Dual Inhibition of Bruton’s Tyrosine Kinase and Phosphoinositide-3-Kinase p110δ as a Therapeutic Approach to Treat Non-Hodgkin’s B Cell Malignancies


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ABSTRACT

Although new targeted therapies, such as ibrutinib and idelalisib, have made a large impact on non-Hodgkin’s lymphoma (NHL) patients, the disease is often fatal because patients are initially resistant to these targeted therapies, or because they eventually develop resistance. New drugs and treatments are necessary for these patients. One attractive approach is to inhibit multiple parallel pathways that drive the growth of these hematologic tumors, possibly prolonging the duration of the response and reducing resistance. Early clinical trials have tested this approach by dosing two drugs in combination in NHL patients. We discovered a single molecule, MDVN1003 (1-(5-amino-2,3-dihydro-1H-inden-2-yl)-3-(8-fluoro-3,4-dihydro-2H-benzo[b][1,4]oxazin-6-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine), that inhibits Bruton’s tyrosine kinase and phosphatidylinositol-3-kinase δ, two proteins regulated by the B cell receptor that drive the growth of many NHLs. In this report, we show that this dual inhibitor prevents the activation of B cells and inhibits the phosphorylation of protein kinase B and extracellular signal-regulated kinase 1/2, two downstream mediators that are important for this process. Additionally, MDVN1003 induces cell death in a B cell lymphoma cell line but not in an irrelevant erythroblast cell line. Importantly, we found that this orally bioavailable dual inhibitor reduced tumor growth in a B cell lymphoma xenograft model more effectively than either ibrutinib or idelalisib. Taken together, these results suggest that dual inhibition of these two key pathways by a single molecule could be a viable approach for treatment of NHL patients.

Introduction

Non-Hodgkin’s lymphomas (NHLs) are among the most common of human cancers, and despite advancements of medical treatments and improvements in patient outcomes, the disease has a 30% mortality rate within the first five years after diagnosis (Howlader et al., 2014; Swerdlow et al., 2016). Activation of B cell receptor (BCR) signaling is a significant mechanistic driver of the development and growth of B cell–derived lymphoid tumors (Buchner and Muschen, 2014; Koehrer and Burger, 2016). Basal BCR signaling is necessary for the survival of B cells (Verkoczy et al., 2007; Wang et al., 2013), and signaling through the complex pathway is amplified during B cell activation (Woyach et al., 2012). The BCR is comprised of a membrane immunoglobulin complex, the ligation of which results in the phosphorylation of the cytoplasmic immunoreceptor tyrosine-based activation motif of BCR coreceptors by two kinases, Lyn (Lck/Yes tyrosine kinase) and Syk (spleen tyrosine kinase). Propagation of the signal occurs via several parallel and interconnected pathways. Two important

**ABBREVIATIONS:** AKT, protein kinase B; BCR, B cell receptor; BTK, Bruton’s tyrosine kinase; CLL, chronic lymphocytic leukemia; ERK 1/2, extracellular signal-regulated kinase 1/2; MCL, mantle cell lymphoma; MDVN1001, 1-(5-amino-2,3-dihydro-1H-inden-2-yl)-3-(8-fluoro-3,4-dihydro-2H-benzo[b][1,4]oxazin-6-yl)-7-(5-piperidin-4-yl)-2,3-dihydro-1H-inden-2-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine; MDVN1002, 1-(5-amino-2,3-dihydro-1H-inden-2-yl)-3-(8-fluoro-3,4-dihydro-2H-benzo[b][1,4]oxazin-6-yl)-7-(5-piperidin-4-yl)-2,3-dihydro-1H-inden-2-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine; MDVN1003, 1-(5-amino-2,3-dihydro-1H-inden-2-yl)-3-(8-fluoro-3,4-dihydro-2H-benzo[b][1,4]oxazin-6-yl)-7-(5-piperidin-4-yl)-2,3-dihydro-1H-inden-2-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine; NHL, non-Hodgkin’s lymphoma; pAKT, phosphorylated AKT; PCI-29732, 1-Cyclopentyl-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; pErk, phosphorylated Erk; PKGII, phosphatidylinositol-3-kinase δ; PK, pharmacokinetic; SW13, 2-(4-amino-3-(3-fluoro-5-hydroxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)(methyl)-5-methyl-3-o-toly(lquinazolin-4(3H)-one.)

