Cancer Therapy: Preclinical

Aurora A Inhibitor (MLN8237) plus Vincristine plus Rituximab Is Synthetic Lethal and a Potential Curative Therapy in Aggressive B-cell Non-Hodgkin Lymphoma

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Abstract

Purpose: Aurora A and B are oncogenic serine/threonine kinases that regulate mitosis. Overexpression of Auroras promotes resistance to microtubule-targeted agents. We investigated mechanistic synergy by inhibiting the mitotic spindle apparatus in the presence of MLN8237 [M], an Aurora A inhibitor with either vincristine [MV] or docetaxel [MD] in aggressive B-cell non-Hodgkin lymphoma (B-NHL). The addition of rituximab [R] to MV or MD was evaluated for synthetic lethality.

Experimental Design: Aggressive B-NHL cell subtypes were evaluated *in vitro* and *in vivo* for target modulation and anti-NHL activity with single agents, doublets, and triplets by analyzing cell proliferation, apoptosis, tumor growth, survival, and mechanisms of response/relapse by gene expression profiling with protein validation.

Results: MV is synergistic whereas MD is additive for cell proliferation inhibition in B-NHL cell culture models. Addition of rituximab to MV is superior to MD, but both significantly induce apoptosis compared with doublet therapy. Mouse xenograft models of mantle cell lymphoma showed modest single-agent activity for MLN8237, rituximab, docetaxel, and vincristine with tumor growth inhibition (TGI) of approximately 10% to 15%. Of the doublets, MV caused tumor regression, whereas TGI was observed with MD (approximately 55%–60%) and MR (approximately 25%–50%), respectively. Although MV caused tumor regression, mice relapsed 20 days after stopping therapy. In contrast, MVR was curative, whereas MDR led to TGI of approximately 85%. Proliferation cell nuclear antigen, Aurora B, cyclin B1, cyclin D1, and Bcl-2 proteins of harvested tumors confirmed response and resistance to therapy.

Conclusions: Addition of rituximab to MV is a novel therapeutic strategy for aggressive B-NHL and warrants clinical trial evaluation. *Clin Cancer Res; 18(8); 2210–9. ©2012 AACR.*

Introduction

Aggressive B-cell non-Hodgkin lymphomas (B-NHL) includes diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), Burkitt's lymphoma, and transformed follicular lymphoma (TFL) that have disparate responses to chemoimmunotherapies. A significant number of patients (approximately 50%–60%) failing frontline therapies have few therapeutic options (1). Therefore, the development of novel safe and effective treatments based on biologically validated targets is urgently needed for these therapy-resistant patients.

Aurora kinase A has received great attention in recent years as a potential therapeutic target for a variety of hematologic and solid malignancies (2–6). Aurora A is a serine/threonine kinase that plays a key role in mitotic initiation, progression, and spindle assembly checkpoint (SAC) activity during the mammalian cell cycle. Aurora A localizes to centrosomes and functions in centrosome maturation and the proper formation of mitotic spindle (7–9). Suppression of its activity results in defects in centrosome maturation and separation, mitotic spindle formation, and chromosome alignment (10–14). Aurora A is able to transform rodent cells leading to tumor formation in xenograft mice (15–17). In humans, Aurora A is overexpressed in numerous solid (breast, colorectal, pancreas, ovary, gastric, and prostate) and hematologic (acute myeloid leukemia and B-NHL) malignancies (18–21). Knockdown of Aurora A protein in tumor cells delays mitotic entry and progression, resulting in the accumulation of cells in G_2/M, spindle defects, polyploid cells, and apoptosis (22–25). In addition, overexpression of Aurora A overrides the SAC and results in resistance to microtubule-targeted agent (MTA, e.g., taxanes, vinca alkaloids) treatment (26, 27). Indeed,

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Translational Relevance

Targeting synergistic mechanisms within the context of the hallmarks of cancer may yield novel effective therapies with minimal toxicity for B-cell non-Hodgkin lymphoma (B-NHL). Auroras (A and B) are a family of mitotic serine/threonine kinases that are involved in high-fidelity cell division. Aberrant overexpression of Auroras is oncogenic, leading to genetic instability, tumor initiation, and progression. Overexpression of Auroras has been shown to promote resistance to microtubule-targeting agents (MTA) such as taxanes and vinca alkaloids. Inhibition of Auroras with siRNA knockdown or pharmacologic intervention with a small molecule inhibitor [MLN8237] leads to enhanced sensitivity of cancer cells to MTAs. Here, we showed that MLN8237 plus vincristine (MV) is synergistic and addition of rituximab to MV (MVR) is synthetic lethal and curative in established B-NHL xenograft mice. These findings suggest that MVR may represent a novel therapeutic strategy in aggressive B-NHL, and a phase I/II trial is enrolling patients on the basis of our data.

inhibition of Aurora A has shown broad therapeutic potential with chemotherapeutics and synergy with MTA in several human tumor models (28–32).

MLN8237 is a second-generation small molecule inhibitor (SMI) of Aurora A kinase. It is orally bioavailable and is a highly selective inhibitor of Aurora A with antineoplastic activity (33–35). MLN8237 binds to and inhibits Aurora A kinase, which may result in disruption of the assembly of the mitotic spindle apparatus, disruption of chromosome segregation, and inhibition of cell proliferation. Several studies show that MLN8237 has significant activity *in vitro* and *in vivo* against numerous tumor models including multiple myeloma (36), T-cell leukemia (37), chronic myeloid leukemia (38), neuroblastoma, and acute lymphoblastic leukemia (39). Recently, MLN8237 has entered phase II clinical investigation in several hematologic malignancies.

