Combined Administration of Rituximab and ON 013105 Induces Apoptosis in Mantle Cell Lymphoma Cells and Reduces Tumor Burden in a Mouse Model of Mantle Cell Lymphoma

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Abstract

Purpose: Mantle cell lymphoma (MCL) is an incurable B-cell lymphoma, and new therapeutic strategies are urgently needed.

Experimental Design: The effects of ON 013105, a novel benzylstyryl sulfone kinase inhibitor, alone or with doxorubicin or rituximab, were examined in Granta 519 and Z138C cells. For in vivo studies, CB17/SCID mice were implanted subcutaneously with Z138C cells and treated with various combinations of ON 013105, doxorubicin, and rituximab. Tumor burden and body weight were monitored for 28 days.

Results: ON 013105 induced mitochondria-mediated apoptosis in MCL cells. Death was preceded by translocation of tBid to the mitochondria and cytochrome c release. In addition, ON 013105–treated cells exhibited reduced levels of cyclin D1, c-Myc, Mcl-1, and Bcl-xL. Using nuclear magnetic resonance (NMR) spectroscopy, we showed specific binding of ON 013105 to eIF4E, a critical factor for the initiation of protein translation. We proffer that this drug–protein interaction preferentially prevents the translation of the aforementioned proteins and may be the mechanism by which ON 013105 induces apoptosis in MCL cells. Efficacy studies in a mouse xenograft model showed that ON 013105 inhibited MCL tumor growth and that combining ON 013105 with rituximab reduced tumor burden further with negligible unwanted effects.

Conclusions: Our findings suggest that ON 013105, alone or in combination with rituximab, may be a potent therapeutic agent to treat MCLs.

Introduction

Mantle cell lymphoma (MCL) is a non–Hodgkin B-cell lymphoma, characterized by a t(11;14) (q13; q32) chromosomal translocation (1, 2). The translocation results in cyclin D1 overexpression which correlates with increased tumor cell proliferation and decreased patient survival (3, 4).

MCL is associated with poor clinical outcomes and inevitable relapses. Relapsed MCL responds poorly to standard therapies, and median overall survival is 5 to 7 years (5–7). New treatments including monoclonal antibodies, combination chemotherapy, and high-dose chemotherapy followed by stem cell transplant have yielded minimal success (8–10).

Benzylstyryl sulfones are novel kinase inhibitors, under development as potential anti-cancer agents. These compounds induce G2–M cell-cycle arrest and apoptosis (11–13). These compounds selectively target tumor cells. Therapeutic levels induce no hepatic, hematologic, or neurologic toxicity in test animals (11, 13). Previously, we showed that ON 013100, ON 01370, and ON 01910.Na inhibited MCL proliferation by modulating cyclin D, cyclin-dependent kinase 4 (CDK4), p53, and MDM2 (12). ON 013100 and ON 01370 are, however, poorly soluble in water. Because water-soluble compounds are easier to formulate and provide superior bioavailability as therapeutics, we developed a water-soluble analogue, ON 013105 (Fig. 1A; ref. 13). ON 013105 retained the anti-cancer activity of ON 013100. We also developed ON 013101 (Fig. 1A), which had negligible effects on MCL cells (data not shown), and used it as a negative control.

In eukaryotic cells, protein translation begins when translation initiation factor 4E (eIF4E) binds to mRNA transcripts and targets them to the eIF4F translation initiation complex. eIF4E is the least abundant component of the complex, and its availability is rate limiting (14, 15).
Translational Relevance

Here, we show that ON 013105 reduces levels of cyclin D1, c-Myc, Mcl-1, and Bcl-xL, and induces mitochondria-mediated apoptosis in cultured mantle cell lymphoma (MCL) lines. Furthermore, we show specific binding of ON 013105 to eIF4E, a protein critical for the initiation of eukaryotic protein translation. Importantly, eIF4E availability preferentially affects the translation of select tumorigenic proteins. We proffer that the binding of ON 013105 to eIF4E inhibits its incorporation into the translation initiation complex, thereby triggering mitochondria-mediated apoptosis. Combined administration of ON 013105 and rituximab effectively inhibits tumor growth in a mouse xenograft model of MCL with no discernible off-target effects. These studies provide a foundation upon which we can better understand and further optimize MCL therapeutic response.

