Targeting Wnt signaling in acute myeloid leukemia stem cells

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Title:
Targeting Wnt signaling in acute myeloid leukemia stem cells

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Conflict of interest:
The authors declare no conflict of interest.

Contribution:
RG conceived the idea; RG and FP designed the study; FP, MK, EN performed the in vitro experiments; FP, MB and KS performed the in vivo experiments; FP, DP, PR, AD and RG analyzed and interpreted the data; RG supervised the study; FP, AD and RG drafted the manuscript.

In this work we tested if targeting Wingless and Int-1 (Wnt) signaling in acute myeloid leukemia (AML) leukemic stem cells (LSCs) by using a porcupine inhibitor (WNT974) is a good strategy to eradicate LSCs. Wnt/β-catenin is an evolutionarily conserved pathway that is involved in embryonic development and stem cells by regulating cell fate and differentiation decisions.¹ In normal conditions, the Wnt/β-catenin pathway mediates normal hematopoietic stem cells self-renewal, proliferation and differentiation and is tightly controlled.²,³ While β-catenin is highly expressed in normal hematopoietic stem cells, it is downregulated during myeloid differentiation.²,⁴ Aberrant activation of this pathway gives rise to the accumulation of β-catenin in the nucleus, and promotes the transcription of many oncogenes such as c-Myc (MYC). β-catenin is highly expressed in AML patients and overexpression of β-catenin in normal CD34⁺ hematopoietic precursors in vitro leads to myeloproliferative disorder of immature cells.⁵,⁶ Further work, identified a crucial role of the Wnt/β-catenin pathway in the development of LSCs,⁷ and different authors proposed to target the Wnt pathway in AML blasts.⁸¹¹ Thus, we hypothesize that targeting Wnt in LSCs using a novel Wnt inhibitor called WNT974, could be an efficient strategy to eradicate LSC, prevent relapse and improve overall outcomes in AML. WNT974 is an inhibitor of the enzyme porcupine, which is a membrane-bound O-acyltransferase located in the endoplasmic reticulum, and it is required for the palmitoylation of Wnt ligands. The inhibition of porcupine prevents the secretion and activity of Wnt ligands outside the cell, leading to a decrease in Wnt ligand cell surface receptor phosphorylation and a reduction in the expression of Wnt target genes.¹²,¹³ Several studies observed that WNT974 is able to target the Wnt pathway in solid tumors and the LSCs in chronic myeloid leukemia.¹⁴ Based on these assumptions, we hypothesized that Wnt inhibition by using WNT974 may block the aberrant Wnt activation in AML LSCs and prevent leukemogenesis. To verify this hypothesis, we first evaluated whether WNT974 treatment can inhibit the Wnt signaling pathway using the K562 cell line, which is the cell line used initially to report the activity of this drug in myeloid leukemias. Treatment of K562 cells with DMSO (control) or WNT974 at different concentrations (0.5, 2.5 and 10µM) for 24 and 48 hours showed a reduction of Wnt pathway targets such as ROR2, LRP6 and GSK3B protein (Figure 1A), and AXIN2 mRNA expression (Figure 1B). We also confirmed that the drug efficiently targets the Wnt pathway by using a luciferase reporter vector in HCT116, a colorectal cancer cell line that shows a constitutive activation of the Wnt pathway (Figure 1C). We also confirmed Wnt targeting by WNT974 in primary AML samples (Online Supplemental Table S1 for patient details). As shown in Figure 1D, WNT974 decreased the mRNA expression of the MYC gene, which is
considered one of the main oncogenes driven by abnormal Wnt/β-catenin signaling. We also observed a similar reduction in mRNA expression of other Wnt target genes such as CTNNB1 and GSK3B after WNT974 treatment of primary AML samples (Online Supplementary Figure S1A). Next, we wanted to investigate if the treatment was able to affect LSCs functions in primary AML patient samples. To assess whether targeting Wnt pathway affects leukemia cell self-renewal, bone marrow (BM) CD34+ selected cells from five AML patients were treated with WNT974 or controls (DMSO) and used in colony forming unit (CFU) assays. We found no significant differences in the number of colonies in primary CFUs after two weeks of culture (Figure 2A and Online Supplementary Figure S1B, left panels). Primary colonies were harvested and then replated in methylcellulose for an additional 14 days. We found significant decreases in the number of secondary colonies