Brief report

The role of phosphatidylinositol 3-kinase-δ in the immunomodulatory effects of lenalidomide in chronic lymphocytic leukemia

*Sarah E. M. Herman,[1,2] *Rosa Lapalombella,[2] Amber L. Gordon,[2] Asha Ramanunni,[2] Kristie A. Blum,[2] Jeffrey Jones,[2] Xiaoli Zhang,[3] Brian J. Lannutti,[4] Kamal D. Puri,[4] Natarajan Muthusamy,[1,2] †John C. Byrd,[1,2,5] and †Amy J. Johnson[1,2,5]

[1]Integrated Biomedical Science Graduate Program, The Ohio State University Medical Center, Columbus, OH; [2]Division of Hematology, Department of Internal Medicine, The Ohio State University, Columbus, OH; [3]Center for Biostatistics, The Ohio State University, Columbus, OH; [4]Calistoga Pharmaceuticals Inc, Seattle, WA; and [5]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH

In patients with chronic lymphocytic leukemia (CLL), lenalidomide can promote humoral immune responses but also induces a distinct disease-specific toxicity of tumor flare and cytokine release. These CLL-specific events result from increased expression of costimulatory molecules on B cells. Here we demonstrate that lenalidomide activation of CLL cells depends on the phosphatidylinositol 3-kinase p110δ (PI3K-δ) pathway. Inhibition of PI3K-δ signaling by the PI3K-δ-inhibiting drug, CAL-101, or by siRNA knockdown of p110δ, abrogates CLL cell activation, costimulatory molecule expression, and vascular endothelial growth factor and basic fibroblast growth factor gene expression that is induced by lenalidomide. In addition, CAL-101 attenuates lenalidomide-mediated increases in immunoglobulin M production by normal B cells. Collectively, these data demonstrate the importance of PI3K-δ signaling for lenalidomide immune modulation. These findings may guide development of strategies for the treatment of CLL that combine lenalidomide with CAL-101, with other inhibitors of the PI3K-δ pathway, or with other agents that target downstream kinases of this signaling pathway. (*Blood*. 2011;117(16): 4323-4327)

Introduction

Lenalidomide is an immune modulatory agent currently approved for marketing in multiple myeloma and myelodysplasia. Lenalidomide is also clinically active in lymphoma, acute myeloid leukemia, and chronic lymphocytic leukemia (CLL).[1,2] The potential mechanisms of action of lenalidomide in these different diseases are multiple.[3] Application of lenalidomide in CLL has been associated with development of antitumor antibodies and reversal of hypogammaglobulinemia[4] but can also induce a disease-specific side effect of tumor flare and cytokine release.[3,5] The downstream manifestations of cytokine release, including increased serum basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), have previously been associated with promoting CLL survival[6-11] and also with decreased response to lenalidomide in CLL.[2] Understanding the mechanisms of these responses to lenalidomide is relevant to the development of this agent in CLL and to the logical design of future combination studies.

We have recently demonstrated that lenalidomide can increase surface expression of CD154 on CLL cells while promoting normal B cells to produce immunoglobulin G (IgG) and IgM.[4] After clinical treatment with lenalidomide, we were also able to demonstrate a similar phenotype in CLL cells in patients receiving CD154 gene therapy,[12] including up-regulation of DR5 and BID. In one patient with pretreatment evidence of residual normal B cells, lenalidomide induced the development of antibodies, including generation of antitumor-directed ROR-1 antibodies.[4] The mechanism by which this occurred was shown in vitro to involve upstream activation of the phosphatidylinositol 3-kinase (PI3K) pathway.[4] Immune activation of CLL cells with up-regulation of costimulatory molecules, such as CD40, CD80, and CD86,[4,5,13] may also be responsible for lenalidomide-induced tumor flare. Given that CLL cell activation could have both favorable immune-modulating effects and detrimental clinical effects from tumor flare and also production of antiapoptotic cytokines, such as bFGF and VEGF, we sought to characterize whether a specific PI3K isoform was responsible for CLL activation by lenalidomide. This work is highly relevant given the observed preclinical[14] and clinical activity[15] of the PI3K p110δ (PI3K-δ) inhibitor, CAL-101, in the treatment of CLL, the development of other compounds targeting B-cell receptor signaling,[16,17] and the potential interest in administering these newer drugs with lenalidomide as therapy for CLL.

