The Novel IKK2 Inhibitor LY2409881 Potently Synergizes with Histone Deacetylase Inhibitors in Preclinical Models of Lymphoma through the Downregulation of NF-κB

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Abstract

Purpose: To evaluate the pharmacologic activity of a novel inhibitor of IkB kinase β (IKK2), LY2409881, in preclinical models of B- and T-cell lymphoma, as a single agent and in combination with histone deacetylase (HDAC) inhibitors.

Experimental Design: The in vitro activity of LY2409881 was determined using an ATP-based growth inhibition assay and flow cytometric assay of apoptosis in lymphoma cell lines. The in vivo activity of LY2409881 was determined using SCID-beige xenograft mouse model. The mechanism of action was determined using immunoblotting, immunofluorescence, and electrophoretic mobility shift assay. Synergy of LY2409881 with other drugs active in lymphoma was determined by calculating relative risk ratio (RRR) and combination index (CI).

Results: LY2409881 inhibited constitutively activated NF-κB, and caused concentration- and time-dependent growth inhibition and apoptosis in lymphoma cells. In models of diffuse large B-cell lymphoma (DLBCL), the cytotoxicity of LY2409881 correlated with the overall activation status of NF-κB, but not simply in a pattern predicted by the cell-of-origin classification of these cell lines. LY2409881 was safe to mice at three dose levels, 50, 100, and 200 mg/kg, all of which caused significant inhibition of tumor growth. LY2409881 suppressed the activity of the NF-κB subunit p65 in lymphoma cells treated by the HDAC inhibitor romidepsin, underlying a potential mechanism of the marked synergy observed of these two drugs.

Conclusion: Collectively, these data strongly suggest that targeting the NF-κB pathway in combination with romidepsin could represent a novel and potent regimen for the treatment of B- and T-cell lymphoma.

Introduction

NFκB is a family of inducible transcription factors that comprises five different proteins, RelA (p65), cRel, RelB, NFκB1 (p50/p105), and NFκB2 (p52/p100; refs. 1, 2). In their inactive state, the proteins are sequestered in the cytoplasm through their binding to the inhibitory protein IkB. In the classical pathway of NF-κB activation, stimulation by a variety of signals such as B-cell receptor engagement leads to activation of the IkB kinase β (IKK2). IKK2 phosphorylates IkB, which renders it a substrate for polyubiquitination and subsequent degradation by the 26S proteasome. Degradation of IkB releases the p50-RelA dimer, which is then translocated into the nucleus, where it regulates expression of genes important in inflammation, proliferation, survival, and DNA-damage repair. Among the target genes regulated by NFκB are IkB, cyclin D1, Bcl-xL, survivin, BCL2, and ATM. In the alternative pathway of NF-κB activation, upstream stimuli such as TNF superfamily cytokine B cell–activating factor (BAFF) activate NF-κB–inducing kinase (NIK). NIK, in turn, phosphorylates IkB kinase α (IKK1), leading to phosphorylation of p100. Subsequent polyubiquitination and degradation of p100 allows translocation of the RelB–p52 dimer into the nucleus, where it activates the expression of prosurvival genes.

Constitutive activation of NFκB is frequently observed in a number of lymphoma subtypes, including cutaneous T-cell lymphoma (CTCL; refs. 3, 4), mantle cell lymphoma (MCL; ref. 5), diffuse large B-cell lymphoma (DLBCL; refs. 6–8), and adult T cell leukemia lymphoma (ATLL; ref. 9). The pathologic significance of NFκB is perhaps best established in DLBCL, as the activated B cell (ABC) subtype of DLBCL is characterized by constitutive activation of NFκB and a worse prognosis compared with those DBCL derived from the germinal center B cell (GCB; refs. 10, 11).
Translational Relevance

Targeting histone deacetylases (HDAC) has become a validated therapeutic strategy for the treatment of select subtypes of lymphoma, though the rationale remains strong across the diversity of subtypes. In the peripheral T-cell lymphomas (PTCL), HDAC inhibitors on the whole produce a response rate of only 25% to 30%, albeit in heavily treated patient populations, and at present are only approved for the treatment of relapsed or refractory PTCL and cutaneous T-cell lymphoma. The HDAC inhibitors are recognized as highly pleiotropic drugs. Although in balance they have therapeutic properties, they also can produce a host of potentially "unfavorable effects" on growth and survival pathways that might well explain their restricted range of activity, and somewhat modest response rate in the clinical setting. The hypothesis of this work is based on understanding some of the undesirable effects of this important class of drugs, and using that information to develop rational drug–drug combinations to mitigate the unfavorable effects, in order to enhance their therapeutic effect. Our results suggest HDAC inhibitors activate some elements of the NF-kB pathway, which likely contributes to some of the undesirable effects of this class of drugs. Simultaneous targeting of the NF-kB pathways with Btk kinase inhibitor BCL6 is found to be highly synergistic in models of diffuse large B-cell lymphoma (DLBCL). The mechanistic basis of the synergy was attributed to the mitigation of the unfavorable effects of HDAC inhibitors on the NF-kB pathway. We propose that by understanding the unfavorable therapeutic features of pleiotropic drugs, rational and synergistic combinations can be identified that can markedly enhance the efficacy of HDAC inhibitor–based therapy across most forms of cancer.