in WNT974-treated cells compared with cells treated with controls, suggesting that WNT974 targeting has an impact on LSC self-renewal (Figure 2A and Online Supplementary Figure S1A, right panels). We performed the same experiment using BM CD34+ cells from three adult healthy donors and from three cord bloods. As shown in Figure 2B and Online Supplementary Figure S1C, the treatment did not affect normal cells. Next, we analyzed whether WNT974 has a role in regulating LSC quiescence by using cell membrane labeling retention assays. For these experiments, CD34+ selected samples from three AML patients were stained with cell trace violet (CTV) dye, which incorporates into the cell membrane and can be detected by flow cytometry. CD34+ selected AML patient cells were isolated, labeled with CTV, and treated for three days with WNT974 and control. The number of viable (7-AAD−, quiescent (CTVhi/CD34+) cells was then determined by flow cytometry. CTVhi/CD34+ cells have been shown previously to increase leukemia initiating ability, and enhanced engraftment in NSG mice. However, in all analyzed patient samples, we did not find a significant decrease in the number of CTVhi/CD34+ cells after treatment with WNT974, compared with controls, except for patient 2 at the highest WNT974 dose (Figure 2C, p-value 0.0077 (t-test)). Next, to assess if the treatment can affect the bulk cell population, we performed Annexin-V/PI assay on the same CD34+ AML samples treated with WNT974 after 72 hours, and found only a slightly increase of the apoptosis at the higher concentration (Figure 2D). Finally, we investigated whether WNT974 could also effectively target LSCs in vivo. Therefore, to test the effects of WNT974 in vivo, we used our well characterized MycRd10/WT/Fli1Rd/WT double knock-in CN-AML mouse model. This model develops an aggressive AML with 100% penetrance and leads to death within five to eight weeks in secondary BM transplantation. First, we confirmed that WNT974 treatment has a similar effect on the primary murine AML cells in this model by conducting a CFU in vitro assay on CD117+ murine cells from BM of AML mice. We observed that the treatment was able to reduce the proliferation after the re-plating (Figure 3A) similar to the treatment in human AML cells. Next, we transplanted MycRd10/WT/Fli1Rd/WT leukemic cells (CD45.2) into lethally irradiated wild-type (WT)-BoyJ (CD45.1) mice together with whole bone marrow cells from WT-BoyJ donors (CD45.1). Two weeks after engraftment, mice were treated with WNT974 or Vehicle (control) at 5 mg/Kg twice a day by oral gavage for one week. The experiment flow chart is shown in Figure 3B. After the last dose, mice were sacrificed and BM and spleen cells were obtained. We measured the Wnt target gene MYC as a surrogate for Wnt pathway targeting in leukemic cells in vivo. We found significant downregulation of murine Myc in spleen cells that were used during the primary transplants (Online Supplementary Figure S2A). Next, we determined whether Wnt pathway knock-down by WNT974 affected leukemia engraftment in secondary BM transplantation, a key feature of stem cells. Leukemic donor cells obtained from mice spleen treated with WNT974 or vehicle were transplanted into lethally irradiated BoyJ recipients. Despite we observed a reduction of Wnt pathway target gene expression in the spleens of secondary transplanted mice (Online Supplementary Figures S2B-E), we did not observe any differences between overall survival (Figure 3C), or engraftment at 1 month (Figure 3D), after re-transplantation. In conclusion, the data shows that the WNT974 treatment was able to reduce Wnt pathway activity in leukemic cells and decreased self-renewal of primary AML LSCs in vitro. However, WNT974 treatment alone was unable to impact LSC functions in vivo. For these experiments, we have used high doses of WNT974, higher than the equivalent doses used in human clinical trials with this drug. Thus, it is unlikely that the lack of in vivo effect is due to poor pharmacokinetics or pharmacodynamics. This is supported by the fact that we observed Wnt target downregulation in leukemic cells in the treated mice. Another possibility is that other pathways or even Wnt reactivation could compensate for the initial Wnt downregulation in LSC, and this could be driven by
the microenvironment. It is likely that perhaps combination of WNT974 with other active agents in AML could lead to a sustained targeting and eradication of LSCs in AML as it was shown in CML 14.

