Lenalidomide Inhibits The Proliferation Of Chronic Lymphocytic Leukemia Cells Via A Cereblon/p21\textsuperscript{WAF1/Cip1}-Dependent Mechanism Independent Of Functional p53

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Short title: Cytostatic effect of lenalidomide in CLL

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Key Points

Lenalidomide inhibits CLL proliferation in a cereblon/p21-dependent manner

Treatment with lenalidomide induces p21 in CLL independent of p53
Abstract

Lenalidomide has demonstrated clinical activity in patients with chronic lymphocytic leukemia (CLL), even though it is not cytotoxic for primary CLL cells in vitro. We examined the direct effect of lenalidomide on CLL-cell proliferation induced by CD154-expressing accessory cells in media containing interleukin (IL)-4 and IL-10. Treatment with lenalidomide significantly inhibited CLL-cell proliferation, an effect that was associated with the p53-independent upregulation of the cyclin-dependent kinase inhibitor p21\textsuperscript{WAF1/Cip1} (p21). Silencing p21 with small interfering RNA (siRNA) impaired the capacity of lenalidomide to inhibit CLL-cell proliferation. Silencing cereblon (CRBN), a known molecular target of lenalidomide, impaired the capacity of lenalidomide to induce expression of p21, inhibit CD154-induced CLL-cell proliferation, or enhance the degradation of Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3). We isolated CLL cells from the blood of patients before and after short-term treatment with low-dose lenalidomide (5 mg per day) and found the leukemia cells also were induced to express p21 in vivo. These results indicate that lenalidomide can directly inhibit proliferation of CLL cells in a CRBN/p21-dependent, but p53-independent, manner at concentrations achievable in vivo, potentially contributing to the capacity of this drug to inhibit disease-progression in patients with CLL.
**Introduction**

Lenalidomide is a second-generation immunomodulatory (IMiD®) drug\(^1-3\) that has both direct tumoricidal as well as immunomodulatory activity in patients with multiple myeloma.\(^4\) This drug also has clinical activity in patients with chronic lymphocytic leukemia (CLL), even though it is not directly cytotoxic to CLL cells *in vitro*.\(^5,6\) As such, its clinical activity in CLL is presumed secondary to its immune modulatory activity.\(^7\) Indeed, lenalidomide indirectly modulates CLL cell survival *in vitro* by affecting supportive cells, such as nurse-like cells,\(^8\) found in the microenvironment of lymphoid tissues. Lenalidomide also can enhance T-cell proliferation\(^1\) and interferon-gamma (IFN-\(\gamma\)) production\(^9\) in response to CD3-crosslinking *in vitro* and dendritic-cell-mediated activation of T-cells.\(^10\) Moreover, lenalidomide can reverse noted functional defects of T cells of patients with CLL.\(^11\)\(^12\) Finally, lenalidomide also can induce CLL B-cells to express higher levels of immunostimulatory molecules, such as CD80, CD86, HLA-DR, CD95, and CD40 *in vitro*,\(^5,13\) thereby potentially enhancing their capacity to engage T-cells in cognate interactions that lead to immune activation in response to leukemia-associated antigen(s).\(^14\)

However, lenalidomide also may have direct antiproliferative effects on CLL cells that account in part for its clinical activity in patients with this disease. This drug can inhibit proliferation of B-cell lymphoma lines\(^15\) and induce growth arrest and apoptosis of mantle-cell lymphoma cells.\(^16\) Although originally considered an accumulative disease of resting G\(_{0/1}\) lymphocytes, CLL increasingly is being recognized as a lymphoproliferative disease that can have high rates of leukemia-cell turnover, resulting from robust leukemia cell proliferation that is offset by concomitant cell-death. Indeed, CLL cells can undergo robust growth in so-called "proliferation centers" within lymphoid tissues in response to signals received from accessory cells within the leukemia...
microenvironment. *In vivo* heavy-water labeling studies have demonstrated that some patients can have relatively high rates of leukemia-cell turnover, generating as much as 1% of their total leukemia-cell population each day, presumably in such tissue compartments.\(^{17}\) Inhibition of leukemia cell proliferation could offset the balance between CLL cell-proliferation and cell-death, resulting in reduction in tumor burden over time. Herein we examined whether lenalidomide could inhibit the growth of CLL cells that are induced to proliferate, an effect that potentially could contribute to its noted clinical activity in patients with this disease.
Methods

Reagents

Lenalidomide was provided by Celgene Corporation (San Diego, CA, USA) and solubilized in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA), which was used as a vehicle control in all experiments. Between 0.01 - 30 μM of lenalidomide was added every 3 days to long-term cultures, unless otherwise indicated.

CLL cell samples

Blood samples were collected from CLL patients at the University of California San Diego (UC San Diego) Moores Cancer Center who satisfied diagnostic and immunophenotypic criteria for common B-cell CLL and who provided written, informed consent, in compliance with the Declaration of Helsinki and the institutional review board of UC San Diego. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), resuspended in 90% fetal calf serum (FCS) (Omega Scientific, Tarzana, CA, USA) and 10% DMSO for viable storage in liquid nitrogen. Alternatively, viably frozen CLL cells were purchased from AllCells (Emeryville, CA, USA) or Conversant Biologicals (Huntsville, AL, USA). Samples with greater than 95% CD19⁺CD5⁺ CLL cells were used without further purification throughout this study.