Moreover, the ABC subtype of DLBCL appears to be addicted to the constitutively activated NF-kB signals in a number of preclinical studies. Indirectly, the importance of NF-kB is also supported by a recent clinical study of a Bruton tyrosine kinase (BTK) inhibitor, ibrutinib (Imbruvica; refs. 12, 13). BTK is a kinase that mediates B-cell receptor signaling, which also leads to activation of NF-kB. As a single agent, ibrutinib produced rare partial response in GC B-cell lymphoma, and an impressive response rate of 40% in the ABC lymphoma, including 8% of complete response (12, 13). These results demonstrate that inhibition of NF-kB is an important strategy to treat lymphoma, particularly those lymphoma types where constitutively activated NF-kB plays critical pathologic roles.

Another common pathologic feature of DLBCL involves deregulation of the acetylation and deacetylation state of critical proteins. For example, BCL6 is a transcription repressor involved in lymphomagenesis (14), and acetylation of BCL6 at the KKKK (376–379) motif inhibits its function as transcription repressor and oncogene (15). The balance of acetylation and deacetylation is carefully orchestrated through the activity of two opposing classes of enzymes, the histone/protein lysine acetyltransferases (HAT) and histone deacetylases (HDAC). Inactivating mutations of two of the HAT family genes, CBP and p300, are found in 40% DLBCL patient samples (16). These mutations prevent BCL6 acetylation, leading to aberrantly activated BCL6 (14). From the therapeutic perspective, lymphomas represent a disease entity where there is a strong rationale to target HDAC, as three HDAC inhibitors are now approved for the treatment of T-cell lymphoma. On a whole, however, this class of drugs produces a response rate of only 25% to 30% across a diverse group of mature, albeit chemotherapy-resistant T-cell lymphoma. The HDAC inhibitors are recognized as highly pleiotropic drugs that obviously in balance, effectively kill T-cell lymphoma. In reality, the pleiotropic effects of these drugs have been long recognized to potentially include a host of "unfavorable effects" that might well explain the restricted range of activity observed across the known HDAC inhibitors in diverse subtypes of lymphoma. We hypothesize that if mechanistic strategies can be found to mitigate these unfavorable effects of HDAC inhibitors, then the efficacy of HDAC inhibitors could be potently enhanced. For example, recent studies have reported that acetylation of p65/RelA is promoted by HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA) and MS-275, resulting in increased transcriptional activity of NF-kB (17). If the acetylation of RelA is an important mechanism to limit the anticancer activity of HDAC inhibitors, then combining HDAC inhibitors and NF-kB inhibitors will be synergistic and may become a promising therapeutic strategy.

Because of the central role of IKK2 in the activation of NF-kB, IKK2 has taken the central stage as a drug target for cancer and inflammatory disorders over the past two decades. Two of the well-published IKK2 inhibitors in preclinical models include MLN120B (18–21) and AS602868 (4, 22), both of which have been shown to possess moderate activity as single agents in models of multiple myeloma and CTCL, respectively. However, no IKK2 inhibitors have reached clinical development, primarily because of the concern over hepatic toxicity seen in IKK2 knockout mice. Novel IKK2 inhibitors that are structurally different from previously developed inhibitors remain an important area of drug development, and may be particularly effective when combined with HDAC inhibitors and other chemotherapeutic drugs.

Our results demonstrate that a novel IKK2 inhibitor, LY2409881, inhibited the growth and survival of lymphoma in vitro and in vivo. LY2409881 induced higher levels of cytotoxicity in lymphoma models known to have constitutively activated NF-kB signals. LY2409881 was well tolerated in vivo. Furthermore, LY2409881 potently synergized with antilymphoma drugs. In particular, LY2409881 and the HDAC inhibitor romidepsin were highly synergistic in every cell line model of T- and B-cell lymphoma examined. The synergy of LY2409881 and romidepsin may derive from the antagonistic effect of LY2409881 on romidepsin-mediated activation of NF-kB. These data provide new insights into this synergistic interaction and suggest a therapeutic strategy for the treatment of lymphoma that is associated with constitutively activated NF-kB.

Materials and Methods

Materials

LY2409881 was obtained from Lilly Research Laboratories. Merck IKK2 inhibitor VIII was ordered from Merck. Bay 11-7082 was obtained from Sigma. Romidepsin, belinostat, and vorinostat were ordered from Selleck Chemicals. All chemicals were dissolved in dimethyl sulfoxide (DMSO) for in vitro studies. DMSO in untreated control samples matched its concentrations in the treated samples.

Cell lines

OCI-Ly1, OCI-Ly7, and Su-DHL4 are GCB DLBCL cell lines; OCI-Ly3, OCI-Ly10, HBL1, and Su-DHL2 are ABC DLBCL lines.

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These cell lines were grown in Iscove Modified Dulbecco Medium with 10% FCS. H1 and H9 are CTCL cell lines. MT1 and TL are ATL cell lines that do not express Tax; MT2 and C5M were HTLV1-transformed cell lines that express Tax. These cell lines were grown in RPMI medium with 10% FCS. Fresh medium was added every 2 to 3 days, and the cells were kept at a cell concentration of 0.1 to 1 x 10^6/mL.

