Antitumoral activity of lenalidomide in \textit{in vitro} and \textit{in vivo} models of mantle cell lymphoma involves the destabilization of cyclin D1/p27\textsuperscript{kip1} complexes

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**Conflicts of interest**

The authors have no conflicts of interest to declare.
STATEMENT OF TRANSLATIONAL RELEVANCE

Mantle cell lymphoma (MCL) is an aggressive hematological neoplasm which lacks effective therapy. MCL cells express high level of cyclin D1, a major cell cycle regulator recently shown to be involved in MCL resistance to chemotherapeutic-induced cell death. However, the pharmacological targeting of cyclin D1/cyclin dependent kinase (CDK) complexes has shown modest activity in the clinical settings. Here, we find a tight correlation between contents of cyclin D1 and the CDK inhibitor p27\(^{\text{KIP1}}\), underlying the sequestration of this latest in cyclin D1/CDK4 complexes, and protection from degradation. We show this phenomenon to be associated with increased tumorigenicity of MCL cells \textit{in vivo}, and describe the possibility to counteract this process by using the thalidomide derivative, lenalidomide. Lenalidomide efficiently disrupts cyclin D1/p27\(^{\text{KIP1}}\) complexes, thus reducing tumor growth and angiogenesis and inducing apoptosis in a MCL xenotransplant model. This study thus provides evidences of lenalidomide efficacy in aggressive, cyclin D1/p27\(^{\text{KIP1}}\)-driven MCL tumors.
ABSTRACT

Purpose: Clinical responses to the immunomodulatory drug lenalidomide have been observed in patients with relapsed/refractory mantle cell lymphoma (MCL), although its mechanism of action remains partially unknown. We investigated whether the expression and subcellular localization of cyclin D1, a major cell cycle regulator overexpressed in MCL, and the cyclin-dependent kinase inhibitor p27KIP1, could identify MCL cases sensitive to lenalidomide, and whether the compound could modulate cyclin D1/p27KIP1 complexes in MCL cells.

Experimental Design: MCL primary samples and cell lines were analyzed for subcellular levels of cyclin D1/p27KIP1 complexes by Western blot, immunohistochemistry, immunoprecipitation and flow cytometry. Activity of lenalidomide in vitro and its effect on cyclin D1/p27KIP1 complexes were evaluated by real time PCR, immunoprecipitation, immunofluorescence and Western blot. In vivo validation was carried out in a mouse xenograft model of human MCL.

Results: We found cyclin D1 and p27KIP1 to be coordinately expressed in all the MCL samples tested. Immunoprecipitation analyses and siRNA assays suggested a direct role of cyclin D1 in the regulation of p27KIP1 levels. The nuclear accumulation of both proteins correlated with MCL cell tumorigenicity in vivo, and sensitivity to lenalidomide activity in vitro and in vivo. Lenalidomide mechanism of action relied on cyclin D1 downregulation and disruption of cyclin D1/p27KIP1 complexes, followed by cytosolic accumulation of p27KIP1, cell proliferation arrest, apoptosis, and angiogenesis inhibition.

Conclusions: These results highlight a mechanism of action of lenalidomide in MCL cases with increased tumorigenicity in vivo, which is mediated by the dissociation of cyclin D1/p27KIP1 complexes, and subsequent proliferation blockade and apoptosis induction.
INTRODUCTION

Mantle cell lymphoma (MCL) is an aggressive lymphoid neoplasm which accounts for 5% to 10% of all B-cell non-Hodgkin lymphomas. It is genetically characterized by the chromosomal translocation t(11;14)(q13;q32) resulting in the overexpression of cyclin D1. Moreover, high levels of chromosomal instability due to the disruption of the DNA damage response pathway associated with the abnormal activation of cell survival mechanisms, may confer an aggressive clinical course to the disease (1). Standard chemotherapy approaches are frequently used, but long-term remissions are rare. After failure of first- or second-line treatments, various single agents are used despite limited response rates (2). Thus, there is still a strong unmet medical need for new treatment options in MCL.

Promising results were achieved in a subset of relapsed MCL patients with a selective cyclin-dependent kinase (CDK) 4/6 inhibitor. However, this strategy appears to be insufficient for long-term disease control (3). In this line, preclinical studies have proposed that CDK-independent functions of cyclin D1 may account for its oncogenic and anti-apoptotic properties (4), suggesting that other(s) partner(s) of cyclin D1 could represent attractive therapeutic targets. Among these partners, the CDK inhibitor p27KIP1 is strongly expressed in the highly proliferative and aggressive blastoid MCL variants while is only present in a minority of the good prognosis and low proliferative MCL tumors (5-7). It was thus proposed that mantle cell lymphomagenesis may result, at least in part, from the ability of the overexpressed cyclin D1 to buffer changes of p27KIP1 levels, thereby rendering ineffective the p27KIP1-mediated inhibition of cellular growth (8). Therefore, the role of p27KIP1 in the pathogenesis of MCL remains controversial.