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Dual Inhibition of BTK and PI3K to Treat B Cell Cancers

Materials and Methods

Reagents and Tumor Cell Lines. Ibrutinib (CAS 936563-96-1) and idelalisib (CAS 870281-82-6) were purchased from ChemShuttle (Union City, CA). Compounds MDVN1003, MDVN1001 (5-(8-fluoro-3,4-dihydro-2H-inden-2-yl)-3-(8-fluoro-3,4-dihydro-2H-benzo[b][1,4]oxazin-6-yl)-1H-inden-2-y1)-7H-pyrrolo[2,3-d]pyrimidin-4-amine), and MDVN1002 (1-(5-amino-2,3-dihydro-1H-inden-2-yl)-3-(3-fluoro-4-isopropoxypyrenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine) were synthesized at Integral Biosciences, Pvt. Ltd. (Noida, India). All tumor cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were tested for mycoplasma by polymerase chain reaction. All revived cells were used within 20 passages and cultured for less than 6 months. Ramos and DOHH-2 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with penicillin and streptomycin.

Enzymatic Kinase Assays. In vitro kinase activity assays were performed by Reaction Biology Corporation (www.reactionbiology.com) as described on the Web site and as described previously (Pujala et al., 2016).

Assessment of BCR-Dependent Signaling Levels. Ramos cells were pretreated for 30 minutes with compounds (0.1 or 1 μM final concentration) and then stimulated with α-human IgM (1.3 μg/ml) (#109-006-129; Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA) for 5 minutes. Levels of phosphorylated Akt (pAkt) (#4060; Cell Signaling Technology, Danvers, MA), Akt (#9272; Cell Signaling Technology), phosphorylated Erk (pErk; T202, Y204) (#4377; Cell Signaling Technology), and ERK (#4396; Cell Signaling Technology) were detected by western blot using the ChemiDoc Imaging System (Bio-Rad, Hercules, CA).

Measuring B Cell Activation by CD69 Expression. Splenocytes (1 x 10⁶ cells/well) were seeded in a 24-well plate and pretreated for 30 minutes with compounds at indicated concentrations and then activated for 4 hours with α-mouse IgD (3 μg/ml) (YULLOMD6-05; Accurate Chemical, Westbury, NY). Cells were stained with α-B220 (1/200, BD Biosciences, San Jose, CA), α-CD69 APC (1/100, BD Biosciences) and a live/dead fixable aqueous dead cell kit (#L34957; ThermoFisher Scientific, Grand Island, NY) and analyzed by flow cytometry using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Gladbach, Germany). At each concentration of compound tested, the inhibition of B cell activation was calculated as the percentage of live B cells expressing CD69 in the presence of the compound divided by the percentage of live B cells expressing CD69 in the vehicle control. Splenocytes from three individual mice were treated independently per condition tested. The IC₅₀ was calculated from the average of the three independent experiments, and curve fitting was done by nonlinear regression using GraphPad Prism (GraphPad Software, La Jolla, CA).

Viability Assay. DOHH-2 or HEL 92.1.7 cells were seeded in a 96-well white plate overnight at a density of 5000 cells/well. Cells were treated with compounds at the indicated concentrations for 72 hours. Cell viability was measured using a Cell Titer-glo kit (Promega, Madison, WI) as described by the manufacturer. The IC₅₀ was calculated from the curve fitted to the data points by nonlinear regression using GraphPad Prism.

kinases downstream of the BCR are Bruton’s tyrosine kinase (BTK) and phosphatidylinositol-3-kinase δ (PI3Kδ) (Seda and Mraz, 2015).

BTK is a member of the Tec family of tyrosine kinases and is recruited to the cell membrane after the activation of the BCR. Together with Syk, BTK phosphorylates phospholipase C-γ2, producing the classic second messengers diacylglycerol and inositol-1,4,5-triphosphate from phosphatidylinositol-4,5-bisphosphate. Diacylglycerol activates protein kinase C, and inositol-1,4,5-triphosphate triggers the release of intracellular calcium, resulting in the activation of several downstream signaling molecules, including extracellular signal-regulated kinase 1/2 (ERK 1/2) (Tomlinson et al., 2001). PI3K is involved in recruiting BTK to the cell membrane and phosphorylates and activates several downstream signaling molecules, including protein kinase B (AKT) (Fruman and Rommel, 2014). The BTK and PI3K signaling pathways are not insulated from one another, and there is evidence of cross-talk between them (Puri et al., 2013). Although the approval of these targeted therapies by the Food and Drug Administration was a significant advance, it has been reported that about 30% of MCL and CLL patients show primary resistance to ibrutinib (Tucker and Rule, 2015). Additionally, MDVN1003 induces apoptosis and decreases viability of DOHH-2 cells, an NHL cell line. Finally, MDVN1003, an orally bioavailable molecule in mice, rats, and dogs, showed significant antitumor effects in an NHL xenograft model in mice. This effect was similar to that seen with combination dosing of ibrutinib and idelalisib in the same model and greater than each of these drugs dosed as single agents. Our results suggest that a dual inhibitor of BTK and PI3Kδ could be an effective treatment strategy for B cell lymphoma patients.
Apoptosis Assay. DOHH-2 cells were seeded in a 24-well plate at 0.5 × 10⁵ cells/well. Cells were then treated with compounds at 1 µM for 4 hours, and apoptosis was measured by flow cytometry (MACSQuant Analyzer 10) using an Annexin V FITC apoptosis detection kit (#556547; BD Biosciences).

