Lenalidomide and CC-4047 Inhibit the Proliferation of Malignant B Cells while Expanding Normal CD34+ Progenitor Cells


Abstract

Clinical studies involving patients with myelodysplastic syndromes or multiple myeloma have shown the efficacy of lenalidomide by reducing and often eliminating malignant cells while restoring the bone marrow function. To better understand these clinical observations, we investigated and compared the effects of lenalidomide and a structurally related analogue, CC-4047, on the proliferation of two different human hematopoietic cell models: the Namalwa cancer cell line and normal CD34+ progenitor cells. Both compounds had antiproliferative effects on Namalwa cells and pro-proliferative effects on CD34+ cells, whereas p21WAF-1 expression was up-regulated in both cell types. In Namalwa cells, the up-regulation of p21WAF-1 correlated well with the inhibition of cyclin-dependent kinase (CDK) 2, CDK4, and CDK6 activity leading to pRb hypophosphorylation and cell cycle arrest, whereas in CD34+ progenitor cells the increase of p21WAF-1 did not inhibit proliferation. Similarly, antiproliferation results were observed in two B lymphoma cell lines (LP-1 and U266) but interestingly not in normal B cells where a protection of apoptosis was found. Finally, CC-4047 and lenalidomide had synergistic effects with valproic acid [a histone deacetylase (HDAC) inhibitor] by increasing the apoptosis of Namalwa cells and enhancing CD34+ cell expansion. Our results indicate that lenalidomide and CC-4047 have opposite effects in tumor cells versus normal cells and could explain, at least in part, the reduction of malignant cells and the restoration of bone marrow observed in patients undergoing lenalidomide treatment. Moreover, this study provides new insights on the cellular pathways affected by lenalidomide and CC-4047, proposes new potential clinical uses, such as bone marrow regeneration, and suggests that the combination of lenalidomide or CC-4047 with certain HDAC inhibitors may elevate the therapeutic index in the treatment of hematologic malignancies. [Cancer Res 2007;67(2):746–55]

Introduction

Myelodysplastic syndromes and multiple myeloma are clonal hematopoietic cell disorders characterized by the accumulation of malignant blasts in the bone marrow compartment as a consequence of abnormalities in proliferation, differentiation, and apoptosis of hematopoietic precursors and their progeny (1, 2). Myelodysplastic syndrome patients present peripheral cytopenias and hypocellular bone marrow, and for the majority of patients, survival is dependent on blood transfusion. Lenalidomide (CC-5013), a drug recently approved by the Food and Drug Administration under the commercial name of Revlimid, has shown remarkable efficacy in myelodysplastic syndrome and multiple myeloma clinical studies (3–6). A high percentage of myelodysplastic syndrome patients enrolled in lenalidomide clinical trials experienced a significant erythroid response leading to transfusion independence, cytogenetic reduction of the malignant clone, and restoration of bone marrow function (4). Lenalidomide and a structurally related analogue, CC-4047, belong to a new class of proprietary compounds referred to as IMiDs immunomodulatory drugs being developed by Celgene Corp. (7–12).

To better understand how lenalidomide reduces abnormal cells and restores bone marrow function in patients, we investigated lenalidomide and CC-4047 effects on the proliferation of two different human hematopoietic cell models: the Namalwa cancer cell line obtained from a Burkitt’s lymphoma patient (13) and CD34+ progenitor cells obtained from human cord blood. We extended our findings to two other B lymphoma cells, the multiple myeloma cell lines LP-1 and U266, and compared them with primary B cells from healthy donors.

In agreement with a previous report indicating that lenalidomide induces G2-G1 growth arrest in multiple myeloma cell lines and in cells from multiple myeloma patients, Gandhi et al. reported that Namalwa cells are particularly sensitive to lenalidomide in arresting cell cycle progression in G1 (3, 12).

Cell cycle is regulated by general mechanisms that involve phosphorylation of specific proteins. For instance, the phosphorylation of the retinoblastoma gene product pRb represents a critical checkpoint of the G1-S transition. When underphosphorylated, pRb sequesters the E2F family transcription factors, which regulate genes encoding proteins required for S-phase DNA synthesis. Phosphorylation of pRb releases E2F that permits the induction of E2F-dependent genes and therefore the entrance into S phase (14). pRb contains 16 potential phosphorylation sites that have been described to be selectively phosphorylated by cyclin-dependent kinase (CDK) 2, CDK4, and CDK6 complexes (15). The activation and inhibition of CDK activity is dependent on interactions formed with the temporarily regulated cyclins and CDK inhibitors (CDKI), respectively. The D-type cyclins activate CDK4 and CDK6, whereas cyclin E and cyclin A mediate CDK2 kinase activity (15). Among the CDKIs, p21WAF-1 inhibits a broad range of CDKs, including CDK2, CDK4, and CDK6 (16–18). The current dogma suggests that, in the presence of p21WAF-1, there is a reduction of pRb phosphorylation leading to inhibition of E2F (19).
To study the restoration of the bone marrow, we used human CD34+ hematopoietic stem cells (HSC) that are capable of self-renewal and differentiating into a variety of hematopoietic lineages as a model. In vivo CD34+ expansion represents an important strategy to restore damaged and depleted bone marrow (20). Furthermore, ex vivo expansion of HSC while retaining their self-renewal capacity and their multipotency is the subject of intense research because of the potential of HSCs for numerous cellular therapeutic applications (21).

