

MYELOID NEOPLASIA

Targeting the leukemia cell metabolism by the CPT1a inhibition: functional preclinical effects in leukemias

Maria Rosaria Ricciardi,¹ Simone Mirabilii,² Matteo Allegretti,² Roberto Licchetta,² Anna Calarco,³ Maria Rosaria Torrì,¹ Robin Foà,² Raffaella Nicolai,⁴ Gianfranco Peluso,³ and Agostino Tafuri¹

¹Hematology, "Sant'Andrea" Hospital–Sapienza, University of Rome, Department of Clinical and Molecular Medicine, Rome, Italy; ²Department of Cellular Biotechnologies and Hematology, Sapienza University of Rome, Rome, Italy; ³Italian National Council of Research (NCR), Institute of Biosciences and BioResources–CNR, Naples, Italy; and ⁴Sigma-Tau Pharmaceutical Industries Pomezia S.p.A., Pomezia, Italy

Key Points

- FAO is a crucial metabolic pathway for leukemic cell proliferation and apoptosis.
- FAO inhibitors represent a novel targeted approach for leukemia treatment.

Cancer cells are characterized by perturbations of their metabolic processes. Recent observations demonstrated that the fatty acid oxidation (FAO) pathway may represent an alternative carbon source for anabolic processes in different tumors, therefore appearing particularly promising for therapeutic purposes. Because the carnitine palmitoyl transferase 1a (CPT1a) is a protein that catalyzes the rate-limiting step of FAO, here we investigated the in vitro antileukemic activity of the novel CPT1a inhibitor ST1326 on leukemia cell lines and primary cells obtained from patients with hematologic malignancies. By real-time metabolic analysis, we documented that ST1326 inhibited FAO in leukemia cell lines associated with a dose- and time-dependent cell growth arrest, mitochondrial damage, and apoptosis induction.

Data obtained on primary hematopoietic malignant cells confirmed the FAO inhibition and cytotoxic activity of ST1326, particularly on acute myeloid leukemia cells. These data suggest that leukemia treatment may be carried out by targeting metabolic processes. (*Blood*. 2015;126(16):1925-1929)

Introduction

Scientific evidence shows that the aberrant activation of signal transduction cascades is directly involved in the acquisition of new metabolic characteristics by tumor cells.¹⁻⁹ Recent studies have focused on the key role of fatty acid oxidation (FAO), as a way to produce adenosine triphosphate, reduced nicotinamide adenine dinucleotide phosphate, and acetyl-coenzyme A in neoplastic cells.¹⁰ The carnitine palmitoyl transferase 1a (CPT1a) catalyzes the rate-limiting step for the entry of fatty acids into the mitochondria, by loading fatty acyl-groups onto carnitine. It has been previously demonstrated that CPT1a interacts with members of the apoptotic machinery, such as Bcl-2 and t-Bid, and its inhibition can cause an accumulation of the toxic metabolite palmitate, resulting in mitochondrial damage and cell death.^{11,12} Inhibition of CPT1a has already been applied in cancer research with positive results. ST1326 is an agent characterized by a reversible inhibitory activity toward the liver isoform of the enzyme CPT1a. ST1326 was initially selected by Sigma-Tau laboratories as a candidate for diabetes and ketoacidosis therapy.^{13,14} Results reported by Pacilli et al¹⁵ demonstrated that the pharmacologic inhibition of FAO by ST1326 impairs cancer cell survival and inhibits tumor cell proliferation in in vitro and in vivo models of Burkitt lymphoma. Therefore, in this study we aimed to expand this approach, investigating the in vitro antileukemic activity of ST1326 on human leukemia cell lines and primary cells obtained from patients with different hematologic diseases.

Study design

Cell lines

Details are reported in supplemental Materials and methods (see the *Blood* Web site).

Samples and treatment

Human normal bone marrow (BM) derived CD34⁺ cells were purchased from StemCell Technologies. Peripheral blood and/or BM aspirates samples were obtained from 31 leukemia patients (supplemental Table 1), who provided written informed consent, in accordance with the Helsinki Declaration and approved by the Institutional Review Board of the Sapienza University of Rome. Cells were suspended in complete medium to a starting concentration of 1.0×10^6 /mL and exposed to ST1326 (1 to 50 μ M) and, for selected experiments, to etomoxir (50 to 200 μ M). Details of cell culture of combination experiments are reported in supplemental Materials and methods.

