



Dual PAK4-NAMPT Inhibition Impacts Growth and Survival, and Increases Sensitivity to DNA-Damaging Agents in Waldenström Macroglobulinemia

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Abstract

Purpose: p21-activated kinase 4 (PAK4) plays a significant biological and functional role in a number of malignancies, including multiple myeloma (MM). On the basis of our promising findings in MM, we here characterize PAK4 expression and role in WM cells, as well effect of dual PAK4-NAMPT inhibitor (KPT-9274) against WM cell growth and viability.

Experimental Design: We have analyzed mRNA and protein expression levels of PAK4 in WM cells, and used loss-of-function approach to investigate its contribution to WM cell viability. We have further tested the *in vitro* and *in vivo* effect of KPT-9274 against WM cell growth and viability.

Results: We report here high-level expression and functional role of PAK4 in WM, as demonstrated by shRNA-mediated knockdown; and significant impact of KPT-9274 on WM cell

growth and viability. The growth inhibitory effect of KPT-9274 was associated with decreased PAK4 expression and NAMPT activity, as well as induction of apoptosis. Interestingly, in WM cell lines treated with KPT-9274, we detected a significant impact on DNA damage and repair genes. Moreover, we observed that apart from inducing DNA damage, KPT-9274 specifically decreased RAD51 and the double-strand break repair by the homologous recombination pathway. As a result, when combined with a DNA alkylating agents bendamustine and melphalan, KPT-9274 provided a synergistic inhibition of cell viability in WM cell lines and primary patient WM cells *in vitro* and *in vivo*.

Conclusions: These results support the clinical investigation of KPT-9274 in combination with DNA-damaging agent for treatment of WM.

Introduction

Waldenström macroglobulinemia (WM) is characterized by lymphoplasmacytic cell infiltration of bone marrow (BM) and secretion of a serum monoclonal immunoglobulin M (IgM) protein. Recent genomic studies have contributed to a better understanding of the molecular mechanisms supporting WM pathogenesis and progression (1–3). These findings have been

rapidly translated from laboratory to clinical setting, with the identification of novel therapeutic approaches and the initiation of several clinical trials for the treatment of patients with relapsed/refractory WM (4, 5). However, despite this substantial progress in treating patients with WM, only a few patients achieve complete remission whereas others develop drug resistance. Therefore, there is a need for identifying novel targets as well as for innovative therapeutic regimens to effectively treat patients with WM.

p21-activated kinase 4 (PAK4) is a member of PAK family of serine/threonine kinases with a role in cytoskeleton reorganization, cell proliferation and survival, and drug resistance (6–8). We have recently reported the oncogenic potential of PAK4 in multiple myeloma (MM) cells and provided evidence of the significant impact of the PAK4 inhibitor KPT-9274 on MM cell growth and viability, representing a potential novel therapeutic intervention in this malignancy (9). KPT-9274 is an orally administered small molecule, currently in clinical trial for the treatment of patients with advanced solid malignancies or non-Hodgkin's lymphoma (NCT02702492). It allosterically binds to, destabilizes and causes the degradation of PAK4. In addition to which, we have also reported that KPT-9274 depletes the synthesis of nicotinamide adenine dinucleotide (NAD) by blocking the activity of nicotinamide phosphoribosyl transferase (NAMPT), the rate-limiting enzyme in the NAD biosynthesis salvage pathway.

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Translational Relevance

Our study provides the framework for the clinical evaluation of novel allosteric PAK4-NAMPT inhibitor in patients with WM, alone or in combination with DNA-damaging agents.

NAD is an essential metabolite required for sustaining energy production (TCA cycle) and regulating various cellular processes. Cancer cells are characterized by higher NAD⁺ turnover than normal cells due to the increased energy required for their cell proliferation and metabolism, as well as regulation of transcription, chromatin dynamics, and DNA repair-processes (10). As NAD⁺ is rapidly consumed and converted to nicotinamide, NAMPT plays a crucial role in replenishing the intracellular NAD⁺ pool. Aberrant activation of NAMPT has been reported in a number of solid and hematologic malignancies, including leukemia, MM, and importantly WM (11, 12).

Here, for the first time we describe a role for PAK4 in WM cell viability and report a significant impact of dual PAK4-NAMPT inhibition via KPT-9274 on WM cell growth and survival. At the molecular level, we identified the DNA damage and repair pathway to be significantly impacted by KPT-9274 leading to synergistic activity in combination with DNA-damaging agents *in vitro* and *in vivo*.

Materials and Methods

Cells and Reagents

The WM cell lines (BCWM-1, MWCL-1, and RPC1WM-1) were cultured in RPMI containing 10% FBS (GIBCO, 10437028), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (GIBCO, 15140122). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient sedimentation. Bone marrow stromal cells (BMSCs) were isolated from BM aspirate of patients with WM and were cultured as previously described (13). Primary WM cells were separated from BM samples by antibody-mediated positive selection using anti-CD19⁺ magnetic-activated cell separation microbeads (Miltenyi Biotech, 130-050-301). A purity of 95% CD19⁺ cells was obtained. KPT-9274 was provided by Karyopharm Therapeutics Inc.; melphalan and bendamustine were purchased from Selleckchem (Selleck Chemicals, S1212).

Cell viability and apoptosis assay

Cell viability was analyzed by CellTiter-Glo (CTG; Promega, G7572). Apoptosis was evaluated by flow cytometric analysis following FITC Annexin-V (BD Biosciences, 556419), PE-Annexin-V (Biolegend, 640947), DAPI (BD Biosciences, 564907), and PI (BD Biosciences, 51-6621E) staining, according to the manufacturer's instructions. Caspase activities were evaluated by specific Caspase-Glo assays (Promega).

NAMPT activity

NAMPT activity was analyzed by NAD/NADH Assay Kit (Colorimetric; Abcam, ab65348) according to the manufacturer's instructions.