Co-culture of CLL cells with HeLa\textsubscript{CD154}, fibroblasts\textsubscript{CD154} or CpG stimulation

HeLa cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). CD154-expressing HeLa cells (HeLa\textsubscript{CD154}) were generated as described. Fibroblasts\textsubscript{CD154} were provided by Dr. Ralph Steinman. For experiments using HeLa\textsubscript{CD154} cells, CLL cells were plated at 1.5x10⁶ cells per well (per mL) in a 24-well tray
on a layer of irradiated HeLa\textsubscript{CD154} (8000 Rad) cells at a CLL:HeLa\textsubscript{CD154} cell ratio of 15:1 in RPMI-1640 medium supplemented with 10% FCS, 10 mM HEPES (Life Technologies, Grand Island, NY, USA), penicillin (100 U/ml)-streptomycin (100 µg/ml) (Life Technologies), 5 ng/mL of recombinant human interleukin (IL)-4 (R&D Systems, Minneapolis, MN, USA) and 15 ng/mL recombinant human IL-10 (Peprotech Inc, Rocky Hill, NJ, USA). In earlier studies we noted that expression of CD154 on the supportive cells combined with exogenous IL-4 and IL-10 provided for optimal CLL-cell proliferation (Figure S1A-B). These cells also were stained with fluorochrome-conjugated monoclonal antibodies specific for CD19, CD5, or ROR1 to confirm via flow cytometry that the proliferating cells were CLL cells (Figure S1C). For co-culture on Fibroblasts\textsubscript{CD154}, 0.8-1x10\textsuperscript{6} CLL cells were plated on 6x10\textsuperscript{5} cells/well mitomycin C treated fibroblasts (10 µg/mL; 3 hrs) in 1 mL per well in the media described above. For long-term cultures, half of the media was renewed every 3 days. For CpG stimulation, CLL cells were plated at 1.5x10\textsuperscript{6} cell/mL in RPMI-1640 supplemented with FCS, HEPES, penicillin and streptomycin as above, to which we added 2.5 µg/mL CpG (InvivoGen, San Diego, CA, USA), 10 ng/mL rhIL-2 and 15 ng/mL rhIL-10 both from Peprotech Inc.

Descriptions of CLL cell proliferation and viability measurements, flow cytometry, cell cycle analysis, gene expression and \textit{TP53} mutation analysis, transfection, immunoblot and statistical analysis are provided in the supplemental methods.
Results

Lenalidomide inhibits CLL cell proliferation

We induced CLL cells to undergo proliferation by co-culturing them with accessory cells made to express CD154 in the presence of exogenous interleukin(IL)-4 and IL-10. CLL cells co-cultured with CD154-expressing HeLa cells (Figure 1A-B) or human fibroblasts (Figure 1C-D) were induced to proliferate, as detected by the reduction in green fluorescence of dividing cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). Moreover, the CD154-expressing cell lines supported CLL-cell proliferation through several rounds of division, as shown by the dilution of CFSE fluorescence over time (Figure 1A-D). Induction of CLL-cell proliferation was associated with changes in the distribution of cells in the different phases of the cell cycle, decreasing the numbers of cells in G0/G1 and concomitantly increasing the numbers of cells in S or G2/M phases of the cell cycle, without affecting the fraction of cells in sub-G1 (Figures 1E-H). Consistent with the induced cellular proliferation, we noted increased numbers of viable CLL cells over time in cultures with exogenous cytokines and CD154-expressing cells, but not in control cultures exposed to supportive cells not expressing CD154 (Figure 1I).

The capacity to induce CLL-cell proliferation allowed us to examine the impact of lenalidomide on dividing CLL cells in vitro. First, CLL cells were co-cultured on CD154-expressing accessory cells in media containing IL-4 and IL-10 for 2 days. Subsequent to this we added lenalidomide to the concentrations listed and cultured the cells for 7 days. We observed a dose-dependent inhibition of CLL-cell proliferation with lenalidomide (Figure 2A), which led to a significant decrease in the fraction of dividing CLL cells, starting at concentrations as low as 0.3 µM (Figure 2B). The inhibition of proliferation
was confirmed by cell-cycle analysis using PI staining (Figure 2C). The fraction of cells in G₀/G₁ phases of the cell cycle were significantly elevated in a dose-dependent fashion with increasing amounts of lenalidomide. The increases in the fraction of cells in G₀/G₁ were accompanied by a significant decrease in the fraction of cells in S or G₂/M phase in cells treated with lenalidomide relative to cultures without added drug. We also observed comparable cytostatic effects, measured via CFSE labeling, viable cell counts, BrdU incorporation, or cell cycle analysis, when lenalidomide was added at the initiation of the co-cultures at drug concentrations ≥ 3 µM (Figure S2A-F). Using this regimen, we observed that lenalidomide could inhibit the induced proliferation of CLL cells of almost all patients examined (19/22), significantly inhibiting the induced increase in numbers of CLL cells noted after 6 days in co-culture (Figure S2G). There were no distinctive characteristics that were shared by the 3 non-responsive samples. On the other hand, we did not observe direct cytotoxic effects of lenalidomide on the proliferating CLL cells, as noted in other studies focusing on resting cells,⁵,⁶ even when the drug was added to the CLL cells at the initiation of co-culture (Figure S2H) or after 2 days of co-culture (Figure 2D). Of note, the level of CD154 expressed on accessory cells was not altered by treatment with lenalidomide (Figure S3A-B), suggesting that the effect of the drug was not due to an indirect effect on the accessory cells used to induce CLL-cell proliferation. Finally, lenalidomide also inhibited CLL-cell proliferation induced by co-culture with accessory cells expressing CD154, in the absence of IL-4 and IL-10, suggesting that the inhibitory effects of lenalidomide on CLL cell proliferation is not mediated through inhibition of the signaling induced by cytokines, such as IL-4 and/or IL-10 (Figure S3C). Consistent with this notion, lenalidomide also displayed a cytostatic effect on CLL cells stimulated to divide following treatment with CpG oligonucleotides in the presence of IL-2 and IL-10 (Figure S4), suggesting that the inhibition of proliferation was not restricted to inhibition of CD40-signaling.
**p21\textsuperscript{WAF1/Cip1} is necessary for lenalidomide-induced inhibition of CLL-cell proliferation**

