Pomalidomide and Lenalidomide Induce p21WAF-1 Expression in Both Lymphoma and Multiple Myeloma through a LSD1-Mediated Epigenetic Mechanism

Laure Escoubet-Lozach,¹ I-Lin Lin,¹ Kristen Jensen-Pergakes,¹ Helen A. Brady,¹ Anita K. Gandhi,² Peter H. Schafer,² George W. Muller,² Peter J. Worland,¹ Kyle W.H. Chan,¹ and Dominique Verhelle¹

¹Celgene, San Diego, California and ²Celgene Corporation, Summit, New Jersey

Abstract

Lenalidomide and pomalidomide have both been evaluated clinically for their properties as anticancer agents, with lenalidomide being available commercially. We previously reported that both compounds cause cell cycle arrest in Burkitt’s lymphoma and multiple myeloma cell lines by increasing the level of p21WAF-1 expression. In the present study, we unravel the molecular mechanism responsible for p21WAF-1 up-regulation using Namalwa cells as a human lymphoma model. We show that the increase of p21WAF-1 expression is regulated at the transcriptional level through a mechanism independent of p53. Using a combination of approaches, we show that several GC-rich binding transcription factors are involved in pomalidomide-mediated up-regulation of p21WAF-1. Furthermore, we report that p21WAF-1 up-regulation is associated with a switch from methylated to acetylated histone H3 on p21WAF-1 promoter. Interestingly, lysine-specific demethylase-1 (LSD1) silencing reduced both pomalidomide and lenalidomide up-regulation of p21WAF-1, suggesting that this histone demethylase is involved in the priming of the p21WAF-1 promoter. Based on our findings, we propose a model in which pomalidomide and lenalidomide modify the chromatin structure of the p21WAF-1 promoter through demethylation and acetylation of H3K9. This effect, mediated via LSD1, provides GC-rich binding transcription factors better access to DNA, followed by recruitment of RNA polymerase II and transcription activation. Taken together, our results provide new insights on the mechanism of action of pomalidomide and lenalidomide in the regulation of gene transcription, imply possible efficacy in p53 mutated and deleted cancer, and suggest new potential clinical uses as an epigenetic therapy. [Cancer Res 2009;69(18):7347–56]

Introduction

Lenalidomide (Revlimid) and pomalidomide are IMiDs immunomodulatory compounds developed by Celgene Corp. that possess multiple cellular activities including direct inhibition of hematopoietic tumor growth, immunomodulation via T-cell and natural killer cell stimulation, enhancement of erythropoiesis, and microenvironmental effects such as antiangiogenic activity (1–6). These compounds have negative effects on hematopoietic tumor cell proliferation and positive effects on CD34+ cell expansion (1–6). Lenalidomide has been approved by the Food and Drug Administration for the treatment of patients with transfusion-dependent anemia due to low-risk or intermediate-1-risk myelodysplastic syndromes associated with a deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities, and in combination with dexamethasone for the treatment of multiple myeloma patients who have received at least one prior therapy (7, 8). Myelodysplastic syndrome patients receiving lenalidomide therapy experience a significant erythroid response characterized by a cytogenetic reduction of the malignant clone and restoration of bone marrow function that leads to transfusion independence (7, 9). Pomalidomide is currently under evaluation for the treatment of hematologic cancers (10). We previously reported that lenalidomide and pomalidomide induce G0-G1 growth arrest in the Burkitt’s lymphoma Namalwa cells and multiple myeloma LP-1 and U266 cells by increasing the levels of p21WAF-1 RNA and protein. An increase in p21WAF-1 expression was also observed in patient primary multiple myeloma cells on treatment with IMiDs immunomodulatory compounds (1). In addition, we presented evidence that p21WAF-1 up-regulation inhibits cyclin-dependent kinase (CDK)-2, CDK4, and CDK6 kinase activity, which leads to the loss of pRb phosphorylation and the inability of these cells to progress into S phase (11). In this report, we investigate the mechanism by which lenalidomide and pomalidomide up-regulate p21WAF-1 expression.

