

Characterisation of the novel apoptotic and therapeutic activities of the histone deacetylase inhibitor romidepsin

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Abstract

Histone deacetylase inhibitors (HDACi) are compounds that target the epigenome and cause tumor cell-selective apoptosis. A large number of these agents that have different chemical structures and can target multiple HDACs are being tested in clinical trials and vorinostat is now an approved drug for the treatment of cutaneous T-cell lymphoma. Although these agents are showing promise for the treatment of hematologic malignancies, it is possible that different drugs may have different mechanistic, biological, and therapeutic activities. When comparing an HDACi belonging to the hydroxamic acid class of compounds (vorinostat) with a cyclic tetrapeptide (romidepsin), we showed that these agents regulate the expression of a common set of cellular genes, but certain genes specifically responded to each agent. Using the E μ -myc mouse model of B-cell lymphoma, we showed previously that overexpression of the prosurvival proteins Bcl-2 and Bcl-X_L inhibited the apoptotic and therapeutic activities of the vorinostat. Herein, we compared and contrasted the apoptotic-inducing activities of the hydroxamic acid oxamflatin with romidepsin. Like vorinostat, oxamflatin was unable to kill lymphomas overexpressing Bcl-2 and Bcl-X_L, indicating that these proteins can generally protect cells against this class of HDACi. In contrast, romidepsin was able to induce

apoptosis in lymphomas overexpressing Bcl-2 with delayed kinetics of cell death and could mediate therapeutic responses against these lymphomas. However, romidepsin was inactive when Bcl-X_L was overexpressed. These data provide strong support that HDACi of different chemical classes may have subtle yet potentially important differences in their molecular and biological activities. [Mol Cancer Ther 2008;7(5):1066–79]

Introduction

Histone deacetylase inhibitors (HDACi) are an exciting new class of anticancer agents that are showing great promise in clinical trials especially for the treatment of hematologic malignancies (1–3). At least 12 different HDACi have been or are presently being tested in clinical trials (2, 3). In general, these agents are well tolerated, and even in early phase trials, clinical responses have been observed in patients with leukemias and lymphomas, and even some solid tumors such as mesothelioma, prostate carcinoma, and non-small cell lung carcinoma have responded to treatment (2, 3). HDACi can elicit a range of biological responses including induction of cell death via apoptosis, autophagy, or necrosis; inhibition of cell proliferation by activating cell cycle progression at G₁-S or S-G₂-M checkpoints; induction of cellular differentiation; modulation of immune responses; and suppression of angiogenesis (1). It is possible that more than one of these effects may be required for the observed therapeutic responses to HDACi. The canonical view of HDACi activity was that these agents inhibited the activity of class I (HDACs 1–3 and 8), class II (HDACs 4–7, 9, and 10), and class IV (HDAC11) HDACs causing chromatin remodeling and altered gene expression, resulting in the various biological effects that are deleterious to tumor cell growth and survival (4). However, it was soon realized that the HDACs can target substrates other than histones and that the post-translational modification of other cellular proteins via acetylation may indeed play an important role in the biological activities of HDACi (5).

A range of naturally occurring or synthetic HDACi have been tested for their anticancer activities in preclinical studies. Six broad classes of HDACi have been defined based on the chemical structures (1). These include the short-chain fatty acids (butyrate and valproic acid), hydroxamates [suberoylanilide hydroxamic acid (vorinostat), trichostatin A, LBH589, oxamflatin, and PDX101], benzamides (MS-275, CI-994, and MGCD-0103), cyclic tetrapeptides [depsipeptide (romidepsin), trapoxin A, and apicidin], electrophilic ketones (trifluoromethylketone), and miscellaneous (depudecin). It is apparent that not all HDACi have the same specificity or affinity for the

Received 11/4/07; revised 2/5/08; accepted 2/21/08.

Grant support: R.W. Johnstone is a Pfizer Australia research fellow and is supported by National Health and Medical Research Council program grant 251608, Cancer Council Victoria, and Leukemia Foundation of Australia.

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doi:10.1158/1535-7163.MCT-07-2256

11 different target HDACs (1). Accordingly, some HDACi such as vorinostat, LBH589, and PXD101 are broadly acting and likely inhibit most if not all class I, II, and IV HDACs (1). By comparison, other HDACi are more selective inhibitors. For example, romidepsin is reported to more potently inhibit HDACs 1 and 2 than HDACs 4 and 6 (6), whereas tubacin selectively inhibits HDAC6 and does not mediate histone hyperacetylation (7).

It is not yet known if targeting a single specific HDAC is sufficient to mediate a given antitumor response and/or if this will depend on the cell type. Moreover, as crystal structures for all mammalian class I, II, and IV HDACs are not available, molecular modeling to predict the selective inhibitory activity of a given compound against target HDACs has not been possible. As such, empirical screens are necessary at this stage to compare and contrast the activities of different HDACi. We have established a model system using primary B-cell lymphomas from E μ -myc transgenic mice to identify the proteins and pathways necessary for anticancer agents to induce apoptosis and elicit therapeutic responses (8). Using this system, we determined that the HDACi vorinostat mediated apoptosis of E μ -myc lymphomas via the intrinsic apoptotic pathway and used the BH3-only proteins Bim and Bid to mediate this effect (8). Moreover, we showed that overexpression of the pro-survival protein Bcl-2, which inhibits activation of the intrinsic apoptotic pathway, completely suppressed the apoptotic and therapeutic activities of vorinostat. Herein, we utilize this system to compare and contrast the activities of structurally diverse HDACi. Consistent with our previously published results using vorinostat (8), we showed that overexpression of Bcl-2 inhibited the apoptotic activity of the hydroxamate-based HDACi oxamflatin. In contrast, we found that the cyclic tetrapeptide romidepsin had the unique ability to kill E μ -myc lymphomas that overexpress Bcl-2, although with extended kinetics compared with that seen with E μ -myc lymphomas. Moreover, unlike vorinostat, romidepsin was able to induce a therapeutic benefit against E μ -myc lymphomas overexpressing Bcl-2. However, we also determined that the expression of endogenous Bcl-X_L could determine relative resistance of E μ -myc/Bcl-2 lymphomas to romidepsin, and romidepsin was unable to kill E μ -myc cells that were transduced to overexpress Bcl-X_L. We conclude that HDACi that belong to different structural classes can have subtle yet potentially important differences in activity that may be exploited clinically to stratify patients for treatment using these different agents.

Materials and Methods

E μ -myc Lymphomas, Cell Culture, and Reagents

E μ -myc, E μ -myc/Bcl-2, and E μ -myc/Bcl-X_L lymphomas were developed as described previously (8) and cultured in six-well plates (Greiner Bio-One) in high-glucose DMEM supplemented with 10% FCS, penicillin/streptomycin, 0.1 mmol/L L-asparagine, and 50 μ mol/L 2-mercaptoethanol. HDACi were dissolved in DMSO for the prepara-

tion of stock solutions (10 mmol/L). Romidepsin was kindly provided by Gloucester Pharmaceuticals and oxamflatin was produced by Dr. Anthony Dear. Tetramethylrhodamine ethyl ester was purchased from Molecular Probes.

Western Blot Analysis

E μ -myc lymphoma cells were lysed in lysis buffer [0.15 mol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 1% Triton X-100] supplemented with protease inhibitors (leupeptin, pepstatin, and phenylmethylsulfonyl fluoride; Sigma-Aldrich) as described previously (8). Proteins (30–50 μ g) were separated on 10% or 15% SDS-polyacrylamide gels electrophoretically transferred onto Immobilon-P nylon membranes (Millipore). Membranes were incubated with the following antibodies: anti-mouse Bcl-2 (BD PharMingen), anti-mouse Bcl-X_L (BD PharMingen), anti-mouse Bcl-w (Chemicon Australia), anti-mouse Mcl-1 (Rockland), anti-mouse A1 (Sapphire Biosciences), anti-Flag tag (Sigma-Aldrich), anti-acetylated histone H3 and anti-acetylated histone H4 (Upstate Biosystems), anti- β actin (Sigma-Aldrich), and anti-tubulin (Sigma-Aldrich) overnight at 4°C followed by subsequent incubation with horseradish peroxidase-conjugated secondary antibodies (DAKO). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham).