Rituximab is a chimeric mouse anti-human CD20 monoclonal antibody used for the treatment of CD20^{+} B-NHLs. The overall response in FL patients is approximately 50% when it is used as a single agent, and the response rate is significantly increased when rituximab is used in combination with chemotherapy (40, 41). The mechanisms of antitumor effect of rituximab include apoptosis, complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP; ref. 42). Our previous study showed that MLN8237 inhibited Aurora A kinase activity and induced apoptosis in aggressive B-NHL cell lines. Moreover, MLN8237 plus docetaxel showed a significant tumor growth inhibition (TGI) with an associated improved overall survival in a mouse MCL xenograft model (32). On the basis of the efficacy of rituximab in inhibiting B-cell proliferation with chemotherapy, we hypothesized

that addition of rituximab to an Aurora A inhibitor plus an MTA (e.g., docetaxel or vincristine) would enhance synergistic activity in B-NHL cells and mouse xenograft models. Here we show that MLN8237 plus vincristine plus rituximab (MVR) has superior anti–B-NHL activity and is curative in mice bearing MCL compared with MLN8237 plus docetaxel plus rituximab (MDR). These finding are highly correlated with harvested tumor analysis of markers of proliferation and cell-cycle regulation.

Materials and Methods

Cells and reagents

B-NHL cell lines used in this study (RL, Granta-519, and SUDHL-4) were from Drs. S. Grant (Virginia Commonwealth University, Richmond, VA) and C. Jordan (University of Rochester, Rochester, NY) and maintained in RPMI 1640 medium (Mediatech) supplemented with 10% FBS, 2 mmol/L sodium pyruvate, and 100 units/mL penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. MLN8237 was kindly provided by Millennium Pharmaceuticals Inc. Rituximab, vincristine, and docetaxel were a kind donation by the Arizona Cancer Center Clinic. The compounds were dissolved at 10 mmol/L in dimethyl sulfoxide as a stock solution and then further diluted to desired concentrations for *in vitro* experiments. Anti-Aurora B (ab2254) antibody was purchased from Abcam. Anti-PCNA (proliferation cell nuclear antigen) and anti-Cyclin B1 (V152) antibodies were purchased from Cell Signaling Technology. Anti-Cyclin D1 (sc-718) and anti-β-actin antibodies were from Santa Cruz Biotechnology and Sigma, respectively. Anti-Bcl2 antibody was purchased from Cell Signaling Technology (Danvers, MA).

Cell proliferation assay

Lymphoma cells (Granta-519 or RL) were seeded at 10,000 per well in 96-well culture plates and allowed to grow for 24 hours followed by the desired treatment with increasing concentrations of the indicated agents (MLN8237, vincristine, or docetaxel) for 4 days. Viable cell densities were determined using a CellTiter 96 Cell Proliferation Assay (Promega). Absorbance readings at 490 nm were analyzed against the control group for each drug treatment to determine cell viability. The studies were carried out in triplicates × 4 and IC_{50} values were estimated by Calcusyn software. For combination studies of MLN8237 plus vincristine or docetaxel, an equipotent ratio was calculated to determine a combined graded combination treatment. The equipotent ratio is the ratio of the median effects resulting from the single-dose treatments of MLN8237 and vincristine or docetaxel. A control group was established for each drug treatment in 6 replicates. The effects of the combined treatments were determined by the combination index (CI) and isobologram methods derived from the median–effect principle of Chou and Talalay.

Apoptosis assay

Using Annexin V staining to detect apoptosis, treated cells were harvested and rinsed with cold PBS once. After

centrifugation for 5 minutes, cells were resuspended in 500 μL of 1× Annexin V–binding buffer [BioVision; Annexin V–fluorescein isothiocyanate (FITC) Reagent Kit, catalog no. 1001-1000] and then added 5 μL of Annexin V–FITC and 5 μL of Propidium Iodide (BioVision; Annexin V–FITC Reagent Kit). After incubation for 5 minutes at room temperature in the dark, the samples were analyzed by flow cytometry.

Immunoblotting

The cells were lysed in NP-40 lysis buffer containing 50 mmol/L Tris.Cl (pH 7.4), 0.15 mol/L NaCl, 0.5% NP-40, 1 mmol/L DTT, 50 mmol/L sodium fluoride, and 2 μL/mL Protease inhibitor cocktail (Sigma). Protein concentrations were determined using the BioRad Protein Assay Kit and 50 μg of protein was resolved by electrophoresis on a 10% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and nonspecific binding was blocked by incubating with 5% nonfat milk in TBST buffer (0.01 mol/L Tris-Cl, 0.15 mol/L NaCl, 0.5% Tween-20, pH 8.0) at room temperature for 1 hour. The membrane was subjected to the indicated antibodies and the proteins were detected by a LI-COR Odyssey Infrared Imaging System.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and rehydrated to distilled water. Antigen unmasking was carried out by bringing slides to a boil in 1 mmol/L EDTA pH 8.0 followed by 15 minutes at a subboiling temperature. After washing in dH_2O 3 times, the slides were incubated in 3% hydrogen peroxide for 10 minutes and then blocked with blocking solution (0.2% bovine serum albumin, 0.01% saponin, and 1% normal rabbit serum in PBS) for 1 hour at room temperature. The slides were then immunostained using anti-PCNA antibody at the dilution 1:1,000 in blocking solution. The reaction was incubated overnight at 4°C. After washing 3 times with PBS, the secondary antibody conjugated horseradish peroxidase (KPL) was applied for 30 minutes at room temperature. The signal was checked using AEC Chromogen Kit (Sigma) following the manufacturer's protocol. Primary or secondary antibody replacement with normal serum from the same animal species was used as the negative controls.

MCL mouse xenograft model

Animal care and treatment were carried out at Arizona Cancer Center's experimental mouse shared services (EMSS) core facility. Severe-combined immunodeficient (SCID) mice were injected with 1×10^7 Granta-519 MCL cells subcutaneously into the right hind flank. When tumors reached a volume of approximately 60 to 120 mm^3, mice were divided randomly (pair matched) into different groups with 12 mice per cohort. The mice were treated with MLN8237 [M], rituximab [R], vincristine [V], and docetaxel [D] alone at indicated dosages or different combinations [MR, MV, MD, MVR, and MDR]. MLN8237 was given orally once a day for 3 weeks, whereas R, V, and

D intravenously once a week for 4 weeks. The length (L) and width (W) of the subcutaneous tumors were measured by calipers and the tumor volume (T_V) was calculated as follows: $T_V = (L \times W^2)/2$. Mice were sacrificed at the end of treatment (3 mice per cohort), end of study, or if they reached more than 2,000 mm^3 at any time during the study. Excised tumors (end of the treatment) were either fixed in paraffin for immunohistochemical (IHC) analysis or snap frozen for Western blotting and DNA microarray studies. Overall survival for each cohort was analyzed by Kaplan–Meier method.

