane staining was performed with Hoechst 33342 and red-fluorescent Alexa Fluor 594 wheat germ agglutinin (WGA) for 10 minutes at 37°C. Scale bar = 5 μm.

(Fig. 1C). We next examined the subcellular localization of WT and TM TAT-FOXO3 fusion proteins. Nuclear and cytoplasmic proteins were extracted from transduced Jurkat cells for different times and the expression of TAT-FOXO3 proteins were assessed by Western blot. As shown in Fig. 1D, WT and TM TAT-FOXO3 proteins localize to the cytosol and to a lesser extent to the nucleus. However, whereas TAT-FOXO3 TM proteins could be found in the nucleus after 2 hours of treatment, the localization of TAT-FOXO3 WT within the nucleus appears more transient as the protein was undetectable in the nuclear fraction after 2 hours of treatment (Fig. 1D).

To assess whether incubation with TAT fusion proteins would lead to intracellular penetration, TAT-Co, WT and TM TAT-FOXO3 fusion proteins were fluorescently labeled. Different leukemic (Jurkat, K562, EHEB) and lymphoblastic (Raji, RPMI 8866, SKW6.4) cell lines were then treated with unlabeled or FITC-labeled TAT-Co, TAT-WT, and TAT-TM fusion proteins for 1 hour at 37°C, extensively washed and analyzed using flow cytometry. Figure 2A shows that FITC-labeled TAT-Co, TAT-WT, and TAT-TM fusion proteins efficiently bound up to

100% of all leukemic and lymphoblastic cells. To confirm the intracellular accumulation of TAT fusion proteins, Jurkat cells incubated for 1 and 6 hours with FITC-coupled TAT-WT and TAT-TM proteins were analyzed by fluorescence microscopy. Costaining of cell membranes and nucleus showed that FITC-labeled TAT-FOXO3 recombinant proteins efficiently accumulated within the cell, reaching cytoplasmic and nuclear compartments (Fig. 2B).

TAT-FOXO3 TM, but not TAT-FOXO3 WT, induces leukemic cell death

To assess whether TAT-FOXO3 fusion proteins could affect leukemic cell viability, we first incubated Jurkat cells with increasing amounts of TAT-FOXO3 TM and TAT-Co control protein for 24 hours at 37°C, and cell viability was determined by flow cytometry using staining of exposed phosphatidylserine by fluorescent Annexin V probe. We found that TAT-TM, but not TAT-Co, induced phosphatidylserine externalization on Jurkat cells, in a dose-dependent manner from 200 to 1 μmol/L, affecting 45% to 80% of the cell population (Fig. 3A). A time-course analysis of phosphatidylserine externalization on Jurkat cells incubated with 500 nmol/L of TAT-FOXO3 TM or TAT-Co revealed that the effect was rapid (Fig. 3B). Within 2 hours, 50% of the cells were positive for Annexin V staining that reached a maximum of 90% after 8 hours. The percentage of cell affected by the transduction of TAT-FOXO3 TM slightly decreased to 75% after 24 hours. This may be explained by the degradation of TAT-FOXO3 TM proteins observed following 16 hours of incubation (Fig. 1D), which may allow the expansion of the small percentage of unaffected cells. In contrast to the effect of recombinant TAT-FOXO3 TM proteins on cell viability, TAT-FOXO3 WT showed nearly no capacity to induce exposition of phosphatidylserine on Jurkat and K562 cells (Fig. 3C), despite similar levels of cell penetration (Fig. 2A) and purity of the recombinant proteins (Fig. 1A). To examine the phosphorylation status of the transduced proteins, WT and TM TAT-FOXO3 fusion proteins were immunoprecipitated from Jurkat cell lysates using anti-HA antibody. Immunoprecipitates were then immunoblotted using phospho-Thr32 FOXO3 and HA antibodies. As shown in Fig. 3D, TAT-FOXO3 WT, but not TAT-FOXO3 TM, fusion proteins were phosphorylated on the Thr32 residue, a major Akt target site (2).