Pharmacokinetic Analysis. All animal studies were done as per protocols approved by the Institutional Animal Care and Use Committee at Medivation or its subsidiaries. See Supplemental Material for experimental details of mouse, rat, and dog pharmacokinetic (PK) studies.

Measuring B Cell Activation by CD69 Expression In Vivo. BALB/c mice were orally dosed with compounds (n = 3 per group) at indicated concentrations for 30 minutes, and then mice were injected intravenously in the tail vein with 100 µg of α-IgD (YULLOMD6-05; Accurate Chemical, Westbury, NY) for 5 hours. Splenocytes were isolated and stained as described earlier. The percentage of live B cells that express CD69 from mice treated with α-IgD and compound was normalized to the percentage of live B cells that express CD69 from mice treated with α-IgD alone.

Mouse Xenograft Model. All animal studies were done as per protocols approved by the Institutional Animal Care and Use Committee at Medivation or its subsidiaries.

DOHH-2 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and were passaged twice weekly. While in the exponential growth phase, 5 million DOHH-2 cells in 0.1 ml of phosphate-buffered saline (1:1 ratio with matrigel) were inoculated into the right flanks of 6- to 7-week-old female CB17/SCID mice (denoted as day 0). When the average tumor volume reached 118 mm³, mice were randomly grouped into eight groups (n = 10 per group). Tumor volume and body weight were measured twice a week. The experiment was terminated when the average tumor volume of the vehicle group reached >2000 mm³, and plasma and tumor samples were collected from each mouse (n = 3 mice per group were sacrificed 5 minutes before the final dose, n = 3 mice per group were sacrificed 30 minutes post final dose, and n = 4 mice per group were sacrificed 6 hours post final dose). Statistical analysis (Kruskal-Wallis corrected for multiple comparisons) was done on the tumor volumes of the groups over time.

Results

As described (Pujala et al., 2016), we aimed to discover reversible dual BTK and PI3Kδ inhibitors based on the similarities between a reversible BTK inhibitor, PCI-29732 (1-Cyclopentyl-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidine-4-amine) (Pan et al., 2007; Marcotte et al., 2010), and a PI3Kδ/γ dual inhibitor, SW15 (2-(4-amino-3-(3-fluoro-5-hydroxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)methyl)-5-methyl-3-(o-tolyl)quinazolin-4(3H)-one) (Berndt et al., 2010). We identified three compounds (Fig. 1), two of which are more potent against one of the two receptors and one compound that is a more potent dual inhibitor (Table 1; synthesis in Supplemental Methods). On the one hand, MDVN1001 is a potent BTK inhibitor (IC₅₀ 0.9 nM) and a relatively weak PI3Kδ inhibitor (IC₅₀ 149 nM). On the other hand, MDVN1002 strongly inhibited PI3Kδ (IC₅₀ 25.9 nM) more than it did BTK (IC₅₀ 695 nM). However, the dual inhibitor, MDVN1003, potently inhibited both kinases (BTK with an IC₅₀ of 32.3 nM, and PI3Kδ with an IC₅₀ of 16.9 nM). As expected, the control compounds, idelalisib and ibrutinib, inhibited one kinase more strongly than the other. Idelalisib, an ATP competitive, reversible inhibitor of PI3Kδ, inhibited PI3Kδ with an IC₅₀ of 1.2 nM, whereas it did not measurably inhibit BTK at any concentration tested. Ibrutinib, an irreversible inhibitor of BTK, potently inhibited BTK (IC₅₀ 0.142 nM) and weakly inhibited PI3Kδ (IC₅₀ 640 nM).