We observed that CLL cells exposed to lenalidomide accumulate in the G\textsubscript{0}/G\textsubscript{1} phase of the cell cycle when stimulated with CD154 and IL-4/IL-10. Prior studies on the B-cell line Namalwa showed that lenalidomide could upregulate the expression of p21\textsuperscript{WAF1/Cip1},\textsuperscript{15} a protein that could inhibit the activity of cyclin dependent kinases (CDKs) involved in G\textsubscript{1}/S progression.\textsuperscript{21,22} We therefore monitored the expression levels of p21\textsuperscript{WAF1/Cip1} in CLL cells stimulated to divide and exposed to lenalidomide. We observed an upregulation of p21\textsuperscript{WAF1/Cip1} mRNA levels after 24 hours of exposure to lenalidomide (Figure 3A). A dose-dependent increase of p21\textsuperscript{WAF1/Cip1} protein also was observed, regardless of whether there was detectable expression of p53 (Figure 3B-C), suggesting that p21\textsuperscript{WAF1/Cip1} may be upregulated via a p53-independent mechanism. To test this hypothesis, we measured the effect of lenalidomide on CLL cells lacking functional p53, of which 99.5% had del(17p) on one allele and an inactivating mutation in exon 5 (at protein codon 174 (R → W)) in the retained allele. In contrast to CLL cells with wild-type p53, we observed that these CLL cells could not be induced to express higher levels of p53 or p21\textsuperscript{WAF1/Cip1} following exposure to γ-irradiation (Figure 3D). However, such CLL cells could be induced to express p21\textsuperscript{WAF1/Cip1} by treatment with lenalidomide, indicating that lenalidomide-induced upregulation of p21\textsuperscript{WAF1/Cip1} in CLL cells does not require functional p53.

We next monitored the expression levels of p21\textsuperscript{WAF1/Cip1} in a larger group of patient samples after treatment with 3 \textmu{M} lenalidomide. We observed that lenalidomide induced upregulation of p21\textsuperscript{WAF1/Cip1} in most samples tested (13/16) (Figure 3E-F). Of note, the 3 samples that failed to upregulate p21\textsuperscript{WAF1/Cip1} expression were induced to...
proliferate in the co-culture system, but were not sensitive to the inhibition mediated by 3 µM lenalidomide (2.6±0.7 versus 2.4±0.7 fold expansion from day 1 to day 6 in control-treated cells versus lenalidomide treated-cells, respectively). Furthermore, we observed that p21WAF/Cip was upregulated in a fashion that correlated with the inhibition of proliferation induced by lenalidomide (Figure 3G), suggesting that p21WAF/Cip1 is involved in the growth inhibitory effects of lenalidomide.

We used siRNA technology to silence p21WAF/Cip1 in primary CLL cells and monitored the activity of lenalidomide on the transfected cells stimulated to divide by co-culture with CD154-expressing cells and exogenous cytokines. We observed reduced expression of p21WAF/Cip1 following silencing (Figure 4A-B). Upon exposure to lenalidomide, the p21-silenced cells still showed an increase in p21WAF/Cip1 protein, but its levels were reduced compared to that of control-treated cells (Figure 4C-D). CLL cells transfected with siRNA specific for p21WAF/Cip1 were significantly less sensitive to the growth inhibitory effects of lenalidomide than CLL cells transfected with control siRNA (Figure 4E-F). These results indicate that expression of p21WAF/Cip1 contributes to the cytostatic activity of lenalidomide on CLL cells.

Lenalidomide mediates its cytostatic activity via a cereblon/p21WAF/Cip1 dependent mechanism in CLL cells

The only known direct molecular target of lenalidomide is cereblon (CRBN), which is part of the Cul4A-DDB1 E3 ligase complex. CRBN also plays a role in the cytostatic and/or cytotoxic activity of lenalidomide in multiple myeloma cells and in the neoplastic B cells of patients with ABC diffuse large B cell lymphoma. To evaluate the role of CRBN in the cytostatic activity of lenalidomide on CLL cells, we used siRNA technology to silence CRBN in primary CLL cells. We observed a significant reduction
of CRBN protein levels in CLL cells 48 hours after transfection with CRBN siRNA, but not with control siRNA (Figure 5A-B). CLL cells transfected with CRBN siRNA expressed substantially lower amounts of p21\textsuperscript{WAF1/Cip1} following treatment with lenalidomide than CLL cells transfected with control siRNA (Figure 5C-D), indicating that CRBN contributed to the lenalidomide-induced expression of p21\textsuperscript{WAF1/Cip1}. Furthermore, we observed that the CRBN-silenced cells were less susceptible to the cytostatic activity of lenalidomide than control-treated CLL cells (Figure 5E-F). These results indicate that the cytostatic activity of lenalidomide involves induction of p21\textsuperscript{WAF1/Cip1} in CLL cells in a CRBN-dependent manner.