Notably, eIF4E availability selectively affects mRNA translation. "Weak mRNAs," that is, those with long, highly structured, G/C-rich 5’ untranslated regions (UTR), are more resistant to ribosome loading than mRNAs with shorter, less structured 5’ UTRs (14–19). While limited eIF4E has little effect on the translation of most mRNAs, weak RNAs, which typically encode tumorigenic proteins (e.g., cyclin D1, c-Myc, Bcl-xL, Mcl-1, VEGF, and survivin), are infrequently translated under these conditions (14–19). An excess of eIF4E preferentially enhances the translation of these proteins, whereas a scarcity disproportionately inhibits their expression (14). In fact, eIF4E is highly expressed in approximately 30% of cancers, including many leukemias and lymphomas (15, 20). Moreover, a recent study showed that eIF4E expression positively correlated with shorter overall survival in patients with MCL undergoing high-dose combinational therapy (hyper-CVAD; ref. 21); therefore, we evaluated potential interplay between ON 013105 and eIF4E.

Combining rituximab (RTX), a CD20-specific antibody, with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), significantly improves response in patients with aggressive, non–Hodgkin lymphoma (22). Rituximab can sensitize cancer cells to the cytotoxic effects of bortezomib and suberoylanilide hydroxamic acid (SAHA; refs. 23, 24). When rituximab binds to CD20, it modulates signaling through NF-κB, mitogen-activated protein kinases (MAPK), and Akt, which are important for the survival and growth of cancer cells (25, 26). In this study, we investigated the combined effects of ON 013105 and rituximab or doxorubicin on MCL cell lines and on tumor burden in a mouse model of MCL. Combining ON 013105 and rituximab significantly reduced tumor burden with minimal impact on overall well-being and supports further examination of this combination for MCL treatment.

Materials and Methods

Cell culture and reagents

Granta 519 and Z138C cells were kindly provided by Dr. Geoffrey Shapiro, Dana-Farber Cancer Institute (Boston, MA) and cultured as previously described (27). Jeko1 and JVM-2 cells were purchased from American Type Culture Collection (ATCC) and cultured per ATCC recommendations. ON 013105 and ON 013101 solutions were prepared in water and dimethyl sulfoxide (DMSO), respectively.

Antibodies were purchased from Santa Cruz Biotechnology, Inc., Cell Signaling Technology, Inc., and Invitrogen. Details supplied as Supplementary Data. Rituximab was manufactured by Genentech and doxorubicin was purchased from Sigma-Aldrich.

Cross-linking of rituximab on MCL cells

Cells were incubated in RPMI-1640, 10 μg/mL rituximab for 30 minutes at 37°C, washed, and seeded in 24-well plates (2 × 10⁶ cells per well). Anti-human IgG antibody was added to 15 μg/mL (final) to cross-link rituximab (RTX-CL) on the cell surface.

Cell viability was analyzed using Cell Titer 96 Cell Proliferation Assay (MTS; Promega Corp.). Granta 519, Z138C, Jeko1, and JVM-2 cells were treated with ON 013105 and processed per manufacturer’s instructions. Absorbance was measured at 490 nm. IC₅₀ values for the effects of ON 013105 on each cell line were determined using CalcuSyn software (Biosoft).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was conducted using Cell Death Detection Kit, Fluorescein (Roche Diagnostics Corp.). Cells were treated with 10 μg/mL RTX-CL or 0.1 μmol/L doxorubicin and various concentrations of ON 013105 for 24 hours and stained with TUNEL reaction mixture per manufacturer’s instructions. Staining was quantified using a FACS Vantage flow cytometer (BD Biosciences).

Mitochondrial and cytosolic fractions were isolated as previously described (28).

Western blot analysis

Granta 519 and Z138C cells were treated with ON 013101, ON 013105, or ON 013105 + RTX-CL (10 μg/mL), and proteins levels assessed by Western blot analysis, as previously described (28).

