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Ritonavir Exhibits Limited Efficacy as a Single Agent in Treating Aggressive Mantle Cell Lymphoma

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Abstract

Background: Mantle Cell Lymphoma (MCL) is an aggressive B cell malignancy accounting for 6% of non-Hodgkin's lymphoma cases in the US. While various therapies are available to treat MCL, patients relapse within 3 to 4 years following treatment from therapy-resistant MCL, making MCL carry one of the worst prognoses of all non-Hodgkin's B cell lymphomas. A better understanding of the biological mechanisms of relapse and therapy-resistance in MCL is vital for developing mechanisms to target relapsing MCL, and providing better care for patients. Recent studies implicate the NFκB pathway and survivin in promotion of aggressive, therapy-resistant MCL. Therefore, we tested the efficacy of inhibiting this pathway in three MCL lines (GP, recently-developed GRL, and JVM2) using the protease inhibitor ritonavir (Abbott Laboratories), which has been shown to downregulate NFκB targets, including survivin, in other hematological malignancies.

Methods: MCL cells were incubated with ritonavir then assessed for changes in proliferation, apoptosis, and activation of NFκB transcriptional targets. In addition, *in vivo* studies were performed to assess ritonavir's utility as a single agent in MCL treatment using an immune-deficient mouse model of human MCL.

Results: When MCL cell lines were incubated with ritonavir *in vitro*, they exhibited reduced proliferation, increased apoptosis, and downregulation of NFκB pathway targets. However, no effect was seen when testing ritonavir as a single agent *in vivo*. Although, treatment with ritonavir plus vincristine *in vitro* revealed significant reduction in the proliferation of MCL compared to either treatment alone.

Conclusions: These studies suggest ritonavir is not suitable as a single-agent therapy for MCL. However, studies combining ritonavir plus vincristine *in vitro* suggest ritonavir may be effective in multi-pronged treatment approaches for MCL. These findings necessitate further studies to determine ritonavir's utility within a multi-pronged treatment approach for treating therapy-resistant MCL.

Keywords: Mantle cell lymphoma; Ritonavir; Refractory lymphoma; Protease inhibitors; NFκB; Survivin

Introduction

Mantle Cell Lymphoma (MCL) is an aggressive non-Hodgkin's B cell lymphoma. MCL is characterized by $CD5^{+}CD23^{-}$ monoclonal B cells containing a t (11; 14) (q13; q32) translocation causing overexpression of cyclinD1, a protein involved in cell proliferation [1]. Accounting for about 6% of all non-Hodgkin's lymphomas, MCL has multiple treatment options, including several chemotherapy regimens with and without rituximab, radio-immunotherapies, small molecule inhibitors, and stem cell transplantation. While these treatments are reasonably effective in lowering tumor burden in patients [2], relapse ultimately occurs due to the failure of these treatments to eliminate residual tumor cells. Consequently, even with current therapeutic options, median patient survival with MCL is only 3 to 4 years. MCL therefore carries the worst survival prognosis of all B cell lymphomas [3]. New treatment regimens are needed to not only target primary tumor burden, but to specifically target residual tumor, thus offering a greater survival benefit to patients.

Recent studies have suggested that aberrant activation of the NFκB pathway including upstream signaling modulators PI3K and Akt may be contributing to the aggressive nature of MCL [4-7]. These signaling patterns, which are broadly involved in cell growth and proliferation [8], have been shown to be constitutively active in MCL, leading to a further upregulation of certain cell cycle-related proteins, including MCL's characteristic driver of malignancy, cyclin D1 [9]. Inhibition of key molecules in this pathway through the use of NFκB pathway inhibitors has shown promise in lowering cyclin D1 expression, leading to a decrease in MCL proliferation [9]. These findings make the NFκB pathway an interesting target for combined therapy designs.

Overexpression of survivin—a target molecule in the NFκB pathway—has been implicated in promoting the proliferation an apoptosis-resistance of MCL [1]. Survivin, which is a member of the inhibitor-of-apoptosis protein family, is involved in preventing apoptosis by directly binding and inhibiting the executioner caspases 3 and 7 [10]. Additionally, survivin plays a role in cell cycle regulation by associating with proteins in the mitotic spindle apparatus [11]. Survivin is not expressed in most adult tissue but is commonly overexpressed in malignancies, making it a unique tumor-specific marker and antigen [12]. Therefore, the use of NFκB/survivin-targeting therapeutics may prove to be successful inhibitors of survivin, thus sensitizing MCL to more conventional therapeutics [11-15]. Other NFκB transcriptional targets also have roles in proliferation and survival that may be

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relevant to the progression, therapy-resistance, and/or aggressiveness of MCL. These include the anti-apoptotic protein Bcl2 and the cell cycle regulator Cyclin D2 [8,16,17]. Therefore, targeting the NFκB pathway in MCL may significantly impair the progression of this aggressive disease.