In vitro Cell Death Analysis

E μ -myc lymphoma cells (5×10^5 /mL) were incubated in the presence of the indicated compounds for 20 h in 1 mL cell culture medium in 24-well plates (Greiner Bio-One). Viability of cells as measured by trypan blue exclusion assay, propidium iodide uptake, Annexin V staining, cell cycle analysis, or tetramethylrhodamine ethyl ester staining were done as described (8).

Mice

C57BL/6 mice (6–8 weeks old) were used for *in vivo* apoptosis assays and therapy studies. The Peter MacCallum Cancer Centre Animal Ethics Committee approved all mouse protocols used in this study. PCR-based genotyping and Western blotting analysis were used to validate lymphoma genotypes (data not shown).

In vivo Apoptosis and Therapy Assays

For *in vivo* apoptosis assays, C57BL/6 mice were injected with E μ -myc lymphomas (5×10^5 cells per animal) and after 10 to 15 days on which lymph nodes became well-palpable romidepsin (5.6 mg/kg) was administered *i.v.* After the indicated time points, mice were sacrificed and cells were harvested from brachial lymph nodes for fluorescence-activated cell sorting-based assays to measure apoptotic signaling (8).

To assess therapeutic efficacy of romidepsin, C57BL/6 mice were injected with E μ -myc lymphomas of the indicated genotypes *i.v.* (5×10^5 cells per animal). Peripheral WBC counts were then monitored until they exceeded $13 \times 10^3/\mu$ L (Sysmex Hematology Analyzer K-1000) and romidepsin was administered at 5.6 mg/kg *i.v.* once every 4 days for a total of four injections. Previously, we had determined that this regimen represented the maximum tolerated dose in lymphoma-bearing

mice (data not shown). Mice in the control cohort received the corresponding amount of vehicle. Cohorts consisted of 8 to 11 mice each, 2 to 3 independently derived lymphomas per genotype. Peripheral WBC counts and body weights were recorded weekly. On signs of major distress or when lymphomas were relapsing as indicated by enlarged brachioaxial lymph nodes, mice were euthanized and a necropsy was done. For analysis of therapeutic efficacy, tumor-induced mortality "events" were recorded. Kaplan-Meier analysis was done and comparisons made using the log-rank (Mantel-Cox) test (MedCalc software version 8.0.2.0).

Results

Romidepsin Can Overcome the Antiapoptotic Effects of Bcl-2 *In vitro*

We and others have determined previously that overexpression of prosurvival Bcl-2 proteins can inhibit the apoptotic activity of HDACi *in vitro* using human tumor cell lines (9–13). Moreover, using E μ -myc lymphomas that had been retrovirally transduced with control vector (MSCV) or with a vector encoding murine Bcl-2, we showed that overexpression of Bcl-2 conferred resistance to the apoptotic and therapeutic effects of vorinostat (8). Three different primary E μ -myc lymphomas overexpressing Bcl-2 and control vector-transduced E μ -myc cells were tested for sensitivity to the HDACi oxamflatin and romidepsin. Both agents could effectively kill E μ -myc but not E μ -myc/Bcl-2 lymphomas in a 24-h dose-response assay as assessed by outer cell membrane damage (Fig. 1A-F) and loss of mitochondrial membrane potential (Fig. 1B).

To determine if the HDACi-inhibitory effects of Bcl-2 were long lasting, we did a time course experiment using doses of oxamflatin (0.1 μ mol/L) and romidepsin (3.0 nmol/L) that were sufficient to kill E μ -myc lymphomas in 24 h. Overexpression of Bcl-2 conferred resistance to oxamflatin even following 72 h of continuous exposure of the cells to this HDACi (Fig. 2A). In contrast, romidepsin could kill two of the four E μ -myc/Bcl-2 lymphomas (4242E μ -myc/Bcl-2 and 229E μ -myc/Bcl-2) over time, whereas another two independently derived E μ -myc/Bcl-2 lymphomas (102E μ -myc/Bcl-2 and 226E μ -myc/Bcl-2) remained relatively insensitive to romidepsin. The primary function of prosurvival Bcl-2 proteins is to inhibit the activity of Bak and Bax proteins and thereby protect the mitochondrial membrane from damage (14). We therefore wished to determine if the induction of apoptosis mediated by romidepsin in 4242E μ -myc/Bcl-2 and 229E μ -myc/Bcl-2 lymphomas was via perturbation of the mitochondrial membrane or through some other mechanism. We quantitated HDACi-induced mitochondrial outer membrane permeabilization (MOMP) by staining with tetramethylrhodamine ethyl ester. Consistent with the data shown in Fig. 2A, oxamflatin and romidepsin induced robust MOMP in all four E μ -myc/MSCV lymphomas following 24-h treatment that increased over time (Fig. 2B). However,

oxamflatin did not mediate any substantial change in MOMP in any of the E μ -myc lymphomas that overexpress Bcl-2. In contrast and consistent with the data shown in Fig. 2A, romidepsin induced MOMP in 4242E μ -myc/Bcl-2 and 229E μ -myc/Bcl-2 and this effect was greatly attenuated or completely lost in the 226E μ -myc/Bcl-2 and 102E μ -myc/Bcl-2 lymphomas.

We have shown previously that cells resistant to HDACi-induced apoptosis through overexpression of prosurvival Bcl-2 proteins undergo arrest in either the G₁ (8) or G₂-M phases of the cell cycle following HDACi treatment depending on the cell type (10, 12). We therefore assessed the cell cycle profiles of E μ -myc/Bcl-2 lymphomas treated with oxamflatin and romidepsin over 3 days. Treatment of 226E μ -myc/Bcl-2 (Supplementary Table S1)⁵ or 102E μ -myc/Bcl-2 (data not shown) lymphomas with oxamflatin or romidepsin over 3 days resulted in a decrease in the percentage of cells in S phase and increase of cells in G₁ (Supplementary Table S1).⁵ Using loss of 2n DNA content (sub-G₁) as readout for DNA fragmentation and thus apoptosis, neither oxamflatin nor romidepsin induced substantial cell death even following 3 days of continuous exposure to replenished agent. Similar results were seen when 4242E μ -myc/Bcl-2 (Supplementary Table S1)⁵ and 229E μ -myc/Bcl-2 (data not shown) lymphomas were treated with oxamflatin. In contrast, treatment of 4242E μ -myc/Bcl-2 (Supplementary Table S1)⁵ and 229E μ -myc/Bcl-2 (data not shown) lymphomas with romidepsin resulted in an increase in the percentage of cells showing DNA fragmentation indicative of apoptosis. Taken together, these data show that overexpression of Bcl-2 robustly inhibits the apoptotic activities of the hydroxamate-based HDACi oxamflatin. In contrast, two of the E μ -myc/Bcl-2 lymphomas that were completely resistant to oxamflatin-induced apoptosis were sensitive to romidepsin-mediated apoptosis following >24-h exposure to drug.

To ensure that romidepsin and oxamflatin induced equivalent histone hyperacetylation at doses of each compound that could kill E μ -myc lymphomas, we did Western blot analysis to assess the acetylation status of histones H3 and H4. As shown in Fig. 2C, treatment of 4242E μ -myc lymphomas with 3.0 nmol/L romidepsin and 0.1 mmol/L oxamflatin induced equivalent acetylation of histones H3 and H4 over a 24-h time course. Moreover, addition of 3.0 nmol/L romidepsin to 4242E μ -myc/Bcl-2 and 226E μ -myc/Bcl-2 lymphomas resulted in an equivalent increase in histone acetylation in a time-dependent manner. These data indicate that the differential sensitivity of 4242E μ -myc/Bcl-2 and 226E μ -myc/Bcl-2 to romidepsin is not related to variations in HDACi-inhibitory activity of the compound in lymphomas that are relatively resistant or sensitive to romidepsin-induced apoptosis.

