RESEARCH LETTER

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TO THE EDITOR:

Wnt inhibitors reduce the unfolded protein response and enhance bortezomib-induced cell death in multiple myeloma

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The Wnt pathway is one of the main regulators of cell growth and is essential for embryonic development and tissue homeostasis.[1] Dysregulation of the Wnt pathway is involved in the pathogenesis of many types of carcinomas and is associated with certain hematological cancers, including multiple myeloma (MM).[2-4] Several studies, primarily using cell lines and mouse models, reported that MM plasma cells (PCs) carry a functional Wnt/β-catenin signaling pathway that promotes cell survival.[3-7] Despite new and successful treatment options, MM remains incurable because of clinical, genetic, and transcriptomic heterogeneity, which ultimately results in therapy resistance and relapse. Thus, an unmet need exists to find new therapeutic targets and improve responses to approved drugs.

In the canonical Wnt pathway, Wnt ligands bind and activate Frizzled receptors and LRP5/6 coreceptors to recruit components of the β-catenin destruction complex.[1,3] Subsequent stabilization of nonphosphorylated β-catenin results in the transcription of Wnt target genes. Here, we targeted 2 pathway components to inhibit Wnt signaling in MM cells. Inhibition of tankyrase promotes degradation of β-catenin owing to stabilization of Axin, which is the concentration-limiting component of the β-catenin destruction complex.[8] Inhibition of porcupine, an enzyme required for the secretion of all human Wnt ligands, was shown to reduce Wnt signaling in vitro and in a mouse model.[9] We demonstrate that targeting Wnt signaling promotes cell death of patient-derived MM cells and downregulates genes involved in the unfolded protein response (UPR). Subsequently, we show that Wnt inhibitors enhance bortezomib (BTZ)-induced cell death in primary MM cells.

First, we tested the functionality of tankyrase inhibitor (TNKSi) XAV939 and porcupine inhibitor (PORCi) C59 in MM cell lines. Single-drug exposure in MM1.S blocked β-catenin upregulation by Wnt3a-conditioned medium in case of TNKSi and downregulated β-catenin expression in case of PORCi (Figure 1A). Treatment with TNKSi caused only moderate apoptosis in MM1.S and L363, ranging from 0.2% to 3.9% specific apoptosis at 10 to 40 μM TNKSi and 3.9% to 29.1% specific apoptosis at 5 to 10 μM PORCi (supplemental Figure 1A). The inhibitor combination induced more than additive cell death in MM1.S, with specific apoptosis increasing to 57.1% for 20 μM TNKSi and 10 μM PORCi (supplemental Figure 1A,B). A significant impact on the number of viable cells was observed after 7 days of treatment (supplemental Figure 1C). We also demonstrated a significant reduction in transcriptional Wnt reporter activity after treatment with both inhibitors (Figure 1B). In addition to MM1.S and L363, the combination of Wnt inhibitors was effective in MM cell lines INA-6 and KMS12-PE (supplemental Figure 1D). In summary, these data show that the Wnt pathway can be inhibited by a combination of TNKSi and PORCi and affects cell survival in MM cell lines.

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The scRNAseq data has been deposited into the Gene Expression Omnibus database (accession number GSE219133).

Data are available on request from the corresponding author, Victor Peperzak (v.peperzak@umcutrecht.nl).

The full-text version of this article contains a data supplement.