The transcriptional regulation of p21WAF-1 has been extensively studied. p21WAF-1 is a transcriptional target of p53 and plays a crucial role in mediating growth arrest when cells are exposed to DNA-damaging agents (12, 13). However, it has been shown that p21WAF-1 is up-regulated in cells where p53 is mutated or silenced, indicating that other transcription factors such as Sp1/Sp3, Egr1, and others are involved (14, 15). The regulation of chromatin structure has been described as a second level of transcriptional regulation for p21WAF-1. DNA methylation of its promoter has been reported in cancer and plays a role in protein regulation (16, 17). It has been suggested that DNA methylation of p21WAF-1 promoter affects promoter accessibility for the transcription factors. Indeed, Zhu and colleagues hypothesized that hypermethylation of p21WAF-1 promoter around the consensus Sp1 binding sites may directly reduce Sp1/Sp3 binding, thereby decreasing p21WAF-1 expression. A second epigenetic mechanism shown to regulate p21WAF-1 transcription is histone acetylation. Both histone acetylases (HAT) and histone deacetylases (HDAC) are key enzymes that catalyze the reversible acetylation/deacetylation of core histone tails (18). Recent evidence indicates that selected HDAC inhibitors may arrest human tumor cells in G1 phase of the cell cycle by increasing the mRNA and protein levels of p21WAF-1 (19, 20).
Finally, histone methylation has been linked to p21WAF-1 promoter regulation. Duan and colleagues (21) reported that the repression of p21WAF-1 transcription was associated with dimethylation of H3K9 by the histone methyltransferase G9a. These observations imply that the H3K9me2 mark present on p21WAF-1 promoter should be demethylated to have the gene transcribed. Originally, it was assumed that histone methylation was an irreversible epigenetic event; however, a few years ago, the cloning of the first lysine demethylase, named lysine-specific demethylase 1 (LSD1), was reported (22, 23). LSD1 has a dual effect depending on the residue it demethylates. When associated with the CoRest complex, LSD1 demethylates the activator mark H3K4 and participates in gene repression (22). When associated with coactivators, LSD1 is involved in transcription activation through demethylation of H3K9me2.

In this study, we show that pomalidomide and lenalidomide regulate p21WAF-1 expression at the transcriptional level through a mechanism involving GC-rich DNA binding transcription factors such as Sp1, Sp3, Egr1, and Egr2. Further experiments indicate that our compounds improve DNA accessibility through a decrease of histone methylation on H3K9 and an increase of histone acetylation. We propose that both pomalidomide and lenalidomide facilitate histone acetylation by an LSD1-dependent mechanism inducing histone demethylation. This study provides new insights on the mechanism of action of pomalidomide and lenalidomide to regulate gene transcription and suggests their new potential clinical uses as epigenetic therapy.

Materials and Methods

Materials. Namalwa CSN.70 and LP-1 cells were purchased from DSMZ. Lenalidomide and pomalidomide (Celgene Corp.) were dissolved in DMSO. Antibodies were purchased from the following suppliers: p21WAF-1 (Cell Signaling Technologies, Inc.); Sp1 (Abcam for chromatin immunoprecipitation assays and Millipore for Western blot), Sp3, LSD1, H3K9/K14ac, H3K9me1, H3K9me2, H3K9me3, histone H3, and LSD1 (Millipore); and β-actin (Sigma-Aldrich). Mithramycin A (MTM) was purchased from Sigma-Aldrich.

Cell culture. Namalwa cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (Namalwa) or 15% (LP-1) fetal bovine serum. Cells were treated with lenalidomide or pomalidomide with a final DMSO concentration of 0.1%.

siRNA-mediated silencing. Two million Namalwa or LP-1 cells were transfected by electroporation (Amaxa Nucleofactor system, Amaxa, Inc.) with 0.2 to 0.4 nmol of Sp1, Sp3, or LSD1 SMRTpool siRNA (Dharmacon) or with 0.4 nmol of Egr1 or Egr2 Stealth siRNA (Invitrogen). Once the silencing of the target gene was obtained, cells were treated with pomalidomide or lenalidomide for 24 h.

Real-time PCR analysis. Total RNAs were purified from cells using the RNeasy Kit (Qiagen). Reverse transcription-PCR and quantification of RNA transcripts were done as previously described (10).