Plasmid constructs and eIF4E protein expression

Full-length human eIF4E (GST tagged) was cloned into pGEX-4T1 (GE Healthcare Biosciences), and eIF4E (GB1 tagged) was cloned into pET24a(+) (Novagen, EMD Millipore Biosciences). Proteins were expressed in Escherichia coli.

Details of GST-eIF4E and GB1-eIF4E protein preparation and purification; assessing protein/compound binding by saturation transfer difference (STD), protein titration, 1H-NMR (nuclear magnetic resonance) spectra, and fluorescence intensity quenching are provided as Supplementary Data.
**Mice**

BALB/c and CB17/SCID mice were from Charles River Laboratories, Inc. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), and all experiments were carried out in accordance with IACUC standards.

Pharmacokinetic analysis and routes of ON 013105 administration are provided as Supplementary Data.

**In vivo efficacy studies**

Female, 6- to 8-week-old CB17/SCID mice were implanted subcutaneously with Z138C cells ($5.0 \times 10^6$). When tumor volumes reached approximately 100 mm$^3$, animals were randomly assigned to treatment groups. Treatments were administered intraperitoneally (i.p.) over 28 days. Every 3 days, mice were weighed and tumors measured.

**Statistical analysis**

Statistical significance was determined using the 2-tailed Student $t$ test. Data represent the mean $\pm$ SD of at least 3 independent experiments, with $P \leq 0.05$ considered statistically significant.

**Results**

**ON 013105 induces mitochondria-mediated apoptosis in MCL cells**

We analyzed the effects of ON 013105 on the viability of 4 MCL cell lines: Granta 519, Z138C, JVM-2, and Jeko1 and calculated IC$_{50}$ values for each (Fig. 1B). Cells were treated with 0 to 10 $\mu$mol/L ON 013105 for 48 hours. While ON 013105 significantly decreased cell viability of all 4 cell lines in a dose-dependent and time-dependent manner (Fig. 1B), we executed the remainder of our in vitro experiments with 2 of the best-characterized MCL cell lines, Granta 519 and Z138C.

To investigate the mechanism by which ON 013105 kills MCL cells, we examined cleavage of PARP, a marker for early apoptosis (29). In untreated Granta 519 and Z138C cells, cleaved PARP was observed with 1.0 $\mu$mol/L ON 013105 or ON 013101 for 0, 8, and 24 hours. For representative Western blot analyses of Mcl-1 and Bcl-xL in the mitochondrial fraction (MF) and cytochrome $c$ (Cytc) in the cytosolic fraction (CF) of untreated Granta 519 and Z138C cells (UN) or treated with 1.0 $\mu$mol/L ON 013105 or ON 013101 for 24 hours, $\beta$-Actin was used as a loading control.

Figure 1. ON 013105 induces dose-dependent apoptosis and reduces viability in MCL cells. A, molecular structures of ON 01305, ON 01301, and ON 01300. B, cell viability by MTS. Z138C, Granta 519, JVM-2, and Jeko1 cells were treated with 0 to 2.0 $\mu$mol/L ON 013105 for 48 hours. Data represent the mean $\pm$ SD of 3 independent experiments. C, representative Western blot analyses of pro-PARP and cleaved PARP in Granta 519 and Z138C cells treated with 0 to 1.0 $\mu$mol/L ON 013105 for 24 and 48 hours. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. D, representative Western blot analyses of caspase-3, caspase-9, pro-PARP, and cleaved caspase-3, caspase-9, and PARP in Granta 519 and Z138C cells, treated with ON 01305 or ON 01301 for 0, 1, 4, 24, and 48 hours. GAPDH was used as loading control. E, representative Western blot analyses of Mcl-1 and Bcl-xL in the mitochondrial fraction (MF) and cytochrome $c$ (Cytc) in the cytosolic fraction (CF) of untreated Granta 519 and Z138C cells (UN) or treated with 1.0 $\mu$mol/L ON 013105 or ON 013101 for 24 hours. $\beta$-Actin was used as a loading control.
PARP cleavage, indicating apoptosis induction (Fig. 1C). Because PARP cleavage occurs downstream of caspase activation in apoptotic signaling, we also examined the effects of ON 013105 on procaspase and PARP cleavage in these cells. We observed a time-dependent increase in the cleaved forms of caspase-3, caspase-9, and PARP in ON 013105–treated cells (Fig. 1D). These data show that ON 013105 induces apoptosis in at least 2 MCL cell lines and suggest that other MCL cell lines may be similarly affected. ON 013101, our negative control, did not produce these effects.