Cytotoxicity assays
Cytotoxicity was evaluated using the CellTiter-Glo Reagent (Promega) according to the manufacturer's manual, as reported previously (23). Experiments were carried out in 96-well plates, with each treatment in triplicate. Samples were taken at typically 24, 48, and 72 hours after treatment. Cytotoxicity was expressed by the decreasing percentage of live cells in each treatment relative to the untreated control from the same experiment, as a function of time. IC_50 (half the maximal inhibitory concentration) for each cell line untreated control from the same experiment, as a function of time.

Flow cytometry
To study apoptosis, Yo-Pro-1 and propidium iodide (Vybrant apoptosis assay kit #4; Invitrogen) were used, as previously described (23). A minimum of 1 x 10^4 events were acquired from each sample. The fluorescence signals acquired by a FACS-Calibur System were resolved by detection in the conventional FL1 and FL3 channels. Cells were considered early apoptotic if Yo-Pro-1 positive but PI-negative, late apoptotic if Yo-Pro-1- and PI-positive, and necrotic if only PI-positive. Alternatively, dead cells were detected by flow cytometry using the Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit from Invitrogen.

Western blotting
Western blotting was performed according to standard protocols, using the chemiluminescence detection system from Thermo Scientific. The following primary antibodies from Cell Signaling Technology were used: anti-p105/p50, anti-p100/p52, antiphosphorylated IkB, anti-IkB, anti-p65, c-Rel, anti-Ku80, anti-actin, anti-HDAC1, anti-HDAC2, anti-HDAC3 anti-HDAC4, anti-HDAC6, and anti-HDAC8 (Sigma). Anticetlated p65 was obtained from Abcam. Goat anti-rabbit or anti-mouse secondary antibodies (Santa Cruz Biotechnology) were used.

Immunofluorescence
Immunofluorescence was performed as previously described (24). After incubation with LY2409881 for 4 hours, the samples were placed on the slides using the Cytospin. After fixation in 10% formalin and 100% methanol, the slides were transferred to the blocking buffer (10% nonfat dry milk) and incubated with primary antibody in the humidity chamber overnight. The slides were then incubated with fluorescein-conjugated secondary antibody for 45 minutes and mounted with 4',6-diamidino-2-phenylindole (DAPI) with the VECTASHIELD Mounting Media from Vector Labs. Anti-p105/p50 primary antibody (Cell Signaling Technology) and anti-HDAC1 anti-rabbit IgG (Jackson Immunoresearch Laboratories) secondary antibody were used. The images were collected using Nikon Eclipse TE 2000-E inverted epifluorescent microscope and a Nikon Photometrics Coolsnap HQ2 camera. The images were analyzed using NIS-Elements AR 3.2 software.

Electrophoretic mobility shift assay
Nuclear extract was made using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's manual. The NF-kB consensus DNA was purchased from Promega, and labeled using the Biotin 3' End DNA Labeling Kit (Thermo Scientific). The nuclear extracts from different treatment groups were incubated with the biotin-labeled NF-kB DNA, and resolved on a native gel, rapidly (30 minutes) transferred to a positive nylon membrane, UV cross-linked, and probed with streptavidin–HRP conjugate using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific). Competition reactions containing a 200-fold molar excess of unlabeled duplex were performed to confirm the specificity of the protein–DNA interactions.

In vivo tumor model
Mouse experiments were carried out in accordance with the principles of laboratory animal care under an Institutional Animal Care and Use Committee–approved protocol. Five- to 7-week-old SCID beige mice (Taconic) were injected with 10^7 Ly10 cells mixed in Matrigel in the posterior flank subcutaneously. When the tumors approached 150 mm^3, the mice were divided into four groups of 8 mice: (i) control group, which received 5% dextrose in water; (ii) LY2409881 at 50 mg/kg in D5W; (iii) LY2409881 at 100 mg/kg in D5W; and (iv) LY2409881 at 200 mg/kg in D5W. The drug or D5W was administered intraperitoneally on day 1 and 4 of every week for 4 weeks. The data were expressed as average tumor volume (mm^3) per group as a function of time. Tumor volume was calculated using the formula: (4/3)pi*r^2, where r = (length + width)/4. Care of mice was done according to institutional guidelines, as previously described (23).

Statistical analysis
Relative risk ratio (RRR) was calculated to measure the degree of drug–drug synergism, and was previously shown to correlate well with combination index (CI, refs. 23, 24). RRR is based on calculating the ratio between the actual value and expected value (EV) of percentage of surviving cells after treatment. EV is calculated by formula: EV = NA x NB/100, where NA represents the percentage of viable cells in the sample treated with drug A and NB represents the percentage of viable cells in the sample treated with drug B. RRR values below 1 represent synergistic effect of the two drugs, values equal to 1 indicate additive effect of the drugs; and values above 1 represent antagonistic effect of the two drugs. Calculation of the CI is based on the Chou and Talalay method, using the free software at www.combosyn.com.

For evaluation of tumor growth in vivo, the generalized estimating equation (GEE) was used to assess the difference in the tumor volume among the treatment groups. It was assumed that the tumor volume follows the model Volume_ij = B0 + B1*time_i + B2*treatment_j + B3*time_i*treatment_j, + epsilon_i for the ith mouse at time t. The model assumes that the tumor volume is linear in time and allows different intercepts and slopes for different treatment groups. The error term epsilon_i is uncorrelated between different mice but correlated between same individual mouse at different times to account for within-mouse correlation.

