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The WNT/β-catenin signaling pathway as a potential therapeutic target in Acute Myeloid Leukemia

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The WNT/β-catenin signaling pathway as a potential therapeutic target in Acute Myeloid Leukemia

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Abstract

Acute Myeloid Leukemia (AML) is the most common acute leukemia in adults. Recent advances in therapies directed to specific molecular targets allowed a substantial improvement in the rates of remission in this disease. However, several cases of resistance have been reported, which leads to a necessity to investigate new therapeutic targets. Constitutive activation of the WNT/β-catenin pathway has been observed in samples from patients with AML, being associated with worse prognosis.

Therefore, the aim of this study was to evaluate the therapeutic potential of IWR-1, a WNT/β-catenin pathway inhibitor, in two *in vitro* models of AML with different genetic profiles.

To achieve this purpose, we used two AML models: the NB-4 and HL-60 cell lines. The expression of *AXIN2* (a specific target of IWR-1) was determined by real time PCR (qPCR). Cells were cultured in absence and presence of different concentrations of IWR-1 that ranged from 10μM to 50μM. The effect of IWR-1 on cell viability was determined using the resazurin assay. Cell death and cell cycle analysis were determined by flow cytometry, using the annexin V/propidium iodide double staining, and propidium iodide/RNase protocol, respectively. Morphological analysis was performed by optical microscopy after May-Grünwald-Giemsa staining. The data were analyzed using the Graphpad Prism software.

Both cell lines expressed the IWR-1 target, *AXIN2*. However, HL-60 cells had higher expression levels than NB-4 cells. Our results showed that IWR-1 reduces metabolic activity in a time, dose and cell line dependent manner, with IC_{50} values of 32.8μM for HL-60 cells and 23.0μM for NB-4 cells, after 48h of treatment. Apoptosis was the main mechanism of cell death activated by IWR-1 in both cell lines, which was confirmed by flow cytometry and morphological analysis. In NB-4 cells, IWR-1 also induced cell cycle arrest in G_0/G_1 phase.

The results suggest that the WNT pathway could constitute a potential therapeutic target in AML. Furthermore, the efficacy of the inhibitor IWR-1 seems not dependent on the expression levels of the target, *AXIN2*. The differences between the effects observed in both cell lines may be due to the differences between their genetic profiles.

Keywords

Acute Myeloid Leukemia, WNT Beta Catenin Signaling Pathway, IWR-1, *AXIN2*.

Resumo

A Leucemia Mielóide Aguda (LMA) é a leucemia aguda mais comum nos adultos. Os avanços recentes nas terapias dirigidas a alvos moleculares específicos permitiram uma melhoria substancial nas taxas de remissão desta doença. Contudo, foram reportados vários casos de resistência às terapias atuais, o que conduz à necessidade de investigar novos alvos terapêuticos. A ativação constitutiva da via WNT/β-catenina foi observada em amostras de pacientes com LMA, tendo sido associada a um pior prognóstico.

Neste contexto, o objetivo deste estudo foi avaliar o potencial terapêutico do IWR-1, um inibidor da via WNT/β-catenina, em dois modelos *in vitro* da LMA, com diferentes perfis genéticos.

Para atingir esse objetivo, utilizámos dois modelos de LMA: as linhas celulares NB-4 e HL-60. A expressão de *AXIN2* (o alvo específico do IWR-1) foi determinada por PCR em tempo real (qPCR). As células foram cultivadas na ausência e na presença de diferentes concentrações de IWR-1, que variaram de 10µM a 50µM. O efeito do IWR-1 na viabilidade celular foi determinado através do ensaio da resazurina. A avaliação da morte celular e do ciclo celular foram determinadas pela citometria de fluxo, utilizando a dupla marcação de anexina V/iodeto de propídeo, e pelo protocolo com iodeto de propídeo/RNAse, respetivamente. A análise morfológica foi realizada por microscopia ótica, utilizando a coloração de May-Grünwald-Giemsa. Os dados foram analisados recorrendo ao software Graphpad Prism.

Ambas as linhas celulares expressaram o alvo do IWR-1, *AXIN2*. Contudo, as células HL-60 apresentaram níveis superiores de expressão em relação às células NB-4. Os resultados demonstraram que o IWR-1 induz uma diminuição da atividade metabólica de uma forma dependente da concentração do fármaco, do tempo de exposição e da linha celular, com valores de IC_{50} de 32,8µM para as células HL-60 e de 23,0µM para as células NB-4, após 48

horas de tratamento. A apoptose foi o principal mecanismo de morte celular ativado pelo IWR-1 em ambas as linhas celulares, o que foi confirmado por citometria de fluxo e análise morfológica. Nas células NB-4, o IWR-1 também induziu paragem do ciclo celular na fase G_0/G_1.