⁵ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

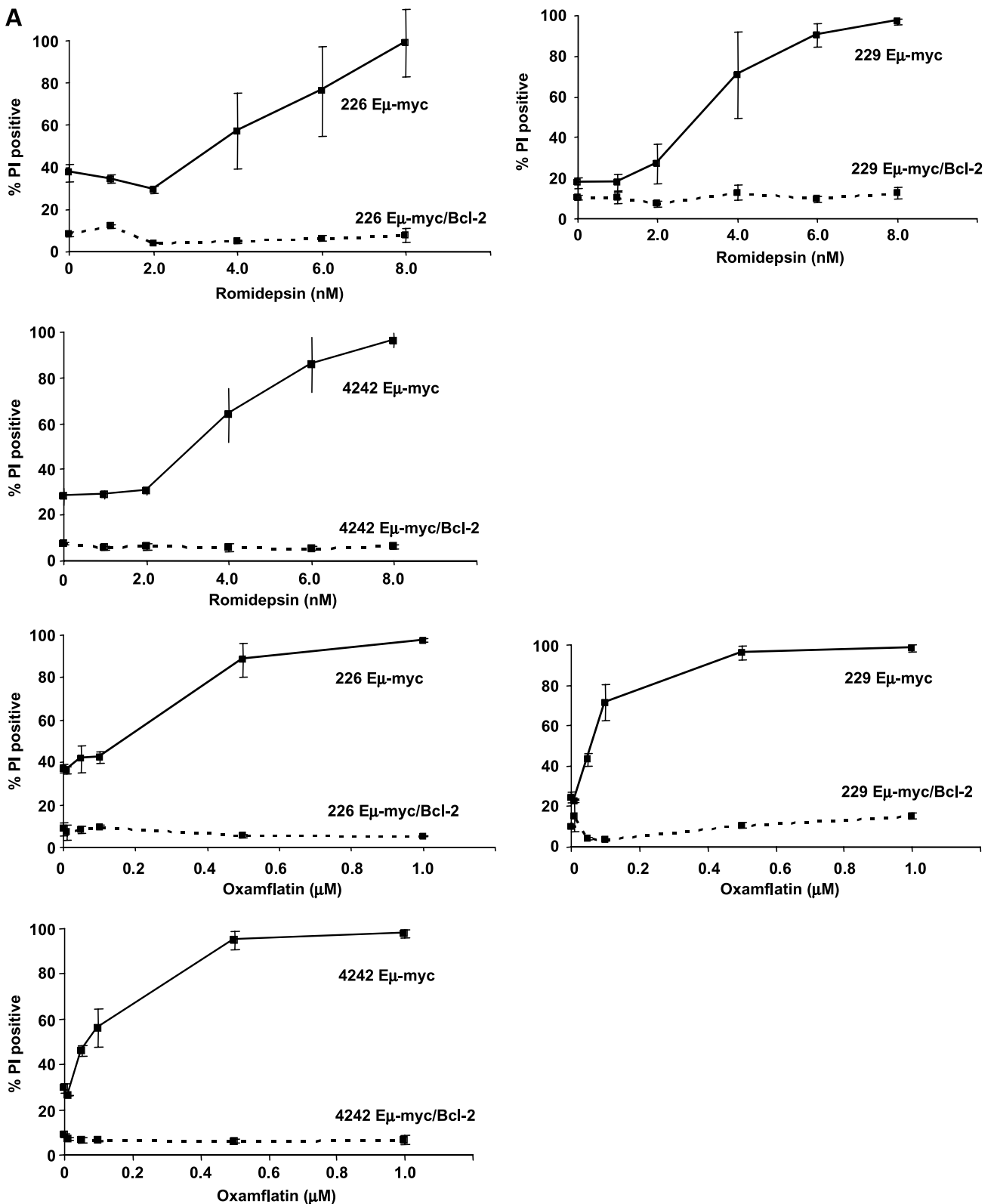


Figure 1. Eμ-myc lymphomas overexpressing Bcl2 are resistant to romidepsin *in vitro* in short-term assays. 4242Eμ-myc, 4242Eμ-myc/Bcl-2, 229Eμ-myc, 229Eμ-myc/Bcl-2, 226Eμ-myc, and 226Eμ-myc/Bcl-2 lymphomas were incubated with the indicated concentrations of romidepsin or oxamflatin for 24 h. Cell viability was assessed by (A) propidium iodide staining and (B) loss of MOMP. Bars, SE of at least three independent experiments.

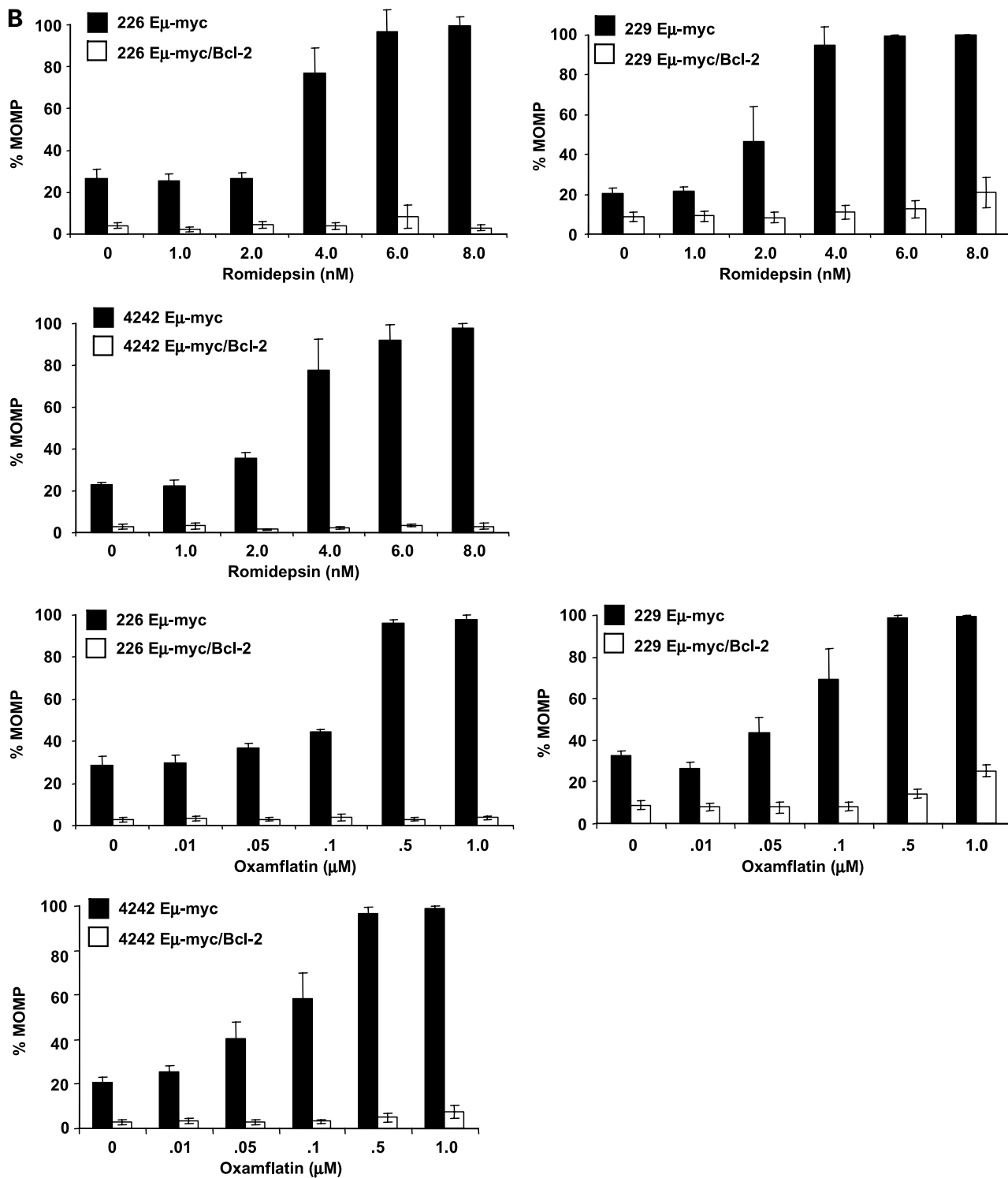


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Apoptotic and Therapeutic Activity of Romidepsin against Eμ-myc and Eμ-myc/Bcl-2 Lymphomas *In vivo*

Our *in vitro* data indicated that romidepsin was capable of rapidly killing Eμ-myc lymphomas and could kill 229Eμ-

myc/Bcl-2 and 4242Eμ-myc/Bcl-2 lymphomas over time but could not kill 226Eμ-myc/Bcl-2 or 102Eμ-myc/Bcl-2 lymphomas. We therefore sought to determine if similar results were observed *in vivo*. For this purpose, we did