In this study, we showed that lenalidomide and CC-4047 (a) inhibit the proliferation of B malignant cell lines, Namalwa, LP-1, and U266 cells, and expand CD34+ progenitor cells; (b) up-regulate p21WAF-1; (c) induce cell cycle arrest in malignant cell models but not in normal CD34+ progenitor cells; and (d) protect normal B cell from apoptosis. In addition, we found that CC-4047 and lenalidomide have synergistic effects with valproic acid [VPA; a histone deacetylase (HDAC) inhibitor] in increasing apoptosis of Namalwa cells and enhancing CD34+ cell expansion, suggesting that certain HDAC inhibitors may be ideal candidates for combination therapy for the treatment of hematologic malignancies.

Materials and Methods

Materials. Lenalidomide and CC-4047 (Celgene Corp., Summit, NJ) were dissolved in DMSO (final concentration, 0.1%). Antibodies were purchased from the following suppliers: p21 WAF-1, phosphorylated pRb Ser795, phosphorylated pRb Ser608, phosphorylated pRb Ser780, and mouse and goat horseradish peroxidase (HRP)-conjugated IgG (Cell Signaling Technology, Inc., Danvers, MA); CDK2, CDK4 (for Western blot), and CDK6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); CDK4 (for immunoprecipitation; Upstate USA, Inc., Lake Placid, NY); phosphorylated pRb Ser621 and phosphorylated pRb Thr286 (BioSource International, Camarillo, CA); pRb (Calbiochem, San Diego, CA); and β-actin (Sigma-Aldrich, St. Louis, MO). Recombinant pRb was purchased from Santa Cruz Biotechnology, VPA was purchased from Sigma-Aldrich, and Nu6140 was purchased from Calbiochem. Goat anti-human IgM was purchased from Jackson ImmunoResearch (West Grove, PA).

Cell culture. Namalwa CXX.70 and LP-1 cells were purchased from DSMZ (Braunschweig, Germany). U266 was purchased from the American Type Culture Collection (Manassas, VA), and cord blood CD34+ cells were purchased from ARCellS (Emeryville, CA). Namalwa, primary B cells, LP-1, and U266 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with penicillin (100 units/mL) and streptomycin (100 µg/mL) and 10% (Namalwa and primary B cells) or 15% (LP-1 and U266) fetal bovine serum. Primary B cells were cultured in the presence of 2 ng/mL of goat anti-human IgM to minimize the percentage of apoptosis usually observed after 3 days of in vitro culture. CD34+ cells were cultured in serum-free condition in Iscove's DMEM (Invitrogen) supplemented with 20% serum substitute B1T (Stern cell Technologies, Vancouver, British Columbia, Canada) in the presence of stem cell factor (100 ng/mL), FLt3 ligand (100 ng/mL), and interleukin-3 (20 ng/mL; BioSource International).

Cell cycle analysis. Cells, cultured with compounds for 24 or 72 h, were fixed and stained according to the Guava Cell Cycle Reagent protocol (Guava Technologies, Hayward, CA). The cell cycle distribution was determined using the FACSArray BD Immunocytometry Systems, San Jose, CA) and FlowJo cytometry software (Tree Star, Ashland, OR).

Analysis of cell division. Cells were labeled with carboxyfluorescein diacetate (CFDA) succinimidyl ester (Vibrant CFDA SE, Molecular Probes, Eugene, OR) at a final concentration of 1.25 µmol/L following the manufacturer’s directions. Cells were then extensively washed and resuspended in the appropriate culture medium. Namalwa and CD34+ cells were plated at 10,000 per well in 24-well plates and incubated for 6 and 7 days, respectively. At 3 days and every day after, an aliquot was removed and analyzed by flow cytometry for CFDA staining.

Cell proliferation and apoptosis analysis. After each indicated time points, cells treated with compounds were counted and phenotypically analyzed by flow cytometry using the FACSArray. The percentage of apoptotic cells and CD34+ cells were monitored using propidium iodide staining (BD PharMingen, San Diego, CA) and the antibody anti-CD34 [phycoerythrin (PE) conjugated; BD PharMingen], respectively, according to the manufacturer’s experimental procedure.