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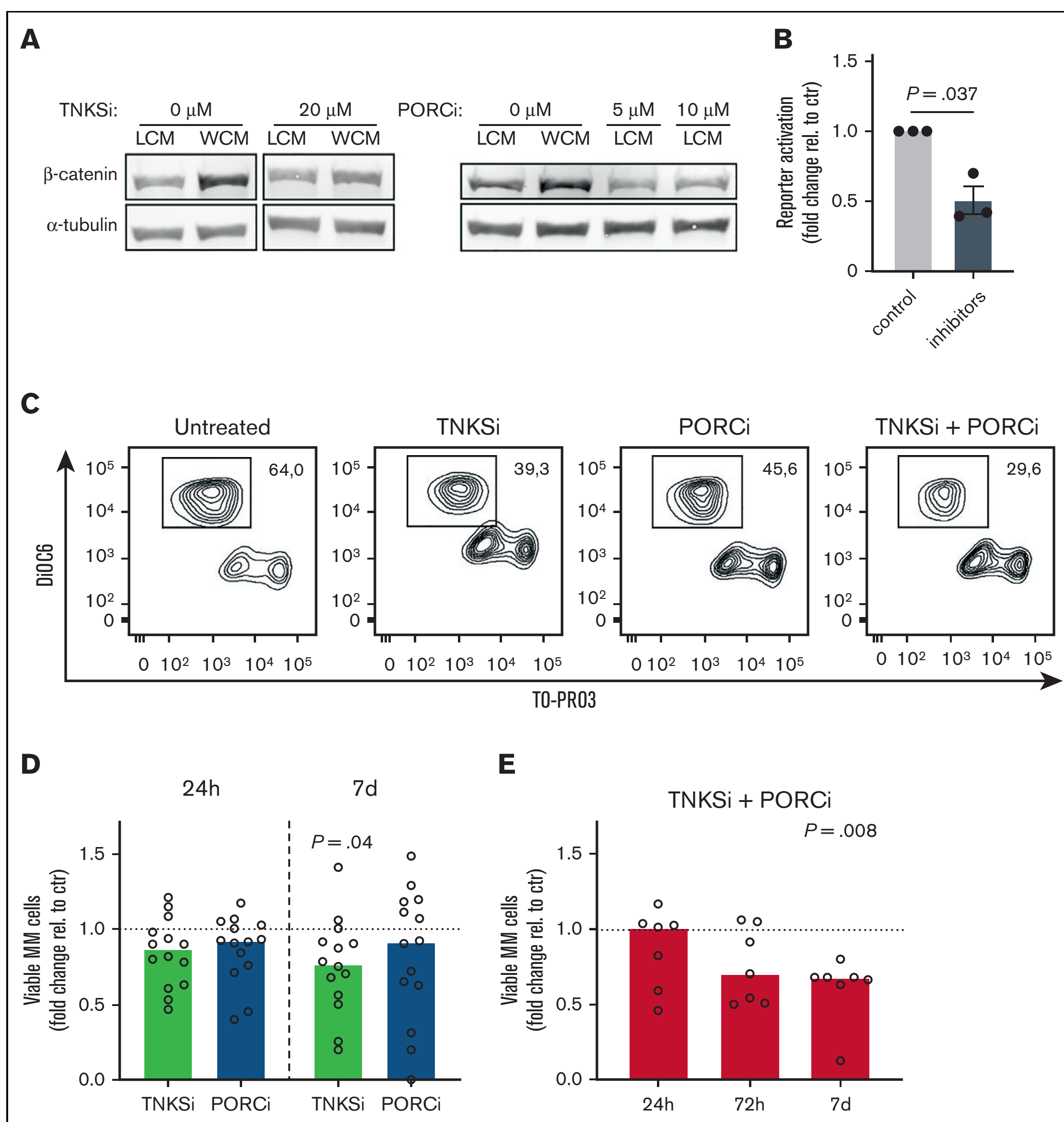

Figure 1. Inhibition of tankyrase and porcupine downregulates Wnt signaling in MM1.S and promotes cell death of primary MM cells. (A) Representative western blots showing β-catenin protein expression in MM cell line MM1.S after 24 hours exposure to indicated concentrations of tankyrase inhibitor (TNKSi) XAV939 or porcupine inhibitor (PORCi) C59, in the presence of 50% Wnt3a-conditioned medium (WCM) or 50% L-cell control medium (LCM). Alpha-tubulin was used as a loading control. (B) Transcriptional Wnt reporter activity as determined by TopFlash after 24 hours of exposure to the combination of 20 µM TSNKi and 5 µM PORCi in MM1.S. Data is normalized to internal Renilla and FOP negative control, and shown as fold change to untreated control. Bars show the mean of 3 individual experiments, error bars represent the standard error of the mean and statistical significance was determined by unpaired *t*-test. (C) Representative flow cytometry analysis plots of viability in $CD38^+$ cells of a primary MM sample after 72 hours of exposure to 20 µM TNKSi, 5 µM PORCi, and the combination thereof, or untreated control cells, in the presence of IL-6 and APRIL. Gates represent viable ($DiOC6^+$/$TOPRO3^-$) cells. (D) Absolute number of viable $CD38^+$ primary MM cells, represented as fold change relative to untreated control per time point, after 24 hours or 7 days of exposure to 20 µM TNKSi or 5 µM PORCi, in the presence of IL-6 and APRIL. Viable cells were identified as $DiOC6^+$/$TOPRO3^-$ and cell counts were determined using flow cytometry beads. Bars indicate the mean of 14 included MM samples (MM1-MM14); dots represent individual MM samples. The dashed line represents the untreated control per time point. Statistical significance was determined by one-way analysis of variance (ANOVA) using Dunnett correction for multiple testing. (E) Absolute viable $CD38^+$ primary MM cells, represented as fold change relative to untreated control per time point, after 24 hours, 72 hours and 7 days of exposure to the combination of 20 µM TNKSi and 5 µM PORCi, in the presence of IL-6 and APRIL. Viable cells were identified as $DiOC6^+$/$TOPRO3^-$ and cell counts were determined using flow cytometry beads. Bars indicate the mean of the 7 included MM samples (MM7, MM9-MM14); dots represent individual MM samples. The dashed line represents the untreated control per time point. Statistical significance was determined by one-way ANOVA using Dunnett correction for multiple testing.