To determine whether ON 013105 induced apoptosis via the mitochondria-mediated or the membrane death receptor–mediated pathway, we examined the expression of Bcl-2, Bcl-xL and Mcl-1, prosurvival proteins, which prevent mitochondria-mediated apoptosis (30, 31), in Granta 519 and Z138C cells. ON 013105 significantly reduced the levels of Mcl-1 and Bcl-xL in a time-dependent manner (Fig. 1E). This is consistent with existing data that show loss of mitochondrial membrane potential when Mcl-1, a survival protein highly expressed in myeloid leukemia cells (32), is inhibited and suggest that ON 013105 induces mitochondria-mediated apoptosis. There was no change in Bcl-2 expression (data not shown).

Bid, a cytosolic pro-apoptotic protein, is activated after proteolytic cleavage to truncated Bid (tBid), which localizes to the mitochondria and enhances mitochondrial membrane permeability. This results in the release of cytochrome c (Cyt c) into the cytosol, which initiates mitochondria-mediated apoptosis (30, 31, 33). To confirm that ON 013105 induced apoptosis by mitochondria-mediated signaling, we examined levels of tBid and Cyt c in the mitochondrial and cytoplasmic fractions of Granta 519 and Z138C cells. ON 013105 increased tBid levels in the mitochondrial fraction, and Cyt c levels in the cytosol of both cell lines (Fig. 1F). ON 013101 did not affect these changes. These data support our finding that ON 013105 induces programmed cell death in MCL cells through the mitochondria-mediated pathway.

ON 013105 inhibits cyclin D1 and c-Myc expression in MCL cells

Constitutive overexpression of cyclin D1 is a hallmark of MCL, and its levels positively correlate with the proliferative rate of malignant cells (3, 4). Cyclin D1 also cooperates with c-Myc to initiate B-cell lymphomas (34–36). Hence, these proteins are considered targets for the development of novel MCL therapeutics. We examined the effects of ON 013105 on cyclin D1 and c-Myc expression in Granta 519 and Z138C cells. We observed a significant dose-dependent decrease in the expression of both proteins in the ON 013105–treated cells (Fig. 2A). To confirm these results, we examined cyclin D1 and c-Myc expression after incubation with ON 013105 or ON 013101 for 1, 4, 8, and 24 hours. There was a significant time-dependent decrease in cyclin D1 and c-Myc levels in both cell lines after ON 013105 treatment as compared with the controls (Fig. 2B). Taken together, these data indicate that ON 013105 reduces levels of cyclin D1 and c-Myc in MCL cells.

ON 013105 binds specifically to eIF4E in MCL cells.

Mcl-1, Bcl-xL, cyclin D1, and c-Myc are among the proteins whose translation is disproportionately inhibited by restricted eIF4E levels and preferentially enhanced by increased eIF4E expression (14–19). Because ON 013105 significantly reduced levels of the aforementioned proteins in MCL cells, we postulated that an interaction between ON 013105 and eIF4E might be involved.