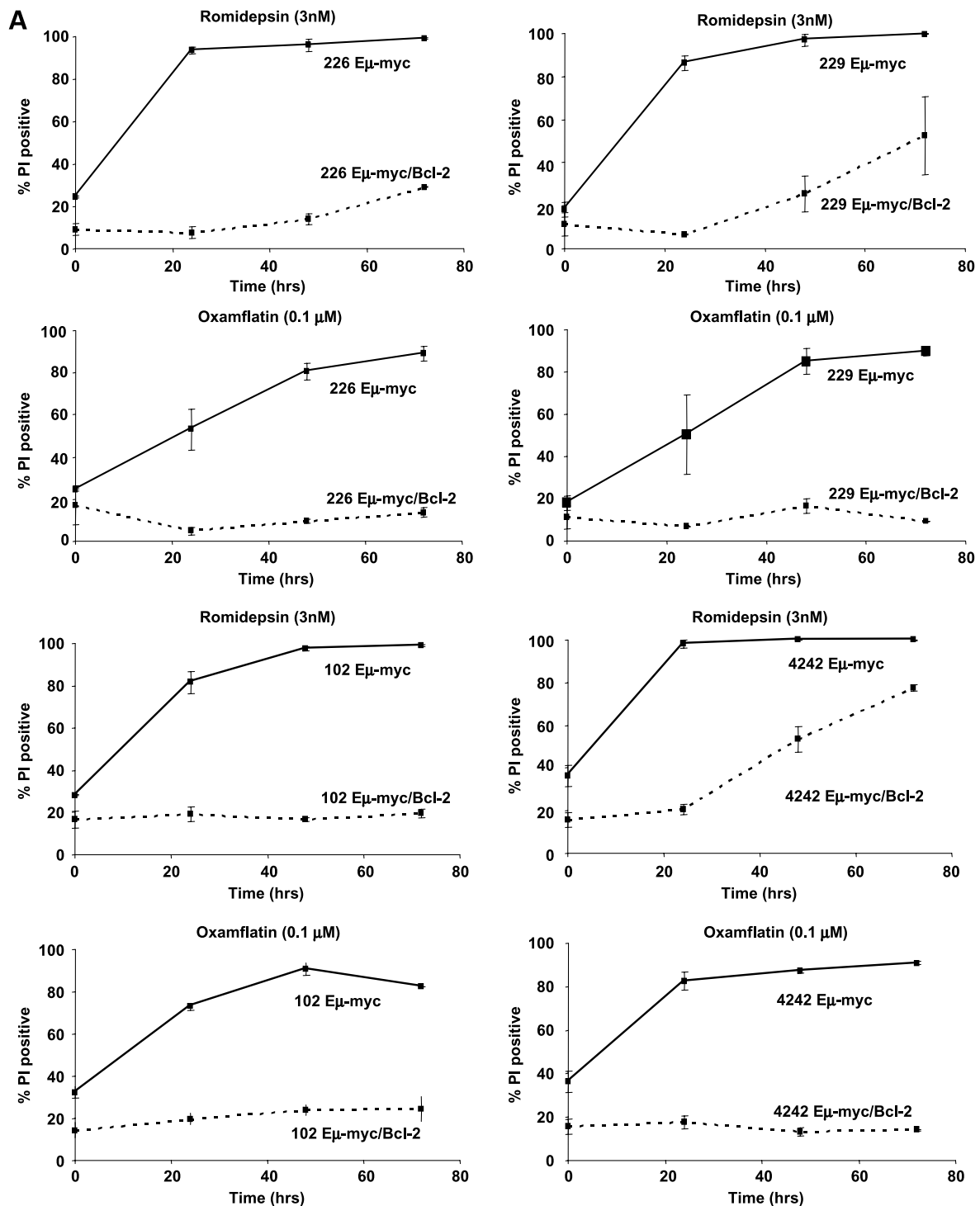


Figure 2. Romidepsin can kill E μ -myc/Bcl2 lymphomas over time. 4242E μ -myc, 4242E μ -myc/Bcl-2, 229E μ -myc, 229E μ -myc/Bcl-2, 226E μ -myc, 226E μ -myc/Bcl-2, 102E μ -myc, and 102E μ -myc/Bcl-2 lymphomas were incubated for up to 72 h with the concentration of HDACi required to kill ~70% of E μ -myc lymphomas following 24-h treatment (3 nmol/L romidepsin or 0.1 μ mol/L oxamflatin). Cell viability was assessed by (A) propidium iodide staining and (B) loss of MOMP. Bars, SE of at least three independent experiments. C, 4242E μ -myc cells were treated with 3.0 nmol/L romidepsin, 0.1 μ mol/L oxamflatin or vehicle (lanes 7-9) for 2 h (lanes 1, 4, and 7), 8 h (lanes 2, 5, and 8), and 24 h (lanes 3, 6, and 9). Whole-cell lysates were used for Western blot analysis using antibodies specific for acetylated histones H3 and H4. Blots were reprobbed with anti-tubulin polyclonal antibody to assess protein loading. D, 4242E μ -myc/Bcl-2 and 226E μ -myc/Bcl-2 cells were treated with 3.0 nmol/L romidepsin for 2 h (lanes 1 and 4), 8 h (lanes 2 and 5), and 24 h or vehicle for 24 h (lanes 7 and 8). Whole-cell lysates were used for Western blot analysis using antibodies specific for acetylated histones H3 and H4. Blots were reprobbed with anti- β actin polyclonal antibody to assess protein loading.