Recent reports have shown the FDA-approved protease inhibitor ritonavir (Abbott Laboratories) may be able to interact and abrogate signaling through the NFκB pathway, resulting in downregulation of survivin and cyclin D2, and alteration of NFκB binding abilities. These effects result in decreased growth, induction of apoptosis, and/or increased survival when ritonavir is tested against both hematological and non-hematological malignancies, including breast cancer, prostate cancer, lung adenocarcinoma, lymphoblastoid B cells, and T cell leukemia [14,18-21]. Therapies that incorporate targeting of survivin in aggressive non-Hodgkin's lymphoma have shown some therapeutic promise when combined with conventional combinatorial treatments *in vitro* and *in vivo* due to their multiple-target approaches [13,22]. Furthermore, mounting evidence confirms the necessity for multi-pronged, multiple target treatment approaches in the treatment of MCL [23,24]. Recent reports have shown ritonavir in combination with other therapies may enhance certain cancer treatments, including extra-nodal marginal zone B cell lymphoma (mucosal-associated lymphoid tissue type), human sarcoma, and various solid tumors, along with enhancing Human Immunodeficiency Virus (HIV) treatments for which it was originally designed [25-28]. Thus, the potential for ritonavir as a treatment for MCL may be promising.

Therefore, we chose to target the NFκB pathway and its targets, including survivin, using ritonavir to assess the potential of this therapy in inhibiting the proliferation and survival of MCL. We utilized three MCL cell lines: JVM2, Granta-519 (GP), and a therapy-resistant MCL cell line, GRL [29], which was derived from GP and represents relapsing MCL and represents highly-aggressive MCL. Briefly, we tested the ability of ritonavir alone or in combination with vincristine to inhibit proliferation and induce apoptosis in the MCL cell lines. Alteration of the NFκB transcriptional targets BCL2, CCND2 (cyclin D2), and BIRC5 (survivin) following ritonavir exposure was also assessed, and the protein levels and localization of the NFκB subunit p65 was analyzed. In addition, *in vivo* studies were performed to assess ritonavir's utility as a single agent in treating MCL. We found that the use of ritonavir in treating MCL as a single agent has little efficacy. However, further studies are warranted to test ritonavir's efficacy when used in a multi-pronged treatment approach.

Materials and Methods

Cell culture

The MCL cell lines, Granta 519 and JVM2, were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). The derivative cell line, GRL was developed from Granta 519, as described below. These cell lines were maintained in DMEM media (Invitrogen, CA) containing 10% fetal bovine serum (FBS) (U.S. Bio-Technologies Inc., PA), 1X penicillin/streptomycin (Invitrogen, CA) and 1X L-Glutamine (Invitrogen, CA), termed DF-10.

Isolation of therapy-resistant cell line GRL from NOD/SCID mice inoculated with GP and treated with CHOP + bortezomib therapy

The GRL cell line was developed as previously described [29]. Briefly, at Day 0 of the study, NOD/SCID mice were irradiated with 2.15 gray irradiation using the cobalt-60 source at the UNMC Experimental Irradiation Facility. On Day 1, mice were inoculated via tail vein injection with 2 x 10^6 GP cells suspended in 100 µl sterile media. On Day 11, mice were given three doses administered at every-other day intervals consisting of 30 mg/kg cyclophosphamide (Sigma-Aldrich, MO) (via intravenous injection), 0.375 mg/kg vincristine (Sigma-Aldrich, MO) (via intravenous injection), 2.5 mg/kg doxorubicin (Sigma-Aldrich, MO) (via intravenous injection), and 0.15 mg/kg prednisone (Sigma-Aldrich, MO) (given orally). On Day 24, mice were administered four doses of 0.5 mg/kg bortezomib given intravenously on every-other day intervals. When mice became moribund following treatment regimen (evidenced by weight loss, hunching back, ruffled fur, excessive dehydration, and/or hind-limb paralysis), mice were euthanized via CO_2 chamber method. Livers were harvested from mice and minced into single cell suspensions. Cells were washed at least three times in DMEM media, then cultured in DF10 in 5% CO_2 at 37°C until murine cells in cultures were eliminated and pure cultures of human tumor cells were obtained. If necessary, cultures were treated with 50 µg/ml gentamycin (Sigma-Aldrich, MO) to avoid/eliminate contamination.

In vitro growth assay

Ten thousand GP, GRL1, and JVM2 cells were cultured in 96-well plates in 200 µl DF10 medium, and the growth of these cells were determined at 24, 48 and 72 hours by MTT assays. Briefly, 25 µl of MTT reagent (Sigma-Aldrich, MO) (5 mg/ml in PBS) was added to the culture and incubated for 2 hours before the respective time point, and the cells were lysed using an SDS-based reagent. The intensity of the color developed was determined at 570 nm using a plate reader. To determine the effects of ritonavir alone, or in combination with vincristine on the GP, GRL, and JVM2 cell lines, ten thousand cells were cultured in the presence of 10 µM ritonavir (Abbott Laboratories, IL), 25 nM doxorubicin, and/or 5 µM vincristine for 24, 48, or 72 hours. The growth rate was measured by MTT and assays as explained above. The change of growth of treated MCL cells was determined relative to untreated cells. Statistical significance was determined using student's T test analyses.