RNA isolation and gene expression profiling

Total RNA was extracted from harvested tumors at the end of treatment using the RNeasy Mini Kit (Qiagen). After checking for RNA quality, DNA microarray was done using the human HG-U133A Affymetrix genechip consisting of 22 277 "probe sets" (Genomic Core Facility at Arizona Cancer Center). Data analysis was carried out using BioConductor libraries (http://www.bioconductor.org) and R programming (http://www.r-project.org). A quality control analysis for each array was carried out using Affymetrix console methods and the affyQCReport library. The array data was background corrected and quantile normalized using the affy Bioconductor library. Probe set values for the same gene were summarized by averaging. To compare conditions, log ratios of expression values were calculated. For each comparison, the mean ± 2 SD of the log fold changes were used as cut-offs to determine outlying differentially expressed genes. Each list of genes was tested for overrepresentation of KEGG and GO terms by counting the number of genes in the list with each pathway or term and using the Fisher exact test to determine overrepresentation. All terms with a P value of 0.05 or less were reported.

Statistical analysis

All *in vitro* experiments were conducted in triplicate. The data were expressed as mean ± SD. The difference between 2 mean values were evaluated using the Student t test and considered to be statistically significant when $P < 0.05$. Statistical analysis of the mouse xenograft model data was carried out by estimating the tumor growth for each mouse by fitting the least squares regression line of the tumor volume by day. The cube root of the observed tumor volumes was used to induce linearity in the raw data values. The slope of the regression line measures the tumor growth rate. ANOVA was used to test for the overall treatment effects on TGI. Tukey's studentized range test was used to assess the significance of pairwise differences between the groups adjusted for multiple comparisons. Survival of the mice was measured from the date of pair matching to sacrifice (event) or end of study (censored). The Kaplan–Meier method was used to estimate survival. The log-rank test was used to compare survival between the respective treatment groups. Statistical adjustments were made for multiple comparisons. Analysis was carried out using Prism

Figure 1. Cell proliferation (MTS) assays of Granta-519 treated with MLN8237 or vincristine or both. A, the IC_{50} for MLN8237 was determined to be 109.8 nmol/L and when combined with vincristine (V), decreased to 2.65 nmol/L. B, IC_{50} for vincristine was determined to be 2.74 nmol/L and when combined decreased to 0.067 nmol/L with a CI value for ED_{50} to be 0.048.
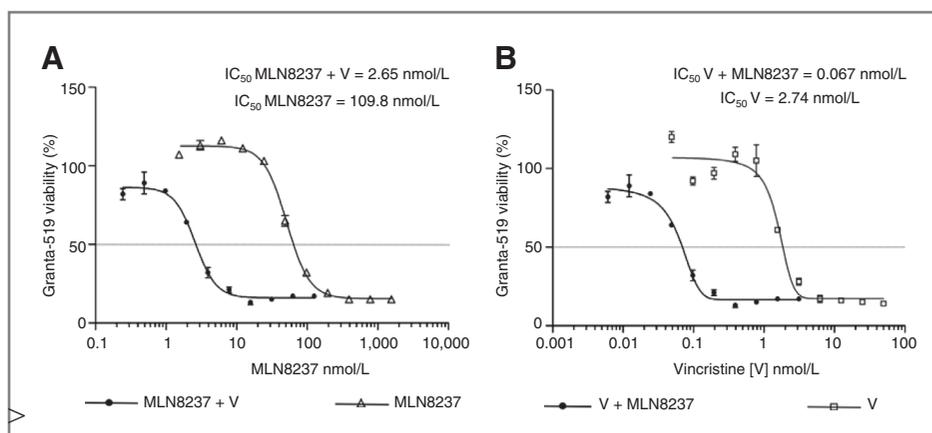
(Graphpad). All *P* values less than or equal to 0.05 were considered statistically significant.

Results

A synthetic lethal interaction between spindle assembly dynamics and inhibition of Aurora A with MLN8237 in aggressive B-NHL cell lines

B-NHL cells (Granta-519 and RL) were treated in serial dilution with [M] or [V], or [D]. The IC_{50} values for the B-NHL cells (Granta-519) determined for MLN8237, vin-

cristine, and docetaxel were 109.8, 2.74, and 4.53 nmol/L, respectively. For combination studies of MLN8237 plus vincristine, the dosage ratio was approximately 40:1 (M:V) for Granta-519 and 5.5:1 for RL cells. Both cell lines experienced a synergistic response with the combination of MLN8237 plus vincristine. The CI values for ED_{50} were determined to be 0.048 and 0.24 for Granta-519 and RL, respectively. Therefore, these interactions can be described as exhibiting very strong synergism. The combined dose median effect was 2.65 nmol/L (in relation to MLN8237)