To investigate the effects of these molecules on signaling through the BCR pathway, phosphorylation of ERK 1/2 and AKT was measured in the Ramos Burkitt’s B cell lymphoma cell line. Ramos cells were pretreated with vehicle or 0.1 µM idelalisib, ibrutinib, MDVN1001, MDVN1002, MDVN1003, or an equimolar cotreatment of idelalisib and ibrutinib (subsequently referred to as “combo”). Cells were treated with α-human IgM for 5 minutes to cross-link and activate the BCR. Levels of phosphorylated ERK 1/2 and AKT were detected by western blot (Fig. 2). Treatment with α-human IgM increased the levels of phosphorylated AKT and ERK 1/2 as compared with the vehicle alone, indicating the BCR signaling pathway was activated (Fig. 2, lanes 1 and 2). Ibrutinib and idelalisib treatments significantly reduced the levels of pERK 1/2 and pAKT. Neither MDVN1001 nor MDVN1002 significantly affected levels of pAKT or pERK 1/2 when tested at 0.1 µM. However, these compounds did inhibit the phosphorylation of AKT and ERK 1/2 at higher concentrations (Supplemental Fig. 1). Combination treatment of idelalisib and ibrutinib potently inhibited levels of both pAKT and pERK 1/2 (Fig. 2). The MDVN1001 and MDVN1002 combination treatment (combo MDVN) also inhibited the
phosphorylation of AKT and ERK1/2, although not as potently as the combination treatment of the two approved inhibitors (Supplemental Fig. 1). Treatment with the dual BTK/PI3Kδ inhibitor compound MDVN1003 inhibited the phosphorylation of AKT and ERK1/2 at both the low concentration (0.1 μM) and the high concentration (1 μM) and did so more potently than the combination of MDVN1001 and MDVN1002 (Fig. 2; Supplemental Fig. 1). Taken together, these data show that MDVN1003 is a more potent inhibitor of BCR signaling than either MDVN1001 or MDVN1002 dosed individually or in combination and suggest that the dual inhibition of BTK and PI3Kδ may have a synergistic effect on downstream signaling molecules.

We wanted to further investigate the potential synergy of inhibiting both BTK and PI3Kδ in B cell lymphomas using ibrutinib, idelalisib, and MDVN1003 as tool compounds. These compounds provided us with the necessary tools to study treatment with monotherapies and dual inhibition. MDVN1003 is more potent than either MDVN1001 or MDVN1002 (or the combination of both) in the cell-based assay and allowed us to study dual inhibition in a single molecule.

To understand the selectivity profile of MDVN1003 and how it compares to those of ibrutinib and idelalisib, we tested these compounds in a kinase panel at 1 μM. Idelalisib is a highly selective PI3Kδ inhibitor and inhibited four other kinases (out of 374 tested) greater than 50%. Ibrutinib inhibited 34 kinases (out of 374 tested) more than 50% (Fig. 3). MDVN1003 behaved similarly to ibrutinib in the kinase panel and inhibited 50 kinases (out of 374 tested) greater than 50%, with a preference for tyrosine kinases (Fig. 3). Among the kinases most potently inhibited by MDVN1003 were BTK, PI3Kδ, and the related Tec- and PI3K-family kinases (Fig. 3; Table 2).

We next investigated the effects of MDVN1003 on the activation of primary B cells ex vivo. Mouse splenocytes were pretreated with ibrutinib, idelalisib, combo, or MDVN1003 for 30 minutes. Cells were treated with α-IgD for 4 hours to activate B cells. Anti-IgD cross-links the surface immunoglobulin and activates the B cell, which upregulates the early B cell activation marker CD69 (Sancho et al., 2005). Activated B cells were detected by flow cytometry as B220+CD69+ in a gate of live cells. The inhibition of B cell activation was measured by the percentage of live B cells that expressed CD69 on the cell surface in treated samples as compared with the α-IgD control. Ibrutinib and idelalisib potently inhibited B cell activation, with IC50 values of 6.9 and 5.4 nM, respectively (Fig. 4A; Table 3). Idelalisib, but not ibrutinib, produced full inhibition of B cell activation. The combo treatment of both compounds showed a modest additive effect with an IC50 of 1.1 nM (Fig. 4A; Table 3) and produced full inhibition of B cell activation. MDVN1003 inhibited B cell activation with an IC50 of 25.2 nM (Fig. 4A; Table 3). Although less potent, MDVN1003 was equally as efficacious as idelalisib and the combo treatment and fully inhibited B cell activation.

