Novel Treatment for Mantle Cell Lymphoma Including Therapy-Resistant Tumor by NF-κB and mTOR Dual-Targeting Approach

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Abstract

Mantle cell lymphoma (MCL) is one of the most aggressive B-cell non-Hodgkin lymphomas with a median survival of approximately five years. Currently, there is no curative therapy available for refractory MCL because of relapse from therapy-resistant tumor cells. The NF-κB and mTOR pathways are constitutively active in refractory MCL leading to increased proliferation and survival. Targeting these pathways is an ideal strategy to improve therapy for refractory MCL. Therefore, we investigated the in vitro and in vivo antilymphoma activity and associated molecular mechanism of action of a novel compound, 13-197, a quinoxaline analog that specifically perturbs IκB kinase (IKK) β, a key regulator of the NF-κB pathway. 13-197 decreased the proliferation and induced apoptosis in MCL cells including therapy-resistant cells compared with control cells. Furthermore, we observed downregulation of IκBα phosphorylation and inhibition of NF-κB nuclear translocation by 13-197 in MCL cells. In addition, NF-κB-regulated genes such as cyclin D1, Bcl-XL, and Mcl-1 were downregulated in 13-197–treated cells. In addition, 13-197 inhibited the phosphorylation of S6K and 4E-BP1, the downstream molecules of mTOR pathway that are also activated in refractory MCL. Further, 13-197 reduced the tumor burden in vivo in the kidney, liver, and lungs of therapy-resistant MCL-bearing nonobese diabetic severe-combined immunodeficient (NOD/SCID) mice compared with vehicle-treated mice; indeed, 13-197 significantly increased the survival of MCL-transplanted mice. Together, results suggest that 13-197 as a single agent disrupts the NF-κB and mTOR pathways leading to suppression of proliferation and increased apoptosis in malignant MCL cells including reduction in tumor burden in mice. Mol Cancer Ther; 12(10); 2006–17. ©2013 AACR.

Introduction

Mantle cell lymphoma (MCL) is one of the most aggressive B-cell malignancies with a median survival of approximately 5 years due to relapse from therapy-resistant tumor cells (1–5). Despite availability of new agents to combine with high-dose therapy followed by stem cell transplantation, the rate of disease-free survival in MCL is disappointing (5–8). Therefore, identification of key cellular/molecular pathways that can be targeted to refractory state of MCL and the development of an effective therapeutic strategy for targeting those pathways is needed.

MCL has a characteristic chromosomal translocation t(11;14)(q13;q32), a hallmark of this disease that results in cyclin D1 overexpression, which is believed to contribute to deregulated proliferation in MCL (9). Although it is widely accepted that cyclin D1 has an important role in the pathogenesis of MCL, accumulating evidence suggests that MCL often has defects in many other cellular processes, such as those involved in cell-cycle regulation, apoptosis, and DNA repair. MCL is known to be resistant to apoptosis with existing therapy. Recent studies have revealed a number of biochemical defects that may contribute to its relatively high resistance to apoptosis due to overexpression of several antiapoptotic proteins. Several lines of evidence indicate that the NF-κB pathway is constitutively active in MCL and leads to the overexpression of several antiapoptotic molecules (9–15). In addition, gene expression analyses of samples from patients of MCL and therapy-resistant MCL cells derived from different tissue sites have frequently shown high expression of NF-κB target genes (12, 16, 17). Consequently, inhibition of this constitutive activation of NF-κB has been shown to elicit cell-cycle arrest and cell death in MCL cells (10, 13–15, 18). Strong evidence implicating the NF-κB pathway in the onset and progression of MCL has made it an attractive
Novel Treatment for Refractory Mantle Cell Lymphoma

target for therapeutic intervention. Aberrant cellular signaling, such as in the phosphoinositide 3-kinase (PI3K)/mTOR pathway, may also contribute to the chemoresistance of MCL (19, 20). Targeting these pathways can be a novel approach for developing a new therapeutic strategy for improved treatment of refractory MCL.

IkB kinase δ (IKKδ) is a key upstream kinase in the NF-κB pathway that is activated by immune and inflammatory responses regulating cell growth and survival in the pathogenesis of various solid and hematologic malignancies (21). In general, NF-κB is transcriptionally inactive in the cytoplasm of most cells through interaction with its cytoplasmic inhibitor protein called IκBα. On activation, the IKKδ becomes phosphorylated and active. Activated IKKδ phosphorylates NF-κB-bound IκBα and targets it for polyubiquitination and proteasome-mediated degradation (21–23). As a consequence, free NF-κB is further activated through posttranslational modifications and translocates to the nucleus, resulting in transcriptional activation of several hundred survival-, proliferation-, and apoptosis-associated genes (21–25). In addition, the IKKδ is known to result in the activation of tuberous sclerosis 1 (TSC1). The phosphorylation-mediated suppression of TSC1 results in the activation of the mTOR pathway (26, 27). Together, these data suggest that IKKδ inhibitors without toxicity in clinics is needed.

