HDM-2 Inhibition Suppresses Expression of Ribonucleotide Reductase Subunit M2, and Synergistically Enhances Gemcitabine-induced Cytotoxicity in Mantle Cell Lymphoma

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Abstract

Mantle cell lymphoma (MCL) usually responds well to initial therapy but is prone to relapses with chemoresistant disease, indicating the need for novel therapeautical approaches. Inhibition of the p53 E3 ligase HDM-2 with MI-63 has been validated as one such strategy in wild-type (wt) p53 models, and our genomic and proteomic analyses demonstrated that MI-63 suppressed the expression of the ribonucleotide reductase (RNR) subunit M2 (RRM2). This effect occurred in association with induction of p21 and cell cycle arrest at G1/S, and prompted us to examine combinations with the RNR inhibitor gemcitabine. The regimen of MI-63/gemcitabine induced enhanced, synergistic anti-proliferative and pro-apoptotic effects in wtp53 MCL cell lines. Addition of exogenous dNTP’s reversed this effect, while shRNA-mediated inhibition of RRM2 was sufficient to induce synergy with gemcitabine. Combination therapy of MCL murine xenografts with gemcitabine and MI-219, the in vivo analogue of MI-63, resulted in enhanced anti-tumor activity. Finally, synergy was seen with MI-63/gemcitabine in primary patient samples, which were found to express high levels of RRM2 compared to MCL cell lines. These findings provide a framework for translation of the rational combination of an HDM-2 and RNR inhibitor to the clinic for patients with relapsed wtp53 MCL.
Introduction

Mantle cell lymphoma (MCL) is a B-cell lymphoma that accounts for 6-8% of lymphoid malignancies, and typically harbors the t(11;14) translocation resulting in aberrant cyclin D1 expression and cell cycle dysregulation. MCL patients present with a spectrum of disease types ranging from slow, indolent growing malignancies, to more aggressive variants, such as the blastoid phenotype. It is characterized clinically by good initial responses to induction chemotherapy, but later almost invariably relapses with a more aggressive course, which has made it an attractive model for novel drug development. One such novel agent is the proteasome inhibitor bortezomib, which was shown to be active against MCL in a phase I trial, and then approved for treatment of relapsed disease after multi-center studies fully demonstrated its activity. Other drugs that have shown promise against MCL include novel agents such as the mammalian target of rapamycin inhibitors temsirolimus and everolimus, and the immunomodulatory drug lenalidomide, as well as more traditional cytotoxics such as gemcitabine.

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC) is an effective, broad-spectrum anticancer agent with activity in a number of malignancies, including as a single agent and in combination for non-Hodgkin lymphoma. It is transported into the cell mainly through the human equilibrative nucleoside transporter (hENT), where it is metabolized by deoxycytidine kinase (dCK) into its two active forms, gemcitabine-diphosphate (dFdCDP) and gemcitabine-triphosphate. The cytotoxic effects of gemcitabine are due to the ability of dFdCDP to inhibit the function of ribonucleotide reductase (RNR), and of dFdCTP to compete with dCTP for incorporation into DNA. dFdCP is identified by RNR as a substrate, metabolized within the active site, and generates mutated products which inactivate the R1 subunit (RRM1), and induce the loss of the tyrosyl radical essential for action of the R2
subunit (RRM2). This produces a global decrease in the level of available cellular dNTP’s for DNA replication. Also, the dFdCTP moiety competes with cellular dCTP for insertion into DNA, after which DNA chain termination occurs and replication ceases. Gemcitabine is highly effective in part due to this dual action, particularly as RNR over-expression is correlated with enhanced invasive potential, malignant transformation, and metastasis, and over-expression by malignant cells conveys a selectivity that helps to reduce toxicity to normal cells.

Another attractive target for MCL therapy may be the p53 pathway, since DNA damage responses are altered in up to 75% of cases, in part through mutations of ataxia telangiectasia mutated (ATM) or p53. Over-expression of HDM-2, the human homologue of the murine double minute protein-2, which is seen in 22% or more of MCL cases, has also been associated with a more aggressive course and decreased survival. This may be due in part to its ability to decrease p53 levels through its activity as the major E3 ubiquitin ligase responsible for p53 ubiquitination prior to its proteasome-mediated degradation. Consistent with the possibility that non-genotoxic stabilization of p53 may be a valid approach to therapy of MCL, we and others have previously demonstrated the activity of HDM-2 inhibitors such as the Nutlins, and MI-63 and MI-219 against wild-type (wt) p53 MCL models both in vitro and in vivo. In this current work, we report the development of a mechanism-based combination regimen, which started with the observation that MI-63 decreased the expression levels of RRM2. By combining an HDM-2 inhibitor with gemcitabine, we were able to demonstrate that this regimen showed enhanced activity against MCL cell lines and xenografts compared to either agent alone. This approach may prove fruitful for the treatment of patients with relapsed, wt p53 MCL, and is ready for translation to the clinic.
Materials and Methods

Reagents. The HDM-2 inhibitors MI-63 and MI-219 were provided by Ascenta Therapeutics (Malvern, PA). Nutlin-3 (NUT), gemcitabine (GEM), for in vitro work, doxorubicin (DOX), and deoxyribonucleotides (dNTP’s) were purchased from Sigma-Aldrich (St. Louis, MO), while GEM for in vivo work was from the M. D. Anderson Cancer Center Clinical Pharmacy (Houston, TX).

