

Small Molecule Inhibitors of I κ B Kinase Are Selectively Toxic for Subgroups of Diffuse Large B-Cell Lymphoma Defined by Gene Expression Profiling

Lloyd T. Lam,¹ R. Eric Davis,¹ Jackie Pierce,² Michael Hepperle,² Yajun Xu,² Maria Hottet,² Yuhua Nong,² Danyi Wen,² Julian Adams,² Lenny Dang,² and Louis M. Staudt¹

¹Metabolism Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland and ²Millennium Pharmaceuticals, Cambridge, Massachusetts

ABSTRACT

Constitutive activation of the NF- κ B pathway is required for survival of the activated B cell–like (ABC) subgroup of diffuse large B-cell lymphoma (DLBCL). Here we show that a small molecule I κ B kinase (IKK) inhibitor, PS-1145, and related compounds are toxic for ABC DLBCL cell lines but not for cell lines derived from the other prevalent form of DLBCL, germinal center B cell–like DLBCL. Treatment of ABC lines with these inhibitors rapidly induced a series of gene expression changes that were attributable to cessation of constitutive IKK activity, similar to changes induced by acute expression of genetic inhibitors of NF- κ B, confirming the effectiveness and specificity of this compound. Before cell death, inhibition of IKK also induced features of apoptosis and an arrest in the G₁ phase of the cell cycle. To test further the specificity of this toxicity, an inducible form of NF- κ B was created by fusing the p65 NF- κ B subunit with the ligand-binding domain of the estrogen receptor (p65-ERD). In the presence of tamoxifen, p65-ERD reversed the toxicity of IKK inhibition and restored expression of many NF- κ B target genes. Another subgroup of DLBCL, primary mediastinal B-cell lymphoma (PMBL), also expresses NF- κ B target genes, and treatment of a PMBL cell line with an IKK inhibitor was toxic and induced gene expression changes of a distinct group of NF- κ B target genes. These studies validate the NF- κ B pathway as a promising therapeutic target in ABC DLBCL, PMBL, and other lymphomas that depend on the activity of NF- κ B for survival and proliferation.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL), an aggressive non-Hodgkin's lymphoma, has been shown to consist of at least three molecular subgroups that differ in the expression of several hundred genes (1–5). Several lines of evidence suggest that these DLBCL subgroups can be regarded as distinct diseases that cannot be distinguished by morphology. First, the DLBCL subgroups seem to derive from distinct stages of B-cell differentiation. The germinal center B cell–like (GCB) subgroup shares a broad gene expression program with normal germinal center B cells, which are therefore the presumed cell of origin for this subgroup (1, 3–5). The activated B cell–like (ABC) subgroup has lost expression of most germinal center B-cell genes and instead has gained expression of some plasma cell–associated genes, suggesting that it may be derived from a cell that is developmentally between the germinal center B cell and the plasma cell (1, 3–5). Finally, primary mediastinal B-cell lymphoma (PMBL) is a distinct subgroup of DLBCL that is presumed to originate from a thymic B cell because this tumor type characteristically arises within the thymus. The PMBL, GCB, and ABC subgroups of DLBCL have significantly different survival rates following conventional multiagent chemotherapy, with 5-year survival rates of 63%, 59%, and 30%, respectively (1–5).

The DLBCL subgroups also differ strikingly in the oncogenic pathways that they engage (2–5). In particular, the NF- κ B pathway has been shown to be constitutively active in ABC DLBCL but not GCB DLBCL (6). In mammals, the NF- κ B protein family consists of five distinct subunits (p50/p105, p65/RelA, c-rel, RelB, and p52/p100) that exhibit a conserved central region called the Rel homology domain important for DNA binding and dimerization (7). NF- κ B activity plays a crucial role in B-cell development and is regulated mainly by members of the I κ B family (8). Upon activation of the upstream I κ B kinase (IKK) complex, I κ B is phosphorylated, ubiquitinated, and targeted for proteasomal degradation (9). This releases NF- κ B from the NF- κ B-I κ B complex in the cytoplasm and allows its translocation into the nucleus for transcriptional activity. Deregulation of the NF- κ B pathway has been shown in many cancer types to facilitate cell survival and/or cell proliferation (for review, see refs. 10–12). Although the mechanism mediating cell survival is not completely clear, several NF- κ B–regulated proteins block programmed cell deaths, including members of the Bcl-2 family (BCL2, BCLXL, and A1/Bfl-1), the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, and XIAP), and GADD45 β (10, 13–17). The NF- κ B pathway also influences proliferation, which may involve NF- κ B target genes such as *cyclin D2*, *c-myc*, and *IL-6* (18–20).

Cell lines of the ABC DLBCL type, but not GCB DLBCL cell lines, were found to have constitutive IKK activity, which caused nuclear accumulation of NF- κ B factors (6).

Received 6/21/04; revised 9/3/04; accepted 9/13/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: J. Pierce is currently at Merck Research Laboratories, Boston, MA. J. Adams is currently at Infinity Pharmaceuticals, Inc., Cambridge, MA. **Requests for reprints:** Louis M. Staudt, 9000 Rockville Pike, Building 10, Room 5A/02, Bethesda, MD 20892. Phone: 301-402-1892; Fax: 301-496-9956; E-mail: lstaudt@mail.nih.gov.

©2005 American Association for Cancer Research.

Dominant-negative inhibitors of the NF- κ B pathway were shown to induce cell death of ABC DLBCL cells but not GCB DLBCL cells, suggesting that this pathway may be an attractive therapeutic target for certain DLBCL subgroups (6). More recently, PMBL has been shown characteristically to express a number of known NF- κ B target genes and have nuclear NF- κ B (2, 21). Unexpectedly, the gene expression profile of PMBL was found to overlap significantly with that of Hodgkin lymphoma, a lymphoma type in which the NF- κ B pathway is frequently activated (2, 21). The dependence of PMBLs on the NF- κ B pathway for survival has not been reported.

Given the potential of the NF- κ B pathway as a therapeutic target in DLBCL, we are interested in investigating whether small molecule inhibitors of this pathway should be developed for this indication. Many NF- κ B pathway inhibitors have been described in the literature (for review, see refs. 22–24). These molecules have been reported to decrease NF- κ B DNA binding, block I κ B degradation in the proteasome, inhibit phosphorylation of I κ B by IKK or block nuclear translocation of NF- κ B. However, for most of these inhibitors, the specificity of the inhibition and the potential for off-target effects have not been fully investigated.

In the present report, we investigated whether IKK inhibitors of the β -carboline class have potential as specific cytotoxic agents for DLBCL. We show that these agents are selectively toxic for certain DLBCL cell lines that have NF- κ B activity and induce gene expression changes that are similar to those caused by inhibition of NF- κ B using a dominant-active form of I κ B. These studies provide support for the future development of this class of IKK inhibitors for the therapy of DLBCL.

MATERIALS AND METHODS

Materials. PS-1145 and MLX105 were obtained from Millennium Pharmaceuticals. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide were purchased from Sigma (St. Louis, MO). Tamoxifen (4-HT) was purchased from Calbiochem (San Diego, CA). Recombinant human tumor necrosis factor α (TNF α) was purchased from R&D Systems (Minneapolis, MN). Poly-D-Lysine plates were purchased from BD Biocoat (Bedford, MA). Blasticidin was purchased from Life Technologies (Gaithersburg, MD). Q-VD (non-omethylated)-Oph (QVD) was purchased from Enzyme Systems Products (Livermore, CA).

OCI cell lines (OCI-Ly3, OCI-Ly7, OCI-Ly10, and OCI-Ly19), PMBL cell line K1106, primary effusion lymphoma cell line BC-1, and adult T-cell leukemia cell line HUT102, were maintained in Iscove's modified Dulbecco's medium with β -mercaptoethanol (55 μ mol/L), penicillin (50 units/mL), streptomycin (50 μ g/mL), and 20% heparinized normal human plasma. SUDHL-6 cells were maintained in RPMI 1640 with penicillin (50 units/mL), streptomycin (50 μ g/mL), and 10% fetal bovine serum. 293 Cells were maintained in DMEM with penicillin (50 units/mL), streptomycin (50 μ g/mL), and 10% fetal bovine serum. Cells were grown in a 37°C incubator in the presence of 5% carbon dioxide. PS-1145 and MLX105 were used at 25 μ mol/L except in the dose response experiments in Figs. 1, 2, 3, and 6.

Human PBMC Assays. Heparinized human whole blood was obtained from normal donors. Peripheral blood mononuclear

cells (PBMC) were separated on a Ficoll gradient (Amersham, Piscataway, NJ) and cultured in AIM-V media (Life Technologies). PS-1145 and MLX105 were serially diluted 1:1 in DMSO with final concentration ranging from 20 to 0.04 μ mol/L; 4×10^5 cells per well were seeded in a 96-well plate and incubated in the presence of 5% CO₂ at 37°C. Cells were preincubated with compound for 1 hour followed by stimulation with 100 ng/mL lipopolysaccharide for 5 hours. Supernatant was collected and was sent to PerBio (Pierce, Rockford, IL) for multiplex cytokine analysis.

IKK Assay to Determine the IC₅₀ of MLX105.

MLX105 is a close analogue of PS-1145, a member of the β -carboline class of IKK inhibitors (25, 26). The specificity of MLX105 inhibition was shown by testing MLX105 against IKK and a panel of 19 purified kinases. The following kinases were expressed and purified as recombinant protein in-house: His-tagged AurA (murine), His-tagged CDK2/cyclinE, glutathione S-transferase (GST)-tagged extracellular signal regulated kinase 2, His-tagged c-Jun NH2-terminal kinase 1, GST-tagged LCK, mitogen-activated protein kinase 2, GST-p38 δ , polo-like kinase 1, His-tagged p70S6 kinase, casein kinase II, PhosK (rabbit), PRAK (or MAPKAP kinase 5), Rho kinase α (rat), serum glucocorticoid regulated kinase 1, and His-tagged GSK3 β were generous gifts from University of Dundee, United Kingdom. DNA-PK was purified from HeLa S3 cells and activated with 10 ng/mL dsDNA. The following were purchased from commercial sources: protein kinase A (Biomol, Plymouth Meeting, PA), PKC α (EMD Biosciences, San Diego, CA), CamKII (Upstate Biotech, Lake Placid, NY). The IC₅₀ value against the IKK complex was determined using assay methods described previously (26). In short, Aurora A kinase assay measured the phosphorylation of biotinylated peptide with phospho-antibodies and streptavidin-coated plates using DELFIA as the readout method. All other assays measure kinase activity via the incorporation of ³³P into biotinylated polypeptides using a streptavidin-coated FlashPlate as the readout method.