Figure 2. Rituximab and MTAs enhance MLN8237-induced apoptosis in B-cell NHL cells. A, RL, Granta-519, and SUDHL-4 cells were treated with docetaxel at 5 nmol/L, rituximab at 10 µg/mL and MLN8237 at 5 nmol/L alone or the combinations as indicated at same doses for 72 hours. B, Granta-519 cells were treated as same as (A) except that vincristine was substituted for docetaxel at the dose of 0.1 nmol/L. Apoptosis was analyzed by flow cytometry after Annexin V and PI staining. The graph represents the mean percentage of apoptosis ± SD (*n* = 3). *, *P* < 0.05; **, *P* < 0.001. C, Granta-519 cells treated with AT9283 (5 nmol/L; pan-Aurora inhibitor) and vincristine (0.1 nmol/L) ± rituximab (10 µg/mL). Apoptosis was analyzed by flow cytometry after Annexin V and PI staining. The graph represents the mean percentage of apoptosis ± SD (*n* = 3).
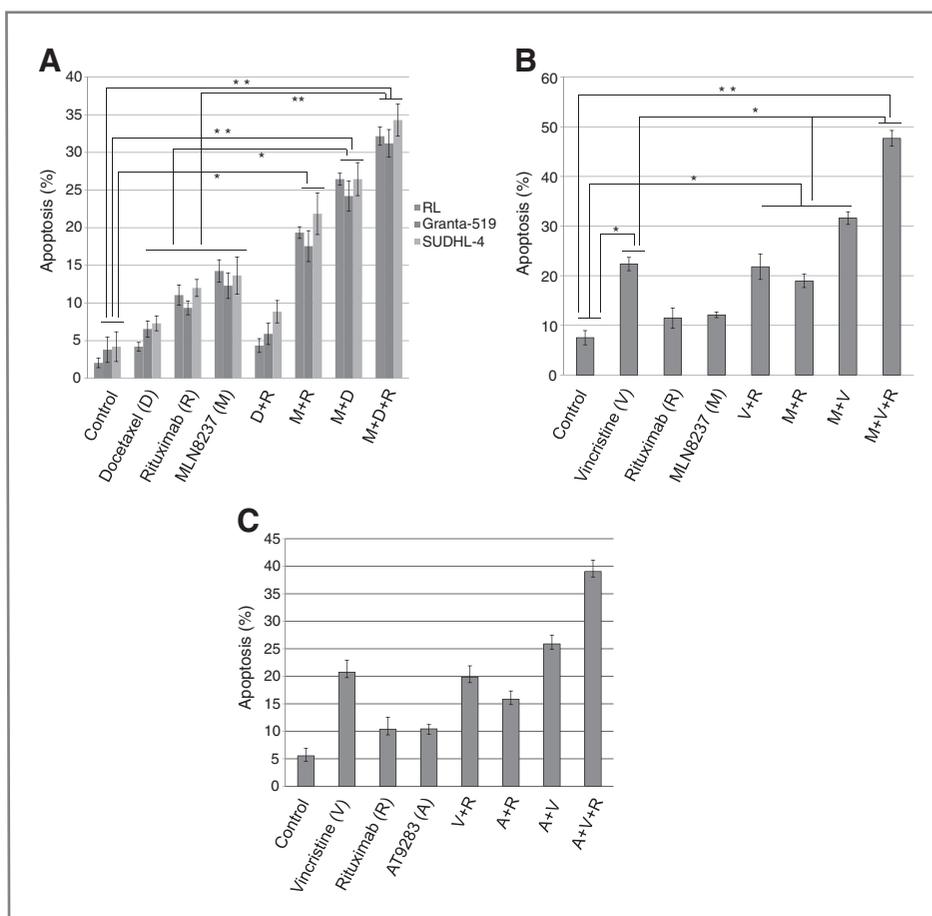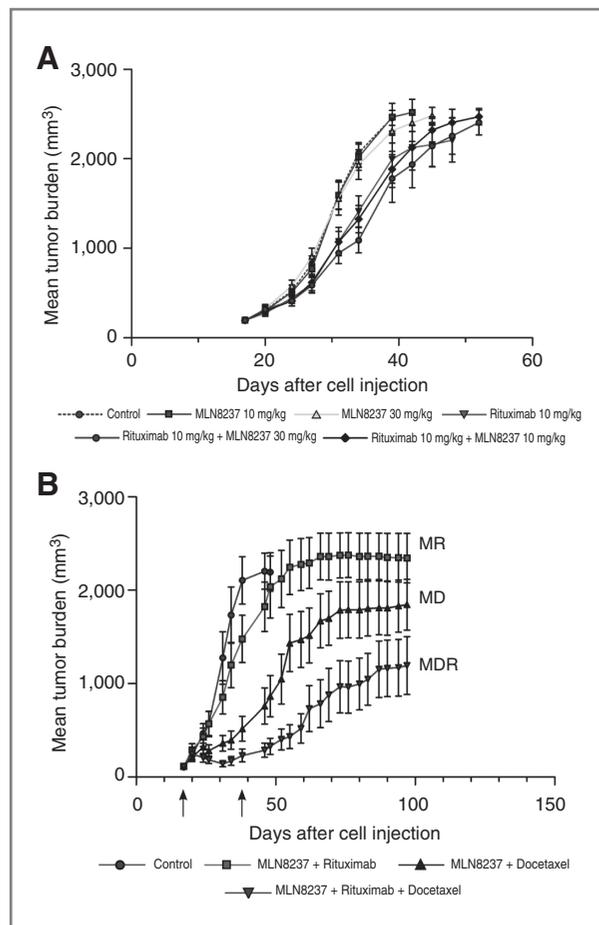
Figure 3. Rituximab increases *in vivo* anti-NHL activity of MLN8237 and MLN8237 plus docetaxel in a Granta-519 MCL xenograft mouse model. A, evaluation of *in vivo* therapeutic activity of [M], [R], and MR in a MCL xenograft mouse model. SCID mice bearing Granta-519 tumors ($n = 12$) were treated with saline (control), MLN8237 at 10 mg/kg or 30 mg/kg, rituximab at 10 mg/kg, MLN8237 10 mg/kg + rituximab 10 mg/kg and MLN8237 30 mg/kg + rituximab 10 mg/kg. Tumor burden were measured 3 times a week and graphed. All values are presented as mean ± SEM. B, evaluation of tumor growth inhibition of M + R versus M + [D] versus M + D + R. Granta-519 xenograft mice were treated with MLN8237 30 mg/kg plus rituximab 10 mg/kg, MLN8237 30 mg/kg plus docetaxel 10 mg/kg and MLN8237 30 mg/kg + docetaxel 10 mg/kg + rituximab 10 mg/kg. Tumor volume versus time is presented as mean ± SEM. The arrows represent the start and the end of treatment.

and 0.067 nmol/L (in relation to vincristine) for Granta-519 cells (Fig. 1), and this was consistent with tumor regression observed in the Granta-519 xenograft model (Fig. 4A). These results indicated that the combination of MLN8237 plus vincristine is synergistic. Hence, vincristine an agent that inhibits microtubule assembly plus Aurora A inhibitor [M] (inhibits centrosome assembly) is synthetic lethal.