Immunohistochemistry

Normal and tumor tissue specimen sections of formalin-fixed, paraffin-embedded BM biopsies were prepared and processed for

immunohistochemistry to detect PAK4 and p-PAK4 (Ser474) protein expression by using specific antibodies (LifeSpan BioSciences, lsc-287254, and Santa Cruz Biotechnology, sc135774).

Immunoblotting

Western blotting (WB) was performed to evaluate the expression levels of total protein and phospho-specific isoforms using following antibodies: PAK4 (LifeSpan BioSciences, lsc-287254); FANCD2 (Santa Cruz Biotechnology, sc-20022), RAD51 (Santa Cruz Biotechnology, sc-398587), RAD51C (Santa Cruz Biotechnology, sc-56214), p-PAK4 (Ser 474; Santa Cruz Biotechnology, sc135774); NAMPT (Cell Signaling Technology, 86634), total and cleaved form of Caspase3 (Cell Signaling Technology, 9662), Caspase7 (Cell Signaling Technology, 9692), Caspase8 (Cell Signaling Technology, 9746), Caspase9 (Cell Signal, 9502s), PARP (Cell Signaling Technology, 9542s), Bcl-xl (Cell Signaling Technology, 2764s), BIM (Cell Signaling Technology, 2819), γ -H2AX (Ser139; Cell Signaling Technology, 9718s), p-CHK1 (Ser 345; Cell Signaling Technology, 2341), p-BAD (Ser112; Cell Signaling Technology, 5284s), p-P53(Ser15; Cell Signaling Technology, 9284s). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, 2118), Histone3 (Cell Signaling Technology, 4499), Vinculin (Santa Cruz Biotechnology, sc25336), and α -Tubulin (Santa Cruz Biotechnology, sc8035) were used as loading control.

PAK4 knockdown

Human GIPZ PAK4 shRNA vectors were purchased from Dharmacon (Lafayette). Scramble: non-silencing Lentiviral shRNA control (Catalog #RHS4346), shRNA: 396# V3LHS_646396, 682# V2LHS_197682, 934# V3LHS_643934, 937# V3LHS_643937.

RT2 Profiler PCR Array

Human DNA Damage Signaling Pathway RT² Profiler PCR Array (PAHS-029Z, Qiagen) was used to evaluate the impact of KPT-9274 on mRNA levels of 84 related genes. Relative expression was calculated using the comparative $\delta\delta(C_t)$ method, using untreated cells as control. Fold-change [$2^{(-\delta\delta C_t)}$] is the normalized gene expression [$2^{(-\delta C_t)}$] in the treated cells divided the normalized gene expression [$2^{(-\delta C_t)}$] in the control cells. Fold-change values greater than 1 indicate a positive or an upregulation; fold-change values less than 1 indicate a negative or downregulation.

HR activity

In vitro HR assay was carried out using FRET-based DNA strand exchange assay (14). Briefly, the BCWM1 cells were treated with different combinations of drugs. After 24 hours, cells were harvested and lysed in chaps buffer. Cell lysate (20,000 cells) was incubated for 45 minutes with homologous ssDNA (Oligo 25; 48-mer; denoted as "Homologous DNA") to form the nucleoprotein filament. Then, fluorescently labeled dsDNA (Oligo 25-FLU and 26-BHQ1) was added to the filament to initiate DNA strand exchange. After 30 minutes of incubation, fluorescence intensity was measured.

Murine xenograft model of human WM

CB17-SCID mice were purchased from Charles River Laboratories. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana-Farber

Cancer Institute. Mice were irradiated (200 cGy) and then inoculated subcutaneously in the right flank with 10×10^6 BCMW-1 cells in 100- μ L RPMI-1640. Following detection of tumor (~3 weeks after the injection), mice were treated intraperitoneally with: KPT-9274 (100 mg/kg body weight) daily for 5 consecutive d/wk for 3 weeks; or bendamustine (25 mg/kg) 1 single dose/wk for 2 consecutive weeks; or the combination with the same dosing regimen used for the individual agents. The control group received the vehicle at the same schedule as the combination group. Tumor volume was evaluated in 2 dimensions by caliper measurements performed approximately twice a week using the following formula: $V = 0.5a \times b^2$, where "a" and "b" are the long and short diameter of the tumor, respectively.

In situ detection of apoptosis and proliferation

Mice tumor sections were subjected to immunohistochemical (IHC) staining for hematoxylin and eosin (H&E) and caspase-3 activation to detect apoptotic cell death.

Statistical analysis

All values are expressed as mean plus or minus the standard deviation (SD). The statistical significance of differences between treatments was analyzed using the Student *t* test using GraphPad Prism analysis software; differences were considered significant when *P* was less than or equal to 0.05. Drug interactions were assessed by CalcuSyn 2.0 software (Biosoft), which is based on the Chou–Talalay method. Combination index (CI) = 1, indicates additive effect; CI < 1 indicates synergism; CI > 1 indicates antagonism.

Results

PAK4 is expressed in WM cells and its targeting by dual PAK4-NAMPT inhibitor significantly impacts WM cell viability

We have analyzed and observed high expression of both PAK4 and phosphorylated (p)-PAK4 Ser474 in primary cells from patients with WM and WM cell lines, with significantly lower expression in normal B lymphocytes (CD19⁺ cells) and CD19[−] cells isolated from four normal donors (Fig. 1A and B and Supplementary Fig. S1A). Undetectable levels of PAK4 were also previously observed in BM specimens from healthy donors (9). Moreover, expression of PAK4 has been confirmed by RNA-seq in a large cohort of patients with WM (Supplementary Fig. S1B). Genetic depletion of PAK4 via stable lentiviral knockdown decreased tumor cell survival proportional to the reduction in PAK4 levels in the BCMW1 cell line (Fig. 1C); this effect was accompanied by induction of apoptosis (Fig. 1D).