Cell culture and patient samples. JVM-2, Granta-519, REC-1, and JeKo-1 MCL cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and validated through The M. D. Anderson Cancer Center Characterized Cell Line Core Facility. Cells were grown in RPMI 1640 (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich). Primary patient samples were obtained from the M. D. Anderson Department of Lymphoma and Myeloma Satellite Lymphoma Tissue Bank. Informed consent was obtained from each patient in compliance with the Declaration of Helsinki according to an M. D. Anderson Cancer Center Institutional Review Board-approved protocol, and samples were purified as previously described.30

Semi-quantitative and real-time PCR. Total RNA was isolated using an RNeasy Plus kit (Qiagen; Valencia, CA), and cDNA was synthesized using Superscript II (Invitrogen). Polymerase chain reaction was performed for 30 cycles annealing at 54.4°C, for RRM2 (GenBank™ accession number: NM_001165931) using sense primer 5’-GAAGGCAGAGGCTTCCTTTT-3’ and anti-sense primer 5’-AGAAACAGCGGGCTTCTGTA-3’; and for the p53-inducible R2 subunit (R2p53, or RRM2B; GenBank™ accession number: NM_015713) using sense primer 5’-GGGGATTCTGTGGTCAGATG-3’, and anti-sense primer
5'-GGCCAGCTTTTTCCAATCTT-3’. Primers and conditions for β2-microglobulin (β2M), which was used as a control, were as previously described.34 Real-time PCR for RRM1, RRM2, RRM2B, dCK, and hENT-1 was performed on a Stepone PCR analyzer (Applied Biosystems; Carlsbad, CA) using inventoried real time Taqman-FAM and GAPDH-VIC probes. Relative transcript expression (RQ) was determined using JVM-2 cells as a calibrator using the ΔΔCT method according to the manufacturer’s instructions.

p53 sequencing. Genomic DNA was extracted from MCL patient samples, and all exons, introns, and regulatory regions of p53 were sequenced by the M. D. Anderson DNA Analysis Core Facility. Exons were amplified using custom PCR primers, and Sanger sequencing was performed on a 3730xl DNA Analyzer using BigDye™ Terminator v3 chemistry (Applied Biosystems). Mutations analysis was performed using SeqScape® Software v2.5 (Applied Biosystems).

Gene expression profiling. RNA was extracted from MI-63- or vehicle-treated cells using TRIzol® (Invitrogen) in triplicate, and RNA was submitted to Genome Explorations (Memphis, TN) for gene expression profiling analysis using the Affymetrix (Fremont, CA) human genome U133 plus 2.0 Array platform.

Immunoblotting. Protein expression was measured by immunoblot analysis performed as previously described.34 Antibodies to RRM1, RRM2, RRM2B, and p53 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), the anti-poly-(ADP-ribose) polymerase (PARP) antibody was from Cell Signaling Technology (Danvers, MA), anti-p21 and anti-HDM-2 antibodies were purchased from EMD4Biosciences (Gibbstown, NJ), and the anti-β-actin antibody was from Sigma-Aldrich.
Cell cycle and cell death analysis. Cells were treated with drug for 24 hours, then fixed in 70% ethanol and stained with propidium iodide (Sigma-Aldrich). Cell cycle data were analyzed on a CANTO II flow cytometer (Becton-Dickson; Franklin Lakes, NJ) using FlowJo v.7.6.1 (Tree Star, Inc.; Ashland, OR). Cell death was also measured using Annexin-V Pacific Blue and TO-PRO-3 (Invitrogen) on a CANTO II flow cytometer using FlowJo v 7.6.1.

Cell proliferation assay. The WST-1 reagent (Roche Diagnostics; Indianapolis, IN) was used to determine the effects of chemotherapy agents on cell proliferation and to calculate their relative IC₅₀'s as previously published.³⁵

Lentiviral shRNA knockdown of RMM2. Lentiviral shRNA particles (Santa Cruz Biotechnologies) containing either a scrambled-sequence shRNA, or shRNAs targeting RRM2, were transduced overnight at a multiplicity of infection of 2 into REC-1 MCL cells. These were then drug selected with 2 μg/ml puromycin (Sigma-Aldrich) to generate stable cell lines.

Drug synergy assays. To evaluate for the presence of synergistic interactions, the methods of Chou and Talalay were used.³⁶,³⁷ Briefly, MI-63 or gemcitabine was added to cells, and the IC₅₀ of each drug individually was determined using the WST-1 assay. A range of serial dilutions was made across the IC₅₀ dose range, with the IC₅₀ set as 1x and dilutions made relative to this value. The agents were then added simultaneously to the cells, or in a sequence-specific order for 3 days, and WST-1 assays were performed. Data were then analyzed using CalcuSyn software (Biosoft; Cambridge, United Kingdom), and combination indices (CI) were calculated.
**In vivo xenograft model.** Experiments were performed in accordance with procedures and protocols approved by the M. D. Anderson Cancer Center Animal Care and Use Committee. Six to eight week old male CB-17 severe combined immunodeficiency (SCID) mice (Harlan Laboratories, Inc.; Indianapolis, IN) were inoculated in the right flank subcutaneously with $5 \times 10^6$ Granta-519 cells. Tumor burden was monitored by measuring the length and width of visible masses using calipers three times per week, and tumor volumes were calculated using the equation $0.4 \times L \times W^2$. When tumor burdens reached approximately 100 mm$^3$ in volume, mice were randomized into four groups of five mice each to intraperitoneal (IP) injections of vehicle (10% polyethylene glycol, 3% Cremophor EL, 87% phosphate-buffered saline), MI-219 (100 mg/kg), gemcitabine (60 mg/kg), or both agents simultaneously at fifty percent of the single-agent dose (50 mg/kg MI-219, 30 mg/kg gemcitabine). Mice were sacrificed by CO$_2$ asphyxiation when tumor size reached greater than 15 mm in any one direction according to institutional guidelines.