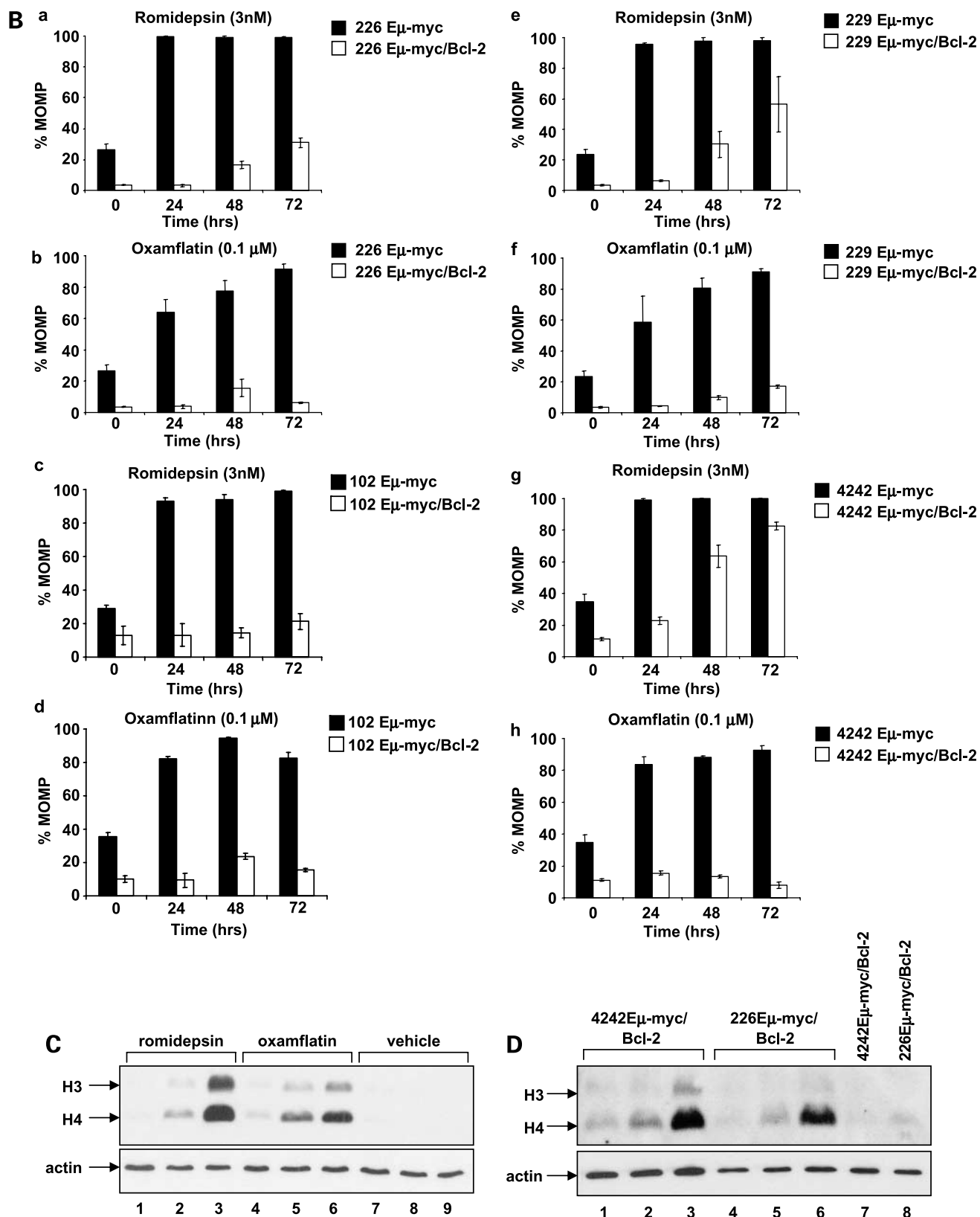


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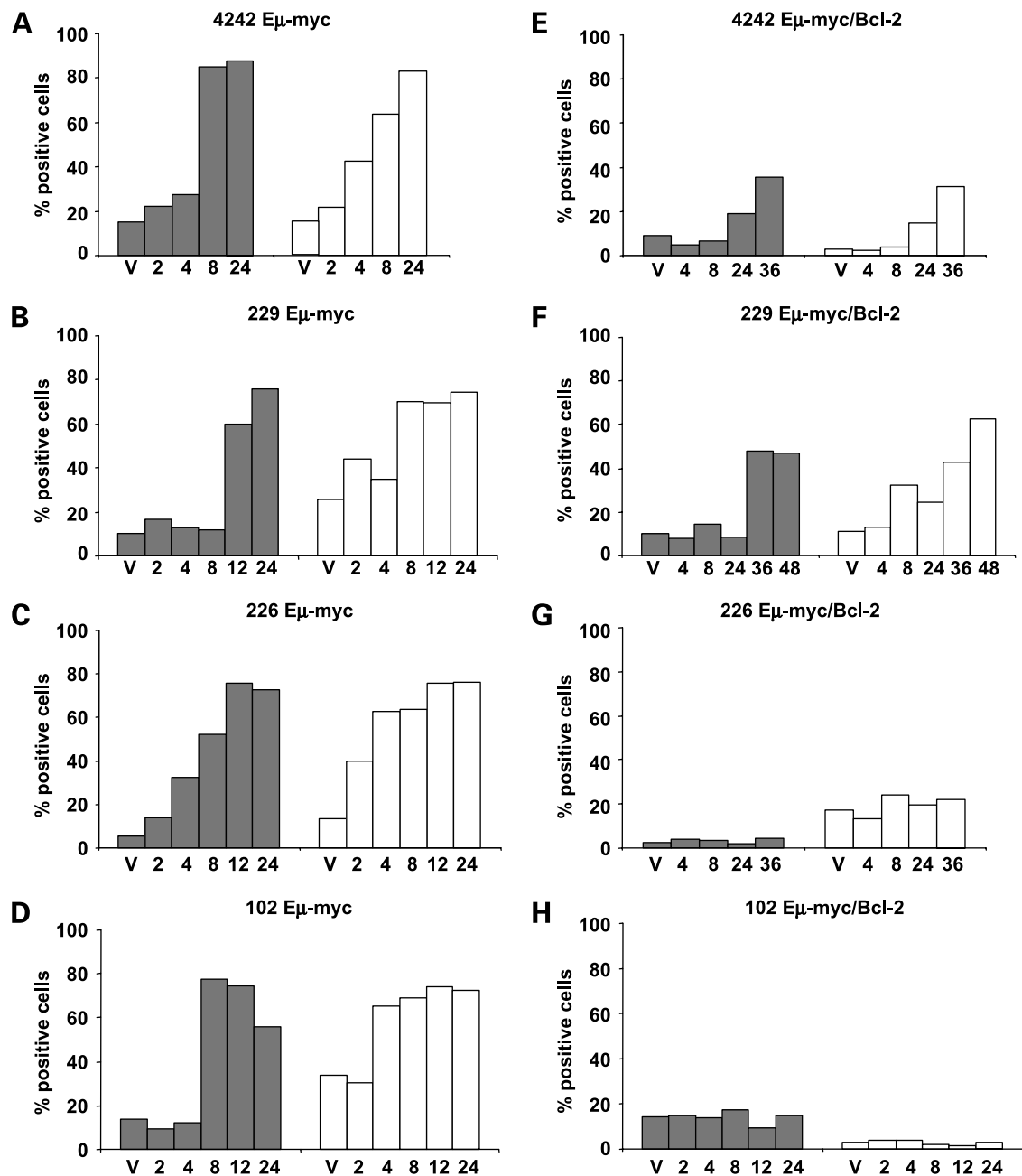


Figure 3. Romidepsin can kill E μ -myc/Bcl2 lymphomas *in vivo*. C57BL/6 mice bearing (A) 4242E μ -myc, (B) 229E μ -myc, (C) 226E μ -myc, (D) 102E μ -myc, (E) 4242E μ -myc/Bcl-2, (F) 229E μ -myc/Bcl-2, (G) 226E μ -myc/Bcl-2, and (H) 102E μ -myc/Bcl-2 lymphomas were injected with romidepsin (5.6 mg/kg i.v.) or vehicle. Lymphoma cells were harvested at the time points (hours) indicated following romidepsin treatment or 24 h following vehicle treatment (v). Apoptosis was measured by either Fluorogold staining for outer cell membrane permeabilization (gray columns) or DNA fragmentation (white columns).

in vivo apoptosis assays that involved treatment of lymphoma-bearing mice with romidepsin, harvesting of tumors over time, and assessment of apoptosis using fluorescence-activated cell sorting-based assays. All four E μ -myc lymphomas grown in the lymph nodes of C57BL/6 mice were sensitive to romidepsin with an increase in apoptotic cells over background detected at 8 to 12 h following addition of romidepsin (Fig. 3A-D). The

percentage of apoptosis increased over the 24-h time course using readouts for outer cell membrane damage and DNA fragmentation (Fig. 3A-D). Consistent with the results seen *in vitro*, all four E μ -myc/Bcl-2 lymphomas were resistant to romidepsin-induced apoptosis 24 h after exposure to the HDACi (Fig. 3E-H). The 226E μ -myc/Bcl-2 and 102E μ -myc/Bcl-2 lymphomas remained insensitive to romidepsin-induced apoptosis *in vivo*, even at the 36 and 48 h time

points, respectively (Fig. 3G and H). However, consistent with our *in vitro* data, 4242E μ -myc/Bcl-2 (Fig. 3E) and 229E μ -myc/Bcl-2 (Fig. 3F) lymphomas did undergo apoptosis at later time points following exposure to romidepsin, although as with the *in vitro* assays the level of apoptosis achieved in these Bcl-2-overexpressing lymphomas at most time points was substantially less than that observed in the parental E μ -myc lymphomas.

We next assessed the therapeutic effects of romidepsin against E μ -myc and E μ -myc/Bcl-2 lymphomas to determine if the induction of apoptosis by romidepsin *in vivo* translated into a therapeutic benefit. For therapy experiments, E μ -myc lymphomas were transplanted into C57BL/6 mice and treatment with romidepsin or vehicle commenced when WBC counts in the peripheral blood reached a pathologic threshold ($>13 \times 10^3/\mu\text{L}$). The survival of mice bearing E μ -myc lymphomas treated with romidepsin was significantly extended compared with vehicle-treated mice (Fig. 4A-D). Interestingly, romidepsin also significantly extended the survival of mice bearing 229E μ -myc/Bcl-2 and 4242E μ -myc/Bcl-2 lymphomas but provided little or no therapeutic benefit in mice bearing 102E μ -myc/Bcl-2 or 226E μ -myc/Bcl-2 lymphomas (Fig. 4E-H).