B-cell purification. Primary B cells were obtained from healthy donor buffy coats (San Diego Blood Bank). B cells were purified using Ficol-Paque (GE Healthcare, Piscataway, NJ) and CD19 microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

Small interfering RNA transfection. Namalwa cells were transfected with p21WAF-1 siGENOME SMARTpool or RISC-free small interfering RNA (siRNA) #1 as a negative control (Dharmacon, Lafayette, CO) at a final concentration of 200 nmol/L. Transfections were done using DharmaFECT 2 transfection reagent (Dharmacon) following the manufacturer's protocol. A total of 5.2 µL of DharmaFECT 2 was used for 0.25 millions of cells in 1.2 mL of transfection volume. After 4 h of incubation, 1 volume of RPMI 1640 supplemented with 20% FBS was added and the cells were incubated for 24 h with the compounds.

Western blot analysis and immunoprecipitation. Whole-cell lysates were prepared according to Cell Signaling Technology method. For Western blot assay, protein extracts were subjected to SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were probed with primary antibody overnight followed by HRP-conjugated appropriate secondary antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) detection system. For immunoprecipitation assay, whole-cell extracts were incubated with CDK2, CDK4, and CDK6 antibodies at 4°C for 3 h and then incubated with protein A agarose beads for 1 h. In each immunoprecipitation assay, protein extracts were incubated with IgG as a negative control. The immunoprecipitates were resolved by SDS-PAGE, and the membranes were probed with p21WAF-1 and CDK2, CDK4, or CDK6 antibodies.

In vitro kinase assay. Total cellular protein was used to immunoprecipitate CDK2 complex in the condition described above. CDK2 kinase activity was assayed as described previously (22). As positive control, CDK2 complexes immunoprecipitated from DMSO-treated cell extracts were incubated with 50 µmol/L Nu6140 [an inhibitor of CDK2 (23)] for 30 min before doing the assay. The kinase reactions were resolved on a SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was first subjected to autoradiography to detect the phosphorylation of recombinant pRb and then immunoblotted with CDK2 and p21WAF-1 antibodies.

Real-time PCR analysis. Total RNAs were purified from cells using RNeasy kit (Qiagen, Valencia, CA). Reverse transcription-PCR from 100 ng of total RNA was done using the SuperScript III One-Step Quantitative PCR Reagent System (Invitrogen) and Taqman PCR probes specific for the gene of interest (Applied Biosystems, Foster City, CA) according to standard methods. The quantity of product obtained was calculated using the standard curve predetermined and normalized to β-actin.

Results

CC-4047 and lenalidomide inhibit Namalwa cell proliferation in regulating cell cycle progression. To better understand the antiproliferative effect of a selected number of IMiDs immunomodulatory drugs in hematopoietic tumor cells (3, 12), Namalwa cells were treated with increasing concentration of CC-4047 and lenalidomide for 72 h, harvested, counted, and subjected to cell cycle analysis by flow cytometry (Fig. 1A). Both lenalidomide and CC-4047 showed a strong effect on cell cycle progression by increasing, in a dose-dependent manner, the percentage of cells arrested in G0-G1 phase and decreasing the percentage of cells arrested in S and G2-M phase. After 3 days of culture, both compounds decreased Namalwa cell proliferation, in a dose-dependent manner, without significantly affecting cell apoptosis (Fig. 1B). The antiproliferative effect was even stronger when the cell count was
monitored after 4 and 5 days of culture [a 4- and 2-fold decrease with 10 μmol/L of CC-4047 and lenalidomide at 5 days, respectively (Fig. 1C and D)]. Taken together, these results showed that lenalidomide and CC-4047 inhibit Namalwa cell proliferation by arresting cells in G0-G1 phase and that CC-4047 is more potent than lenalidomide in regulating cell cycle progression and apoptosis.

**CC-4047 and lenalidomide up-regulate the CDKI p21WAF-1.**

Next, we examined the effects of lenalidomide and CC-4047 on the expression of proteins controlling the G1-S cell cycle transition. Affymetrix gene array analysis from total RNA obtained from Namalwa cells cultured with CC-4047 and lenalidomide showed that, as early as 6 h, many genes were affected (data not shown). Among them, p21WAF-1 was found to be up-regulated by both compounds. Because p21WAF-1 has been described to directly regulate the activity of CDKs involved in the G1-S progression (16-18), we sought to determine its role in CC-4047- and lenalidomide-induced cell cycle arrest. The confirmation of p21WAF-1 mRNA and protein up-regulation was done by quantitative real-time PCR and Western blot, respectively. We observed a dose-dependent and incubation time-dependent increase of p21WAF-1 mRNA (Fig. 2A) and protein (Fig. 2B) in response to CC-4047 and lenalidomide. As early as 8 h, the protein level of p21WAF-1 was increased with CC-4047 and lenalidomide and further enhanced after 24 and 72 h. The expression of other CDKIs has been examined. The mRNA and protein levels of p27KIP-1, p16INK4A, and p15INK4B were not affected in Namalwa cells treated with CC-4047 or lenalidomide for 24 h (data not shown).