Constructs and Stable Cell Lines. 293 cells were transfected with pNF- κ B-TA-Luc (Clontech, Palo Alto, CA) engineered to confer blasticidin resistance. After transfection, blasticidin was added for selection of positive clones. These clones were then evaluated by their responsiveness to phorbol 12-myristate 13-acetate/ionomycin or TNF α stimulation.

To assemble the I κ B-luciferase reporter, the Kozak and I κ B sequence (lacking a stop codon) were first excised from pI κ B-EGFP (Clontech) with *HindIII* and *BamHI* and inserted into the same sites of pBluescript. To remove a potential stop codon and place the *Photinus* luciferase sequence in the proper frame, pGL3 (Promega Co., Madison, WI) was modified by blunt religation after opening the *HindIII* and *NcoI* sites, followed by blunt religation of the *XhoI* site. The luciferase sequence and stop codon were then excised with *NheI* and *XbaI* and inserted into the pBluescript/I κ B construct opened with *SpeI* and *XbaI*. The majority of the 5' I κ B-3' luciferase sequence was then excised via *NotI* and a unique *XhoI* site within I κ B, and then joined to a 5' FLAG-tagged sequence of I κ B already contained in the vXY-IRES-puromycin retroviral plasmid (6) opened with the same enzymes. All junctions and changes were verified by DNA sequencing. Retroviral transduction of the reporter into B cell lines was by spin infection as previously described (6).

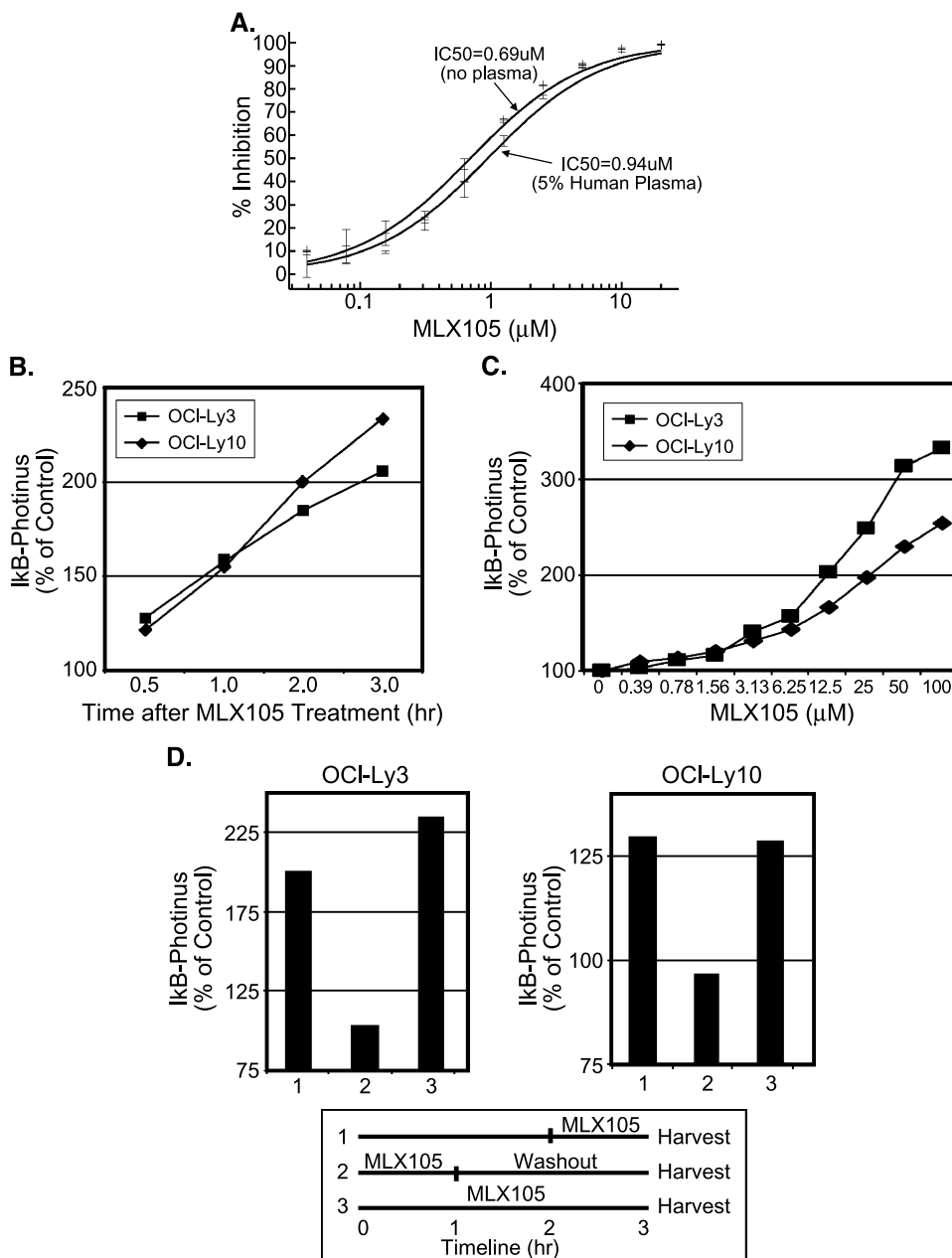


Fig. 1 Characterization of MLX105. *A*, 293 NF- κ B-TA-Luc cells were treated with increasing concentrations of MLX105 (0-20 μ mol/L) for 1 hour before stimulation with TNF α . Inhibition of response to TNF α stimulation, expressed as a % of control value. *B*, ABC DLBCL cell lines (OCI-Ly3 and OCI-Ly10) expressing the I κ B-Photinus luciferase fusion reporter were treated with 25 μ mol/L of MLX105 in a time course study. The luciferase readings at each time point are the % of the luciferase reading obtained with untreated cells. *C*, ABC DLBCL cell lines (OCI-Ly3 and OCI-Ly10) expressing the I κ B-Photinus luciferase fusion reporter were treated with increasing concentrations of MLX105 (0-100 μ mol/L) for 4 hours. The luciferase readings at each time point are the % of the luciferase reading obtained with untreated cells. *D*, ABC DLBCL cell lines (OCI-Ly3 and OCI-Ly10) expressing the I κ B-Photinus luciferase fusion reporter were treated with 25 μ mol/L of MLX105 according to the scheme. The luciferase readings at each time point are the % of the luciferase reading obtained with untreated cells.

Expression of the I κ B super-repressor in OCI-Ly3 cells for microarray analysis was as previously described (6), with modifications to achieve high-efficiency transduction. OCI-Ly3 cells were engineered to stably express the murine ecotropic retroviral receptor (mCAT; ref. 27). A pMSCV-Puro retroviral vector containing the mCAT sequence was modified to confer bleomycin resistance, by excising the PGK promoter and puromycin resistance gene at *Eco*RI and *Cla*I (blunted) sites, and replacing them with an IRES-bleomycin resistance cassette excised from pIRES-Bleo3 (Clontech) at *Eco*RI and *Xba*I (blunted) sites. After transduction of OCI-Ly3 with this retrovirus, bleomycin-resistant clones were isolated by limiting dilution and functionally tested for ecotropic receptor expression, based on their ability to be infected by the MgirL22Y ecotropic

retrovirus conferring green fluorescent protein (GFP) expression. The source of this virus was a stable producer line kindly provided by Cynthia Dunbar (NIH, Bethesda; MD) (28). Acute transductions were then done on an OCI-Ly3/mCAT clone that was highly infectable by ecotropic retrovirus, using a retroviral vector that expresses membrane-localizing enhanced GFP (EGFP) via a downstream internal ribosomal entry site (IRES) element. Three separate spin infections were carried out with empty vector or vector containing a 5' FLAG-tagged coding region of I κ B, mutated in the two serine phosphorylation sites (S32G/S36A; ref. 6). Retroviruses were prepared by calcium phosphate transfection of 293T producer cells, along with a helper plasmid (pCL-Eco) coding for gag, pol, and the murine retroviral envelope (29). Two days after infection, live cells for

microarray analysis were collected by Ficoll density gradient separation. Fluorescence-activated cell sorting analysis showed that >80% of cells were EGFP positive; subsequent culture confirmed the expected death of cells infected with the κ B super-repressor but not the empty vector.

The design of a form of NF- κ B that would be resistant to IKK inhibition was based on previous reports showing the potential consequence of mutating the nuclear localization sequence of p65 or cRel and fusion to the human estrogen receptor (ER) (17, 30, 31). We prepared a similar construct (p65-ERD) using a mutant cDNA of p65 in which the native nuclear localization sequence (KRKR) had been changed to DQNQ (32), from the original preparation (33) kindly provided by Keith Brown (NIH, Bethesda, MD). The wild-type ligand-binding domain of the human ER- β was PCR-amplified using a 5' primer with an in-frame *Bam*HI site before the Kozak sequence and a 3' primer with a FLAG sequence and an *Mfe*I site after the stop codon. Using these enzymes, the PCR product was used to replace the EGFP sequence in p κ B-EGFP. The nuclear localization sequence mutant p65 was PCR-amplified using a 5' primer with *Kpn*I site before the Kozak sequence and a 3' primer replacing the stop codon with an in-frame *Bam*HI site. Using these enzymes, the PCR product was used to replace the κ B sequence in the p κ B-ERD construct. The in-frame fusion and all coding sequences of the 5' mutant p65/3' ERD construct were verified by DNA sequencing. The p65-ERD construct was excised from flanking sites with *Xho*I and *Hpa*I and inserted into *Sal*I and *Eco*RV sites of pBluescript, then reexcised with *Xho*I and *Not*I for insertion into vXY-IRES-puromycin. After spin infection and puromycin selection, cell lines stably expressing p65-ERD were grown in phenol red-free medium containing human plasma or fetal bovine serum that was stripped with charcoal. Activation of p65-ERD was achieved by adding 4-hydroxy-tamoxifen (4-HT, 6 mmol/L in ethanol) at a final concentration of 3 μ mol/L.