Os resultados sugerem que a via WNT/β-catenina poderá constituir um potencial alvo terapêutico na LMA. Além disso, a eficácia do inibidor IWR-1 parece não depender dos níveis de expressão do alvo, *AXIN2*. As diferenças entre os efeitos observados nas duas linhas celulares poder-se-ão dever às diferenças entres os seus perfis genéticos.

Palavras-chave

Leucemia Mielóide Aguda, Via de Sinalização WNT Beta Catenina, IWR-1, *AXIN2*.

Introduction

Acute Myeloid Leukemia (AML) is the most common acute leukemia in adults.[1] The diagnosis is mainly based on the identification of at least 20% of myeloid blasts in the bone marrow and/or peripheral blood. The exceptions, in which the diagnosis is not correlated with the blast percentage, correspond to the presence of the following cytogenetic abnormalities: t(8;21)(q22;q22), inv(16)(p12q22) ort(16;16)(p13;q22), t(15;17)(q22;q12).[2] Currently, the most commonly used system to classify the different types of AML corresponds to the World Health Organization (WHO) classification, which was revised in 2016 (Table 1).[3]

Table 1 – WHO classification of AML and related neoplasms (2016).

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1) - RUNX1-RUNX1T1; AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22) - CBFB-MYH11; Acute Promyelocytic Leukemia (APL) with t(15;17)(q22;q12) - PML-RARα; AML with t(9;11)(p21.3;q23.3) - MLLT3-KMT2A; AML with t(6;9)(p23;q34.1) - DEK-NUP214; AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) - GATA2, MECOM; AML (megakaryoblastic) with t(1;22)(p13.3;q13.3) - RBM15-MKL1; AML with BCR-ABL1 (provisional entity); AML with mutated NPM1; AML with biallelic mutations of CEBPA; AML with mutated RUNX (provisional entity).
AML with myelodysplasia-related changes
Defined by morphologic detection of multilineage dysplasia (defined as the presence of 50% or more dysplastic cells in at least 2 cell lines).
Therapy-related myeloid neoplasms
Occurs in patients previously treated with drugs such as etoposide or alkylating agents.

Table 1 – WHO classification of AML and related neoplasms (2016) (continuation).

AML, Not Otherwise Specified
Group defined by absence of cytogenetic abnormalities, that can be subdivided into: AML with minimal differentiation; AML without maturation; AML with maturation; Acute myelomonocytic leukemia; Acute monoblastic/monocytic leukemia; Pure erythroid leukemia; Acute megakaryoblastic leukemia; Acute basophilic leukemia; Acute panmyelosis with myelofibrosis.
Myeloid sarcoma
Rare solid tumor composed by myeloid blasts.
Myeloid proliferations related to Down syndrome
Two forms are recognized: Transient abnormal myelopoiesis (TAM); Myeloid leukemia associated with Down syndrome.

Adapted from Arber D, *et al* (2016)[3]

Regarding the treatment of AML, the induction therapy with cytarabine and an anthracycline is still considered a standard of care for patients with this disease. The standard combination is the 7+3 regimen, which consists of a 7-day continuous infusion of cytarabine (at a dosage of 100 or 200mg/m^2 per day) from day 1 to 7 and daunorubicin (at a dosage of 60 mg/m^2 per day) from day 1 to 3. For younger patients not undergoing a stem cell transplant, this therapy is usually followed by a high-dose cytarabine (HiDAC) consolidation therapy (twice a day at a 3g/m^2 dose on days 1, 3 and 5). The hematopoietic stem cell transplant is considered one of the best ways of preventing recurrence, but is still associated with high morbidity and mortality.[4]

Other therapies were developed, directed to specific targets of AML cells, as all-trans retinoic acid (ATRA) for AML with *PML-RARα* fusion gene. The disruption of the *RARA* gene results in maturation arrest of myeloid progenitors at the promyelocytic stage. Treatment with ATRA was able to induce differentiation in *in vitro* and *in vivo* models, and induce remission in patients with AML carrying *PML-RARα*. The incorporation of ATRA, and also afterwards, of arsenic trioxide (ATO), into standard chemotherapy of these patients, demonstrated a substantial improvement in clinical outcomes.[5] Nearly 90% of these patients can be cured with a combination of ATRA and ATO.[6,7] Nevertheless, drug resistance to conventional therapeutic drugs and to specific target therapies, such as ATRA and ATO, has been recognized as a critical problem.[8,9] In order to overcome the challenge of drug resistance in this disease, there is a need to investigate potential new drugs, that can overcome resistance and increase remission, as well as decrease the relapse rate in AML.