**Statistical Analyses**

Data were subjected to statistical analyses by calculation of the standard error of the mean (SEM). The significance of drug effect relationships was further determined using one tailed unpaired t tests using Excel software (Microsoft Corporation; Redmond, WA). For the *in vivo* studies, an analysis of co-operative effects of MI-219 and GEM on tumor growth data was performed using a Bayesian Bootstrapping approach. We calculated $Pr(\min(\mu_M, \mu_G) < \mu_C | data)$, the posterior probability that the minimum of the two (posterior) means of the response, as evidenced by either final tumor volume, or average tumor growth rate, for MI-219 alone, $\mu_M$, or GEM alone, $\mu_G$, was less than the (posterior) mean response for the combination, $\mu_C$. This probability calculates the likelihood that average response in the combination arm will be less than the minimum of the two single agent arms. Cooperative effects are shown if this posterior
probability is large. The statistical software R (www.r-project.org) was used for all the analyses using 10,000 bootstrap samples.
Results

MI-63 induces down-regulation of RRM2 in MCL cells. We previously demonstrated that inhibition of HDM-2 by MI-63 was an effective strategy against MCL, and sought to further explore the consequences of HDM-2 inhibition at the genomic level. Gene expression profiling of wtp53 REC-1 cells exposed to MI-63 for 24 hours revealed enhanced expression of known p53 target genes (Table 1), such as p21 and Sestrin-1. Also, strong up-regulation of the apoptotic mediators tumor necrosis factor receptor superfamily, member 6 (Fas) and Bcl-2-associated X protein (Bax) was seen, consistent with stabilization of p53 by MI-63. This coincided with down-regulation of key genes involved in cell cycle progression and cell proliferation, such as E2F transcription factor 8 (E2F8) and thymidine kinase. Of particular interest was the suppression of the RNR M2 polypeptide (RRM2), which was reduced by more than 3-fold based on two different probes relative to the vehicle control. In order to further confirm this decrease, we measured RRM2 transcript levels by RT-PCR in Granta-519 and JVM-2, two other wtp53 MCL cell lines. Compared to β2M as a control, exposure of REC-1, Granta-519, or JVM-2 to either MI-63, or the cis-imidazoline HDM-2 inhibitor Nutlin, resulted in decreased RRM2 transcript levels in all cell lines (Figure 1A). Conversely, and as expected, HDM-2 inhibition increased transcript levels for the p53-inducible RNR subunit M2B (RRM2B), which supplies excess dNTP’s to cells undergoing genotoxic stress. Suppression of transcription of RRM2 also resulted in a profound decrease in RRM2 expression at the protein level in Granta-519 and JVM-2 cells (Figure 1B) compared to β-actin as a control, and compared to RRM2B, which increased modestly after exposure to MI-63.

Gemcitabine is synergistic with MI-63. We next considered the possibility that MI-63-mediated suppression of RRM2 could enhance sensitivity to gemcitabine, since RRM2
over-expression correlates with enhanced clinical gemcitabine resistance. Over-expression correlates with enhanced clinical gemcitabine resistance. Exposure of MCL cell lines to MI-63 alone at 5 μM for 24-hours resulted in the induction of cell death in 35-50% of wtp53 cells (Figure 2A), while gemcitabine at 10 nM produced apoptosis in 20-50% of cells. When both agents were added simultaneously for 24 hours, this increased the proportion of cells undergoing cell death to 90% in REC-1, 80% in Granta-519, and 60% in JVM-2.

Statistical analysis using an unpaired t-test indicated that the combination of MI-63 and gemcitabine induced a significantly greater reduction in viability (p<0.05) compared to either MI-63 or GEM alone. The sole exception to this pattern were mutant (mut) p53 JeKo-1 cells, in which small amounts of cell death were seen due to their relative resistance to low doses of gemcitabine, and complete lack of sensitivity to MI-63.