We further extended our analysis by assessing by flow cytometry that how TAT-FOXO3 TM fusion proteins affected survival of different leukemia/lymphoma cells. Over 6 tested cell lines, only Jurkat T-cell leukemia Jurkat and K562 chronic myelogenous leukemia transduced with recombinant TAT-FOXO3 TM fusion proteins underwent massive cell death as shown by Annexin V/7-AAD double staining (Fig. 4A). These observations were consistent with the effect of plasmid-based expression of FOXO3 TM proteins in Jurkat (ref. 2; data not shown) and K562 leukemic cells (29). On other tested cell lines including the B-cell lymphoblast Raji, RPMI 8866, SKW6.4, and the CLL EHEB, TAT-FOXO3 TM had a significant but weaker effect on cell death induction as compared with TAT-Co control proteins (20%–30% of Annexin V–positive cells; Fig. 4A). We previously reported that survival of B-cell CLL is partly mediated by Akt-dependent inhibition of FOXO3 and showed that

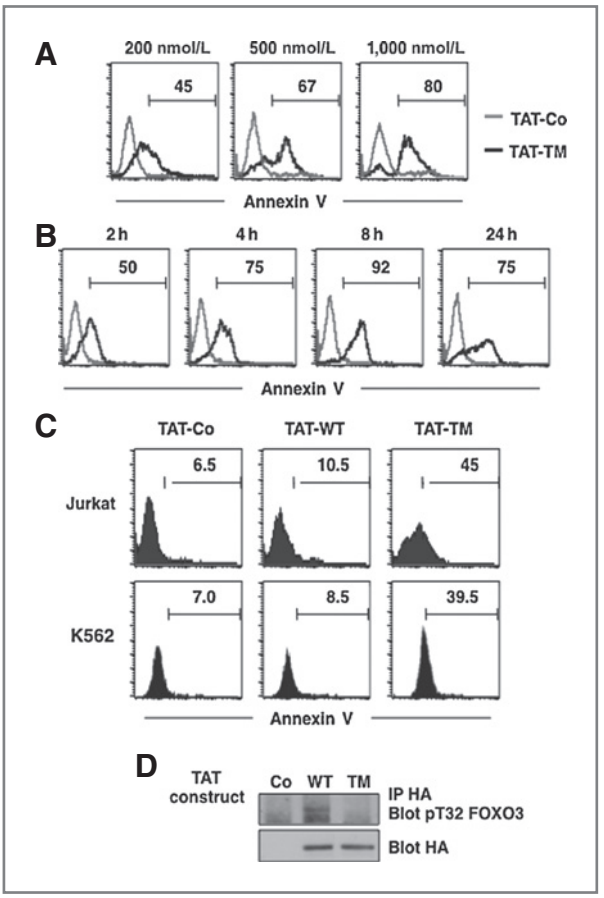

Figure 3. TAT-FOXO3 TM, but not TAT-FOXO3 WT, induces the externalization of phosphatidylserine on Jurkat cells. A, Jurkat cells were treated with the indicated concentrations of TAT-HA control proteins (TAT-Co; gray lines) and TAT-FOXO3 TM fusion proteins (TAT-TM; dark lines) for 24 hours at 37°C. Detection of phosphatidylserine was performed after Annexin V-PE staining and analyzed by flow cytometry. The histograms show 1 experiment representative of 3 independent experiments. B, Kinetics of TAT-TM–induced externalization of phosphatidylserine on Jurkat cells. Cells were incubated with 500 nmol/L of TAT-TM (dark lines) or TAT-Co (gray lines) for the indicated times. Annexin V-PE staining was performed as above. The histograms show 1 experiment representative of 3 independent experiments. C, TAT-TM, but not TAT-WT, induced externalization of phosphatidylserine on Jurkat and K562 cells. Cells were treated with 500 nmol/L of the indicated TAT fusion proteins for 24 hours at 37°C. Annexin V-PE staining was performed as above. One experiment representative of 3 independent experiments is shown. D, Jurkat cells were incubated with 500 nmol/L of TAT-WT and TAT-TM fusion proteins for 2 hours at 37°C. Protein expression and phosphorylation were analyzed on anti-HA immunoprecipitates followed by Western blot analysis with phospho-Thr32 FOXO3 and HA antibodies.