We next evaluated the effect of pharmacological inhibition of PAK4 using KPT-9274, a dual PAK4-NAMPT inhibitor. We observed a significant dose- and time-dependent decrease of WM cell viability (Fig. 2A); and suppression of WM-BMSC interaction-mediated growth of WM cells (Supplementary Fig. S2A) after treatment with KPT-9274, whereas no effect was observed on BMSCs (data not shown). Moreover, PAK4-related biological processes such as migration, invasion and adhesion to BMSCs were negatively affected by the inhibitor (Supplementary Fig. S2B and data not shown). The inhibition of cell viability observed after treatment with KPT-9274 was associated with a dose- and time-dependent induction of apoptosis (Fig. 2B) via activation of

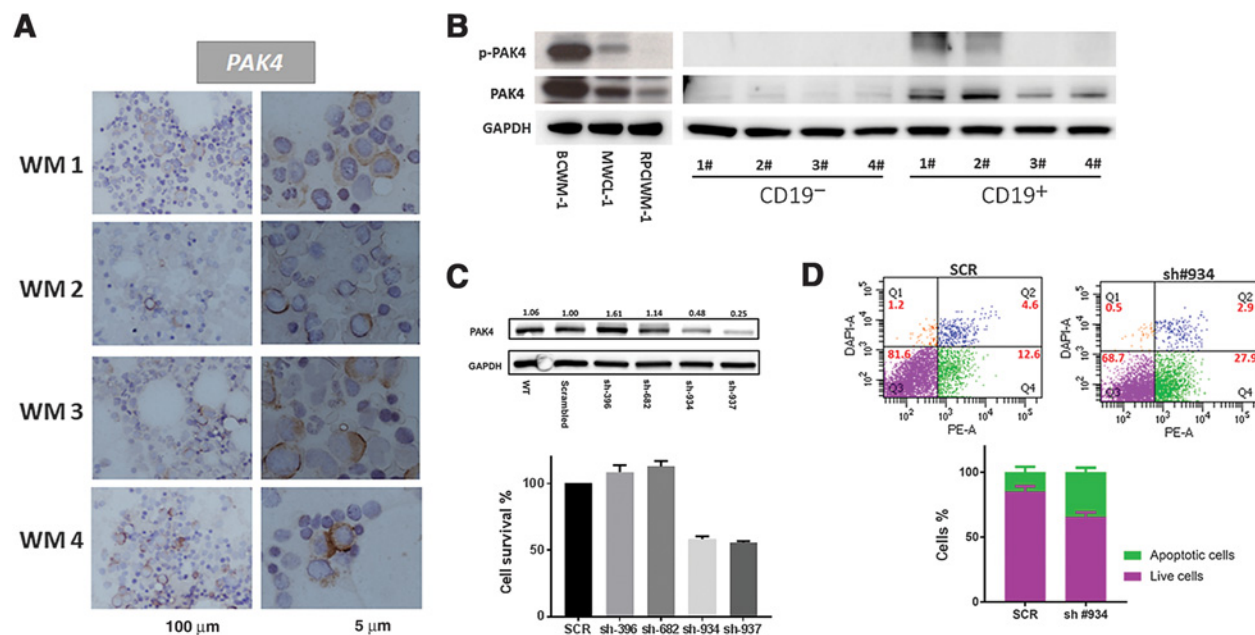
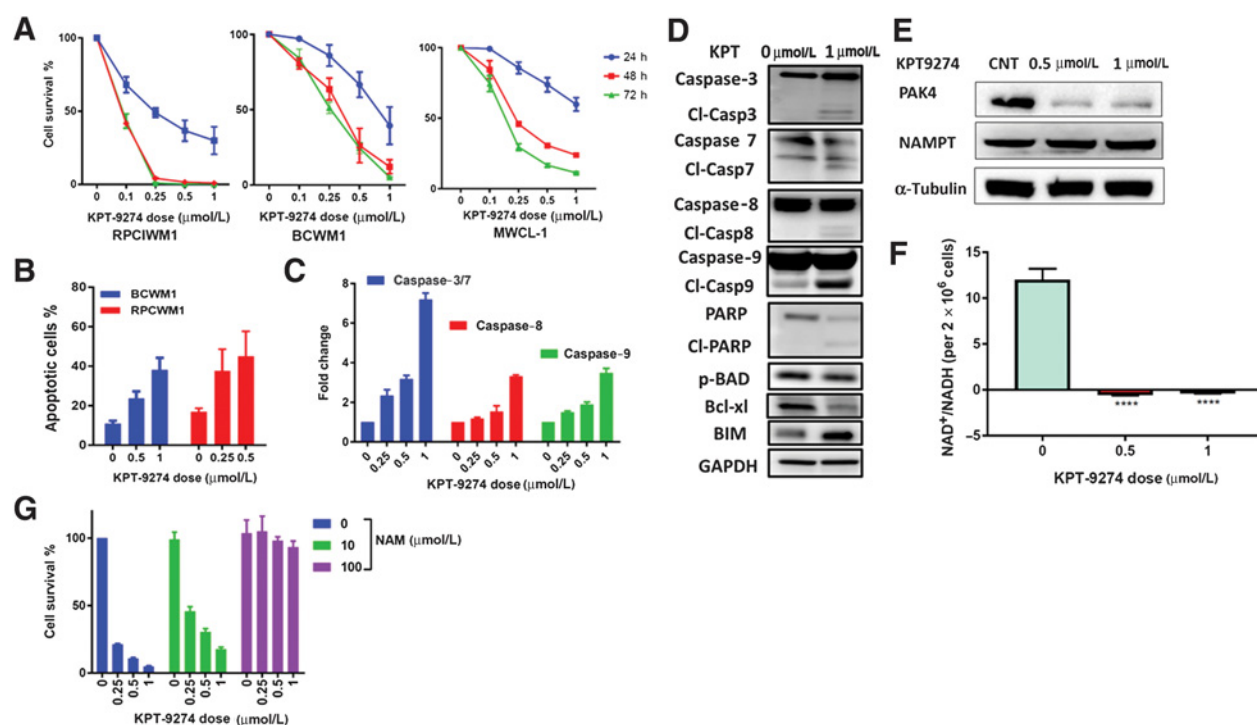


Figure 1.