Combination chemotherapy regimens can have different efficacies depending on the sequence of addition of the drugs of interest. We therefore titrated MI-63 and gemcitabine against a panel of MCL cell lines, and sought to determine what sequence of administration could be superior. We first determined the IC₅₀ of each cell line with both MI-63 and GEM at 3 days (Supplementary Figure 1A and B, respectively). These calculated IC₅₀’s allowed us to dose the agents in a 1:1 ratio based on the fold IC₅₀ (0.25x, 0.5x, 1x, 2x, and 4x), as suggested for synergy calculations as set out in the methods of Chou and Talay. Simultaneous administration of the two agents was compared with a schedule that allowed for pre-treatment with one agent for 24 hours, followed by 48 hours with the second agent, for a total time period of 72 hours. In Granta-519 cells, all three of these approaches resulted in enhanced cell killing compared to either agent alone at the lower drug concentrations (Figure 3B). Combination index (CI) analysis yielded CI values of 0.32-0.970, consistent with the presence of strong synergy for all three sequences (Table 2). Simultaneous addition of MI-63 and GEM also showed synergy in REC-1 (CI 0.33-0.93) and JVM-2 cells (0.71-0.81)(Table 2). Pre-treatment with MI-63 was synergistic in all three cell lines, particularly at low concentrations of 0.25x up to 1x of the IC₅₀, with CI values ranging as low as 0.28 in REC-1.
However, synergy was lost at the higher concentrations tested of 2x and 4x the IC₅₀. Also, pre-treatment with gemcitabine demonstrated synergy in REC-1 cells (CI range 0.32-0.74), though this was lost at higher concentrations. In contrast, JVM-2 and Granta-519 cells showed synergy across the concentration range, with CI ranges of 0.67-0.91 and 0.41-0.97, respectively.

Expression of RNR is regulated in a cell cycle-dependent manner³⁹,⁴⁰, and it was therefore of interest to analyze the impact of simultaneous addition of MI-63 and gemcitabine on wtp53 MCL cells. Cell cycle analysis indicated that MI-63 as a single agent induced a G₁ cell cycle arrest (Supplementary Figure 2), whereas gemcitabine induced a predominantly S-phase arrest. When both agents were combined, they generated a G₁ arrest in REC-1 and JVM-2 cells, and a G₁/S arrest in Granta-519 cells. These findings suggest that MI-63 was able to override the S-phase arrest induced by gemcitabine, and that the loss of RRM2 expression was due to a G₁ cell cycle arrest, during which this subunit is known to be depleted due in part to its short half-life.³⁹ At the protein level, MI-63 as a single agent induced an accumulation of p53, p21, and HDM-2 in all three wtp53 MCL cell lines compared to the vehicle control (Figure 2C). The same was true for the MI-63/gemcitabine combination, although the levels of these targets typically did not quite reach those seen with MI-63 alone. Enhanced levels of p21 would be expected to repress cyclin-dependent kinase 2, which would cause hypo-phosphorylation of Retinoblastoma protein and E2F, the latter of which is necessary for RRM2 transcription³⁹,⁴⁰, providing another mechanism for reduced RRM2 expression. Consistent with this mechanism, MI-63 alone decreased RRM2 levels in all three wtp53 MCL cell lines (Figure 2C), while gemcitabine seemed to mildly induce RRM2, suggesting the involvement of an inducible chemoresistance pathway. When the two were combined, cells exposed to the MI-63/gemcitabine regimen showed reduced levels of RRM2 compared to gemcitabine alone, possibly accounting for the enhanced pro-apoptotic activity.
Synergy between MI-63 and gemcitabine is related to RRM2. HDM-2 inhibition triggers a p53-dependent death program, and since gemcitabine also activates apoptotic signaling in part through p53\textsuperscript{41}, it was possible that the MI-63/gemcitabine combination was working through enhanced p53 induction. Western blotting of MCL cell lines treated with the combination did not reveal increased levels of p53, p21, or HDM-2 compared with either MI-63 or gemcitabine alone (Figure 2C), suggesting this was not the case. However, to test this more directly, we added an excess of dNTP’s to the culture media of cells exposed to either agent or the combination, and evaluated the effects on cell death and signaling. Addition of dNTP’s to MI-63-treated cells did not reduce cell death (Figure 3A), which was actually increased (p value 0.003), whereas dNTP’s slightly attenuated the degree of cell death induced by gemcitabine alone (p value 0.03). When dNTP’s were added to cells exposed to the combination, the synergistic effect was substantially blunted, with a reduction in cell death from 75% to 50% (p value <0.01), which was comparable to that induced by MI-63 alone (Figure 3A). This was associated with a decreased level of cleavage of poly (ADP-ribose) polymerase (PARP) in the presence of dNTPs compared to that without supplemental nucleotides (Figure 3B). Notably, the levels of p53, p21, and HDM-2 that accumulated after exposure to the MI-63/gemcitabine combination were not dramatically changed by the presence of dNTPs, suggesting that p53 signaling was not affected. To further evaluate the role of suppression of RRM2 in the synergy of this combination, we generated REC-1 cells in which expression of this polypeptide was reduced by over 90% using a targeted shRNA (Figure 3C). Knockdown of RRM2 enhanced cell death to some extent due to both MI-63 and gemcitabine (Figure 3D), but the combination regimen was no longer able to show synergistic induction of apoptosis. These findings support the possibility that the MI-63/gemcitabine regimen is active through the ability of the HDM-2 inhibitor to reduce RRM2, thereby sensitizing to the ribonucleotide reductase inhibitor.
Activity of MI-63/gemcitabine against an MCL xenograft model. Since the combination of MI-63 and gemcitabine showed promising efficacy in vitro, we evaluated its activity against established MCL xenografts in an immunodeficient murine model using MI-219, the in vivo formulation of MI-63. The latter agent alone, when given daily for 2 weeks at a dose of 100 mg/kg, slowed tumor growth (p<0.05) (Figure 4, upper panel) to an average of 30 mm³/day (Figure 4, lower panel), compared to 74 mm³/day for the vehicle control (p=0.002). Gemcitabine as a single agent, given at 60 mg/kg every three days for a total of only 2 weeks, also delayed tumor growth (p<0.05) (Figure 4, upper panel), slowing it to 20 mm³/day (p=0.09; Figure 4, lower panel). For the combination treatment, each agent was reduced in dose by 50%, and MI-219 at 50 mg/kg with gemcitabine at 30 mg/kg for two weeks slowed tumor growth to 5 mm³/day (p=0.049). Analysis of the cooperative effects between MI-219 and GEM in terms of the final tumor volume indicated that the posterior probability of co-operative effect was found to be 0.9951, indicating that there was less than a 50 in 10,000 chance that the combination did not have a cooperative effect. For the average tumor growth rate, the cooperative effect was greater than 0.9977, demonstrating that there was less than a 25 in 10,000 chance that the combination did not have a cooperative effect, and a greater than 9,975 in 10,000 chance that such an effect did exist. These analyses indicated a high level of evidence for the presence of co-operative effects of the combination treatment as compared to the single agents. No toxicities were observed with either the single agents or the combination at the doses used in these cohorts.