Rituximab enhances apoptosis in B-cell NHL cells treated with MLN8237 plus microtubule-targeting agents

MCL patients with high Aurora A expression showed significantly worse survival than low expressers, suggesting
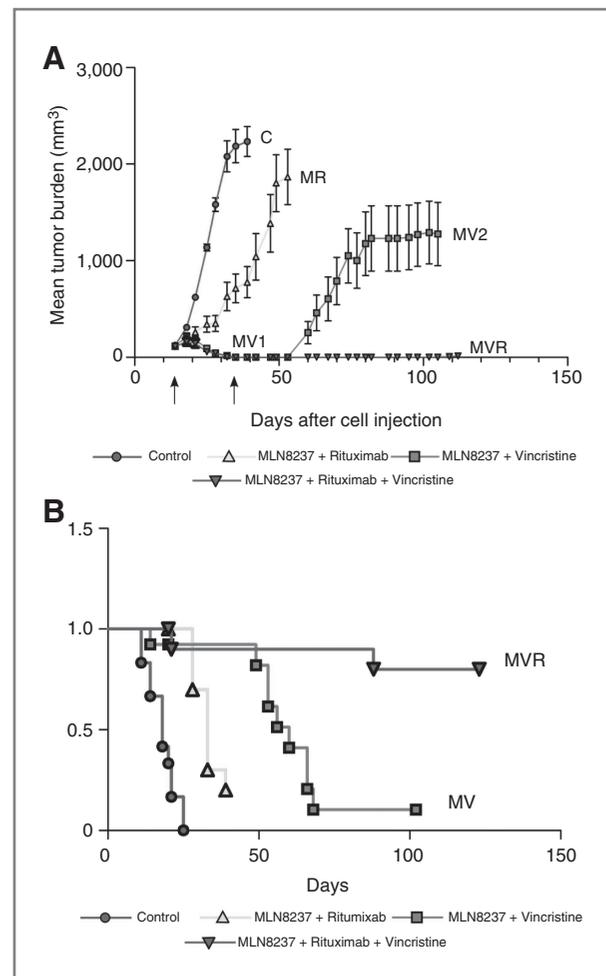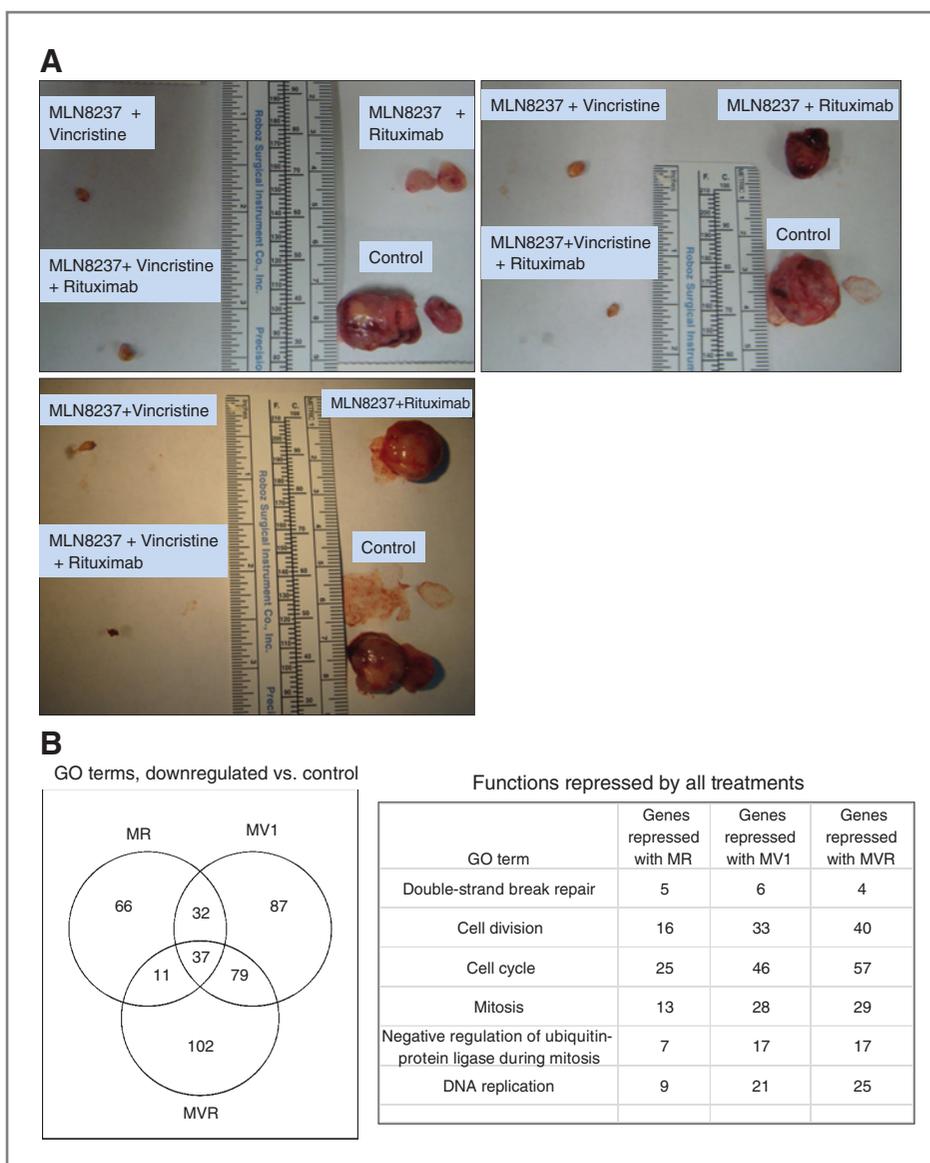

