A cyclin-D1 interaction with BAX underlies its oncogenic role and potential as a therapeutic target in mantle cell lymphoma

Elena Beltran1, Vicente Fresquet2, Javier Martinez-Useros3, Jose A. Richter-Larrea4, Ainara Sagardoy5, Izaskun Sesma6, Luciana L. Almada2, Santiago Montes-Moreno2, Reiner Siebert6, Stefan Gesskopf7, Maria J. Calasanz8, Raquel Malumbres6, Melissa Rieger9, Felipe Prosper3, Iziore S. Lossos9, Miguel Angel Piris4, Martin E. Fernandez-Zapico9, and Jose A. Martinez-Climent1,9

1Oncology Division, Center for Applied Medical Research, University of Navarra, 31008 Pamplona, Spain; 2Schulze Center for Novel Therapeutics, Mayo Clinic, Rochester, MN 55905; 3Molecular Pathology Program, National Cancer Research Center, 28029 Madrid, Spain; 4Institute of Human Genetics, Christian-Albrechts University Kiel and University Hospital Schleswig-Holstein, Campus Kiel, 24118 Kiel, Germany; 5Department of Genetics, University of Navarra, 31008 Pamplona, Spain; 6Department of Hematology and Cell Therapy, Clinica Universidad de Navarra, 31008 Pamplona, Spain; and 7Division of Hematology-Oncology, Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL 33136-1002

Edited* by Janet D. Rowley, University of Chicago, Chicago, IL, and approved June 21, 2011 (received for review December 29, 2010)

The chromosomal translocation t(11;14)(q13;q32) leading to cyclin-D1 overexpression plays an essential role in the development of mantle cell lymphoma (MCL), an aggressive tumor that remains incurable with current treatment strategies. Cyclin-D1 has been postulated as an effective therapeutic target, but the evaluation of this target has been hampered by our incomplete understanding of its oncogenic functions and by the lack of valid MCL murine models. To address these issues, we generated a cyclin-D1–driven mouse model in which cyclin-D1 expression can be regulated externally. These mice developed cyclin-D1–expressing lymphomas recapitulating features of human MCL. We found that cyclin-D1 inactivation was not sufficient to induce lymphoma regression in vivo; however, using a combination of in vitro and in vivo assays, we identified a novel prosurvival cyclin-D1 function in MCL cells. Specifically, we found that cyclin-D1, besides increasing cell proliferation through deregulation of the cell cycle at the G0–S transition, sequences the proapoptotic protein BAX in the cytoplasm, thereby favoring BCL2’s antiapoptotic function. Accordingly, cyclin-D1 inhibition sensitized the lymphoma cells to apoptosis through BAX release. Thus, genetic or pharmacologic targeting of cyclin-D1 combined with a prosurvival BH3 mimetic synergistically killed the cyclin-D1–expressing murine lymphomas, human MCL cell lines, and primary lymphoma cells. Our study identifies a role of cyclin-D1 in deregulating apoptosis in MCL cells, and highlights the potential benefit of simultaneously targeting cyclin-D1 and survival pathways in patients with MCL. This effective combination therapy also might be exploited in other cyclin-D1–expressing tumors.

Mantle cell lymphoma (MCL) is a distinct lymphoma entity that accounts for 6–8% of all cases of lymphoma (1, 2). MCL is thought to be derived from naïve pregerminal center B lymphocytes localized in primary follicles or in the mantle region of secondary follicles, and thus most tumors do not show somatic hypermutation of the Ig heavy-chain coding (IGH) genes. Cytologically, two major MCL subsets can be distinguished, the classical and blastoid/pleomorphic variants, which share a characteristic CD19+CD5−CD23− immunophenotype (2). Almost all MCL cases show the chromosomal translocation t(11;14)(q13;q32), which juxtaposes the CCND1 gene with IGH gene enhancers and causes overexpression of the cyclin-D1 protein. The best-known function ascribed to cyclin-D1 is in positive regulation of cell cycling. In MCL cells, constitutive cyclin-D1 activation maintains retinoblastoma (RB) protein in a phosphorylated state and promotes cell proliferation, thus likely initiating tumorigenesis (3). However, whether cyclin-D1 has additional oncogenic functions in the lymphoma cells has not been well addressed. In B lymphocytes, cyclin-D1 deregulation seems insufficient to induce neoplastic transformation, given that other genetic changes are required for the development of malignancy (3, 4). Numerous genes have been postulated as candidates that cooperate with cyclin-D1 to promote MCL development, including the cell-cycle regulators P16INK4a and CDK4; the DNA-damage sensor and repair genes ATM, TP53, and ARF; and components of the apoptotic machinery, such as BCL2L11 and BCL2 (3, 5–7). However, the functional mechanisms underlying the interplay of cyclin-D1 with these oncogenic proteins and their impact on MCL pathogenesis remain unexplored.