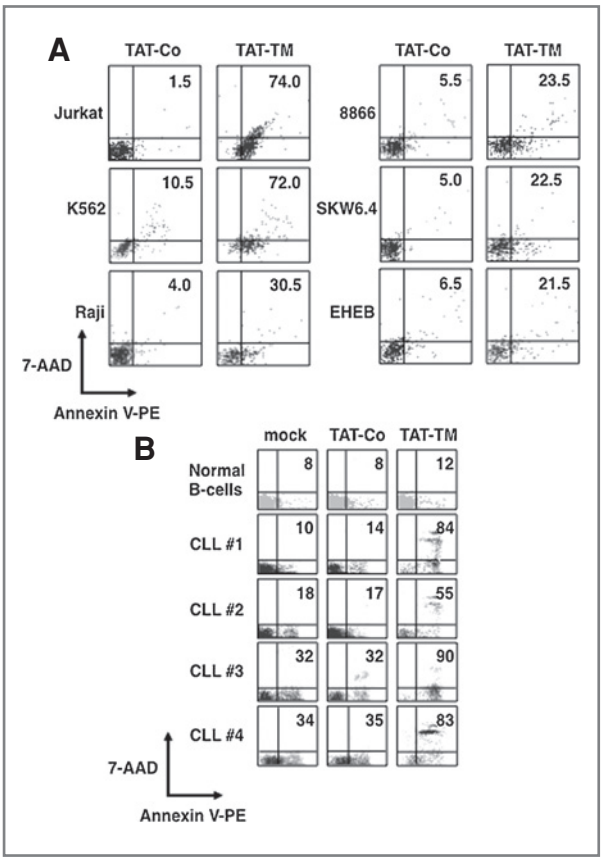

Figure 4. Effects of TAT-FOXO3 TM transduction on phosphatidylserine externalization on leukemic and lymphoblastic cells. A, the indicated leukemic (Jurkat, K562, EHEB) and lymphoblastic (Raji, RPMI 8866, SKW6.4) cell lines, were incubated with 500 nmol/L of TAT-FOXO3 TM (TAT-TM) or TAT-control (TAT-Co) proteins for 24 hours. Cell survival was then measured by Annexin V-PE/7-AAD staining and flow cytometry analysis. The percentage of apoptotic cells (Annexin-V positive) is shown in the top right side of each diagram. B, normal B cells and primary leukemic B cells obtained from 4 different CLL patients were either left untransduced (mock) or incubated with 500 nmol/L of TAT-FOXO3 TM (TAT-TM) or TAT-control (TAT-Co) proteins for 24 hours. Cell survival was measured as described above. The percentage of apoptotic cells (Annexin-V positive) is shown in the top right side of each diagram. Histograms show 1 experiment representative of 2 independent experiments.

CLL transfection with a plasmid encoding a constitutively active form of FOXO3, FOXO3 TM, dramatically reduced cell survival. We therefore examined whether transduction of primary leukemic cells from CLL patients could also reduce their viability. As shown in Figure 4B, incubation with TAT-FOXO3 TM, but not TAT-Co, caused massive death of CLL cells purified from 4 different patients; whereas, the viability of primary B cells purified from a healthy donor was not significantly affected. Of note, the small amount of cell death observed in TAT-Co treated cells was due to the background level of death in tissue culture as indicated by the levels of viability observed in untransduced control conditions (mock; Fig. 4B).