Compounds containing the quinoxaline core are found in a number of natural products as validated hits from high-throughput screens, clinical candidates, and drugs on the market (34). Synthesis and screening of a focused library of quinoxaline compounds led to a well-defined structure–activity relationship and the identification of a quinoxaline urea analogue that inhibited growth of cancer cells with low micromolar potency (35, 36). In addition, recent studies by us suggest that the quinoxaline urea analog 13-197 inhibits the constitutively active form of IKKβ which is an emerging target for therapeutic development (28–30). In cell-based NF-κB-driven dual luciferase assay, the potency of 13-197 is comparable with the reported NF-κB inhibitors, parthenolide, curcumin and noscapine (37). Considering that IKKβ and the NF-κB pathway are constitutively active in the therapy-resistant MCL, we speculated that 13-197 might be a therapeutic option for refractory MCL.

The effect of this novel inhibitor 13-197 and its molecular mechanism(s) against MCL have not been described. Therefore, we evaluated the efficacy and potency of 13-197 as a single agent targeting NF-κB and mTOR pathways in MCL and therapy-resistant MCL cell lines. We found that 13-197-treated cells showed inhibition of IκB phosphorylation and phosphorylation of S6K and 4E-BP1 which are downstream of IKKβ in the NF-κB and mTOR pathways, respectively. This resulted in significant suppression of proliferation and increased apoptosis in MCL cells including therapy-resistant cells, as well as reduction of tumor burden in mice. Our data strongly supports 13-197 as a novel and potential therapeutic agent against malignant MCL to be further explored to take to clinic.

Materials and Methods

Cell lines and maintenance

The MCL cell line Granta 519 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) and Rec-1 and Mino were obtained from the American Type Culture Collection (ATCC). These cell lines were authenticated by the ATCC in 2012. These cell lines were maintained in RPMI media (Invitrogen) containing 10% FBS (Atlanta Biologicals), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 2 mmol/L L-α-Glutamine (Invitrogen). This medium with supplements is referred to as the RF-10 medium in this article. The cultures were maintained in a humidified incubator adjusted with 5% CO2 and 95% air atmosphere at 37°C. All cultures were split twice weekly, cell counting was conducted using a hemocytometer, and cell viability was assayed by Trypan blue staining method.

Isolation of therapy-resistant MCL cells

The therapy-resistant MCL cell lines were characterized and established after transplanting the Granta 519 into the nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice following treatment with CHOP chemotherapy and bortezomib as described previously (16, 38). These therapy-resistant tumor cells derived from liver, kidney, and lungs were described as Granta 519 resistant from liver (GRL), Granta 519 resistant from kidney (GRK), and Granta 519 resistant from lungs (GRR), respectively, and compared with parental Granta 519 cells (GP). These cells have been shown to be resistant to chemotherapy, as confirmed both in vitro and in vivo studies. The additional properties of these therapy-resistant cell lines have been recently published (16, 38).

The therapeutic agent 13-197

In this study, we used a quinoxaline urea analogue called 13-197, which inhibits NF-κB and mTOR pathways via IKKβ in pancreatic cancer cell lines in vitro and in vivo (28). The molecular structure of 13-197 is described in Fig. 1A. The toxicity and pharmacokinetic (PK) properties of this compound have been reported by Gautam and colleagues (39). IC_{50} of 13-197 in different MCL cell lines are described in Supplementary Table S1.

In vitro growth assay

Ten thousand GP, GRL, GRK, GRR, Rec-1, and Mino MCL cells were cultured in RPMI media containing 0.5,
Effect of 13-197 on therapy-resistant MCL cells growth/proliferation in vitro. Ten thousand of each MCL cells indicated were cultured in RF-10 media containing 1, 5, 10, and 20 µmol/L 13-197 in 96-well plates for 24, 48, and 72 hours. A, the chemical structure and molecular properties of 13-197. B to G, MTT assay was used to determine the cell viability in control and treated cells. The values represent the means ± SD from triplicate wells of the 96-well plates. H to M, the proliferation levels of control and treated cells were determined using [3H]-thymidine uptake method. The values represent the means ± SD from triplicate wells of the 96-well plates. Similar results were obtained from three sets of independent experiments.