Methods

Cell culture and treatment reagents

Written, informed consent was obtained in accordance with the Declaration of Helsinki to procure cells from patients with CLL following a blood collection protocol approved by The Ohio State University Institutional Review Board,[18] and selection was performed as previously described.[19] CAL-101 was supplied by Calistoga Pharmaceuticals. Lenalidomide (Revlimid; Celgene) was obtained as previously described.[5] The 0.5μM dose of lenalidomide and 1μM dose of CAL-101 used in the in vitro experiments
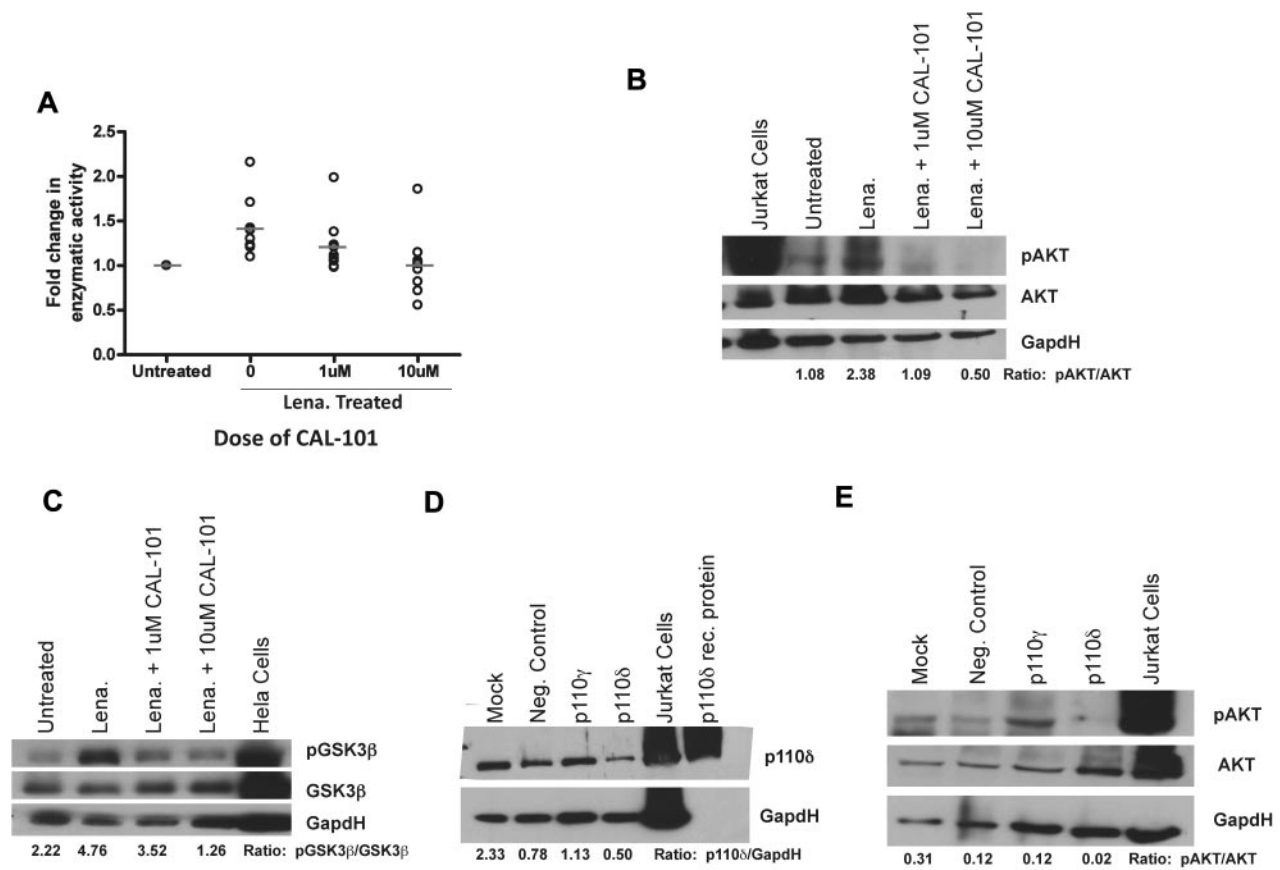
Figure 1. Lenalidomide leads to activation of the PI3K pathway via a PI3K-δ-dependent mechanism. (A) CD19$^+$ cells from CLL patients (N = 9) treated with or without 0.5μM lenalidomide were examined for PI3K activity with and without the addition of 1 or 10μM CAL-101 to the lysate. Results were calculated relative to micrograms of protein. (B) CD19$^+$ cells from CLL patients (N = 6) were incubated with or without 0.5μM lenalidomide and/or CAL-101 for 48 hours. AKT phosphorylation at Ser473 was assessed by immunoblot. Results are shown from one of 6 experiments. (C) CD19$^+$ cells from CLL patients (N = 4) were incubated with or without 0.5μM lenalidomide and/or CAL-101 for 48 hours. GSK3β phosphorylation at Ser9 was assessed by immunoblot. Results are shown from one of 4 experiments. (D) CD19$^+$ cells from CLL patients (N = 3) were transfected with siRNA targeted to PI3K-δ, PI3K-γ, or a nonsense target. p110δ protein expression was assessed by immunoblot. Results are shown from one of 3 experiments. (E) CD19$^+$ cells from CLL patients (N = 3) were transfected with siRNA targeted to PI3K-δ, PI3K-γ, or a nonsense target and then incubated with or without 0.5μM lenalidomide for 48 hours. AKT phosphorylation at Ser473 was assessed by immunoblot. Results are shown from one of 3 experiments. (B-E) Quantification was done using the Alpha Innotech FluorChemQ MultiImage III System.