TAT-FOXO3 TM induces apoptotic cell death and $p27^{KIP1}$ and Bim expression on leukemic cells

To investigate how TAT-FOXO3 TM induces cell death in Jurkat and K562 leukemic cells, we examined the cell morphology and the nature of the biochemical pathways that were induced following incubation with TAT-FOXO3 TM. As shown in Fig. 5A, the incubation of Jurkat cells with TAT-FOXO3 TM for 24 hours induced cytoplasm and nucleus condensation, as well as membrane blebbing. These results, together with the Annexin V/7-AAD staining data, were reminiscent of an apoptotic cell death. We therefore examined whether treatment with TAT-FOXO3 TM could induce the activation of effector caspases. Caspase-3 and caspase-7 are the main effector caspases that are activated in response to both intracellular and extracellular death signals (30). Jurkat cells were transduced with mock buffer, TAT-Co or TAT-FOXO3 TM fusion proteins for 24 hours, and cell lysates were subjected to Western blot analysis using antibodies against procaspase-3 and procaspase-7. Analysis of total cell lysates from mock and TAT-Co–treated cells showed the presence of 2 polypeptides with an apparent molecular mass of 32 and 35 kDa, representing procaspase-3 and procaspase-7, respectively (Fig. 5B). These bands disappeared in cells incubated with TAT-FOXO3 TM proteins, indicating that both caspase-3 and caspase-7 were cleaved. Consistently, TAT-FOXO3 TM–mediated activation of caspase-3 and caspase-7 was associated with the cleavage of their major substrate, PARP (Fig. 5B). The induction of a caspase-dependent apoptosis by TAT-FOXO3 TM was further confirmed by the observation that cell death induction by TAT-FOXO3 TM was reduced when Jurkat cells were incubated with TAT-FOXO3 TM in the presence of the caspase inhibitor Z-VAD-fmk (Fig. 5C). FOXO3 has been shown to activate a variety of genes including $p27^{KIP1}$ and Bim (1). Luciferase assays using a Bim promoter reporter construct transfected in Jurkat cells showed that TAT-TM, but not TAT-WT, fusion proteins could increase the activity of the Bim promoter (Fig. 5D, left). Western blot analysis of Jurkat and K562 cells left untransduced (mock) or incubated with TAT-Co or TAT-FOXO3 TM fusion proteins revealed that TAT-FOXO3 TM induced the expression $p27^{KIP1}$ and Bim in both cell types (Fig. 5D). Overall, our data indicate that TAT-FOXO3 TM reduced cell viability in transduced leukemic cells through the induction of a caspase-dependent apoptotic cell death and expression of $p27^{KIP1}$ and Bim.

TAT-FOXO3 TM blocks primary T-cell proliferation through $p27^{KIP1}$ and IκBβ induction

Several studies have shown that primary T-cell activation and proliferation passes through Akt-mediated inactivation of FOXO transcription factors (18, 19) and genetic deletion of FOXO3 in mice led to spontaneous T-cell lymphoproliferation associated with hyperactive NF-κB pathway (21). Because recombinant TAT-FOXO3 TM proteins induced apoptotic cell death of the T-cell leukemia