Gene expression microarrays. Total RNA was isolated from Namalwa or LP1 cells using RNAeasy Plus (Qiagen). Affymetrix human U133A 2.0 or human U133 2.0 plus gene chips were used for gene expression analysis. The GC-RNA algorithm was used for analysis and all analysis was done using GeneSpring 7.3 (Agilent Technologies). Results from replicate chips were averaged for calculating the fold differences.

To evaluate the significance of the presence of Sp1 binding sites among the promoters of pomalidomide-regulated genes identified, we used the NextBio software. Genes differentially expressed were ranked according to their response to pomalidomide or lenalidomide, and a list of genes containing Sp1 binding sites in their promoter was made. Significance scores were evaluated as previously described (24, 25). Briefly, the null hypothesis was that the distribution of up-regulated or down-regulated genes containing Sp1 binding sites in their promoter is not significantly different from what would be randomly expected given the platform; the alternative hypothesis is that the genes containing Sp1 binding sites are enriched in the upper rankings among up-regulated or down-regulated genes compared with what would be expected in random samplings from the microarray platform.

Western blot. Protein extracts were subjected to SDS-PAGE (Invitrogen) and transferred onto a nitrocellulose membrane. Membranes were probed with primary antibody overnight followed by Alexa-conjugated secondary antibody. Immunoreactive bands were visualized and quantified with Odyssey Infrared Imaging (LI-COR Biosciences). Representative images from at least three independent experiments are presented in the figures.

Sp1 binding assay. The TransAM Sp1 Kit (Active Motif) was used to assay the DNA-binding activity of Sp1 in nuclear extracts from Namalwa cells. For competitive binding experiments, 5 µg of nuclear extract from the stimulated cells were assayed in the presence of wild-type or mutated competitor oligonucleotides.

Chromatin immunoprecipitation assays. RNA polymerase II and p30 chromatin immunoprecipitation assays were carried out by GenPathway, Inc., as described by Labhart and colleagues (26). H3K9ac chromatin immunoprecipitation assays were done in-house as previously described (27, 28) using antibodies directed against acetylated histone H3 on lysines 9 and 14 (Millipore). H3K9me2 and Sp1 chromatin immunoprecipitation assays were done according to Squazzo and colleagues (29) using antibodies directed against dimethylated histone H3 on lysine 9 (Millipore) and Sp1 (Abcam), respectively. A complete protocol can be found online.

Enrichment in the proximal region of p21WAF-1 promoter was quantified by quantitative PCR using the primers 5'GCCAGATTGTGGCTCATTTCGTTGG-3' and 5'CTGCCAGCCGCGAGGCAGCTGAG-3', and enrichment of optic actrophy-1 (OPA-1) promoter was quantified by quantitative PCR using the primers 5'-CCGCGTACAGGAGGTGGAAGCT-3' and 5'-CAGGATGACCCGAGGATG-3'. Data were normalized to input DNA. Error bars represent SDs calculated from triplicate quantitative PCR determinations.

HDAC assay. Twenty micrograms of HeLa nuclear extract (Upstate) were incubated with pomalidomide or lenalidomide for 1 h. HDAC activity was measured using a HDAC colorimetric assay kit (Upstate).

Histone methylation. Five micrograms of purified histones were subjected to SDS-PAGE on a 16% Tris-glycine gel (Invitrogen), transferred onto a nitrocellulose membrane, and blotted with antibodies against H3K9me1, H3K9me2, H3K9me3, histone H3, and LSD1 (Millipore); and β-actin (Sigma-Aldrich).

Statistics. Statistical analysis was done using Student’s t test, P < 0.05 was considered statistically significant. In the figures, P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)) indicate significant differences as compared with DMSO.

Results

Pomalidomide and lenalidomide activate p21WAF-1 transcription. We previously reported that p21WAF-1 RNA expression was induced by pomalidomide and lenalidomide in Namalwa cells after 8 hours of treatment (11). To investigate the early mechanistic events involved, we first analyzed the regulation of p21WAF-1 RNA expression and the recruitment of RNA polymerase II on p21WAF-1 gene in response to pomalidomide and lenalidomide in time-course experiments.