1, 5, 10, 20, and 50 µmol/L 13-197 or dimethyl sulfoxide (DMSO; vehicle) in 96-well plates, and the growth of these cells were determined at 24, 48, and 72 hours using MTT and [3H]-thymidine uptake assays. Briefly, 25 µL of MTT reagent (5 mg/mL in PBS) was added to the culture and incubated for two hours before the respective time point, and the cells were lysed using an SDS-based lysis reagent. The intensity of the color developed was determined at 570 nm using a plate reader (Biotek). In another set of experiments, 0.5 µCi of [3H]-thymidine was added 15 hours before cell harvest. The cells were harvested at 24, 48, and 72 hours using a PHD cell harvester (Cambridge Technologies). The incorporated radioactivity was counted using a liquid scintillation counter (Packard Instruments).

Apoptosis assay

The MCL cell lines were cultured at a concentration of 1 x 10^6 cells/ml in RF-10 media containing 10 µmol/L 13-197 or DMSO for 48 hours. The percentage of cells

Chemical formula: C_{22}H_{15}BrN_4O_3
Molecular mass: 474.03275
undergoing apoptosis was then assessed using the Annexin-V-FITC apoptosis assay kit (BD Biosciences) according to the manufacturer’s instructions and flow cytometry.

**Cytomorphology**

Control and 13-197–treated cells were washed twice with PBS. Cytospin preparations were made from different MCL cells used in this study and stained with Wright-Giemsa stain in the University of Nebraska Medical Center (UNMC) pathology core laboratory, and the cytomorphology was compared by light microscopy.

**Western blotting**

Western blot analysis was conducted using a standardized protocol in the laboratory. Briefly, the cells were harvested after the indicated time, washed with ice-cold PBS, and lysed in a buffer containing 50 mmol/L Tris–HCl (pH 7.0), 150 mmol/L NaCl, 0.1% SDS, 1% Na-deoxycholate, 1% NP-40, and a complete protease and phosphatase inhibitor cocktail (Pierce). For cytoplasmic and nuclear protein extraction, a NE-PER kit (Pierce) was used according to the manufacturer’s instructions. Protein concentration was determined with a protein assay kit (Bio-Rad). These protein lysates were subjected to 10% to 12% SDS–polyacrylamide gel electrophoresis. After electrophoresis and protein transfer to a polyvinylidene difluoride (PVDF) membrane, the membrane was blocked with 5% nonfat dry milk and probed with specific primary antibodies. The primary antibodies used in this study included NF-κB (p65), McI-1, β-actin (Santa Cruz Biotechnology), IκBα, phospho-IκBα, phospho-NF-κB (p65), Bcl-XL, S6K, phospho-S6K, 4E-BP1, phospho-4E-BP1, PARP, α-tubulin (Cell Signaling Technology), and an anti-cyclin D1 (BD Biosciences). Immunoreactivity was detected using appropriate peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized using an enhanced chemiluminescence (ECL) detection system (Pierce, IL).

**MCL patient blood samples**

Primary MCL cells were obtained from four MCL patients in leukemic phase using an UNMC Institutional Review Board-approved protocol and informed consent. Peripheral blood mononuclear cells (PBMC) from patients of MCL with high lymphocyte count were isolated using a lymphocyte separation medium (Accurate Chemical and Scientific) as described previously (40) and used to evaluate the efficacy of 13-197. The immunophenotypic analysis of these cells using flow cytometry showed more than 90% CD5 and CD20 positivity. These MCL cells were treated with vehicle control (DMSO) or different concentrations of 13-197 for 24 and 48 hours to determine the efficacy of 13-197 as described earlier for the established cell lines. As an additional control, normal B cells from three healthy donors purified using Miltenyi magnetic bead separation method were used. These healthy donor samples were provided by the apheresis core unit of the UNMC clinic.

**In vivo studies**

All animal experiments were approved by the UNMC Institutional Animal Care and Use Committee (IACUC). For in vivo studies, six- to eight-week-old NOD.CB17-Prkd<sup>−/−</sup> J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the UNMC Comparative Medicine Animal Facilities. The NOD/SCID mice were irradiated with 215 Gy using the UNMC experimental irradiator facility. One million of therapy-resistant MCL (GRL) cells suspended in 100 μL sterile media were transplanted into NOD/SCID mice via lateral tail vein injection. After seven days, one set of seven mice was treated with cremophor (vehicle) orally and served as a control group, and another set of 10 mice was treated with 30 consecutive daily oral doses of 13-197 (120 mg/kg/body weight) and served as an experimental group. When mice became moribund as evidenced by weight loss, hunching back, ruffled fur, excessive dehydration, and/or hind-limb paralysis, they were euthanized using the CO2 chamber method. Liver, lungs, and kidneys were harvested and fixed in 10% buffered formalin solution for further histologic and immunohistochemical analyses. The survival of the vehicle or 13-197–treated mice was determined by the Kaplan–Meier method and analyzed using the log rank test.