CC-4047 and lenalidomide promote the interaction between p21WAF-1 and CDK2, CDK4, and CDK6 and reduce pRb phosphorylation in Namalwa cells. It has been shown that p21WAF-1 suppresses cell cycle progression by binding to and inhibiting the kinase activity of the CDK-cyclin complex (19). To verify this effect in our model, CDK2, CDK4, and CDK6 complexes were immunoprecipitated from extracts of Namalwa cells incubated for 24 h with increasing concentrations of CC-4047 and lenalidomide and any bound p21WAF-1 was detected by Western blotting. CC-4047 and, to a lesser extent, lenalidomide treatment resulted in a dose-dependent enhancement of p21WAF-1 binding to CDK2, CDK4, and CDK6 that correlates with the potency of CC-4047 and lenalidomide to increase p21WAF-1 expression (Fig. 2C).

To determine if p21WAF-1 binding to CDK2, CDK4, and CDK6 correlated with functional effects in CDK activity, we analyzed the pRb residues described to be phosphorylated by the cyclin D/CDK4/CDK6 and by the cyclin E/CDK2 complexes (15). Total lysates from Namalwa cells incubated for 24 h with increasing concentrations of CC-4047 and lenalidomide were subjected to immunoblotting with antibodies against phosphorylated pRb.
Ser<sup>795</sup>, phosphorylated pRB Ser<sup>608</sup>, phosphorylated pRB Ser<sup>780</sup>, phosphorylated pRB Ser<sup>612</sup>, phosphorylated pRB Thr<sup>821</sup>, pRB, and β-actin. As expected, we observed a dose-dependent decrease of pRB phosphorylation with no modification of pRB protein level (Fig. 3A).

**CC-4047 and lenalidomide indirectly reduce CDK2 kinase activity.** We verified that the decrease of pRB phosphorylation was due to the inhibition of CDK2 kinase activity. As presented in Fig. 3B, the in vitro kinase assay done with lysates from Namalwa cells cultured for 24 h with CC-4047 and lenalidomide showed a reduction of CDK2 kinase activity correlating with the increase of p21<sup>WAF-1</sup> binding to CDK2. As a positive control, CDK2 kinase activity was reduced when the immunoprecipitated complex was incubated with Nu6140, an inhibitor of CDK2 (23).

**p21<sup>WAF-1</sup> is required for CC-4047 to induce cell cycle arrest in Namalwa cells.** To show that p21<sup>WAF-1</sup> is required for CC-4047 to induce Namalwa cell cycle arrest, we inhibited p21<sup>WAF-1</sup> expression with siRNA. As shown in Fig. 3C, a decreased percentage of cells was arrested in G<sub>0</sub>-G<sub>1</sub> when cells were transfected with siRNA targeting p21<sup>WAF-1</sup> mRNA and incubated for 24 h with CC-4047 compared with cells transfected either with a siRNA control or siRNA against an unrelated mRNA (58.5% versus 67.6% for siRNA control and 66.7% for siRNA unrelated).

Collectively, these results suggest that, in Namalwa cells, the up-regulation of p21<sup>WAF-1</sup> induced by CC-4047 and lenalidomide plays an important role in the inhibition of CDK2, CDK4, and CDK6 activity, leading to the reduction of pRB phosphorylation that results in cell cycle arrest in G<sub>1</sub> phase and inhibition of cell proliferation.

**CC-4047 and lenalidomide inhibit U266 and LP-1 cell proliferation (similarly to Namalwa cell) and protect normal B cells from apoptosis.** To show that this mechanism is not restricted to Namalwa cells, we studied CC-4047 and lenalidomide effects on two other B malignant cell models: LP-1 and U266 multiple myeloma cell lines. We found that the compounds up-regulated p21<sup>WAF-1</sup> mRNA and protein level in both cell lines (data not shown; Fig. 4A, left). In addition, the CDK2 kinase assay done with lysates from LP-1 and U266 treated for 24 h with CC-4047 and lenalidomide showed a reduction of CDK2 activity correlating with the increase of p21<sup>WAF-1</sup> binding to CDK2 (Fig. 4A, right). To correlate CDK2 inhibition to cell proliferation, we monitored the viable cell count and the cell cycle progression. After 3 days of