Figure 1A shows that p21WAF-1 RNA expression was up-regulated by pomalidomide as early as after 2 hours of treatment. After 24 hours of treatment, the RNA expression of p21WAF-1 was increased by 7-fold as compared with cells treated with DMSO. Chromatin
immunoprecipitation experiments indicated that pomalidomide induced the recruitment of RNA polymerase II on the coding region of p21WAF-1 gene (Fig. 1B) in a time-dependent manner and correlated with the up-regulation of p21WAF-1 RNA expression. The same correlation was observed with Namalwa cells treated with lenalidomide for 6 and 24 hours (Fig. 1C and D).

**Identification of the transcription factors involved in pomalidomide-induced p21WAF-1 up-regulation.** The transcription of p21WAF-1 is under the control of a dozen proteins, but two major transcription factors have been reported to play a critical role in the regulation of p21WAF-1 promoter: p53 and Sp1. p53 binds p21WAF-1 promoter in its distal region (at −2301 and −1394 bp relative to the transcription start site), whereas Sp1 binds p21WAF-1 promoter in its proximal GC-rich region (between −119 and +1 bp relative to the transcription start site; Fig. 2A; ref. 14).

In Namalwa cells, it is known that p53 contains a mutation in its DNA binding domain (30). This mutation was confirmed in the Namalwa cell line we used by both sequencing and ELISA-based gel shift assays, in which no specific binding of p53 extracted from Namalwa nuclei to an oligonucleotide containing a p53 binding site was observed (data not shown). We also performed chromatin immunoprecipitation assays using a p53 antibody in Namalwa cells. As a positive control, we used A549 cells, in which p53 is known to up-regulate p21WAF-1 in response to actinomycin D. Whereas p53 strongly binds p21WAF-1 promoter in actinomycin D–treated A549 cells, no recruitment of p53 was detected in pomalidomide-treated Namalwa cells (Fig. 2A). These results were observed for both p53 binding sites described on p21WAF-1 promoter (regions A and B in Fig. 2A). Taken together, these data exclude a role of p53 in pomalidomide- and lenalidomide-induced up-regulation of p21WAF-1.

The second transcription factor we studied belongs to the Sp transcription factor family. Sp1 is known to interact with GC-rich promoter sequences and to have its binding inhibited by MTM (31). The treatment of Namalwa cells by MTM abolished both the up-regulation of p21WAF-1 RNA expression (Fig. 2B) and the recruitment of RNA polymerase II on p21WAF-1 gene (Fig. 2C), suggesting that, in contrast to p53, Sp1 is involved in the activation of p21WAF-1 promoter.

To confirm this hypothesis, we analyzed by chromatin immunoprecipitation assay the recruitment of Sp1 on p21WAF-1 promoter in response to pomalidomide. Figure 2D shows that, as early as at 2 hours, Sp1 recruitment is induced. This effect was time dependent, with more than 2-fold increase of Sp1 binding detected at 2, 6, and 24 hours.

To expand these findings to other genes up-regulated by pomalidomide, an expression profiling experiment was done in Namalwa cells using Affymetrix gene expression microarrays. The analysis of the promoters of genes up-regulated by pomalidomide after 4 and 8 hours showed a significant enrichment for genes with...
Sp1 binding sites \( (P = 0.0035 \text{ and } P = 0.0007, \text{ respectively}) \), whereas down-regulated genes did not show any enrichment (Fig. 3A). Sp1 binding site enrichment was also found in genes up-regulated by pomalidomide in the multiple myeloma cell line LP-1 cells, described to be responsive to pomalidomide (11). Based on this in silico analysis of promoters, we conclude that pomalidomide preferentially up-regulates genes whose promoters contain Sp1 binding sites.

**Pomalidomide does not regulate Sp1 expression or Sp1 binding activity.** To assess by which mechanism Sp1 regulates \( p21_{\text{WAF-1}} \) transcription in response to pomalidomide, we studied the effect of this compound on Sp1 RNA and protein levels, as well as on Sp1 binding activity.