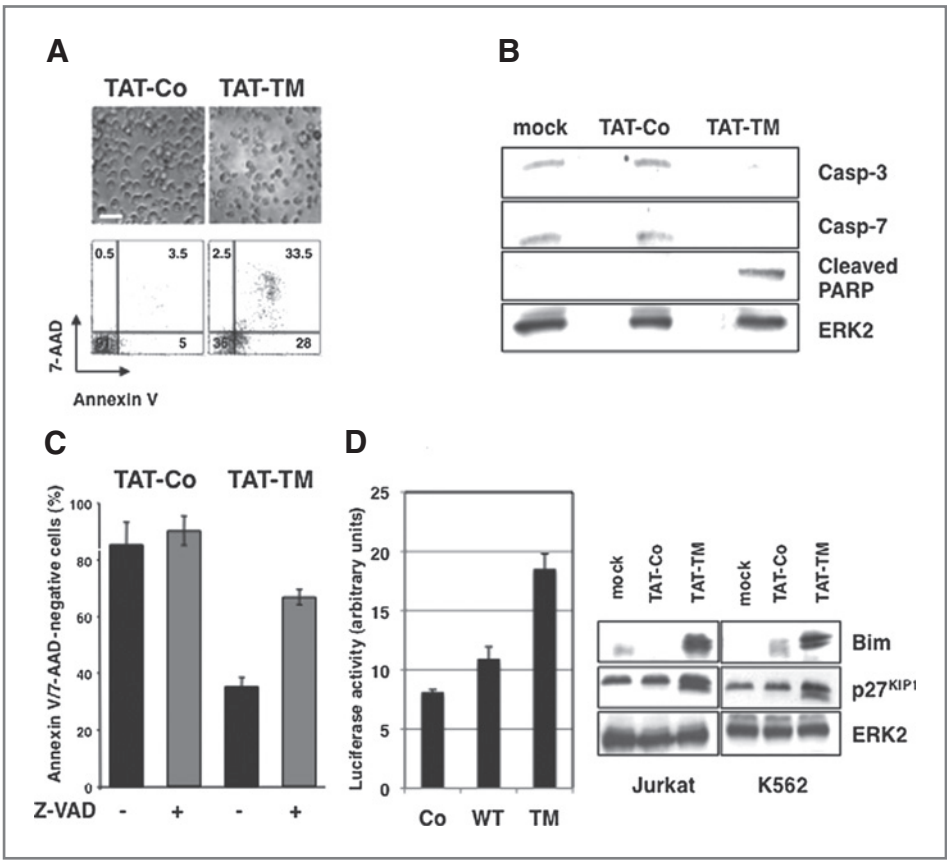

Figure 5. TAT-FOXO3 TM fusion proteins induce apoptotic cell death and $p27^{KIP1}$ and Bim expression on leukemic cells. A, Jurkat cells were treated with 500 nmol/L of the indicated TAT fusion proteins for 24 hours at 37°C. Cell morphology was then analyzed by photon microscopy (top) and cell survival was evaluated by Annexin V/7-AAD staining and flow cytometry analysis (bottom). Scale bar = 25 μm. B, whole-cell lysates of the above cells were analyzed for caspase cleavage by Western blotting using antiprocaspase-3 (casp-3) and antiprocaspase-7 (casp-7) antibodies. PARP cleavage was assessed using antibodies against the cleaved form of PARP. ERK2 immunoblot was used as loading control. C, Jurkat cells were treated with 500 nmol/L of the indicated TAT fusion proteins for 24 hours at 37°C in the presence or not of 50 μmol/L of caspase inhibitor Z-VAD-fmk. Cell survival was then evaluated by Annexin V/7-AAD staining and flow cytometry analysis. Means ± SD of 3 independent determinations are shown. D, (left) Jurkat cells (5×10^6) were electroporated with 5 μg of Bim-luciferase reporter plasmid, transferred to 37°C for 24 hours and incubated for 16 hours with TAT-Co, TAT-WT or TAT-TM fusion proteins. Normalized luciferase activities were then determined in triplicate and expressed as the mean ± SD of arbitrary units. Histograms show 1 experiment representative of 2 independent experiments. Right, Jurkat and K652 cells were either left untransduced (mock) or treated with 500 nmol/L of the indicated TAT fusion proteins for 24 hours at 37°C. Whole-cell lysates were analyzed by Western blot with anti-Bim and anti-$p27^{KIP1}$ antibodies. ERK2 immunoblot was used as loading control.