Although inactive in most adult tissues, the WNT/β-catenin signaling pathway has been shown to be critically involved in embryogenesis and organs development. In the canonical WNT pathway, β-catenin is a signal transducer that is necessary for the formation of the mesoderm, from which the hematopoietic system is derived. Multiple *WNT* genes are expressed in bone marrow, including *WNT2b, WNT3a, WNT5a, and WNT10b*. This pathway is known to regulate cell proliferation and plays an important role in chemoresistance.[10] The constitutive activation of the canonical WNT/β-catenin pathway has been observed in samples from patients with AML, and β-catenin expression is associated with worse prognosis.[11,12]

WNT proteins activate at least three types of intracellular signaling pathways: the canonical WNT pathway, the non-canonical pathway of planar polarity and non-canonical WNT/Ca^{2+} pathway (Figure 1)[13]. The WNT/β-catenin pathway (also known as the canonical WNT pathway) is centered on the latent gene regulatory protein β-catenin; the non-canonical pathway of planar polarity coordinates the polarization of planar cells in a developing

epithelium and depends on the GTPases of the Rho family, thus regulating the cytoskeleton and being responsible for the shape of the cell; the non-canonical WNT/Ca^{2+} pathway stimulates calcium increase, regulating its intracellular concentration. [14]

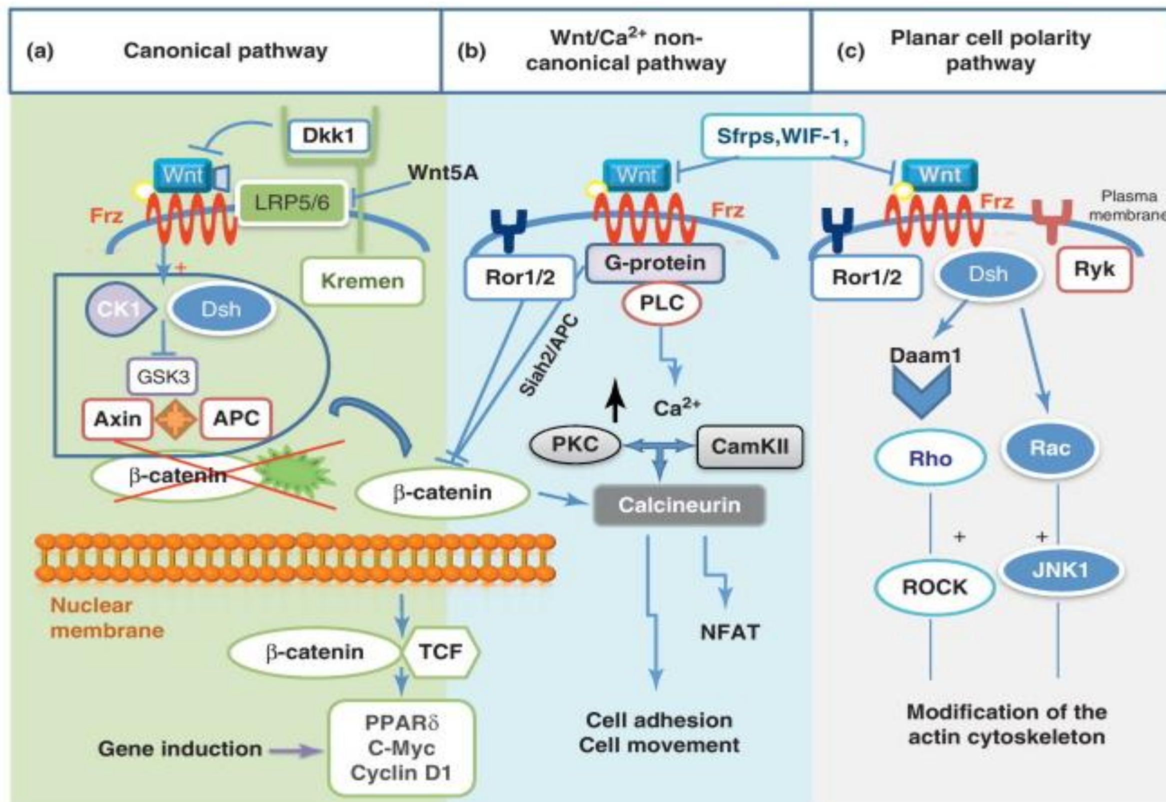

Figure 1 - Schematic diagram representing the components of the WNT/β-catenin signaling pathways. From Marinou k, *et al.* (2012).[13]