Luciferase Assays. NF- κ B-TA-Luc stable 293 cells were seeded in black poly-D-Lysine 96-well plates 1 day before the assay at 0.5×10^6 cells per well. MLX105 was serially diluted (1:2) in DMSO to obtain a range of concentrations (0.04-20 μ mol/L). The medium was changed to AIM-V (with or without 5% human plasma) before adding the serially diluted MLX105 solution. After incubating with MLX105 at 37°C for 1 hour, the cells were stimulated with 10 ng/mL TNF α at 37°C for 1.5 hours. Cells were lysed with 1X passive lysis buffer and assayed with the luciferase assay system (Promega) according to the manufacturer's instructions. Triplicate assays were done to calculate the IC₅₀ using the Xfit software.

For the κ B-luciferase reporter, cells growing in culture medium were directly dispensed, in duplicate or triplicate, into V-bottomed 96-well plates. Additional medium containing MLX105 or DMSO control was added, and the plate was returned to the cell culture incubator for up to 4 hours before harvest. Cells were pelleted by centrifugation, the medium was removed by aspiration, and 50 μ L of Glo Lysis buffer (Promega) was added to each well. After lysis at room temperature for 15 minutes, or storage at -80°C, the lysate was mixed with 50 μ L of Bright Glo stable substrate (Promega) and transferred to an opaque flat plate for measurement of light

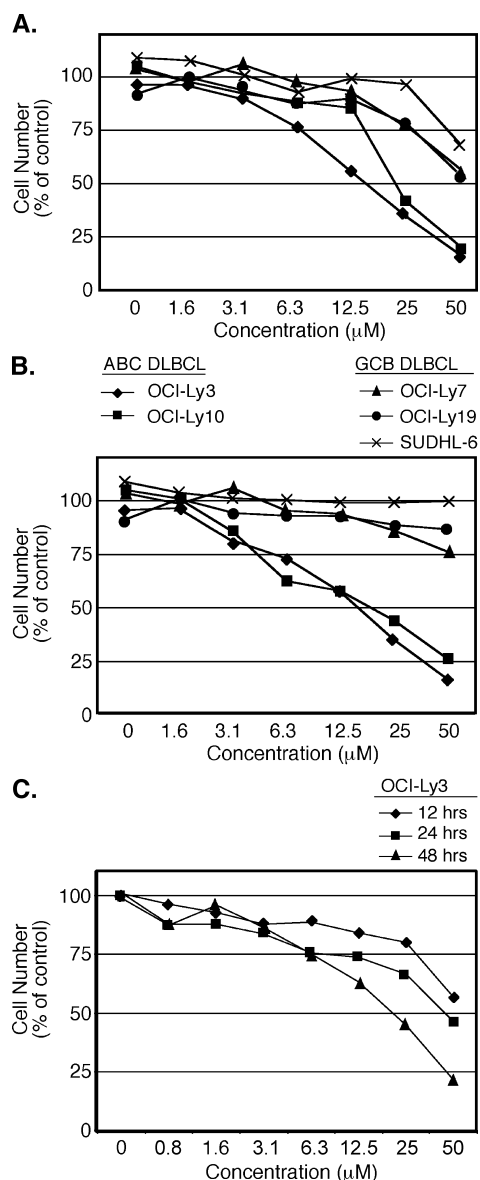


Fig. 2 Selective toxicity of MLX105 for ABC DLBCL cell lines. *A*, ABC DLBCL cell lines (OCI-Ly3 and OCI-Ly10) and GCB DLBCL cell lines (OCI-Ly7, OCI-Ly19, and SUDHL-6) were treated with increasing concentrations of PS-1145 (0-50 μ mol/L) for 48 hours and assigned for viability by MTT. The cell numbers at each drug dose are expressed as the % of cell numbers obtained with untreated cells cultured in parallel. *B*, ABC DLBCL cell lines (OCI-Ly3 and OCI-Ly10) and GCB DLBCL cell lines (OCI-Ly7, OCI-Ly19, and SUDHL-6) were treated with increasing concentration of MLX105 (0-50 μ mol/L) for 48 hours and assigned for viability by MTT. The cell numbers at each drug dose are expressed as the % of cell numbers obtained with untreated cells cultured in parallel. *C*, time course of MLX105 toxicity for ABC DLBCL cells. OCI-Ly3 cells were treated with increasing concentration of MLX105 (0-50 μ mol/L) for 12, 24, or 48 hours and assigned for viability by MTT. The cell numbers at each drug dose and time point are expressed as the % of cell numbers obtained with untreated cells cultured in parallel.

emission in a luminometer. The values obtained for untreated controls were taken as 100% and the treated samples were expressed as percentage of the corresponding controls.

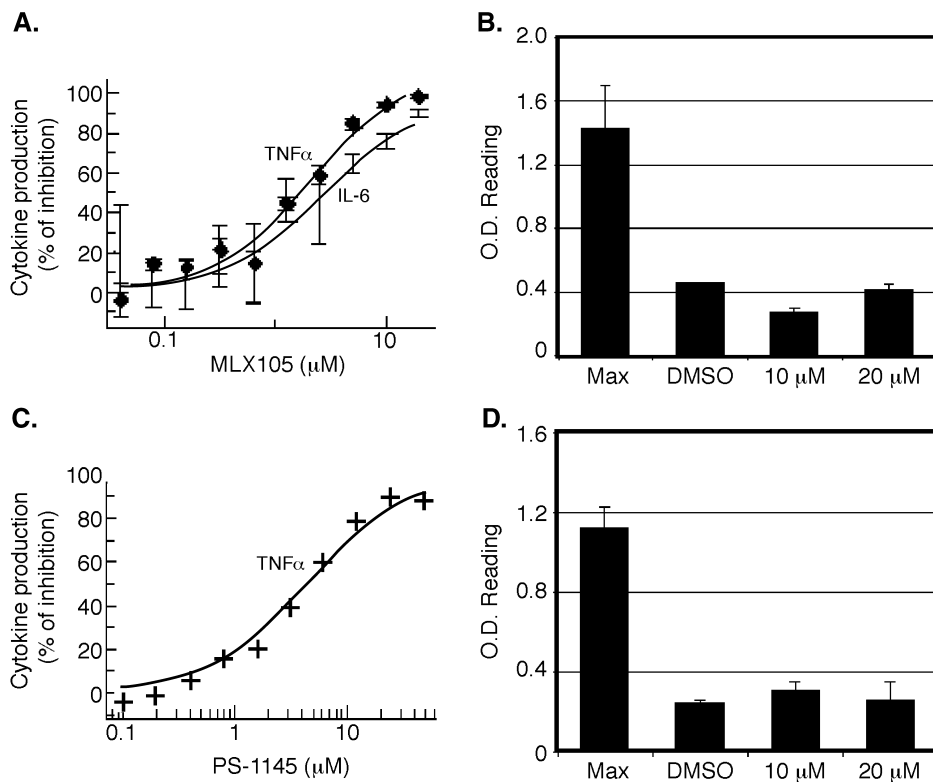


Fig. 3 No significant cytotoxicity of MLX105 and PS-1145 for PBMCs. *A*, effect of MLX105 on LPS-stimulated $\text{TNF}\alpha$ and IL-6 production in PBMCs. *B*, cytotoxicity assay (lactate dehydrogenase release) in PBMCs treated with 10 or 20 $\mu\text{mol/L}$ MLX105 for 6 hours. *C*, effect of PS-1145 on LPS-stimulated $\text{TNF}\alpha$ production in PBMCs. *D*, cytotoxicity assay (lactate dehydrogenase release) in PBMCs treated with 10 or 20 $\mu\text{mol/L}$ PS-1145 for 6 hours.

Lactate Dehydrogenase Assays. The cytotoxicity of MLX105 and PS-1145 was monitored by the measurement of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant. PBMCs were treated with MLX105 or PS-1145 for 6 hours. Supernatant from human PBMCs assay was collected and lactate dehydrogenase activity was measured following the instructions of the Cytotoxicity Detection Kit (lactate dehydrogenase; Roche, Nutley, NJ). The control (Max) was obtained by making a cell lysate using 2% Triton X-100.

MTT Assays. MTT assays were done as described (34). In brief, cells grown in 96-well plate were treated with increasing concentration of MLX105 (0-50 $\mu\text{mol/L}$) for 12, 24, or 48 hours. MTT was added to the cells 2 hours before harvesting. Cells were lysed in isopropanol with 1% hydrochloric acid. The plate was read with a 96-well spectrometer using a 570-nm filter. The background was subtracted using a dual-wavelength setting of 570 and 630 nm.

Gene Expression Profiling and Data Analysis. DNA microarray analysis was done as described (34). Total RNA was prepared using the Trizol reagent (Life Technologies) according to the manufacturer's instruction. For each sample, 40 μg of total RNA were used for the fluorescent probe preparation reaction. The raw gene expression data from each DNA microarray hybridization were normalized as described (35). Gene expression data are available at http://lymphochip.nih.gov/IKK_inhibitor/LLweb/Homepage.html. Hierarchical clustering was done using Cluster software and visualized using the Tree View software (36).