Jurkat cells, we, therefore, wished to examine the effect of TAT-FOXO3 TM on primary T cells purified from blood mononuclear cells using magnetic negative depletion. T cells were then activated with a combination of immobilized anti-CD3 plus soluble anti-CD28 antibodies for 48 hours at 37°C. As shown in Fig. 6A, stimulation of TAT-Co–treated cells by CD3/CD28 dramatically increased T-cell growth, leading to the formation of large blastic cells (top right). By contrast, activated T cells in the presence of TAT-FOXO3 TM proteins generated fewer blasts (bottom right), indicating that TAT-FOXO3 TM impaired T-cell proliferation or activation. Analysis of Annexin V/7-AAD staining on CD3/CD28-stimulated T cells showed that neither TAT-Co nor TAT-FOXO3 TM proteins induced cell death (Fig. 5B). However, the quantification by flow cytometry of T-cell proliferation using the CFSE dilution method revealed that the treatment with TAT-FOXO3 TM decreased the number of cycling T cells from 70% in control conditions to 41% in TAT-FOXO3 TM-treated cells (Fig. 6C). Consistent with this, Western blot analysis of lysates from CD3/CD28-stimulated T cells showed that the incubation with TAT-FOXO3 TM increased the expression of the NF-κB inhibitor IκBβ and of the cell-cycle inhibitor $p27^{KIP1}$ (Fig. 6D). These data indicate that in contrast to their effect on leukemic T cells, TAT-FOXO3 TM proteins did not affect primary T-cell viability but impaired T-cell proliferation.

Discussion

The Akt pathway is commonly activated in several human cancers, leading to growth factor independence, impairment of cell death pathways, and uncontrolled proliferation. Critical effectors downstream of Akt are the tumor suppressor genes of the FOXO family of transcription factors (1), and several studies have shown that

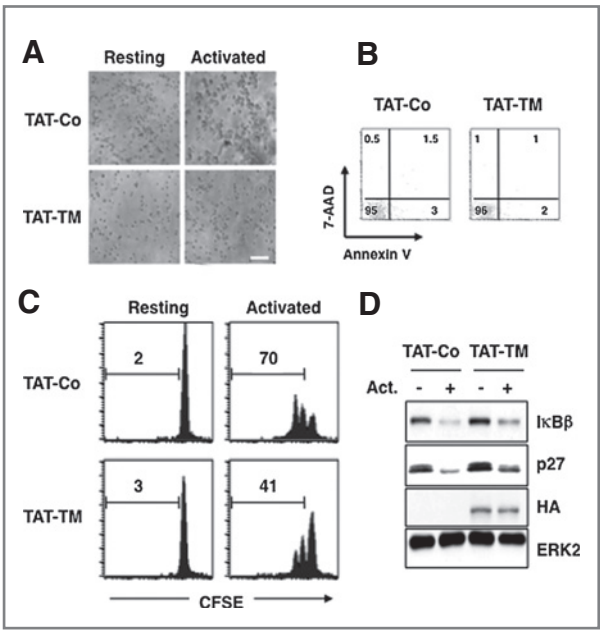

Figure 6. Effects of TAT-FOXO3 TM fusion proteins on primary T-cell survival and proliferation. A, primary T cells purified from blood mononuclear cells were activated or not with a combination of immobilized anti-CD3 (5 μg/mL) plus soluble anti-CD28 (5 μg/mL) antibodies for 48 hours at 37°C. Morphology of resting and activated T cells was then analyzed by photon microscopy (Bar, 25 μm). B, flow cytometry analysis of Annexin V/7-AAD staining on CD3/CD28-stimulated T cells. C, purified T cells were stained with 3 μmol/L of CFSE for 10 minutes at 37°C. Cells were washed twice, resuspended in culture medium, and activated or not with a combination of immobilized anti-CD3 (5 μg/mL) plus soluble anti-CD28 (5 μg/mL) antibodies for 48 hours at 37°C in the presence of 500 nmol/L of TAT-FOXO3 TM (TAT-TM) or TAT-control (TAT-Co) proteins. Cell proliferation was analyzed by flow cytometry. D, purified T cells were activated as described above for 2 hours at 37°C. Cells were collected, washed, and whole-cell lysates were then analyzed by Western blot using antibodies against IκBα, $p27^{KIP1}$, and HA tag. ERK2 immunoblot was used as loading control. Experiments are representative of 2 independent experiments.