![Figure 2](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-06-2306-10.1158/0008-5472.CAN-06-2306)
culture, CC-4047 and lenalidomide significantly decreased the number of viable LP-1 and U266 cells and increased the percentage of cells arrested in G₀-G₁ phase compared with DMSO (Fig. 4B). In contrast to malignant cells, we did not find any modulation of p21WAF-1 expression or pRb phosphorylation in normal B cells, obtained from healthy donors, cultured for 24 or 72 h with CC-4047 or lenalidomide (data not shown; Fig. 4C). Interestingly, after 3 days of culture with CC-4047 and lenalidomide, we observed a dose-dependent increase of viable cells (Fig. 4D). This effect was not due to the activation of cell proliferation because 95% of the B cells, after 3 days of culture, were arrested in G₀-G₁ phase but was the result of a decrease in apoptosis (18% and 24% compared with 35% with 1 μmol/L CC-4047 and 10 μmol/L lenalidomide, respectively; Fig. 4D). These findings show that CC-4047 and lenalidomide affect the proliferation of different B malignant cells (Burkitt’s lymphoma and multiple myeloma) while protecting normal B cells from apoptosis.

**CC-4047 and lenalidomide enhance CD34+ cell expansion.** To understand how lenalidomide may play a role on the restoration of patient bone marrow observed in clinical studies (4, 24), we investigated the effects of lenalidomide and CC-4047 in a hematopoietic progenitor cell model (CD34+ cells). Cord blood–derived CD34+ cells were cultured in the expansion medium supplemented with increasing concentrations of CC-4047 and lenalidomide. In contrast to our B malignant models, both compounds increased, in a dose-dependent manner, the total number of CD34+ cells after 6 days of culture (Fig. 5A). This effect was the resultant of both an increase of the total number of cells and the percentage of CD34+ cells. This enhancement was observed with three different CD34+ expansion cocktails and from CD34+ cell isolated from bone marrow as well as from steady-state and granulocyte colony-stimulating factor–mobilized peripheral blood (data not shown). To make sure that the opposing effects obtained between Namalwa cells and CD34+ cells were compound and not culture medium
dependent, we cultured the Namalwa cells for 3 and 6 days with the CD34+ expansion medium and CC-4047. A decrease in proliferation as described above was found (data not shown), indicating that the contrary effects were cell and not culture medium dependent. We validated the opposing effect of CC-4047 on Namalwa and CD34+ cells in analyzing the rate of cell division using CFDA labeling technique. Following the loss of CFDA fluorescence level by fluorescence-activated cell sorting (FACS) analysis, we monitored the cell division rate of Namalwa and CD34+ cells cultured with 1 μmol/L CC-4047 for 3 to 7 days. As shown in Fig. 5B, the loss of fluorescence in CD34+ cells incubated with CC-4047 from 4 to 7 days was higher compared with the vehicle control, suggesting a greater cell division rate. In contrast, and in support of our data described above, the cell division rate in CC-4047-treated Namalwa cells was lower than the control.

CC-4047 and lenalidomide up-regulate p21WAF1 without affecting CDK2 kinase activity in CD34+ cells. Because CC-4047 and lenalidomide lead to an increase of CD34+ cell expansion, we investigated the regulation of p21WAF1 by our compounds. The level of p21WAF1 mRNA and protein was increased with both CC-4047 and lenalidomide compared with DMSO in CD34+ cells cultured for 3 days (Fig. 5C). This increase was detected by real-time PCR as early as 24 h and was sustained at 48 and 72 h of cell expansion (data not shown). After several days of CD34+ cell culture, the percentage of CD34+ cells typically decrease as cells start to differentiate and/or die (25). Depending on the cell context, increases of p21WAF1 have been functionally linked to cell cycle arrest, cell senescence, cell cycle progression, apoptosis, and cell differentiation (26-28). To determine whether the increase of p21WAF1 is subpopulation selective, we did real-time PCR with RNAs isolated from CD34+ and CD34- cells populations separated by magnetic beads after 72 h of CD34+ expansion with CC-4047. We detected a 2-fold increase of p21WAF1 in both populations, indicating that CC-4047-induced p21WAF1 up-regulation observed in CD34+ cell expansion was not due to the small portion of dying and differentiated cells (data not shown). Next, we determined whether p21WAF1 induction by CC-4047 and lenalidomide resulted in the loss of CDK2 kinase activity as shown for malignant B cells in Figs. 3 and 4. The in vitro kinase assay and the coimmunoprecipitation experiment done with lysates from CD34+ cells incubated for 72 h with CC-4047 or lenalidomide showed neither reduction of CDK2 kinase activity (Fig. 5D) nor p21WAF1 binding to CDK2 complex (data not shown). In addition, we examined the phosphorylation of pRb. No modification on CDK4/CDK6- and