Cell cycle and apoptosis analysis

Cell cycle distribution, annexin V (Ann V) binding, and mitochondrial transmembrane potential ($\Delta\Psi_m$) were performed as previously published.¹⁶⁻¹⁸

FAO assay

FAO rates were measured in real time by the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were suspended in unbuffered Dulbecco's modified Eagle medium supplemented with 2.5 mM glucose and 0.5 mM

Submitted December 16, 2014; accepted August 6, 2015. Prepublished online as *Blood* First Edition paper, August 14, 2015; DOI 10.1182/blood-2014-12-617498.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2015 by The American Society of Hematology

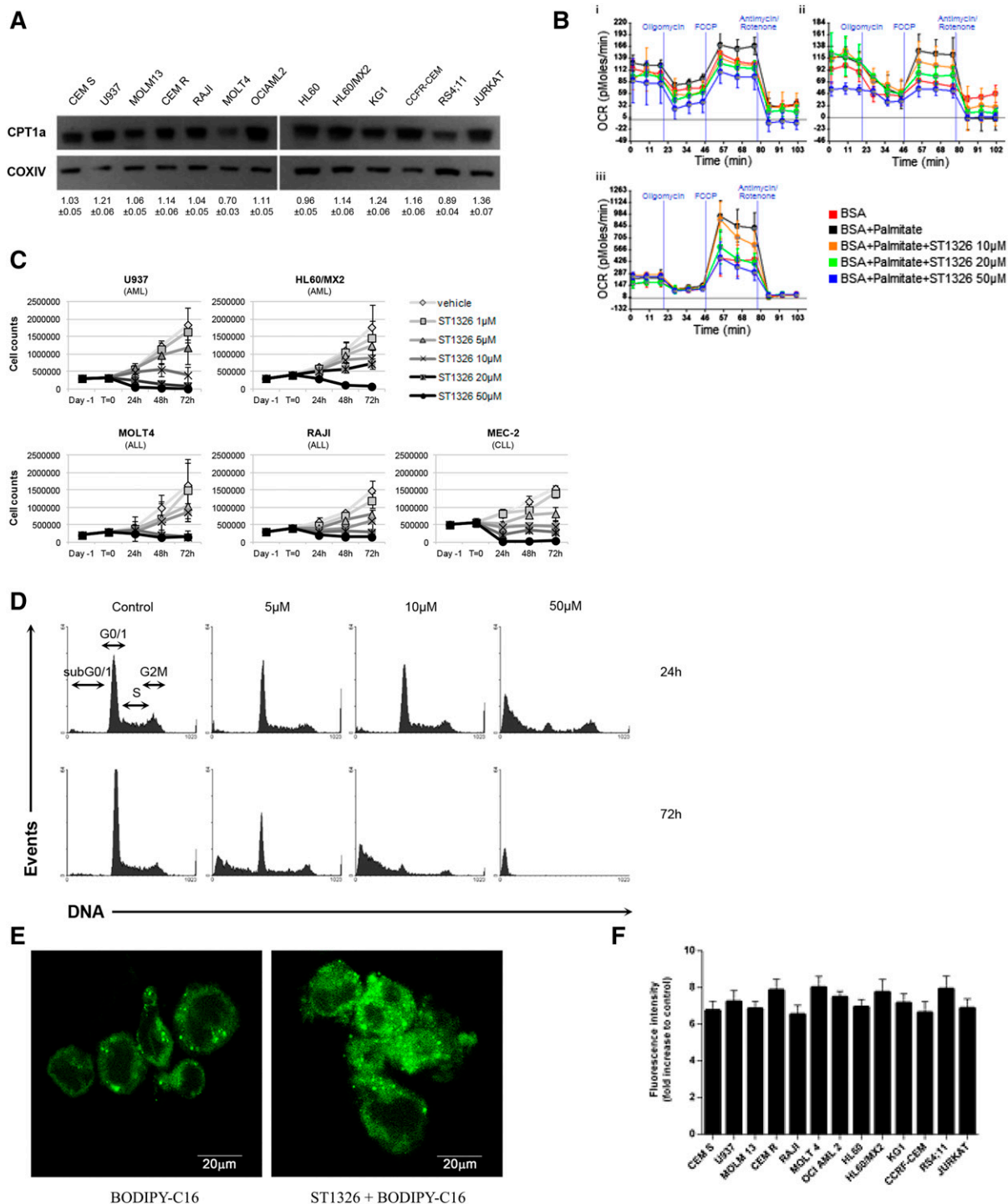


Figure 1. ST1326 induces FAO decrease, palmitate accumulation, and apoptosis in leukemia cell lines. (A) CPT1a expression in isolated mitochondria of leukemia cell lines. Cytochrome c oxidase subunit IV (COXIV) was used as housekeeping. Densitometry values of CPT1a/COXIV ratio are reported for each cell line as mean \pm standard deviation (SD) of 3 independent experiments. (B) ST1326 induces FAO decrease in cell line and primary AML cells. Time course of a representative FAO experiment on U937 (i), MOLT4 (ii), and MEC-2 (iii) cell lines exposed to increasing concentrations of ST1326. BSA, bovine serum albumin; OCR, oxygen consumption rate. (C) ST1326 induces cell growth arrest in dose- and time-dependent fashion in hematopoietic cell lines with different ontogenesis. Dose-response growth curves: several