drugs used to treat cancers lead to FOXO3 activation essentially through Akt inhibition (31). Akt-mediated phosphorylation of FOXO proteins leads to nuclear exclusion and inactivation of their transcriptional activity (1), preventing the activation of genes involved in cell-cycle and cell death regulation such as Bim and $p27^{KIP1}$ (1). Consistently, it has been shown that both adenovirus-mediated gene transfer and plasmid-based transfection of FOXO3 mutated on the Akt sites Thr32, Ser253, and Ser315, FOXO3 TM promotes cell-cycle arrest and apoptotic cell death in cancer cells (2, 29, 32, 33). We have also shown that plasmid-based expression of FOXO3 TM reduced both spontaneous and chemokine-induced cell survival of CLL cells (10). Thus, increasing FOXO activity appears as a promising therapeutic strategy (22). However, most anticancer drugs cause a variety of side effects, whereas gene-based therapy needs to overcome several technical and ethical problems. Furthermore, DNA transfection techniques in human cells are generally poorly efficient, resulting in low protein expression. In contrast, penetrating peptides, such as the TAT-PTD, have been shown to deliver large and active proteins into cultured cells, allowing fast and concentration-dependent transduction of PTD fusion proteins into nearly 100% of cells. The TAT-PTD was also shown to be useful *in vivo*, to successfully transduce bioactive proteins into the cells of mouse models of human diseases including cancer (23, 34). Consistently, we observed a concentration-dependent accumulation of recombinant TAT-FOXO3 fusion proteins into leukemic cells. Cell transduction was achieved in less than 1 minute and fluorescence microscopy analysis using FITC-labeled TAT-FOXO3 TM proteins showed that TAT-FOXO3 proteins efficiently accumulated within the cell, reaching cytoplasmic and nuclear compartments. This mechanism of TAT-FOXO3 TM uptake was compatible with fluid phase endocytosis, which is the current mechanism explaining how macromolecules fused to the TAT-PTD penetrate the cell (23, 35).

The treatment of leukemic cells with TAT-FOXO3 TM fusion proteins resulted in apoptotic cell death that is characterized by typical morphologic changes, phosphatidylserine exposition to outer membrane leaflet, activation of effector caspases and PARP cleavage, expression of Bim, and death blockade by the caspase inhibitor Z-VAD (30). It is important to note that WT TAT-FOXO3 showed no or very weak induction of apoptosis, despite similar purification procedures, molecular size, and cell transduction ability. This observation excludes the possibility that the effect of TAT-FOXO3 TM on cell survival resulted from nonspecific toxic effects of the transduced proteins. The reason why WT TAT-FOXO3 showed almost no activity is unclear. However, Jurkat and K562 cells displayed high constitutive Akt activity (17, 36), and we found that WT TAT-FOXO3, but not TAT-FOXO3 TM, was subjected to phosphorylation on the Akt site Thr32, an event involved in nuclear exclusion (2). Consistently, cell fractionation experiments showed that TAT-FOXO3 WT proteins only transiently accumulated into the nucleus, in contrast to TAT-FOXO TM proteins. FOXO transcription factors activate several genes, including Bim and $p27^{KIP1}$, that in turn, regulate critical cellular processes such as cell-cycle arrest, DNA repair, stress response, and cell death (1). However, a recent study showing that the cytosolic form of FOXO1 can promote autophagy in cancer cells in response to stress (37) suggests that FOXO proteins might regulate protein expression independently of gene transcription. Although it remains unclear whether TAT-FOXO3 TM proteins directly activate gene transcription, our data support this notion. The microscopy analysis showed accumulation of TAT-FOXO3 TM within the nucleus at times compatible with the activation of a transcription program. In addition, incubation of Jurkat cells with the transcription inhibitor actinomycin D reduced cell death induced by TAT-FOXO3 TM (data not shown). Also, the treatment with TAT-FOXO3 TM increased the activity of a Bim promoter reporter construct and induced the protein