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**Figure 4.** CC-4047 and lenalidomide inhibit the proliferation of U266 and LP-1 cells and protect normal B cells from apoptosis. **A,** whole-cell extracts from LP-1 and U266 cells treated for 24 h with 1 μmol/L of CC-4047, lenalidomide, or vehicle control (DMSO) were prepared. Left, cell extracts were subjected to Western blot analysis using p21WAF1 and β-actin antibodies. Visualization of proteins was done using the ECL detection system. Right, 150 μg of cell extracts were immunoprecipitated with CDK2 antibody or IgG as a negative control. The kinase reaction assay was done as described in Fig. 3B. **B,** cells were cultured with 1 μmol/L of compounds or DMSO. After 72 h, viable cells were counted using propidium iodide staining and subjected to cell cycle analysis by flow cytometry. **C,** primary B cells were incubated for 72 h with increasing concentrations of CC-4047 and lenalidomide. **C,** whole-cell extracts were subjected to Western blot analysis using p21WAF1, phosphorylated pRb Ser785, and β-actin antibodies. Visualization of proteins was done using the ECL detection system. **D,** viable and apoptotic cells were monitored using propidium iodide staining. **D,** number of viable cells and the percentage of apoptotic cells. Points, mean obtained in 200 μL of triplicate; bars, SE. Representative of three independent experiments.

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CDK2-specific phosphorylation sites was detected when CD34+ cells were cultured with both compounds for 72 h (data not shown). These results indicate that, in contrast to the malignant B-cell models used, despite an increase of p21 WAF-1 expression, CC-4047 and lenalidomide are unable to reduce CDK2 kinase activity, pRb phosphorylation, and cell proliferation in our hematopoietic progenitor model.

VPA is synergistic with CC-4047 and lenalidomide in increasing Namalwa cell apoptosis and CD34+ progenitor cell expansion. VPA, a drug that belongs to the HDAC inhibitor family, possesses antitumoral activities correlated with p21 WAF-1 up-regulation (29–31) and stimulates proliferation and self-renewal of HSCs (32). Because both VPA and CC-4047/lenalidomide present similar differential effects on the growth of hematopoietic cancer cells and progenitor cells, we sought to assess whether a combined treatment would show additive or synergistic effects on our cell models. First, we monitored by FACS analysis the percentage of apoptotic cells when Namalwa cells were incubated for 3 days with CC-4047 or lenalidomide combined with VPA. We found that, depending on the concentration used, VPA can be additive or synergistic with CC-4047 in inducing cell apoptosis. Indeed, at 0.1 mmol/L, VPA was additive to CC-4047, whereas, at 1 mmol/L, VPA was synergistic with CC-4047 (Fig. 6A). To a lesser extent, we found that VPA possesses synergistic effects when combined with lenalidomide (1.7-fold increase in the percentage of apoptotic cells with 1 mmol/L VPA combined with 10 μmol/L lenalidomide; Fig. 6A). Next, we monitored by FACS analysis the total number of CD34+ cells obtained after 6 days of CC-4047 or lenalidomide and VPA. Additive effects on the total number of CD34+ cells were observed for both CC-4047 and lenalidomide (Fig. 6B), and for some concentration combinations (e.g., 0.3 mmol/L VPA + 0.001 μmol/L CC-4047 and 0.3 mmol/L VPA + 1 μmol/L lenalidomide), synergism was achieved (Fig. 6B). Collectively, these results suggest that the combination of VPA with CC-4047 and lenalidomide not only increases apoptosis in tumor cell line but also enhances CD34+ progenitor cell expansion, which has a significant effect in terms of elevating the therapeutic index versus either treatment alone. This beneficial combination might not be specific to VPA but HDAC inhibitors in general because we obtained identical results with trichostatin A (another HDAC inhibitor; data not shown).

**Discussion**

Several cellular activities for selected IMiDs immunomodulatory drugs have been described, including antiangiogenic activities, anti-inflammatory functions through regulation of cytokine production and T-cell and natural killer cell stimulation (3, 8–12), and antiproliferative effects on hematopoietic tumor cells (3, 12). In this study, we showed that CC-4047 and lenalidomide directly
represents the complement of these compounds in helping the repopulation responsible for cell cycle arrest in G\textsubscript{1} phase in Namalwa cells. The regulated by CC-4047 and lenalidomide and is, at least in part, correlated with the cell cycle arrest in G\textsubscript{1} in multiple myeloma and CDK6 activity is not totally understood. In some scenarios, increased p21 WAF-1 level induces an increase in the formation of B cells but protect them from apoptosis (Fig. 4).