hematopoietic cell lines were exposed to increasing concentrations of ST1326 for the indicated periods of time. Cell counts and viability were then assessed by trypan blue exclusion counting. (D) ST1326 induces a dose- and time-dependent cell cycle modulation and apoptosis in U937 cells. U937 cells were exposed to the indicated concentrations of ST1326 or vehicle control up to 72 h. Distribution of cells in the different phases of cell cycle and in the sub-G0/1 peak was assessed by acridine orange DNA/RNA staining as described.^{16,17} DNA histograms show results of 1 representative experiment performed on U937 cells. (E) ST1326 induces intracellular palmitate accumulation. Representative images of U937 cells stained with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (BODIPY-C16) with or without ST1326 treatment as described in "Study design." Images were acquired with laser scanning confocal microscope (TCS SP2 AOB5; Leica Microsystems, Mannheim, Germany) using a $\times 40$ (numerical aperture = 1.25) oil immersion lens with optical pinhole at 1 AU. Scale bar indicates 20 μ m. (F) Effects of ST1326 on palmitate accumulation. Relative BODIPY-C16 uptake is expressed as fluorescence intensities (mean \pm SD of 3 independent experiments) in leukemia cell lines exposed to ST1326 as compared with the respective untreated controls. The increase of fluorescence intensities in ST1326-exposed cells is proportional to the intracellular palmitate accumulation (details in supplemental Materials and methods).