We compared the 1-dimensional (1D) 1H-NMR spectra of 30 μmol/L ON 013105, 30 μmol/L ON 013101, and a mixture of 1 mmol/L ON 013105, 1 mmol/L ON 013101, and 1 μmol/L GST-eIF4E, with the corresponding STD 1D 1H-NMR spectrum [Fig. 3A (i)–(iv), respectively]. Peaks shared by the STD spectrum and the spectra of ON 013105 or ON 013101 indicate binding of the drug(s) to eIF4E, whereas peaks in the individual drug spectra that do not correspond to a peak in the STD spectrum indicate a lack of interaction. We observed that peaks *, §, and ¶ were shared.
Figure 3. Examining the potential interactions between eIF4E and ON 013105 or ON 013101 using NMR spectroscopy, saturation transfer difference, and fluorescence intensity quenching. A, 1D $^1$H-NMR spectra of (1) 30 μmol/L ON 013105, (2) 30 μmol/L ON 013101, and (4) a mixture of 1 mmol/L ON 013105, 1 mmol/L ON 013101, and 1 μmol/L GST-eIF4E. (3) Corresponding STD 1D $^1$H-NMR spectrum. (+), peak present in ON 013105 spectrum, but not in STD spectrum; (−), peak shared by ON 013105 and STD spectra, but not ON 013101 spectrum; (+), peak from buffer component; (++, peak from protein stock solution; #, peak from internal calibration standard.) B, 1D $^1$H-NMR spectra of 30 μmol/L ON 013105 (1), titrated with increasing concentrations of GST-eIF4E [0.06 μmol/L (2), 0.25 μmol/L (3), 1.0 μmol/L (4), and 4.0 μmol/L (5)] (final). (+), peak from buffer component; (++, peak from protein stock solution; #, peak from internal calibration standard.) C, 1D $^1$H-NMR spectra of 30 μmol/L ON 013101, incubated with 0 μmol/L GST-eIF4E (1), and 4 μmol/L GST-eIF4E (2). 1D $^1$H-NMR spectrum of buffer alone (3). #, peak from internal calibration standard. D, fluorescence intensity quenching of tryptophan residues in 0.3 μmol/L GB1-eIF4E/100 mmol/L sodium phosphate, titrated with increasing concentrations of ON 013105.
by the ON 013105 spectrum [Fig. 3A (i)] and the STD spectrum [Fig. 3A (iii)]. These peaks did not appear in the ON 013101 spectrum [Fig. 3A (ii)]. Moreover, peak \( \ddagger \) prominent in the ON 013101 spectrum [Fig. 3A (ii)], was not expressed in the STD spectrum [Fig. 3A (iii)]. Together, these data indicate that ON 013105 binds to eIF4E, and there is no detectable interaction between eIF4E and ON 013101.

Another method of exploring small-molecule–protein interaction is by monitoring changes in the 1D \(^1\)H-NMR spectrum of the small molecule with increasing concentrations of its target protein. If the molecule binds to the protein, the height of individual NMR peaks will decrease as protein concentrations increase (37). We recorded the spectrum of 30 \( \mu \)mol/L ON 013101 alone [Fig. 3B (i)] and with increasing concentrations of GST-eIF4E [Fig. 3B (ii)–(v)]. Peak “+” was the result of a buffer component and was stable in all spectra. Peak “++” was derived from the protein stock solution, and its height increased as the protein concentration increased. Peak “*” was the internal calibration standard. All peaks derived from ON 013105 proportionately decreased in amplitude as the concentration of GST-eIF4E increased, indicating direct binding of ON 013105 and GST-eIF4E.

To rule out an interaction between GST-eIF4E and our negative control, we compared the 1D \(^1\)H-NMR spectrum of 30 \( \mu \)mol/L ON 013101 alone and together with 4 \( \mu \)mol/L GST-eIF4E [Fig. 3C (i) and (ii)]. No discernible differences in the amplitude of the NMR peaks were observed in these samples, indicating that ON 013101 does not bind to GST-eIF4E.

The eIF4E protein contains many tryptophan residues, and their intrinsic fluorescence has been exploited to assess the binding of small molecules to eIF4E (38). To confirm binding of ON 013105 to eIF4E, we examined the fluorescence intensity of GB1-eIF4E, with increasing concentrations of ON 013105 and ON 013101 (Fig. 3D and data not shown). There was a significant, dose-dependent decrease in GB1-eIF4E fluorescence as ON 013105 concentrations increased (Fig. 3D), and no change in GB1-eIF4E fluorescence with increasing concentrations of ON 013101 (data not shown). These data indicate that ON 013105 binds specifically to eIF4E and ON 013101 does not.