Although no drug has been shown so far to specifically modulate p27KIP1 expression/activity in MCL, the immunomodulatory agent lenalidomide, has been
shown to regulate p27\textsuperscript{KIP1} levels, in multiple myeloma (MM) cell lines and patient cells (9). This structural analogue of thalidomide approved by the US Food and Drug Administration for the treatment of MM, myelodysplastic syndromes and relapsed/refractory MCL, is also currently under active investigation with promising results for the treatment of chronic lymphocytic leukemia and non-Hodgkin lymphomas including MCL, in combination with steroids or rituximab/bendamustine (10). Lenalidomide treatment leads to enhanced proliferative and functional capacity of T-lymphocytes, activation of effector responses and suppression of inflammation (11). Although lenalidomide shows superior safety and efficacy relative to thalidomide, its mechanism of action remains partially unknown.

We reported previously that the overexpression of cyclin D1 in pro-B leukemic cells does not induce p27\textsuperscript{KIP1} relocalization from the nucleus to the cytoplasm but rather induces its sequestration of in trimeric complexes with cyclin D1 and CDK4, thereby regulating its stability (12). Here, we investigated whether the CDK inhibitor exerted similar functions in MCL cells, and the impact of cyclin D1/p27\textsuperscript{KIP1} complex formation on MCL cell growth and response to lenalidomide in both \textit{in vitro} and \textit{in vivo} settings.

\textbf{PATIENTS, MATERIALS AND METHODS}

\textbf{Isolation and culture of primary cells}

Cells from 13 previously untreated MCL patients, diagnosed according to the World Health Organization classification criteria (13), were used. Informed consent was obtained from each patient in accordance with the guidelines of the Ethical Committee of the Hospital Clínica in Barcelona, Spain, the CHU Côte de Nacre, Caen, France, and the Declaration of Helsinki. The clinical characteristics of patients are listed in Table 1.
Mononuclear cells from peripheral blood samples (PBMCs) were isolated by Ficoll/hypaque sedimentation (GE Healthcare), and conserved within the Biobank of our institutions (Hematopathology collection from IDIBAPS Biobank and the tumor library of Basse-Normandie, CHU Caen). Cells were either used directly or cryopreserved in liquid nitrogen in the presence of 10 % dimethyl sulfoxide, 60 % FBS and 30 % RPMI 1640. All samples contained more than 80 % of CD19+/CD5+ malignant lymphocytes, as assessed by flow cytometry.

Cell lines
Six previously described human MCL cell lines (Z-138, ZBR, Jeko-1, JBR, Rec-1 and JVM-2) (14) were used in this study. Cell lines were grown in RPMI 1640 or Dulbecco’s Modified Eagle’s Medium, supplemented with 10 % to 20 % heat-inactivated FBS, 2 mmol/L glutamine, 50 μg/mL penicillin-streptomycin (Life Technologies) and maintained in a humidified incubator at 37 ºC with 5 % carbon dioxide. All cultures were routinely tested for Mycoplasma infection by PCR and the identity of all cell lines was verified by using AmpFISTR identifier kit (Life Technologies).

Detection of p27KIP1 and cyclin D1 proteins in MCL primary samples by flow cytometry
Primary MCL cells (10^6) were washed in PBS and permeabilized with Perm & Stab reagent (Beckman Coulter). Cells were labeled with anti-cyclin D1 (M-20) or anti-p27KIP1 (C-19) (Santa Cruz Biotechnology) antibodies (Abs) for 30 min, washed in PBS and then labeled with Alexa Fluor 647-conjugated goat anti-rabbit IgG (Life Technologies) before being washed and fixed in PBS/PFA 0.5 % solution. As a negative control, cells were labeled only with the secondary antibody. Cellular fluorescence of at least 10^4 events was analyzed with a Gallios cytometer and data with the Kaluza software (Beckman Coulter).
Apoptosis detection by annexin V labeling

Cells were treated with 1 μmol/L lenalidomide (kindly provided by Celgene) for 72 h, and the percentage of apoptotic cells was quantified by staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Bender Medsystems) as described (15). A total of 10⁴ stained cells per sample were acquired and analyzed in an Attune acoustic focusing cytometer using Attune software (Life Technologies).

Immunohistochemical analysis of MCL cell lines

MCL cell cultures (5 x 10⁷ cells per line) were washed once in PBS and resuspended in 200 μL of a mix (v/v) of human thrombin (Sigma) and freshly isolated human plasma. Once solidified, cell pellets were formalin-fixed before paraffin embedding on silane-coated slides in a fully automated immunostainer (Bond Max, Leica Microsystems). Immunohistochemical studies were performed on consecutive tissue sections as previously described (14) using anti-phospho(p)-histone H3 (Epitomics), anti-cyclin D1 (EP12) or anti-p27^KIP1^ (sx53g8) (Dako) primary Abs. Preparations were evaluated with an Olympus DP70 microscope by means of a 40X/0.75 NA objective and DPManager software v2.1.1 (Olympus).