Results
LY2409881 is a novel IKK2 inhibitor that inhibits TNF-induced activation of NF-kB. LY2409881 is the trihydrochloride salt of a novel pyrimidinyl benzothiophene compound (Fig. 1A). Using kinase profiling in more than 300 kinases, LY2409881 was highly selective for IKK2.
By in vitro kinase assay, LY2409881 potently inhibited IKK2, with a IC_{50} of 30 nmol/L. In contrast, the IC_{50} for IKK1 and other common kinases was at least one log higher. The specificity of LY2409881 for NF-κB signaling was further studied in a cell-based assay, by examining the effect of LY2409881 in the TNFα-dependent antiapoptosis function. TNFα is a well-characterized upstream stimulus of NF-κB. TNFα stimulates both antiapoptotic signals, mediated by NF-κB, and proapoptotic signals, mediated by TNF receptor–associated death domain (TRADD) and FAS-associated death domain (FADD) cascade pathways (25). As a result, TNFα is not a potent anticancer drug. In the ovarian cancer cell line SKOV3, LY2409881 demonstrated moderate cytotoxicity.
LY2409881 on other subunits of NF-κB was inactivated by LY2409881. We further evaluated the effects of GCB subtype. As shown in Fig. 1E, LY2409881 inhibited the lines: LY10, representing the ABC subtype of DLBCL, and LY1, the

When treated by LY2409881 at 10 μmol/L, the nuclear signals of NF-κB in two more DLBCL cell lines, LY10 and LY3, were found to be the most sensitive cell lines (Fig. 3A and Supplementary Table S1). Two other ABC cell lines, HBL1 and SUDHL2, were significantly more resistant to LY2409881 than LY10 and LY3. In fact, HBL1 and SUDHL2 were even more resistant to LY2409881 than the three GCB cell lines (Fig. 3A and Supplementary Table S1). We reasoned that the two resistant ABC cell lines might have different pathways of activation of NF-κB from the sensitive ABC cell lines, and interrogated four key components of the NB-κB pathway: (i) nuclear p50, which reflects activation of the classical pathway, (ii) p65/RelA, which is required for the classical pathway, (iii) nuclear p52, which reflects activation of the alternative pathway, and (iv) IκB, which negatively regulates both p50 and p52.

Figure 3B demonstrates that all four ABC cell lines (bold and italicized) had a higher level of activation of NF-κB through the classical pathway, represented by a substantially higher ratio of p50/p105 in the nucleus, than the three GCB cell lines (underlined). Similarly, the four ABC cell lines had higher level of nuclear RelA than the three GCB cell lines. Note that the lower band in the immunoblot of the nuclear protein extract was RelA/p65, and the upper band was nonspecific. In the cytoplasmic protein extracts, there was only one band detected by the anti-p65 antibody, corresponding in size to the lower band in the nuclear protein extract. Nuclear p52 was abundantly present in all four ABC cell lines, and only present in one of the three GCB cell lines, LY7. The level of IκB was lower in the two most sensitive cell lines, LY10 and LY3, and one of the most resistant cell lines, HBL1, than the other four cell lines. The above results demonstrate that the ABC cell lines consistently activate NF-κB through both the classical and alternative pathways. The GCB cell lines have relatively lower level of activation through the classical pathway, and are infrequently activated through the alternative pathway. Different DLBCL cell lines may use complex and distinct mechanisms to activate NF-κB signaling, and nevertheless remain sensitive to the IKK2 inhibitor LY2409881.

LY2409881 is well tolerated and inhibits tumor growth \textit{in vivo}

We used a well-established xenograft model of DLBCL to confirm the activity of LY2409881 \textit{in vivo}. SCID-beige mice implanted with LY10 cell-derived tumors were given intraperitoneal injections of LY2409881 twice weekly at three different doses: 50, 100, and 200 mg/kg. The treatments were well tolerated, resulting in no death or severe morbidity of the mice. The average tumor volume was graphed as a function of time for each
IKK2 Inhibition Potently Synergizes with HDAC Inhibitors

If NF-κB responsive genes are involved in the proliferation and survival of cancer cells, then inhibiting the classical pathway of NF-κB using the IKK2 inhibitor LY2409881 should theoretically potentiate the effect of other chemotherapeutic drugs. We investigated the drug–drug interaction of LY2409881 with doxorubicin and cyclophosphamide in DLBCL cell lines. LY2409881 was highly synergistic with doxorubicin (Fig. 4A) and cyclophosphamide (Fig. 4C) in the ABC cell line SUDHL2. In the GCB cell line, LY1, the drug LY2409881 demonstrated no synergy with either doxorubicin (Fig. 4B) or cyclophosphamide (Fig. 4D). Supplementary Table S2 summarizes the drug–drug interaction of LY2409881 with doxorubicin and cyclophosphamide in a panel of lymphoma cell lines representing different disease entities, indicating that synergy was uncommon in DLBCL cell lines when LY2409881 was combined with these two chemotherapeutic drugs. In contrast, LY2409881 and the pan-HDAC inhibitor, romidepsin, were potently synergistic in both SUDHL2 and LY1 (Fig. 4E and F). In fact, marked and consistent synergy was observed for LY2409881 and romidepsin in all seven DLBCL cell lines irrespective of the ABC and GCB classification, with RRR values often below 0.1 at mildly or moderately cytotoxic concentrations of each drug as single agent (Table 1). The values of CI were calculated for results in Fig. 4 and presented in Supplementary Table S3, demonstrating highly concordant results with the RRR values.