For MLX105 treatment experiments, mRNA from untreated cells was labeled with the Cy3 dye and mRNA from MLX105 treated cells was labeled with the Cy5 dye. Data was selected such that the Cy3 signal intensity was >250 relative fluorescent units. For p65-ERD rescue experiments, mRNA from cells treated with tamoxifen and MLX105 was labeled with the Cy5 dye and mRNA from MLX105-treated cells was labeled with the Cy3 dye. Data was selected such that the Cy5 signal intensity was >250 relative fluorescent units for the Cy5 channel. For the $\text{I}\kappa\text{B}$ super-repressor experiments, mRNA from cells transduced with retroviruses expressing the $\text{I}\kappa\text{B}$ super-repressor was labeled with the Cy5 dye and mRNA from cells transduced with the control retrovirus was labeled with the Cy3 Dye. Data were selected such that the Cy3 signal intensity was >500 relative fluorescent units. For PS-1145 treatment experiments, mRNA from untreated cells was labeled with the Cy3 dye and mRNA from PS-1145 treated cells was labeled with the Cy5 dye. Data was selected such that the Cy3 signal intensity was >250 relative fluorescent units.

In the MLX105 time course studies of the ABC DLBCL cell lines, a gene was selected as an NF- κB target gene in both OCI-Ly3 and OCI-Ly10 cells if MLX105 decreased expression of the gene by >1.4-fold at ≥ 2 time points in both the OCI-Ly3 and OCI-Ly10 time courses. Genes that were affected by PS-1145 were selected from among the NF- κB target genes in the MLX105 time course studies. Genes were selected if PS-1145 decreased their expression by >1.4-fold at ≥ 2 time points. In the p65-ERD experiments, p65 target genes were selected from the set of NF- κB target genes identified in the

MLX105 time course experiments. A gene was selected to be a p65 target gene in both OCI-Ly3 and OCI-Ly10 if it was >1.4-fold more highly expressed in tamoxifen-treated cells than in untreated cells at ≥ 3 time points within the OCI-Ly3 and OCI-Ly10 time series. Genes that were affected by the I κ B super-repressor were selected from among the NF- κ B target genes in the MLX105 time course studies. Genes were selected if I κ B super-repressor decreased their expression by >1.4-fold at ≥ 2 time points and MLX105 decreased their expression by >1.4-fold at ≥ 1 time point. In the MLX105 time course studies of the PMBL cell line, a gene was selected as a NF- κ B target gene in K1106 cells if MLX105 decreased expression of the gene by >1.4-fold in at least one time point.

DNA Content Analysis after MLX105 Treatment. Cells were treated with 25 μ mol/L MLX105 for 72 hours. After pelleting of cells and resuspension in 0.5 mL PBS, 6 mL of 80% ethanol were added while vortexing. Cells were fixed and stored at 4°C until analysis. Cells were washed once with buffer (1% fetal bovine serum in PBS), incubated for 30 minutes at 37°C in the same buffer containing propidium iodide (10 μ g/mL) and RNaseA (0.25 mg/mL), and then analyzed by fluorescence-activated cell sorting without washing. ModFit LT software (Verity Software House, Inc., Topsham, ME) was used to quantitate relative proportions of each phase of the cell cycle.

Detection of Apoptosis. Activation of caspase 3 and/or caspase 7 was measured in the presence (20 μ mol/L) or absence of QVD using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. Annexin V staining was done with the Annexin V-PE apoptosis detection kit I (BD PharMingen, San Diego, CA) according to the manufacturer's instructions. 7-Aminoactinomycin D was used to discriminate between live and dead cells. Mitochondrial membrane potential was quantified with the methyl ester of tetramethylrhodamine (Molecular Probes, Eugene, OR). Cells were loaded with tetramethylrhodamine (final concentration, 20 nmol/L) in culture for 30 minutes, then stained with 7-aminoactinomycin D for live and dead cells discrimination and kept in medium with tetramethylrhodamine at one third of the loading concentration during fluorescence-activated cell sorting analysis.

RESULTS

Selective Toxicity of IKK Inhibitors for ABC DLBCL Cells. Previously, gene expression profiling showed that two DLBCL cell lines, OCI-Ly3 and OCI-Ly10, are excellent models for the ABC DLBCL subgroup since they resemble primary ABC DLBCL tumors in gene expression (4). Similarly, several DLBCL cell lines (e.g., OCI-Ly7, OCI-Ly19, and SUDHL-6) serve as models of GCB DLBCL because they resemble primary GCB DLBCL tumors in gene expression (4). The ABC DLBCL cell lines have constitutive nuclear NF- κ B due to constitutive IKK activation, and this is not a feature of the GCB DLBCL cell lines. Given that dominant interference with the NF- κ B pathway was toxic to the ABC DLBCL cell lines (6), we evaluated whether small molecule inhibitors of IKK would show similar toxicity.

PS-1145 is a small molecule inhibitor of IKK in the β -carboline family (25) that has been shown to be effective in blocking the NF- κ B pathway in multiple myeloma cell lines (26). Various structural analogues of PS-1145 have since been

reported to have similar abilities to inhibit IKK (25). One PS-1145 analogue, MLX105, has less affinity for human serum proteins than PS-1145 (data not shown), a feature that proved useful in our cell culture systems since the ABC DLBCL cell lines require human plasma for optimal growth *in vitro*. MLX105 inhibited IKK complex activity with an IC₅₀ of 25 nmol/L and had negligible cross-reactivity to other kinases tested (Table 1).

We investigated the performance of MLX105 as an IKK inhibitor in several cell-based assays. One assay used 293 cells stably transfected with a transcriptional reporter in which a promoter construct of four tandem copies of NF- κ B binding site was used to drive expression of a luciferase gene. MLX105 inhibited the increase in transcriptional activity caused by TNF α treatment of these cells in a dose-dependent fashion, with an IC₅₀ of 0.69 μ mol/L in the absence of human plasma and 0.94 μ mol/L in the presence of 5% human plasma (Fig. 1A).

To measure IKK activity more directly in a cell-based assay, we created a nontranscriptional reporter by fusing *Photinus* luciferase to I κ B α . A retroviral vector was used to stably express the I κ B-*Photinus* fusion protein in cells under the control of the retroviral long-terminal repeat promoter, and its level was easily quantified in cell lysates by a luciferase assay. Long-terminal repeat promoter function is considered to be relatively independent of NF- κ B activity, and this is supported by our experience with multiple cell lines used in this study. For example, expression of long-terminal repeat-driven unmodified *Photinus* or *Renilla* luciferase proteins is unaffected by manipulations expected to change NF- κ B activity (data not shown). In contrast, it was expected that the I κ B-luciferase protein could be degraded as a result of IKK activity, because this has been reported for an I κ B-EGFP reporter (37). Extensive testing of the I κ B-*Photinus* reporter in GCB DLBCL lines showed that stimulation with phorbol 12-myristate 13-acetate/ionomycin or CD40 ligation, known to activate IKK, produced a rapid and substantial decrease in luciferase activity. Because I κ B-*Photinus* is degraded as a result of IKK activity, but its transcription is relatively independent of NF- κ B activity, the level of the reporter protein serves as an indicator of IKK activity: a decrease in luciferase activity is observed as IKK activity increases, and vice versa. In ABC DLBCL reporter lines, the I κ B-*Photinus* reporter level rose after exposure to MLX105 in a time- and dose-dependent fashion (Fig. 1B and C). Similar rises were produced by MLX105 in other lymphoid lines with constitutive IKK activity but not in lines in which the NF- κ B is not active (data not shown). These reporter studies confirmed the presence of constitutive IKK activity in ABC DLBCL lines, and its inhibition by MLX105. In addition, the I κ B-*Photinus* reporter showed that MLX105 has a rapid onset of action and that its effect on constitutive IKK activity is reversible by washing (Fig. 1D).

To determine if PS-1145 and MLX105 are specifically toxic to the ABC DLBCL cell lines, we treated the DLBCL cell lines with either molecule and used an MTT assay to measure live cells over time. Both ABC DLBCL cell lines were susceptible to PS-1145 and MLX105, with IC₅₀s of ~ 18 μ mol/L, whereas the three GCB DLBCL cells were less affected by these agents (IC₅₀ not reached at 50 μ mol/L; Fig. 2A and B). This result showed that PS-1145 and MLX105 have selective toxicity for ABC DLBCLs, which is consistent with the fact that NF- κ B activity is

characteristic of this DLBCL subgroup. Figure 2C shows the dose- and time-dependent toxicity of OCI-Ly3 cells treated with increasing concentrations of MLX105 (0–50 $\mu\text{mol/L}$) for 12, 24, and 48 hours.

To determine the efficacy of PS-1145 and MLX105 in inhibiting NF- κ B–dependent cytokine production and whether these agents are toxic to normal cell types, we have treated PBMCs with these inhibitors. We first determined the effective concentration of these inhibitors in inhibiting lipopolysaccharide-stimulated TNF α or IL-6 production in PBMCs. MLX105 inhibited TNF α or IL-6 production with IC₅₀ of 2 and 2.8 $\mu\text{mol/L}$, whereas PS-1145 inhibited TNF α production with IC₅₀ of 4.7 $\mu\text{mol/L}$ (Fig. 3A and C). Cytotoxicity of these inhibitors towards these cells was determined by lactate dehydrogenase release from the cytosol of damaged cells. As shown in Fig. 3B and D, no significant cytotoxicity was observed in PBMCs with 20 $\mu\text{mol/L}$ treatment of either MLX105 or PS-1145, suggesting that these compounds are pharmacologically specific.