Clinically, there is no curative therapy for MCL; all treatment modalities, including combined immunochemotherapy and radiotherapy or intensive high-dose chemotherapy with stem cell transplantation, have failed to prevent disease recurrence and progression (8–10). In attempts to improve this poor outcome, attention has turned to novel therapies targeting specific regulatory pathways that are essential for the growth and maintenance of the transformed phenotype, some of which are currently undergoing clinical testing (8, 10, 11). However, a rigorous evaluation of the molecular targets that may be suitable for these compounds has not been conducted. Cyclin-D1, which plays a critical role in MCL development, has emerged as one of the most promising therapeutic targets, but its analysis has been hampered by the lack of useful MCL mouse models (10).

To investigate the function of cyclin-D1 in MCL development and its potential role as a therapeutic target, we generated a cyclin-D1–driven mouse model in which cyclin-D1 expression can be externally regulated. These mice developed lymphomas recapitulating some features of human MCL. Our study identifies a novel role for cyclin-D1 in deregulating apoptosis in MCL cells and highlights the potential benefits of simultaneously targeting cyclin-D1 and survival pathways in patients with MCL.

Results

Inhibition of Cyclin-D1 Has Moderate Effects on MCL Cell Growth but Enhances Sensitivity to Apoptosis. Cyclin-D1 has been postulated as a promising therapeutic target in MCL, based on its critical role in tumor development and its overexpression in virtually all


Conflict of interest statement: R.S. has received honoraria from Abbott.

This Direct Submission article had a prearranged editor.

Data deposition: Gene expression microarray data reported in this paper have been submitted to Gene Expression Omnibus (accession no. GSE25613).

1To whom correspondence should be addressed. E-mail: jmcliment@unav.es.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018941108/DCSupplemental.
cases. However, inhibition of cyclin-D1 in human MCL cell lines by siRNA resulted in a moderate effect on cell growth, leading to accumulation of cells in G1 phase of the cell cycle and to a minor increase in the apoptotic rates (Fig. S1A) (15). These data suggest that additional genetic pathways are contributing to the transformed phenotype in MCL cells. To identify the genes participating in lymphomagenesis and search for valid therapeutic targets, we developed a combined cellular-genomic-proteomics screen. In this approach, the cytotoxicity to compounds targeting cancer-related molecular pathways was tested in MCL cell lines, and was correlated with their genomic, gene expression, and proteomic profiles (Fig. S1B). Although the MCL cell lines showed variable sensitivity to the drugs included in the screening (Fig. S1C), the BH3-only mimetic ABT-737, a small molecule that binds to BCL2, BCL-XL, and BCL-W (13), was selectively effective in some MCL cell lines, whereas other remained resistant (Fig. L4 and Fig. S1D). The reduced cell viability in the sensitive cells after exposure to the BH3 mimetic was associated with a marked increase in the apoptotic rates and with cleavage of caspase 9, indicating the involvement of the intrinsic apoptotic pathway (Fig. 1B and Fig. S1E). Similarly, ABT-737–sensitive cells were killed by this drug in vivo after i.v. inoculation into immunodeficient RAG2−/−γc−/− mice (lacking B, T, and dendritic cells) (14), whereas ABT-737–resistant MCL cells were not (Fig. 1C). Accordingly, longer survival of mice carrying the ABT-737–sensitive HBL2 cells was accompanied by a reduction in lymphoma volume and attenuation of tumor lytic activity (Fig. S1 F and G). We next checked whether cyclin-D1 silencing might influence ABT-737 sensitivity. Notably, simultaneous siRNA-mediated knockdown of cyclin-D1 and ABT-737 exposure were associated with a partial reversion of tumor resistance in ABT-737–resistant MCL cell lines (Fig. 1D and Fig. S1H). These data suggest that inhibition of cyclin-D1 may functionally interact with the apoptotic machinery to facilitate ABT-737–mediated apoptosis in MCL cells.