References

Figures legend

Figure 1. Validation of WNT974 activity in cell lines
(A) Western blot assay of ROR2, LRP6 and GSK3β after WNT974 treatment or control (DMSO) for 24 and 48 hours. Densitometry analysis was conducted using ImageJ software. Band intensity is reported relative to β–Actin (ACTB). (B) AXIN2 mRNA fold change expression of K562 cells treated with WNT974 at different concentrations, or control (DMSO), for 24 hours. P values represent *<0.05, **<0.01 (t-test). (C) Luciferase assay of HCT116 cells transfected with a wild type or mutant β-catenin activity reporter vector. After 30 hours from transfection, the cells were treated with Wnt3a ligand (200ng/ml), WNT974 5µM, or combination for 24 hours. Data are normalized to the control wild type. P values represent *<0.05, **<0.01, ***<0.001 (t-test). (D) MYC mRNA relative expression of BM-CD34+ selected primary AML treated with WNT974 1uM for 24 hours (*: p<0.05, **: p<0.01 by t-test). GAPDH was used as a normalizer.

Figure 2. WNT974 treatment of primary CD34+ AML patient samples decreased self-renewal but not apoptosis or quiescence.
(A) Colony forming unit (CFU) assay of CD34+ primary AML cells treated with WNT974 drug at different concentration or vehicle (DMSO 0.1% volume). After 2 week, colonies were scored and replated. A second scoring was done after 2 weeks of replating. In the graph we reported mean and standard deviation from a technical triplicate. P values represents *<0.05, **<0.01, ***<0.001 (t-test). (B) CFU assay of CD34+ cells from bone marrow of adult healthy donors treated with WNT974 drug at different concentration or vehicle (DMSO 0.1% volume). After 2 weeks, colonies were scored and replated. A second scoring was done after 2 weeks of replating. All tests (t-test) were non-significant. (C) CellTrace Violet assay of three CD34+ selected primary AML cells treated with WNT974 at various concentrations, or vehicle (DMSO 0.1%). 7-AAD staining was conducted to select only the living cells. In the graphs are reported the different counts at day 0 (dark blue, only control), and at day 6 (3 days of incubation, plus 3 days of treatment or vehicle). All values are not significant except for patient 2 at the higher dose of WNT974, p-value 0.0077, t-test. (D) Annexin V-FITC and PI analysis by flow cytometry of CD34+ selected primary AML cells treated with WNT974 or control at different concentrations for 72 hours. Living (Annexin-, PI-), Early apoptosis (Annexin+, PI-), Late apoptosis (Annexin+, PI+) and Necrosis (Annexin-, PI+). Each analysis was conducted in technical triplicate. P values represents *<0.05, **<0.01 (t-test).

Figure 3. WNT974 activity in a primary murine model of AML.
(A) CFU assay of CD117+ m MLLPTD/WT/Flt3ITD/WT BM cells treated with WNT974 at 0.5 and 1µM or vehicle (DMSO 0.1%) for 2 weeks. Results from first scoring are shown in left panel. After scoring, the cells were replated in fresh Methocult media and scored after another 2 weeks (right panel). P-value obtained with Student’s t-test analysis: *<0.05, **<0.01 and ***<0.001. (B) Flow chart of in vivo re-transplantations experiment. (C) Overall survival of secondary transplanted mice (n=5 mice for group). (D) Evaluation of engraftment at 1 month. P values represents *<0.05 (t-test).
Supplementary Table S1.
Cytogenetics and molecular features of the primary AML samples.