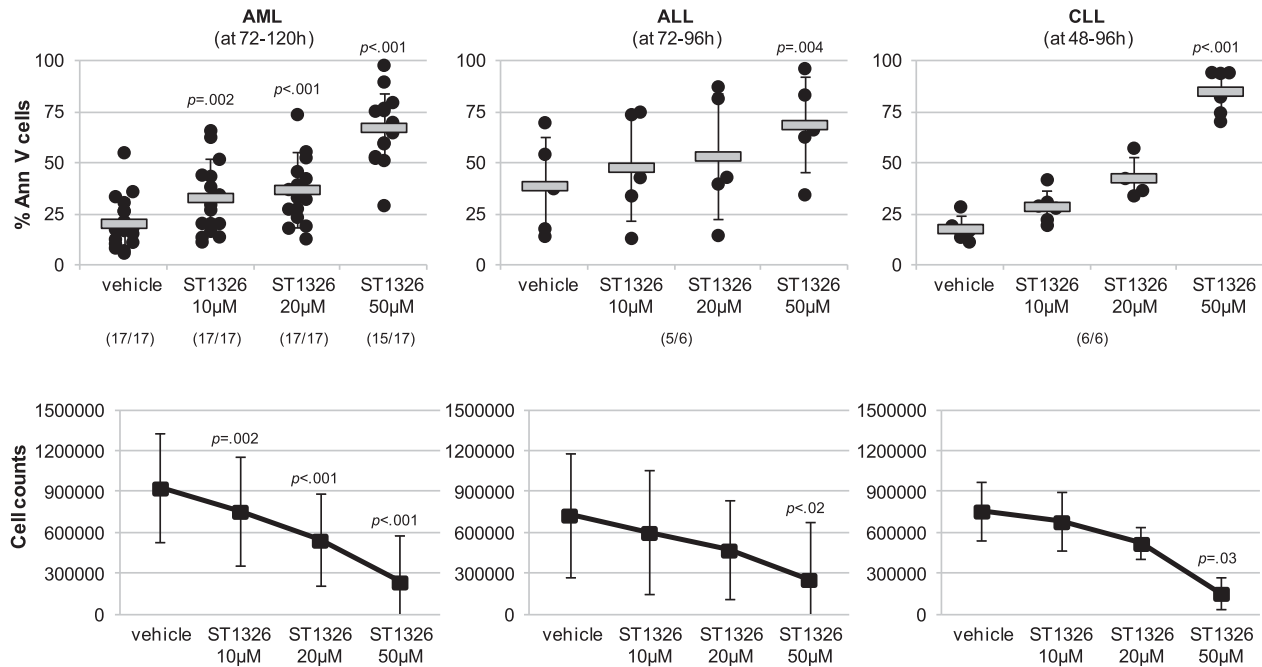


Figure 2. ST1326 induces a dose- and time-dependent apoptosis in primary cells from hematopoietic disease. AML, acute lymphoblastic leukemia (B-ALL), and chronic lymphoblastic leukemia (CLL) primary cells were exposed to the indicated concentrations of ST1326. Apoptosis induction was assessed by Ann V/propidium iodide (PI) staining (see supplemental Materials and methods). Percentages of Ann V-positive cells and Ann V-positive/PI cells are shown. Cell counts and viability were assessed at the indicated time points by trypan blue exclusion counting.

L-carnitine (both from Sigma-Aldrich) adjusted to pH 7.35, transferred in CellTak (BD Bioscience) coated plates, and incubated for 30 minutes at 37°C in a CO₂-free incubator. ST1326 or etomoxir (Sigma-Aldrich) were added 15 minutes before the assay to the indicated concentrations. Bovine serum albumin alone or conjugated to palmitate (Seahorse Bioscience) were added to each well just prior the assay, to a final concentration of 33 and 200 μM, respectively. Oxygen consumption rates were measured for basal state and following the sequential injection of oligomycin (1 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (0.4-0.8 μM), and a mix of antimycin A (1 μM) and rotenone (1 μM) (all from Seahorse Bioscience) in each well. After the assay, cells were detached and manually counted to assess cell viability.

CPT1a expression and activity and measurement of fluorescent palmitate accumulation

Details are reported in supplemental Materials and methods.

Results and discussion

We first demonstrated that all leukemia cell lines constitutively expressed CPT1a (Figure 1A) and that this enzyme activity was significantly inhibited by 2 μM ST1326 (supplemental Results and supplemental Table 2). Primary acute myeloid leukemia (AML) samples similarly to AML cell models express CPT1a: the mean densitometry values of CPT1a/COXIV ratio were 1.18 ± 0.12 and 1.12 ± 0.06 , respectively (data not shown).

We next documented that ST1326 was effective in inducing dose-dependent FAO inhibition as demonstrated by real-time metabolic analysis in cell lines (Figure 1B) and primary samples (supplemental Figure 1). Conversely, 50 μM etomoxir induced only minor effects (supplemental Figure 2).

We then analyzed the activity of ST1326 in 3 different leukemia models demonstrating that the CPT1a inhibitor induced dose- and time-dependent cell growth arrest, cell cycle modulation, and apoptosis (Figure 1C-D and supplemental Figure 3). In particular, a marked depletion of the S phase, associated with an increase in G2M phase and sub-G0/1 peak, was documented after 24 hours of exposure to 50 μM ST1326. The same effects occurred also at low ST1326 concentrations over longer times of exposure (Figure 1D). The cytotoxic activity of the molecule was also confirmed by using Ann V (supplemental Figure 3). Etomoxir failed to reproduce similar significant results (supplemental Figure 3). By monitoring the retention of chloromethyl-X-rosamine (CMXRos), we demonstrated that ST1326 induced a significant reduction of $\Delta\Psi_m$ (supplemental Figure 4). The loss of $\Delta\Psi_m$ preceded the phospholipid exposure and the formation of sub-G0/1 peak on DNA histogram demonstrating that ST1326 induced apoptosis through activation of the mitochondrial pathway. Drug washout experiments demonstrating the irreversible cytotoxic activity of ST1326 are reported in supplemental Results and supplemental Figure 5.