**BCL2 Genomic Amplification and Protein Expression Determine Responses to ABT-737.** Analysis of the mechanisms underlying ABT-737 sensitivity revealed that the MCL-responsive cells were distinguished by a gene expression signature composed of 93 overexpressed genes, 13 of which (14%) mapped to chromosome bands 18q21-q22 (hypergeometric test, \( P = 6.85 \times 10^{-7} \)), including the BCL2 gene (Fig. 2A and Fig. S2A). Microarray-based comparative genomic hybridization (a-CGH) and fluorescence in situ hybridization (FISH) studies detected genomic amplification of chromosome 18q21 including the BCL2 gene locus in the four sensitive cell lines but not in any of the resistant tumors (Fig. 2B, Fig. S2B, and ref. 15). In addition, BCL2 protein was expressed in all but one of the MCL cell lines, at fivefold greater levels in those with 18q21 amplification (Fig. 2C and Fig. S2C). However, the expression of cyclin-D1 and of other proteins commonly altered in MCL and/or involved in apoptosis regulation was not correlated with ABT-737 sensitivity (Fig. 2 C and D). These data strongly indicate that BCL2 expression levels determine the response to ABT-737 in cyclin-D1–expressing MCL cells. To evaluate the clinical significance of our findings, we investigated the genomic and expression status of BCL2 in 183 primary cyclin-D1+ MCL specimens. Twenty-seven of the 183 specimens (15%) showed genomic gain or amplification of chromosome 18q21, including the BCL2 gene locus (Fig. 2E and Fig. S2D). BCL2 gain/amplification was correlated with higher levels of BCL2 protein expression, as determined by Western blot analysis (Fig. 2F). Immunohistochemistry (IHC) studies showed that almost all MCL biopsy specimens showed expression of BCL2, ranging from very low to high levels (Fig. 2G). Quantitative measurement of BCL2 expression assessed by IHC revealed that MCL cases with genomic gain/amplification of the BCL2 gene had a greater number of cells with BCL2 expression compared with nonamplified lymphomas (mean per tumor ± SEM, 13,000 ± 1,100 cells vs. 10,800 ± 540 cells; \( P = 0.05 \), Wilcoxon signed-rank test) (Fig. S2E and Table S1). Together, these data indicate that both cyclin-D1 and BCL2 are coexpressed in most patients with MCL, highlighting these molecules as potential targets for directed therapies.

**Generation of Cyclin-D1–Expressing Lymphomas in Mice.** The foregoing results prompted us to explore the putative cyclin-D1/BCL2 interaction and their role as therapeutic targets in vivo. We generated a cyclin-D1–driven tumor model in mice in which cyclin-D1...
expression could be regulated by doxycycline (Dox). For this, the human CCND1 gene was cloned into the CombTA vector (16) and stably transfected into mouse IL-3-dependent BaF3 pro-B lymphocytes, which were selected because of their similarity to the putative MCL cells of origin—naïve B lymphocytes with an active VDJ recombination program (Fig. S3A and B). In two single-cell isolated cyclin-D1-expressing clones, CyD1-1 and CyD1-4, cyclin-D1 expression could be silenced within 48 h after exposure to Dox (Fig. S3C). In vitro cyclin-D1 overexpression did not give these cells the ability to grow independently of IL-3, nor did it substantially modify cell cycle or apoptotic rates, although it did increase cell proliferation. However, i.v. inoculation of CyD1-1 and CyD1-4 cells in Rag2−/− mice did not induce tumor development (Fig. S3 D and E). We next tested whether additional genetic alterations induced by ionizing irradiation could promote transformation of cyclin-D1–expressing cells (17). Irradiated CyD1-1 and CyD1-4 cells were cultured without IL-3 and injected into immunodeficient mice. One of the CyD1-4 cell clones (obtained after irradiation with 1 Gy and hereinafter referred to as CyD1-4-1Gy) consistently developed tumors after 3–4 wk (median OS, 21 ± 4 d) (Fig. 3A). Genomic analysis of CyD1-4-1Gy cells with a-a-CHG revealed visible genomic alterations that were not present in the original CyD1-4 cells (see below), indicating that these acquired changes might have promoted cell transformation.