Namalwa cells treated with pomalidomide for different time periods did not show any modulation of Sp1 RNA levels, as quantified by real-time PCR (Fig. 3B), or Sp1 protein levels (Fig. 3C). Moreover, MTM treatment did not modify Sp1 expression (Fig. 3C), excluding the hypothesis that MTM would affect pomalidomide-induced up-regulation of \( p21_{\text{WAF-1}} \) transcription by regulating Sp1 expression. These results indicate that pomalidomide does not up-regulate \( p21_{\text{WAF-1}} \) by modifying Sp1 expression.

To determine whether pomalidomide modulates Sp1 activity, we performed ELISA-based Sp1 binding assays using nuclear extracts from Namalwa cells treated with 10 \( \mu \text{mol/L} \) pomalidomide and 1 \( \mu \text{mol/L} \) MTM for different times. Although Sp1 extracted from Namalwa nuclei was able to specifically bind to a Sp1 binding site containing oligonucleotide (Fig. 3D, controls), no change in Sp1 binding was observed in response to pomalidomide treatment for 2, 4, or 6 hours. This result indicated that pomalidomide up-regulates \( p21_{\text{WAF-1}} \) expression without modifying Sp1 activity.

To assess the importance of Sp1 in the regulation of \( p21_{\text{WAF-1}} \) by pomalidomide, we used siRNA technology to silence Sp1. Figure 4A shows that Sp1 protein levels were significantly decreased in Sp1 siRNA–transfected cells as compared with control siRNA–transfected cells and that Sp1 silencing blunted pomalidomide-induced \( p21_{\text{WAF-1}} \) up-regulation by 35%.

Whereas this result shows that Sp1 plays an important role in the process, it also reveals that other transcription factors that also bind GC-rich regions may be involved. Among GC-rich DNA binding site transcription factors described to be involved in \( p21_{\text{WAF-1}} \) transcription regulation, we tested the role of Sp3, Egr1, and Egr2. As observed for Sp1, the silencing of Sp3, Egr1, and Egr2 individually by siRNA reduced the up-regulation of \( p21_{\text{WAF-1}} \) by pomalidomide by 44%, 57%, and 36%, respectively (Fig. 4B and C). Taken together, these results indicate that several GC-rich DNA binding transcription factors are involved in \( p21_{\text{WAF-1}} \) up-regulation by pomalidomide.

**Pomalidomide induces chromatin modifications on \( p21_{\text{WAF-1}} \) promoter.** We first analyzed the DNA methylation status of the \( p21_{\text{WAF-1}} \) promoter. Three CpG-rich regions were identified in the

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**Figure 2.** Sp1 is involved in the up-regulation of \( p21_{\text{WAF-1}} \) gene in response to pomalidomide. A, chromatin immunoprecipitation assay evaluating the recruitment of p53 on regions A and B of \( p21_{\text{WAF-1}} \) promoter in Namalwa cells in response to 10 \( \mu \text{mol/L} \) pomalidomide. Right, \( p21_{\text{WAF-1}} \) promoter regions A and B, amplified by quantitative PCR. The recruitment of p53 on \( p21_{\text{WAF-1}} \) promoter in A549 cells in response to actinomycin D (Act D) treatment was used as a positive control. B, \( p21_{\text{WAF-1}} \) RNA expression in response to 1 \( \mu \text{mol/L} \) MTM and 10 \( \mu \text{mol/L} \) pomalidomide treatment for 24 h. C, chromatin immunoprecipitation assay evaluating the recruitment of RNA polymerase II on \( p21_{\text{WAF-1}} \) gene in response to 1 \( \mu \text{mol/L} \) MTM and 10 \( \mu \text{mol/L} \) pomalidomide treatment for 24 h. D, recruitment of Sp1 on \( p21_{\text{WAF-1}} \) promoter in response to pomalidomide.
promoter using EMBOSS CpG plot. Bisulfite sequencing experiments showed that none of these regions was methylated in Namalwa cells treated with 0.1% DMSO (Supplementary Fig. S1). Thus, we extrapolate that pomalidomide-mediated up-regulation of p21WAF-1 does not act through this mechanism.

We then focused on the acetylation of histone H3 on lysines 9 and 14 (H3K9/14ac), which has been largely described to be associated with open chromatin and transcriptional activation (18, 32). Using chromatin immunoprecipitation assays, we showed that pomalidomide treatment increased H3K9/14 acetylation in a time-dependent manner (Fig. 5A). Indeed, as early as 30 minutes, the increase in the acetylation of H3K9 was observed, reaching more than 2-fold after 3 and 6 hours of treatment.