Enhanced Expression of Bcl-X_L in 226E μ -myc/Bcl-2 and 102E μ -myc/Bcl-2 Lymphomas Correlates with Resistance to Romidepsin-Induced Apoptosis

To determine why 226E μ -myc/Bcl-2 and 102E μ -myc/Bcl-2 lymphomas remain resistant to romidepsin-induced apoptosis compared with 229E μ -myc/Bcl-2 and 4242E μ -myc/Bcl-2 lymphomas, we assessed the expression of prosurvival Bcl-2 proteins in the cells. All cells overexpressed approximately equivalent amounts of exogenous Bcl-2 (Fig. 5A). We next assessed the endogenous expression of prosurvival Bcl-2 family members in these lymphomas (Fig. 5B). The expression of Bcl-w, Mcl-1, and A1 was approximately equivalent in all E μ -myc/Bcl-2 lymphomas. In contrast, the levels of Bcl-X_L were significantly higher in 226E μ -myc/Bcl-2 and 102E μ -myc/Bcl-2 lymphomas compared with 229E μ -myc/Bcl-2 and 4242E μ -myc/Bcl-2 lymphomas.

To determine if increased expression of Bcl-X_L could confer resistance to romidepsin, we developed 4242E μ -myc/Bcl-X_L lymphomas and tested these for sensitivity to HDACi. Treatment of 4242E μ -myc and 4242E μ -myc/Bcl-X_L lymphomas with increasing concentrations of romidepsin or oxamflatin over 24 h resulted in dose-dependent loss of plasma membrane integrity and mitochondrial function in 4242E μ -myc lymphomas, whereas 4242E μ -myc/Bcl-X_L lymphomas were unaffected (Fig. 6A and B). Moreover, cell cycle analysis revealed that DNA fragmentation occurred in E μ -myc lymphomas in response to increasing doses of oxamflatin and romidepsin, whereas E μ -myc/Bcl-X_L lymphomas arrested in the G₁ phase of the cell cycle (data not shown). Similar results were seen using 102E μ -myc/Bcl-X_L and 229E μ -myc/Bcl-X_L lymphomas (data not shown).

Treatment of 4242E μ -myc/Bcl-X_L lymphomas with romidepsin or oxamflatin over a 72-h time course resulted

in little or no outer cell membrane permeabilization nor any significant decrease in mitochondrial membrane potential (Fig. 6C and D). In contrast, parental E μ -myc lymphomas were effectively killed by romidepsin and oxamflatin within the first 24 h (Fig. 6C and D). Similar results were observed using 102E μ -myc/Bcl-X_L and 229E μ -myc/Bcl-X_L lymphomas (data not shown).

Discussion

HDACi are showing promise in the clinic as anticancer agents, particularly for the treatment of hematologic malignancies (1–3). A large number of HDACi have been synthesized or purified from natural sources and there are clear differences in the chemical composition of the different compounds and their relative affinities for different class I, II, and IV HDACs (1, 15, 16). Although HDACi have variously been shown to induce tumor cell apoptosis, inhibit cell cycle progression, augment antitumor immune responses, or suppress angiogenesis depending on the tumor cell type, concentration of HDACi used, or cellular context of the experiment, very few comparative studies aimed at identifying different molecular or biological activities of structurally diverse HDACi have been done to identify subtle yet potentially important unique activities of the different compounds.

Herein, we have directly compared the apoptotic activities of the cyclic tetrapeptide romidepsin with the hydroxamic acid oxamflatin using primary lymphomas derived from E μ -myc transgenic mice. The ability to genetically manipulate E μ -myc lymphomas through the use of retroviral gene transduction and perform both *in vitro* and *in vivo* studies on independently derived primary tumors makes this an ideal preclinical system to identify proteins and molecular pathways that may be important for HDACi activity. Using this system, we showed previously that the hydroxamic acid vorinostat mediated E μ -myc lymphoma cell apoptosis via the intrinsic apoptotic pathway and overexpression of either Bcl-2 or Bcl-X_L rendered E μ -myc cells resistant to the apoptotic and therapeutic activities of vorinostat (8). We showed herein that overexpression of Bcl-2 similarly protects E μ -myc lymphomas against apoptosis induced by oxamflatin even following 72-h continuous exposure to the compound. In contrast, we identified E μ -myc lymphomas overexpressing Bcl-2 that were resistant to apoptosis induced by oxamflatin (shown here) or vorinostat (8) but were sensitive to romidepsin-induced apoptosis following extended (that is, >24 h) exposure to the compound. Importantly, we showed that these lymphomas were sensitive to romidepsin *in vivo* and the compound retained therapeutic efficacy against these cells. However, we also identified E μ -myc/Bcl-2 lymphomas that were insensitive to romidepsin-induced apoptosis *in vitro* and *in vivo*. One apparent difference between the romidepsin-resistant and romidepsin-sensitive E μ -myc/Bcl-2 lymphomas was the relative expression of Bcl-X_L and we showed that overexpression of Bcl-X_L conferred resistance to romidepsin in all lymphomas