**Immunohistochemistry**

The H&E-stained histologic sections and immunohistochemical analyses for CD20, phospho-p65, and phospho-S6K expression of tissues harvested from control and 13-197–treated mice were conducted at the Histology Core Facility of the Nebraska Medical Center. For tumor burden analyses, the histologic sections were scanned under virtual microscopy and the tumor area was quantitated using Neuroinformatika software.

**Statistical analysis**

Each experiment was conducted in triplicate and repeated for an additional two to three times and the mean and standard error values were calculated. The significance of difference (P value) was calculated using independent Student t test and P values less than 0.05 were considered significant.

**Results**

**Growth inhibitory effect of 13-197 against MCL and therapy-resistant MCL cells**

As 13-197 has been reported to be effective in micromolar (μmol/L) ranges of concentration to kill pancreatic cancer cells in vitro (28), we used the micromolar concentrations of 13-197 to determine its efficacy in MCL cell lines. To examine the ability of 13-197 to inhibit proliferation of therapy-resistant MCL cells in vitro, the MCL cells were incubated with 1, 5, 10, and 20 μmol/L 13-197 for 24, 48, and 72 hours, and the growth of the cells was assessed using MTT and 3[H]-thymidine uptake assays. The MTT result showed a dose- and time-dependent growth inhibition of MCL cells as shown in Fig. 1B–G. Similarly, the
3[H]-thymidine uptake assay also showed significant inhibition of MCL cells proliferation at 1 μmol/L 13-197. A dose- and time-dependent inhibition of MCL cells was observed as shown in Fig. 1H–M. At 10 μmol/L, 13-197 completely suppressed the proliferation in all MCL cell lines used in the present study. Together, these results suggest that 13-197 suppresses growth/proliferation in vitro of aggressive MCL and therapy-resistant MCL cells at the micromolar potency. To address the toxicity issue of this compound, we also determined the effect of 13-197 on the viability of normal B cells of healthy donors (n = 3). The MTT assay results clearly showed no significant effect of 13-197 (ranges, 1–100 μmol/L) on the viability of normal B cells (Supplementary Fig. S1), suggesting 13-197 specificity to kill tumor cells only, not normal or untransformed cells.

**Induction of increased apoptosis by 13-197 in MCL and therapy-resistant MCL cells**

To investigate the ability of 13-197 to induce apoptosis in GP, GRL, GRK, GRR, Rec-1, and Mino MCL cells, cytomorphologic analyses and AnnexinV staining method were used in 13-197–treated cells for 48 hours. Light microscopic appearance of Wright–Giemsa stained control and treated cells are shown in Fig. 2A. Results from cytomorphology of therapy-resistant MCL cells clearly showed a significant increase in apoptotic cells in therapy-resistant MCL cells compared with vehicle (DMSO)-treated cells and further confirms the ability of 13-197 to induce apoptosis in these cell lines. These results are consistent with previous studies (Fig. 1) exhibiting the ability of 13-197 to inhibit proliferation and growth in malignant cells of MCL.

**Downregulation of NF-κB signaling molecules in MCL cells following treatment with 13-197**

It has been established that NF-κB is constitutively active in malignant MCL (9–13, 41). Therefore, initially, we confirmed the constitutive activation of NF-κB pathway using Western blot analysis. Our results clearly showed the increased level of nuclear NF-κB (p65) and phosphorylation of IκBα in MCL and therapy-resistant MCL cell lines as well as primary cells of a MCL patient compared with normal B cells (Supplementary Fig. S2). We then explored whether 13-197 downregulates NF-κB signaling molecules in the MCL and therapy-resistant MCL cells. Initially, we examined the effect of 13-197 on IκBα phosphorylation, a major target molecule of the NF-κB signaling pathway, using Western blot method. The results indicated a significant downregulation of IκBα phosphorylation at the 5 μmol/L concentration of 13-197, and the downregulation of IκBα phosphorylation followed a dose- and time-dependent manner in MCL cell lines including the therapy-resistant cell lines.