**ON 013105 and RTX-CL induce apoptosis in MCL cells**

Combining therapeutics that kill cancer cells via different molecular mechanisms can improve MCL treatment outcomes (5, 39). For example, combining rituximab with CHOP yields significant clinical benefits in the treatment of CD20+ B-cell lymphomas (22). Moreover, significant levels of apoptosis are induced in Burkitt lymphoma cell lines when cell surface–bound rituximab is cross-linked with an antibody, that is, F(ab’)2 goat anti-human IgG, whereas rituximab treatment alone has no \textit{in vitro} effect on apoptosis (40). We examined the effects of ON 013105 alone, or combined with cross-linked rituximab (RTX-CL) or doxorubicin, on the viability of Granta 519 and Z138C cells. We observed a significant decrease in viability when cells were treated with ON 013105 and RTX-CL, as compared with either single reagent or no treatment (Fig. 4A). While the combination of doxorubicin and ON 013105 significantly decreased viability (Fig. 4B), doxorubicin induced a considerable effect as a single agent (Fig. 4B). We also examined the combined effects of ON 013105 with RTX-CL or doxorubicin on apoptosis in Granta 519 and Z138C cells. We incubated cells for 24 hours with 0 to 1.0 \( \mu \)mol/L ON 013105 alone or combined with RTX-CL (Fig. 4C). Apoptosis was significantly higher in cells treated with both ON 013105 and RTX-CL than in just ON 013105 (Fig. 4C). Consistent with the viability data (Fig. 4B), doxorubicin induced considerable apoptosis as a single agent, and the addition of ON 013105 did not significantly enhance these levels (Fig. 4D). Therefore, we focused on the combined effects of ON 013105 and RTX-CL in our remaining \textit{in vitro} experiments. To confirm the apoptosis data, we incubated cells with ON 013105 alone, or combined with RTX-CL, and examined levels of pro-PARP and cleaved PARP by Western blot analysis (Fig. 4E). Consistent with Fig. 4C, the combination of RTX-CL and ON 013105 induced PARP cleavage at much lower concentrations than ON 013105 alone, in both Granta 519 and Z138C cells (Fig. 4E).

**Molecular mechanisms underlying the effects of ON 013105 and RTX-CL on MCL cells**

The molecular mechanisms through which rituximab directly induces apoptosis or sensitizes CD20+ B cells to apoptosis induced by other anti-cancer agents is not well understood; however, rituximab can modulate intracellular signaling of prosurvival molecules such as NF-κB, MAPK, Akt, and the Bcl-2 family of proteins (25, 26, 41, 42). We analyzed the effect of ON 013105 alone, and combined with RTX-CL, on the expression of Bcl-2 and Mcl-1 in Granta 519 and Z138C cells by Western blot analysis (Fig. 5A), and quantified average Mcl-1 expression by densitometry (Fig. 5B). While there was no effect on the expression of Bcl-2 (Fig. 5A), we observed a significant decrease in Mcl-1 expression in cells treated with ON 013105 + RTX-CL as compared with ON 013105 alone (Fig. 5A and B). These results indicate that ON 013105 and rituximab act together to silence Mcl-1, a protein vital to the growth and survival of MCL (43).

**Pharmacokinetic analysis of ON 013105**

Preliminary experiments comparing the pharmacokinetics of ON 013105 in mice following intraperitoneal, intravenous, and oral gavage (\textit{per os}) administration indicated that plasma levels of ON 013105 were comparable when delivered i.p. and i.v. (but not \textit{per os}), and at concentrations sufficient to anticipate a therapeutic effect (Supplementary Fig. S1). We chose i.p. for ease of administration. To better characterize pharmacokinetics by i.p., mice were given 100 mg/kg ON 013105. Blood was collected at various points after treatment, fractionated, and ON 013105 plasma levels analyzed using liquid chromatography/mass spectrometry. Average plasma concentrations of 32 µg/mL ON 013105 were attained 10 and 20
minutes postadministration (Fig. 6A). When measured after 40 and 60 minutes, plasma levels of ON 013105 decreased to 2.9 μg/mL (Fig. 6A). Significant levels of ON 013100, an active metabolite of ON 013105, were also detected in the plasma (Fig. 6A).