In this report, we present additional data indicating that CC-4047 and lenalidomide inhibit the proliferation of two multiple myeloma cell lines, LP-1 and U266, through the same mechanism of action as Namalwa cells. These results corroborate those of Hideshima et al. (3), who reported that p21 WAF-1 up-regulation was correlated with the cell cycle arrest in G\textsubscript{1} in multiple myeloma patient cells induced by thalidomide and three different IMiDs immunomodulatory drugs. Therefore, the role of p21 WAF-1 in the antitumor activity of CC-4047 and lenalidomide can be extended to some other B-cell malignancies. Interestingly, CC-4047 and lenalidomide do not possess antiproliferation activity in normal B cells but not in CD34\textsuperscript{+} cells because of the different concentration of p21 WAF-1 in malignant cells versus CD34\textsuperscript{+} cells. p21 WAF-1 interaction with CDK4/CDK6 complex might have opposite effects on cell proliferation than lenalidomide. Our investigation led us to identify p21 WAF-1 as an important player in lenalidomide and CC-4047 mechanism of action. We found that p21 WAF-1 expression is up-regulated by CC-4047 and lenalidomide and is, at least in part, responsible for cell cycle arrest in G\textsubscript{1} phase in Namalwa cells. The increased p21 WAF-1 level induces an increase in the formation of CDKs complexes (CDK2, CDK4, and CDK6; Fig. 2C), inhibiting their kinase activity and leading to the loss of pRB phosphorylation that enables the cell cycle progression to S phase (Fig. 3A and B).

Figure 6. Combination of VPA with CC-4047 or lenalidomide in Namalwa cells and CD34\textsuperscript{+} cells. A, Namalwa cells were treated with VPA at 0.1, 0.3, or 1 mmol/L combined with the indicated concentrations of CC-4047 (left) or lenalidomide (right) for 72 h. Apoptotic cells were monitored by flow cytometry using propidium iodide staining. Points, mean of the percentage of apoptotic cells obtained of triplicates; bars, SE. Representative of three independent experiments. B, CD34\textsuperscript{+} cells were plated into 96-well plates at 2,000 per well in a volume of 200 μL and treated with VPA at 0.1 or 0.3 mmol/L, combined to the indicated concentrations of CC-4047 (left) or lenalidomide (right). After 6 d, the total number of CD34\textsuperscript{+} cells obtained was monitored by flow cytometry with the antibody anti-CD34 (PE conjugated). Points, mean of triplicates of the number of CD34\textsuperscript{+} cells obtained in 200 μL; bars, SE. Representative of three independent experiments.

It has been suggested that p21 WAF-1 interaction with CDK2 and CDK4/CDK6 complexes might have opposite effects on cell proliferation (33). We showed that the reduction of pRB phosphorylation at Ser\textsuperscript{780}, Ser\textsuperscript{795}, and Ser\textsuperscript{608}, described to be CDK4/CDK6-specific phosphorylation sites (15), is correlated with p21 WAF-1 interaction with CDK4 and CDK6 (Figs. 2C and 3A) in Namalwa cells. The functional effect of p21 WAF-1 interaction with CDK4 and CDK6 activity is not totally understood. In some scenarios, p21 WAF-1 interaction with CDK4/CDK6 is thought to be promitogenic in stabilizing cyclin D/CDK4/CDK6 complexes or in sequestering p21 WAF-1 from cyclinE/CDK2 complex, allowing CDK2 to be active on pRB phosphorylation (33). The differential effect on CDK4/CDK6 activity has been described to be dependent on p21 WAF-1 concentration; at low concentration, p21 WAF-1 might enhance CDK4/CDK6 kinase activity, whereas at high concentration p21 WAF-1 inhibits CDK2, CDK4, and CDK6 activities (33, 34). Our findings would support a model in which p21 WAF-1 inhibits CDK2, CDK4, and CDK6 activities in malignant B cells but not in CD34\textsuperscript{+} cells because of the different concentration of p21 WAF-1 in malignant cells versus CD34\textsuperscript{+} cells. p21 WAF-1-induced CDK4/CDK6 kinase inhibition has been proposed as part of the mechanism of action for several antitumor drugs, such as conjugated linoic acid, silymarin, genistein, epigallocatechin-3-gallate, and berberine (35–37).

Our study indicates that lenalidomide and CC-4047 regulate p21 WAF-1 expression by increasing mRNA (Fig. 3A) through a mechanism that we are currently investigating. In addition to transcription regulation, multiple posttranscriptional mechanisms maintain and sustain p21 WAF-1 protein level (38). Gandhi et al. (12) have shown that lenalidomide inhibits Namalwa cell proliferation by affecting the AKT pathway. Lenalidomide-induced AKT inhibition may participate in sustaining p21 WAF-1 protein levels in the nucleus through reduction of p21 WAF-1 phosphorylation, which results in its cytoplasmic retention (39).