PAK4 is expressed and functional in WM cells. **A**, Representative images of PAK4 immunocytochemistry stain in BM from 4 different patients with WM. Positive staining appears in a dark brown color; scale bars, 100 and 5 μ m. **B**, Protein lysates from 3 WM cell lines, CD19⁺ and CD19[−] cells isolated from healthy individuals were analyzed for p-PAK4 and PAK4 expression by WB. GAPDH was used as loading control. **C**, Genetic depletion of PAK4 was achieved using 4 different pGIPZ lentiviral vectors (Dharmacon) containing the target sequence or scrambled control. Cells were collected 3 days after puromycin selection. Protein lysates were analyzed by WB, confirming decreased PAK4 protein levels in cells expressing PAK4 shRNAs compared with scrambled cells. Cell viability was evaluated by CellTiter-Glo Luminescent Cell Assay on puromycin-selected shRNA-expressing cells in culture for 48 hours. **D**, Apoptotic cell death was assessed by flow cytometric analysis following DAPI and PE-AnnexinV staining in cells expressing scramble or PAK4-targeting shRNA (construct #934).

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**Figure 2.**

KPT-9274 inhibits WM cell viability and triggers apoptotic cell death. **A**, WM cell lines were treated with different concentrations of KPT-9274. Cells viability was evaluated with CellTiter-Glo at indicated time (24–48–72 hours) and expressed as the percentage of change from untreated cells. Data represent mean $\pm$ SD of five experiments performed in triplicates. **B**, BCWM1 and RPCIWM1 were cultured in the absence or presence of several concentrations of KPT-9274 for 48 hours. Apoptotic cell death was assessed by flow cytometry analysis following Annexin-V and propidium iodide (PI) staining. The percentage of AnnexinV⁺ cells is shown in the graphs. **C**, Indicated caspase activities were evaluated in BCWM1 cells after KPT-9274 treatment for 48 hours using luminescence assay. **D**, Whole-cell lysates from BCWM1 cells treated with control vehicle or KPT-9274 for 48 hours were subjected to Western blot analysis and probed with antibodies against indicated proteins, with GAPDH as loading control. **E**, BCWM1 cell were treated with different doses of KPT-9274 for 48 hours. Protein lysates were assessed by WB analysis for expression of PAK4 and NAMPT using specific Abs. α -Tubulin was used as loading control. **F**, Cellular NAD⁺ levels in BCWM1 treated with KPT-9274 were measured using an enzyme cyclic assay and normalized to total cell number. **G**, BCWM1 cells were treated with KPT9274 (1 μ mol/L) in absence or presence of NAM (10 and 100 μ mol/L) for 48 hours. Cell viability was tested with CellTiter-Glo Luminescent Cell Assay and expressed as the percentage of change from untreated cells. ****, $P = 0.0001$.

effector caspases, mainly through the intrinsic apoptotic pathway and PARP cleavage (Fig. 2C and D). Moreover, increased BIM levels and BCL-2 antagonist of cell death (BAD) activation, via inhibition of serine 112 phosphorylation, as well as decreased Bcl-xL levels were observed in BCWM1 cells treated with the inhibitor compared with control cells (Fig. 2D).

KPT-9274 allosterically binds to and destabilizes PAK4 causing its degradation. In addition, KPT-9274 also inhibits the activity of nicotinamide phosphorybosyltransferase (NAMPT), the rate limiting enzyme for NAD⁺ production from nicotinamide in mammalian cells. We confirmed that treatment with KPT-9274 significantly decreases PAK4 expression in WM cells, and while not affecting NAMPT protein levels, has a profound effect on cellular NAMPT activity (Fig. 2E and F). Moreover, NAMPT expression was not affected by PAK4 knockdown in BCWM1 cells (Supplementary Fig. S1C); however, we observed a slight (but not significant) reduction of NAMPT activity in PAK4 depleted cells as compared with control cells (data not shown).

The effect of KPT-9274 treatment on NAMPT inhibition can be rescued by repletion of NAD⁺ through biosynthesis from nicotinamide (NAM); consistently, we found that exogenous NAM rescued KPT-9274-induced WM cell death in a dose-dependent

manner (Fig. 2G), further confirming the importance of NAD⁺ depletion in the antitumor effect of KPT-9274 on WM cells.

Aside NAMPT, alternative NAD⁺ sources available to tumors have been reported, including nicotinate phosphorybosyltransferase (NAPRT), which mediates NAD⁺ production from nicotinic acid (NA). We have evaluated the expression of NAPRT1 in BCWM1 cells and observed no significant impact after treatment with KPT-9274 (Supplementary Fig. S2C); importantly, KPT-9274-mediated inhibition of WM cell growth was largely abolished by supplementation with NA (Supplementary Fig. S2D).

Dual PAK4-NAMPT inhibition exerts a potent activity against primary WM cells *in vitro* and *in vivo* in a xenograft model of human WM

Primary WM cells were obtained from BM aspirates of patients with WM following CD19-positive selection. Treatment with KPT-9274 significantly inhibited cell viability and triggered apoptosis in primary WM cells from both newly diagnosed as well as relapsed patients, in a time- and dose-dependent manner (Fig. 3A and B and Supplementary Fig. S2E). Sensitivity to KPT-9274 was also observed in primary cells resistant to the BTK inhibitor Ibrutinib (Fig. 3C), and in the context of the

patient-derived BM microenvironment (data not shown). Finally, we investigated the anti-WM effect of KPT-9274 *in vivo* in a murine xenograft model of human WM using BCWM1 in SCID mice. Following detection of tumors, mice were treated with either 100 mg/kg KPT-9274 or vehicle orally 5 d/wk for 3 weeks. As shown in Fig. 3D, treatment with KPT-9274, compared with vehicle alone, significantly inhibited WM cell tumor growth. No related toxicity was observed in mice, as determined by daily evaluation of activity and overall body weight change during the course of treatment (data not shown). Histologic examination of tumors retrieved from BCWM1-bearing mice confirmed significant tumor cell apoptosis (caspase-3 staining; Fig. 3E). Moreover, WB analysis of protein lysates from retrieved tumor cells confirmed significant induction of caspase-7 as well as inhibition of p-BAD and decreased PAK4 expression (Fig. 3F).