Next, we determined whether other IKK2 inhibitors also synergize with romidepsin, and examined the interaction of romidepsin with three other IKK2 inhibitors, including the Merck IKK2 inhibitor IV, Merck IKK2 inhibitor VIII, and Bay 11-7082 in the LY1 cell line. As shown in Fig. 4G, the Merck IKK2 inhibitor VIII and romidepsin were markedly synergistic in the LY1 cell line, similar to the synergy of LY2409881 and romidepsin demonstrated in Fig. 4F. In contrast, neither Bay 11-7082 (Fig. 4H) nor IKK2 inhibitor IV (not shown) was highly synergistic with romidepsin in the LY1 cell line. We then sought to determine whether the synergy observed is a class effect of HDAC inhibitors and LY2409881. As summarized in Table 1, high-level synergy was rarely observed for LY2409881 and another pan-HDAC inhibitor belinostat, suggesting that the synergistic effect of IKK2 inhibitors and HDAC inhibitors may not be class-dependent, but rather unique to the specific drug romidepsin.

LY2409881 and romidepsin act antagonistically on p65/RelA

We explored the mechanism of the synergy of LY2409881 and HDAC inhibitors by determining how they differentially affect the p65 protein binding to the DNA probe containing the NF-κB consensus sequence. As shown in Fig. 5A, LY2409881 decreased the binding of p65 to the NF-κB consensus sequence, and romidepsin increased the binding to the NF-κB consensus sequence in a concentration-dependent manner in the DLBCL cell line LY10. Similarly, two other HDAC inhibitors, belinostat and vorinostat
SAHA, also increased the binding capacity of p65 to the NF-κB DNA (Fig. 5B). Figure 5C demonstrated that LY2409881 markedly reduced binding of p65 to its substrate DNA. In contrast, romidepsin increased the binding of DNA and NF-κB in a concentration-dependent manner. Combining LY2409881 and romidepsin, on the other hand, was able to suppress the increased binding of DNA and p65 caused by romidepsin. The amount of loaded nuclear protein, as demonstrated by HDAC1, was relatively constant across the samples, as shown by the quantification using densitometry (Fig. 5D). We also compared the protein levels of p65 in the samples used for the electrophoretic mobility shift assay (EMSA) assay. Compared with the untreated control, LY2409881 reduced the level of p65 by about 40%, whereas romidepsin at the 5-nmol/L concentration did not change the level of nuclear p65; and decreased it by about 30% at the 10-nmol/L concentration. The fact that romidepsin increased the binding of DNA and p65 in the setting of unchanged or even reduced nuclear protein level of p65 strongly suggests that romidepsin increased the affinity of p65 for the NF-κB DNA.

As acetylation of p65 has been shown to promote DNA-binding activity of p65, we investigated the effect of romidepsin on the acetylation of p65 in DLBCL cell lines. Treatment with romidepsin resulted in increased acetylation of p65 in the nucleus in all of four DLBCL cell lines, irrespective of the ABC or GCB subtype to which they belong (Fig. 5E). The induction of acetylated p65 by romidepsin was concentration dependent, and was least in the ABC cell line HBL1. These results demonstrated that treatment with romidepsin was associated with increased acetylation of p65 and increased binding of p65 to the NF-κB consensus DNA, all of which might limit the antitumor properties of romidepsin due to transcription of NF-κB-dependent genes. Treatment with LY2409881 was able to inhibit nuclear localization of p65 and suppress romidepsin-
Figure 4.
LY2409881 was synergistic with doxorubicin, cyclophosphamide, and HDAC inhibitors. Synergy was determined by calculating the RRR values, labeled in each graph. The CI values were calculated in the Supplementary Table S3, demonstrating the same level of synergy as revealed here by the RRR values. A, SU-DHL2 cells were treated by the following drugs: LY2409881 at 4, 8, and 15 μmol/L (IK1, IK2, and IK3, respectively), doxorubicin at 40, 160, and 800 nmol/L (D1, D2, and D3, respectively), and the two drugs in combination. B, LY1 cells were treated with LY2409881 at 2, 5, and 10 μmol/L (IK1, IK2, and IK3, respectively); doxorubicin at 100, 400, and 750 nmol/L (D1, D2, and D3, respectively), and the two drugs in combination. C, SU-DHL2 cells were treated by the following drugs: LY2409881 at 4, 8, and 15 μmol/L (IK1, IK2, and IK3, respectively), 4-hydroxycyclophosphamide at 2, 10, and 16 μmol/L (C1, C2, and C3, respectively), and the two drugs in combination. D, LY1 cells were treated with LY2409881 at 2, 5, and 10 μmol/L (IK1, IK2, and IK3, respectively), 4-hydroxycyclophosphamide at 2.5, 3, and 4 μmol/L (C1, C2, and C3, respectively), and the two drugs in combination. E, SU-DHL2 was treated with LY2409881 at 4, 8, and 15 μmol/L (IK1, IK2, and IK3, respectively), romidepsin at 0.8, 1.5, and 2.8 nmol/L (R1, R2, and R3, respectively), and the two agents in combination (IK1 + R1, IK2 + R2, and IK3 + R3, respectively) for 48 hours. F, OCI-LY1 cells were treated with LY2409881 at 2, 5, and 10 μmol/L (IK1, IK2, and IK3, respectively), romidepsin at 3, 4.5, and 6 nmol/L (R1, R2, and R3, respectively), and the two agents in combination (IK1 + R1, IK2 + R2, and IK3 + R3, respectively) for 48 hours. Note that the concentrations of drugs used for A and B were different, as the two cell lines differed in their sensitivity to these drugs. G and H were conducted in the LY1 cell line, as in F. Romidepsin was given at the concentrations of 3, 4.5, and 6 nmol/L (R1, R2, and R3, respectively), same as in F. Note that there was a small difference in the cytotoxicity of romidepsin in the experiments done on different days. In G, the Merck IKK2 inhibitor VIII was given at 8, 10, and 15 μmol/L (Mk1, Mk2, and Mk3, respectively), replacing LY2409881 in F. In H, the IKK2 inhibitor BayII-7082, in place of LY2409881, was given at 0.25, 0.375, and 0.75 μmol/L (Bay1, Bay2, and Bay3, respectively).
mediated enhancement of p65-DNA binding, thereby mitigating an undesired feature of the pleiotropic HDAC inhibitor romidepsin.