are reached clinically. In addition, as we reported,[14] CAL-101 has a modest effect on cell viability; thus, the conditions used in these experiments show little effects on viability.

Flow cytometry

Surface staining with antibodies to CD20, CD40, CD80, CD86, or IgG1 (BD Biosciences) was done as previously described.[4]

Immunoblot analysis

Immunoblots were performed as previously described.[20] Antibodies included: anti-AKT, antiphospho-AKT (Ser473), anti-GSK3β, antiphospho-GSK3β (Ser 9; Cell Signaling), anti-p110δ (Santa Cruz Biotechnology), and anti-GAPDH (Millipore).

Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was prepared using a SuperScript First-Strand Synthesis System (Invitrogen) as previously described.[21] Real-Time PCR was performed using predesigned TaqMan Gene Expression Assays and an ABI Prism 7700 sequence detection system (Applied Biosystems).

PI3K assay

The PI3K assay was performed on whole-cell lysates from CLL cells. The enzyme-linked immunosorbent assay was performed according to the manufacturer's instructions as previously described[14] (Echelon Biosciences).

siRNA transfection

CLL cells were transfected as previously described.[22] PI3K-δ-specific siRNA (Ambion) was used at a final concentration of 50nM.

Ig detection

Quantitation of IgM was determined as previously described.[4] Briefly, lenalidomide-treated or vehicle-treated CLL cells were irradiated and placed in culture with target, normal B cells in the absence or presence of pokeweed mitogen (5 μg/mL).