MI-63 and gemcitabine are active against primary MCL cells. Regimens such as MI-63 and gemcitabine that show synergy pre-clinically may be attractive candidates for translation to the clinic, and we therefore evaluated the activity of this mechanism-based combination against patient-derived MCL cells. Nine primary samples were obtained, six of which harbored two wt p53 alleles based on genomic DNA sequencing, while two were
heterozygous, with one \emph{wt}p53 and one mutant p53 allele (Supplementary Table 1). The majority of these samples were from patients with relapsed disease that had been previously treated with up to four prior lines of therapy (Supplementary Table 1), while two were from patients with newly diagnosed disease. Exposure of these samples to MI-63 for 24 hours induced cell death in 20-80% of cells (Figure 5A). Gemcitabine alone for 24 hours showed greater variability, in that while some samples were quite responsive, with cell death seen in up to 53%, such as the cells from patient 3, others were relatively resistant, with values as low as 3%, such as in patient 8. When the combination regimen was used for 24 hours, substantial enhanced effects consistent with synergy were seen in the samples from patients 1 through 4 (p<0.05 relative to MI-63 alone or gemcitabine alone). Weakly additive effects were seen in patients 5, 7, and 9 (p<0.05 relative to gemcitabine alone), while patients 6 and 8 showed no enhancement over what had been seen with MI-63 alone (Figure 5A).

To gain further insights into the sensitivity of these primary samples, we evaluated the expression levels of hENT-1, the gemcitabine transporter, dCK, which activates and metabolizes gemcitabine, and RRM1 and RRM2 by quantitative real-time PCR. Primary samples 1 through 4 expressed both hENT-1 and dCK (Figure 5B), suggesting they could readily transport and metabolize gemcitabine, and therefore respond well to the single-agent and combination therapy. Samples 5, 7, and 9, in contrast, had variable but generally lower expression levels of these genes (Figure 5B), possibly accounting for the lower level of enhanced cell death with the MI-63/gemcitabine regimen. Finally, samples 6 and 8, which showed no additive effects, also both expressed no hENT-1 (Figure 5B), though the former did show some sensitivity to single-agent gemcitabine (Figure 5A). All of the samples showed at least some expression of RRM1, but this was lower than that seen in some of the MCL cell lines (Figure 5B), and did not appear to correlate with sensitivity to gemcitabine. Similarly, RRM2 expression levels, which were more consistent between samples (Figure 5C), also did not predict for sensitivity to either gemcitabine or MI-63/gemcitabine. However, in
comparison to dCK, RRM1, and hENT-1 in the MCL cell lines, primary samples did express a relative abundance of RRM2 (Figure 5C). These findings support the translation of strategies to the clinic that suppress RRM2 levels in combination with gemcitabine, such as HDM-2 inhibition, for patients with relapsed mantle cell lymphoma that harbor wild-type p53 and express the human equilibrative nucleoside transporter and deoxycytidine kinase.
Discussion

Mantle cell lymphoma remains a challenging entity for the clinician, since there is as yet no widely accepted standard of care.\textsuperscript{1-3} Immunochemotherapy strategies incorporating rituximab are commonly used in the front-line settings.\textsuperscript{43,44} Younger patients may receive standard dose cyclophosphamide, doxorubicin, vincristine, and prednisone, followed by autologous stem cell transplantation, or more aggressive approaches with hyper-fractionated cyclophosphamide regimens without transplantation.\textsuperscript{43,44} These more intense approaches appear to increase complete response rates and overall survivals, but cannot be applied in all cases given that the median age at the time of diagnosis is typically in the mid-60’s. In the latter patients, and even in a substantial proportion of those who can receive aggressive therapy, the disease is still not cured, and at the time of relapse is more aggressive, less chemosensitive, and has a poor post-relapse survival.\textsuperscript{43,44} As a result, there is a strong imperative to develop new therapeutic agents and combinations, and to also utilize them in a molecularly adapted fashion, so that only those who would be most likely to benefit are exposed to the accompanying risks.