![Figure 2. Effect of 13-197 on therapy-resistant MCL cells morphology/apoptosis. Exponentially growing therapy-resistant and other MCL cells were treated with 10 μmol/L 13-197 for 48 hours. Following treatment, cells were stained with Wright-Giemsa staining using a cytopsin preparation. The cells were examined under bright field microscope for the apoptotic cells and images were captured at ×40 magnification. A, cytomorphology of different MCL cells indicated above following treatment with 13-197 specifically examining apoptotic bodies. B, Annexin-V apoptosis detection assay was used to access percentage of cells undergoing apoptosis in those MCL cell lines following treatment with 10 μmol/L 13-197 for 48 hours. The values represent the means ± SD of three separate experiments.](image-url)
examined (Fig. 3A and C). It is well known that phosphorylated IkBα is rapidly ubiquitinated, which leads to its proteasomal degradation, and this allows the release of NF-κB (p65) from the complex. Released p65 undergoes posttranslational modifications including phosphorylation, which leads to its nuclear translocation (25). We next conducted Western blot analysis for NF-κB phosphorylation and nuclear translocation in DMSO- (vehicle) and 13-197–treated MCL cells following cytoplasmic and nuclear fractionation of the cells. Fig. 3B and D shows a decreased level of NF-κB (p65) phosphorylation and inhibition of its nuclear translocation.

Figure 3. Effect of 13-197 on the NF-κB pathway–associated molecules in therapy-resistant MCL cells. MCL cells were cultured in RF-10 media containing vehicle (DMSO) or indicated concentration of 13-197 for different time points. After incubation, cells were harvested and whole-cell lysate or cytoplasmic/nuclear fraction was prepared and subjected to Western blot for the expression of NF-κB pathway–associated proteins. A, the levels of IkBα phosphorylation by 13-197 in GP cells in a dose-dependent manner. B, decreased levels of NF-κB nuclear translocation in GP MCL cells following treatment with different concentrations of 13-197. PARP and α-tubulin were also detected to confirm cytoplasmic and nuclear fractionation of proteins. C, phosphorylation status of IkBα by 13-197 in therapy-resistant and their parental GP cells in a time- and dose-dependent manner. D, status of IkBα and p65 phosphorylations, p65 nuclear translocation, Mcl-1, and Bcl-XL in therapy-resistant and their parental GP cells following treatment with 10 μmol/L 13-197. E, levels of IkBα and p65 phosphorylations, p65 nuclear translocation, Mcl-1, Bcl-XL, and cyclin D1 in Rec-1 and Mino MCL cell lines following treatment with 10 μmol/L 13-197. F, expression levels of cyclin D1 in therapy-resistant and parental GP cell lines following treatment with different concentrations of 13-197. β-Actin was used as an internal control in all these experiments. The results shown are representative of three sets of independent experiments.
Retention by 13-197 in the therapy-resistant MCL cells. The inhibition of p65 nuclear level by 13-197 was also dose dependent (Fig. 3B). Further, our results of Western blot analysis showed decreased expression of NF-xB-regulated antiapoptotic molecules including Bcl-XL, a member of the Bcl-2 antiapoptotic gene family, overexpressed in MCL, and Mcl-1 in all 13-197-treated MCL cells (Fig. 3D and E). Cyclin D1 is a molecule which is overexpressed in MCL and is also regulated by NF-xB (9). We next examined the expression level of cyclin D1 by Western blot analysis following treatment of MCL cells with 13-197. As expected, the expression level of cyclin D1 was significantly decreased by 13-197 at 10 µmol/L in MCL cells examined, and the decreased level of cyclin D1 followed in a dose-dependent fashion, as shown in Fig. 3F. These results were consistent with other MCL cell lines including Rec-1 and Mino (Fig. 3E). Overall, these results suggest that 13-197 suppresses the cell growth and induced the apoptosis by abrogating NF-xB signaling pathway in MCL and therapy-resistant MCL cell lines.