Statistical analysis

All reported statistical evaluations were performed with methods previously described.[14] Because the same patient samples were treated under different conditions, linear mixed effects models were used for all analyses to account for the correlations of observations from the same patient. Reverse-transcribed polymerase chain reaction (RT-PCR) data were first normalized to internal controls before analysis. Log transformation was also used for some experiments to reduce variance and skewness. The Holm procedure was used to adjust for multiple comparisons. P values $\leq .05$ (including those adjusted for multiple comparisons) were considered significant.
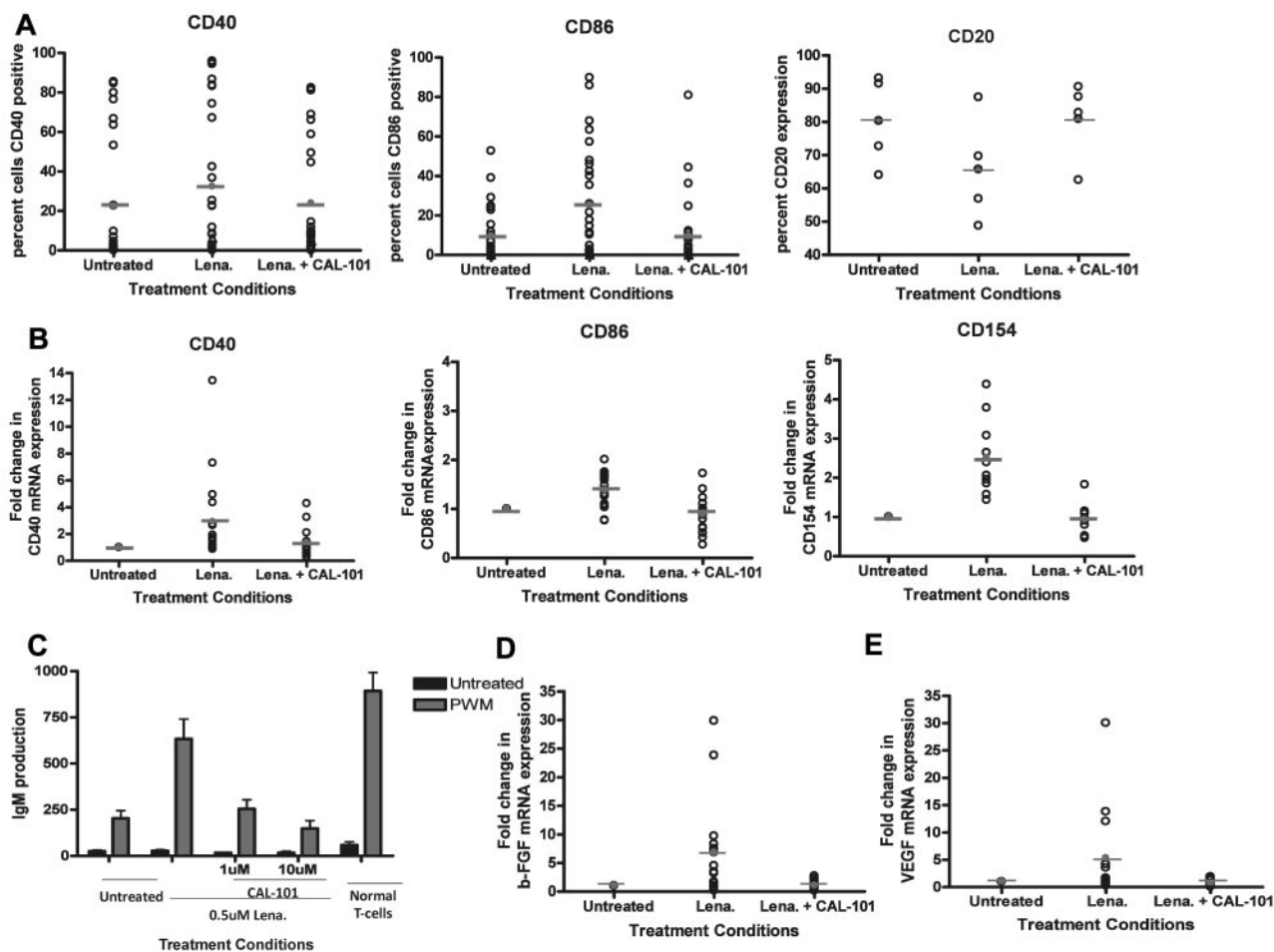
Figure 2. Inhibition of PI3K-δ prevents CLL cell immune activation induced by lenalidomide. (A) CD19^{+} cells from CLL patients (N = 25) were treated with or without 0.5μM lenalidomide and/or 10μM CAL-101 for 48 hours. Surface expression of CD20, CD40, or CD86 was evaluated by flow cytometry using CD20-phycoerythrin, CD40-phycoerythrin, or CD86-phycoerythrin antibodies and IgG1-phycoerythrin isotype control. (B) CD19^{+} cells from CLL patients (N = 15) were treated with or without 0.5μM lenalidomide and/or 10μM CAL-101 for 48 hours. RNA was extracted and converted to cDNA, and RT-PCR analysis was done to determine quantities of CD40, CD86, and CD154 mRNA. (C) CD19^{+} cells from CLL patients (N = 6) were treated with or without 0.5μM lenalidomide and/or CAL-101 for 48 hours. CLL cells were irradiated (20 Gy) and placed in culture with purified B cells, in the absence or presence of 5 μg/mL pokeweed mitogen. Quantification of IgM was determined by enzyme-linked immunosorbent assay. (D) CD19^{+} cells from CLL patients (N = 15) were treated with or without 0.5μM lenalidomide and/or 10μM CAL-101 for 48 hours. RNA was extracted and converted to cDNA, and RT-PCR analysis was done to determine quantities of bFGF. (E) CD19^{+} cells from CLL patients (N = 13) were treated with or without 0.5μM lenalidomide and/or 10μM CAL-101 for 48 hours. RNA was extracted and converted to cDNA, and RT-PCR analysis was done to determine quantities of VEGF.