Immunofluorescence

Cyclin D1 and p27^KIP1^ expression was determined by fluorescence microscopy in Rec-1 cells untreated or treated with 1 μmol/L lenalidomide. Cells (5 x 10⁵) were fixed on poly-L-lysine-coated glass coverslips with 4 % paraformaldehyde, permeabilized with a solution containing 0.1 % saponin and 10 % FBS, and labeled with anti-cyclin D1 (DCS-6, Cell Signaling Technology), anti-p27^KIP1^ (C-19, Santa Cruz Biotechnology), or anti-p27^KIP1^-phospho(p)Thr198 (R&D Systems) Abs, followed by appropriate anti-mouse-tetramethylrhodamine isothiocyanate (TRITC) or anti-rabbit-FITC (Sigma).
secondary Abs. Coverslips were mounted on glass slides with DAPI-containing Fluoroshield mounting medium (Sigma) and visualized on a Nikon H5505 microscope by means of a 100X/1.30 NA oil objective (Nikon) with the use of Isis Imaging System v5.3 software (MetaSystems GmbH).

Immunoprecipitation and Western blotting

Whole protein extracts were obtained by cell lysis in Triton buffer (20 mmol/L Tris-HCL pH 7.6, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 % Triton X-100) supplemented with protease and phosphatase inhibitors (10 mg/mL leupeptin, 10 mg/mL aprotinin, 1 mmol/L phenylmethanesulfonyl fluoride, 5 mmol/L NaF, and 2 mmol/L Na3VO4). For cyclin D1 and p27(KIP1) immunoprecipitation, 3 x 10^7 cells were resuspended in immunoprecipitation (IP) buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 20 % glycerol, and protease/phosphatase inhibitors as above) and incubated for 15 min on ice. Cells were centrifuged for 15 minutes at 16,000 g and the supernatants were recovered. Then, 500 μg of proteins were incubated overnight at 4 ºC with 2 μg cyclin D1 (M-20) or p27(KIP1) (C-19) (Santa Cruz Biotechnology) Abs. Protein A-agarose beads (50 μL, Roche) were then added and the mixture was incubated for 1 h at 4 ºC. Beads were washed 3 times in IP buffer, resuspended in 20 μL of Laemmli buffer before SDS-PAGE. Cytosolic and nuclear extracts were prepared from 5 x 10^6 cells with the BioVision nuclear/cytosol fractionation kit as recommended by the supplier. Fifty μg proteins were loaded onto 12 % to 15 % SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Membranes were probed with Abs against p27(KIP1) (C-19) and CDK4 (C-22) (Santa Cruz Biotechnology), cyclin D1 (DSC-6) and active caspase-3 (5A1E) (Cell Signaling Technology), p27(KIP1)-pThr198 (R&D Systems). Equal protein loading was confirmed by analyzing β-tubulin or β-actin (Sigma) expression. Purity of cytosolic and nuclear fractions was checked by incubating membranes with anti-Grp78/BiP (Cell Signaling Technology) and anti-PARP (Roche) Abs, respectively. Membranes were incubated with horseradish peroxidase-labeled anti-mouse (Sigma),
anti-rabbit (Cell Signaling Technology) or anti-rat (Santa Cruz Biotechnology) secondary Abs. Chemiluminescence detection was done by using ECL system (Pierce) in a mini-LAS4000 (Fujifilm) or FluorSImager (Bio-Rad) device and densitometry analyses were performed with Image Gauge (Fujifilm) or QuantityOne software (Bio-Rad), respectively.

**Determination of protein half-life**

MCL cells were cultured in the presence of 50 ng/mL cycloheximide (CHX, Sigma) for 0 to 180 min and then harvested. Whole cell extracts were analyzed by SDS-PAGE as previously. The density of each band was measured with FluorSImager and QuantityOne software (Bio-Rad). P27\(^\text{KIP1}\) or cyclin D1 half-life was deduced from the semi-log curve: time/f(p27\(^{\text{KIP1}}\) or cyclin D1/β-actin or β-tubulin ratio).

**RNA interference assay**

Jeko-1 cells (10\(^7\)) were electroporated (250 V, 950 μF, Gene Pulser II, Bio Rad) with 750 nM control (sc-37007, Santa Cruz Biotechnology) or cyclin D1 siRNAs (r(UGA CCC CGC ACG AUU UCA U)d(TT); r(AUG AAA UCG UGC GGG GUC A)d(TT), Qiagen) in RPMI 1640 medium without FBS, cultured 48 h in complete medium and harvested thereafter. Whole cell lysates were obtained and analyzed by immunoblotting with anti-cyclin D1, anti-p27\(^\text{KIP1}\) and anti-β-tubulin Abs as before. Densitometry analyses were performed as before.

**Real-time PCR**

Total RNA was extracted using TRIZOL (Life Technologies) following manufacturer’s instructions. One microgram of RNA was retrotranscribed to cDNA with M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Roche). Cyclin D1 and p27\(^\text{KIP1}\) mRNA expression was analyzed in duplicate by quantitative real-time PCR on the Step
one system (Applied Biosystems) by using predesigned Assay-on-Demand probes (Applied Biosystems). The relative expression of each gene was quantified by the comparative cycle threshold (Ct) method (∆∆Ct) by using β-actin as endogenous control. Expressions levels are given using control untreated cells as a calibrator.