The synergy of belinostat and LY2409881 varies in different cell lines with distinct expression level of HDAC isoforms

Unlike romidepsin, belinostat synergized with LY2409881 only in select DLBCL cell lines, including LY10, LY7, and SUDHL2 (Table 1). In contrast, belinostat and LY2409881 were antagonistic in HBL1 and LY1. We asked whether the difference in synergy in different cell lines may be secondary to different expression levels of HDAC isoforms that are targets of belinostat. Class I HDACs HDAC1, HDAC2, and HDAC3 have been shown to localize primarily to the nucleus, and are potently inhibited by HDAC1 was lower in HBL1 than the other three cell lines. In contrast, the cytosolic level of HDAC4 was highest in HBL1, and HDAC6 is much higher in HBL1 than the other cell lines. It is conceivable that the distinct expression pattern of the HDAC isoforms in HBL1 may be responsible for the lower level of acetylated p65 induced by romidepsin (Fig. 5E), and ultimately the weak synergy in the combination LY2409881 and romidepsin and lack of synergy between LY2409881 and belinostat (Table 1).

Discussion

NF-kB has been a logical target for cancer treatment for many years due to its frequent activation in many cancer models and its capability to promote cell proliferation and survival (2). Because the classical pathway has been shown to be the predominant pathway involved in tumorigenesis, targeting NF-kB has historically focused on finding potent and selective inhibitors of IKK2. Although dozens of IKK2 inhibitors have been reported, none have yet made it to the clinic. The long-standing concern over potential hepatotoxicity of IKK2 inhibitors originated from the observation that the IKK2 knockout mice died during embryogenesis from massive liver necrosis. However, recent data demonstrated that hepatocyte-specific ablation of IKK2 did not lead to increased apoptosis after TNFα stimulation (28, 29), suggesting that adult liver may not be prone to necrosis when exposed to IKK2 inhibitors. Novel IKK2 inhibitors that are structurally different from the previously developed inhibitors remain an important goal for the treatment of many diseases that are pathogenetically associated with highly activated NF-kB signaling, such as many types of cancer.

Here, we demonstrate that the novel IKK2 inhibitor LY2409881 is active in both B- and T-cell lymphoma models in which NF-kB is established to play important pathogenetic roles. For example, the sensitivity of DLBCL cells to LY2409881 correlate with the activation status of NF-kB, with two ABC cell lines, LY3 and LY10, being most sensitive to LY2409881 (Fig. 3A and Supplementary Table S1). Paradoxically, two other ABC cell lines, SUDHL2 and HBL1, were actually more resistant than the GCB cell lines studied (Fig. 3A and Supplementary Table S1). We believe these results may underscore the complexity of the NF-kB pathway, and suggest that the cell of origin (COO) classification of DLBCL cell lines may not necessarily correlate well with the status of NF-kB as in patients with DLBCL. For example, we demonstrated that the classical pathway was activated in all four ABC cell lines in a higher degree and in all three GCB cell lines to a relatively lower degree, as demonstrated by the presence of p50 and p65 in the nucleus (Fig. 3B). The alternative pathway was activated in all four ABC cell lines and only one of three GCB cell lines, as demonstrated by the presence of p52 in the nucleus (Fig. 3B). Of note, both the classical and alternative pathways have previously been reported to be involved in DLBCL (30). We also observed substantial difference in the protein level of nuclear kB, which was least abundant in the most sensitive ABC cell lines LY3 and LY10, and the more resistant ABC cell line HBL1. Collectively, these results suggest that sensitivity to the IKK2 inhibitor cannot be simply predicted by the COO classification, or any of the subunits of NF-kB. Rather, the sensitivity of the different cell lines to IKK2 inhibition may also be explained by how NF-kB is activated using available genetic data. For example, the LY3 cell line is known to harbor a homozygous activating mutation of CARD11, leading to activation of IKK2 and the NF-kB pathway (8). Therefore, LY3 is expected to be highly dependent on IKK2-induced NF-kB–responsive prosurvival signals. Consistently, LY3 is the most sensitive DLBCL cell line. In contrast, SUDHL2 has homozygous inactivating mutation of TNFAIP3/A20 (7), which results in constitutive activation of upstream regulatory proteins that stimulate the IKK1/IKK2/IKK3 kinases (31). Although LY2409881 potently inhibits IKK2, it is not active against IKK1. The leaky inhibition of the NF-kB pathway by LY2409881 in SUDHL2 therefore may result in relative resistance of the cell line to LY2409881. Similarly, the activation NF-kB in HBL1 is a result of the heterozygous missense mutation Y196F in the immunoreceptor tyrosine-based activation motif of CD79B (32). Mutation of Y196 in