Results and discussion

Previous studies have demonstrated that lenalidomide up-regulates CD154 protein expression on CLL cells by activation of AKT, I-κ kinase, and by nuclear factor-κB nuclear translocation with subsequent increased mRNA transcription and stabilization without inducing cell death.[4] Treatment of CLL cells with the pan-PI3K inhibitor LY294002 antagonized this CD154 up-regulation.[4] PI3K signaling by lenalidomide could occur through any of the 4 catalytic isoforms of PI3K: p110α, p110β, p110γ, and p110δ. We therefore sought to determine whether a specific isoform of PI3K was responsible for this. We first confirmed that lenalidomide directly increased the enzymatic activity of PI3K ($P = .0012$; Figure 1A). We found that inhibition of PI3K-δ via the p110δ-specific small-molecule inhibitor CAL-101 prevented the increase in PI3K enzymatic activity ($P = .0152$; Figure 1A). Next, we sought to determine whether inhibition of PI3K-δ with CAL-101 could prevent the increase in downstream phosphorylation of AKT induced by lenalidomide; we found that inhibition of PI3K-δ by CAL-101 prevented phospho-AKT formation (Figure 1B). To

confirm these results, we evaluated the phosphorylation of GSK3β, another downstream protein in the PI3K pathway. We found that lenalidomide provoked an increase in phospho-GSK3β that was preventable by cotreatment with CAL-101, again suggesting a link between PI3K-δ and lenalidomide-dependent PI3K activity (Figure 1C). To confirm that these results were indeed the result of PI3K-δ inhibition, we knocked down PI3K-δ in CLL cells (Figure 1D). We were able to show that lenalidomide was unable to induce phosphorylation of AKT when PI3K-δ-specific siRNA was expressed (Figure 1E). Given our desire to focus on selectively expressed hematopoietic isoforms, we did not target p110α and p110β. Such investigation with pan-PI3K inhibitors warrants future studies. These findings demonstrate that lenalidomide-mediated PI3K activation and subsequent effects on nuclear factor-κB and CD154[4] in CLL cells use a PI3K-δ-dependent pathway.

We have previously shown that lenalidomide activates CLL cells. To extend our findings, we sought to determine whether inhibition of PI3K-δ prevented lenalidomide-induced up-regulation of other costimulatory molecules important to B-cell antigen presentation.[4] We found that treatment with the PI3K-δ inhibitor CAL-101 was able to prevent the up-regulation of CD40

and CD86 induced by lenalidomide ($P = .0102$ and $< .0001$, respectively; Figure 2A). Similarly, we found that inhibition of PI3K-δ could also prevent the increase in mRNA of CD40, CD86, CD154, and CD80 promoted by lenalidomide ($P < .009$ for all genes; Figure 2B; and data not shown). Activation of CLL cells by lenalidomide has also been shown to induce internalization of the CD20 antigen on CLL cells[21] similar to that observed in activated normal B cells[23,24]; concurrent treatment with CAL-101 prevented this effect ($P = .0057$; Figure 2A). The CD40-CD154 axis is important in allowing lenalidomide-treated CLL cells to promote Ig production by normal B cells; thus, we next assessed whether CAL-101 could prevent the occurrence of this effect. As we have also previously shown,[4] lenalidomide treatment of CLL cells increased IgM levels. However, pretreatment of CLL cells with CAL-101 could completely prevent production of IgM by normal B cells ($P < .0001$; Figure 2C). Cytokines, such as bFGF[6-8] and VEGF,[6,9-11] support the survival of CLL cells. Moreover, it has been observed that circulating levels of bFGF can correlate with response or nonresponse to therapy for CLL.[2] Thus, we sought to determine whether lenalidomide treatment of CLL cells in culture could promote conditions favoring enhanced tumor cell production of these factors. We found that lenalidomide treatment increased bFGF mRNA in all patients ($P = .0004$) and that coincubation with CAL-101 prevented this up-regulation ($P < .001$; Figure 2D). Similarly, we found that lenalidomide treatment increased VEGF mRNA in a subset (8 of 13) of patients and that this effect was also reversed by CAL-101 cotreatment ($P = .002$; Figure 2E).