We lastly monitored for changes in the expression of Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3), transcription factors found to be degraded in a CRBN-dependent manner and required for the activity of lenalidomide on multiple myeloma cells and T cells.\textsuperscript{26-28} For this, we transfected CLL cells with either control siRNA or CRBN-specific siRNA, co-cultured the transfected cells on CD154-expressing supportive cells in media without (control) or with various concentrations lenalidomide for 24 hours, after which time we prepared cell lysates, which were examined via immunoblot analysis. We observed reduced expression levels of IKZF1 and IKZF3 in control CLL cells treated with lenalidomide at concentrations as low as 0.3 \(\mu\text{M}\) (Figure 5 G-H). However, CLL cells silenced for CRBN failed to experience enhanced degradation IKZF1 or IKZF3 upon treatment with lenalidomide, even at concentrations as high as 30 \(\mu\text{M}\) (Figure 5G-H). These results suggest that changes in expression levels of IKZF1 and IKZF3 may be involved in the CRBN-dependent mechanism of action of lenalidomide on CLL cells.
Lenalidomide induces p21WAF1/Cip1 expression in vivo in CLL patients

To assess whether lenalidomide could induce p21WAF1/Cip1 in CLL cells at concentrations that could be achieved in vivo, we examined CLL cells of patients before and after starting therapy with low-dose lenalidomide (e.g. 5 mg PO QD). CLL blood mononuclear cells were isolated from patients (n = 5) before and 15 days after starting treatment with lenalidomide at 2.5 mg per day for the first week, and 5 mg per day for the second week. For each of these samples, the proportion of CD19+CD5+ cells exceeded 91%. Patients providing samples CLL1, CLL2, CLL3, CLL4, or CLL5 experienced reduction from pretreatment values in their blood absolute lymphocyte counts of 41%, 22%, 40%, 88%, or 55%, respectively, following lenalidomide therapy. We examined the CLL cells for expression of p21WAF1/Cip1 by immunoblot analysis (Figure 6). For all patient samples examined, we observed induced-expression of p21WAF1/Cip1 in the CLL cells of patients following initiation of therapy with lenalidomide. These results indicate that lenalidomide can induce the expression of p21WAF1/Cip1 in CLL cells of patients receiving doses as low as 5 mg per day.
Discussion

This paper describes for the first time a mechanism by which lenalidomide directly affects CLL cells, potentially contributing to its noted clinical activity in patients with CLL. Recent studies have found that patients with CLL may have a high rate of leukemia-cell turnover, with cell-death rates balancing out the birth-rates of new cells, which may represent approximately 1% of the leukemia-cell clone each day. Agents that interfere with the capacity of leukemia cells to grow could tilt the balance in favor of cell-death, resulting in clearance of leukemia cells, even when such agents lack direct cytotoxic activity. Lenalidomide potentially could act through such a mechanism. We found that lenalidomide could cause a dose-dependent inhibition of CLL-cell proliferation that resulted in the accumulation of cells in the G_{0/1} phase of the cell cycle. This activity was dependent upon the induced expression of the cell cycle regulator p21^{WAF1/Cip1} by a mechanism dependent on CRBN, a known molecular target of lenalidomide. Importantly, we observed these effects at concentrations as low as 0.3 µM, below the 0.6 µM estimated concentration of drug found in the plasma of patients treated daily with 5 mg of lenalidomide. Consistent with this, we found that the CLL cells of patients treated with lenalidomide at such doses were induced to express p21^{WAF1/Cip1} in vivo. As such, the capacity of lenalidomide to inhibit the proliferation of CLL cells may account in part for its observed clinical activity.

We found that lenalidomide could induce p21^{WAF1/Cip1} in leukemia cells harboring del(17p) and a single dysfunctional mutant allele of p53, indicating that the cytostatic activity of this drug did not require functional p53. This may account in part for the observed clinical activity of lenalidomide in patients with CLL cells with del(17p). It also could support a notion of treating patients with lenalidomide to mitigate the risk of disease.
progression. Our results are consistent with the p53-independent mechanism by which lenalidomide upregulates $p21^{WAF1/Cip1}$ in the Namalwa cell line, into which a mutation prevents the binding of p53 to the $p21^{WAF1/Cip1}$ promoter. In these cells, the upregulation of $p21^{WAF1/Cip1}$ is mediated by a LSD1-dependent epigenetic mechanism, leading to a reduction in histone methylation and an increase in histone acetylation of the $p21^{WAF1/Cip1}$ promoter, which facilitates the access to DNA of transcription factors, such as sp1, sp3, Egr1 and Egr2. Silencing experiments in multiple myeloma cells identified the contribution of sp1, sp3, Egr1 and Egr2 to the upregulation of $p21^{WAF1/Cip1}$ induced by lenalidomide or pomalidomide in plasma cells, even though none of these factors alone appeared to be physiologic regulators of $p21^{WAF1/Cip1}$ expression. Rather it is suggested that a combination of these factors, and possibly others from the abundant array of $p21^{WAF1/Cip1}$ regulators, may be involved. A comparable complex network of factors also may be responsible for the lenalidomide-induced upregulation of $p21^{WAF1/Cip1}$ in CLL.