Next, PCs from patients with newly diagnosed MM were studied for response to Wnt signaling inhibitors (supplemental Table 1). All primary samples were obtained after written informed consent, and protocols were approved by the local ethics committee of the Utrecht University Medical Center and contributing partners of the Dutch Parelsnoer Project. Patient-derived MM cells were

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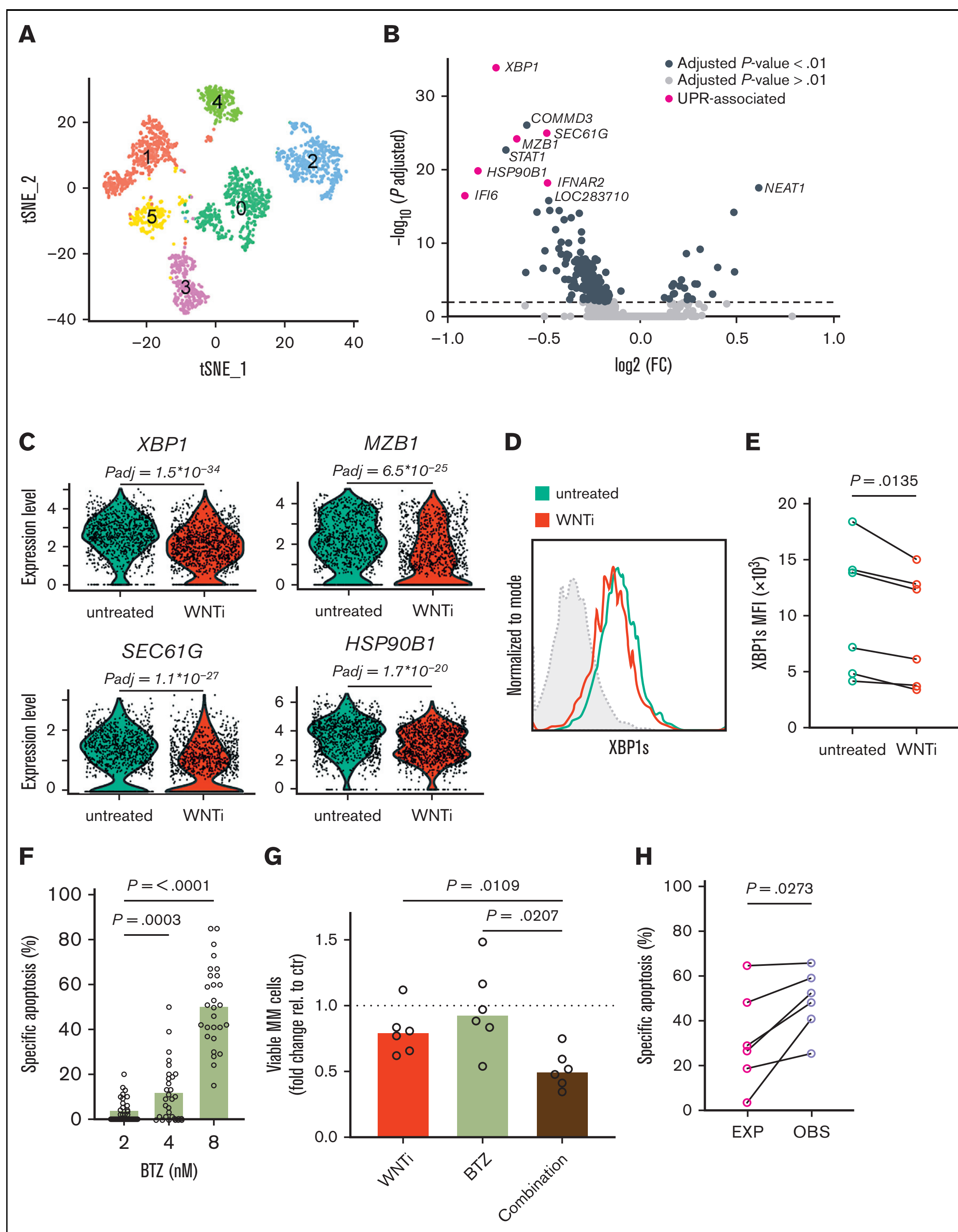