Signaling through the BCR is often a driver of tumor growth in B cell malignancies (Buchner and Muschen, 2014), and it has been reported that both ibrutinib and idelalisib reduce cell viability and induce apoptosis in NHL B cell lines (Honigberg et al., 2007; Lannutti et al., 2011; Qu et al., 2015). To determine whether MDVN1003 behaved similarly to the approved drugs, we measured the effect of MDVN1003 on the viability of DOHH-2 cells, a non-Hodgkin’s B cell lymphoma line that expresses BTK and PI3Kδ. After 72 hours of treatment, MDVN1003 effectively killed DOHH-2 cells with an IC50 of 1.34 μM, whereas the IC50 values for ibrutinib and idelalisib were 0.023 and 0.86 μM, respectively (Fig. 4B; Table 3). The IC50 for the combo treatment was 0.0084 μM, suggesting an additive effect on cell viability in inhibiting both BTK and PI3Kδ. The cytotoxicity induced by these compounds was determined to be apoptosis, as indicated by the significant population of Annexin V+ propidium iodide–cells in treatment groups as compared with the vehicle control (P < 0.0001, one-way analysis of variance) (Fig. 4C). Combination treatment of MDVN1001, the BTK inhibitor, and MDVN1002, the PI3Kδ inhibitor, also showed an additive effect on cell viability of DOHH-2 cells with an IC50 of 0.87 μM, as compared with IC50 values of 1.47 μM with MDVN1001 alone and 2.75 μM with MDVN1002 alone (Supplemental Fig. 2). Although a modest effect, these data suggest a benefit of dual inhibition of BTK and PI3Kδ.

To understand if the cytotoxicity of these compounds was dependent on inhibition of BTK and PI3Kδ, we tested the effects of the compounds on viability of HEL 92.1.7 erythroblast-like cells that do not express these kinases. The IC50 values of ibrutinib, idelalisib, combo, and MDVN1003 were all greater than 10 μM (Table 3), suggesting that these compounds are preferentially cytotoxic in cells that express BTK and PI3Kδ.

<table>
<thead>
<tr>
<th>IC50 (μM)</th>
<th>BTK (μM)</th>
<th>PI3Kδ (μM)</th>
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<tbody>
<tr>
<td>ibrutinib</td>
<td>&gt;100,000</td>
<td>1.2</td>
</tr>
<tr>
<td>idelalisib</td>
<td>0.142</td>
<td>640</td>
</tr>
<tr>
<td>MDVN1001</td>
<td>9.0</td>
<td>149</td>
</tr>
<tr>
<td>MDVN1002</td>
<td>695</td>
<td>25.9</td>
</tr>
<tr>
<td>MDVN1003</td>
<td>32.3</td>
<td>16.9</td>
</tr>
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</table>
To study the pharmacology of MDVN1003 in vivo, we investigated the PK properties of this molecule across species. The PK profiles of MDVN1003 in mice, rats, and dogs after a single oral dose are shown in Fig. 5. The noncompartmental analysis PK parameters of MDVN1003 in mice, rats, and dogs are summarized in Table 4. Following oral administration in mice, MDVN1003 showed rapid absorption, with a time of maximum concentration in plasma (tmax) of 0.25 hours, and then declined with a biexponential decay and an elimination half-life (t1/2) of 1.3 hours (Fig. 5). The absolute oral bioavailability (F) was acceptable at 40%. Following intravenous administration in mice, MDVN1003 showed low systemic clearance (4.94 l/h/kg) that was 150% of hepatic blood flow in the rat (QH = 3.31 l/h/kg (Davies and Morris, 1993)) and a moderate volume of distribution (Vd = 2.0 l/kg), as shown in Table 4. Following oral administration in dog, MDVN1003 showed rapid absorption, with a tmax of 0.42 hours, and then declined with a biexponential decay and a t1/2 of 1.2 hours (Fig. 5). The absolute oral bioavailability (F) was an acceptable 31%.

### Table 2

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Idelalisib</th>
<th>Ibrutinib</th>
<th>MDVN1003</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi3Kδ</td>
<td>nM</td>
<td>nM</td>
<td>nM</td>
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<tr>
<td>PI3Kγ</td>
<td>354</td>
<td>&gt;100,000</td>
<td>275</td>
</tr>
<tr>
<td>PI3Kβ</td>
<td>90.2</td>
<td>&gt;100,000</td>
<td>708</td>
</tr>
<tr>
<td>BMX/ETK</td>
<td>30</td>
<td>&gt;1000</td>
<td>107</td>
</tr>
<tr>
<td>ITC</td>
<td>&gt;100,000</td>
<td>19.3</td>
<td>&gt;300</td>
</tr>
<tr>
<td>TEC</td>
<td>&gt;100,000</td>
<td>&lt;0.1</td>
<td>211</td>
</tr>
</tbody>
</table>

**Notes:**

- BMX/ETK, epithelial and endothelial tyrosine kinase; ITC, interleukin-2–inducible T-cell kinase dimethyl sulfoxide; TEC, Tec protein tyrosine kinase.
decay and a $t_{1/2}$ of 1.5 hours (Fig. 5). The absolute oral bioavailability ($F$) was a moderately high 62%. Following intravenous administration in dog, MDVN1003 showed moderate systemic clearance (1.23 l/h/kg) that was 66% of hepatic blood flow in the dog [QH = 1.85 l/h/kg (Davies and Morris, 1993)] and a moderate volume of distribution ($V_d$ = 1.0 l/kg), as shown in Table 4.