Effects of ON 013105, rituximab, and doxorubicin in a mouse model of MCL

To determine the in vivo efficacy of ON 013105 alone, or combined with doxorubicin or rituximab, we used a mouse model of MCL. CB17/SCID mice were implanted subcutaneously with Z138C cells and assigned to various treatment groups when tumor volumes reached about 100 mm³. First, we analyzed the efficacy of ON 013105 as a single agent. Tumor burden in mice treated with ON 013105 (22.5 and 75 mg/kg, every 2 days) was significantly less than that of the untreated, control mice (Fig. 6B). While tumor volume was also significantly less in the mice treated with ON 013105 (75 mg/kg, once a week) versus the control-treated mice, there was no statistically significant advantage in this group versus ON 013105 (22.5 mg/kg, every 2 days; Fig. 6B). To minimize potential, off-target effects and enhance efficacy, we chose to investigate the effects of the lower dose of ON 013105 (22.5 mg/kg, every 2 days), in combination with doxorubicin or rituximab.

ON 013105 (22.5 mg/kg, every 2 days)–treated mice had significantly smaller tumors than control-treated mice (Fig. 6C). While doxorubicin alone (3.5 mg/kg, once a week) and ON 013105 + doxorubicin reduced tumor volume even further (Fig. 6C), all doxorubicin-treated mice experienced severe off-target effects, including significant weight loss (Fig. 6E), and death. Although treatment with rituximab alone (2.5 mg/kg, every 3 days) moderately inhibited tumor growth, only ON 013105 was significantly more effective at controlling tumor volume than rituximab as a single agent (Fig. 6D). Overall, combining ON 013105 with rituximab was the optimal treatment; increases in tumor volumes were...
negligible (Fig. 6D), and the mice in this treatment group suffered no weight loss (Fig. 6E).

Discussion

Previously, we showed that ON 01910.Na blocked the translation of cyclin D1 in MCL cells by inhibiting PI3K/Akt/mTOR/eIF4E-BP signaling and induced apoptosis through the mitochondria-mediated pathway (44). Here, we used a more potent, water-soluble compound, ON 013105, to explore further apoptotic signaling in MCL cells, and the molecular mechanisms by which ON 013105 inhibits cyclin D1 and c-Myc expression (1, 3, 35). We also evaluated the effects of ON 013105 alone, and combined with doxorubicin or rituximab, in a mouse model of MCL.

ON 013105 significantly decreased the viability of 4 MCL cell lines. In Granta 519 and Z138C cells, ON 013105, but not ON 013101, induced the cleavage of PARP, caspase-3, and caspase-9, decreased the expression of the prosurvival proteins, Mcl-1 and Bcl-xL, induced the truncation of Bid and its translocation to the mitochondria, and induced the release of Cyt c into the cytosol, indicating mitochondria-mediated apoptosis. ON 013105 also decreased levels of cyclin D1 and c-Myc proteins that were critical for MCL initiation and growth.

Furthermore, we showed that ON 013105 bound directly to eIF4E. We hypothesize that the binding of ON 013105 to eIF4E inhibits its incorporation into the translation initiation complex, preferentially blocking translation of the aforementioned oncogenic proteins. The resulting decrease in these proteins induces apoptosis.

Rituximab is a monoclonal antibody to the CD20 antigen, which is expressed on normal and malignant B cells, including MCLs. While rituximab kills both normal and malignant B cells, the normal B-cell population can be replenished through differentiation of lymphoid stem cells (45). As a monotherapy, rituximab had moderate effects on MCLs in several clinical trials (46); however, combined with CHOP, it yielded significant benefits for patients with non–Hodgkin B-cell lymphoma (22). Doxorubicin is a component of both CHOP and hyper-CVAD therapies. It is commonly used to treat breast, esophageal, and Kaposi sarcoma; and Hodgkin and non–Hodgkin lymphomas, including MCL (47).