Figure 2. Targeting Wnt signaling downregulates expression of genes involved in the UPR and enhances BTZ-induced cell death in primary MM cells. Transcriptome analysis by single-cell RNA sequencing of viable CD38+CD138+ primary MM cells of samples MM9-MM13 after 72 hours of exposure to a combination of 20 μM tankyrase inhibitor XAV939 and 5 μM porcupine inhibitor C59, or untreated control cells, in the presence of IL-6 and APRIL. (A) Dimensionality reduction and clustering of transcriptome analysis by tSNE, and cluster distribution of the individual MM samples. The 6 clusters are denoted by digits 0 to 5 and corresponding colors. (B) Volcano plots of genes differentially expressed between MM cells exposed to the inhibitor combination vs control. Dark gray data points indicate significant differentially expressed (DE) genes with an adjusted *P* value of <.01; light gray data points indicate nonsignificant DE genes with an adjusted *P* value of >.01. The top 15 DE genes have been annotated with their corresponding gene name, whereas the genes within the top 15 that are functionally related to the UPR pathway have been colored red. (C) Violin plots showing expression of several important mediators of the UPR pathway: *XBP1*, *MZB1*, *SEC61G*, and *HSP90B1*, in MM cells exposed to control of the inhibitor combination. Individual data points

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cultured in the presence of growth factors IL-6 and APRIL, which were shown to benefit survival of PCs ex vivo.[10]

Exposure to either TNKSi or PORCi in patient-derived MM did not significantly affect the number of viable PCs after 24 hours. TNKSi did significantly reduce the mean number of viable MM cells compared with the untreated control after 7 days (Figure 1C,D). Next, we tested the inhibitor combination on PC survival. There was no significant effect after 24 or 72 hours of incubation, but after 7 days, the mean number of viable MM cells was significantly reduced compared with the untreated control (Figure 1E). Similar to the MM cell lines, there was a high variability in response to the inhibitors between individual samples, especially when given as single drugs. We were unable to correlate genetic aberrations with sensitivity to the inhibitors, both in cell lines and primary samples. We also analyzed a publicly available cohort (depmap.org) of 14 MM cell lines with known genetic backgrounds that were treated with PORCi C59 or IWP-2. There were no significant associations between C59 or IWP-2 sensitivity and the expression of MM differentiation and survival genes in these cell lines.

To elucidate the mechanism underlying this combinatorial effect, PC from samples MM09-MM13 (supplemental Table 1) were subjected to single-cell RNA sequencing after 72 hours of exposure to the Wnt inhibitor combination or untreated control. Clustering of the transcriptome by tSNE analysis showed that the samples cluster individually, with sample MM12 divided over clusters 1 and 5 (Figure 2A; supplemental Figure 2). These results reflect the high degree of interpatient heterogeneity in MM. Analysis of treated and untreated cells of all samples identified 153 differentially expressed genes with an adjusted P value of $<.01$ (Figure 2B). Interestingly, most of the top hits, including *XBP1*, *MZB1*, *SEC61G*, and *HSP90B1,* were associated with the UPR, and their expression was significantly reduced in treated vs control cells (Figure 2B-C). Because 6 out of the 10 most differentially expressed genes were functionally associated with the UPR, we hypothesized that inhibition of the UPR is involved in MM cell apoptosis upon combined TNKSi and PORCi treatment.[11-17] Furthermore, unbiased pathway analysis of the top 50 downregulated genes revealed the UPR as the most significantly affected pathway after treatment with TNKSi and PORCi (supplemental Figure 3). In addition, protein expression of spliced XBP1 (XBP1s) was measured to validate RNA sequencing results. Intracellular expression of XBP1s was significantly lower after treatment with both Wnt inhibitors compared with the untreated control (Figure 2D-E).