Xenograft mouse model and tumor phenotyping

With the use of a protocol approved by the animal testing ethical committee of the University of Barcelona, CB17-severe combined immunodeficient (SCID) mice (Charles River) were inoculated subcutaneously into their lower dorsum with the indicated MCL cell line (10^7 cells per mice) in Matrigel (1:1) (Becton Dickinson). Twenty-three days post-inoculation, animals were killed according to institutional guidelines, and tumor xenografts were extirpated. For the lenalidomide protocol, mice bearing JBR tumors were randomly assigned into cohorts of 4 mice each at day 8, when tumors were palpable. Then, animals received a daily intraperitoneal injection of 10 mg/kg lenalidomide or an equal volume of vehicle for 3 weeks. The shortest (s) and longest (l) diameters of the tumor were measured with external calipers and tumor volume (in mm³) was calculated with the use of the following standard formula: s² x l x 0.5. Tumor samples were snap-frozen in OCT medium (Sakura Tissue Tek) or formalin fixed and included in paraffin. Tissue sections were stained for p-histone H3, cyclin D1 and p27KIP1 as above, or using anti-platelet endothelial cell adhesion molecule-1 (PECAM-1, M20, Santa Cruz Biotechnology) or anti-caspase-3 cleaved (5A1E, Cell Signaling Technology) primary Abs, followed by evaluation with an Olympus DP70 microscope. Total protein extracts from cryopreserved tumor samples were obtained as previously described, (14) and analyzed by SDS-PAGE as above for p27^KIP1-pThr198 and activated caspase-3 levels.

Statistical analysis
Presented data are the means ± SD or SEM of 3 independent experiments. All statistical analyses were done by using GraphPad Prism 4.0 software (GraphPad Software). Comparison between 2 groups of samples was evaluated by nonparametric Mann–Whitney test to determine how response is affected by 2 factors. Pearson test was used to assess statistical significance of correlation. Results were considered statistically significant when \( p \) value < 0.05.

RESULTS

p27KIP1 and cyclin D1 are simultaneously expressed and interact in MCL cells

We assessed the protein levels of p27KIP1 and cyclin D1 by Western blot in a set of six MCL samples, including a blastoid case. As shown in Fig. 1A, while both cyclin D1 and p27KIP1 were present at variable levels in all the patients analyzed, the blastoid case (Pt #3) harbored higher levels of the two proteins, when compared to the conventional cases. The nuclear accumulation of the two proteins was detectable by immunohistochemistry in tumor specimens from both conventional and blastoid cases, but at increased levels in cells from the blastoid MCL patient (25 % versus 9 % cyclin D1-positive nuclei and 94 % versus 34 % p27KIP1-positive nuclei between Pt#3 and Pt#1, respectively) (Fig. 1B, supplemental Fig. S1A). We developed an immunolabelling protocol for accurate detection and quantification of these proteins by flow cytometry and observed a significant correlation \( (r=0.774, \text{ ** } p=0.009) \) between intracellular p27KIP1 and cyclin D1 protein contents in an extended series of 10 MCL primary cases (Fig. 1C, Supplemental Figure S2A). Additionally, the calculation of the ratio \( (r) \) between the mean fluorescence intensity of cyclin D1- and p27KIP1-positive cells and isotypic control-stained cells, allowed observing differences between patients with apparent similar levels of both proteins by Western blot. This could be observed between the representative patients #4 and #6 (Fig. 1D compared to Fig. 1A). Independently of the level of each protein, p27KIP1 protein was present in cyclin...
D1/CDK4 complexes in 3 representative cases (Pts #4-6, Fig. 1E), in accordance with previous studies (8). Altogether, these results confirm the existence of a physical interaction of cyclin D1 and p27\textsuperscript{KIP1} that may underlie the co-expression of both proteins in MCL primary samples, independently of the MCL subtype.

**High levels of cyclin D1 and p27\textsuperscript{KIP1} are associated with increased tumorigenicity of MCL in vivo**

Previous studies have shown that increased nuclear levels of both cyclin D1 and p27\textsuperscript{KIP1} are encountered mainly in the blastoid MCL cell lines albeit with no direct relation with the proliferation rates (8). We assessed the expression and subcellular localization of these two proteins by immunohistochemistry in a set of 6 MCL cell lines, including 2 cell lines with induced drug resistance to bortezomib (JBR and ZBR) (14). As shown in Fig. 2A, all cell lines expressed both proteins in the nucleus and the cytoplasm. The nuclear contents of cyclin D1, p27\textsuperscript{KIP1} as well as the mitotic associated antigen phospho-histone H3 revealed two main groups of tumors. The first group includes Rec-1, JBR and ZBR cells where the 3 proteins were highly expressed, mainly in the nucleus. In these cells, cyclin D1 and p27\textsuperscript{KIP1} were detected in 95 % and 76 % of the nuclei, respectively, when compared to the second group composed by Jeko-1, JVM-2 and Z-138 showing an average of 55 % cyclin D1- and 41 % p27\textsuperscript{KIP1}-positive nuclei, respectively (Fig. 2A, supplemental Fig. S1B). The interaction between cyclin D1 and p27\textsuperscript{KIP1} was similar between the two groups of cell lines, as shown in the representative Jeko-1 and JBR cell lines (Fig. 2B). To assess the possible implication in vivo of increased cyclin D1/p27\textsuperscript{KIP1} complexes, this set of cell lines was subcutaneously inoculated in immunosuppressed mice and tumor burden was evaluated after 23 days. As shown in Fig. 2C, a significant increase in tumor volume was observed in mice inoculated with the subgroup of cells with higher cyclin D1/p27\textsuperscript{KIP1} content. Representative tumor sections from Jeko-1 and JBR cells were studied by immunohistochemistry and demonstrated a remarkable nuclear
accumulation of cyclin D1 and p27^KIP1 in JBR tumors together with an increased mitotic index, as illustrated in Fig. 2D. Interestingly, this was associated with a higher tumor vascularization as shown by increased labeling of PECAM-1 (also known as CD31), in accordance with the previously reported role of cyclin D1 in tumor angiogenesis (16). Altogether, these data suggest that, by contrast with solid tumors (17;18), the overexpression of cyclin D1 in MCL cells is not associated with the cytoplasmic relocalization of p27^KIP1, and that cases with higher contents in both proteins are more susceptible to generate fast growing tumors in vivo.