We found that lenalidomide's cytostatic activity and capacity to induce $p21^{WAF1/Cip1}$ in CLL cells was dependent upon CRBN. We observed that the silencing of CRBN in primary CLL cells mitigated the cytostatic effect of lenalidomide and inhibited its capacity to induce leukemia-cell expression of $p21^{WAF1/Cip1}$. This is consistent with the notion that reducing the expression levels of a drug target reduces its activity, and also consistent with the data presented by Zhu and colleagues, showing that the suppression of CRBN renders multiple myeloma cell lines less responsive to lenalidomide. CRBN is the adaptator protein part of a Cul4A-DDB1 E3 ubiquitin ligase complex and is the subunit responsible for substrate recognition. Our results suggest that the interaction of lenalidomide with this E3 ligase complex via binding to CRBN increases $p21^{WAF1/Cip1}$ expression, which ultimately contributes to cell-cycle arrest of CLL cells.
Recent studies identified the transcription factors IKZF1 (Ikaros) and IKZF3 (Aiolos) as being substrates of CRBN, which are rapidly degraded upon exposure to lenalidomide, and required for its anti-proliferative and immunomodulatory activity.\(^{26-28}\) IKZF1 and IKZF3 are transcriptional regulators involved in hematopoiesis, and in the development of the adaptive immune system.\(^{36}\) Work performed in mutant mouse models showed that IKZF1 is required at different levels in hematopoiesis, such as in the generation of chronic lymphoid precursors, while IKZF3 appears more restricted to B cells and is required for the generation of peritoneal, marginal, and recirculating B cells, and for the generation of long-lived plasma cells.\(^{36}\) These transcription factors may act as transcriptional repressors by forming complexes with proteins from the mSin3 family of co-repressors and histone deacetylases.\(^{37}\) Disruption of these complexes via lenalidomide-induced degradation of IKZF1 and IKZF3 could alleviate the repression of p21\(^{\text{WAF1/Cip1}}\) transcription that may be mediated by factors such as Runx-1.\(^{38}\) This would be in line with the recently described mechanism of IL-2 de-repression in T cells following lenalidomide exposure that was dependent on IKZF1/IKZF3 degradation.\(^{26,27}\) Additional studies are required to evaluate whether these proteins are responsible for the cytostatic activity of lenalidomide in CLL cells.

Collectively, this study sheds new light on the mechanism of action of lenalidomide, which may inhibit CLL-cell proliferation. Such activity might mitigate the activity of drugs that are most active against cycling cells, accounting in part for the unfavorable results of clinical trials coupling lenalidomide with cytotoxic agents, such as fludarabine monophosphate.\(^{39-41}\) This mechanism also could account in part for the lack of disease progression generally observed during lenalidomide maintenance-therapy, regardless of whether the patient has leukemia cells that harbor del(17p) and/or have dysfunctional p53.\(^{42}\) As lenalidomide is not directly cytotoxic to CLL cells, this drug might tilt the
balance between leukemia cell growth and cell death,\textsuperscript{17} conceivably resulting in a faster decline in tumor burden of patients who have relatively high rates of leukemia cell turnover. Clinical trials combining heavy-water-labeling assessment of leukemia-cell turnover and subsequent treatment with lenalidomide would be required to formally test this hypothesis.
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Authorship

J.-F.F. designed and performed research, analyzed data and wrote the paper; E.M.G. performed research, analyzed data and revised the paper; S.G.: designed and performed research and analyzed data; D.F., I.S.B., M.S. and B.Cui performed research and analyzed data; L.G.C., B.Cathers and A.L.-G. designed research, supervised the study and revised the paper; D.M. designed research, supervised the study and revised the paper; T.J.K. designed research, supervised the study, provided patient samples, and wrote the paper.

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Conflict-of-interest Disclosures

L.G.C., S.G., B.Cathers and A.L.-G. are employees of Celgene Corporation. T.J.K. and D.M. received financial support from Celgene Corporation. The remaining authors declare no competing financial interests.
References