Because the increase of acetylation could result from an inhibition of HDACs, we measured the ability of pomalidomide and lenalidomide to inhibit the deacetylation of histones in an in vitro assay. In contrast to the HDAC inhibitor trichostatin A, pomalidomide and lenalidomide did not inhibit HDAC activity at any dose tested (Fig. 5B), suggesting that these compounds do not up-regulate the acetylation of H3K9/14 on p21WAF-1 promoter by directly inhibiting HDACs.

Figure 3. Effect of pomalidomide on Sp1 expression and binding activity. A, the enrichment of genes whose promoters contain Sp1 binding sites among the genes up-regulated by 10 μmol/L pomalidomide in Namalwa and LP-1 cells was evaluated using NextBio software. P values establishing the significance for this enrichment for up-regulated and down-regulated genes are represented in the chart. Genes up-regulated by pomalidomide in Namalwa and LP1 cells were clustered and color-coded according to their degree of up-regulation as compared with DMSO-treated cells. Dark green squares, genes whose promoters contain Sp1 binding sites. B, real-time PCR assessing Sp1 RNA expression in response to 10 μmol/L pomalidomide. C, Western blot evaluating Sp1 protein levels in response to 10 μmol/L pomalidomide and 1 μmol/L MTM treatment for 24 h. D, nuclear extracts from Namalwa cells treated with MTM and pomalidomide for the indicated times were assayed using the TransAM Sp1 assay kit. Controls were done using nuclear extracts from pomalidomide-treated Namalwa cells and wild-type or mutated Sp1 binding site–containing oligonucleotides.
Finally, we analyzed by chromatin immunoprecipitation assay the dimethylation of histone H3 on lysine 9 (H3K9me2), a mark which is associated with transcriptional repression, on \( p21^{WAF-1} \) promoter in the presence of DMSO or 10 \( \mu \)mol/L pomalidomide. As shown in Fig. 5C, H3K9 was dimethylated in basal conditions, which is consistent with the absence of \( p21^{WAF-1} \) transcription. Interestingly, pomalidomide led to a 6-fold decrease of H3K9me2 after 6 hours. Interestingly, no modification was observed on the promoter of OPA-1, a gene that is not regulated by pomalidomide in Namalwa cells.

To determine whether pomalidomide specifically regulates H3K9 methylation on \( p21^{WAF-1} \) promoter, we assessed the methylation of total histone H3. The Western blot method shown in Fig. 5D indicates that Namalwa cells treated with pomalidomide or lenalidomide for 1, 6, and 24 hours did not present any change in tri-, di-, and mono-methylation of H3K9. These data suggest that pomalidomide regulates \( p21^{WAF-1} \) transcription through specific demethylation of H3K9 on its promoter.

LSD1 is a histone demethylase described to participate in gene transcription through demethylation of H3K9me2. By chromatin immunoprecipitation assay, we confirmed that the presence of LSD1 on several promoters was concomitant with the presence of the H3K9ac mark and the absence of the H3K9me2 mark (data not shown). Using siRNAs, we tested the involvement of LSD1 in \( p21^{WAF-1} \) up-regulation. Figure 6A shows that LSD1 silencing reduced both pomalidomide- and lenalidomide-induced \( p21^{WAF-1} \) up-regulation. The statistical analysis of several independent experiments indicates that this effect is significant at \( P < 0.05 \) for 1 and 10 \( \mu \)mol/L of the compounds (Fig. 6B).

The up-regulation of \( p21^{WAF-1} \) by pomalidomide and lenalidomide is not restricted to Namalwa cells, as we have previously shown that \( p21^{WAF-1} \) was up-regulated in the multiple myeloma cell lines LP-1 and U266 (11). Figure 6C shows that this up-regulation of \( p21^{WAF-1} \) is also decreased in LP-1 cells silenced for LSD1, extending the role of this demethylase in the mechanism of action of pomalidomide and lenalidomide in a myeloma cell line.