expression of 2 known FOXO3 targets, Bim and $p27^{KIP1}$, in Jurkat and K562 leukemic cells. Of note, these events were not observed in cells transduced with TAT-FOXO3 WT (Fig. 5D and data not shown). These data correlate with previous studies showing that DNA transfection of constitutively activated FOXO3 mutants in leukemia/lymphoma cell lines increased transcription and expression of Bim and $p27^{KIP1}$ and led to cell-cycle arrest and/or apoptosis (2, 10, 11, 29). Furthermore, our data obtained with protein-based delivery of a constitutively activated FOXO3 in leukemic cells from CLL patients are congruent with our previous study based on a plasmid-based transfection of green fluorescent protein–tagged FOXO3 TM mutants (10). FOXO transcription factors share some transcriptional targets (38). Whether TAT-FOXO3 TM also impacts on pathways regulated by other FOXO family members, such as FOXO1 and FOXO4, remains an important issue that requires further investigations.

Some neoplastic cell lines used in our study were not fully sensitive to the induction of apoptosis by TAT-FOXO3 TM treatment, including the B-cell lymphoblastic cell lines Raji, RPMI 8866, SKW6.4, and the chronic leukemia EHEB. The discrepancy was not due to a poorer efficiency of cell penetration by the TAT fusion proteins on those cell types because we found that FITC-labeled TAT-FOXO3 fusion proteins exhibit similar transduction efficiencies in all cell lines. Thus, the minimal effects on cell death induction by TAT-FOXO3 TM observed on some cell lines could implicate different functional outcomes of FOXO3 activation in those cells or a certain degree of resistance to cell death. This latter explanation seems unlikely in regard to the reported sensibility to apoptosis of the majority of the studied cell lines. Another possibility could be a difference in the cellular background that affects the response to the fusion proteins. Further studies aimed at increasing the efficiency of protein transduction in hematologic malignancies are clearly required.

Finally, we found that treatment of primary activated T cells with TAT-FOXO3 TM did not affect their survival. Similar data were obtained using primary unaffected B cells, which in contrast to leukemic B cells from CLL, were not induced to apoptosis by TAT-FOXO3 TM. However, TAT-FOXO TM proteins were able to partially block both blast formation and proliferation of CD3/CD28-stimulated T cells. Consistent with a transcriptional effect on activated T cells, TAT-FOXO3 TM was shown to increase the expression of the cell-cycle regulator $p27^{KIP1}$ and of the NF-κB inhibitor, IκBβ. These data correlate with previous studies showing that T cell-cycle progression requires Akt-mediated inactivation of FOXO transcription factors which otherwise contribute to quiescence by promoting the expression of the cell-cycle inhibitor $p27^{KIP1}$ (18, 19), along with the induction of NF-κB inhibitory factors pathway (39). On the basis of our observations, it would be interesting to investigate the effect of protein-based transduction of active FOXO3 on primary B-cell proliferation that has been described to depend on Akt-dependent inactivation of FOXO (20). These data suggest that in addition to targeting some hematologic malignancies, TAT-FOXO3 TM may also reduce the uncontrolled cell proliferation associated with lymphoproliferative disorders such as autoimmune lymphoproliferative syndromes or posttransplant lymphoproliferative disorder.

In conclusion, our data show that cell-penetrating TAT-FOXO3 TM fusion proteins not only are antilymphoproliferative agents but also display antileukemic properties by inducing apoptotic cell death. Several studies in mouse models of human diseases using TAT-PTDs to deliver bioactive proteins and compounds have already been described (23). Improvement of protein-based delivery of active FOXO proteins allowing preclinical studies in mouse models of hematologic malignancies and lymphoproliferative disorders may demonstrate the potential of TAT-FOXO fusion proteins as novel therapeutic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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