Real-time PCR

RNA was isolated from fresh passages or following treatment with ritonavir of GP, GRL and JVM2 cells using Trizol reagent (Invitrogen, CA). cDNA was synthesized from RNA, and real-time PCR was performed for transcripts associated with the NFκB signaling pathway using an ABI PRISM 7000 Sequence Detection System, as described previously [30]. Statistical significance was determined using student's T- test analyses. Primers used in these experiments are as follows:

BIRC5: F: 5'-ACCTGAAGCTTCCTCGACA-3', R: 5'-AACCCTTCCCAGACTCCACT-3'; BCL2: F: 5'- GCATGCG-GCCTCTGTTTGATTTCT-3', R: 5'-AGGCATGTTGACTTCACTT-GTGGC-3'; CCND2: F: 5'-CTTCGCTTCTGGTATCTGGC-3', R: 5'-TGAGGAATGTTGTGATGGG-3'; HPRT: F: 5'-AGGGT-GTTATTCCTCATGGAC-3', R: 5'-GTAATCCAGCAGGTCAG-CAAAG-3'.

Apoptosis assay

GP, GRL, and JVM2 cells were cultured at a concentration of 1 x 10^6 cells/ml in 10 µM, 20 µM, or 40 µM ritonavir for 24, 48, or 72 hours. The amount of the cell population undergoing apoptosis was then assessed using the AnnexinV:FITC apoptosis assay kit (BD Pharmingen, CA),

performed according to manufacturer's instructions. Flow cytometry was performed in the flow cytometry core facilities of the University of Nebraska Medical Center, Omaha, NE. Statistical significance was determined using student's T test analyses.

Immuno-cytochemistry

Immunocytochemistry for the NFκB subunit p65 was performed to assess changes in protein level expression and localization at 24 hours following 10 μM ritonavir treatment in the GP, GRL, and JVM2 cell lines. Briefly, cells were adhered to poly-L-lysine-coated slides using a Shandon Cytospin III. Cells were fixed in paraformaldehyde, rinsed in Phosphate-buffered Saline (PBS), and then permeabilized with a Triton-X solution. Cells were washed, then blocked in 1% BSA in PBS, then incubated with primary NFκB p65 antibody (Santa Cruz Biotech, CA) at a 1:100 dilution. Cells were washed and incubated in goat α-mouse IgG-HRP antibody (Santa Cruz Biotech, CA) at a 1:1000 dilution. The VectaShield DAB kit (Vector Labs, CA) was used to develop slides, which were then cover-slipped and viewed via light microscopy.

Western blotting

Western blotting for the NFκB subunit p65 was performed to assess changes in protein level expression at 24 hours following 10 μM ritonavir treatment in GP, GRL, and JVM2 cell lines. Protein was isolated from cells using Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, IL). Proteins were run on 4-20% Ready Gels (Bio-rad, CA) and transferred onto PVDF membranes. Membranes were probed with mouse α-human p65 antibody (Santa Cruz Biotechnology, Inc, CA) and mouse α-human actin antibody (Novus Biologicals, CO) at 1:400 dilutions. Secondary antibody utilized was goat α-mouse IgG-HRP antibody (Santa Cruz Biotech, CA) at a 1:1000 dilution. VectaShield DAB kit was used to develop the membrane, which was then imaged using a PowerShot S X 200 camera.

In vivo studies

For *in vivo* studies, 6-8 week old NOD.CB17-*Prkdc*^{scid}/J mice were purchased from (Jackson Laboratories, ME) and housed in the UNMC Comparative Medicine Animal Facilities.

Studies assessing efficacy of ritonavir in NOD/SCID mice inoculated with GP

On Day 0 of the studies, NOD/SCID mice were irradiated with 2.15 gray using the UNMC cobalt irradiator facility. On Day 1, mice were inoculated with 1 x 10^6 GP cells suspended in 100 μl sterile media by tail vein injection. In one study, mice were then treated with 7 consecutive daily doses of 30 mg/kg ritonavir (administered orally, suspended in 50 μl milk vehicle) beginning on Day 11. This dosing is consistent with that used in previous studies [14,20]. In a second study, mice were treated with 30 consecutive daily doses of 30 mg/kg ritonavir beginning on Day 8. For ritonavir treatment, 30 mg/kg doses were given for 30 consecutive days. Mice were monitored for survival. When mice became moribund (as evidenced by weight loss, hunching back, ruffled fur, excessive dehydration, and/or hind-limb paralysis), mice were euthanized using the CO_2 chamber method. Statistical significance was determined using Kaplan-Meier Log-Rank test analyses.

Results

Effects of ritonavir on the proliferation of MCL cell lines *in vitro*

In order to be an effective treatment for MCL, ritonavir must inhibit the proliferation of this aggressive malignancy. Ritonavir has previously shown success in the inhibition of proliferation of other cancer types [18-20]. In order to assess the ability of ritonavir to inhibit proliferation *in vitro*, the GP, GRL, and JVM2 cell lines were incubated with 10 μM ritonavir for 24, 48, or 72 hours, and the proliferation of the cells was assessed using MTT assays. The 10 μM ritonavir concentration was chosen as this is the calculated approximate concentration present in the serum of a patient on a daily regimen of ritonavir, as currently approved by the FDA. At all time points, the GP, GRL, and JVM2 cells treated with 10 μM ritonavir exhibited significantly lower proliferation than control (untreated) cells, as shown in (Figure 1 Left Panel). This suggests ritonavir is successful in inhibiting the growth of aggressive, refractory MCL *in vitro*.