KPT-9274 impairs HR and induces DNA damage in WM cells

Our reanalysis of transcriptomic data in MM cells following KPT-9274 treatment (Gene Expression Omnibus accession number GSE93745) revealed a significant enrichment of DNA damage response and repair (DDR) genes in KPT-9274-treated cells compared with untreated cells (Supplementary Fig. S3A). We have therefore used a targeted human PCR array to elucidate the impact of KPT-9274 on the transcriptional regulation

of DNA damage and repair genes in WM cells. We observed repression of DNA damage and repair genes in WM cell lines treated with KPT-9274 compared with control cells (Fig. 4A). Specifically, we observed significant inhibition of Fanconi Anemia (FA)/BRCA pathway-related genes (FANCD2 and BRCA1 among others) as well as DDR genes such as RAD51. These observations were also confirmed by qPCR (Supplementary Fig. S3B) and at the post-transcriptional level by WB analysis, as observed for RAD51 and other components of the homologous recombination (HR) repair machinery (Fig. 4B). This effect was accompanied by inefficient HR-mediated repair activity (Fig. 4C), along with induction of DNA damage (assessed by γ H2AX) and p53 activation in both KPT-9274-treated (Fig. 4D). These observations correlate with the described activity of NAD on DNA damage and repair (10, 15, 16). Consistent with these findings, we also observed that nicotinamide and nicotinic acid supplementation partly rescued the effect of KPT-9274 on DNA damage and repair (Fig. 4C–E and Supplementary Fig. S3C–S3D).

Dual PAK4-NAMPT inhibition potentiates sensitivity to bendamustine via synergistic modulation of DDR markers

Fanconi anemia and DDR pathways have been implicated in resistance to DNA-alkylating agents (17–20). Because

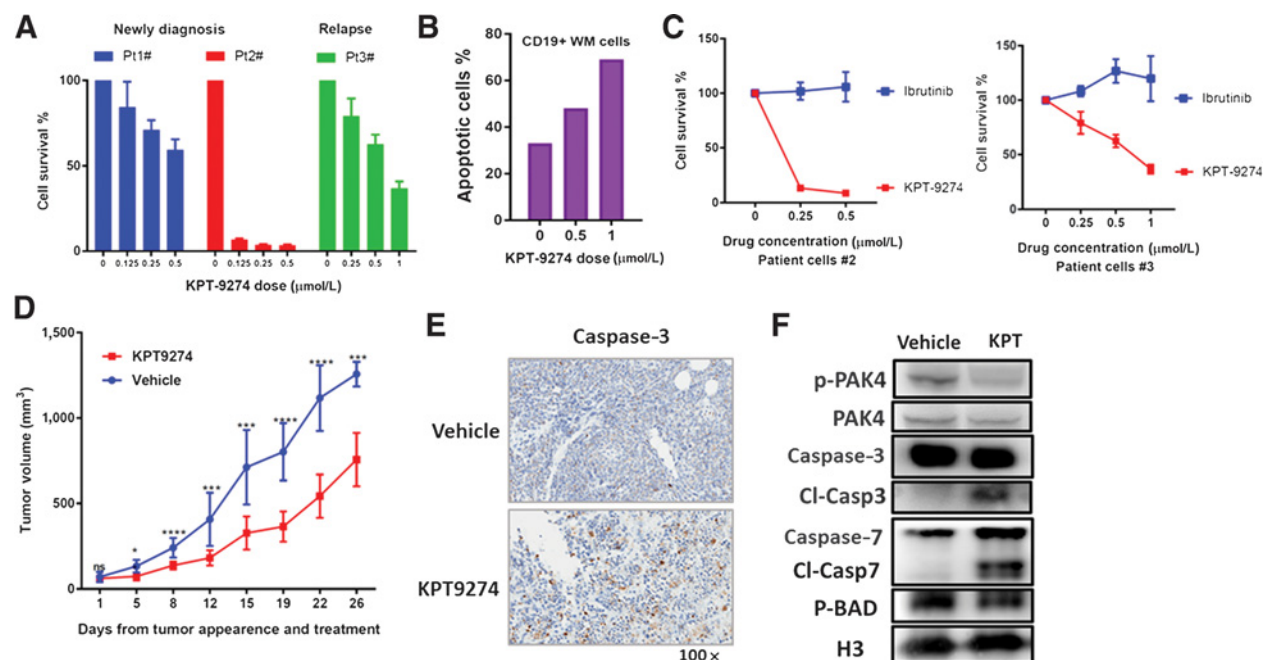
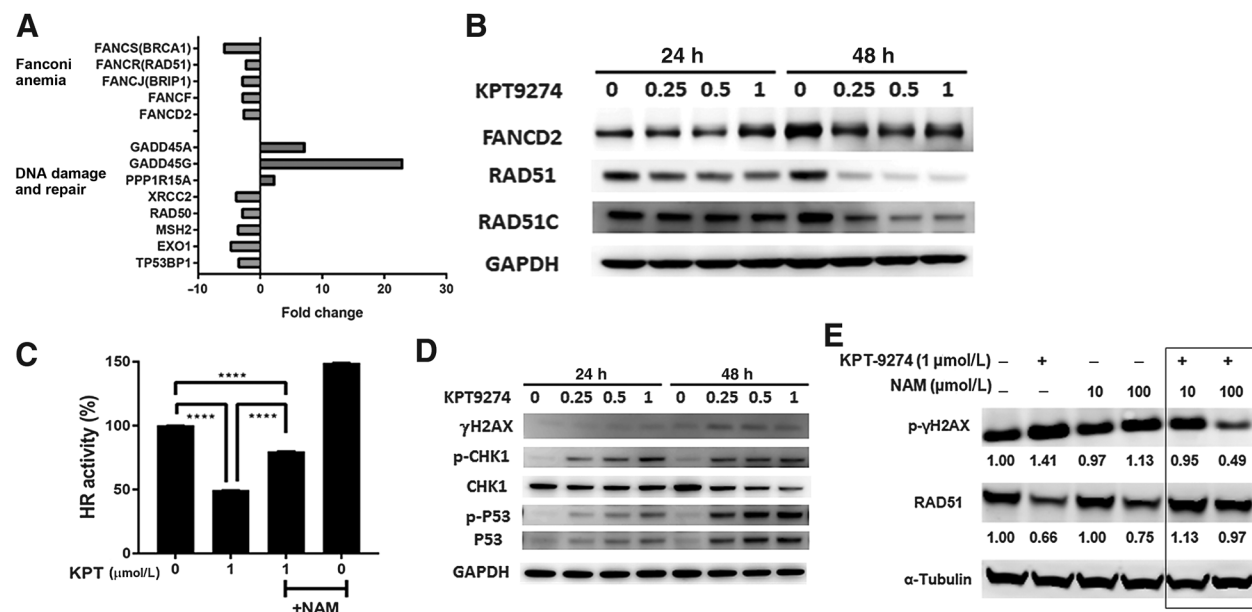