To investigate whether BCL2 overexpression could similarly cooperate with cyclin-D1 to transform B lymphocytes, CyD1-4 cells were transfected with the human BCL2 gene cloned in the pcDNA3.1 vector (hereinafter, CyD1-4–BCL2 cells). Injection of 2.5 × 10^4 cells into immunodeficient mice was associated with tumor development starting at week 6 (median OS, 57 ± 11 d) (Fig. 3B). Isolated cell suspensions from cyclin-D1–driven lymphomas grew independently of IL-3 and could be transplanted into secondary Rag2−/− recipients (Fig. S3F). Mouse lymphomas consistently involved bone marrow, peripheral blood, spleen and liver (Fig. S3G). Histopathologically, tumors were composed of an infiltrate of large and pleomorphic cells with a CD19+CD5+CD23+IgM+ phenotype (confirmed by flow cytometry analysis of cell suspensions) and with a high proliferative rate, shown by Ki67 index of 100% and abundant mitoses, thus resembling the blastoid/pleomorphic variant of human MCL. The lymphomas showed coexpression of cyclin-D1 and BCL2 proteins. BCL2 expression was greater in CyD1-4–BCL2 lymphomas, whereas cyclin-D1 expression was fivefold greater in CyD1-1–1Gy1 lymphomas. P53 expression was detected by IHC in 5–30% of the tumor cells (Fig. 3C, Fig. S3 H and I, and Table S2). In addition, Western blot analysis identified changes typically found in blastoid MCL, such as overexpression of CDK4, P27kip, MYC, and MCL1 proteins (Fig. 3D) (18–21). Moreover, a-CHG studies of mouse lymphomas identified genomic alterations common to human MCL, such as gains of mouse chromosomes 6 and 9q (syntetic with gains of human chromosomes 3q21, 7p11-p22.3, and 15q21-q25) and deletions of chromosome 3q (syntetic with loss of human chromosome 19q13.2). A chromosomal deletion is associated with human MCL1, which harbors PSEN (Fig. 3E and Fig. S3J) (7, 19).

In summary, cyclin-D1–driven lymphomas recapitulated some of the cellular, histopathological, and genetic features of the blastoid/pleomorphic variants of human MCL, qualifying them as valid experimental models for testing directed therapies in vivo.

**Combined Targeting of Cyclin-D1 and BCL2 Effectively Kills Lymphoma Cells.** Administering Dox to the cyclin-D1–expressing lymphoma cells in culture or to the drinking water of mice led to down-regulation of cyclin-D1 levels by >95% within 48–72 h (Fig. 4 A and B and Fig. S4A). However, mouse lymphomas showed moderate differences in growth rate, cell cycle, and apoptotic indices after Dox-induced cyclin-D1 silencing or after exposure to ABT-737 in vitro (Fig. 4 B and C and Fig. S4B). Remarkably, simultaneous cyclin-D1 silencing and ABT-737 exposure induced prominent proliferative arrest and apoptosis more efficiently than cyclin-D1 inhibition or ABT-737 treatment alone, killing the lymphoma cells synergistically (Fig. 4 C and Fig. 4 D).
B\textsuperscript{1}Gy and CyD1-4 wk (nine mice per group). (B) The expression levels of the apoptotic modulators BCL2 and BCL-XL were observed in the expression levels of the apoptotic modulators BCL2 and BCL-XL.