Induction of apoptosis following ritonavir treatment in MCL cell lines

Ritonavir has been previously shown to inhibit the proliferation and induce apoptosis in several malignancies, including hematologically-relevant adult T cell leukemia and lymphoblastoid B cells [14,20]. Therefore, to test the ability of ritonavir to induce apoptosis in MCL, AnnexinV:FITC apoptosis assays were performed. GP, GRL, and JVM2 cells were incubated with 10 μM, 20 μM, and 40 μM ritonavir for 48 and 72 hours and analyzed flow cytometrically for the binding of AnnexinV to indicate apoptosis induction. As shown in (Figure 1 Right Panel), treatment with ritonavir for 48 or 72 hours induces apoptosis in the GP, GRL, and JVM2 cell lines in a dose-dependent manner. This corroborates with previous studies exhibiting the ability of ritonavir to inhibit proliferation and induce apoptosis in malignant cells of hematopoietic lineage.

Changes in transcript level of NFκB targets following ritonavir treatment

Mechanistically, ritonavir has been shown to act on malignancies via modification of NFκB pathway activity [14, 18-19]. Therefore, to ascertain the mechanisms of proliferation inhibition and induction of apoptosis following ritonavir treatment in MCL, transcriptional changes to NFκB pathway targets were assessed. GP, GRL1, and JVM2 cell lines were treated with 10 μM ritonavir for 6 hours then harvested for RNA. Real-time PCR was performed to assess the effects of ritonavir on transcript levels of selected NFκB targets relevant to MCL. Transcript levels of the pathway targets BIRC5 (survivin transcript), BCL2 (anti-apoptotic Bcl2 protein), and CCND2 (cyclin D2 transcript) were compared between treated versus untreated groups. As seen in (Figure 2), at 6 hours following 10 μM ritonavir treatment, the transcript levels of BCL2 and CCND2 were significantly down-regulated in the treated cells as compared to untreated cells. Interestingly, the transcript levels of BIRC5 were significantly down-regulated in GP and GRL1 at 6 hours, but not in JVM2, suggesting this MCL line may have signaling interactions that differ from those of the Granta 519-type lines. Overall, the downregulation of the BCL2, CCND2, and BIRC5 (except in JVM2) transcripts in ritonavir-treated cells suggest ritonavir may be acting on or reducing the level of NFκB signaling in these treated cells.

Alterations in protein levels of p65 following ritonavir treatment

Due to implications of ritonavir acting through modulation of NFκB signaling, the protein levels and localization of the NFκB subunit p65 were evaluated in GP, GRL, and JVM2 cell lines following 24 hours treatment with 10 μM ritonavir. Figure 3 exhibits the protein levels of p65 seen in GP, GRL, and JVM2 at 24 hours following ritonavir