In the canonical pathway, several proteins are involved, like WNT, Frizzled receptor (Frz) and LRP5/6 (LDL-receptor-related protein) which regulate the proteolysis of β-catenin, which acts on cell-cell adhesion and gene regulation. In the absence of WNT interaction (situation A on figure 2) with Frizzled and LRP, cytoplasmic β-catenin is sequestered in a large protein-degrading complex which includes the following proteins: APC, Axin, Glycogen synthetase kinase 3 (GSK -3β) and Casein Kinase 1 (CK1). While Axin and APC stabilize the complex, the GSK-3β and CK1 are responsible for β-catenin phosphorylation. After this, β-catenin is recognized by the β-Trcp, which signals β-catenin to ubiquitination and subsequent degradation in proteasome.[15,16]

On the other hand, when WNT binds to the Frizzled receptor (situation B on figure 2), it induces the phosphorylation of LRP 5/6 through the membrane binding of GSK-3β and CK1γ. Then, Axin is recruited to the membrane (the intracellular Disheveled protein is required for this recruitment), where it interacts with the phosphorylated tail of the LRP 5/6 protein, disrupting this complex and leading to β-catenin release. Once stabilized, β-catenin is translocated to the nucleus where it interacts with members of the TCF (T-cell factor)/LEF (Lymphoid Enhancer Factor) transcription factor family to induce gene expression (Figure 1).[15,16] This cellular pathway induces, among others, the active transcription of c-MYC and cyclin D1[17], which control the transition from G_1 to S phase of the cell cycle.

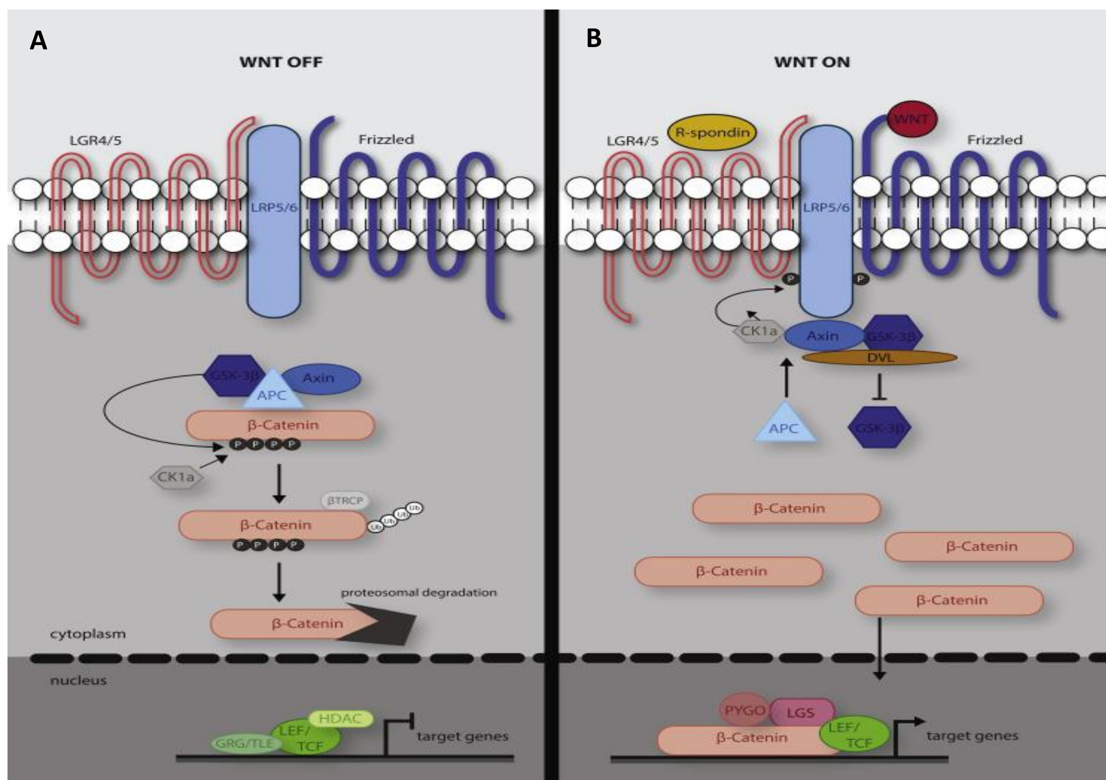

Figure 2 - Schematic diagram representing the components of the WNT/β-catenin canonical pathway. A - WNT off; B - WNT on. From Staal FJ, *et al*. (2016)[15]

Given the importance of WNT/β-catenin signaling to the tumorigenesis and chemorresistance, as well as other processes of cell proliferation in numerous diseases, several inhibitors of this pathway have been developed in order to be used in clinical

research.[18] IWR-1 is an inhibitor of the PARP (Poly ADP-ribose polymerase) activity of Tankyrases 1 and 2 [19], which are proteins that signal the Axin protein to be degraded by ubiquitin-proteasome system. Axin is a protein that promotes the stability of the β-catenin degrading complexes, preventing the translocation of β-catenin to the nucleus. Mechanistically, with administration of IWR-1 it is expected that Axin levels will increase and consequently, there will be a decrease of β-catenin-mediated transcription of genes involved in proliferation.