p27^KIP1 is stabilized upon its binding to cyclin D1

p27^KIP1 is an instable protein which is rapidly degraded by the proteasome pathway after its dissociation from cyclin/CDK complexes (19). As reported previously in another B-cell model (12), we hypothesized that p27^KIP1 could be stabilized by its binding to cyclin D1 in MCL cells. To explore this hypothesis, Jeko-1 cells were transfected with either non-relevant, scramble siRNA or cyclin D1-specific siRNA, and levels of both proteins were assessed 48 h post-transfection. As shown in Fig. 3A, a 35 % decrease of cyclin D1 level resulted in a remarkable downregulation of p27^KIP1 level closed to 60%. In line with this, in the 4 representative cell lines Jeko-1, Rec-1, JBR, and Z-138, cyclin D1 half-life was always shorter than p27^KIP1 half-life, suggesting that the degradation of p27^KIP1 occurred after the degradation of cyclinD1 (Fig. 3B and 3C, supplemental Fig. S2B). This was confirmed in a B-cell lymphoma cell line transduced with a TAT-cyclin D1 fusion protein, where we found that the degradation of p27^KIP1 occurred after the degradation of cyclin D1 (supplemental Fig. S2C). Altogether, these data argued in favor of a direct role of cyclin D1 in p27^KIP1 protection from proteasomal degradation resulting in p27^KIP1 stabilization.

Lenalidomide disrupts cyclin D1/p27^KIP1 complexes in vitro
The inhibition of β-catenin/cyclin D1 signaling has been recently involved in the mechanism of action of the immunomodulatory drug lenalidomide in MM cells (20). To gain insights into the role of cyclin D1/p27KIP1 interaction in MCL cell proliferation and tumor growth, we assessed the capacity of lenalidomide to downregulate cyclin D1 in MCL. Rec-1 cells were treated for 72 h with 1 μmol/L lenalidomide and cyclin D1 complexes were analyzed for their content in p27KIP1 and CDK4. As shown in Fig. 4A, while the drug induced a 16 % increase in annexin V-positive apoptotic cells when compared to control condition, it also provoked a substantial decrease of cyclin D1-bound p27KIP1. No alteration in CDK4 interaction with cyclin D1 was noticed. This effect did not result from a transcriptional modulation of CCND1 and CDKN1B genes, as no significant modification of their respective transcripts could be observed by quantitative RT-PCR upon lenalidomide treatment (Fig. 4B). More interestingly, the analyses of cyclin D1 and p27KIP1 subcellular localization by dual immunofluorescence showed that, while both proteins mainly co-localized in the nuclei of Rec-1 cells in control condition, cell exposure to lenalidomide led to a decreased cyclin D1 expression, as well as a reduced p27KIP1 nuclear amount, leading to a perinuclear cytosolic redistribution of the protein (Fig. 4C, left panels). Accordingly, lenalidomide-treated cells became positive for the cytosolic, Thr198-phosphorylated form of p27KIP1 (Fig. 4C, right panels). Western blots performed with nuclear and cytosolic extracts from control and lenalidomide-treated Rec-1 cells confirmed this remarkable increase in cytosolic p27KIP1-pThr198 levels after lenalidomide treatment (Fig. 4D). Of note, this cytosolic relocation of the CDK inhibitor, together with the induction of caspase-dependent apoptosis, could represent a specific hallmark of the activity of the drug in vitro, as illustrated by the screening of proliferation and cell death markers using an antibody array and by the detection of distinct apoptotic features by multispectral imaging flow cytometry (Supplemental Fig. 3A and 3B). Altogether, these results suggest that, in in vitro settings, lenalidomide is able to overcome the nuclear sequestration of p27KIP1 by
cyclin D1, leading to cytosolic relocalization of the CDK inhibitor and consequent induction of MCL cell death.