### Table 1. Synergy of LY2409881 with HDAC inhibitors

<table>
<thead>
<tr>
<th></th>
<th>IC_{50}</th>
<th>IC_{40}</th>
<th>IC_{20}</th>
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<tbody>
<tr>
<td>(A) Synergy of LY2409881 and romidepsin</td>
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<tr>
<td>SUDHL2</td>
<td>0.81</td>
<td>0.4</td>
<td>0.03</td>
</tr>
<tr>
<td>RIVA</td>
<td>0.75</td>
<td>0.13</td>
<td>0.31</td>
</tr>
<tr>
<td>HBL1</td>
<td>1.02</td>
<td>0.51</td>
<td>0.42</td>
</tr>
<tr>
<td>LY10</td>
<td>0.92</td>
<td>0.84</td>
<td>0.39</td>
</tr>
<tr>
<td>LY7</td>
<td>0.48</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>LY8</td>
<td>0.75</td>
<td>0.05</td>
<td>0.01</td>
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<tr>
<td>(B) Synergy of LY2409881 and belinostat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUDHL2</td>
<td>0.52</td>
<td>0.63</td>
<td>0.39</td>
</tr>
<tr>
<td>RIVA</td>
<td>1.03</td>
<td>0.84</td>
<td>1.09</td>
</tr>
<tr>
<td>HBL1</td>
<td>1.33</td>
<td>3.23</td>
<td>5.81</td>
</tr>
<tr>
<td>LY10</td>
<td>0.88</td>
<td>0.84</td>
<td>0.98</td>
</tr>
<tr>
<td>LY7</td>
<td>1.18</td>
<td>1.67</td>
<td>2.95</td>
</tr>
<tr>
<td>LY8</td>
<td>0.93</td>
<td>0.36</td>
<td>0.07</td>
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<tr>
<td>RRR</td>
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<td>0.5–0.99</td>
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<tr>
<td>0.3–0.49</td>
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<tr>
<td>&lt;0.1</td>
<td>High</td>
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NOTE: (A) DLBCL cells were treated with LY2409881 or romidepsin at the concentrations that were expected to cause 20%, 40%, and 60% inhibition, i.e., IC_{50}, IC_{40}, and IC_{20}, respectively. In addition, cells were treated by the combination of the two drugs at IC_{50}/IC_{50}, IC_{40}/IC_{40}, and IC_{20}/IC_{20} concentrations. RRR was calculated as described in Materials and Methods, with lower RRR values indicating higher levels of synergy. (B) same as in (A), except belinostat replaced romidepsin. Four cell lines belong to the ABC subtype of DLBCL, including SUDHL2, RIVA, HBL1, and LY10. Three cell lines belong to the GCB subtype of DLBCL, including LY1, LY7, and LY8.
IKK2 Inhibition Potently Synergizes with HDAC Inhibitors

Figure 5.
The distinct effects of LY2409881 and HDAC inhibitors on p65/RelA, and the expression of HDACs in DLBCL. A–C, DNA-binding activity of p65 determined by EMSA. A, LY10 cells were treated by LY2409881 (LY), DMSO, or romidepsin (Romi) for 22 hours and prepared for nuclear extract and EMSA. B, same as in A, except that LY10 cells were treated with DMSO, belinostat (Bel), or vorinostat (Vor) for 22 hours. C, same as in A, except cells were treated with DMSO, LY2409881, and romidepsin at 5 or 10 nmol/L in combination with LY2409881 (R5 + LY or R10 + LY, respectively) for 8 hours to avoid excessive cell killing by the combination at 22 hours. D, nuclear protein samples, same as those used in C, were run for Western blot analysis probed by the indicated antibodies. The numbers represented normalized signal intensity with the HDAC1 level in the control DMSO sample as 1.00. RelA level in the control sample was also set to 1.00, then compared with HDAC1 level to derive a relative RelA level after normalized for the loading error, which was negligible. E, acetylation of p65 determined by Western blot analysis. Two ABC (LY10 and HBL1) and two GCB (LY7 and LY1) subtype DLBCL cells were treated by romidepsin at 2.3 and 10 nmol/L for 22 hours. Nuclear (nuc) and cytoplasmic (cy) extracts were electrophoresed and probed with the antiacetylated p65 antibody. F, the expression of HDAC isoforms varied in different cell lines. Four DLBCL cell lines were harvested in log phase. The nuclear and cytoplasmic extracts were separated by electrophoresis, and probed with the indicated antibodies. The numbers represented the fold change in expression relative to the control. Actin was used as an internal control. The data represent at least two independent experiments.