To show that the toxicity of MLX105 in ABC DLBCL cell lines was due to inhibition of the NF- κ B pathway, we tested whether enforced expression of the p65 NF- κ B subunit (RelA) in an IKK-independent fashion could rescue ABC DLBCL cells from MLX105 toxicity. To this end, we constructed an inducible form of NF- κ B p65, termed p65-ERD, in which the ligand-binding domain of the estrogen receptor (ERD) is fused to the COOH terminus of a mutant form of p65 (Fig. 4C). Binding of estrogen or estrogen analogues (e.g., 4-hydroxytamoxifen, 4-HT) to ERD fusion proteins is thought to induce a conformational change that can alter their function and/or subcellular localization. In addition, we mutated the p65 nuclear localization sequence in the p65-ERD fusion protein so that I κ Bs cannot bind, thereby allowing p65-ERD to enter the nucleus irrespective of the amount of I κ B in the cell, and therefore irrespective of the level of IKK activity. A retroviral vector was used to stably express p65-ERD in ABC and GCB DLBCL lines. Tight control and inducibility of p65-ERD were shown in the GCB line BJAB, in which significant amounts of CD83, a known target of NF- κ B, was induced within 3 hours after addition of 4-HT (data not shown). In the absence of 4-HT, MLX105 killed the p65-ERD-transduced forms of both OCI-Ly3 and OCI-Ly10, but not OCI-Ly7 (Fig. 4A and B), similar to previous observations in wild-type cells (Fig. 2A). Activation of the p65-ERD fusion protein with 4-HT substantially rescued OCI-Ly3 and OCI-Ly10 cells from MLX105 toxicity (Fig. 4A and B). This result supports the view that MLX105 kills ABC DLBCLs by inhibiting the NF- κ B pathway.

Gene Expression Changes Caused by IKK Inhibition.

Given the biochemical and functional specificity of MLX105, we next used this compound to identify which genes are activated by NF- κ B signaling in the ABC DLBCL cell lines. We used Lymphochip DNA microarrays to profile gene expression changes in OCI-Ly3 or OCI-Ly10 cells that were treated with PS-1145 or MLX105 for the indicated times (Fig. 5A). We found that PS-1145 and MLX105 inhibit the expression of many known NF- κ B target genes in both cell lines (e.g., *A1*, *A20*, *c-IAP2*, *I κ B α* , *TNF α* , and *IRF4*; refs. 38, 39). Many of these are important antiapoptotic genes (e.g., *A1*, *A20*, *c-IAP2*, and *GADD45 β*) and the down-regulation of these proteins may contribute to the cytotoxic effect of PS-1145 and MLX105 on

these cells. Since p65/RelA could rescue the cells from MLX105-induced cell death (Fig. 4), we have studied the genes that might play a role in this rescue by gene expression profiling. We found that certain antiapoptotic genes (e.g., *A1*, *A20*, *c-IAP2*, and *GADD45 β*) are regulated by p65/RelA (Fig. 5B). Whether one of these genes is the crucial determinant of cell survival, or multiple genes, is currently unclear. We next compared gene expression changes in cells acutely expressing the super-repressor form of I κ B α with gene expression changes induced by MLX105 (Fig. 5C). OCI-Ly3 cells were transduced with high efficiency with a retrovirus expressing the super-repressor form of I κ B α , and cells were harvested for gene expression profiling 48 hours later. Figure 5C shows that NF- κ B target genes that were down-regulated by MLX105 treatment for 36 to 48 hours were also inhibited by the super-repressor form of I κ B α . These results provide further support for the specificity of MLX105 inhibition of the NF- κ B pathway.

Cell Cycle Arrest and Apoptosis. At these later time points, we noted that a large number of genes related to cellular proliferation were down-regulated (data not shown). These genes belong to the proliferation gene expression signature, which includes genes expressed more highly in dividing cells than in quiescent cells (38). To illustrate this observation, we averaged the expression levels of six proliferation signature genes that are involved in G₂-M phase cell cycle progression (i.e., *AURKB*, *PLK1*, *BIRC5*, *CENPF*, *STK6*, and *ASPM*). The expression of this G₂-M phase gene expression signature was decreased by 75% upon treatment with MLX105 for 48 hours (Fig. 6A). In addition, both ABC DLBCL cell lines were blocked in the G₁ phase of the cell cycle by MLX105 treatment (Fig. 6B), in keeping with anti-proliferation effects of the super-repressor form of I κ B α reported previously (6). To determine if apoptosis was induced with MLX105 treatment, we measured the activity of “executioner” caspase 3 and/or caspase 7 in these cells (Fig. 6C). Caspase activation was observed with MLX105 treatment in both ABC DLBCL cell lines and was blocked by incubating the cells with QVD, a broad caspase inhibitor. In addition, we confirmed that MLX105 induces apoptosis by staining with Annexin V (Fig. 6D), and with tetramethylrhodamine to measure the loss of mitochondria membrane potential (Fig. 6E). We conclude that inhibiting IKK activity in the ABC DLBCL cell lines with MLX105 leads to cell cycle arrest and apoptosis.

Toxicity of IKK Inhibitors for Other Lymphoma Types.

Recently, another subgroup of DLBCL, primary mediastinal B cell lymphoma, was studied by gene expression profiling and shown to express a number of known NF- κ B target genes (2, 21). The cell line K1106 is a good model of PMBL because it expresses highly many of the genes that are characteristically expressed in primary tumor samples from PMBL patients, including many NF- κ B target genes (2). Treatment with MLX105 killed K1106 cells, although with an IC₅₀ that was ~4-fold greater than that observed for OCI-Ly3 cells (Fig. 7A). MLX105 treatment of K1106 cells resulted in decreased expression of a discrete set of genes (Fig. 5D), many of which were also identified as NF- κ B target genes in ABC DLBCL cell lines (Fig. 5A). However, some NF- κ B target genes were restricted to either the PMBL cell line K1106 or the ABC DLBCL cell lines. Whereas this could reflect idiosyncrasies of

Table 1 Kinase selectivity data for MLX105

Kinase	IC ₅₀ ($\mu\text{mol/L}$)	Kinase	IC ₅₀ ($\mu\text{mol/L}$)
AURORA-A	>100	MAPKAPK2	>100
CAMKII	>100	P38	>100
CDK2/cyclinE	>100	PHOSK	>100
CKII	22	PKA	>100
DNA-PK	12	PKC- α	98
ERK2	>100	PLK	>300
GSK3	>100	PRAK	>100
IKK2	0.025	P70S6K	>100
JNK1-CJUN	>100	ROKA	>100
LCK	>100	SGK1	>100

the cell lines used, it is possible that NF- κ B activity in DLBCLs of different subgroups may regulate distinct genes. In support of this hypothesis, some of the NF- κ B target genes identified in the DLBCL cell lines were differentially expressed between primary tumor biopsies derived from ABC DLBCL and PMBL (2, 3). In particular, *IRF4*, *PIM1*, and *DIFF48* were identified as NF- κ B target genes in the ABC DLBCL cell lines but not in K1106, and these three genes were more highly expressed in ABC DLBCL tumor biopsies than in PMBL tumor biopsies ($P < 0.001$; data not shown). Conversely, *SPI1* and *RAFTLIN* were identified as NF- κ B target genes in K1106 but not in the ABC DLBCL cell lines, and these two genes were more highly expressed in PMBL tumors than in ABC DLBCL tumors ($P < 0.001$; data not shown).

Several other lymphoma types have previously been characterized as having constitutive NF- κ B activity, including primary effusion lymphoma (40) and adult T-cell leukemia/lymphoma (41). We treated the primary effusion lymphoma cell line BC-1 with MLX105 and found that the cells were killed with an IC₅₀ that was comparable to that of the ABC

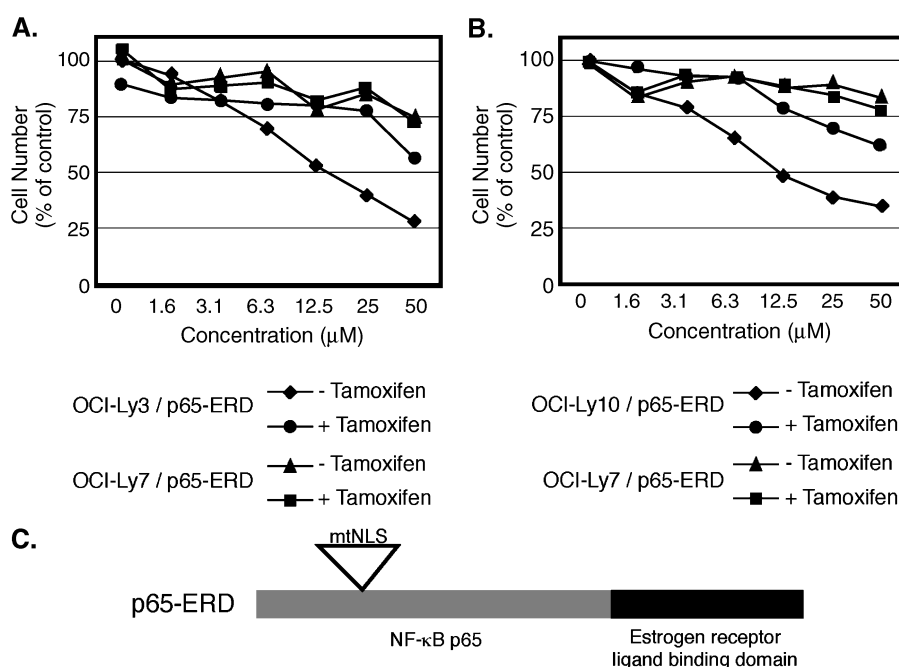
cell line OCI-Ly3 (Fig. 7B). Similarly, MLX105 treatment was toxic for HUT102, a human T-cell leukemia virus type I-infected adult T-cell leukemia cell line. These findings show that a broad range of lymphoma types depend on NF- κ B signaling for survival.

DISCUSSION

The NF- κ B pathway was first implicated in the regulation of immune and inflammatory mechanisms but recently has also been linked to cell survival, proliferation, and oncogenesis in many cell types (10–12). In the present report, we show that inhibition of the NF- κ B pathway using small molecule IKK inhibitors is toxic for certain subgroups of DLBCL that are defined by gene expression profiling. These findings, together with previous studies (6), validate the NF- κ B pathway as a therapeutic target for some DLBCL subgroups. The present results provide support for the continued development of small molecule IKK inhibitors for the treatment of lymphomas.