**Antitumoral activity of lenalidomide in vivo involves cyclin D1/p27KIP1 inhibition**

In addition to its known direct pro-apoptotic activity toward MCL cells (21;22), several studies appointed for an important anti-angiogenesis activity of lenalidomide in multiple cancer models (23). As MCL tumors with high contents of cyclin D1 and p27KIP1 also presented a high level of vascularization, we next determined whether lenalidomide could target in vivo cyclin D1/p27KIP1 complexes, as well as tumor angiogenesis and progression. CB17-SCID mice were inoculated with JBR cells to generate a cyclin D1high-p27high-MCL xenograft animal model. Within one week of inoculation, animals developed palpable tumors that increased rapidly over the next two weeks (Fig. 5A). On day 8, when the tumor size reached 5 mm in diameter, mice were randomized into lenalidomide-treated (10 mg/kg) and control (vehicle) groups, receiving the corresponding administration diary, and tumor volume was calculated 23 days post-inoculation. As shown in Fig. 5A, lenalidomide therapy achieved a significant MCL tumor regression (*p<0.05), when compared to vehicle groups. At this moment, tumor size was reaching about 10% of body-weight in control mice, with no evidence of tumor-related toxicity and no differences in mean body weight between vehicle and lenalidomide groups (data not shown). Tumors isolated from control and drug-treated MCL-bearing mice revealed a 40% reduction in tumor burden in the lenalidomide-receiving group (Fig. 5B). As exemplified in Fig. 5B and 5C, a remarkable decrease in the mitotic index, together with the activation of caspase-3 and the tightening of blood vessels was observed in tumors from lenalidomide-receiving mice. Lenalidomide treatment induced a substantial decrease of cyclin D1 and p27KIP1, that was associated with the phosphorylation of the CDK inhibitor at threonine 198 (Fig. 5B and 5C). Taken together, these data confirm the in vitro observation that lenalidomide is able to impede...
the growth of MCL tumors with high cyclin D1 and p27\textsuperscript{kip1} contents, its antitumor effect being related to the cytosolic redistribution p27\textsuperscript{kip1} and subsequent apoptosis induction.

**DISCUSSION**

Current treatment strategies for MCL rely on poly-immunochemotherapy (24) that provides high overall response rates although the responses are not durable and sequential therapies are thus necessary. In this context, preclinical and clinical studies have proved that MCL is sensitive to a range of novel agents that may become useful adjuvants to standard regimens. Among them, the second generation immunodulatory compound lenalidomide, has recently demonstrated single-agent efficacy in relapsed and refractory MCL patients (25). Mechanistically, lenalidomide has been found in preclinical studies to enhance NK- and T-cell activities against tumor cells, to alter the balance of pro- and anti-inflammatory cytokines, to regulate tumor-stroma interaction, to inhibit angiogenesis, and albeit to a lesser degree, to induce cell cycle arrest and apoptosis (23). However, it is presently unclear which of these mechanisms are responsible for its clinical activity in responding MCL patients. In the present study, we show that lenalidomide antitumoral activity in \textit{in vitro} and \textit{in vivo} preclinical models of MCL involves both a tumoricidal role against malignant B cells and an anti-angiogenic activity in MCL cultures and xenografts in agreement with previous results obtained in MCL and MM models (21;22;26).

Of special interest, we demonstrate herein that the drug is able to target the interaction between cyclin D1 and its inhibitor p27\textsuperscript{kip1}. According to our results, this interaction may physiologically underlie the parallelism between p27\textsuperscript{kip1} and cyclin D1 levels, as well as in the elevated levels of both proteins in aggressive MCL subtypes. We report here that p27\textsuperscript{kip1} is detected in all MCL samples with significantly higher levels in blastoid MCL. Moreover the levels of the protein correlated with cyclin D1 in all MCL tumors and cell lines analyzed. Conversely, previous studies have reported an overall
inverse correlation between these two proteins in most neoplastic models and a low frequency of p27\textsuperscript{KIP1} expression in MCL (5-7). In these studies, the methods used for p27\textsuperscript{KIP1} detection did not distinguish tumor cells from residual normal cells and their sensitivity was remarkably weak. The presence of the CDK inhibitor in all our primary cases and its correlation with cyclin D1 expression, were confirmed by a more selective and sensitive cytofluorimetric approach in tumor cell-enriched primary samples.

Our data reveal that increased mitotic index in MCL cell lines is associated with increased tumorigenic potential \textit{in vivo}. This also suggests that, although p27\textsuperscript{KIP1} alone is not recognized as a proliferation marker in gene expression studies, elevated contents of both cyclin D1 and p27\textsuperscript{KIP1} may be used as a surrogate for the identification of aggressive MCL tumors (27-29).

While p27\textsuperscript{KIP1} is an instable protein which is rapidly degraded after its dissociation from cyclin D1/CDK complexes by the proteasome pathway (19), we and others (8) have demonstrated by co-immunoprecipitation experiments that p27\textsuperscript{KIP1} and cyclin D1 are bound in MCL cell lines in basal conditions, independently of their expression levels. The calculated half-life of cyclin D1 and p27\textsuperscript{KIP1} in all MCL cell lines showed that they were similar among the cell lines analyzed although cyclin D1 half-life was always shorter than p27\textsuperscript{KIP1} half-life. By means of a validated model of transduction of TAT-cyclin D1 fusion protein (30), we found that the presence \textit{de novo} of cyclin D1 in mature B-cell was accompanied by the upregulation of p27\textsuperscript{KIP1} and that the degradation of p27\textsuperscript{KIP1} followed that of cyclin D1. Finally, we noticed that p27\textsuperscript{KIP1} half-life was shortened in the cells in which cyclin D1 was transiently downregulated. Altogether, these data strongly suggest that, in MCL cells, p27\textsuperscript{KIP1} degradation could not occur as long as it is sequestrated by cyclin D1. Our results further explains, at least in part, the co-existence of both proteins at high levels in aggressive MCL, associated with elevated mitotic index, high tumorigenicity \textit{in vivo} and with reported drug resistance.