Our earlier pre-clinical studies had supported the possibility that HDM-2 inhibition was a rational strategy for therapy of MCL, and we therefore sought to develop combination regimens that could enhance this activity further. We first demonstrated that treatment of \textit{wtp53} MCL models with MI-63 resulted in a profound decrease in expression of RRM2 (Table 1, Figure 1). This was associated with an arrest at G1/S, and accumulation of p53 and its downstream target p21. RRM1, which is transcribed in S phase, has a long half-life of 20 hours, and remains at a constant level throughout the cell cycle.\textsuperscript{45} In contrast, while RRM2 is also regulated in a cell cycle-dependent manner, and transcribed during S phase, it has a much shorter half-life of 3 hours\textsuperscript{46}, and expression levels are therefore much more sensitive to
interruption of its transcription and translation. The decrease in RRM2 with MI-63 indicated that the combination of MI-63 and gemcitabine could be synergistic, particularly as dFDCP is known to inhibit RRM2\textsuperscript{12}, and in lung cancer patients decreased levels of RRM2 conveyed sensitivity to gemcitabine.\textsuperscript{47} Consistent with this possibility, the combination of MI-63 and gemcitabine was effective against MCL, and reached the statistical criteria for synergistic interactions (Table 2). Furthermore, studies into the influence of the sequence dependence of gemcitabine and MI-63 demonstrated that simultaneous use of the two agents, as well as pre-treatment with either one or the other, was effective (Figure 2). Also, MI-63 appeared to overcome the S-phase arrest effect of gemcitabine, and the expected subsequent enhanced transcription of RRM2. A synergistic interaction was further supported by the MCL xenograft model, where a combination of MI-63 and gemcitabine showed enhanced tumor growth delay, even at a 50% dose reduction compared to either of the single agents (Figure 4).

Treatment of primary samples \textit{ex-vivo} also yielded promising results despite the fact that six of the nine patients had received prior dose-intensive chemotherapy in the form of either stem cell transplantation, or hyper-fractionated cyclophosphamide (Supplementary Table 1). Notably, the efficacy against patient samples also showed some dependency on the expression of the nucleoside transporter hENT-1, and on dCK (Figure 5). Of particular interest was that the levels of hENT-1, dCK, and RRM1 were substantially higher in the MCL cell line REC-1, and lower in the MCL patient samples. Conversely, RRM2 levels were substantially higher in the primary lymphoma cells than in the REC-1 model, and both of these would be expected to negatively impact upon the sensitivity of MCL \textit{in vivo} to gemcitabine. Indeed, the expression of hENT-1 has been shown to be a mediator of sensitivity to gemcitabine in MCL, and a good correlation was found between hENT-1 expression and uptake and sensitivity to this nucleoside analog in previous studies.\textsuperscript{39} Similarly, RRM2 overexpression has been reported as a mechanism of resistance to gemcitabine in cell lines\textsuperscript{13}, and
over expression was correlated with poor survival in lung adenocarcinoma patients treated with docetaxel and gemcitabine.\textsuperscript{47} We observed a synergistic effect between MI-63 and gemcitabine in some of the MCL patient samples, while others were resistant to this combination. The resistance may be linked to other cellular factors beyond those we evaluated here, since gemcitabine can be transported, metabolized, and inactivated by additional factors, including the human equilibrative nucleoside transporter-2, the human concentrative nucleoside transporters-1 and -2, cytidine deaminase, and 5'-nucleotidase, among others. For example, one MCL patient who was resistant to nucleoside analogues demonstrated abundant hENT-1, dCK, and minimal 5'-nucleotidases, but was still resistant on presentation to fludarabine and gemcitabine.\textsuperscript{48} Moreover, alterations in genes such as ATM and p53 may influence resistance to nucleoside analogues\textsuperscript{49}, particularly in MCL given the high rate of changes in DNA damage pathways.\textsuperscript{21,22} It is also likely that the prior chemotherapy regimens patients received will have had an effect on the sensitivity to the MI-63/gemcitabine combination, particularly in those who received regimens incorporating DNA damaging agents.

The cis-imidazoline HDM-2 inhibitor RO5045337 is currently undergoing phase I trials targeting patients both with solid tumors (NCT00559533), and with hematologic malignancies such as chronic lymphocytic leukemia (NCT00623870). With regard to the latter study, a recent preliminary report indicated that 47 patients had been treated to date, and pharmacodynamic studies had shown induction of p53 target genes such as p21, Bax, and Fas.\textsuperscript{50} Dose escalation was continuing upward in two strata from 810 and 1920 mg/m$^2$/day, respectively, and clinical activity was seen in one patient with acute myeloid leukemia at 360 mg/m$^2$ who had achieved an ongoing complete remission for more than 9 months. These preliminary results of the first clinical trial of an HDM-2 inhibitor provide a proof of principle that the p53/HDM-2 axis can be manipulated with specific inhibitors not only pre-clinically, but also
clinically. In combination with our current data, which provide a molecular rationale for combining an HDM-2 inhibitor and gemcitabine in models of MCL, especially if they have wild-type p53 and express hENT-1 and dCK, these findings provide a strong framework for translation of this approach to the clinic. Moreover, since nucleoside analogues are used in other subtypes of non-Hodgkin lymphomas, as well as other hematologic malignancies, including acute and chronic leukemias, both in the relapsed and in some front-line settings, investigation of this regimen may be warranted pre-clinically and clinically in those settings as well.
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Authorship

Contribution: R.J.J. designed and performed all of the research, animal experiments and wrote the manuscript; L.E.F, J.E.R. and M.W. consented patients and obtained their samples; S.R and SN were essential for helping with processing of patient samples and maintaining the lymphoma tissue bank; D.Y. provided the MI-63 and MI-219 and method; VB performed statistical support and analysis; and R.Z.O. supervised all the research completed herein, and offered valuable suggestions and manuscript editing.