Figure 4. MLN8237 plus vincristine plus rituximab is synthetic lethal and curative in a mouse xenograft model of MCL. A, Granta-519 xenograft mice ($n = 12$ per cohort) were treated with saline (control), MLN8237 30 mg/kg + rituximab 10 mg/kg, MLN8237 30 mg/kg + vincristine 0.375 mg/kg, and MLN8237 30 mg/kg + vincristine 0.375 mg/kg + rituximab 10 mg/kg. MLN8237 was given by once daily × 3 weeks orally, vincristine and rituximab by once every week × 4 weeks intravenously. MV1 represents the phase of tumor regression whereas MV2 represents the phase of lymphoma relapse. Tumor burdens were measured, graphed, and represented as mean ± SEM. B, Kaplan–Meier survival curves show overall survival differences between MVR in comparison with control, MR, and MV.

that Aurora A kinase is a potential target for aggressive B-cell NHL therapy (32). In a MCL (Granta-519) mouse xenograft model, single-agent MLN8237 [M] activity was modest with a TGI of approximately 10% to 15% (32). Recently, it was observed that MTAs such as [D], paclitaxel, and [V] had synergistic activity with an Aurora A kinase small molecular inhibitor or Aurora A transcriptional silencing in solid tumors (28–31). In a mouse MCL xenograft model, we showed enhance TGI (approximately 55%–60%) when M was combined with docetaxel compared with docetaxel alone (approximately 10%–15%) with an enhanced overall survival (32). Rituximab [R] is an effective treatment in B-NHL when combined with chemotherapy. To determine

Figure 5. Reactivation of cell-cycle regulators and antiapoptosis as a mechanism of resistance to MV abrogated by MVR therapy. A, tumor sizes of 3 mice per cohort sacrificed 3 hours after end of the last treatment are shown for control, MR, MV, and MVR therapy. B, gene expression profiling identified unique and common gene ontologies repressed by individual drug treatments compared with control and each other (Venn diagram and list of repressed functionalities).

Functions repressed by all treatments

GO term	Genes repressed with MR	Genes repressed with MV1	Genes repressed with MVR
Double-strand break repair	5	6	4
Cell division	16	33	40
Cell cycle	25	46	57
Mitosis	13	28	29
Negative regulation of ubiquitin-protein ligase during mitosis	7	17	17
DNA replication	9	21	25

whether R enhances cytotoxicity of MLN8237, docetaxel, or vincristine and doublet combinations, we evaluated degree of apoptosis by flow cytometry in RL, Granta-519, and SUDHL-4 cell lines using sublethal doses (docetaxel 5 nmol/L, rituximab 10 μg/mL, MLN8237 5 nmol/L). As shown in Fig. 2A, MLN8237, docetaxel, or rituximab alone had no significant effect on inducing apoptosis compared with control. However, the combination treatments of MR, MD, and MDR significantly induced apoptosis compared with control ($P < 0.05$ and $P < 0.001$) in all 3 B-NHL cell lines. Importantly, rituximab significantly enhanced apoptosis of MD therapy (Fig. 2A). Interestingly, apoptosis was not increased by RD therapy. However, superior results were observed with vincristine (0.1 nmol/L) and combinations MV and MVR at sublethal doses in Granta-519 cells (Fig. 2B) that correlate well with the MTS cell viability data (Fig. 1).

Rituximab increases *in vivo* anti-NHL activity of MLN8237 and MLN8237 plus docetaxel

On the basis of our *in vitro* data that targeting Aurora A in combination with rituximab [R] was more effective in inducing apoptosis, we evaluated this combination in a SCID mouse xenograft model of MCL (Granta-519). There were 6 cohorts of 12 mice: vehicle control, MLN8237 at 10 mg/kg and 30 mg/kg p.o. once a day for 3 weeks, rituximab at 10 mg/kg i.v. once per week × 4, MLN8237 at 10 mg/kg or 30 mg/kg for 3 weeks + rituximab 10 mg/kg i.v. once per week × 4. The dose of rituximab was based on a clinically relevant dose used in mouse xenograft tumor models. Treatments with MLN8237 or rituximab alone showed an approximately 10% to 15% TGI compared with vehicle control. However, MLN8237 (10 mg/kg or 30 mg/kg) plus rituximab showed significant TGI of approximately 50% compared
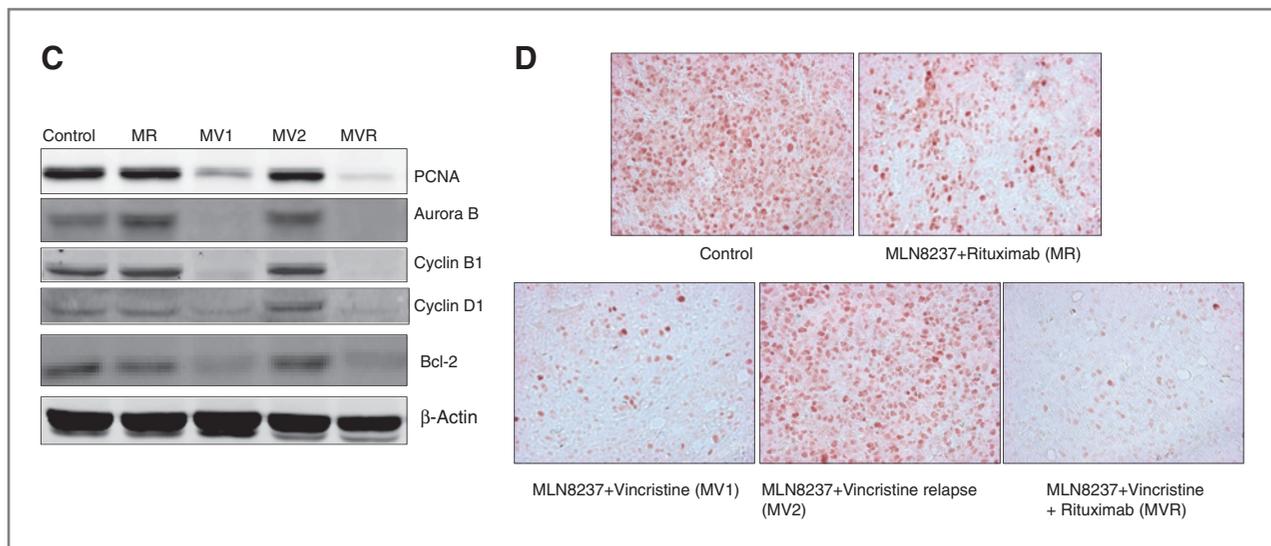
Figure 5. (*Continued*) C, protein was isolated from the tumors 3 hours after the end of last treatment as indicated and at relapse (MV2). Western blotting analysis showed that PCNA, Aurora B, cyclin B1 cyclin D1, and Bcl-2 were inhibited by MV1 (tumor regression phase) and MVR but not MR and MV2 (tumor relapse phase). D, PCNA was evaluated by immunohistochemistry from tumors 3 hours after the end of last treatment for control, MR, MV1, MVR, and MV2 (at relapse).