To investigate whether lipid accumulation is part of the mechanism that induces apoptosis in leukemia cells, we used BODIPY-C16 to stain intracellular palmitate accumulation. A representative microphotograph in Figure 1E showed an increase in green fluorescence by accumulated palmitate when cells were treated with 10 μM ST1326. The quantification of the fluorescence intensities in all cells revealed a significant block of palmitate metabolism (Figure 1F), which reinforces the idea that ST1326 can reduce β-oxidation in leukemia cells.

The results obtained from the analysis of AML primary samples indicated that ST1326 exerts a dose- and time-dependent cytotoxic activity in all (17/17) samples from de novo patients (Figure 2). Induction of apoptosis was associated with a significant reduction of

cell counts (Figure 2). Activity of ST1326 was confirmed by viability/ carboxyfluorescein diacetate succinimidyl ester (CFSE) staining/ CD34⁺ leukemic cells (supplemental Figure 6). ST1326 induced a significant increase in apoptosis also in samples from 2 relapsed AML patients: Ann V⁺ cells increased from 21.18% ± 7.75% to 25.23% ± 6.53% (*P* = not significant), 30.20% ± 3.27% (*P* = not significant), and 73.09% ± 4.11% (*P* = .00018) in the presence of 10, 20, and 50 μM ST1326, respectively (data not shown). Data demonstrating the favorable interaction of ST1326 with cytarabine (AraC) in AML cell lines and primary samples are reported in supplemental Results and supplemental Figure 7.

A time- and dose-dependent increase in the levels of apoptosis was observed in 5/5 samples from B-ALL and in 6/6 samples from chronic lymphoblastic leukemia analyzed after exposure to ST1326 (Figure 2). The apoptosis increase was associated with a significant (at 50 μM) cell count reduction (Figure 2). The sample obtained from a relapsed B-ALL patient was equally sensitive to ST1326 (data not shown).

In summary, our results show that there is a variability within and among leukemias in sensitivity to ST1326, which is not unexpected given the heterogeneity of these leukemias. These observations may support the perspective of leukemia patient stratification according to metabolic phenotypes.

No cytotoxic effects were detected on normal BM CD34⁺ cells as reported in the supplemental Results.

So far, lipid metabolism in cancer cells has been investigated mainly from the anabolic perspective, as fatty acid synthase is overexpressed in many cancers (reviewed in Menendez and Lupu¹⁹). Conversely, the importance of FAO in cancer cell bioenergetic balance has been poorly established. Our results agree with the data previously reported by Samudio et al,²⁰ reinforcing the idea that β-oxidation represents a crucial metabolic pathway for leukemic cells, not only for energy production but also for controlling cell growth, survival, and chemoresistance. Moreover, these observations have been strengthened by subsequent works on other hematologic malignancies.²¹⁻²³

Taken together, these findings emphasize the roles of FAO as a potential target for leukemia treatment and of ST1326 as a promising agent able to effectively inhibit FAO on leukemia cells, leading to cell growth reduction and apoptosis.