C30 and C210 were detected in the expression of the cyclin-D1 and BCL2 expression in mouse lymphomas ((human), Trisomy of 7p. In addition, other genomic changes that are characteristic of MCL variants, such as overexpression of CDK4, P27kip1, MYC, and MCL1 proteins. (E) Example of whole-genome aCGH analysis of a mouse lymphoma, showing genomic alterations that overlap with those observed in the blastoid/pleomorphic variants of human MCL, such as the gains of human chromosomes 3q and 7p. In addition, other genomic changes that are characteristic of MCL cells, such as the loss of chromosome 1p22 and 1q22, were identified.

S4 B and C). However, this synergy was not observed after cyclin-D1 silencing and exposure to the BH3 mimetic TW37 (22), bortezomib, or doxorubicin (Fig. S4D). In mouse carrying cyclin-D1–induced lymphomas, the combination of ABT-737 with Dox-induced cyclin-D1 inhibition was associated with better responses, including a statistically significantly longer OS and clearance of lymphoma cells detected by imaging systems compared with control mice (Fig. 4D and Fig. S5). In vivo individual cyclin-D1 or BCL2 blocking did not modify cell morphology or proliferation rate, and did not induce signs of apoptosis. In contrast, cell proliferation and apoptotic changes were visible in the tumor biopsy specimens with the use of the combined therapy (Fig. S6). These data indicate that simultaneous inhibition of cyclin-D1 and BCL2 has synergistic antitumor activity on mouse lymphomas, recapitulating our previous results in human MCL.

**Cyclin-D1 Sequestrates BAX and Inhibits ABT-737-Mediated Apoptosis.** Based on the foregoing findings, we decided to investigate the mechanisms responsible for the enhanced therapeutic efficacy of ABT-737 in MCL cells after cyclin-D1 inhibition. Consistent with its role in cell-cycle regulation, a decrease in cyclin-D1 led to decreased phosphorylation of RB and increased P27kip1 expression in mouse lymphomas (Fig. S7A). However, no apparent changes were observed in the expression levels of the apoptotic modulators Bcl2, Mc1, Bcl-xL, Bax, and Bak, among others (Fig. S7B). Notably, using flow cytometry, we found that the unbound fraction (active conformation) of BAX, a protein that functions as a final effector of the apoptotic cascades, was increased after cyclin-D1 silencing in the mouse lymphomas and in ABT-737–resistant MCL cell lines (Fig. 5A). Further analysis demonstrated that cyclin-D1 can complex with BAX in the cytoplasm of the lymphoma cells. Using immunofluorescence (IF) studies coupled with Western blot analysis of nuclear/cytoplasmic cellular protein fractions, we observed that most cyclin-D1 protein was present in the cytoplasm of both human and murine lymphomas, whereby it colocalized with BAX (Fig. 5 B and C). Accordingly, in the human MCL cell line Jeko1, the presence of BAX (but not of BAK) was detected by immunoblotting after cyclin-D1 immunoprecipitation. Likewise, in the cyclin-D1–expressing lymphomas, cyclin-D1 formed complexes with Bax, but not with BAK, Puma, Noxa, Bim, or Bad (Fig. 5D and Fig. S7C). As control for the experiment, the immunoprecipitation of a known partner of cyclin-D1, CDK4, was included (Fig. S7D). Silencing of Bax with siRNA in the lymphoma cells abrogated the therapeutic synergy between cyclin-D1 inhibition and ABT-737, indicating a prominent role of Bax in the resistance to the BH3 mimetic (Fig. S7E). These results reveal a mechanism in which the proapoptotic BAX protein is sequestered by overexpressed cyclin-D1, thereby impairing apoptosis after ABT-737 exposure. After therapeutic depletion of cyclin-D1, BAX protein is released, exposing lymphoma cells to the ABT-737 action and facilitating apoptosis.