13-197 inhibits the activation of mTOR pathway molecules

Constitutive activation of mTOR signaling pathway has also been implicated in the development of various malignancies including MCL (9, 19, 42). With regard to constitutive activation of mTOR pathway in MCL, our results of Western blot analysis are also in agreement where we observed the increased phosphorylations of the mTOR downstream molecules such as S6K and 4E-BP1 in MCL cell lines, including therapy-resistant cell lines and primary cells of a patient of MCL compared with normal B cells (Supplementary Fig. S2). Activation of S6K and inactivation of 4E-BP1 through phosphorylation by mTOR led to increased protein synthesis, cell proliferation, and VEGF secretion and, ultimately, to tumorigenesis (26). Because IKKβ is the major upstream kinase in the NF-xB signaling pathway which is known to activate mTOR signaling pathway (27), we examined the ability of 13-197 in modulating mTOR pathway molecules by targeting IKKβ. In this regard, we analyzed the principal molecules of the mTOR pathway, especially S6K and 4E-BP1 phosphorylation, in the six- and 24-hour 13-197-treated MCL cells using Western blot. The results shown in Fig. 4 clearly show that 13-197 treatment for 24 hours, significantly downregulated the phosphorylation of S6K and 4E-BP1 in all MCL cell lines examined. The results for 6-hour treatment with 13-197 in parental GP and therapy-resistant GRL cell lines are shown in Supplementary Fig. S3, where 13-197 significantly decreased the levels of mTOR-activated molecules in both parental and therapy-resistant MCL. These results suggest that 13-197 can abrogate both NF-xB and mTOR signaling pathways by targeting central player IKKβ. Together, the results showed that 13-197 is an effective compound that has all the desirable properties to inhibit the constitutively active NF-xB pathway and results in significantly decreased...
proliferation and increased apoptosis in MCL as well as therapy-resistant MCL variants isolated from liver, lungs, and kidney.

**Effect of 13-197 in MCL primary cells**

To authenticate our previous observations in established MCL cell lines, we further investigated the antilymphoma activity of 13-197 using primary cells of patients with MCL. For this purpose, we treated MCL primary cells with increasing concentrations of 13-197. After 48 hours of treatment, cell growth and apoptosis were determined using MTT/3-H-thymidine uptake and Annexin-V assays, respectively. As shown in Fig. 5A–D, 13-197 treatment induced a decreased cell growth/proliferation and an increase in the apoptotic-appearing cells of primary MCL cells in a dose-dependent manner. Further, we determined the effects of 13-197 on NF-kB signaling molecules using Western blot analyses as shown in Fig. 5E. Following 24-hour treatment with increasing concentration of 13-197, results of Western blot analysis clearly showed that 13-197 at 20 μmol/L concentration decreased the phosphorylation of IkB, reduced the level of

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Figure 5. Effect of 13-197 in primary MCL cells. Primary MCL cells from samples of patients with MCL (n = 4) were cultured in RF-10 medium containing vehicle (DMSO) or indicated concentrations of 13-197 for 24 and 48 hours. Following incubation, cells were subjected to growth/proliferation, apoptosis, and Western blot analyses. A, MTT assay was used to determine the cell viability in control and 13-197–treated combined cells of two MCL patients (MCL-1 and -2) for 48 hours. The values represent the means ± SD from four wells of the 96-well plates. B, MTT assay was used to determine the cell viability in control and 13-197–treated cells of two additional MCL patients (MCL-3 and -4) for 48 hours. The values represent the means ± SD from four wells of the 96-well plates. C, the proliferation levels in control and treated cells of MCL patients (n = 2) were determined using 3[H]-thymidine uptake method following treatment with 13-197 for 48 hours. The values represent the means ± SD from triplicate wells of the 96-well plates. D, Annexin-V apoptosis detection assay was used to access percentage of cells undergoing apoptosis in those MCL primary cells (n = 2) following treatment with 13-197 for 48 hours. The values represent the means ± SD from triplicate wells of the six-well plates. E, following 24 hours of 13-197 treatments, cells were harvested and whole-cell lysate or cytoplasmic/nuclear fraction was prepared and subjected to Western blot for the expression of indicated molecules of NF-κB pathway. β-Actin was used as an internal control in this experiment. This figure shows representative data of two MCL patients.
nuclear NF-κB (p65) by inhibiting its nuclear translocation, and subsequently downregulated the expression level of NF-κB target molecules including Cyclin D1, Bcl-XL, and Mcl-1. Together, these results suggest that 13-197 also suppresses the cell growth and induces apoptosis by abrogating the NF-κB signaling pathway in primary cells of patients with MCL, substantiating our previous finding in established MCL cell lines.