Various lines of evidence support the view that the β -carboline class of IKK inhibitors has high specificity for the NF- κ B pathway. First, these inhibitors show low nanomolar inhibition of IKK2 *in vitro*, but have considerably higher IC₅₀ values for other kinases. Second, MLX105 was cytotoxic for ABC DLBCL and PMBL cells, which have constitutive IKK activation, but not GCB DLBCL cells, which do not. Third, the p50 NF- κ B subunit could block the cytotoxicity of MLX105 for ABC DLBCL cells, which argues that any potential off-target effects of this small molecule do not contribute appreciably to its ability to kill these lymphoma cells. Fourth, treatment of ABC DLBCL cells with these inhibitors decreased the expression of a wide variety of known NF- κ B target genes, and the p50 subunit of NF- κ B was able

Fig. 4 NF- κ B p65 rescues ABC DLBCL cells from toxicity of an IKK kinase inhibitor. A and B, OCI-Ly3, OCI-Ly10, and OCI-Ly7 cells that expressed the p65-ERD fusion protein were treated with increasing concentrations of MLX105 (0–50 $\mu\text{mol/L}$) for 48 hours in the presence or absence of 4-HT to activate p65-ERD. The cells were assayed for viability by MTT. The cell numbers at each drug dose and treatment group are the % of cell numbers obtained with untreated cells cultured in parallel. C, schematic diagram of the p65-ERD fusion protein, showing the location of an introduced mutation in the p65 nuclear localization motif (*mtNLS*).



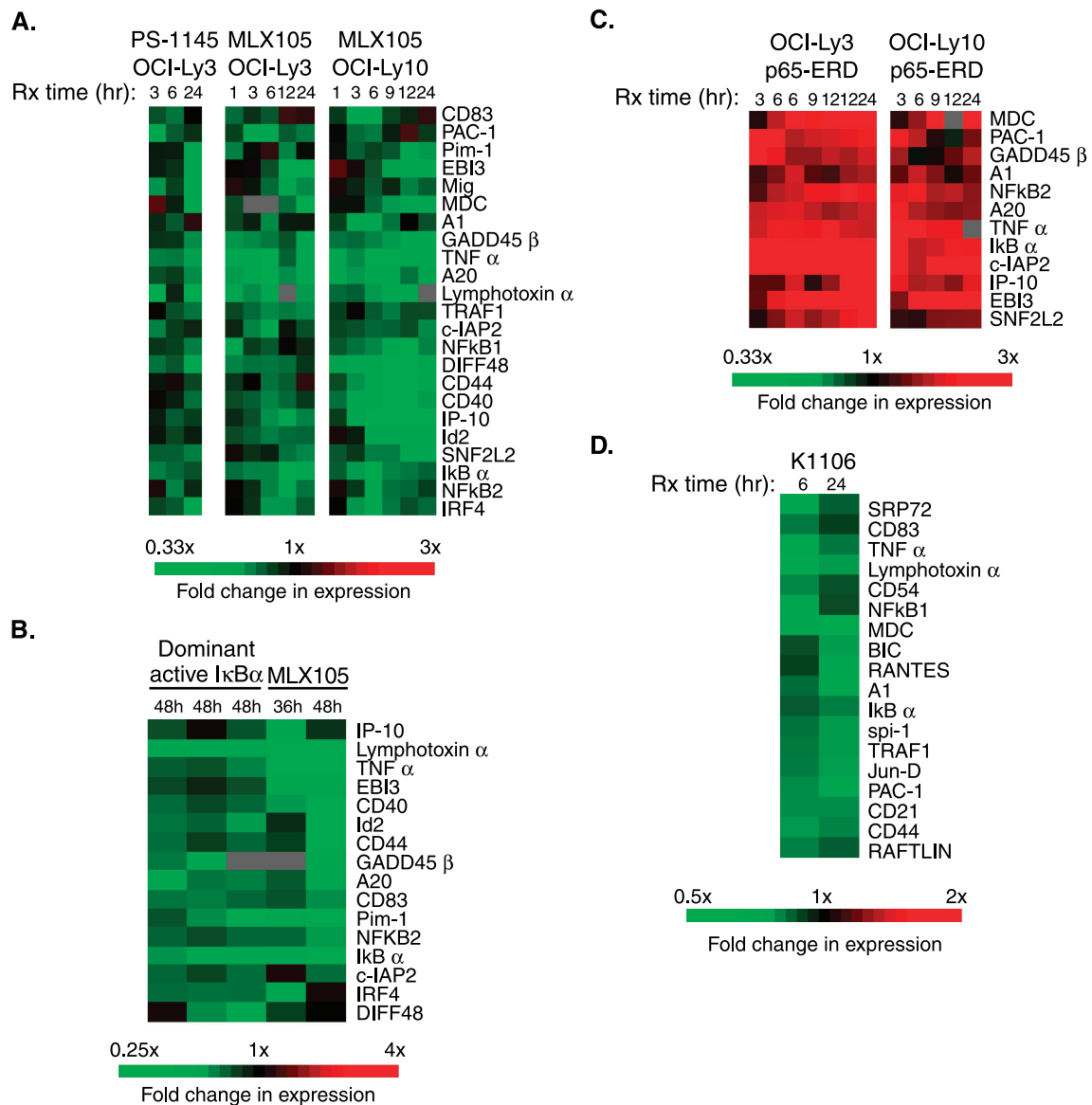


Fig. 5 Gene expression profiling of ABC DLBCL and PMBL cells treated with IKK inhibitors. *A*, genes that were down-regulated in both OCI-Ly3 and OCI-Ly10 cells treated with 25 μ Mol/L PS-1145 or MLX105 for the indicated times. *Row*, data from one gene on the DNA microarray. *Column*, data from a single experiment comparing gene expression in treated cells with gene expression in untreated cells cultured in parallel. *Color intensity*, ratio of gene expression in untreated cells to that in treated cells according to the color scale. *Red*, ratios >1 ; *green*, ratios <1 ; *black*, no significant change in gene expression; *gray*, missing data. *B*, maintenance of NF- κ B target gene expression by ectopic NF- κ B p65 activity in ABC DLBCL cells treated with MLX105. OCI-Ly3 and OCI-Ly10 cells expressing p65-ERD were treated with MLX105 (25 μ Mol/L) for the indicated times in the presence or absence of tamoxifen. The ratio of gene expression in tamoxifen-treated versus tamoxifen-untreated cells is depicted according to the color scale. *Red*, higher expression of genes in cells in which p65-ERD was activated by tamoxifen. *C*, IKK inhibitor MLX105 and an I κ B super-repressor cause similar gene expression changes in ABC DLBCL cells. OCI-Ly3 cells were treated with 25 μ Mol/L MLX105 for 36 or 48 hours or were infected with a retrovirus expressing an I κ B super-repressor and harvested 48 hours later. *Color intensity*, ratio of gene expression in MLX105 treated versus untreated cells or the ratio of gene expression in cells transduced with the I κ B super-repressor retrovirus versus cells transduced with a control retrovirus, according to the color scale. *D*, genes that were down-regulated in K1106 cells treated with 25 μ Mol/L MLX105 for the indicated times. *Color intensity*, ratio of gene expression in MLX105 treated versus untreated cells.

to block the inhibition of many of these genes. Taken together, these data suggest that this class of IKK inhibitors holds considerable promise for development as specific inhibitors of the NF- κ B pathway.

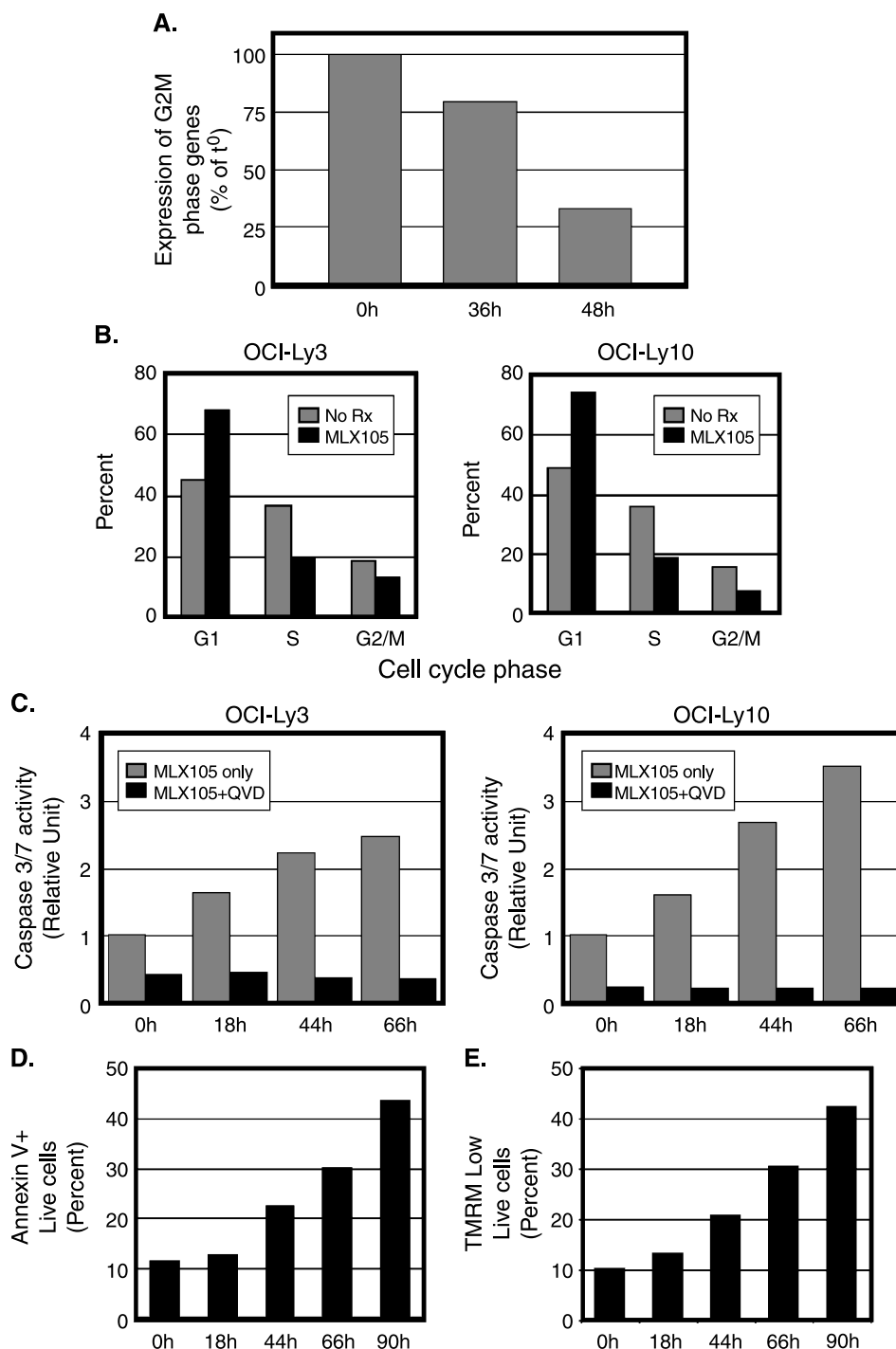
Currently, gene expression profiling analysis has defined three subgroups of DLBCL that can be viewed as distinct diseases that are impossible to distinguish by morphology.