IWR-1 has been widely used for *in vitro* engineering of various tissues, including cardiomyocytes, retinal pigmented epithelial cells, pneumocytes, and dopaminergic neurons.[20] With regard to neoplastic diseases, IWR-1 has commonly been tested in solid tumor cells, as in lung[21] and colorectal cancer[22] cells, with favorable results. A study performed in acute lymphocytic leukemia cells also had promising results.[23]

Therefore, the aim of this work is evaluate the therapeutic potential of IWR-1, a WNT/β-catenin pathway inhibitor, in two *in vitro* models of AML.

Materials and methods

Cell lines and cell culture

In this work, we used two cell lines of AML (NB-4 and HL-60), both obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. HL-60 cells were established in 1976 from peripheral blood of a 35-year-old woman, and represent characteristically a model of AML with maturation, that has the amplification of the *C-MYC* gene. NB-4 cell line was established in 1989 from the blood marrow of a 23-year-old woman with AML, by the time of the second relapse of this disease. This model of acute promyelocytic leukemia presents the fusion gene *PML-RARα*, which results from the translocation t(15:17)(q22;q12). Both cell lines were maintained in RPMI-1640 medium

(Lonza Biowhittaker), containing L-glutamine 2mM, HEPES-Na 25mM, penicillin 100U/mL, streptomycin 100μg/mL, supplemented with 10% of fetal bovine serum (FBS), at 37ºC in an humidified atmosphere containing 5% CO_2. Cells were cultured at an initial cell density of 0.5 x 10^6 cells/mL.

Evaluation of *AXIN2* gene expression

We studied the gene expression levels of *AXIN2*, which encodes the Axin protein that is important in WNT/β-catenin pathway. We isolated total RNA from both cell lines with NZYol reagent (NZYTech) according to the manufacturer's instructions. After RNA extraction, total RNA concentration and purity (OD_{260}/OD_{280}) was quantified using Nanodrop 1000 (Thermo Scientific). Extracted RNA was stored at -80°C. For cDNA synthesis, the samples containing total RNA were reversely transcribed with NZY First Strand cDNA Synthesis Kit from NZYTech, according to manufacturer's protocol. A mixture of oligo$(dT)_{18}$ and random hexamers were used as primers. The cDNA was stored at -20°C until qPCR analysis.

To analyse the *AXIN2* gene expression, 1 μl cDNA was added to SsoFast™ EvaGreen® Supermix (BioRad) with 300nM of primers. We used primers for *AXIN2* (forward: 5'-GCTGAGAGGAACTGGAAGAAGA-3'; reverse: 5'-GGAGGCAAGTCACCAACATAG-3') and for reference gene *GUSB* (forward: 5'-CAGGTGATGGAAGAAGTG-3'; reverse: 5'-AAGTAGTAGCCAGCAGAT-3'). All samples were used in duplicate and no template controls were included. qPCR was carried out in a CFX96 Touch™ Real Time PCR Detection System (BioRad, USA) in 96-well plates. The thermocycling parameters were one cycle of 30 seconds at 95°C and 40 cycles of 5 seconds at 95°C and 20 seconds at 60°C. The relative experience was calculated with the $2^{-\Delta CT}$ (Livak) method.

Metabolic activity assessment

Resazurin is a blue oxidized non-fluorescent compound that is reduced to resorufin (pink compound) by dehydrogenases, mainly from mitochondria. The amount of compound converted is proportional to the metabolic activity of the cells, which in turn is an indicator of the cellular viability.[24]

Both cell lines were incubated in absence and in presence of increasing concentrations of IWR-1 (ApexBio®), ranging from 10 µM to 50µM, in a single dose administration. Resazurin (Sigma-Aldrich®) was added to a final concentration of 10µg/mL and cell viability was assessed every 24 hours during 3 days. Following incubation, absorbance was measured at 570nm and at 600nm in a spectrophotometer (*Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments*) and the metabolic activity was calculated with the following formula:

$$\frac{(A570nm - A600nm)sample - (A570nm - A600nm)blank}{(A570nm - A600nm)control - (A570nm - A600nm)blank} \times 100$$

The results were expressed in percentage (%) ± SEM of at least 6 independent experiments.