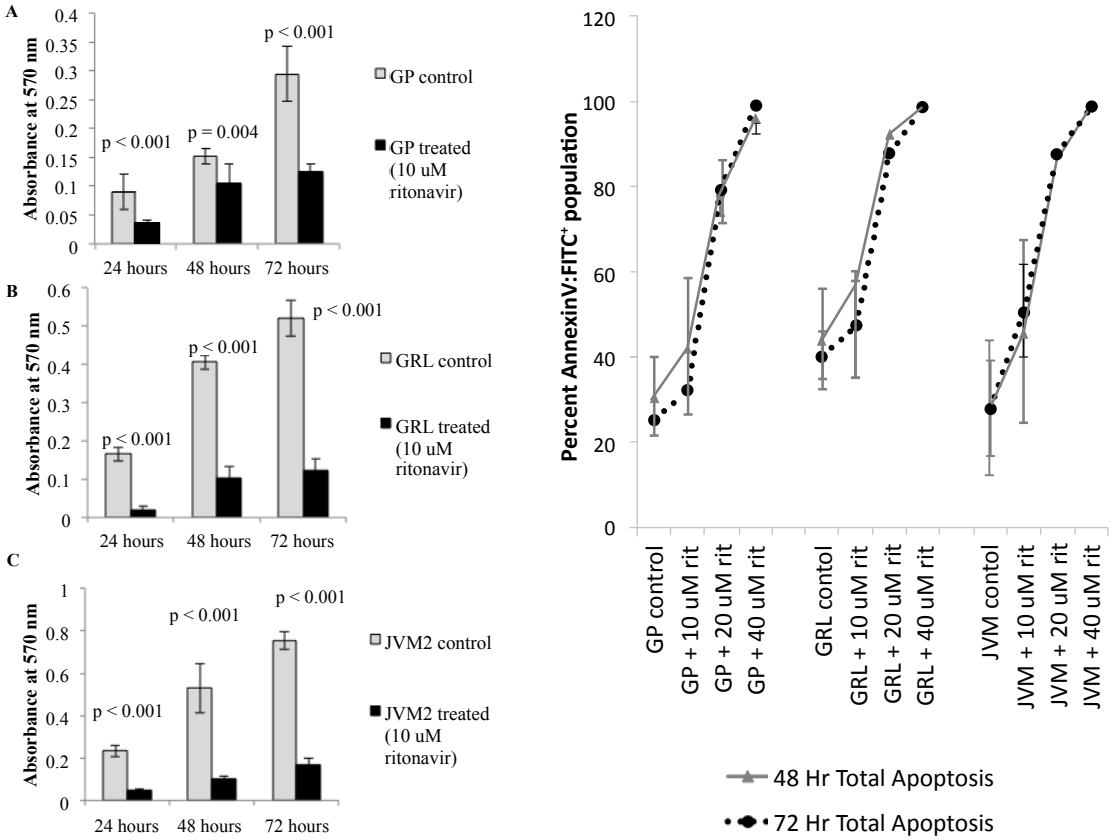

Figure 1: Effects of 10 μM ritonavir treatment on the proliferation and induction of apoptosis in MCL. *Left Panel*: proliferation of GP (A), GRL (B), and JVM2 (C) after 24, 48, or 72 hour incubation periods with 10 μM ritonavir, as assessed using MTT assays. GP, GRL, and JVM2 cell lines were seeded at 10,000 cells per well in a 96 well plate and incubated with 10 μM ritonavir for 24, 48, or 72 hours. Two hours prior to the given time point, 5 mg/ml MTT was added to each well, and cells were harvested at time point using a detergent-based buffer. Proliferation of the cells was assessed calorimetrically using a spectrophotometer. *Right Panel*: Induction of apoptosis in GP, GRL, and JVM2 after 48 or 72 hour incubation periods with 10 μM ritonavir, as assessed using AnnexinV:FITC apoptosis assay. GP, GRL, and JVM2 cells (1 x 10^6 cells) were treated with 10 μM ritonavir for 48 or 72 hours. The AnnexinV:FITC substrate and/or propidium iodide was then added to cells, and cells were analyzed via flow cytometry for positive staining. Cells positive for AnnexinV alone, or both AnnexinV and propidium iodide considered to be apoptotic.

treatment as assessed by immuno-cytochemical staining and western blotting. While western blotting reveals no significant change in total p65 protein levels, the immunocytochemical staining suggests a modest downregulation in nuclear staining of p65 in the GP and GRL cells treated with ritonavir, as compared to control. When activated, p65 will translocate from the cytoplasm to the nucleus. Therefore, the reduction in nuclear staining seen following ritonavir treatment in GP and GRL cells suggests there may be inhibition of NFκB activity as a result of ritonavir treatment.

Efficacy of seven day ritonavir treatment regimen in vivo in NOD/SCID mice inoculated with GP.

In a preliminarily test of ritonavir's efficacy *in vivo* against the aggressive human MCL line GP, 10 immune-compromised NOD/SCID mice were inoculated with 1 x 10^6 GP cells via tail vein injection. Ten days following inoculation, 5 mice were given vehicle only, while 5 mice received 30 mg/kg/day ritonavir for 7 consecutive days. Mice were then observed for survival. Shown in (Figure 4 Left Panel), treatment with ritonavir did not significantly improve overall survival with this treatment regimen, suggesting a longer treatment regimen or combined treatment approach may be necessary for treating MCL with ritonavir *in vivo*.

Efficacy of 30 day ritonavir treatment regimen *in vivo* in NOD/SCID mice inoculated with GP

In determining the efficacy of a longer treatment course similar to those previously shown effective against lymphoblastoid B cells *in vivo* [14], 20 NOD/SCID mice were first inoculated with 1 x 10^6 GP cells via tail vein injection. Seven days following the MCL injections, 10 mice were treated with vehicle only, while 10 mice were treated with 30 mg/kg/day ritonavir for 30 consecutive days. Mice were then observed for survival. As shown in (Figure 4 Right Panel), treatment with ritonavir at this dosing regimen and schedule did not significantly improve overall survival as assessed using Kaplan-Meier Log Rank Test analyses ($p = 0.17$), thus suggesting a combined regimen may be necessary for treating MCL with ritonavir *in vivo*.

Effects of ritonavir treatment in combination with vincristine treatment on the proliferation of MCL cell lines

In the treatment of MCL, multi-pronged approaches incorporating targeting of multiple mechanisms of growth and proliferation have shown greater potential than single treatment therapies [23-24]. In addition, ritonavir treatment has been shown to be particularly effective in enhancing the efficacy of standard treatments in certain cancers [18,26]. Therefore, GP, GRL, and JVM2 cells were treated