Our data demonstrate that the PI3K-δ pathway is involved in lenalidomide-mediated activation of CLL cells. These findings have potential relevance to the clinical use of the combination of lenalidomide with CAL-101, pan-PI3K inhibitors, and potentially other B-cell receptor-signaling agents. Our data suggest that such agents may antagonize the immune-modulating properties of lenalidomide. Thus, if lenalidomide-mediated immune modulation is considered desirable, coadministration of such drugs with lenalidomide should be approached with caution. Conversely, concomitant therapy with agents, such as CAL-101, might be attractive for preventing the immune activation, tumor flare, and cytokine-release syndrome associated with lenalidomide treatment. The addition of CAL-101 or similar drugs to lenalidomide might also reduce the induction of cytokines, such as bFGF and VEGF, which has been associated with poor response to treatment, thus offering the prospect of enhanced antitumor activity with combination therapy.[2]

Authorship

Contribution: S.E.M.H. planned the research, performed experiments, analyzed data, drafted the first and subsequent drafts of the paper, and approved the final version of the paper; R.L. planned the research, performed experiments, analyzed data, assisted in drafting the paper, and approved the final version of the paper; A.L.G. and A.R. were involved in planning components of the research, performed experiments, reviewed drafts, and approved the final version of the paper; K.A.B. and J.J. were involved in recruitment of patients and sample collection, reviewed drafts, and approved the final version of the paper; X.Z. was involved in planning components of the research, did all the statistical analysis, reviewed drafts, and approved the final version of the paper; B.J.L. and K.D.P. provided input and suggestions to the presentation of the data and a critical reagent (CAL-101) essential for completion of the work and reviewed and approved the final version of the paper; and N.M., J.C.B., and A.J.J. planned every aspect of the proposal, supervised the research, analyzed data, reviewed drafts, obtained funding for the research work, and approved the final version of the paper.

Conflict-of-interest disclosure: J.C.B. has financial interest in Calistoga Pharmaceuticals (stock options). B.J.L. and K.D.P. are employees of Calistoga Pharmaceuticals and have financial interest in this company (stock options and salary). The remaining authors declare no competing financial interests.

Correspondence: John C. Byrd, The Ohio State University, B302 Starling-Loving Hall, 320 West 10th Ave, Columbus, OH 43210; e-mail: john.byrd@osumc.edu; and Amy J. Johnson, The Ohio State University Comprehensive Cancer Center Bldg, Rm 455C, 410 West 12th Ave, Columbus, OH 43210; e-mail: amy.johnson@osumc.edu.

References

1. Chanan-Khan A, Miller KC, Musial L, et al. Clinical efficacy of lenalidomide in patients with relapsed or refractory chronic lymphocytic leukemia: results of a phase II study. *J Clin Oncol.* 2006;24(34):5343-5349.

2. Ferrajoli A, Lee BN, Schlette EJ, et al. Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. *Blood.* 2008;111(11):5291-5297.

3. Awan FT, Johnson AJ, Lapalombella R, et al. Thalidomide and lenalidomide as new therapeutics for the treatment of chronic lymphocytic leukemia. *Leuk Lymphoma* 51(1):27-38.

4. Lapalombella R, Andritsos L, Liu Q, et al. Lenalidomide treatment promotes CD154 expression on CLL cells and enhances production of antibodies by normal B cells through a PI3-kinase-dependent pathway. *Blood.* 2010;115(13):2619-2629.

5. Andritsos LA, Johnson AJ, Lozanski G, et al. Higher doses of lenalidomide are associated with unacceptable toxicity including life-threatening tumor flare in patients with chronic lymphocytic leukemia. *J Clin Oncol.* 2008;26(15):2519-2525.

6. Kay NE, Bone ND, Tschumper RC, et al. B-CLL cells are capable of synthesis and secretion of both pro- and anti-angiogenic molecules. *Leukemia.* 2002;16(5):911-919.