As we show here, lenalidomide is effective in these aggressive cases and high cyclin D1 and p27\textsuperscript{KIP1} expression may become a useful surrogate to determine the sensitivity...
in the clinical settings, although more studies are required. In this context, a recent phase I/II trial revealed a very nice overall response rate (62 %) to lenalidomide-based combination in MCL patients with failure to initial treatment (31).

In contrast to solid tumors, where nuclear cyclin D1 overexpression induces the relocalization of p27KIP1 in the cytoplasmic compartment and increases the proliferation of the malignant cells (17;18), our data indicate that both proteins colocalize in the nucleus of MCL cells with high cyclin D1 nuclear contents. We have shown a relationship between high cyclin D1/p27KIP1 levels and high proliferation index and/or aggressive MCL variants, suggesting a possible role of nuclear p27KIP1 in tumorigenesis in vivo. In this sense, the presence of, cyclin D1/p27KIP1 complexes in aggressive B-cell lymphomas was shown to result not only in an abnormal nuclear accumulation of the CDK inhibitor, but also in a loss of its inhibitory activity towards cyclin E/CDK2 complexes (32). Our results suggest that the disruption of p27KIP1/cyclin D1 complexes by lenalidomide, mainly at the nuclear level, may lead to the cytosolic accumulation of a phosphorylated, catalytically inactive form of the CKI (33). Cytoplasmic p27KIP1 has multiple roles and is involved in several cell functions, including cell motility by inhibiting the RhoA pathway and preventing stress actin fiber polymerization (17;34). Recently, cytosolic p27KIP1 has been involved in the control of microtubule stability (35), providing a biological rationale to evaluate new lenalidomide-based combination strategies with microtubule destabilizing agents. However, further studies are required to completely unravel the functions of nuclear and cytosolic p27KIP1 in MCL pathogenesis and its possible role in the tumoricidal activity of lenalidomide in this model.

Reference List


Acknowledgments

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Table 1. Characteristics of MCL patients

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1 M: male; F: female.
2 Source of the cells used in the in vitro analysis; PB, peripheral blood; LN, lymph node.
3 % of tumor cells quantified by flow cytometry.
FIGURE LEGENDS

**Fig. 1. Cyclin D1 and p27^KIP1 interact in MCL primary samples.** (A) Whole cell extracts were prepared and analyzed by SDS-PAGE. Blots were revealed with anti-p27^KIP1, anti-cyclin D1 and anti-β-actin (as loading control) antibodies. Experiments have been repeated three times, a representative one is shown. (B) Immunohistochemical staining for cyclin D1 and p27^KIP1 in consecutive sections of cell-blocks from 2 MCL patients (800X magnification). (C) Primary cells from MCL patients (n=10) were labeled with anti-cyclin D1 or anti-p27^KIP1 antibodies then with anti-rabbit IgG Alexa-Fluor 647. Isotypic control was prepared without primary antibody. Stained cells were analyzed by flow cytometry and mean fluorescence intensity ratios (r values) between p27^KIP1 or cyclin D1 positive cells and their respective isotypic controls were calculated. r values corresponding to p27^KIP1 expression were then plotted against the r values of cyclin D1 staining. Statistical analysis was performed using Pearson’s test. (D) Results from flow cytometry analysis of 2 representative patients, indicating the respective r values from cyclin D1 and p27^KIP1 staining. In gray, isotypic control curves; in black, cyclin D1 or p27^KIP1 curves. (E) Cyclin D1 complexes were immunoprecipitated (IP) from 3 representative primary MCL cultures and analyzed by SDS-PAGE then Western blots for the presence of p27^KIP1, cyclin D1 and CDK4 (as a control of immunoprecipitation) in both cyclin D1-bound (b) and -unbound (u) fractions.

**Fig. 2. Increased cyclin D1 and p27^KIP1 complexes are associated with increased tumorigenicity in vivo.** (A) Immunohistochemical validation of increased mitotic index (p-histone H3), p27^KIP1 and cyclin D1 expression in 6 MCL cell lines (800X magnification). (B) Whole cell extracts were prepared from Jeko-1 and JBR cells and immunoprecipitated with anti-cyclin D1 or anti-p27^KIP1 antibodies or rabbit non-immune serum (NS) as control. Total cell extract was analyzed in parallel (input). Proteins were separated by SDS-PAGE. (C) MCL cell lines were subcutaneously inoculated in SCID.
mice (10^7 cells per mouse) and tumor growth was recorded for 23 days. The tumor volume means from the 6 different MCL cell lines are indicated on the graph. Statistical analyses were performed using Student’s *t* test for matched pairs. (D) Immunohistochemical analysis of increased mitotic index (phospho-histone H3) and tumor angiogenesis (PECAM-1), p27^KIP1^ and cyclin D1 expression in bortezomib-resistant (JBR) vs. bortezomib-sensitive tumors (Jeko-1) (800X magnification).