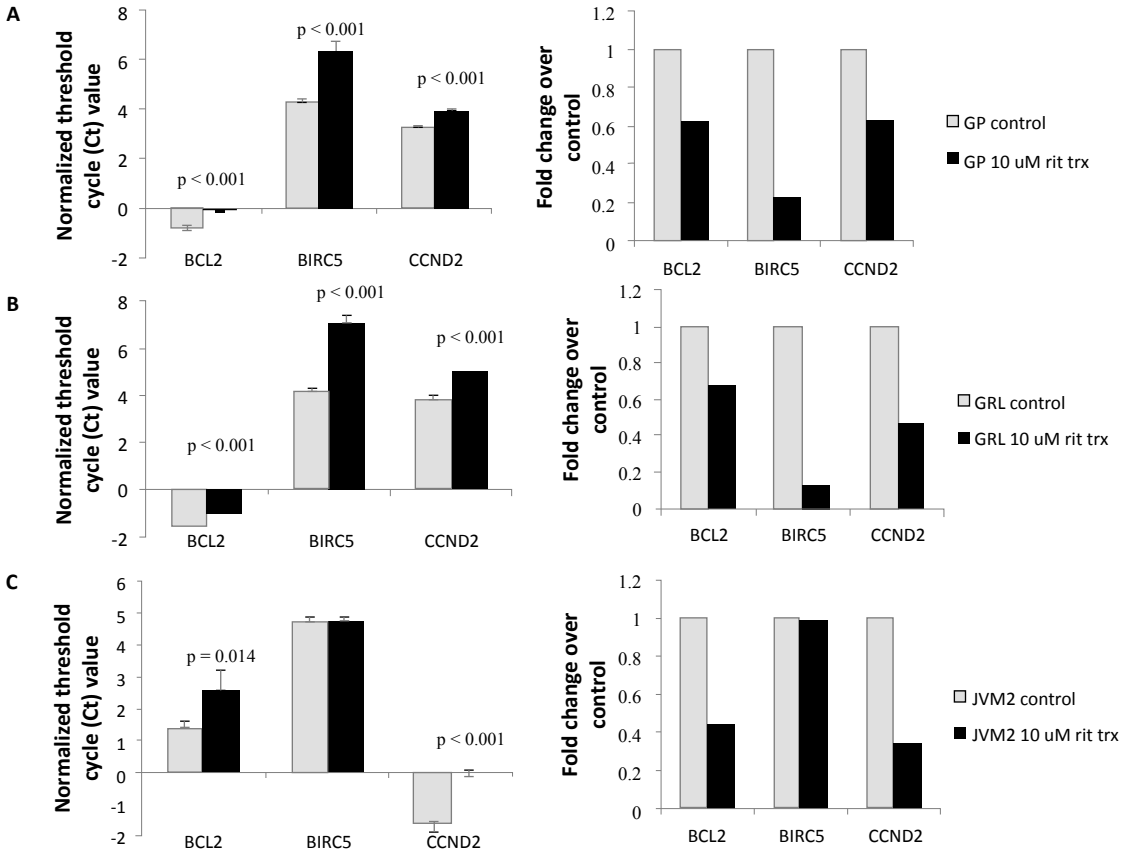

Figure 2: Effects of ritonavir treatment on NFκB target transcripts in MCL. Effects of 6 hours treatment with 10 μM ritonavir on the mRNA expression levels of NFκB targets BCL2, BIRC5 (survivin), and CCND2 (cyclin D2) in GP (A), GRL (B), and JVM2 (C), as assessed using real time PCR. GP, GRL, and JVM2 cells (1 x 10^6 cells) were treated with 10 μM ritonavir for 6 hours. Cells were then spun down and RNA isolated via Trizol. RNA was used for subsequent cDNA preparation and transcript-level analyses. Graphs on the left show threshold cycle (Ct) values of transcripts, normalized to the housekeeping control gene transcript HPRT. Graphs on the right show the correlative fold changes between untreated and treated cells.

with 10 μM ritonavir and/or 5 μM vincristine for 24, 48, or 72 hours. Vincristine, which is a vinca alkaloid and mitotic inhibitor, was utilized as it is represented in the standard MCL therapy of cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP), as well as other often-used clinical treatment regimens [24,31]. It is therefore plausible that targeting different mechanisms of proliferation and survival in MCL through combining the effects of ritonavir plus vincristine may lead to improved efficacies. Changes in proliferation of GP, GRL, and JVM2 cells at 24, 48, and 72 hours following ritonavir and/or vincristine treatments were assessed using MTT assays. As shown in (Figure 5), treating JVM2, GRL, or GP cells with a combination of vincristine and ritonavir resulted in a significant reduction of cell proliferation compared to vincristine alone or ritonavir alone (ritonavir alone to combined treatment: $p < 0.0041$ in GP, $p < 0.0095$ in GRL1, and $p < 0.001$ in JVM2 across all time intervals). This suggests ritonavir may have utility in MCL treatment as a partner in combined therapeutic regimens.

Discussion

The treatment of MCL represents an extremely difficult task for clinicians. While MCL patients often respond well to primary therapy, their period of remission is short-lived and gives way to aggressive disease that is nearly always resistant to currently available therapeutics. This issue presents an immediate and vital need for new therapeutic

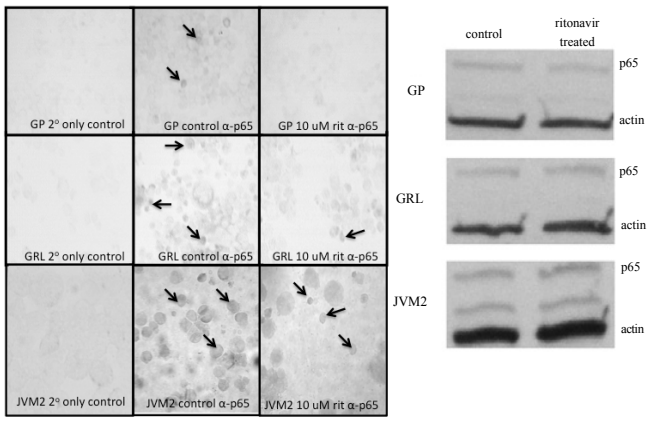

Figure 3: Effects of ritonavir treatment NFκB protein levels in MCL. Effects of 24 hours treatment with 10 μM ritonavir on the protein level expression and localization of NFκB subunit p65 in GP, GRL, and JVM2 cell lines, as assessed using immunocytochemistry (left panel, 40X magnification) and western blotting (right panel). GP, GRL, and JVM2 cells (1 x 10^6 cells) were treated for 24 hours with 10 μM ritonavir. For immunocytochemistry, cytospins were prepared and staining performed for p65. For western blotting, protein was then isolated with cell lysis buffer, run on SDS-PAGE gels, transferred to PVDF membranes, and blotted for p65. Arrows on immunohistochemistry figure indicate representative nuclear staining of p65.