Figure 3.

KPT-9274 impairs cell viability and promotes apoptosis in primary WM cells and displays significant activity *in vivo* in a xenograft murine model of WM. **A**, CD19⁺ WM cells purified from 3 patients with WM, were cultured in the presence of different concentrations of KPT-9274 for 48 hours. Cell viability was assessed by CTG and expressed as the percentage of change from untreated cells. **B**, Apoptosis was evaluated in primary WM cells after 48 hours treatment with KPT-9274 by flow cytometry analysis following Annexin-V and propidium iodide (PI) staining. The percentage of AnnexinV⁺ cells is shown in the graphs. **C**, CD19⁺ primary WM cells were treated with different concentration of either KPT-9274 or Ibrutinib. Cell viability was assessed by CTG after 48 hours of treatment. **D**, Sublethally irradiated SCID mice were injected subcutaneously with BCWM-1 cells. Following detection of tumor, mice were randomized and treated with either KPT-9274 or vehicle orally, for 5 consecutive d/wk for 3 weeks. Tumor volume was evaluated by caliper measurement. Differences between the 2 groups were evaluated using standard *t* test. (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). **E**, Tumors were isolated from KPT-9274-treated and control mice and sections were evaluated by histological examinations following caspase3 staining. One representative experiment is shown. **F**, Tumor cells retrieved from mice were lysed in RIPA buffer and whole-cell lysate subjected to WB analysis and probed with antibodies against p-PAK4, PAK4, caspase-7, and p-BAD. Histone 3 was used as loading control.

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**Figure 4.**

KPT-9274 affects DDR pathways in WM cells. **A**, Total RNA from control or KPT-9274-treated BCWM1 cells was evaluated by Human DNA Damage Signaling Pathway RT² Profiler PCR Array to profile the expression of 84 genes involved in DNA damage signaling pathways. The relative expression level compared with control cells for each gene is plotted relative in the graph. **B–D**, Whole-cell lysates from BCWM-1 cells treated with several concentrations of KPT-9274 for 24 and 48 hours were subjected to WB analysis and probed with indicated antibodies. GAPDH was used as loading control. **C**, Relative HR activity of BCWM-1 cells treated with KPT-9274 in absence or presence of nicotinamide (NAM, 100 μmol/L). **E**, Whole cell lysates from BCWM-1 treated with KPT9274 (1 μmol/L) in absence and presence nicotinamide (10 and 100 μmol/L) for 48 hours, were subjected to WB analysis and probed with indicated antibodies. ****, $P = 0.0001$.