These subgroups differ with respect to their presumptive cell of origin, their oncogenic abnormalities, and their clinical outcome (2, 3, 5). Two DLBCL subgroups, ABC and PMBL, have constitutive activity of the NF- κ B pathway, whereas GCB DLBCL does not. The deficiency of NF- κ B activation in GCB DLBCL is likely to be related to the fact that normal germinal center B cells have particularly low expression of

NF- κ B target genes (38). Germinal center B cells undergo selection in the germinal center microenvironment for those cells that increase the affinity of their antigen receptors through the process of somatic hypermutation. A current view of this process is that the germinal center B cell is poised to die unless it is positively selected by strong antigenic signaling through the B cell receptor or by signaling through CD40 due to interaction with CD40 ligand-bearing T cells.

Thus, low activity of the NF- κ B pathway would favor the default death pathway of germinal center B cells, and the B-cell receptor and CD40 might provide survival signals, in part, by activating NF- κ B. The reasons for constitutive activity of the NF- κ B pathway in ABC DLBCL and PMBL are less clear. One possibility is that this pathway is physiologically engaged at particular stages of B cell differentiation and these lymphoma types represent malignant versions of these B cell

Fig. 6 IKK inhibition in ABC DLBCL cells down-regulates proliferation gene expression and induces cell cycle arrest and apoptosis. **A**, expression of genes encoding G₂-M phase proteins (*AURKB*, *PLK1*, *BIRC5*, *CENPF*, *STK6*, and *ASPM*) in OCI-Ly3 cells treated with MLX105 (25 μ mol/L) for 36 and 48 hours. Expression levels of the individual genes were averaged and the % decrease in this average in treated versus untreated cells is shown. **B**, cell cycle arrest in ABC DLBCL cells induced by IKK inhibition. OCI-Ly3 and OCI-Ly10 cells were treated with or without MLX105 (25 μ mol/L) for 72 hours and assayed for the percentage of cells in each phase of the cell cycle. **C**, activation of caspase 3 and/or caspase 7 in ABC DLBCL cells by IKK inhibition. OCI-Ly3 and OCI-Ly10 cells were treated with MLX105 (25 μ mol/L) for the indicated times and assayed for caspase 3 and/or caspase 7 activity. Results from cells also cultured with the pan-caspase inhibitor QVD confirm the specificity of the assay. Values are normalized to the number of live cells in each sample and expressed relative to the control value. **D**, Annexin V staining of OCI-Ly10 cells treated with MLX105 (25 μ mol/L) for 0, 18, 44, 66, and 90 hours. Values shown at each time are the number of cells positively-stained with Annexin V-PE that exclude the dye 7-AAD, indicative of apoptosis prior to death, and are expressed as the % of the 7-aminoactinomycin D-negative cells. **E**, TMRM staining of OCI-Ly10 cells treated with MLX105 (25 μ mol/L) for 0, 18, 44, 66, and 90 hours. Values shown at each time are the number of cells with subnormal staining by TMRM that exclude 7-aminoactinomycin D, indicating loss of mitochondria membrane potential prior to death, and are expressed as the % of the 7-aminoactinomycin D-negative cells.



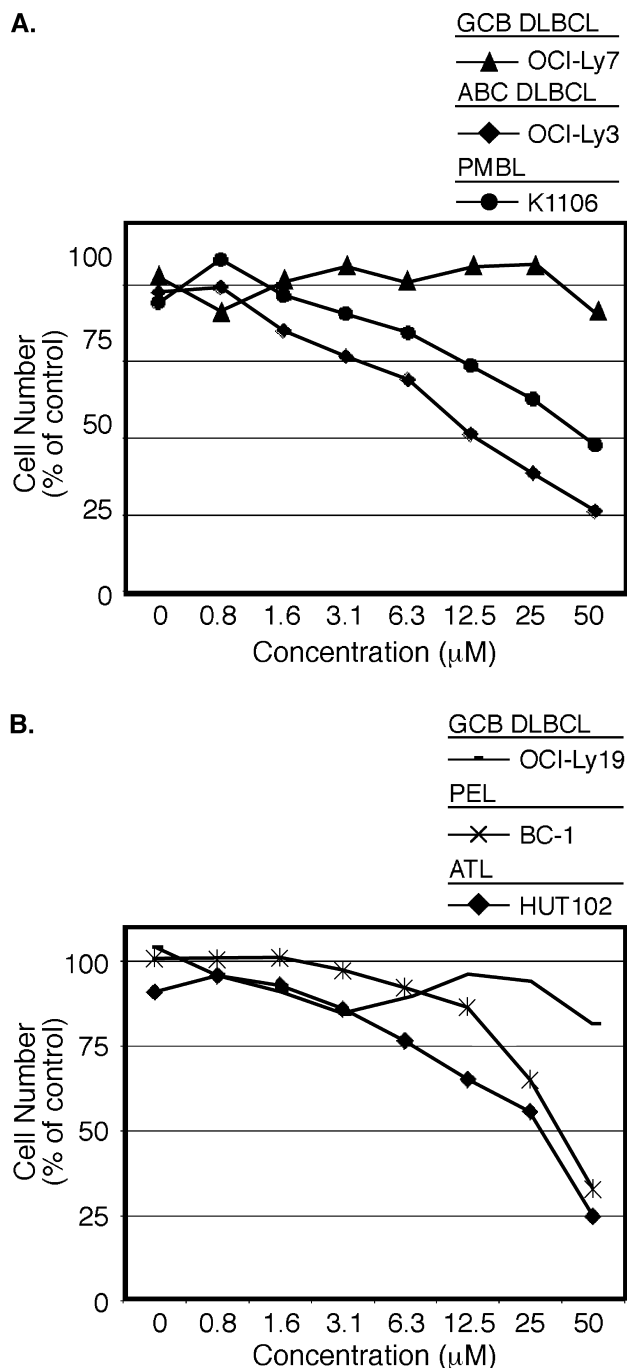


Fig. 7 Selective toxicity of MLX105 for multiple lymphoma types. *A*, OCI-Ly3, OCI-Ly7, and K1106 cells were treated with increasing concentrations of MLX105 (0–50 $\mu\text{mol/L}$) for 72 hours and assayed for viability by MTT. The cell numbers at each drug dose are the % of the cell numbers obtained with untreated cells cultured in parallel. *B*, OCI-Ly19, BC-1, and HUT102 cells were treated with increasing concentrations of MLX105 (0–50 $\mu\text{mol/L}$) for 48 hours and assayed for viability by MTT. The cell numbers at each drug dose are the % of the cell numbers obtained with untreated cells cultured in parallel.

types. The other possibility is that an unknown oncogenic event may trigger the NF- κ B pathway in these lymphoma subgroups.

The killing of the K1106 cell line by an IKK inhibitor provides evidence that this lymphoma type has constitutive activation of the NF- κ B pathway due to constitutive IKK activation. Previous studies showed that this cell line and primary biopsy samples of PMBL express NF- κ B target genes and have nuclear NF- κ B, but did not verify the dependence of this lymphoma type on NF- κ B for survival (2, 21). Gene expression profiling showed an unexpected and pronounced similarity between PMBL and Hodgkin lymphoma. In the present context, this similarity is notable since Hodgkin lymphoma has been shown to have constitutive activation of the NF- κ B pathway, which is required for the survival of these cells (12–46). The activity of the NF- κ B pathway in both PMBL and Hodgkin lymphoma may reflect a common origin of these lymphomas from a thymic B cell, which might have a physiologic engagement of this pathway. Alternatively, PMBL and Hodgkin lymphoma may have a common oncogenic abnormality that activates the NF- κ B pathway. Some Hodgkin lymphomas have mutations in the I κ B α gene that lead to a nonfunctional protein, whereas others have constitutive activation of IKK by Epstein-Barr virus or by an unknown mechanism (42, 46). Epstein-Barr virus is not present in PMBL cells and thus cannot account for the activation of NF- κ B in these cells. Future work will be needed to elucidate the mechanisms underlying NF- κ B activation in PMBL.

Other lymphomas have been reported to exhibit activation of IKK by viruses or intracellular signaling pathways. For example, primary effusion lymphoma is caused by infection by Kaposi's sarcoma-associated herpes virus. The genome of Kaposi's sarcoma-associated herpes virus encodes a viral FLIP protein that leads to the activation of the NF- κ B pathway by interaction with IKK (47, 48). Human T-cell leukemia virus type I, the etiologic agent for adult T-cell leukemia, encodes a protein called Tax that activates the NF- κ B pathway via the IKK complex (41). Chromosomal translocations in marginal zone lymphoma create a c-IAP2/MALT fusion protein or cause MALT1 or Bcl-10 to be overexpressed, leading to IKK activation (49). Our present findings together with previous reports suggest that, at a minimum, therapeutic targeting of IKK may prove effective in two subgroups of DLBCL, primary effusion lymphoma, adult T-cell leukemia, mucosa-associated lymphoid tissue lymphoma and in some Hodgkin lymphomas.