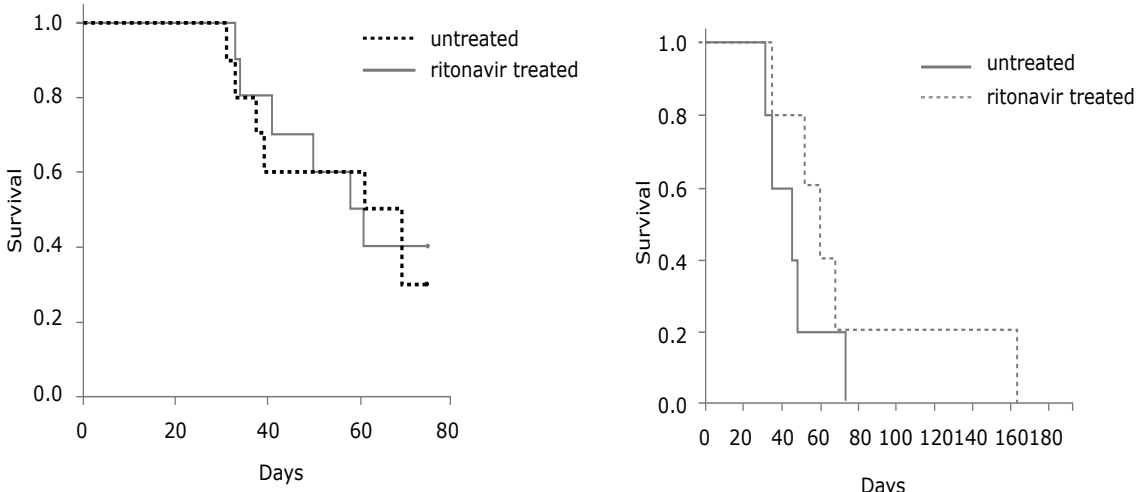

Figure 4: Efficacy of ritonavir as a single agent for treatment of MCL *in vivo*. *Left Panel:* Kaplan Meier Log Rank Test of efficacy with 7 days consecutive daily ritonavir treatment regimen *in vivo* in NOD/SCID mice inoculated with GP. *Right Panel:* Kaplan Meier Log Rank Test of efficacy with 30 days consecutive daily ritonavir treatment regimen *in vivo* in NOD/SCID mice inoculated with GP.

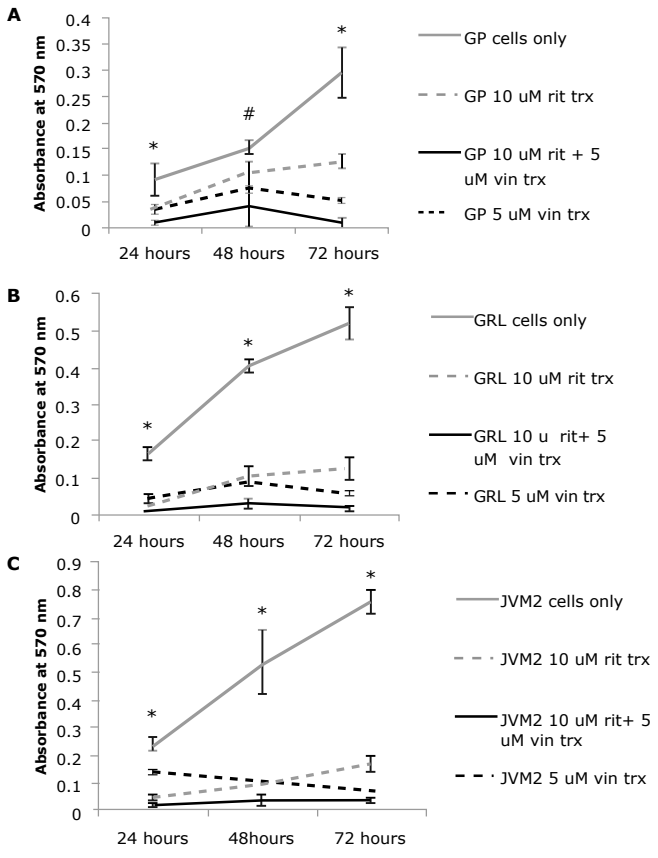

Figure 5: Efficacy of combined ritonavir + vincristine treatment in MCL. Effects of 10 μM ritonavir treatment and/or 5 μM vincristine treatment on the proliferation of GP (panel A), GRL (panel B), and JVM2 (panel C) after 24, 48, or 72 hour incubation periods, as assessed using MTT assays. GP, GRL, and JVM2 cell lines were seeded at 10,000 cells per well in a 96 well plate and incubated with 10 μM ritonavir and/or 5 μM vincristine for 24, 48, or 72 hours. Two hours prior to the given time point, 5 mg/ml MTT was added to each well, and cells were harvested at time point using a detergent-based buffer. Proliferation of the cells was assessed calorimetrically using a spectrophotometer. ** $p < 0.001$; # $p = 0.027$ comparing vincristine treatment alone to combined treatment with ritonavir and vincristine.*

measures for treating this disease. As scientific progress leads to the investigation of drug mechanisms and interactions, the potentials for alternate uses of FDA-approved pharmaceuticals in disparate disease settings has come to light. For example, while the therapeutic monoclonal antibody to CD20, rituximab, revolutionized the treatment of B-cell malignancies upon its FDA approval, rituximab is now well-appreciated and utilized in autoimmune/inflammatory conditions such as rheumatoid arthritis or systemic lupus erythematosus [32].