Conflict of Interest Disclosure

D.Y. is an employee of, and stockholder in, Ascenta Therapeutics, Inc. The remaining authors declare no relevant financial conflicts of interest.
Abbreviations

ATM, ataxia telangiectasia mutated; Bax, Bcl-2-associated X protein; CI, combination indices; 
dCK, deoxycytidine kinase; dFdCDP, gemcitabine diphosphate; dGdC, Gemcitabine (2',2'-difluoro-2’-deoxycytidine); dNTPs, deoxyribonucleotides; DOX, doxorubicin; E2F8, E2F transcription factor 8; Fas, tumor necrosis factor receptor superfamily, member 6; GEM, gemcitabine; HDM, human homologue of MDM; hENT, human equilibrative nucleoside transporter; IP, intraperitoneal; MCL, mantle cell lymphoma; MDM, murine double minute; 
mut, mutant; NUT, Nutlin; PARP, poly-(ADP-ribose) polymerase; RNR, ribonucleotide reductase; RQ, relative transcript expression; RRM1, ribonucleotide reductase subunit M1; 
RRM2, ribonucleotide reductase subunit M2; RRM2B, ribonucleotide reductase subunit M2B, 
or R2p53; SCID, severe combined immunodeficiency; wt, wild-type.
References


Tables

Table 1. Gene expression profiling of *wtp53* REC-1 MCL cells after exposure to MI-63.¹

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<th>Gene</th>
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<th>Description</th>
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<td>NM_024680</td>
<td>E2 transcription factor 8</td>
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<td>BC001886</td>
<td>Ribonucleotide reductase M2 polypeptide</td>
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<td>-3.3</td>
<td>BE966236</td>
<td>Ribonucleotide reductase M2 polypeptide</td>
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<td>CCNE2</td>
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<td>Cyclin E2</td>
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¹REC-1 cells were treated for 24 hours with vehicle or 5 μM MI-63. Total cellular RNA was then extracted from triplicate experiments, and gene expression changes were detected using the Affymetrix human genome U133 plus 2.0 Array. Fold changes in genes were determined with vehicle- versus MI-63-treated cells using Ingenuity Pathway Analysis software. Ten representative up-regulated and down-regulated genes are shown from the profile.
Table 2. Combination index analysis of MCL cell lines treated with MI-63 and gemcitabine.  

<table>
<thead>
<tr>
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<th>Pre-treatment with gemcitabine, followed by MI-63</th>
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<tr>
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<td>Fold X IC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>Granta-519</td>
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</tr>
<tr>
<td>JVM-2</td>
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<tr>
<td>REC-1</td>
<td>0.33*</td>
<td>0.50*</td>
<td>0.93*</td>
</tr>
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</table>

The indicated *wtp53* MCL cell lines were incubated with MI-63 or gemcitabine alone, and their individual IC<sub>50</sub> values determined. Interactions were determined using a 1:1 ratio of MI-63 to gemcitabine by simultaneous addition of both drugs, or in a sequence-specific manner for 72 hours, and combination index values calculated using a range of IC<sub>50</sub>’s ranging from 0.25 to 4 fold the IC<sub>50</sub> of each drug in each cell line. *Denotes a synergistic interaction.
Figure Legends

Figure 1. HDM-2 inhibitors decrease ribonucleotide reductase M2 polypeptide expression.

(A) REC-1, Granta-519, and JVM-2 wtp53 MCL cell lines were treated for 24 hours with either vehicle, 5 μM MI-63, 5 μM Nutlin, or 0.5 μM doxorubicin (DOX) as a positive control for 24 hours. Qualitative PCR was performed to detect RRM2 and RRM2B mRNA levels, as well as β2M as a loading control, and transcripts were visualized by native gel electrophoresis. Lanes marked +RT received 1 μL of cDNA stock solution, while those labeled with 1:10 received 1 μL of 1:10 dilution of +RT. Representative images are shown in both panels of one of three independent experiments.

(B) Protein levels of RRM2 and RRM2B, as well as β-actin as a loading control, were determined in lysates from MCL cells treated as above.

Figure 2. Gemcitabine and MI-63 enhance cell death.

(A) REC-1, Granta-519, and JVM-2, and the mutp53 MCL cell line JeKo-1, were treated for 24 hours with vehicle, 5 μM MI-63, 10 nM gemcitabine, or both. Flow cytometric analysis was then performed after staining with Annexin-V and TO-PRO-3, from which the proportion of cells undergoing apoptosis was calculated and normalized to the vehicle control group. Values represent the mean +/- the standard error from three independent experiments. An unpaired t-test was performed to evaluate for significance, and “*” denotes p values of <0.01 relative to MI-63 alone, while “#” denotes p values of <0.01 relative to GEM alone.