**Pharmacologic Inhibition of Cyclin-D1 and BCL2 Is an Effective Combination for Treating Human MCL.** Our experimental data suggest that therapy with ABT-737 could be selectively active in a fraction of MCL cases with increased BCL2 expression (~15% of patients with MCL, according to Fig. 2 E–G and Fig. S2 D and E), but that with simultaneous cyclin-D1 silencing, the BH3 mimetic might be clinically effective in most patients with MCL irrespective of BCL2 expression. To begin to translate these findings to the clinical setting, we tested the therapeutic activity of the cyclin-D1/CDK inhibitor seliciclib (roscovitine), alone and in combination with ABT-737, in the human MCL cell lines and mouse lymphomas in a dose–dependent manner, increasing the unbound BAX protein fractions from 16% to 33% (Fig. 5 E and F). Thus, in the MCL cell lines as well as in the mouse lymphomas, the combination of rosuvitine and ABT-737 resulted in decreased cell survival and massive apoptosis (Fig. 5G and Table S3). However, MCL cell lines represent advanced models of disease with therapeutic responses that might not be extrapolated to patient lymphomas. Therefore, fresh peripheral blood mononuclear cells were isolated from four unselected patients diagnosed with cyclin-D1 MCL, with leukemic disease and in combination with rosuvitine and ABT-737. Responses to ABT-737 were correlated with BCL2 expression levels in three of the four cases. Nevertheless, the combination therapy of ABT-737 and rosuvitine induced marked growth retardation and massive apoptosis in all cases irrespective of BCL2 expression, with responses comparable to those observed in the human MCL cell lines (Fig. 5H). Taken together, these data demonstrate that concomitant rosuvitine and ABT-737 expression is therapeutically effective in human MCL cell lines and in primary MCL cells. Our results highlight the potential benefit of simultaneously targeting cyclin-D1 and survival pathways for the effective treatment of most cases of human MCL, in agreement with the observations in the mouse lymphoma model. On the basis of these findings, we think that the synergistic combination of rosuvitine and ABT-737 should be tested in a clinical trial with patients with MCL.

**Discussion** Here we have defined a role of cyclin-D1 in deregulating apoptosis by interacting with BAX in the cytoplasm of MCL cells that may have therapeutic implications. A key element in our work is the murine lymphoma model generated by adding secondary changes to cyclin-D1–expressing B lymphocytes that were engrafted in immunocompromised mice. Silencing of cyclin-D1 in these mice showed that cyclin-D1 inhibition did not kill the lymphoma cells, but did sensitize them to apoptosis. Our data

![Fig. 3. Characterization of cyclin-D1-expressing lymphomas in mice. (A) Injection of CyD1-4-16g cells in mice was associated with consistent development of tumors after 3–4 wk (nine mice per group). (B) CyD1-4-BCL2 cells did not grow independent of IL-3 in vitro, but induced tumor development in immunodeficient mice starting at week 6 (nine mice per group). (C) Histopathological and IHC studies of CyD1-4-16g and CyD1-4-BCL2 lymphomas revealed similar profiles to those of the pleomorphic variant of human MCL. HE, H&E staining. (D) Western blot analysis of mouse lymphomas identified common changes that are characteristic of human blastoid/pleomorphic MCL variants, such as overexpression of CDK4, P27kip1, MYC, and MCL1 proteins. (E) Example of whole-genome aCGH analysis of a mouse lymphoma, showing genomic alterations that overlap with those observed in the blastoid/pleomorphic variants of human MCL, such as the gains of human chromosomes 3q and 7p. In addition, other genomic changes that are characteristic of MCL cells, such as the loss of chromosome 1p22-21, were identified.](image-url)
indicate the potential benefit of simultaneously targeting cyclin-D1 and survival pathways in patients with MCL. The role of different oncogenes in initiating and maintaining cancer has previously been investigated in conditional transgenic mouse models. Remarkably, inactivation of some oncogenes was sufficient to rerove tumors, including BCR-ABL–induced leukemias, MYC-induced lymphomas and carcinomas, and lung carcinomas and melanomas after KRAS and HRAS inactivation.
gene alterations mainly occur in highly and 4374.
3461.
27:6738 Blood 280.
6142.
435:677 105:4445 www.pnas.org/cgi/doi/10.1073/pnas.1018941108 cDNA was ampli-
116:2531 494.
2542.
127.
We thank Drs. Saul Rosenberg and Jane Hoff-Smith 22:2097 94:3262 1476.
15. Mestre-Escorihuela C, et al. (2007) Homozygous deletions localize novel tumor sup-
138.–