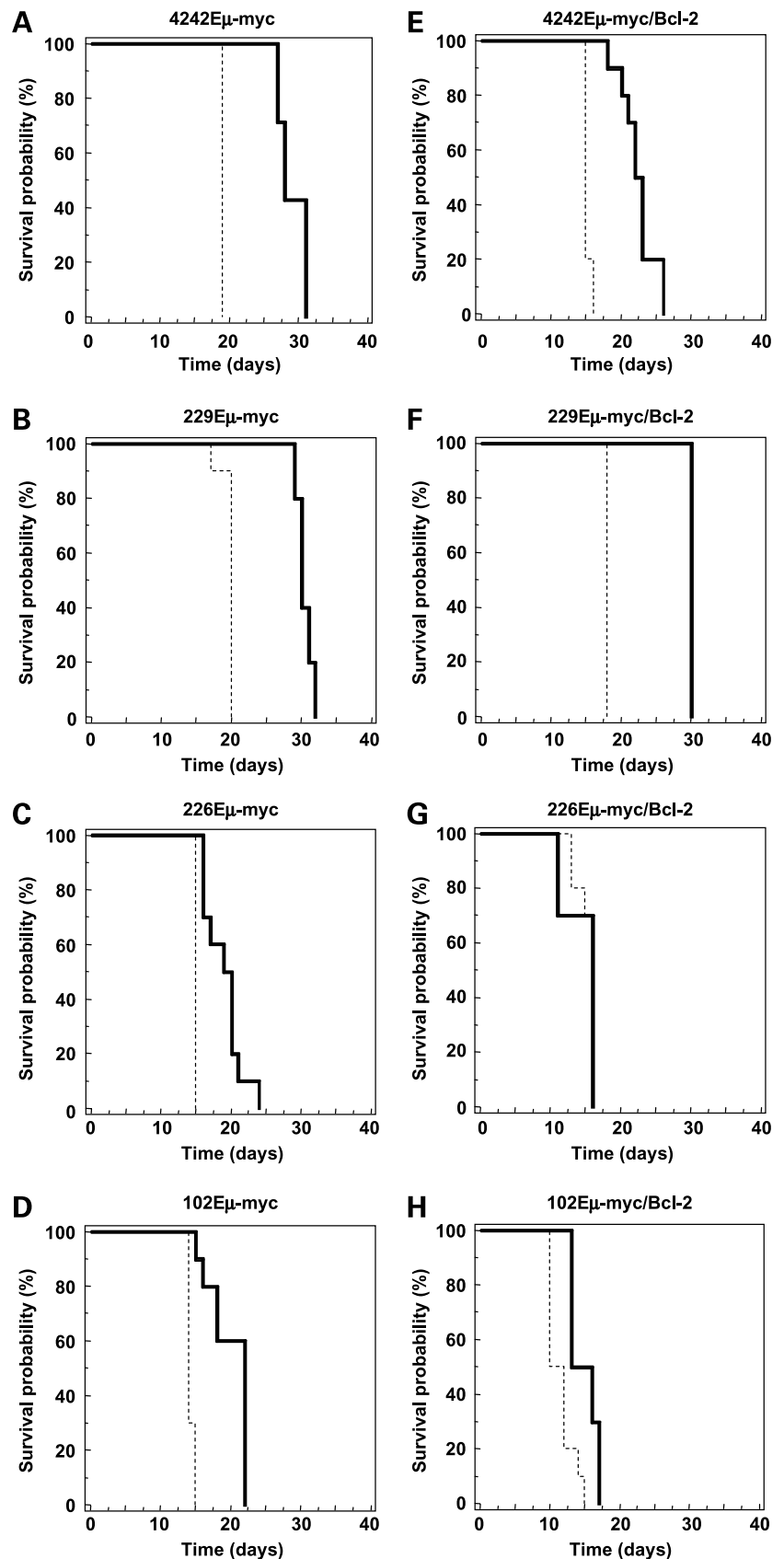


Figure 4. Therapeutic effect of romidepsin *in vivo*. C57BL/6 mice (10 mice per group) bearing (A) 4242E μ -myc, (B) 229E μ -myc, (C) 226E μ -myc, (D) 102E μ -myc, (E) 4242E μ -myc/Bcl-2, (F) 229E μ -myc/Bcl-2, (G) 226E μ -myc/Bcl-2, and (H) 102E μ -myc/Bcl-2, lymphomas were treated with romidepsin or vehicle. Therapy commenced after WBC counts reached $\geq 13 \times 10^3/\mu\text{L}$. Therapy consisted of either 5.6 mg/kg romidepsin (injected i.v. every 4 d for a total of four doses) or vehicle. Kaplan-Meier survival curves of vehicle-treated mice (dashed line) and romidepsin-treated mice (solid line) are shown. Median survival and *P* values for the different lymphomas were as follows: 4242E μ -myc, median survival vehicle 19 d, median survival romidepsin 28 d, *P* < 0.0003; 4242E μ -myc/Bcl-2, median survival vehicle 12 d, median survival romidepsin 22.5 d, *P* < 0.0001; 229E μ -myc, median survival vehicle 20 days, median survival romidepsin 30 d, *P* < 0.0001; 229E μ -myc/Bcl-2, median survival vehicle 18 d, median survival romidepsin 30 d, *P* < 0.0001; 226E μ -myc, median survival vehicle 15 d, median survival romidepsin 19.5 d, *P* < 0.0001; 226E μ -myc/Bcl-2, median survival vehicle 16 d, median survival romidepsin 16 d, *P* = 0.86; 102E μ -myc, median survival vehicle 14 d, median survival romidepsin 22 d, *P* < 0.0001; 102E μ -myc/Bcl-2, median survival vehicle 11 d, median survival romidepsin 14.5 d, *P* < 0.07.

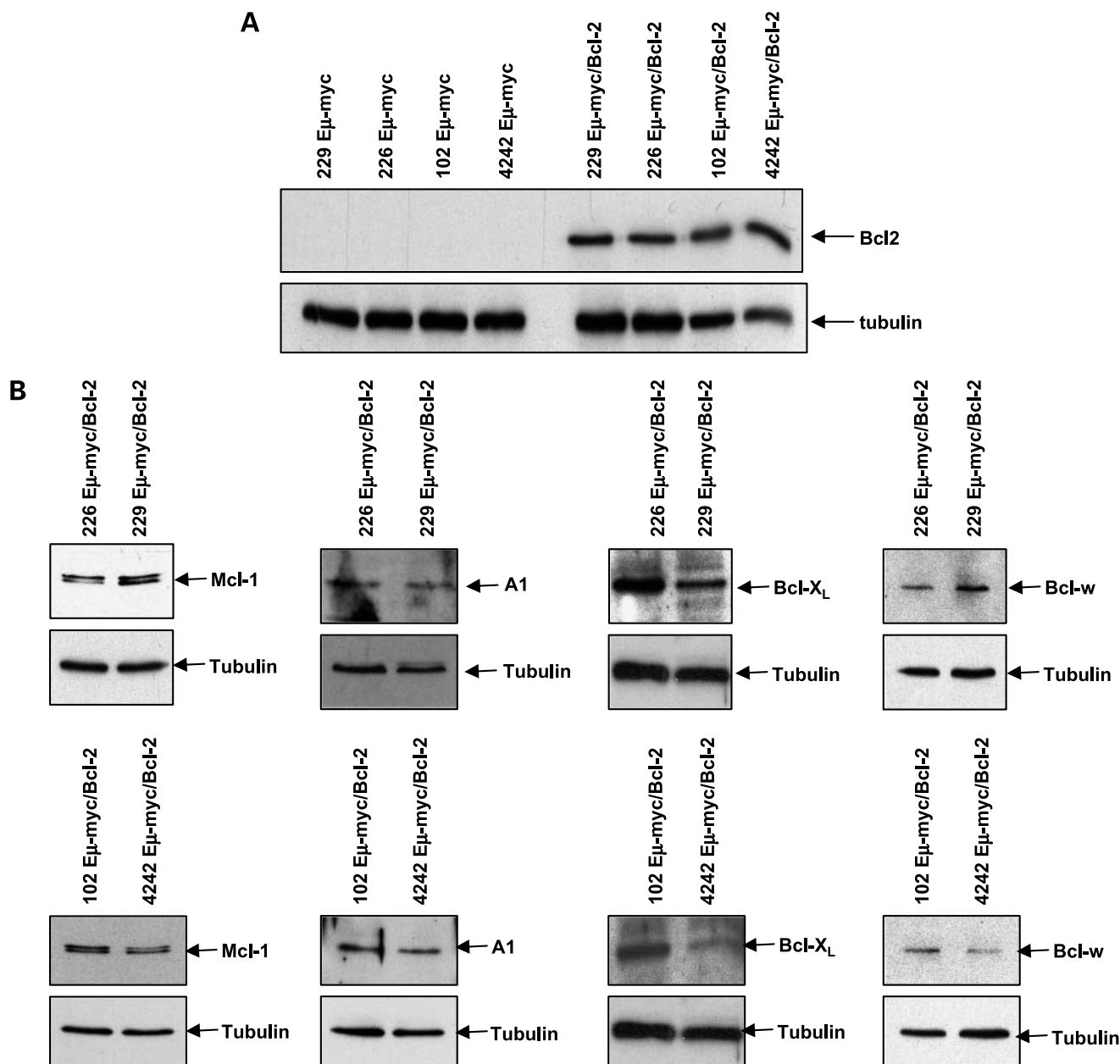


Figure 5. Expression of exogenous Bcl-2 and endogenous prosurvival Bcl-2 family proteins in Eμ-myc and Eμ-myc/Bcl2 lymphomas. **A**, expression of exogenous Bcl-2 was detected by Western blot using whole-cell lysates from 4242Eμ-myc, 4242Eμ-myc/Bcl-2, 229Eμ-myc, 229Eμ-myc/Bcl-2, 226Eμ-myc, 226Eμ-myc/Bcl-2, 102Eμ-myc, and 102Eμ-myc/Bcl-2 lymphomas. Blots were reprobbed with anti-tubulin polyclonal antibody to assess protein loading. **B**, expression of endogenous Bcl-X_L, Mcl-1, Bcl-w, and A1 was detected by Western blot using whole-cell lysates from 4242Eμ-myc/Bcl-2, 229Eμ-myc/Bcl-2, 226Eμ-myc/Bcl-2, and 102Eμ-myc/Bcl-2 lymphomas. Blots were reprobbed with anti-tubulin polyclonal antibody to assess protein loading.

tested. Although Bcl-2 and Bcl-X_L are thought to function in a very similar manner to inhibit the proapoptotic activities of Bax and Bak and thereby protect the mitochondrial outer membrane from damage (14), it appears that these proteins may have distinct roles in certain physiologic or pathologic contexts. It has been shown that Bcl-X_L may indeed be up to 10 times more effective than Bcl-2 in inhibiting apoptosis mediated by chemotherapeutic drugs such as doxorubicin

and etoposide (17). Moreover, knockout of *Bcl-2* has no effect on the latency of myc-induced lymphomagenesis and the severity of the disease was unaltered indicating that other prosurvival Bcl-2 family members play an important role in regulating myc-induced apoptosis (18). Indeed, it appears that Bcl-X is critical to regulate survival of immature B cells, whereas Bcl-2 may play a more important role in regulating apoptosis in mature B cells (18, 19).