MM PCs accumulate misfolded immunoglobulin subunits and therefore rely on a functional UPR response for survival.[18] Because proteasome inhibitors, such as BTZ, induce the accumulation of misfolded proteins in MM cells, we hypothesized that targeting the UPR with Wnt inhibitors could sensitize MM cells to BTZ. Combining Wnt inhibition and BTZ has previously been tested with β-catenin inhibitors, BC2059 and pyrvinium pamoate, and showed synergistic effects in MM cells.[19,20] Using a cohort of 28 patient-derived MM samples, we first determined BTZ's efficacy in vitro (Figure 2F). At a concentration of 2 nM, mean apoptosis in MM cells was 4.3%, which leaves a window to observe the combined effect of BTZ and inhibitors of Wnt signaling. Next, patient-derived samples were exposed to TNKSi and PORCi for 48 hours before adding BTZ for another 24 hours of coincubation. The mean number of viable MM cells after combination treatment was significantly lower compared with single treatment with Wnt inhibitors or BTZ (Figure 2G). The observed percentages of apoptosis by the combination treatment were significantly higher than the expected percentages based on the additive effects of single-drug treatments, indicating a potent combined effect for the combination of TNKSi and PORCi together with BTZ (Figure 2H).

Many drugs that have been approved for MM have toxicities that influence quality of life; for example, BTZ is notoriously associated with polyneuropathy. In addition, MM cells develop therapy-resistant relapses that complicate disease management. Rational and potent combination therapy is a strategy to overcome both the development of therapy resistance and reduce dose-related side effects. Inhibition of the Wnt pathway might be part of a combination therapy regimen including BTZ. Clinical trials in other malignancies demonstrated that inhibitors of Wnt signaling, including vantictumab, rosmantuzumab, ipafricept, and small molecule inhibitors LGK974, ETC-159, BC2059, and PRI-724, are tolerated well by patients, despite the pivotal role of Wnt signaling in stem cell maintenance and tissue homeostasis.[4] Importantly, the combination of Wnt inhibitors and BTZ had no significant effect on the viability of healthy-donor peripheral blood mononuclear cell subsets (supplemental Figure 4).

In conclusion, we examined the therapeutic potential of targeting the Wnt pathway in patient-derived MM cells. Our results demonstrate that TNKSi and PORCi, 2 well-established methods to block the Wnt pathway in other cell types and malignancies, promote cell death of primary MM cells. Single-cell RNA sequencing showed that inhibition of Wnt signaling is likely effectuated via the downregulation of genes involved in the UPR. Combining TNKSi and

Figure 2 (continued) represent the sequenced single cells, and the adjusted P value is annotated as P-adj, for which $<.01$ is considered statistically significant. (D) Representative example of intracellular protein expression of XBP1s measured by flow cytometry. Samples were treated with TNKSi and PORCi for 72 hours (red) or untreated as control (green). The dotted line shows a-specific background staining. (E) Comparison of intracellular XBP1s expression in 6 primary MM samples (MM13, MM18-22) treated with TNKSi and PORCi for 72 hours or untreated as control. XBP1s expression was denoted as mean fluorescence intensity (MFI), as measured by flow cytometry as indicated in (D). Statistical significance was determined with a paired *t*-test. (F) Plot showing specific apoptosis to BTZ at concentrations of 2, 4, and 8 nM in a cohort of 28 newly diagnosed MM samples. Viable cells were identified as DiOC6+/TOPRO3–. Bars indicate the mean of all included MM samples; dots represent individual MM samples. Statistical significance was determined by one-way ANOVA using Dunnett correction for multiple testing. (G) Absolute number of viable CD38+CD138+ cells, represented as fold change relative to untreated control, after 72 hours of exposure to 20 μM TNKSi and 5 μM PORCi, 24 hours exposure to 2 nM BTZ, or the combination thereof, in the presence of IL-6 and APRIL. Viable cells were identified as DiOC6+/TOPRO3- and cell counts were determined using flow cytometry beads. Bars indicate the mean of 6 included MM samples (MM4-5, MM12-13, MM16-17); dots represent individual MM samples. The dashed line represents the untreated control per time point. Statistical significance was determined by one-way ANOVA using Dunnett correction for multiple testing. (H) Plot comparing observed specific apoptosis to expected specific apoptosis. The 6 connected datapoints represent the 6 individual MM samples. Statistical significance was determined with a paired *t*-test. Specific apoptosis was calculated by comparing the number of surviving cells in treated conditions vs untreated control. OBS, observed; EXP, expected.

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PORCi with BTZ resulted in the potent killing of primary MM cells, demonstrating that inhibitors of Wnt signaling might be considered therapeutic targets in future antimyeloma combinatorial strategies.

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