Given the low clearance and acceptable oral bioavailability of MDVN1003 in mice, we aimed to show pharmacological

Fig. 4. MDVN1003 inhibits B cell activation in mouse splenocytes and induces apoptosis in DOHH-2 B cell lymphoma cells. (A) Splenocytes were pretreated with the indicated compounds at different concentrations for 30 minutes and then treated with α-IgD (3 μg/ml) for 4 hours to activate BCR signaling. Activated B cells were identified by flow cytometry as the B220+CD69+ cells in a live gate. To calculate the effect of each compound on the inhibition of B cell activation, the percentage of B220+CD69+ cells in each condition was normalized to the α-IgD vehicle control. The IC$_{50}$ was calculated from the curve fitted to the data points by nonlinear regression using GraphPad Prism. (B) DOHH-2 cells were treated with compounds at the indicated concentrations for 72 hours. Cell viability was measured as described in the Materials and Methods. The IC$_{50}$ was calculated from the curve fitted to the data points by nonlinear regression using GraphPad Prism. (C) DOHH-2 cells were treated with compounds at 1 μM for 4 hours, and apoptosis was measured by flow cytometry using an Annexin V FITC apoptosis detection kit. Apoptotic cells were determined as Annexin V+ propidium iodide (PI)− by flow cytometry. Dot plots from a representative experiment are shown in the top panels, and a summary of the percentages of apoptotic cells from four independent experiments is plotted in the bottom panel (mean ± standard deviation, ****$P < 0.0001$, one-way analysis of variance). PI-A, propidium iodide area.
vehicle group reached 2000 mm³. Mice were placed into one of
dosing was terminated when the average tumor volume of the
treatment of ibrutinib and idelalisib and by treatment
combinations of ibrutinib and idelalisib (15 mg/kg ibrutinib/
MDVN1003 were both dosed twice daily. Mice were
dosed once daily due to its irreversible binding, and idelalisib
25 mg/kg idelalisib or 30 mg/kg ibrutinib/50 mg/kg idelalisib),
and idelalisib at 25 or 50 mg/kg reduced average tumor volume
by 6.6 and 14.2%, respectively, as compared with the vehicle
control group (not statistically significant). When dosed in com-
bination, the average tumor volume of the ibrutinib (15 mg/kg)
and idelalisib (25 mg/kg) low combo group was reduced by 38.1%
as compared with the vehicle group, whereas tumors in the
ibrutinib (30 mg/kg) and idelalisib (50 mg/kg) high combo group
were reduced by 57.9% on average as compared with the vehicle
control group ($P < 0.001$, Kruskal-Wallis corrected for multiple
comparisons). Treatment with MDVN1003 reduced tumor
growth by 45.2% ($P < 0.01$, Kruskal-Wallis corrected for multiple
comparisons).

Discussion

The potential advantage of dual inhibition of BTK and
PI3Kδ for treatment of NHL lies in the possibility of treating
refractory patients or overcoming developed resistance to
either BTK or PI3Kδ single inhibitors due to the synergistic or
additive effects of blocking two BCR pathway targets. Codos-
treatment of JeKo1 cells, an MCL cell line, with ibrutinib and idelalisib
resulted in decreased adhesion to fibronectin, a BCR-dependent
process, as compared with cells treated with each drug
individually (de Rooij et al., 2015). The authors found a strong
synergistic effect with the dual treatment. In another example,
combination treatment of BCWM1 Waldenström’s
cellular pathways with both ibrutinib and the highly
selective second-generation PI3Kδ inhibitor TGR-1202
(2-[1(S)-1-[4-amino-3-(3-fluoro-4-propan-2-yloxyphenyl)pyrazolo[3,4-
d]pyrimidin-1-yl]ethyl]-6-fluoro-3-(3-fluorophenyl)chromen-4-
one TG Therapeutics, New York, NY) resulted in increased cell
death as compared with treatment with the individual drugs
(Davids et al., 2016).

In this report, we corroborate the findings that cotreatment
of B cells with ibrutinib and idelalisib results in effects
superior to those seen with either drug dosed individually.