7. Konig A, Menzel T, Lynen S, et al. Basic fibroblast growth factor (bFGF) upregulates the expression of bcl-2 in B cell chronic lymphocytic leukemia cell lines resulting in delaying apoptosis. *Leukemia.* 1997;11(2):258-265.

8. Menzel T, Rahman Z, Calleja E, et al. Elevated intracellular level of basic fibroblast growth factor correlates with stage of chronic lymphocytic leukemia and is associated with resistance to fludarabine. *Blood.* 1996;87(3):1056-1063.

9. Lee YK, Shanafelt TD, Bone ND, Strege AK, Jelinek DF, Kay NE. VEGF receptors on chronic lymphocytic leukemia (CLL) B cells interact with STAT 1 and 3: implication for apoptosis resistance. *Leukemia.* 2005;19(4):513-523.

10. Farahani M, Treweeke AT, Toh CH, et al. Autocrine VEGF mediates the antiapoptotic effect of CD154 on CLL cells. *Leukemia.* 2005;19(4):524-530.

11. Chen H, Treweeke AT, West DC, et al. In vitro and in vivo production of vascular endothelial growth factor by chronic lymphocytic leukemia cells. *Blood.* 2000;96(9):3181-3187.

12. Wierda WG, Cantwell MJ, Woods SJ, Rassenti LZ, Prussak CE, Kipps TJ. CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. *Blood.* 2000;96(9):2917-2924.

13. Aue G, Njuguna N, Tian X, et al. Lenalidomide-induced upregulation of CD80 on tumor cells correlates with T-cell activation, the rapid onset of a cytokine release syndrome and leukemic cell

clearance in chronic lymphocytic leukemia. *Haematologica.* 2009;94(9):1266-1273.

14. Herman SE, Gordon AL, Wagner AJ, et al. Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood.* 2010;116(12):2078-2088.

15. Furman RRF, Coutre SE, Benson DM, et al. Interim results for a phase I study of CAL-101, a selective oral inhibitor of phosphatidylinositol 3-kinase p110delta isoform, in patients with relapsed or refractory hematologic malignancies. *J Clin Oncol.* 2010;28(suppl):15. Abstract 3032.

16. Friedberg JW, Sharman J, Sweetenham J, et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood.* 2010;115(13):2578-2585.

17. Advani JPS, Smith SM, Pollyea DA, et al. Effect of Btk inhibitor PCI-32765 monotherapy on responses in patients with relapsed aggressive NHL: evidence of antitumor activity from a phase I study. *J Clin Oncol.* 2010;28(suppl):15. Abstract 8012.

18. Keating M, Cheson B, Gribben JG, Robertson B, Nadler L. Innovative strategies for the treatment of CLL. *Leuk Lymphoma.* 1996;22(suppl 2):53-64.

19. Lucas DM, Edwards RB, Lozanski G, et al. The novel plant-derived agent silvestrol has B-cell selective activity in chronic lymphocytic leukemia and acute lymphoblastic leukemia in vitro and in vivo. *Blood.* 2009;113(19):4656-4666.

20. Johnson AJ, Lucas DM, Muthusamy N, et al. Characterization of the TCL-1 transgenic mouse as a preclinical drug development tool for human chronic lymphocytic leukemia. *Blood.* 2006;108(4):1334-1338.

21. Lapalombella R, Yu B, Triantafillou G, et al. Lenalidomide down-regulates the CD20 antigen and antagonizes direct and antibody-dependent cellular cytotoxicity of rituximab on primary chronic lymphocytic leukemia cells. *Blood.* 2008;112(13):5180-5189.

22. Hussain SR, Cheney CM, Johnson AJ, et al. Mcl-1 is a relevant therapeutic target in acute and chronic lymphoid malignancies: down-regulation enhances rituximab-mediated apoptosis and complement-dependent cytotoxicity. *Clin Cancer Res.* 2007;13(7):2144-2150.

23. Valentine MA, Cotner T, Gaur L, Torres R, Clark EA. Expression of the human B-cell surface protein CD20: alteration by phorbol 12-myristate 13-acetate. *Proc Natl Acad Sci U S A.* 1987;84(22):8085-8089.

24. Anolik J, Looney RJ, Bottaro A, Sanz I, Young F. Down-regulation of CD20 on B cells upon CD40 activation. *Eur J Immunol.* 2003;33(9):2398-2409.