Another group of pharmaceuticals that has recently been studied for alternate uses is protease inhibitors. While originally developed as inhibitors of the HIV protease, this class of molecules is now being appreciated for its potential use in anti-cancer treatment regimens [33]. For example, the protease inhibitor ritonavir has demonstrated effects in various cancer types, including breast, prostate, and several hematological malignancies [14,18-20]. Therefore, improved therapy options for patients with MCL may be found in revisiting the varied potentials of FDA-approved drugs such as these protease inhibitors.

The results reported in this study suggest ritonavir may have only limited utility in treating MCL as a single agent. *In vitro* studies found that incubating MCL cell lines (GP, JVM2, and GP-derived therapy-resistant GRL) with a concentration of ritonavir equivalent to serum levels in patients on daily ritonavir regimens significantly reduced cellular proliferation while inducing apoptosis. This data corroborates with previous reports that have shown reduced proliferation and increased apoptosis following ritonavir treatment in other hematological malignancies [14,20,34].

Reports suggesting the anti-cancer mechanisms of ritonavir may be through an alteration of signaling in the NFκB signaling pathways [14,18,19]. Therefore, transcript levels of the NFκB targets BCL2, CCND2, and BIRC5 were assessed following ritonavir treatment. These molecules were of particular interest due to their known roles in modulating survival and proliferation in MCL, amongst other cancers. In fact, disregulation of the BCL2 and survivin molecules has been shown to play a role in the progression, resistance, and/or aggressiveness of MCL, while cyclin D2 appears to serve as a functional replacement for cyclin D1 in rare MCL cases [35-39]. When treated for 6 hours with ritonavir, the transcript levels of BCL2 and CCND2

in the GP, GRL, and JVM2 cell lines were significantly downregulated compared to untreated MCL cells. Interestingly, the BIRC5 transcript was also downregulated significantly in GP and GRL cells, but not in JVM2. This suggests intrinsic signaling mechanisms between the cell lines may be altering the molecular outcomes of ritonavir treatment amongst the cells, although all cell lines exhibited similar apoptosis induction and reduced proliferative outcomes.

In addition to assessing transcript level changes of NFκB pathway targets, the protein levels and expression patterns of p65, a subunit of NFκB, were analyzed in the GP, GRL, and JVM2 cell lines. Although there was no change in the total protein levels of p65 following 24 hours ritonavir treatment in the cells, there appeared to be a modest downregulation of p65 nuclear localization in the ritonavir-treated GP and GRL cells. This suggests ritonavir may be inhibiting some activation of NFκB. This modest decrease in nuclear localization suggests ritonavir action is not likely mediated solely through interaction with the NFκB signaling pathway, implicating a role for ritonavir actions in other signaling networks. Ritonavir interaction has been suggested to occur further upstream within the PI3K/Akt signaling pathway [19], providing insight into alternate pathways for consideration in determining ritonavir's mechanistic actions in MCL.

Due to the promising *in vitro* activity of ritonavir against MCL, we chose to test ritonavir's action *in vivo*. Two *in vivo* studies were performed to preliminarily test the efficacy of ritonavir as a single agent in treating MCL. In one dosing scheme, 30 mg/kg/day ritonavir was administered to mice for 7 consecutive days beginning at 10 days following inoculation with GP. In a second dosing scheme, devised to more closely resemble those previously shown effective against lymphoblastoid B cells *in vivo* [14], mice were administered 30 mg/kg/day ritonavir for 30 consecutive days beginning at 7 days following inoculation with GP. Neither of these dosing schemes testing ritonavir as a single agent yielded significant prolongation in survival of treated mice over sham controls.

While ritonavir as a single agent displays no *in vivo* efficacy against MCL, studies suggest that ritonavir may be most optimal as a therapeutic agent when combined with other therapies, leading to a multi-target treatment approach [18,25,26]. When the GP, GRL1, and JVM2 cell lines were incubated with ritonavir in combination with a standard chemotherapeutic, vincristine, cell proliferation was significantly reduced compared to ritonavir treatment or vincristine treatment alone. This provides strong support for the development and testing of a combined therapeutic regimen with ritonavir and standard MCL therapeutics for *in vivo* testing, particularly due to extensive evidence suggesting multi-pronged therapies are more optimal treatments for MCL than single therapy designs that target only one aspect of the cancer's biology [23,24].

In conclusion, this study provides evidence for only limited utility of ritonavir in treating MCL. While ritonavir appears to have no *in vivo* activity against MCL as a single agent, evidence derived from *in vitro* studies holds promise for possible therapeutic potential of ritonavir utilization in a combined therapeutic regimen for treatment of MCL. Due to ritonavir's status as an FDA-approved drug, these studies warrant future *in vivo* studies for testing ritonavir in combined treatment regimens for more optimized MCL therapies. As MCL is an aggressive disease with a grim prognosis, these studies are vital to providing novel therapies better suited to managing MCL disease, thereby providing patients more optimal care and improved progression-free survival.

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