Cell cycle analysis

Propidium iodide (PI) is the most commonly used marker to perform the cell cycle analysis by flow cytometry. This marker connects to the DNA, intercalating between its double-stranded chains. PI intensity is proportional to the quantity of DNA present in each cell, allowing us to determine the relative proportion of cells in the G_0/G_1 phase, S phase, and G_2/M phase. Based on DNA fragmentation, apoptotic cells with this technique will be identified by the fewest DNA quantity than G_0/G_1 phase, called as Sub-G_1 or apoptotic peak. Since PI also connects to RNA, it is necessary to remove all the RNA with RNAse treatment, for a high resolution in the quantification of DNA.[25]

We performed the evaluation of cell cycle by flow cytometry, using the Immunostep PI/RNAse kit. HL-60 and NB-4 cells were incubated with 30μM and 20μM of IWR-1, respectively, during 48 hours. Afterwards, $1,0x10^6$ cells were collected and washed by centrifugation with PBS, during 5 min at 1000xg. The pellet was resuspended in 200μL of cold ethanol 70%, during vortex agitation, and incubated during 30 min at 4ºC. Then, cells were washed with PBS, resuspended in 400μL of PI/RNase solution and incubated for 15 minutes at room temperature. A six-parameter, four color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used and at least 25.000 events were collected by acquisition using CellQuest software (Becton Dickinson, San Jose, CA). Cell cycle distribution was analyzed using the ModFit LT software (Verity Software House). Results were expressed in percentage (%) of cells in the different cell cycle phases (G_0/G_1, S, and G_2/M) ± SEM of 5 independent experiments.

Cell death assessment by flow cytometry

Cell death was evaluated by flow cytometry using the annexin V (AV) and propidium iodide (PI) double staining. During apoptosis, phosphatidylserine (a negative charged phospholipid) translocates from the internal layer to the external layer of the cellular membrane. Annexin V (AV), attached to a fluorochrome, binds specifically to phosphatidylserine in the presence of calcium, allowing the identification of the cells in apoptosis. In necrosis, cells suffer edema and consequently the membrane becomes permeable. In this context, PI will be able to enter in the cell and intercalate with DNA, enabling the identification of necrotic cells. Shortly, viable cells will be negative for both compounds (AV-/PI-), cells in early apoptosis will be AV+/PI-, cells in late apoptosis/necrosis will be AV+/PI+ and cells in necrosis will be AV-/PI+.[26]

HL-60 and NB-4 cells were incubated with 30μM and 20μM of IWR-1, respectively, during 48 hours. Afterwards, $0,5x10^6$ cells were collected and centrifuged with PBS during 5 minutes at 1000xg. Cells were resuspended in 100μL of cold binding buffer and incubated with 2.5μL of AV-APC (BD Biosciences) and 2μL de PI (BioLegends), for 15 min at room temperature in the dark. After incubation time, cells were diluted in 400μL of binding buffer. We used the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). At least 10.000 events were collected by acquisition using CellQuest software. The results were analyzed with the Paint-a-Gate software and expressed in percentage (%) ± SEM of 5 independent experiments.

Cell death assessment by morphological analysis

The morphological analysis was performed in both cell lines using May-Grünwald-Giemsa staining protocol. Cells were incubated during 48 hours, in absence and in presence of IWR-1, in same doses used in flow cytometry. For the preparation of the smears, the cells were collected and centrifuged during 5 min at 1000xg. To improve the adhesion of the cells to the slide, they were resuspended in a small quantity of FBS. After that, the smears were stained during 3 min with of May-Grünwald solution, and then during 15 min with Giemsa staining. Finally, the smears were washed and the cells were visualized in the optic microscope. We used the *Nikon Eclipse 8Oi* microscope, with a digital camera attached, that allowed the recording and processing of the images, with the programme NIS-Elements D.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (version 7.03 for Windows; GraphPad Software, Inc., San Diego, CA, USA). Mann-Whitney U test was used to determine the statistical significance of gene expression and flow cytometry results,

considering a significance level of $p<0.05$. All data were expressed in mean ± SEM of the number of independent experiments indicated in the figure legend. For half maximal inhibitory concentration (IC_{50}) determination, non-linear curve fit dose–response was performed for each cell line.

Results

AXIN2 gene expression levels

Since *AXIN2* encodes the Axin protein, which is the target of IWR-1, we analyzed the gene expression levels in our models (Fig.3). Both cell lines expressed *AXIN2* gene, being the expression approximately 5 times higher in HL-60 cells than in NB-4 cells.