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* Chronic Myeloid Leukemia in Myeloid Blast Crises
Supplementary Figures

A

Patients

CTNNB1

GSK3β

Patients

DMSO

WNT974 1μM

PT#8  PT#9  PT#10  PT#11

PT#8  PT#9  PT#10  PT#11

B

PT #7

PT #8

WNT974 (μM) 0 0.5 1 0 0.5 1 0 0.5 1

CFU

Replating

CFU

Replating

C

CD34+ Cord blood

#1

CD34+ Cord blood

#2

CD34+ Cord blood

#3

WNT974 (μM) 0 0.5 1 0 0.5 1 0 0.5 1

CFU

Replating

CFU

Replating

CFU

Replating
(A) CTNNB1 (left panel) and GSK3B (right panel) mRNA relative expression on GAPDH of BM-CD34+ selected primary AML treated with WNT974 1uM for 24 hours (*: p<0.05, **: p<0.01, ***: p<0.001, by t-test). (B) Colony forming unit (CFU) assay of BM CD34+ primary AML cells treated with WNT974 drug at different concentration or vehicle (DMSO 0.1% volume). After 2 week, colonies were scored and replated. A second scoring was done after 2 weeks of replating. In the graph we reported mean and standard deviation from a technical triplicate (**: p<0.01, ***: p<0.001 by t-test). (C) Colony forming unit (CFU) assay of CD34+ cells from cord blood, treated with WNT974 drug at different concentration or vehicle (DMSO 0.1% volume). After 2 week, colonies were scored and replated. A second scoring was done after 2 weeks of replating. In the graph we reported mean and standard deviation from a technical triplicate.
Supplementary Figure S2.

(A) Myc mRNA relative expression on Actinb of spleen and whole bone marrow (WBM) from BoyJ transplanted mice with Mll\textsuperscript{PTD/WT} / Flt3\textsuperscript{ITD/WT} murine cells and treated with 5mg/Kg/twice at day with WNT974 by oral gavage for one week before retransplantation. (****: p<0.0001 by t-test). (B) Myc mRNA relative expression on Actinb of spleen and WBM from BoyJ secondary transplanted mice with spleen from Mll\textsuperscript{PTD/WT} / Flt3\textsuperscript{ITD/WT} murine cells and treated with 5mg/Kg/twice at day with WNT974 by oral gavage for one week before secondary transplantation. Spleen and WBM were harvested when natural death occurred (**: p<0.01 by t-test). (C) Axin2 mRNA relative expression on Actinb of spleen and WBM from BoyJ secondary transplanted mice with spleen from Mll\textsuperscript{PTD/WT} / Flt3\textsuperscript{ITD/WT} murine cells and treated with 5mg/Kg/twice at day with WNT974 by oral gavage for one week before secondary transplantation. Spleen and WBM were harvested when natural death occurred (**: p<0.01 by t-test). (D) Ctnnb1 mRNA relative expression on Actinb of spleen and WBM from BoyJ secondary transplanted mice with spleen from Mll\textsuperscript{PTD/WT} / Flt3\textsuperscript{ITD/WT} murine cells and treated with 5mg/Kg/twice at day with WNT974 by oral gavage for one week before secondary transplantation. Spleen and WBM were harvested when natural death occurred (***: p<0.001 by t-test). (E) Gsk3b mRNA relative expression on Actinb of spleen and WBM from BoyJ secondary transplanted mice with spleen from Mll\textsuperscript{PTD/WT} / Flt3\textsuperscript{ITD/WT} murine cells and treated with 5mg/Kg/twice at day with WNT974 by oral gavage for one week before secondary transplantation. Spleen and WBM were harvested when natural death occurred (***: p<0.001 by t-test).