The histone methyltransferase G9a has been reported to be associated with the repression of \( p21^{WAF-1} \) promoter in HL-60 cells (21). Nevertheless, the silencing of G9a in our model did not significantly affect the basal level of \( p21^{WAF-1} \) or its up-regulation by pomalidomide.

![Figure 4](https://example.com/figure4.png)
pomalidomide and lenalidomide (data not shown). These observations suggest that the silencing of G9a is not sufficient to derepress the p21WAF-1 gene and that G9a does not play a critical role in the mechanism of action of pomalidomide and lenalidomide.

From these data, we propose the following model, illustrated in Fig. 6D. Under basal condition, p21WAF-1 promoter is silenced through the dimethylation of H3K9. Through a mechanism to be elucidated, pomalidomide and lenalidomide would lead to the activation of demethylases, such as LSD1, which removes the H3K9me2 mark from p21WAF-1 promoter, allowing the acetylation of this residue by HATs. The change of conformation of the chromatin resulting from the acetylation of H3K9 allows the binding of Sp1, Sp3, Egr1, and Egr2 and the recruitment of RNA polymerase II on p21 WAF-1 promoter, which leads to gene transcription.

Discussion

The transcription factor p53 possesses a critical function as a tumor suppressor gene. p53 is normally activated by cellular stress such as DNA damage, hypoxia, and deficiency of growth factors or nutrients, and mediates a growth-suppressive response that involves cell cycle arrest and apoptosis. In the case of cell cycle arrest, p21WAF-1 seems to be sufficient to block cell cycle progression out of G1 until repair has occurred or cellular stress has been resolved. In more than half of the tumors, p53 is mutated or deleted (33), which is associated with prognosis, progression, and therapeutic response of tumors (34, 35). In our study, we present evidence that lenalidomide and pomalidomide up-regulate p21WAF-1 through a p53-independent pathway by using p53 double-mutant Namalwa cells (36). The fact that lenalidomide and pomalidomide can induce cell cycle arrest through p21WAF-1 by bypassing p53 expands their use in a large panel of tumors independently of p53 status.

In this study, we show that GC-rich DNA binding transcription factors play an important role in pomalidomide-induced-p21WAF-1 transcription. This is suggested by the fact that the treatment of Namalwa cells by MTM, which inhibits all transcription factors binding to GC-rich regions (37), abolished the up-regulation of p21WAF-1. Sp1, whose binding to DNA is inhibited by MTM (31), is involved in the up-regulation of p21WAF-1 in several cell types (14, 38), as well as in the repression of this gene (39–42). In addition, Sp3, Egr1, and Egr2 bind GC-rich regions and have been
described to bind \( p21^{WAF-1} \) promoter and regulate its transcription (20, 43–45). Here, we showed that Sp1 is recruited to \( p21^{WAF-1} \) promoter as early as 2 hours after treatment, and its silencing by siRNA attenuated pomalidomide-induced \( p21^{WAF-1} \) up-regulation. Moreover, Sp1, Sp3, Egr1, and Egr2 silencing experiments showed a slighter \( p21^{WAF-1} \) up-regulation by pomalidomide. However, the silencing of none of these GC-rich DNA binding transcription factors independently was able to completely inhibit \( p21^{WAF-1} \) up-regulation, suggesting that either the level of protein silenced was not sufficient to totally repress pomalidomide up-regulation of \( p21^{WAF-1} \) or/and these transcription factors act in concert to activate \( p21^{WAF-1} \).

Our data suggest that pomalidomide favors the accessibility of GC-rich DNA binding transcription factors to \( p21^{WAF-1} \) promoter. We hypothesized that pomalidomide and lenalidomide might induce \( p21^{WAF-1} \) transcription through epigenetic modifications. Bisulfite sequencing experiments indicated that the CpG regions of \( p21^{WAF-1} \) promoter in Namalwa cells in basal conditions were not methylated, which subsequently ruled out the hypothesis that regulation of DNA methylation was important. Interestingly, we

