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

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Running Title: Lenalidomide Immunomodulation is PI3K-δ dependent

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ABSTRACT

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 co-stimulatory molecules on B-cells. Here we demonstrate that lenalidomide activation of CLL cells depends on the phosphatidylinositol 3-kinase p110-delta (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, co-stimulatory molecule expression, and VEGF and b-FGF gene expression that are induced by lenalidomide. In addition, CAL-101 attenuates lenalidomide-mediated increases in IgM 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.
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 (reviewed in \(^3\)). Application of lenalidomide in CLL has been associated with development of anti-tumor antibodies and reversal of hypogammaglobulinemia\(^4\), but can also induce a disease-specific side effect of tumor flare and cytokine release\(^3,5\). The down-stream manifestations of cytokine release, including increased serum b-FGF and 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 IgG and IgM\(^4\). Following 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 pre-treatment evidence of residual normal B-cells, lenalidomide induced the development of antibodies, including generation of anti-tumor-directed ROR-1 antibodies\(^4\). The mechanism by which this occurred was shown in vitro to involve up-stream activation of the phosphatidylinositol 3-kinase (PI3K) pathway\(^4\). Immune activation of CLL cells with up-regulation of co-stimulatory 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 anti-apoptotic cytokines such as b-FGF and VEGF, we sought to characterize if a specific PI3-kinase isoform was responsible for CLL activation by lenalidomide. This work is highly relevant given the observed pre-clinical\(^14\)and clinical activity\(^15\) of the phosphatidylinositol
3-kinase p110-delta (PI3K-δ) inhibitor, CAL-101, in the treatment of CLL; the development of other compounds targeting B-cell receptor signaling\textsuperscript{16,17}; and the potential interest in administering these newer drugs with lenalidomide as therapy for CLL.

**MATERIALS AND 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 IRB\textsuperscript{18} and selection was performed as previously described\textsuperscript{19}. CAL-101 was supplied by Calistoga Pharmaceuticals (Seattle, WA). Lenalidomide (Revlimid; Celgene, Summit, NJ) was obtained as previously described\textsuperscript{5}. The 0.5\textmu M dose of lenalidomide and 1\textmu M dose of CAL-101 used in the in vitro experiments are reached clinically. In addition as we reported\textsuperscript{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, San Jose CA) was done as previously described\textsuperscript{4}.

*Immunoblot analysis.* Immunoblots were performed as previously described\textsuperscript{20}. Antibodies included: anti-AKT, anti-phospho-AKT (Ser473), anti-GSK3\textbeta, anti-phospho-GSK3\textbeta (Ser9) (Cell Signaling, Danvers, MA), anti-p110\textdelta (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-GAPDH (Millipore, Billerica, MA).

*Quantitative RT-PCR.* RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was prepared using a SuperScript First-Strand Synthesis System (Invitrogen) as previously described\textsuperscript{21}. Real-Time PCR was performed using pre-designed TaqMan\textregistered Gene Expression Assays and an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).
**PI3K Assay.** The PI3K assay was performed on whole-cell lysates from CLL cells. The ELISA assay was performed according to the manufacturer’s instructions as previously described\(^{14}\) (Echelon Biosciences, Salt Lake City, UT).

**siRNA Transfection.** CLL cells were transfected as previously described\(^{22}\). PI3K-δ-specific siRNA (Ambion, Austin, TX) was used at a final concentration of 50nM.

**Immunoglobulin 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 (PWM) (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 condition, linear mixed effects models were used for all analysis to account for the correlations of observations from the same patient. RT-PCR data was first normalized to internal controls before analysis. Log transformation was also used for some experiments to reduce variance and skewness. Holm’s procedure was used to adjust for multiple comparisons. \(P\)-values of \(\leq 0.05\) (including those adjusted for multiple comparisons) were considered significant.

**RESULTS AND DISCUSSION**

Previous studies have demonstrated lenalidomide upregulates CD154 protein expression on CLL cells by activation of AKT, IKK, and by NF-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 upregulation\(^4\). PI3K signaling by lenalidomide could occur through any of the four catalytic isoforms of PI3K: p110\(\alpha\), p110\(\beta\), p110\(\gamma\), and p110\(\delta\). We therefore sought to determine if a specific isoform of PI3K was responsible for this. We first confirmed that lenalidomide directly increased the enzymatic activity of PI3K (\(p\)-value=0.0012) (Fig. 1A). We found that inhibition of PI3K-δ via the p110\(\delta\)-specific small-molecule inhibitor, CAL-101, prevented the increase in PI3K activity.
enzymatic activity (p-value=0.0152) (Fig. 1A). Next we sought to determine if 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 (Fig. 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 co-treatment with CAL-101, again suggesting a link between PI3K-δ and lenalidomide-dependent PI3K activity (Fig. 1C). To confirm that these results were in fact due to PI3K-δ inhibition, we knocked-down PI3K-δ in CLL cells (Fig 1D). We were able to show that lenalidomide was unable to induce phosphorylation of AKT when PI3K-δ-specific siRNA was expressed (Fig 1E). Given our desire to focus on relatively select expressed hematopoietic isoforms, we did not target p110 alpha and beta. Such investigation with pan-PI3-kinase inhibitors warrants future studies. These findings demonstrate that lenalidomide-mediated PI3K activation and subsequent effects on NF-κB and CD154 in CLL cells utilize a PI3K-δ-dependent pathway.