with vehicle control ($P < 0.05$; Fig. 3A) indicating rituximab enhances anti-NHL activity of MLN8237.

Next, we evaluated the effect of MLN8237 plus docetaxel or MLN8237 plus rituximab versus MDR triple combination to increase anti-NHL activity in a MCL xenograft mouse model. SCID mice bearing Granta-519 were treated with vehicle, MLN8237 30 mg/kg + rituximab 10 mg/kg, MLN8237 30 mg/kg + docetaxel 10 mg/kg, and MLN8237 30 mg/kg + rituximab 10 mg/kg + docetaxel 10 mg/kg, respectively. As shown in Fig. 3B, TGI was superior for MDR (approximately 85%) compared with MD (approximately 55%–60%) and MR (approximately 25%). Statistical analysis showed that tumor growth rate for MR, MD, and MDR was significantly less than that for control with $P = 0.004$, $P < 0.0001$, and $P < 0.0001$, respectively. MD and MDR also significantly inhibited tumor growth compared with MR with $P = 0.0024$ and $P < 0.0001$, respectively. Together, these data indicated that docetaxel increased anti-NHL activity of MLN8237 and MR significantly. The overall survival for mice treated with MDR was more than 45 days compared with control versus MD (approximately 30 days) and MR (approximately 15 days; $P = 0.01$). No weight loss of more than 10% was observed in any of the treatment arms.

MLN8237 plus vincristine plus rituximab is curative in a mouse xenograft model of mantle cell lymphoma

Because vincristine is used commonly in B-NHL as part of standard chemotherapy (e.g., CHOP), we replaced docetaxel with vincristine, the former targets microtubule polymerization whereas the latter microtubule depolymerization. We treated xenograft mice ($n = 4$ per treatment arm) bearing Granta-519 with vincristine at 4 different doses (0.0375 mg/kg, 0.07 mg/kg, 0.14 mg/kg, and 0.375 mg/kg) i.v. once per week ×4. The results showed that vincristine alone had a modest TGI of approximately

10% to 15% compared with control ($P > 0.05$; data not shown). However, MLN8237 (30 mg/kg p.o., once a day for 3 weeks) plus vincristine (0.375 mg/kg i.v., once a week ×4) [MV] and MLN8237 (30 mg/kg p.o., once a day for 3 weeks) plus vincristine (0.375 mg/kg i.v., once a week ×4) plus rituximab (10 mg/kg i.v., once a week ×4) [MVR] led to tumor regression [TR] compared with control or MR ($P = 0.0072$ and $P < 0.0001$, respectively; Fig. 4A). Interestingly tumors treated with MV relapsed 20 days after stopping treatment, whereas those treated with MVR did not relapse for more than 100 days after stopping treatment (curative; Fig. 4A). Kaplan–Meier analysis of overall survival showed that the mice treated with MV and MVR had a statistically significant improvement in overall survival when compared with the control ($P < 0.0001$) or MR ($P = 0.0043$; Fig. 4B). The mice in all cohorts tolerated treatment well with no weight loss by more than 10%. In fact mice in the MVR were healthy and some were separated because of in-fighting.

Reactivation of cell-cycle regulators is a mechanism of resistance to MV abrogated by MVR therapy

To gain mechanistic insights for MV- or MVR-driven tumor regression, tumors ($n = 3$) were harvested at end of treatment (3 weeks) for each cohort. Figure 5A shows the relative sizes of control versus MR versus MV versus MVR. These tumors were evaluated for up- and downregulated genes by expression profiling and validated by Western blotting for selected cell-cycle regulators and biomarkers of proliferation. Unique and common Gene Ontologies (GO) were repressed by individual treatments (MR vs. MV vs. MVR) consistent with modulation of cell-cycle regulators (Fig. 5B) that provides mechanistic insight of the effectiveness of MV or MVR therapy. Overexpression cyclin D1 associated with G_1 and S-phase of the cell cycle (a hallmark

of MCL) and overexpression of Bcl-2, continues to be elevated with MR but is suppressed by MV1 (tumor regression phase) and is reexpressed in MV2 (tumor relapse phase), but remains completely suppressed with MVR therapy (Fig. 5C). Cyclin B1 associated with G_2/M phase of the cell cycle follows a similar expression pattern as cyclin D1 implicating an active cell cycle is inhibited by MVR therapy. Overexpression of Aurora B (mitotic phase and SAC) is inhibited by MV1 (regression phase) but is reactivated on relapse (MV2). However, MVR therapy continues to inhibit Aurora B by repressing mitotic sister chromatid segregation by repressed gene expression analyzed by GO. Inhibition of mitotic sister chromatid segregation is unique to MVR therapy and is most likely due to rituximab-mediated inhibition of cell proliferation. This effect is highlighted by PCNA by IHC and Western blot analysis, which showed proliferation to be completely repressed by MVR therapy and correlates with downregulation of cell-cycle activators identified by gene expression profiling (Fig. 5D).