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**Figure 6.** Role of LSD1 in pomalidomide- and lenalidomide-induced \( p21^{WAF-1} \) expression. A and C, LSD1 and \( p21^{WAF-1} \) protein levels in cells (A, Namalwa; C, LP-1) in which LSD1 has been silenced and which have been treated with pomalidomide or lenalidomide for 24 h were assessed by Western blot analysis. B, the histogram shows the average of the effect of pomalidomide and lenalidomide on \( p21^{WAF-1} \) expression, quantified in independent experiments. D, model for sequential steps involved in pomalidomide- and lenalidomide-mediated \( p21^{WAF-1} \) up-regulation.
uncovered the modification of the status of H3K9 on nucleosomes decorating p21WAF1 proximal promoter. Whereas H3K9 acetylation has been described to be an activator mark of transcription (18), the dimethylation of the same residue has been associated with gene repression (21, 23). In our model of pomalidomide-induced p21WAF1 expression, we observed a fine regulation of modification of this mark with a switch of H3K9 methylation to H3K9 acetylation.

The acetylation of histones H3 and H4 correlates with an open state of chromatin, which allows the recruitment of various transcription factors to the promoters. The regulation of histone acetylation, which is maintained by a dynamic balance of HDACs and HATs, plays an important role in the modulation of p21WAF1 expression, as illustrated in previous studies (19, 20, 38, 43, 44). In addition, several synthetic and natural HDAC inhibitors showed a consistent up-regulation of p21WAF1 expression in various cancer cell lines (19, 20, 45, 46). We propose that the pomalidomide-induced increase of H3K9 acetylation, possibly due to the dismissal of HDACs from p21WAF1 promoter, leads to the relaxation of the chromatin structure and allows the full recruitment of GC-rich DNA binding transcription factors including Sp1, Sp3, Egr1, and Egr2. Such a mechanism is in agreement with previous publications on Sp1 and Sp3 (19, 38, 47).

Importantly, although H3K9 acetylation increased on p21WAF1 promoter in response to pomalidomide and lenalidomide, we conclude that none of these compounds were HDAC inhibitors because (a) the global level of histone H3 acetylation was not altered (data not shown) and (b) HDAC assays assessing the effect of pomalidomide and lenalidomide on the inhibition of HDACs in vitro gave negative results.

Our study suggests that pomalidomide and lenalidomide might regulate the activity of the histone demethylase LSD1, which would participate in the demethylation of H3K9. LSD1 demethylates histone through a flavin-dependent mechanism (22). Its activity on promoters can lead to gene transcription or repression, depending on which residue is demethylated. In addition to its repressor role and as observed by Metzger and colleagues (23), LSD1 can act as a transcriptional coactivator by demethylating H3K9. This study done in LNCaP cells showed that LSD1 activity is required for androgen-dependent gene expression. LSD1-dependent transcriptional activation is not restricted to androgen receptor function in prostate cancer cells because a genome-wide location analysis of LSD1 in MCF7 human breast cancer cells reported a surprisingly large number of promoters bound to LSD1, among which 84% were also associated with RNA polymerase II (48), underlining the importance of LSD1 in gene activation. LSD1 has already been reported to up-regulate p21WAF1 through p53 stabilization (49). Our study describes for the first time that LSD1 participates in p21WAF1 transcription in a p53-independent manner.

Several mechanisms by which pomalidomide and lenalidomide activate LSD1 can be considered. In our study, LSD1 was detected only on active H3K9ac-positive promoters and is absent from promoters on which H3K9 is dimethylated. These observations suggest that pomalidomide and lenalidomide induce p21WAF1 up-regulation by mediating the recruitment of LSD1 on the promoter. This could result from a direct mechanism (posttranslational modification of LSD1 protein) or from an indirect mechanism involving the activation of a third partner required for LSD1 activation. Taken together, this study shows for the first time that pomalidomide and lenalidomide are regulating gene transcription through the regulation of acetylation and methylation of H3K9, providing new insights on the mechanism of action of these compounds.

Disclosure of Potential Conflicts of Interest

All authors are employees of Celgene and hold stock options in Celgene.

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Pomalidomide and Lenalidomide Induce p21<sup>WAF-1</sup> Expression in Both Lymphoma and Multiple Myeloma through a LSD1-Mediated Epigenetic Mechanism

Laure Escoubet-Lozach, I-Lin Lin, Kristen Jensen-Pergakes, et al.


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