The IKK inhibitors also blocked the proliferation of ABC DLBCL cells prior to inducing cell death, in keeping with previous observations (6). This antiproliferative effect was associated with a broad decrease in the expression of genes that are expressed in proliferating cells and an arrest in the G₁ phase of the cell cycle. This observation may have implications regarding the combination of IKK inhibitors with other chemotherapeutic agents that induce DNA damage during DNA synthesis. Because IKK inhibitors block entry into S phase, it will be important to investigate whether these inhibitors should be given before, during or after treatment with DNA-damaging chemotherapeutic agents. Because NF- κ B can be activated by certain chemotherapeutic agents (12), IKK inhibitors could synergize with these agents in the killing of tumor cells. However, because IKK inhibitors also block S phase entry, it may prove important to administer these agents only after DNA damage-inducing chemotherapeutic agents.

Our results show that small molecule IKK inhibitors hold promise as a new class of targeted therapeutic agents in lymphoma. If such inhibitors become available for clinical use, it may be important to determine the activity of the NF- κ B pathway in tumor biopsies by gene expression profiling or other quantitative techniques in order to determine which patients might optimally benefit from IKK inhibition. Although IKK inhibitors may compromise immune function due to the importance of the NF- κ B pathway in both innate and acquired immunity, the short-term administration of these inhibitors to cancer patients might be achieved with manageable effects on immune function. Furthermore, given the frequent activation of the NF- κ B pathway in human cancer cells (10–12), IKK inhibitors may have therapeutic potential for a broad range of cancer types.

ACKNOWLEDGMENTS

We thank Keith Brown for the gifts of plasmids, the members of the Staudt laboratory for helpful discussions, and Liming Yang for setting up the web site.

REFERENCES

- Wright G, Tan B, Rosenwald A, et al. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. *Proc Natl Acad Sci U S A* 2003;100:9991–6.
- Rosenwald A, Wright G, Leroy K, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. *J Exp Med* 2003;198:851–62.
- Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med* 2002;346:1937–47.
- Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000;403:503–11.
- Staudt LM. Molecular diagnosis of the hematologic cancers. *N Engl J Med* 2003;348:1777–85.
- Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor κ B activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med* 2001;194:1861–74.
- Ghosh S, May MJ, Kopp EB. NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998;16:225–60.
- Gugasyan R, Grumont R, Grossmann M, et al. Rel/NF- κ B transcription factors: key mediators of B-cell activation. *Immunol Rev* 2000;176:134–40.
- Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF- κ B activity. *Annu Rev Immunol* 2000;18:621–63.
- Kucharczak J, Simmons MJ, Fan Y, Gelinac C. To be, or not to be: NF- κ B is the answer—role of Rel/NF- κ B in the regulation of apoptosis. *Oncogene* 2003;22:8961–82.
- Karin M, Cao Y, Greten FR, Li ZW. NF- κ B in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002;2:301–10.
- Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF- κ B. *J Clin Invest* 2001;107:241–6.
- Wang CY, Guttridge DC, Mayo MW, Baldwin AS Jr. NF- κ B induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* 1999;19:5923–9.
- Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF- κ B antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998;281:1680–3.
- Jin R, De Smaele E, Zazzeroni F, et al. Regulation of the gadd45 β promoter by NF- κ B. *DNA Cell Biol* 2002;21:491–503.
- De Smaele E, Zazzeroni F, Papa S, et al. Induction of gadd45 β by NF- κ B downregulates pro-apoptotic JNK signalling. *Nature* 2001;414:308–13.
- Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev* 1999;13:400–11.
- Grumont RJ, Strasser A, Gerondakis S. B cell growth is controlled by phosphatidylinositol 3-kinase-dependent induction of Rel/NF- κ B regulated *c-myc* transcription. *Mol Cell* 2002;10:1283–94.
- Hinz M, Loser P, Mathas S, et al. Constitutive NF- κ B maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. *Blood* 2001;97:2798–807.
- Hideshima T, Chauhan D, Schlossman R, Richardson P, Anderson KC. The role of tumor necrosis factor α in the pathophysiology of human multiple myeloma: therapeutic applications. *Oncogene* 2001;20:4519–27.
- Savage KJ, Monti S, Kutok JL, et al. The molecular signature of mediastinal large B-cell lymphoma differs from that of other diffuse large B-cell lymphomas and shares features with classical Hodgkin lymphoma. *Blood* 2003;102:3871–9.
- Garg A, Aggarwal BB. Nuclear transcription factor- κ B as a target for cancer drug development. *Leukemia* 2002;16:1053–68.
- Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF- κ B pathway in the treatment of inflammation and cancer. *J Clin Invest* 2001;107:135–42.
- Karin M, Yamamoto Y, Wang QM. The IKK NF- κ B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004;3:17–26.
- Castro AC, Dang LC, Soucy F, et al. Novel IKK inhibitors: β -carboline. *Bioorg Med Chem Lett* 2003;13:2419–22.
- Hideshima T, Chauhan D, Richardson P, et al. NF- κ B as a therapeutic target in multiple myeloma. *J Biol Chem* 2002;277:16639–47.
- Crowder C, Kopantzev E, Williams K, et al. An unusual H-Ras mutant isolated from a human multiple myeloma line leads to transformation and factor-independent cell growth. *Oncogene* 2003;22:649–59.
- Kang E, Giri N, Wu T, et al. *In vivo* persistence of retrovirally transduced murine long-term repopulating cells is not limited by expression of foreign gene products in the fully or minimally myeloablated setting. *Hum Gene Ther* 2001;12:1663–72.
- Naviaux RK, Costanzi E, Haas M, Verma IM. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J Virol* 1996;70:5701–5.
- Beg AA, Ruben SM, Scheinman RI, et al. I κ B interacts with the nuclear localization sequences of the subunits of NF- κ B: a mechanism for cytoplasmic retention. *Genes Dev* 1992;6:1899–913.
- Luque I, Gelinac C. Distinct domains of I κ B α regulate c-Rel in the cytoplasm and in the nucleus. *Mol Cell Biol* 1998;18:1213–24.
- Franzoso G, Carlson L, Brown K, et al. Activation of the serum response factor by p65/NF- κ B. *EMBO J* 1996;15:3403–12.
- Ruben SM, Dillon PJ, Schreck R, et al. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF- κ B. *Science* 1991;254:11.
- Lam LT, Pickeral OK, Peng AC, et al. Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. *Genome Biol* 2001;2:RESEARCH0041.
- Shaffer AL, Yu X, He Y, et al. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity* 2000;13:199–212.
- Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95:14863–8.
- Li X, Fang Y, Zhao X, et al. Characterization of NF- κ B activation by detection of green fluorescent protein-tagged I κ B degradation in living cells. *J Biol Chem* 1999;274:21244–50.

38. Shaffer AL, Rosenwald A, Hurt EM, et al. Signatures of the immune response. *Immunity* 2001;15:375–85.
39. Pahl HL. Activators and target genes of Rel/NF- κ B transcription factors. *Oncogene* 1999;18:6853–66.
40. Keller SA, Schattner EJ, Cesarman E. Inhibition of NF- κ B induces apoptosis of KSHV-infected primary effusion lymphoma cells. *Blood* 2000;96:2537–42.
41. Jeang KT. Functional activities of the human T-cell leukemia virus type I Tax oncoprotein: cellular signaling through NF- κ B. *Cytokine Growth Factor Rev* 2001;12:207–17.
42. Cabannes E, Khan G, Aillet F, Jarrett RF, Hay RT. Mutations in the I κ B α gene in Hodgkin's disease suggest a tumour suppressor role for I κ B α . *Oncogene* 1999;18:3063–70.
43. Emmerich F, Meiser M, Hummel M, et al. Overexpression of I κ B α without inhibition of NF- κ B activity and mutations in the I κ B α gene in reed-sternberg cells. *Blood* 1999;94:3129–34.
44. Krappmann D, Emmerich F, Kordes U, et al. Molecular mechanisms of constitutive NF- κ B/Rel activation in Hodgkin/Reed-Sternberg cells. *Oncogene* 1999;18:943–53.
45. Staudt LM. The molecular and cellular origins of Hodgkin's disease. *J Exp Med* 2000;191:207–12.
46. Bargou RC, Emmerich F, Krappmann D, et al. Constitutive nuclear factor- κ B-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997;100:2961–9.
47. Field N, Low W, Daniels M, et al. KSHV vFLIP binds to IKK- γ to activate IKK. *J Cell Sci* 2003;116:3721–8. Epub 2003 Jul 30.
48. Liu L, Eby MT, Rathore N, et al. The human herpes virus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the I κ B kinase complex. *J Biol Chem* 2002;277:13745–51. Epub 2002 Feb 5.
49. Thome M, Tschopp J. TCR-induced NF- κ B activation: a crucial role for Carma1, Bcl10 and MALT1. *Trends Immunol* 2003;24:419–24.

Clinical Cancer Research

Small Molecule Inhibitors of I κ B Kinase Are Selectively Toxic for Subgroups of Diffuse Large B-Cell Lymphoma Defined by Gene Expression Profiling

Lloyd T. Lam, R. Eric Davis, Jackie Pierce, et al.

Clin Cancer Res 2005;11:28-40.

Updated version Access the most recent version of this article at:
<http://clincancerres.aacrjournals.org/content/11/1/28>

Cited articles This article cites 49 articles, 20 of which you can access for free at:
<http://clincancerres.aacrjournals.org/content/11/1/28.full#ref-list-1>

Citing articles This article has been cited by 78 HighWire-hosted articles. Access the articles at:
<http://clincancerres.aacrjournals.org/content/11/1/28.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://clincancerres.aacrjournals.org/content/11/1/28>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.