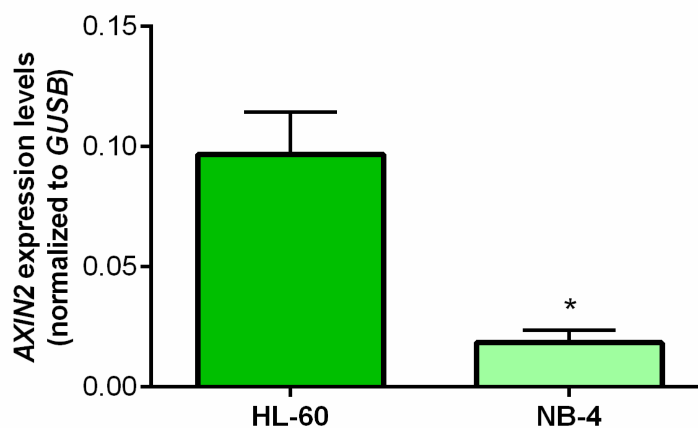

Figure 3 – *AXIN2* gene expression levels in HL-60 and NB-4 cells. Analysis was performed by qPCR, as described in the methods section. Results, normalized to *GUSB* gene, are expressed as mean ± SEM obtained from 3 independent experiments. *$p<0.05$

Metabolic activity assessment

Our results showed that IWR-1 reduces the metabolic activity in a dose, time and cell line dependent manner. As observed in figure 4, IWR-1 decreases metabolic activity in both cell lines with a single dose administration, being the NB-4 cells a little more sensitive than HL-

60. After 48h of exposure to IWR-1, we observed a reduction on metabolic activity in HL-60 cells, being the IC_{50} of the drug between 30µM and 40µM. The decrease in metabolic activity was more evident in NB-4 cells, being the IC_{50} of IWR-1 between 20µM and 30µM at 48h. The effect of incubation time was also more noticed in the cells with the *PML-RARa* fusion gene (NB-4), being the effect more pronounced after 72h of exposure when comparing with HL-60 cells.

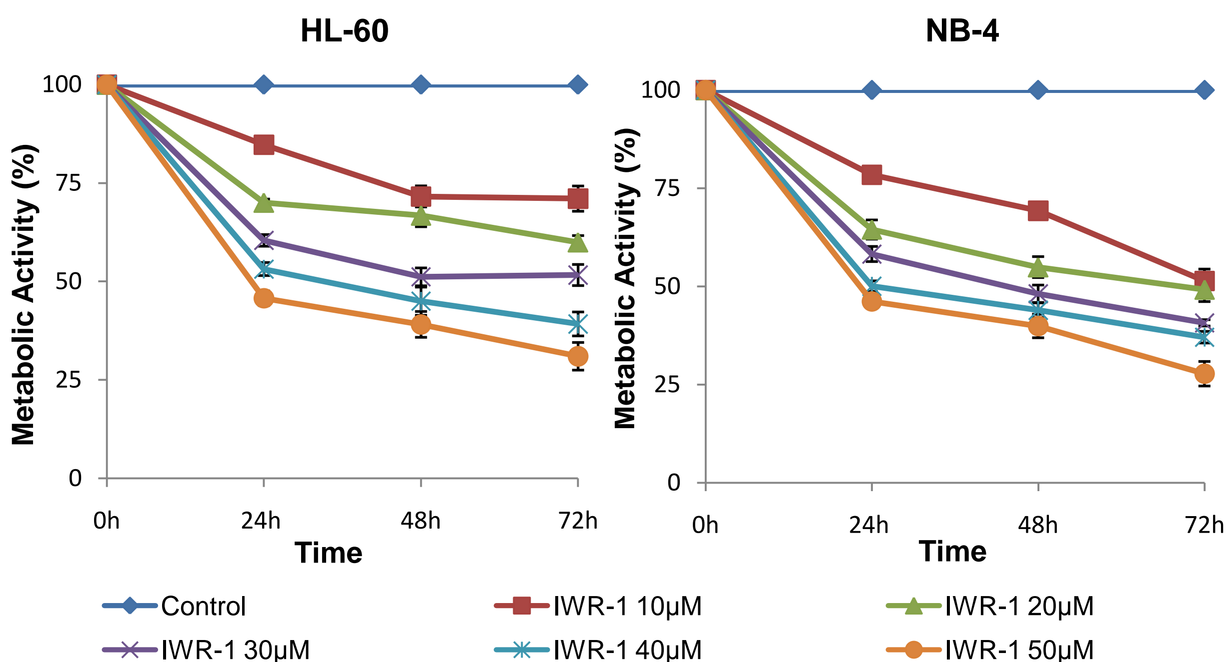

Figure 4 – Dose-response curves of IWR-1 in HL-60 and NB-4 cells. Cells were incubated with IWR-1 in concentrations that ranged from 10µM to 50µM and metabolic activity was evaluated with the resazurin assay, as described in the methods section. Metabolic activity is expressed in cell percentage (%) normalized to control. Results are expressed as mean ± SEM obtained from at least 6 independent experiments.