### Table 3

IC₅₀ values for the inhibition of B cell activation in primary mouse
splenocytes and cytotoxic effects in DOHH-2 lymphoma and HEL 92.1.7
cells

<table>
<thead>
<tr>
<th>Primary B Cell Activation (CD69)</th>
<th>DOHH-2 Cell Viability</th>
<th>HEL 92.1.7 Cell Viability</th>
</tr>
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<tbody>
<tr>
<td>nM</td>
<td>nM</td>
<td>nM</td>
</tr>
<tr>
<td>Ibrutinib</td>
<td>6.9</td>
<td>23</td>
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<tr>
<td>Idelalisib</td>
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<td>860</td>
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<tr>
<td>Combo</td>
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<tr>
<td>MDVN1003</td>
<td>25.2</td>
<td>1340</td>
</tr>
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</table>

![Fig. 5. MDVN1003 concentration-time profile following an oral dose in mouse, rat, and dog.](image-url)
The phosphorylation of downstream signaling molecules AKT and ERK 1/2 was significantly dampened in cells treated with both drugs as compared with each drug individually (Fig. 3). Similarly, in the xenograft model, dosing the two inhibitors more effectively reduced the tumor burden than dosing each compound separately (Fig. 6). These data support the hypothesis that targeting two BCR-controlled pathways could be more efficacious than treatment with a monotherapy.

Preclinical data suggesting the potential synergism of inhibiting both BTK and PI3KΔδ have spurred clinical trials of combination treatments. TG Therapeutics has several ongoing clinical studies with TGR-1202 and ibrutinib for B cell malignancies, including relapsed or refractory CLL and MCL. From their phase I/Ib study, TG Therapeutics has reported interim results showing an overall response rate of 82% in CLL and 60% in MCL, but has yet to show a reduction in the rate of resistance (Davids et al., 2016).

Although combination treatment of two separate BTK and PI3KΔδ inhibitors does provide the advantage of being able to independently control the dose of each inhibitor, we tested the hypothesis that a dual inhibitor in a single molecule could be a potential therapeutic approach for NHL. Having the dual inhibition activity built into a single molecule could ease the toxicology profile since there would be a single set of off-target effects. The cost of treatment of patients may also be lower with a single molecule due to lower manufacturing and formulation costs (Shanafelt et al., 2015). We have previously described our discovery of dual BTK and PI3KΔδ inhibitors (Pujala et al., 2016), and here, we report that one of these dual inhibitors does exhibit properties similar to combination dosing of ibrutinib and idelalisib, albeit not as potent or selective.

MDVN1003, although a dual inhibitor of BTK and PI3KΔδ, is not as potent an inhibitor as idelalisib and ibrutinib are against their primary targets as measured in vitro enzymatic assays (Table 1). Despite the difference in potencies, MDVN1003 inhibits the in vitro phosphorylation of AKT and ERK 1/2 as well as either ibrutinib or idelalisib. MDVN1001 and MDVN1002 are potent single inhibitors of BTK and PI3KΔδ, respectively, but these compounds only inhibited the phosphorylation of AKT or ERK 1/2 at the higher concentration of 1 μM. This could be due to the lower potency of MDVN1002 against PI3KΔδ molecules or possibly due to a difference in the cell permeability of MDVN1001 and MDVN1002. Similar to the combo treatment with ibrutinib and idelalisib, combination treatment with MDVN1001 and MDVN1002 showed an additive effect on the inhibition of the phosphorylation of AKT and ERK1/2 upon BCR activation and on cell viability of DOHH-2 cells. Taken together, the data suggest a synergistic effect of dual inhibition of BTK and PI3KΔδ and by MDVN1003. This is further supported by the superior efficacy of MDVN1003 in the xenograft model as compared with either ibrutinib or idelalisib dosed individually. The difference in potency of MDVN1003 against BTK and PI3KΔδ could explain why treatment with this compound is not as effective as combo dosing of ibrutinib and idelalisib in vitro or in vivo (Figs. 4 and 6). Further investigation of the structure-activity relationships of these compounds would be required to identify more potent dual BTK and PI3KΔδ inhibitors with good oral bioavailability that could be tested for better efficacy in vivo.

Another possibility for the superior performance of MDVN1003 compared with monotherapy in the tumor xenograft model is that the antitumor effect could be unrelated to the inhibition of BTK or PI3KΔδ. MDVN1003 inhibits many kinases at 1 μM (Fig. 3), so it is possible that the antitumor effect is not due to synergy of dual inhibition but instead due to the inhibition of another kinase that is required for the growth of DOHH-2 cells. Treatment with MDVN1001, MDVN1002, or the combo treatment with these molecules was similarly as potent as MDVN1003 in the cell viability assay, which raises the possibility that the effect on cell viability by these three molecules may not be completely driven by the inhibition of BTK and PI3KΔδ. However, MDVN1003 is not generally cytotoxic. This molecule, along with ibrutinib and idelalisib, did not induce cell death in HEL 92.1.7 erythroblasts (Fig. 4C). These cells do not express BTK or PI3KΔδ, and any other kinase inhibited by MDVN1003 was not sufficient to induce cytotoxicity. Further optimization of the tool compound MDVN1003 is necessary to improve the selectivity profile and fully rule out off-target effects.