While ON 013105 induced apoptosis and reduced viability of MCL cells, we tested whether combinations of ON 013105 with rituximab or doxorubicin would enhance these effects or induce them at lower concentrations to minimize potential off-target effects when used for cancer treatment. The combination of ON 013105 and doxorubicin significantly reduced viability and induced apoptosis in MCL cells; however, as a single agent, doxorubicin affected these biologies very efficiently (Fig. 4B and D). These data suggest that combining ON 013105 with doxorubicin would offer no significant advantage over doxorubicin alone. In contrast, RTX-CL alone had little effect on percent viability and apoptosis (Fig. 4A and C); however, when 0.1 μmol/L ON 013105 was added, the combination reduced viability of MCL cells more than doxorubicin alone and more than 0.01 or 0.1 μmol/L ON 013105 alone (Fig. 4A and B). Moreover, when RTX-CL was combined with 0.01 or 0.1 μmol/L ON 013105, the percent apoptosis induced was more than additive (Fig. 4C).

To explore the effects of these drugs in vivo, we used a mouse xenograft model of MCLs. As a monotherapy, all 3
ON 013105 regimens reduced tumor burden significantly more than the control (Fig. 6B). Benzylstyryl sulfones are well tolerated in animals (11, 13). Despite this observation, we chose to explore the effects of the lowest dose of ON 013105 (22.5 mg/kg, every 2 days) with rituximab or doxorubicin to minimize potential side effects. Tumor burden was significantly reduced in the ON 013105 (22.5 mg/kg, every 2 days)–treated mice versus the control group. Treatment with doxorubicin alone and with ON 013105 + doxorubicin resulted in even smaller tumor burden.
volumes (Fig. 6C). It should be noted that all mice treated with doxorubicin experienced severe, deleterious effects, including lethargy, hair loss, significant weight loss (Fig. 6E), and in some instances, sudden death. In contrast, we observed no off-target effects in the mice treated with ON 013105, rituximab, or a combination thereof. In fact, even as their tumor burdens decreased, their average body weight steadily increased (Fig. 6E). Overall, we observed the best response in the mice treated with ON 013105 (22.5 mg/kg, every 2 days) + rituximab (Fig. 6C–E).

In summary, ON 013105 induces mitochondria-mediated apoptosis in MCL cells. Our in vitro experiments indicated that combining a low concentration of ON 013105 with rituximab induced more apoptosis in MCL cells than either agent alone, even at higher concentrations. Our in vivo studies closely mirrored this trend. We found that combining the lower experimental dose of ON 013105 with rituximab worked better than either alone and induced no discernible off-target effects in the mice. Moreover, ON 013105 and rituximab kill MCL cells by different mechanisms of action, which makes the combination less vulnerable to acquired drug resistance. Our findings support continued investigation into ON 013105 as a monotherapy, or in combination with rituximab, to treat MCLs, which is currently an incurable cancer. Importantly, our data suggest that ON 013105 binds to eIF4E, a potent oncogene, which is elevated in approximately 30% of human cancers, including Hodgkin and non–Hodgkin lymphomas, chronic myelogenous leukemia, acute myeloid leukemia, and cancers of the breast, prostate, lung, head and neck, and colon (20); therefore, its efficacy may not be limited to MCLs. By specifically targeting eIF4E, ON 013105 has the potential to treat various cancers that use this mechanism of countering apoptosis.

Disclosure of Potential Conflicts of Interest
M.V.R. Reddy is a consultant for Onconova Therapeutics, Inc. R. Kumar has ownership interest in Onconova Therapeutics, Inc. E.P. Reddy has a commercial research grant from, ownership interest in and is a consultant for Onconova Therapeutics, Inc. J.E. Groopman is on the advisory board for Onconova Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

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Other: Design and synthesis of ON 013105 molecule, M. V. R. Reddy

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References


Combined Administration of Rituximab and ON 013105 Induces Apoptosis in Mantle Cell Lymphoma Cells and Reduces Tumor Burden in a Mouse Model of Mantle Cell Lymphoma

Anil Prasad, Ashutosh Shrivastava, Evangelos Papadopoulos, et al.

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