Exactly how romidepsin can overcome the protective activities of Bcl-2, whereas other compounds such as vorinostat and oxamflatin cannot remain to be determined. Our data showing that romidepsin can induce MOMP in the sensitive E μ -myc/Bcl-2 lymphomas provides evidence that the compound is still mediating its effects via

the intrinsic apoptotic pathway rather than by an alternative or additional pathway. Moreover, as the romidepsin-resistant E μ -myc/Bcl-2 lymphomas and the E μ -myc/Bcl-X_L lymphomas show no loss of MOMP following exposure to romidepsin, we conclude that the ability to protect the mitochondria from damage mediated by romidepsin is the

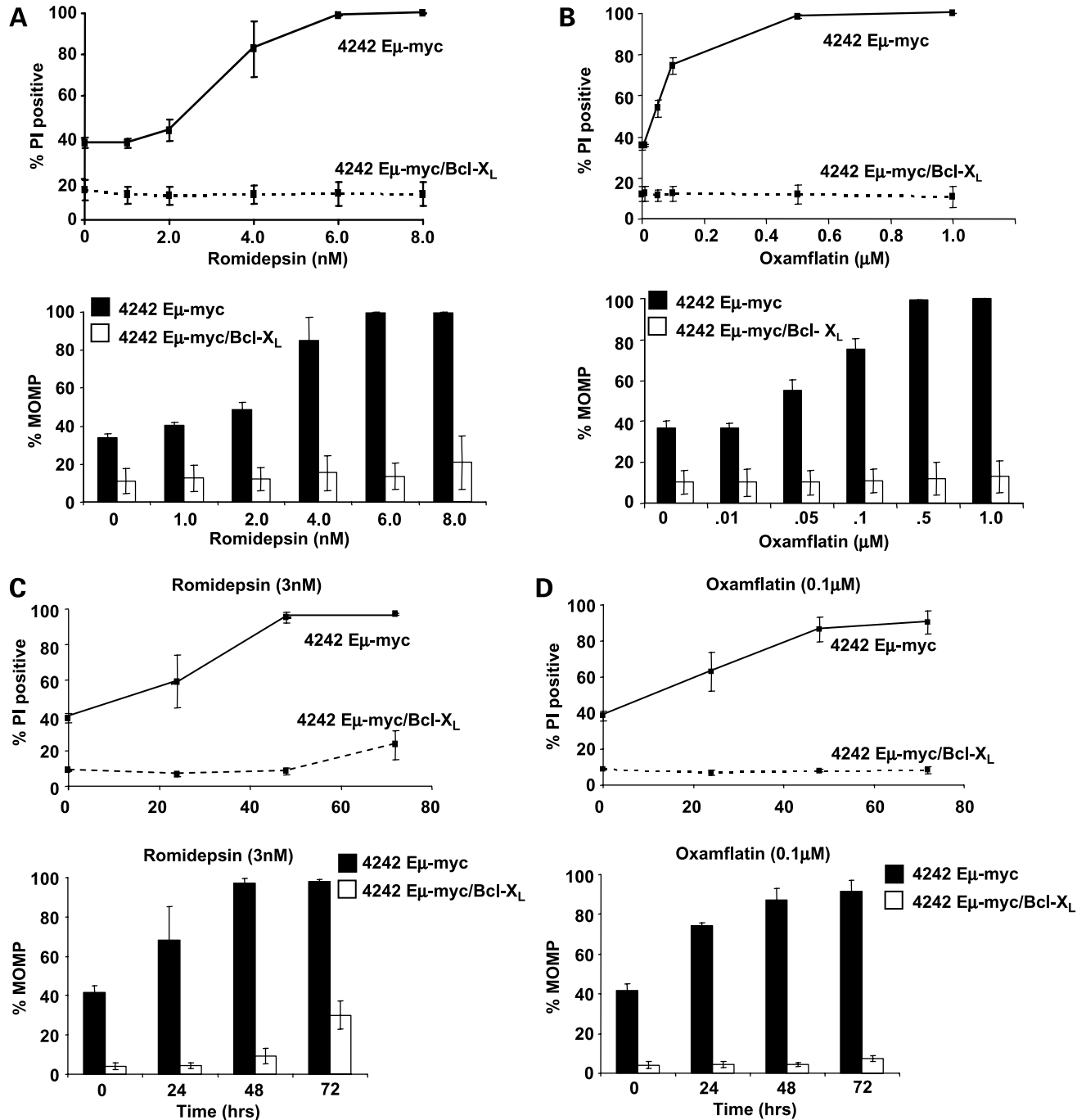


Figure 6. E μ -myc lymphomas overexpressing Bcl-X_L are resistant to romidepsin and oxamflatin *in vitro*. 4242E μ -myc and 4242E μ -myc/Bcl-X_L were incubated with the indicated concentrations of (A) romidepsin or (B) oxamflatin for 24 h or with (C) 3 nmol/L romidepsin or (D) 0.1 μ mol/L oxamflatin for up to 72 h. Cell viability was assessed by propidium iodide staining and by loss of MOMP. Bars, SE of at least three independent experiments.

key event in mediating tumor cell resistance to the compound. Bcl-2 can inhibit the apoptotic activity of many different anticancer agents that have diverse mechanisms of action (20). Interestingly, like romidepsin, aphidicolin has been shown to kill Bcl-2-overexpressing cells following prolonged exposure concomitant with increased DNA fragmentation and outer cell membrane permeabilization (21).

Our studies provide further evidence that structurally different HDACi may have subtle yet potentially important differences in their molecular and biological activities. We have shown previously that tumor cell apoptosis induced by romidepsin is inhibited by the multidrug resistance protein P-glycoprotein, whereas vorinostat and oxamflatin are unaffected by P-glycoprotein expression (12). Moreover, we showed that the cleavage and activation of the BH3-only protein Bid, which is a critical mediator of HDACi-induced apoptosis (8, 10), occurs in a caspase-independent manner following treatment with oxamflatin or vorinostat but is reliant on caspase activity in response to romidepsin (12). Finally, gene expression profiling has revealed that romidepsin and vorinostat equivalently regulate the expression of large numbers of common genes; however, vorinostat-specific and romidepsin-specific genes were identified (22), further showing that these agents do indeed possess some unique molecular activities. Interestingly, we have found that overexpression of Bcl-2 confers resistance to apoptosis mediated by other hydroxamic-based HDACi (LAQ824 and LBH589), the short-chain fatty acid valproic acid and the benzamide MS-275,⁶ indicating that the ability of romidepsin to overcome the effects of Bcl-2 may be unique to this compound. Whether other cyclic tetrapeptides such as trapoxin A or apicidin can also kill lymphomas overexpressing Bcl-2 remains to be determined.

In summary, we have shown that, unlike oxamflatin or vorinostat, romidepsin can overcome the antiapoptotic effects of Bcl-2 *in vitro* and this translates into a therapeutic effect *in vivo*. As vorinostat is already a Food and Drug Administration-approved drug and romidepsin is being tested clinically to achieve Food and Drug Administration approval (2, 23), identifying the molecular events that may inhibit the therapeutic efficacy of each compound may be important for the future stratification of patients being treated with one or other compound. Our data indicate that, in situations where Bcl-2 is overexpressed in a particular tumor, romidepsin may be a more preferred treatment option than vorinostat. In contrast, if P-glycoprotein is overexpressed in a particular tumor, then vorinostat would likely be more efficacious than romidepsin, which is a substrate for P-glycoprotein. However, our data also indicate that the relative expression of a single antiapoptotic protein such as Bcl-2 may not provide a true indication of the relative sensitivity of a tumor to romidepsin. Indeed, we

showed that the relative expression of Bcl-X_L inversely correlated with the apoptotic and therapeutic activities of romidepsin and Bcl-X_L appeared to be a far more potent inhibitor of romidepsin-mediated apoptosis than Bcl-2. Clearly, romidepsin and hydroxamate-based compounds such as vorinostat and oxamflatin have different relative affinities for individual HDACs, with romidepsin being more selective for class I HDACs, whereas vorinostat and related compounds are considered pan-HDACi (1). It remains to be determined if the subtle yet potentially important antitumor effects of structurally diverse HDACi relate to differences in their target specificity resulting in some specific gene regulatory activities or other inherent differences in the activities of the compounds that could be unrelated to HDAC-inhibitory activity.

Disclosure of Potential Conflicts of Interest

R.W. Johnstone: Novartis and Merck grant support. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank David Huang, Andreas Strasser, Clare Scott, Jerry Adams, Suzanne Cory, and Alan Harris (Walter and Eliza Hall Institute) for helpful advice, Eμ-myc mice, and eDNAs for Bcl-2 and Bcl-X_L and Drs. Chris Clarke and Nigel Waterhouse (Peter MacCallum Cancer Centre) for helpful advice and input. Depsipeptide was kindly provided by Gloucester Pharmaceuticals.

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⁶ Unpublished observations.

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