Figure legends

Figure 1. **CLL cells co-cultured with CD154-expressing supportive cells are induced to proliferate.** A-B) CLL cells were labeled with CFSE and co-cultured on HeLa_{CD154} in the presence of IL-4 and IL-10, as described in Methods. Cells were collected on days 2, 3, 6, and 10 for analysis by flow cytometry. The results of assays on 2 representative CLL samples are shown in panel A and the fraction of dividing cells observed in 6 patient samples tested is presented in panel B. One-way ANOVA and Tukey’s multiple comparison test were used to determine statistically significant differences from day 2. ***p<0.001 (mean ± S.E.M.; n=6). C-D) CLL cells were labeled with CFSE and co-cultured on Fibroblasts_{CD154} in the presence of IL-4 and IL-10 as described in Methods. Cells were collected on days 1, 6, and 9 for analysis by flow cytometry. One representative CLL sample out of 4 is shown in panel C and the fraction of dividing cells observed in all 4 patients tested is presented in panel D. One-way ANOVA and Tukey’s multiple comparison test were used to determine statistically significant differences from day 1. ***p<0.001 (mean ± S.E.M.; n=4). E-F) CLL cells were co-cultured on Fibroblasts_{CD154} in the presence of IL-4 and IL-10 and, at the indicated time, subjected to cell cycle analysis following PI staining. One representative CLL sample out of 4 is shown in panel E and the fraction of cells in each phase for all 4 patients tested is presented in panel F. **p<0.01, ***p<0.001 (Student’s t-test, mean ± S.E.M.; n=4). G-H) CLL cells were co-cultured on Fibroblasts_{CD154} in the presence of IL-4 and IL-10 and, at the indicated time, subjected to EdU incorporation for a period of 4 hours as described in Methods. One representative CLL sample out of 4 is shown in panel G and the fraction of Edu+ cells observed in all 4 patients tested is presented in panel H. **p<0.01 (Student’s t-test, mean ± S.E.M.; n=4). I) CLL cells from 3 different patients were co-cultured on HeLa_{CD154} or non transfected HeLa cells in the presence of
IL-4 and IL-10. At the indicated days, live CLL cell counts were assessed by flow cytometry, as described in Methods, and are presented as expansion folds relatively to day 1. *p<0.05 (Student’s t-test, mean ± S.E.M.; n=3).

**Figure 2. Lenalidomide inhibits CLL cell proliferation.** A-B) CFSE-labeled CLL cells from 3 different patients were co-cultured with Fibroblasts$\text{CD}_{154}$ and IL-4/IL-10 for 48 hours and exposed to increasing single doses of lenalidomide or DMSO as vehicle control for 7 days, at which point the cells were harvested, and analyzed by flow cytometry for proliferation. In panel A, CFSE profiles are presented for cells of a representative patient. In panel B, the fraction of dividing CLL cells present with increasing doses of lenalidomide were determined using FlowJo software, by establishing the non-dividing cells based on unstimulated, CFSE-labeled CLL cells. Data from 3 patients are presented. One-way ANOVA and Tukey’s multiple comparison test were used to determine statistically significant differences from day 1. *p<0.05, ***p<0.001 (mean ± S.E.M.; n=3). C) CLL cells from 3 different patients were stimulated with IL-4/IL-10 in the presence of Fibroblasts$\text{CD}_{154}$ and exposed to increasing doses of lenalidomide or DMSO as control. Cell cycle analysis was performed by flow cytometry after 6 days using PI staining as described in Methods. Percentage of cells in G$_0$/G$_1$, S, or G$_2$/M were assessed using the cell cycle analysis tool from FlowJo software and are presented as boxes indicating the median, minimum and maximum values of cell proportions in each phase of the cell cycle. One-way ANOVA and Tukey’s multiple comparison test were used to determine statistically significant differences from control. *p<0.05, **p<0.01, ***p<0.001 (mean ± S.E.M.; n=3). D) CLL cells from 3 patient samples were co-cultured on Fibroblasts$\text{CD}_{154}$ in the presence of IL-4 and IL-10 and increasing doses of lenalidomide from day 2 of co-culture. The fraction of viable CLL
cells were measured after 7 days of treatment by flow cytometry using 7-AAD. Live cells were identified as 7-AAD-negative cells. (mean ± S.E.M.; n=3).

Figure 3. Lenalidomide upregulates p21^{WAF1/Cip1} in CLL cells. A) CLL cells from 4 patient samples were co-cultured with Fibroblasts_{CD154} and IL-4/IL-10 and exposed to 10 μM lenalidomide or equivalent volume of DMSO. After 24 hours, the cells were collected and analyzed for the expression levels of p21^{WAF1/Cip1} mRNA by Quantigene. The expression of Hypoxanthine Guanine Phosphoribosyltransferase (HPRT) was monitored as housekeeping gene and used to normalize p21^{WAF1/Cip1} expression level for each sample. *p<0.05 (Student’s t-test, mean ± S.E.M.; n=4). B-C) CLL cells were co-cultured with HeLa_{CD154} and IL-4/IL-10 and exposed to increasing amounts of lenalidomide or vehicle control. In parallel, HeLa_{CD154} alone were cultured with increasing doses of lenalidomide as a control. After 24 hours, the cells were collected and protein extracted for the analysis of p21^{WAF1/Cip1}, p53 and GAPDH expression by immunoblot. In panel B, the results from 3 CLL samples are shown, along with the HeLa_{CD154} control, for which the same amount of total protein as the CLL cells was run. In panel C, the densitometry analysis of p21^{WAF1/Cip1} and p53 expression is presented for all 3 patients from panel B. The intensity of the target proteins were normalized to GAPDH levels. One-way ANOVA and Tukey’s multiple comparison test were used to determine statistically significant differences from control. *p<0.05, **p<0.001 (mean ± S.E.M.; n=3). D) CLL cells from a sample deficient in functional p53 (p53 def) and from a sample with functional p53 (p53 WT) were exposed to either γ-irradiation (1Gy) or 3 μM lenalidomide followed by 8 hours incubation, at which point the cells were collected for protein extraction and detection of p21^{WAF1/Cip1}, p53, and GAPDH by immunoblot. E-F) CLL cells from 16 patients were co-cultured with HeLa_{CD154} and IL-4/IL-10 and exposed to 3 μM lenalidomide or DMSO. After 3 days, the cells were collected, lysed,
and analyzed for p21\textsuperscript{WAF1/Cip1} and GAPDH expression by immunoblot. Panel E shows the immunoblot results, while panel F presents the densitometry analysis quantifying the expression levels of p21\textsuperscript{WAF1/Cip1} protein for all patients presented in panel E. The expression levels of p21\textsuperscript{WAF1/Cip1} have been normalized to GAPDH. ***p<0.001 (Student’s t-test). G) CLL cells were co-cultured with HeLa\textsubscript{CD154} and IL-4/IL-10 and exposed to 3 µM lenalidomide or equivalent volume of DMSO. At day 3, a fraction of the cells were collected, lysed and analyzed for the expression of p21\textsuperscript{WAF1/Cip1} and GAPDH by immunoblot as described above. At day 6, viable cell counts were performed by flow cytometry as described in Methods. The percent decrease in proliferation for each patient measured at day 6 (100 x (expansion fold\textsubscript{CTRL-treated samples} - expansion fold\textsubscript{lenalidomide-treated samples}) / expansion fold\textsubscript{CTRL-treated samples}) is presented in function of the percent increase in p21\textsuperscript{WAF1/Cip1} protein expression measured by densitometry analysis as above (100 x (p21\textsubscript{CTRL-treated samples} – p21\textsubscript{lenalidomide-treated samples}) / p21\textsubscript{CTRL-treated samples}). Each dot represents data from one CLL patient (n=22; Pearson r = 0.52).