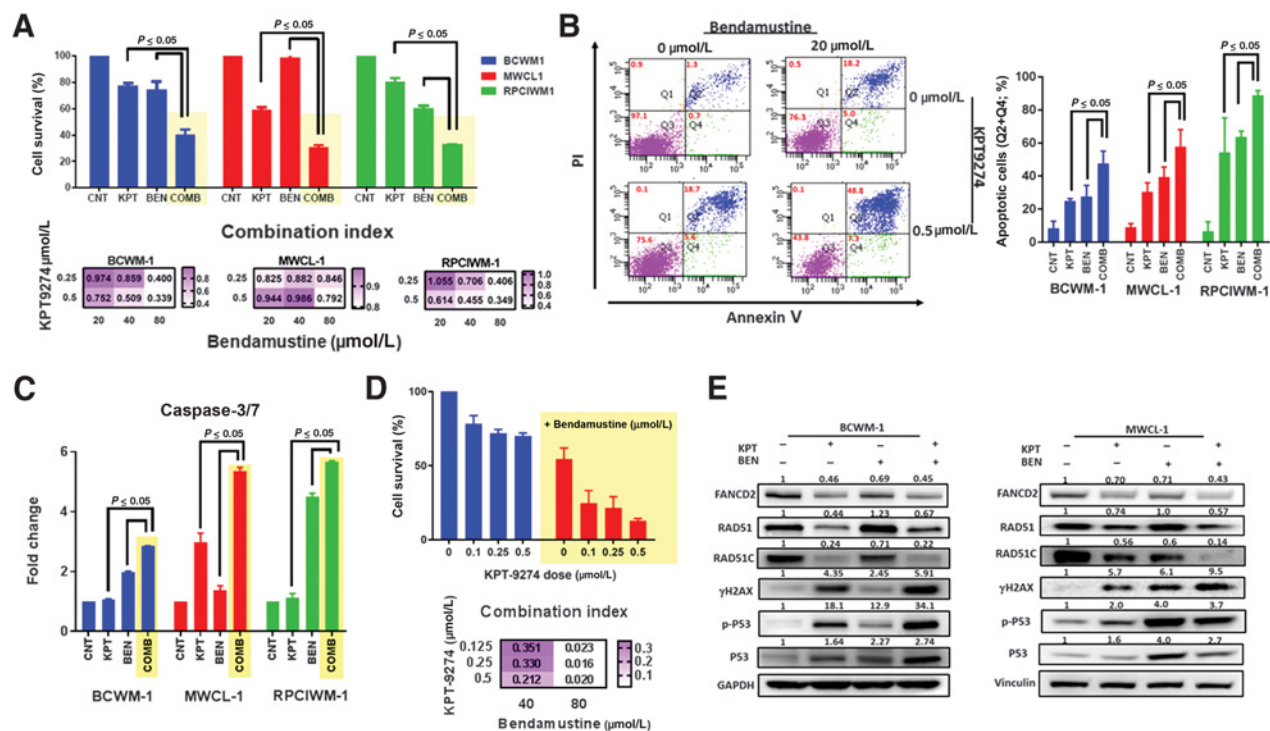
bendamustine is currently one of the main drugs used in patients with WM (21–23), we have explored the potential of KPT-9274 to impact sensitivity of WM cells to bendamustine. WM cells were therefore simultaneously treated with different concentration of KPT-9274 and bendamustine. The combination treatment resulted in significant time- (data not shown) and dose-dependent inhibition of cell viability (Fig. 5A); isobologram and combination index analysis revealed strong synergism of the combination as compare to single agents, with a combination index (CI) < 1.0 at all tested doses (Fig. 5A and Supplementary Fig. S4A). The synergistic inhibition of WM viability was accompanied by significant induction of apoptotic cell death (Fig. 5B) and activation of caspase-3/7 (Fig. 5C) in combined versus single-agent therapy; and the effect was partially rescued by NA supplementation (Supplementary Fig. S4B). Importantly, neither single agent nor the combination triggered death of healthy donor PBMCs, suggesting a favorable therapeutic index (Supplementary Fig. S4C). Interestingly, in PAK4-depleted WM cells, treatment with bendamustine significantly increased the rate of early and late apoptosis compared with scrambled control cells (Supplementary Fig. S4D). Importantly, a potent synergistic anti-WM activity of KPT-9274 in combination with bendamustine was observed in primary WM patient cells, with a CI < 1.0 with all tested doses (Fig. 5D). Finally, DNA damage markers were significantly affected by the combination regimen when compared with either drug alone (Fig. 5E); the effect was partially rescued by NA supplementation (Supplementary Fig. S4E). A similar synergism was observed in WM cells treated with KPT-9274 in combination with the DNA-damaging agent melphalan (Supplementary Fig. S5).

KPT-9274 and bendamustine synergize to suppress human WM cell growth *in vivo*

Having shown that combined KPT-9274 plus bendamustine induced synergistic apoptosis in WM cells *in vitro*, we next examined the *in vivo* efficacy of the combination in a human xenograft mouse model using BCMW.1 cells injected subcutaneously in SCID mice. After tumor development, mice were treated with vehicle, KPT-9274 (100 mg/kg, daily for 5 days a week, administered orally), bendamustine (25 mg/kg, 1 injection intraperitoneally/week for 2 weeks) as single agents or in combination. As seen in Fig. 6A and B, the combination of bendamustine with KPT-9274 induced a significant reduction in tumor growth compared with mice receiving vehicle or either single agents. The combination did not have increased toxicity (data not shown). Furthermore, WB analysis of cell lysates from retrieved tumors confirmed the effect of the combination on the DDR pathway (Fig. 6C).

Discussion

We have recently shown a significant biological and functional role of PAK4, and the therapeutic value of a dual PAK4-NAMPT inhibitor (KPT-9274) in MM. On the basis of the promising findings in MM and other malignancies, including PDAC, Renal Cell Carcinoma (RCC), triple-negative breast cancer, and B-cell acute lymphoblastic leukemia (B-ALL; refs. 9, 24–28), we here characterize and report a significant antitumor effect of KPT-9274 in WM, including ibrutinib-resistant primary patients WM cells. In this study, we also confirmed the dual inhibition properties of KPT-9274, via its impact on both PAK4 protein expression and

**Figure 5.**

KPT-9274 induces synergistic growth inhibitory effects in combination with bendamustine in WM cells *in vitro*. **A**, WM cell lines were simultaneously treated with KPT-9274 and/or bendamustine for 48 hours. Cells viability was tested with CellTiter-Glo and expressed as the percentage of cell viability from untreated cells. Data represent mean $\pm$ SD of three experiments performed in triplicates (top). The interaction between KPT-9274 and bendamustine was analyzed using the CalcuSyn software program (Biosoft) based on the Chou-Talalay method. The Combination index (CI) is represented as heatmaps (bottom). CI = 1 indicates additive effects; CI < 1 indicates synergism and CI > 1 indicates antagonism. **B**, Apoptotic cell death was assessed by flow cytometric analysis following AnnexinV and PI staining in single treated with single agents or combination. One representative experiment is shown on the left panel, whereas the percentage of AnnexinV⁺ in different WM cell lines following treatment is shown on the right. Data represent mean $\pm$ SD of two experiments. **C**, Caspase 3/7 activation was evaluated after combination treatment using luminescence assay. **D**, Primary CD19⁺ cells were cultured in the presence of several concentrations of KPT-9274 in combination with bendamustine for 48 hours. Cell viability was assessed by CTK uptake and presented as the percentage of compared with control cells (bendamustine 40 μmol/L) (top). Drug interactions were assessed by CalcuSyn 2.0 software (Biosoft) and CIs are represented in the heatmap (bottom). **E**, Whole-cell lysates from BCWM-1 (left) and MWCL1 (right) cell lines treated with control, or single agents or combination were subjected to WB analysis and probed with indicated antibodies.

cellular NAD⁺ levels in WM cells. Co-inhibition of these targets via KPT-9274 led to synergistic anti-tumor effects through energy depletion, inhibition of proliferation, and ultimately apoptosis in WM cells. Rescue experiments with nicotinamide and nicotinic acid confirmed the importance of NAD⁺ depletion for the growth inhibitory effect exerted by KPT-9274 on WM cells.