We have previously shown that lenalidomide activates CLL cells. To extend our findings, we sought to determine if inhibition of PI3K-δ prevented lenalidomide-induced upregulation of other co-stimulatory molecules important to B-cell antigen presentation. We found that treatment with the PI3K-δ inhibitor CAL-101 was able to prevent the upregulation of CD40 and CD86 induced by lenalidomide (p-value=0.0102 and <0.0001, respectively) (Fig. 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-value<0.009 for all genes) (Fig. 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 similar to that observed in activated normal B-cells; concurrent treatment with CAL-101 prevented this effect (p-value=0.0057) (Fig. 2A). The CD40-CD154 axis is important in allowing lenalidomide-treated CLL cells to promote
immunoglobulin production by normal B-cells; thus, we next assessed if CAL-101 could prevent the occurrence of this effect. As we have also previously shown⁶, lenalidomide treatment of CLL cells increased IgM levels. However, pre-treatment of CLL cells with CAL-101 could completely prevent production of IgM by normal B-cells (p-value<0.0001) (Fig. 2C).

Cytokines such as basic fibroblast growth factor (b-FGF)⁶-⁸ and vascular endothelial growth factor (VEGF)⁶,⁹-¹¹ support the survival of CLL cells. Moreover, it has been observed that circulating levels of b-FGF can correlate with response or non-response to therapy for CLL². Thus, we sought to determine if lenalidomide treatment of CLL cells in culture could promote conditions favoring enhanced tumor cell production of these factors. We found that lenalidomide treatment increased b-FGF mRNA in all patients (p-value=0.0004) and that co-incubation with CAL-101 prevented this up-regulation (p-value<0.001) (Fig. 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 co-treatment (p-value=0.002) (Fig. 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, co-administration 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 b-FGF and VEGF that has been associated with poor response to treatment, thus offering the prospect of enhanced anti-tumor activity with combination therapy².
ACKNOWLEDGEMENTS

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AUTHOR DISCLOSURES

Dr. John Byrd has financial interest in this company (stock options).

Drs. Brian Lannutti and Kamal Puri are employees of Calistoga Pharmaceuticals and have financial interest in this company (stock options and salary).

AUTHOR CONTRIBUTIONS

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 of the paper, and approved the final version of the paper.

A.L.G. and A.R. were involved in planning components of the research, performing 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.

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.

REFERENCES


Figure Legends

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 0.5 μM lenalidomide were examined for PI3K activity with and without the addition of 1 or 10 μM CAL-101 to the lysate. Results were calculated relative to μg of protein. (B) CD19⁺ cells from CLL patients (N=6) were incubated with or without 0.5 μM lenalidomide and/or 10 μM CAL-101 for 48 hours. AKT phosphorylation at ser473 was assessed by immunoblot. Results are shown from one of six experiments. (C) CD19⁺ cells from CLL patients (N=4) were incubated with or without 0.5 μM lenalidomide and/or 10 μM CAL-101 for 48 hours. GSK3β phosphorylation at ser9 was assessed by immunoblot. Results are shown from one of four 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 three 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 0.5 μM lenalidomide for 48 hours. AKT phosphorylation at ser473 was assessed by immunoblot. Results are shown from one of three experiments. B-E: Quantification was done using the Alpha Innotech FluorChemQ Multimage III System.

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 0.5 μM lenalidomide and/or 10 μM CAL-101 for 48 hours. Surface expression of CD20, CD40 or CD86 was evaluated by flow cytometry using CD20-, CD40- or CD86-PE antibodies and IgG1-PE isotype control. (B) CD19⁺ cells from CLL patients (N=15) were treated with or without 0.5 μM lenalidomide and/or 10 μM 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 0.5 μM lenalidomide and/or 10 μM 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 PWM. Quantification of IgM was determined by ELISA. (D) CD19⁺ cells from CLL patients (N=15) were treated with or without 0.5 μM lenalidomide and/or 10 μM CAL-101 for 48 hours. RNA was extracted and converted to cDNA and RT-PCR analysis was done to determine quantities of b-FGF. (E) CD19⁺ cells from CLL patients (N=13) were treated with or without 0.5 μM lenalidomide and/or 10 μM CAL-101 for 48 hours. RNA was extracted and converted to cDNA and RT-PCR analysis was done to determine quantities of VEGF.
Figure 1

1A

Fold change in enzymatic activity

Untreated Lena Treated Dose of CAL-101

1B

![Phosphorylated AKT and GSK3β in different cell lines and doses of CAL-101](image)

1C

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Figure 2

2A

CD40

percent cells CD40 positive

Untreated Lena. Lena. + CAL-101

Treatment Conditions

CD86

percent cells CD86 positive

Untreated Lena. Lena. + CAL-101

Treatment Conditions

CD20

percent CD20 expression

Untreated Lena. Lena. + CAL-101

Treatment Conditions

2B

CD40

Fold change in CD40 mRNA expression

Untreated Lena. Lena. + CAL-101

Treatment Conditions

CD86

Fold change in CD86 mRNA expression

Untreated Lena. Lena. + CAL-101

Treatment Conditions

CD154

Fold change in CD154 mRNA expression

Untreated Lena. Lena. + CAL-101

Treatment Conditions

2C

IgM production

Untreated 1uM CAL-101 10uM Lena.

0.5uM Lena.

Normal T-cells

Treatment Conditions

2D

Fold change in b-FGF mRNA expression

Untreated Lena. Lena. + CAL-101

Treatment Conditions

2E

Fold change in VEGF mRNA expression

Untreated Lena. Lena. + CAL-101

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