**Figure 4. p21\textsuperscript{WAF1/Cip1} silencing in CLL cells interferes with the anti-proliferative activity of lenalidomide.** A-B) siRNAs specific for p21\textsuperscript{WAF1/Cip1} (p21) or non-specific siRNA control (CTRL) were transfected into CLL cells using HiPerfect reagent as described in Methods, and co-cultured on HeLa\textsubscript{CD154} and IL-4/IL-10. After 48 hrs, the cells were collected and lysed for detection of p21\textsuperscript{WAF1/Cip1} and GAPDH protein by immunoblot. In panel A, data from 2 representative patients are presented and in panel B, densitometry analysis quantifying the levels of p21\textsuperscript{WAF1/Cip1} protein in 5 different CLL samples is shown. The expression of p21\textsuperscript{WAF1/Cip1} has been normalized to GAPDH. **p<0.01 (Student’s t-test, mean ± S.E.M.; n=5). C-D) CLL cells from 4 patient samples were transfected as in panel A with either CTRL siRNA or p21\textsuperscript{WAF1/Cip1} siRNA and co-cultured on HeLa\textsubscript{CD154} and IL-4/IL-10 in the presence of 3 µM lenalidomide or control.
media. After 48hrs, the cells were collected and lysed for detection of p21WAF1/Cip1 and GAPDH protein by immunoblot. In panel C, data from 2 representative patients are presented and in panel D, densitometry analysis quantifying the levels of p21WAF1/Cip1 protein in 4 different CLL samples is shown. The expression of p21WAF1/Cip1 has been normalized to GAPDH. *p<0.05 (Student’s t-test, mean ± S.E.M.; n=4). E) CLL cells from 5 patient samples were transfected as in panel A with either CTRL siRNA or p21WAF1/Cip1 siRNA and exposed to 3 µM lenalidomide or DMSO. After 72 hrs, CLL cell proliferation was measured using BrdU incorporation, which provides absorbances at 450 – 690 nm. *p<0.05; **p<0.01 (Student’s t-test, mean ± S.E.M.; n=5). F) Proliferation data from panel E were used to calculate the percent inhibition of proliferation induced by lenalidomide in p21WAF1/Cip1–silenced cells and in CTRL cells ((AbsorbanceCTRL-treated samples – Absorbancelenalidomide-treated samples) / AbsorbanceCTRL-treated samples x 100). **p<0.01 (Student’s t-test).

Figure 5: CRBN silencing interferes with p21WAF1/Cip1, IKZF1 and IKZF3 expression and the anti-proliferative activity of lenalidomide in CLL cells. A-B) CLL cells were transfected with CRBN siRNA or non specific control siRNA (CTRL) using AMAXA and co-cultured on FibroblastsCD154 with IL-4 and IL-10 for 48hrs, at which point the cells were collected and lyzed for the analysis of CRBN protein expression and β-actin by immunoblot. Data from 2 representative patients are shown in panel A and densitometry analysis quantifying the expression levels of CRBN protein in 3 transfected CLL samples is presented in panel B. The expression of CRBN has been normalized to β-Actin. *p<0.05 (Student’s t-test, mean ± S.E.M.; n=3). C-D) CLL cells were transfected with CTRL siRNA or CRBN siRNA as above, plated on FibroblastsCD154 for 48hrs, at which point increasing doses of lenalidomide were added to the cells. After 5 days of
lenalidomide exposure, CLL cells were collected and lysed to monitor for p21WAF1/Cip1 and β-Actin protein expression by immunoblot. Data from a representative patient is shown in panel C and densitometry analysis quantifying the expression levels of p21WAF1/Cip1 protein in 3 transfected CLL samples is presented in panel D. The expression of p21WAF1/Cip1 has been normalized to β-Actin. *p<0.05 (Student’s t-test, mean ± S.E.M.; n=3). E-F) CLL cells were transfected with CTRL siRNA or CRBN siRNA, co-cultured on FibroblastsCD154 and exposed to lenalidomide as above. After 5 days of lenalidomide exposure, the fraction of CLL cells in S phase of the cell cycle was measured by Edu incorporation and flow cytometry. In panel E, data from each patient sample tested are presented, while panel F shows the combined data for all 3 patients presented in panel E, after being normalized to CTRL cells. *p<0.05, **p<0.01 (Student’s t-test, mean ± S.E.M.; n=3). G-H) CLL cells were transfected with CTRL siRNA or CRBN siRNA, co-cultured on FibroblastsCD154 and exposed to lenalidomide as above. After 24hrs of lenalidomide exposure, CLL cells were collected and lysed to monitor for IKZF1, IKZF3 and β-Actin protein expression by immunoblot. Data from a representative patient is shown in panel G. Densitometry analysis quantifying the expression levels of IKZF1 and IKZF3 obtained using CLL cells from 3 different patients is presented in panel H. The expression of each target protein has been normalized to β-Actin. *p<0.05 (Student’s t-test, mean ± S.E.M.; n=3).