**Fig. 3. Cyclin D1 stabilizes p27^KIP1^.** (A) Jeko-1 cells were transfected by electroporation with cyclin D1 siRNA and non-silencing siRNA. Knockdown of cyclin D1 proteins as well as p27^KIP1^ expression levels were checked by Western blot. β-tubulin was probed as a loading control. Ratio between β-tubulin and cyclin D1 and p27^KIP1^ levels is showed. (B) MCL cells were treated with CHX (50 μg/mL) and harvested at the times indicated. Total proteins were purified, separated on SDS-PAGE, revealed by immunoblotting with anti-p27^KIP1^, anti-cyclin D1 and anti-β-actin or anti-β-tubulin antibodies. The density of each band was measured and p27^KIP1^ or cyclin D1 half-life was deduced from the semi-log curve: time/f(p27^KIP1^/β-actin ratio) (see supplemental data). (C) The calculated half-lives were then plotted on the graph for each cell line.

**Fig. 4. Cyclin D1-mediated stabilization of p27^KIP1^ is disrupted by lenalidomide and leads to apoptotic cell death in Rec-1 cells.** (A) Protein extracts were prepared from either untreated or lenalidomide-treated (1 μmol/L for 72 h) Rec-1 cells and immunoprecipitated with anti-cyclin D1 Ab. Proteins were separated by SDS-PAGE and cyclin D1 and p27^KIP1^ protein amounts within cyclin D1-bound and unbound fractions were analyzed as previously. CDK4 was used as quality control of the immunoprecipitation. (B) RNA was isolated from Rec-1 cells treated with lenalidomide 1 μmol/L for 72h. Cyclin D1 and p27^KIP1^ mRNA levels were quantified by reverse transcription-PCR and real-time PCR using β-actin as an endogen control. (C)
Detection of cyclin D1 (red), total or phosphorylated p27^{kip1} (green) and nuclei (DAPI blue staining) by fluorescence microscopy in Rec-1 cells untreated or treated as above with lenalidomide. (D) Whole cell extracts and cytosol enriched and nucleus enriched extracts from Rec-1 treated as above were analyzed by Western blot for phosphop27^{kip1}. *: unspecific band. PARP and Grp78 were used as a loading and purity control from nucleus and cytosol, respectively.

**Fig. 5.** Lenalidomide targets cyclin D1/p27^{kip1} complexes and reduces tumor growth in MCL xenografts. (A) JBR cells (10^7 cells per mouse) were subcutaneously inoculated into the right flank of SCID mice. Tumor-bearing animals received intraperitoneal injections of 10 mg/kg lenalidomide or equal volume of vehicle. Lenalidomide has been administrated daily for 15 days. (B) The corresponding biopsies harbored several hallmarks of lenalidomide activity in JBR cells such as a decrease in mitotic index (p-Histone H3) and angiogenesis (PECAM-1), decrease in cyclin D1 levels, p27^{kip1} cytosolic accumulation and caspase-3 processing (800X magnification). (C) Western blot analysis of JBR tumor samples from control and lenalidomide-treated groups, confirming the accumulation of p27^{kip1}-pThr198 and processing of caspase-3 in lenalidomide-exposed MCL tumors.
Figure 1

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MCL pts

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B

Pt#1

Pt#3

H&E

C

p = 0.009

r = 0.774

Relative p27\text{KIP1} levels vs. Relative cyclin D1 levels

D

Pt #4

Pt #6

Pt #4

Pt #6

r = 3.4

r = 4.7

r = 3.9

r = 7.0

events
cyclin D1-Alexa fluor 647

p27\text{KIP1}-Alexa fluor 647

E

IP: cyclin D1

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WB

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Figure 2

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B

**Jeko-1**

- **IP: D1**
  - NS
  - b
  - u

- **Input: p27^{kip1}
  - NS
  - b
  - u

**JBR**

- **IP: D1**
  - NS
  - b
  - u

- **Input: p27^{kip1}
  - NS
  - b
  - u

C

**Tumor volume (mm^3 ± SD)**

- **JVM-2** (n=3)
- **Jeko-1** (n=4)
- **Z-138** (n=3)
- **Rec-1** (n=3)
- **JBR** (n=4)
- **ZBR** (n=3)

***p < 0.001

D

- **H&E**
- **p-H3**
- **PECAM-1**
- **p27^{kip1}**
- **cyclin D1**

**Jeko-1**

**JBR**
Figure 3

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B

+ CHX

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C

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Figure 4

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% of AnnexinV+ cells

- control: 9.1
- lenalidomide: 24.9

B

Relative mRNA levels

- control
- lenalidomide 1 μmol/L

C

Control and lenalidomide conditions with fluorescence images for p27KIP1, cyclin D1, DAPI, and overlays.

D

Western blot analysis for lenalidomide treatment in whole cell lysate, nucleus, and cytosol for p27KIP1-phosphoThr198*, PARP, and Grp78.
Antitumoral activity of lenalidomide in in vitro and in vivo models of mantle cell lymphoma involves the destabilization of cyclin D1/p27KIP1 complexes

Alexandra Moros Sanz, Sophie Bustany, Julie Cahu, et al.

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