Discussion

Lymphoma is a malignant transformation of B- or T-lymphocytes and is the most common type of hematologic malignancy in the United States which represents approximately 5% of all cancers and approximately 55% of blood cancers. Although modern treatment options such as R-Hyper-CVAD for MCL, R-CHOP for DLBCL and TFL have improved clinical outcomes (43–45), there are no curative therapies for more than 50% of patients. Here, we show that preclinical *in vitro* and *in vivo* mechanistic investigations with MLN8237 [M], an Aurora A selective SMI combined with Vincristine [V] is potently synergistic. However, B-NHL treated with MV eventually acquires resistance and relapse. In contrast, when Rituximab [R] is combined with MV, there is enhanced apoptosis and complete responses (curative) in a mouse xenograft model of MCL. Hence, MVR is a promising novel therapy and an early phase clinical trial has been initiated with this combination in relapsed/refractory aggressive B-NHL.

Studies using RNA interference and selective SMIs have shown that Aurora A inhibition is characterized by a SAC-induced mitotic arrest with formation of unipolar spindles, a tetraploid phenotype, and biphasic apoptosis. Thus, inhibiting its enzyme activity with specific SMIs to the catalytic domain ATP-binding site is regarded as feasible for targeted cancer therapy, and numerous inhibitors including MLN8237 have been developed and are now in clinical trials (2). Recently, we showed Aurora A to be overexpressed in a number of B-NHL cell lines and in MCL patient tissues increased expression correlated with decreased survival (32). Hence, we hypothesized that Aurora A is an attractive therapeutic target for the treatment of aggressive B-NHL. MLN8237 had potent activity against Aurora A kinase (32) and induced apoptosis in cultured cells (Fig. 2). However, as a single agent TGI is modest (Fig. 3A), suggesting that inhibition of Aurora A kinase alone is insufficient as an

anti-NHL therapy. We (32) and others (26, 27) have shown that Aurora A amplification overrides the SAC leading to paclitaxel resistance. Hence, inhibition of Aurora A abrogates the mitotic delay induced by paclitaxel (27) at sublethal doses. Therefore, Aurora SMIs in combination with MTAs such as the taxanes (32, 46) and [V] show synergy *in vitro* cell culture models of apoptosis and *in vivo* antitumor activity. Furthermore, we show that the combination of MLN8237 plus vincristine (targets microtubule depolymerization and hence inhibits spindle assembly) to have superior synergistic activity (Fig. 1 and 4A) than MLN8237 plus docetaxel (targets microtubule polymerization and inhibits spindle disassembly). The molecular mechanism(s) that determines synergy of Aurora inhibition plus vincristine is due to complete inhibition to form centrosome-driven mitotic spindle formation.

Since U.S. Food and Drug Administration approval in 1997, rituximab [R] has become one of the most widely prescribed therapeutic agents for the B-NHL patients due to the ubiquitous expression of the target protein CD20 on the surface of B lymphocytes and the fact that the vast majority of NHLs are B-cell malignancies (47). CD20 is a B-cell marker of differentiation and proliferation. The mechanisms of antilymphoma effect of R from experimental evidence are apoptosis, CDC, ADCC, ADCP, and vaccinal effect (48). In combination with chemotherapy, rituximab significantly improves survival outcomes for patients with B-NHLs (49, 50). Here, we show that rituximab induced apoptosis in different subtypes of B-NHL cells *in vitro* and importantly, when combined with MLN8237 or AT9283 plus a MTA (particularly vincristine) at sublethal doses induced significantly higher percentage of apoptosis (decreased Bcl-2; Fig. 5C) than each agent alone (Fig. 2). Although MV is synergistic in mice bearing MCL tumors, despite an initial complete response there is eventual tumor relapse (Fig. 4A). However, addition of rituximab to MV completely abrogated this relapse. To gain mechanistic insight gene expression profiling (GEP) and confirmatory Western blotting showed reemergence of expression of Aurora B, cyclin B1, cyclin D1, and Bcl-2 with MV therapy but not with MVR therapy. Comparisons of GEP of MR versus MV and MVR indicate that the latter combination directly and indirectly interferes with all aspects of the cell cycle, including inhibition of sister chromatid segregation (Fig. 5B). In contrast, addition of rituximab to MD led to superior TGI (approximately 85%) but not to tumor regression (Fig. 3B), as was observed with MV and MVR. Gene expression profiling showed several B-cell surface markers including CD19 and CD24 to be downregulated with MVR compared with MV, which could contribute to complete responses observed with the former therapy. We hypothesize that lymphoma progenitor or stem cells to be extremely sensitive to MVR therapy and this concept is under investigation.

In conclusion, collectively our findings indicate that Aurora A is an excellent therapeutic target for aggressive B-NHLs. The potential of a synergistic interaction between Aurora inhibition (MLN8237) and microtubule spindle

assembly (vincristine) is identified as a potent therapy for aggressive B-NHL. However, the MV combination is not curative in mice bearing B-NHL. The addition of R to MV prevents relapse and is synthetic lethal with curative potential, most likely by interfering with cellular components of proliferation not affected by MV therapy alone. Therefore, MLN8237 plus vincristine plus rituximab represents a novel therapeutic strategy in aggressive B-NHL and warrants early phase clinical trial evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Mahadevan, A. Stejskal, L.S. Cooke, D.O. Persky, R.I. Fisher, T.P. Miller, and W. Qi.
Development of methodology: D. Mahadevan, A. Stejskal, L.S. Cooke, D.O. Persky, R.I. Fisher, T.P. Miller, and W. Qi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Mahadevan, D.O. Persky, R.I. Fisher, T.P. Miller, and W. Qi.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Mahadevan, A. Stejskal, L.S. Cooke, A. Manziello, and C. Morales.

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Correction: Aurora A Inhibitor (MLN8237) plus Vincristine plus Rituximab Is Synthetic Lethal and a Potential Curative Therapy in Aggressive B-cell Non-Hodgkin Lymphoma

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Aurora A Inhibitor (MLN8237) plus Vincristine plus Rituximab Is Synthetic Lethal and a Potential Curative Therapy in Aggressive B-cell Non-Hodgkin Lymphoma

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