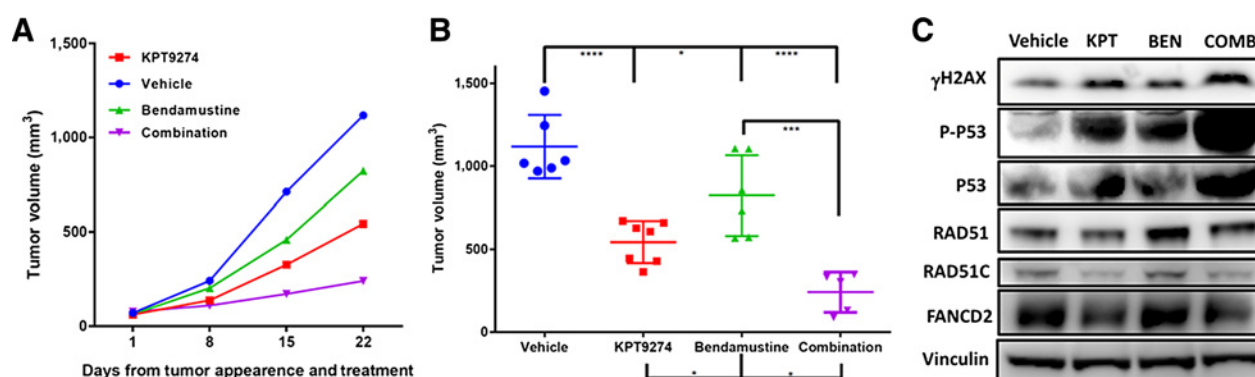
Recent studies have implicated activity of PAK family members as well as NAD⁺ in DNA damage and repair (16, 29–31). We have observed a significant induction of DNA damage along with inhibition of DNA repair in WM cells after treatment with KPT-9274. Interestingly, induction of DNA damage was also observed in PAK4 knockdown cells (data not shown). The intrinsic apoptotic pathway is a major mechanism of cell death in response to DNA damage, and the p53 tumor suppressor is the primary regulator of apoptosis in response to damage (32, 33). We have observed a significant increase in the phosphorylation of Chk1, leading to the activation of p53 in cells treated with KPT-9274. At the same time, KPT-9274 significantly affected expression of genes involved in the FA/BRCA as well as HR-mediated repair sustaining the induction of DNA damage. In line with the hypothesis that defective NAD⁺ synthesis is responsible for the increased DNA damage observed upon KPT-9274 treatment in WM cells, we

found that exogenously added NAD⁺ reduced DNA damage in WM-treated cells, and partly rescued the inhibition of HR.

FA/BRCA and DDR pathways have been shown to affect sensitivity to alkylating agents (17–20), and our mechanistic studies indeed demonstrated that dual PAK4 and NAMPT inhibition by KPT-9274 in WM cells sensitizes them to the activity of alkylating agents, such as melphalan or bendamustine. The combination treatment resulted indeed in a synergistic time- and dose-dependent inhibition of cell viability in cell lines and primary WM cells, while sparing the viability of healthy PBMC. We also observed a synergistic induction of apoptosis and caspase activation following treatment with combined versus single-agent therapies with greater induction of DNA damage. The anti-WM activity of this combination was finally confirmed *in vivo* in mice xenografted with BCWM1. We believe these results are highly significant, considering that bendamustine is an approved agent for treatment of NHL and WM (34, 21–23). Importantly, we show here for the first time the impact of targeting DDR and HR pathways in WM.

In summary, we report significant antiproliferative activity of dual PAK4-NAMPT inhibition in WM cells, with induction of apoptosis, DNA-damage response and FA/BRCA pathway

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**Figure 6.**

Combination therapy results in tumor shrinkage *in vivo* compared with single-agent therapy. **A**, Sublethally irradiated SCID mice were subcutaneously inoculated with BCWM-1 WM cell line. Treatment started following detection of tumor (approximately 2 weeks from cell injection). Mice were treated either with vehicle (100 mg/kg once/daily, 5 d/wk), KPT-9274 (100 mg/kg once/daily, 5 d/wk), bendamustine (25 mg/kg, once/weekly, for 2 weeks) and a combination of KPT-9274 and bendamustine. Tumors were measured in two perpendicular dimensions weekly. **B**, Comparison of tumor volume in control and treated mice 3 weeks after initial assessment of tumor appearance and start of treatment. **C**, Whole-cell lysate from tumor cells retrieved from mice was subjected to WB analysis and probed with antibodies against with indicated antibodies. Vinculin was used as loading control. *, $P = 0.05$; ***, $P = 0.001$; ****, $P = 0.0001$.

disruption and a strong synergistic activity in combination with DNA damaging agents such as bendamustine; suggesting KPT-9274 as a novel therapeutic strategy in WM as monotherapy or in combination with alkylating agents.

Disclosure of Potential Conflicts of Interest

S. Oliva is a consultant/advisory board member for Celgene, Janssen, Amgen, Adaptive Biotechnologies. W. Senapedis holds ownership interest (including patents) in Karyopharm Therapeutics. E. Baloglu is an employee of and holds ownership interest (including patents) in Karyopharm Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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