In practice, ibrutinib is extremely effective, and the 24-month progression-free survival in the phase I and phase II studies of ibrutinib in CLL was greater than 80% (Tucker and Rule, 2015). However, 30% of patients are initially refractory to ibrutinib (Tucker and Rule, 2015), and even more become resistant after long-term dosing (Byrd et al., 2013; Wang et al., 2013; Smith, 2015), so reducing the rate of resistance or increasing the duration of response could be important for treating patients with B cell malignancies. A potential advantage of combination therapy of BTK and PI3KΔδ inhibitors may be to reduce the rate of acquired resistance to either BTK or PI3KΔδ inhibition and potential efficacy in patients initially resistant to treatment of either a BTK or a PI3KΔδ inhibitor. However, the preclinical data reported here and elsewhere (Jones and Byrd, 2014; Mathews Griner et al.,...
2014; Zhang et al., 2014; de Rooij et al., 2015; Koehrer and Burger, 2016) do not directly test whether dual inhibition or codosing is efficacious in the case of resistance. To date, no preclinical or clinical studies have looked at the effects of cotreatment with BTK and PI3Kα inhibitors on the rates of resistance. In fact, there is a dearth of reagents with which to investigate these questions and a lack of knowledge around adaptive resistance to PI3Kα inhibition.

In summary, our results show that dual inhibition of BTK and PI3Kα by treatment with a single dual inhibitor could potentially improve the outcomes of NHL patients and may prevent resistance or extend the duration of response. Although MDVN1003 proved useful as an initial tool compound, further medicinal chemistry efforts are required to improve potency to achieve cytotoxic and tumor inhibitory effects that match the combination treatment with ibrutinib.

Fig. 6. MDVN1003 inhibits B cell activation in vivo and inhibits tumor growth in a B cell lymphoma xenograft model. (A) Scheme of the treatment conditions for the experiment in (B). (B) BALB/c mice were orally dosed with compounds or vehicle (Veh and -) at indicated concentrations for 30 minutes. Mice in all groups except the vehicle control were then dosed intravenously with α-IgD for 5 hours. Splenocytes were isolated and stained with a live/dead marker, αB220, and αCD69 antibodies. Activated B cells were determined as B220+CD69+ in a live gate by flow cytometry. Percentage of activated B cells post compound treatment was normalized to the percentage of active B cells in the α-IgD control (- in graph). (C–E) Compounds were tested in a mouse xenograft model of NHL. Ibrutinib was dosed at 15 mg/kg (light blue) or 30 mg/kg (dark blue); idelalisib was dosed at 25 mg/kg (pink) or 50 mg/kg (red); combo low dosing group was 15 mg/kg ibrutinib and 25 mg/kg idelalisib (ibrutinib/idelalisib low, light green); combo high dosing group was 30 mg/kg ibrutinib and 50 mg/kg idelalisib (ibrutinib/idelalisib high, dark green); MDVN1003 was dosed at 100 mg/kg. Ibrutinib was dosed orally once daily, and the other two compounds were dosed orally twice daily. (C) Average body weight of mice in each dosing group (mean ± standard deviation of n = 10 mice per group). (D) Average tumor volume of mice in each dosing group (mean ± standard deviation, ***P < 0.001, Kruskal-Wallis). (E) The tumor volume of each mouse in the dosing groups on the final day of dosing (day 31 post inoculation). The black line is the average tumor volume of each group, and the colored lines are the standard deviations (***P < 0.001, **P < 0.01, Kruskal-Wallis corrected for multiple comparisons). Cpd, Compound; HPβCD, hydroxypropyl beta-Cyclodextrin; mpk, mg/kg.
and idelalisib. Additional investigation is required to better understand the mechanisms by which resistance to ibrutinib and idelalisib occurs and generate reagents to better elucidate preclinical efficacy. The results presented here provide a proof of concept that a dual inhibitor of BTK and PI3Kδ in a single molecule can be developed and could be used as a viable approach for the treatment of B cell malignancies.

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References

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Dual Inhibition of BTK and PI3Kδ to Treat B Cell Cancers

231


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