In this study, we present strong evidence that CC-4047 and lenalidomide have opposite effects on progenitor cells versus tumor cells. In contrast to our malignant cell models, we found that CC-4047 and lenalidomide strongly enhance CD34\textsuperscript{+} expansion in increasing (a) the overall rate of cell division and (b) the percentage of CD34\textsuperscript{+} cells (Fig. 5). Interestingly, p21 WAF-1 up-regulation is observed with the enhancement of CD34\textsuperscript{+} expansion (Fig. 5C). Despite this apparent discrepancy, the association of p21 WAF-1 with
both proliferation and maintenance of myeloid precursor cells has been described in a previous report (40). Yaroslavskiy et al. (40) have shown that p21\(^{\text{WAF-1}}\) concentration is low in growth-arrested CD34\(^+\) cells but increases in parallel to the cellular expression of CD71 (marker of proliferation) and the decrease of the percentage of cells in G0-G1. Using p21\(^{\text{WAF-1}}\) knockout mice, Mantel et al. (41) have shown the importance of p21\(^{\text{WAF-1}}\) in the maintenance of stem progenitor cells in vivo through a mechanism not fully understood. These pleiotropic effects of p21\(^{\text{WAF-1}}\) may explain the increase of p21\(^{\text{WAF-1}}\) observed in CD34\(^+\) cell expansion and the lack of correlation with cell cycle arrest (26, 33, 38). As shown in Fig. 5D, in CD34\(^+\) cells, p21\(^{\text{WAF-1}}\) does not inhibit CDK2 activity. Indeed, the difference observed might be dependent on p21\(^{\text{WAF-1}}\) concentration as discussed above (33, 34) because the p21 WAF-1 level is at least 100 times lower in CD34\(^+\) cells than in Namalwa cells after CC-4047 or lenalidomide treatment.

HDAC inhibitors are a new class of anticancer agents currently being evaluated in phase I and II clinical trials (42). VPA can induce differentiation of cell lines derived from neuroblastoma, glioma, teratocarcinoma, and leukemic blasts from patients with acute myelogenous leukemia (29–31, 43). Therefore, VPA is being discussed as a promising novel anticancer drug. Recently, several reports showed that VPA and other HDAC inhibitors, such as Laq824 and CG1521, have opposite effects on stem cells compared with blast cells. They prevent the differentiation of bone marrow CD34\(^+\) but enhance proliferation of HSCs in increasing their self-renewal potential (32, 44). These studies indicate that, similarly to lenalidomide and CC-4047, VPA enhances the expansion of hematopoietic progenitor cells in addition to its antitumoral activity. In this report, we show that VPA and trichostatin A are additive and synergistic with CC-4047 and lenalidomide in increasing the apoptosis of Namalwa cells and in enhancing CD34\(^+\) expansion (data not shown; Fig. 6A and B), suggesting that the combination therapy might be extremely beneficial method to treat hematologic malignancies but also to expand ex vivo HSCs for transplantation and gene therapies.

In conclusion, the results of the present study are interesting in several respects. In defining the important role of p21\(^{\text{WAF-1}}\) in the antiproliferative effects of certain IMiDs immunomodulatory drugs, we not only provide new insights on the mechanism of action of CC-4047 and lenalidomide but also identify a new biomarker that could be used to select the responders in patients undergoing lenalidomide therapy. This biomarker could also be used to study the pharmacokinetic/pharmacodynamic relationship in patients treated with lenalidomide or other IMiDs compounds, which could be very useful for dosage optimization. In finding that CC-4047 and lenalidomide have opposite effects on the proliferation of progenitor cells versus tumor cells, we can explain, at least in part, the clinical observations from myelodysplastic syndrome and multiple myeloma patients treated with lenalidomide. Furthermore, the results showing that CC-4047 and lenalidomide enhance CD34\(^+\) cell expansion, without loss of their self-renewal capacity, open a new potential use in ex vivo bone marrow regeneration as well as ex vivo amplification of HSC for transplantation and gene therapies. Finally, our in vitro experiments showing the efficacy of the combination of CC-4047 and lenalidomide with VPA raise the possibility that certain HDAC inhibitors might be ideal candidates for combination therapy by elevating the therapeutic index, versus bone marrow regeneration as open a new potential use for CC-4047 and lenalidomide.

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References


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Lenalidomide and CC-4047 Inhibit the Proliferation of Malignant B Cells while Expanding Normal CD34 + Progenitor Cells

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