CD79B impairs association of the negative regulatory Lyn kinase, leading to activation of the BTK, which is known to positively regulate both the classical and alternative NF-κB pathways (33). It has also been shown that the PI3K pathway is constitutively activated in HBL1 (34). As a result, the HBL1 cells treated by the IKK2 inhibitor may survive using the prosurvival signals from the alternative NF-κB and the PI3K–AKT pathways. It is possible that the cell culture system may not be an ideal tool to study the ABC/GCB distinction and their representative phenotype. It remains unknown whether the complexity of DLBCL sensitivity to IKK2 inhibition seen in the cell lines also applies to patients. Although gene expression profiling is able to define the ABC subtype of DLBCL as characterized by activated NF-κB, there is no method to predict sensitivity to specific NF-κB inhibitors. As a number of drugs in clinical use or development are able to inhibit NF-κB, including BTK inhibitors, bortezomib, carfilzomib, revlimid, and NEDD8 activating enzyme (NAE) inhibitors, it will be important to use primary patient samples to study the relationship of NF-κB status and sensitivity to these drugs. The insight may help us determine which patients with the ABC subtype of DLBCL will respond to these drugs that affect NF-κB. Finally, we demonstrated that LY2409881 was active in models of T-cell lymphoma (Supplementary Table S1 and Fig. 2). As there is no highly effective treatment for ATLL, LY2409881 or similar drugs that inhibit NF-κB may be a promising strategy to improve the outcome of patients with ATLL.

Although LY2409881 is not a highly potent drug as a single agent, we have found that it markedly synergizes with other antitumor drugs. In particular, the synergy of LY2409881 and the HDAC inhibitor romidepsin was substantial and consistently observed in all the tested DLBCL cell lines. The mechanistic basis of the synergy is at least partially explained by how LY2409881 and romidepsin exert opposite effects on the NF-κB pathway on
multiple aspects, particularly p65/RelA. LY2409881 inhibited phosphorylation of IkB (Fig. 1C), leading to accumulation of IkB in the cytoplasm. LY2409881 blocked the accumulation of p50 and p65 in the nucleus (Figs. 1D, 1E, and 5D), and decreased the capacity of p65 binding to its substrate DNA (Figs. 5A and C). Romidepsin, on the other hand, increased the binding capacity of p65 to the NF-kB DNA (Figs. 5A and C). It is worth noting that acetylated p65 was accumulated in the nucleus of four DLBCL cell lines, irrespective of the ABC and GCB subtypes (Fig. 5E), but was least abundant in the HBL1 cell line. It is well established that acetylation of p65 facilitates its nuclear localization (35). Furthermore, acetylation of p65 is facilitated by its phosphorylation at Serine 536, which is mediated by IKK2 in models of multiple myeloma (36). These results demonstrate that romidepsin stimulates acetylation of p65 in DLBCL cells irrespective of the ABC and GCB subtypes, leading to increased p65 and DNA binding and activation of the NF-kB transcription program. LY24098881, on the other hand, inhibits nuclear localization and the DNA-binding activity of p65, likely due to the inhibitory effect of IKK2 inhibition on the phosphorylation, acetylation, nuclear localization, and activity of p65. Collectively, LY2409881 may prevent romidepsin-mediated NF-kB activation either indirectly, by increasing the level of IkB, or directly, by inhibiting the phosphorylation and acetylation of p65. We believe these effects underlie the high level and consistent synergism of LY2409881 and romidepsin in all studied DLBCL cell lines. The observation that the synergism occurred in both the ABC and GCB cell lines obviously raises the possibility that other uncharacterized mechanisms may contribute to the ubiquitous synergy, possibly due to the pleiotropic effects of romidepsin. Other IKK2 inhibitors were not synergistic with romidepsin consistently (Fig. 4H), presumably because they may not cause the same biochemical changes as LY2409881. LY2409881 also synergized with another HDAC inhibitor, belinostat, in some DLBCL cell lines. The protein level of HDAC isoforms may be a potential determinant of the lack of synergy between LY2409881 and belinostat in some cell lines such as HBL1 (Fig. 5F and Table 1). Finally, the IKK2 inhibitors, CYL-19s and CYL-26z, were recently found to increase p53 stability and p21 expression (37). As romidepsin also stimulates the expression of p21, the IKK2 inhibitor LY2409881 and romidepsin may synergistically increase p21, leading to cell-cycle arrest and apoptosis.

In summary, we have demonstrated that a novel IKK2 inhibitor, LY2409881, effectively inhibits models of B- and T-cell lymphoma, where NF-kB is a well-established pathogenetic factor. We validated the activity of LY2409881 using a xenograft mouse model of DLBCL. LY2409881 was demonstrated to directly and indirectly inhibit romidepsin-mediated activation of NF-kB, underlying a strong and consistent synergism with romidepsin in models of DLBCL. We believe combining LY2409881 and romidepsin will be a highly effective strategy in treating lymphoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Deng, M. Lipstein, R. Rodriguez, W.Y. Tsai, A.S. Wasmuth, O.A. O’Connor
Writing, review, and/or revision of the manuscript: C. Deng, M. Lipstein, R. Rodriguez, C. McIntosh, S. Jaken, O.A. O’Connor
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Deng, M. Lipstein, O.A. O’Connor
Study supervision: C. Deng, O.A. O’Connor
Other (chemistry support): A.S. Wasmuth

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IKK2 Inhibition Potently Synergizes with HDAC Inhibitors


The Novel IKK2 Inhibitor LY2409881 Potently Synergizes with Histone Deacetylase Inhibitors in Preclinical Models of Lymphoma through the Downregulation of NF-κB

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