**Figure 6: Lenalidomide therapy induces p21WAF1/Cip1 expression in CLL cells in vivo.** A-B) CLL blood mononuclear cells from 5 different patients were collected prior therapy (Pre) and 15 days after lenalidomide treatment (Post). All patients received 2.5mg QD for the first 7 days, and 5mg QD for the subsequent 7 days. Protein were extracted from all samples and analyzed for p21WAF1/Cip1 and GAPDH expression by
immunoblot. Panel A shows the immunoblot data for all 5 patients tested. Panel B shows the densitometry analysis quantifying the expression of p21<sup>WAF1/Cip1</sup> protein in the 5 patient samples shown in panel A. The expression of p21<sup>WAF1/Cip1</sup> has been normalized to GAPDH. **p<0.001 (Student’s t-test).
**Figure 1**

(A) Flow cytometry analysis showing the percentage of dividing cells over time. (B) Graph depicting the percentage of dividing cells at different time points. (C) Additional flow cytometry analysis with time points d1, d6, and d9. (D) Bar graph showing the percentage of dividing cells over time. (E) DNA content analysis with percentage of cells in G0/G1, S, and G2/M phases. (F) Bar graph comparing the percentage of cells in G0/G1 phase. (G) Edu incorporation analysis showing 0% at d1 and 11% at d6. (H) Bar graph comparing the percentage of Edu+ cells. (I) Cell expansion fold over time, showing a significant difference between HeLa and HeLaCD154.
Figure 2

Graph A: Histograms showing the distribution of CFSE counts for different concentrations of len (0, 0.3, 1, 3, 10, 30 μM).

Graph B: Bar graph showing the percentage of dividing CLL cells at different len concentrations. The graph includes error bars and statistical significance indicated by asterisks.

Graph C: Graphs showing the percentage of CLL cells in G0/G1, S, and G2/M phases at different len concentrations. The graphs include error bars and statistical significance indicated by asterisks.

Graph D: Line graph showing the percentage of viable CLL cells at different len concentrations. The graph includes error bars and statistical significance indicated by asterisks.
Figure 3

(A) Bar graph showing p21WAF1/Cip1 mRNA levels with and without len, indicated by asterisk.

(B) Western blot images comparing p21WAF1/Cip1, p53, and GAPDH expression levels in different conditions.

(C) Bar graph showing p21WAF1/Cip1 protein levels with different len concentrations (0, 0.1, 1, 10 μM).

(D) Western blot images comparing p21WAF1/Cip1, p53, and GAPDH expression levels in different p53 WT and def conditions.

(E) Western blot images comparing p21WAF1/Cip1 and GAPDH expression levels in different CLL conditions with and without HeLaCD154.

(F) Bar graph showing p21WAF1/Cip1 protein levels with and without len, indicated by asterisk.

(G) Scatter plot showing the decrease in cell expansion (%) against the increase in p21WAF1/Cip1 expression (%), with a linear trend line and P value of 0.01.
Figure 5

A. Western blot analysis of CRBN siRNA treatment in CLL1 and CLL2 cells. The expression of CRBN and β-Actin is shown.

B. CRBN protein levels determined by Western blot analysis using CTRL and CRBN siRNA.

C. Western blot analysis of p21^WAF1/Cip1 protein levels with varying concentrations of len (µM).

D. Graph showing p21^WAF1/Cip1 protein levels with CTRL and CRBN siRNA.

E. CLL1, CLL2, and CLL3 cells' percentage in S phase with different len (µM) concentrations.

F. Graphs depicting the percentage of CLL cells in S phase with CTRLsi and CRBN siRNA.

G. Western blot analysis of IKZF1 and IKZF3 proteins with CTRL and CRBN siRNA.

H. Protein levels of IKZF1 and IKZF3 with CTRLsi and CRBN siRNA.
Figure 6

(A) Western blot analysis showing the expression of p21<sub>WAF1/Cip1</sub> and GAPDH in CLL samples before and after treatment.

(B) Graph depicting the increase in p21<sub>WAF1/Cip1</sub> protein expression from pre- to post-treatment.
Lenalidomide inhibits the proliferation of chronic lymphocytic leukemia cells via a cereblon/p21WAF1/Cip1-dependent mechanism independent of functional p53

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