**Antitumor effect of 13-197 against therapy-resistant MCL in vivo**

To determine the antitumor efficacy of 13-197 against therapy-resistant MCL tumor cells in vivo, we used 17 immune-compromised NOD/SCID mice which were transplanted with GRL. We selected GRL for this study because, in a previous study, we showed that, among the stable therapy-resistant MCL cell lines we developed, GRL is the most aggressive cell line (16, 38). Seven days after tumor transplantation, the mice were randomized and divided into two groups. One group of mice (n = 7) were given vehicle (cremophor, p.o.) only, whereas another group of mice (n = 10) received 13-197 (120 mg/kg/day, p.o.) for 30 consecutive days. The dose of 13-197 for the treatment of mice was achieved from the reports described previously (28, 39). The dose and schedule for 13-197 were well tolerated. On days 12 and 20 after initiation of treatment, we randomly selected three mice from each treatment group for necropsy and tumor burden analyses in different organs including liver, kidney, and lungs; this was done using CD20 immunohistochemical analyses. As shown in Fig. 6A, there was significantly decreased tumor burden in the liver, lungs, and kidney from mice treated with 13-197 compared with control. Indeed, 13-197 significantly increased disease-free survival (P < 0.001) of mice transplanted with MCL compared with control mice (Fig. 6B), indicating that 13-197 decreases tumor burden and, thereby, increases the survival of mice. Further, we validated the 13-197 effect on targeting NF-κB and mTOR pathways in vivo. In this regard, our immunohistochemistry results with phosphorylated NF-κB (p-65) and mTOR (p-S6K) molecules showed significantly decreased levels in 13-197–treated liver, kidney, and lungs of MCL-bearing NOD/SCID mice (Fig. 6C), confirming 13-197 antilymphoma efficacy targeting NF-κB and mTOR in vivo as well. Together, these results suggested that 13-197 has potential therapeutic efficacy against therapy-resistant MCL and warrants further preclinical and clinical investigations to take this approach to the clinical setting.

**Discussion**

In the present study, single-agent treatment of MCL with oral compound 13-197 not only suppressed cell growth and induced apoptosis in vitro, but also reduced the tumor burden and increased the survival of MCL-bearing mice. In addition to the significant antilymphoma efficacy as a single agent, the 13-197 also targeted NF-κB and mTOR signaling pathways by specifically inhibiting the nuclear retention of NF-κB (p65) and phosphorylation of IκB, S6K, and 4E-BP1 in MCL cells including therapy-resistant cells. Intracellular signaling pathways that control cell growth and survival mechanisms are complex, interactive, and often cross-talk with each other in various types of human hematologic malignancies. Frequently, however, these pathways including NF-κB and PI3K-mTOR are used and constitutively activated in these tumors (3, 12, 13, 19, 42). Targeting these pathways with new and novel therapeutic agents that have molecular specificity is likely to provide improved treatment outcomes (8, 9, 43). Novel therapies, ideally, should be based on a basic understanding of the biology of therapy-resistant MCL, especially molecular aspects of the disease progression, which translate into the clinical phenotype. Therefore, MCL cells from refractory patients needed to be evaluated, particularly tumor cells from different tissues, to understand the influence of tissue microenvironment on molecular phenotype of therapy-resistance. It is not feasible to conduct all these studies in patients with resistant MCL; therefore, to determine the efficacy and understand the molecular basis of therapy-resistance in MCL, we used the therapy-resistant MCL cell lines that we have already established from relapsed tumors from different organs of MCL-bearing NOD/SCID mice, following high-dose therapy combined with bortezomib, a proteasome inhibitor (16, 38). These therapy-resistant cell lines served as ideal preclinical models in the present study. In this regard, targeting NF-κB and mTOR pathways in these therapy-resistant MCL cells further attest the novelty and therapeutic efficacy of 13-197.

The oral compound 13-197 showed a significant anti-MCL efficacy in our study. Recently, Radhakrishnan and colleagues (28) have shown that 13-197 has strong therapeutic efficacy against human pancreatic adenocarcinoma via targeting of the NF-κB and mTOR pathways. They have identified 13-197 as an IKKβ inhibitor with the screening of 13-197 against a panel of kinases. Their study shows that 13-197 targets IKKβ and inhibits NF-κB and mTOR signaling pathways in pancreatic cancer cell lines. With regard to toxicity and pharmacokinetics properties of 13-197, Gautam and colleagues (39) have shown that 13-197 does not have significant toxicity to normal tissues of mice and rats.