References

- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674.
- Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer*. 2011;11(2):85-95.
- DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab*. 2008;7(1):11-20.
- Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clin Cancer Res*. 2009;15(21):6479-6483.
- Robey RB, Hay N. Is Akt the "Warburg kinase"?—Akt-energy metabolism interactions and oncogenesis. *Semin Cancer Biol*. 2009;19(1):25-31.
- Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. 2006;3(3):177-185.
- Lu CW, Lin SC, Chen KF, Lai YY, Tsai SJ. Induction of pyruvate dehydrogenase kinase-3 by hypoxia-inducible factor-1 promotes metabolic switch and drug resistance. *J Biol Chem*. 2008;283(42):28106-28114.
- Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer*. 2009;9(8):563-575.
- Jones RG, Plas DR, Kubek S, et al. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell*. 2005;18(3):283-293.
- Carracedo A, Cantley LC, Pandolfi PP. Cancer metabolism: fatty acid oxidation in the limelight. *Nat Rev Cancer*. 2013;13(4):227-232.
- Paumen MB, Ishida Y, Han H, et al. Direct interaction of the mitochondrial membrane protein carnitine palmitoyltransferase I with Bcl-2. *Biochem Biophys Res Commun*. 1997;231(3):523-525.
- Giordano A, Calvani M, Petillo O, et al. tBid induces alterations of mitochondrial fatty acid oxidation flux by malonyl-CoA-independent inhibition of carnitine palmitoyltransferase-1. *Cell Death Differ*. 2005;12(6):603-613.
- Giannessi F, Pessotto P, Tassoni E, et al. Discovery of a long-chain carbamoyl aminocarnitine derivative, a reversible carnitine palmitoyltransferase inhibitor with antiketotic and antidiabetic activity. *J Med Chem*. 2003;46(2):303-309.
- Anderson RC. Carnitine palmitoyltransferase: a viable target for the treatment of NIDDM? *Curr Pharm Des*. 1998;4(1):1-16.
- Pacilli A, Calienni M, Margarucci S, et al. Carnitine-acyltransferase system inhibition, cancer cell death, and prevention of myc-induced lymphomagenesis. *J Natl Cancer Inst*. 2013;105(7):489-498.
- Ricciardi MR, Petrucci MT, Gregorj C, et al. Reduced susceptibility to apoptosis correlates with kinetic quiescence in disease progression of chronic lymphocytic leukaemia. *Br J Haematol*. 2001;113(2):391-399.
- Tafari A, Meyers J, Lee BJ, Andreeff M. DNA and RNA flow cytometric study in multiple myeloma. Clinical correlations. *Cancer*. 1991;67(2):449-454.
- Jiffar T, Kurinna S, Suck G, et al. PKC alpha mediates chemoresistance in acute lymphoblastic

Acknowledgments

The authors thank RomaAIL (Associazione Italiana contro le Leucemie-linfomi e mieloma) for providing laboratory space and D. Calef for editing the manuscript.

This work was supported by grants from Sapienza (C26G13WSNF and C26A137XXS), Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2010AX2JX7_003), Fondi strutturali europei "Ricerca e Competitività" (PON01_01802; PON01_02512), Sigma-Tau S.p.A., and Fondazione Internazionale D'AMATO Onlus.

Authorship

Contribution: M.R.R. designed the research, performed experiments, analyzed the data, and wrote the manuscript; S.M. conducted and analyzed FAO assays; R.L. and M.A. performed functional experiments and assisted with cell culture; A.C. performed CPT1a expression and activity experiments and fluorescence assays; R.F. provided samples and clinical data; M.R.T. provided critical revision of the manuscript; R.N. and G.P. provided scientific advice and critical revision of the manuscript; and A.T. designed the research, supervised the project, and wrote the manuscript.

Conflict-of-interest disclosure: R.N. is employed at Sigma-Tau S.p.A. as medical research senior advisor. Sigma-Tau is an Italian pharmaceutical company owner of the patent for ST1326. The co-author declares that there are no conflicts of interest regarding the manuscript because she did not receive any financial supports or benefits, nor did any member of her immediate family, for writing this paper. The remaining authors declare no competing financial interests.

Correspondence: Agostino Tafuri, Hematology, "Sant'Andrea" Hospital—Sapienza, University of Rome, Department of Clinical and Molecular Medicine, Via di Grottarossa 1035, 00189 Rome, Italy; e-mail: agostino.tafuri@uniroma1.it.

- leukemia through effects on Bcl2 phosphorylation. *Leukemia*. 2004;18(3):505-512.
19. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer*. 2007;7(10):763-777.
20. Samudio I, Harmancey R, Fiegl M, et al. Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. *J Clin Invest*. 2010;120(1):142-156.
21. Tirado-Vélez JM, Joumady I, Sáez-Benito A, Cózar-Castellano I, Perdomo G. Inhibition of fatty acid metabolism reduces human myeloma cells proliferation. *PLoS One*. 2012;7(9):e46484.
22. Caro P, Kishan AU, Norberg E, et al. Metabolic signatures uncover distinct targets in molecular subsets of diffuse large B cell lymphoma. *Cancer Cell*. 2012;22(4):547-560.
23. Tung S, Shi Y, Wong K, et al. PPAR α and fatty acid oxidation mediate glucocorticoid resistance in chronic lymphocytic leukemia. *Blood*. 2013;122(6):969-980.