(B) Granta-519 cells were incubated simultaneously with single agent MI-63 or gemcitabine for 72 hours. In parallel, cells were exposed either first to MI-63 for 24 hours followed by gemcitabine and MI-63 for 48 hours, or to gemcitabine first for 24 hours followed later by MI-63 and gemcitabine for 48 hours. Cell viability was determined using the WST-1 reagent, and
results were expressed as the percentage viability relative to the vehicle control, which was arbitrarily set at 100%. The presence of synergistic interactions was determined by calculation of the combination index from the cell viabilities calculated across a serial dilution range of MI-63 or gemcitabine (Table 2). Each panel provides representative data from one of three independent experiments.

(C) Protein levels of HDM-2, p53, RRM1, RRM2, RRM2B, and p21, and β-actin as a loading control, were determined by Western blotting of cellular lysates.

Figure 3. Excess dNTP reverses synergy between MI-63 and gemcitabine.

(A) Granta-519 cells were incubated with vehicle, 5 μM MI-63, 10 nM gemcitabine, or both agents simultaneously for 24 hours, either without or with exogenous dNTPs at 50 μM. Cell death was then determined by flow cytometry using Annexin-V and TO-PRO-3 staining relative to the vehicle control. Each panel provides representative data from one of three independent experiments. An unpaired t-test was performed comparing cells to which dNTPs had been added to those exposed to drug alone, and “**” denotes p values of <0.05.

(B) Cellular lysates were probed for PARP, HDM-2, p53, RRM2, RRM2B, and p21, as well as β-actin as a loading control.

(C) REC-1 cells were infected with Lentiviral particles carrying a scrambled sequence shRNA, or an shRNA targeting RRM2, and stable cell lines were generated by drug selection. Cellular lysates were then probed for their content of RRM2, RRM2B, and β-actin as a loading control.

(D) REC-1 shRNA cells were incubated with vehicle, 5 μM MI-63, 10 nM gemcitabine, or both agents simultaneously for 24 hours, and the proportion of cells undergoing apoptosis was determined by flow cytometry using Annexin-V and TO-PRO-3. Statistically significant differences, defined as a p value of <0.05, are denoted by “**.”
Figure 4. MI-63 and gemcitabine inhibit tumor growth in vivo.

(A) SCID mice were inoculated with Granta-519 cells subcutaneously, and monitored until tumors were established. Five mice per group were then injected intraperitoneally with vehicle, MI-219 daily for two weeks, gemcitabine every third day for two weeks, or both agents using the same schedules but at a 50% dose reduction. Tumor volumes were measured three times per week, and are plotted as a function of time in the upper panel. Statistically significant differences, defined as a p value of <0.05, are indicated by “*” relative to the vehicle control, and by “#” relative to MI-219 alone. In the lower panel, the average tumor growth rate per day was calculated, and the p values of each group are shown relative to the vehicle group, as well as to MI-219 alone, or gemcitabine alone.

Figure 5. Synergistic activity of MI-63 and gemcitabine in MCL patient samples.

(A) MCL cells were purified from the peripheral blood of patients with circulating neoplastic cells using magnetic-activated cell sorting and CD19 microbeads. These were then either exposed to 5 μM MI-63, 10 nM gemcitabine, or both agents simultaneously for 24 hours. Cell death was determined by flow cytometry using Annexin-V and TO-PRO-3 staining relative to the vehicle-treated control, and REC-1 cells were included as an additional control. Each panel provides representative data from one of three independent experiments, and p values which were <0.05 are indicated with “**” denoting significance relative to MI-63 alone, and “#” denoting significance relative to GEM alone.

(B) Aliquots of each of the primary samples analyzed in panel A were also subjected to RNA extraction, cDNA was synthesized, and the levels of RRM1, dCk, hENT-1 and RRM2 were measured by quantitative real-time PCR using the ΔΔCT method with the JVM-2 cell line used
as a relative calibrator. The transcript level in REC-1 cells was also measured as a control, and is plotted on a separate scale due to their high expression of RRM1, dCK and hENT-1.

(C) Real time PCR analysis of RRM2 transcript levels in MCL patient samples is shown, along with REC-1 as a cell line control.
Figure 1.

(A) 

<table>
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<tr>
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(B) 

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- RRM2
- RRM2B
- β₂M
Figure 2

(A) % Cell Death

(B) Granta-519

(C) REC-1, Granta-519, JVM-2, JeKo-1
Figure 3.

(A) Bar graph showing % Cell Death with different drug treatments:
- MI-63
- MI-63 & GEM
- GEM
- MI-63 & GEM

(B) Western blot analysis of PARP, Cleaved PARP, HDM-2, p53, RRM2, RRM2B, p21, and β-actin with different drug treatments:
- Vehicle
- MI-63
- GEM
- MI-63 & GEM

(C) Western blot analysis showing RRM2, RRM2B, and β-actin with different treatments:
- Scramble
- shRNA RRM2

(D) Bar graph showing % Cell Death with different drug treatments:
- 5 μM MI63
- 10 nM GEM
- MI-63 & GEM
Figure 5.

(A) 

(B) 

(C)
HDM-2 inhibition suppresses expression of ribonucleotide reductase subunit M2, and synergistically enhances gemcitabine-induced cytotoxicity in mantle cell lymphoma