Our results and reports by others have shown that NF-κB is constitutively expressed in refractory MCL (9–13, 41). In this regard, our therapy-resistant MCL cell lines showed constitutive activation of IKK-NF-κB (p65) and IκBα. In addition, these MCL cells overexpressed Mcl-1, Bcl-XL, and cyclin D1 that are known to be regulated by NF-κB (9, 13, 14). Treatment of MCL cells with 13-197 downregulated the constitutively active NF-κB and inhibited the phosphorylation of IκBα and p65, and ultimately suppressed the expression of proliferation (cyclin D1) and survival (Bcl-XL, Mcl-1) molecules. Cyclin D1, a NF-κB–regulated gene, is overexpressed in MCL as a result of a t(11;14) chromosomal translocation, a hallmark of MCL (9). Cyclin D1 has been previously shown to be involved
in the regulation of MCL cell proliferation (9, 12). In this article, we show that inhibition of proliferation of MCL correlated with the downregulation of the expression of cyclin D1 protein. The suppression of cyclin D1 by 13-197 resulted in the suppression of MCL cell proliferation. Because NF-κB is well known to mediate antiapoptotic effects (21), we examined whether suppression of NF-κB by 13-197 could lead to apoptosis. We found that 13-197 significantly induced the apoptosis in MCL cells including therapy-resistant cells and primary cells of patients with MCL that were examined (Figs. 2 and 5D). To further elucidate the mechanism of 13-197–mediated apoptosis in MCL cells, we investigated the important apoptotic regulators, that is, Bcl-XL and Mcl-1, which are known to be regulated by NF-κB and overexpressed in MCL cells. Our results show that 13-197 downregulated the antiapoptotic genes Bcl-XL and Mcl-1 in MCL cells including therapy-resistant cells and also in MCL primary cells. Further, our data also showed that 13-197 induced the cleavage of PARP protein, a marker of apoptosis, in these MCL cells (Fig. 3B and E). Together, the results suggest that 13-197 exerts its antiproliferative and apoptotic effects in MCL cells by inhibiting the NF-κB pathway.

IKKβ, the major upstream kinase required for NF-κB activation, is constitutively phosphorylated in refractory MCL (14, 22, 44). We found that 13-197 treatment abrogated
the constitutive IKKβ of NF-κB pathway activation through the inhibition of IκBα phosphorylation. Inhibition of IκBα phosphorylation resulted in the suppression of constitutive phosphorylation of p65 and its nuclear translocation (Figs. 3 and 5). Several lines of evidence indicate that activation of IKKβ leads to the activation of another downstream signaling pathway like mTOR signaling pathway molecules, that is, S6K and 4E-BP1, that increase the protein synthesis and, thereby, abnormal cell proliferation in various malignancies (20, 41, 42). In this context, our results showed that 13-197 can perturb the mTOR signaling pathway by also inhibiting S6K and 4E-BP1 phosphorylation (Fig. 4). These data suggest that 13-197 principally targets IKKβ which leads to abrogation of NF-κB (transcriptionally) and mTOR (translationally) and, thus, suppresses cell proliferation and induces apoptosis in malignant cells of MCL.

Having shown the in vitro effect of 13-197, as a next logical step we examined the efficacy of 13-197 in vivo in a mouse tumor model. NOD/SCID mice bearing therapy-resistant MCL tumor showed a significant decrease in tumor burden following treatment with 13-197 (Fig. 6A). Further, this reduced tumor burden significantly reflected in increasing disease-free survival of NOD/SCID mice transplanted with MCL (Fig. 6B), showing the ability of 13-197 as a single agent to reduce tumor burden and increase survival in an animal model. In addition, 13-197 showed antilymphoma efficacy by dual targeting NF-κB and mTOR pathways against therapy-resistant MCL bearing a mouse model (Fig. 6C). In addition, we observed no significant synergistic effect in reducing tumor burden when 13-197 was used in combination with CHOP chemotherapy (data not shown). This observation reaffirms that NOD/SCID mice bearing GRL tumor are already resistant to CHOP chemotherapy that we developed as a single agent (data not shown). This observation further reinforces the dual targeting of NF-κB and mTOR pathways by which 13-197 modulates proliferation/apoptosis and exerts its antilymphoma activity while also warranting further preclinical and clinical investigations to take this approach to clinical setting.

Disclosure of Potential Conflicts of Interest
R.N. Rajule has ownership interest in WO32012/07414 A2 patent. P. Radhakrishnan, N.K. Chaturvedi, and A. Natarajan have ownership interests (including patents) in a pending patent. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank the core facilities of flow cytometry, histology, and virtual microscopy at the UNMC for their help in these studies.

Grant Support
This work was financially supported by the Lymphoma Research Foundation, New York, NY (to S.S. Joshi) and, in part, by an NIH grant (CA127239 to A. Natarajan). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 28, 2013; revised July 18, 2013; accepted July 31, 2013; published OnlineFirst August 20, 2013.

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Molecular Cancer Therapeutics

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