We performed a non-linear curve fit dose-response to determine more accurately the IC_{50}. After 48h of IWR-1 exposure, the mathematical IC_{50} for HL-60 cells was 32.8µM, being 1.4x higher that in NB-4 cells (23.0µM).

Cell cycle analysis

To assess if IWR-1 presents a cytostatic effect, we conducted the PI/RNAse protocol, as described in the methods section. Cell cycle distribution for untreated and treated cells is represented in figure 5.

Besides no statistical significance was found, our results showed a slight cell cycle arrest in G_0/G_1 phase induced by IWR-1 in NB-4 cells. For this cell line, the percentage of cells in the G_0/G_1 phase increased from 40% to 45.8%, the S phase decreased from 46.2% to 45.4% and the G_2/M phase decreased from 13.4% to 9%, relatively to the control. In HL-60 cells the treatment with IWR-1 did not produce relevant changes in the cell cycle.

Additionally, with this method we can also detect the apoptotic peak (Sub-G_1), which results from the DNA fragmentation that occurs in apoptotic cells. For both cell lines, it was observed that the cells in the apoptotic peak increased in percentage in relation to the control.

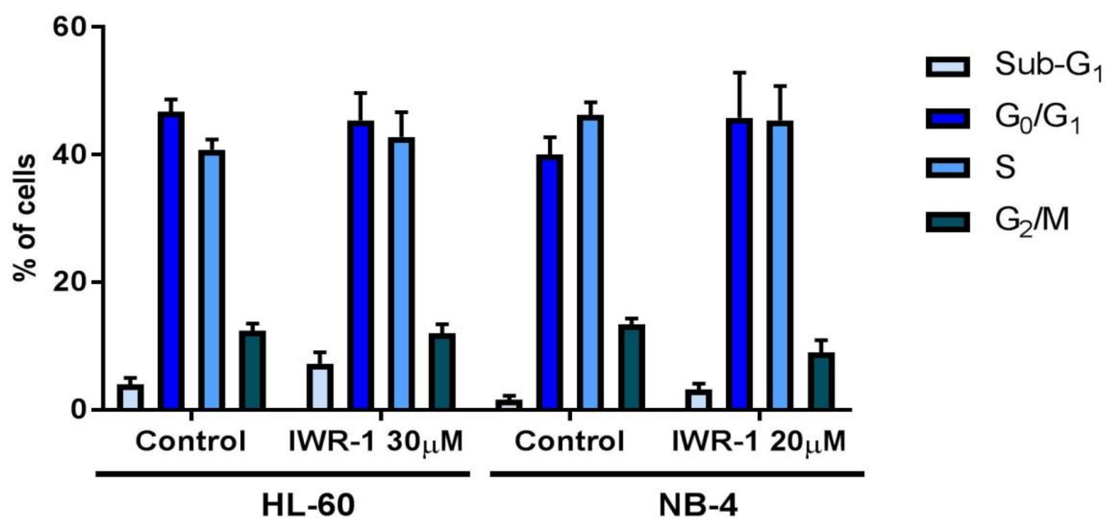

Figure 5 – Effect of IWR-1 on the cell cycle distribution of HL-60 and NB-4 cells. Cells were incubated during 48h with IWR-1 30µM for HL-60 cells and 20µM for NB-4 cells. Cell cycle distribution was assessed by flow cytometry, using propidium iodide/RNAse protocol. Data are expressed as percentage of cells in Sub-G_1 (apoptotic peak), G_0/G_1 phase, S phase and G_2/M phase, and represent mean ± SEM obtained from 5 independent experiments.

Cell death assessment by flow cytometry

We evaluated the type of cell death induced by IWR-1 by flow cytometry with AV/IP staining, as described in the methods section.

As represented in figure 6, our results revealed that IWR-1 induces a cytotoxic effect on both cell lines. The cell death was mainly mediated by apoptosis, but we also observed considerable differences in the percentage of cells in necrosis. The live cells reduced from 86% to 67% in Hl-60 cells and from 85.2% to 54% in NB-4 cells, in comparison to the control.

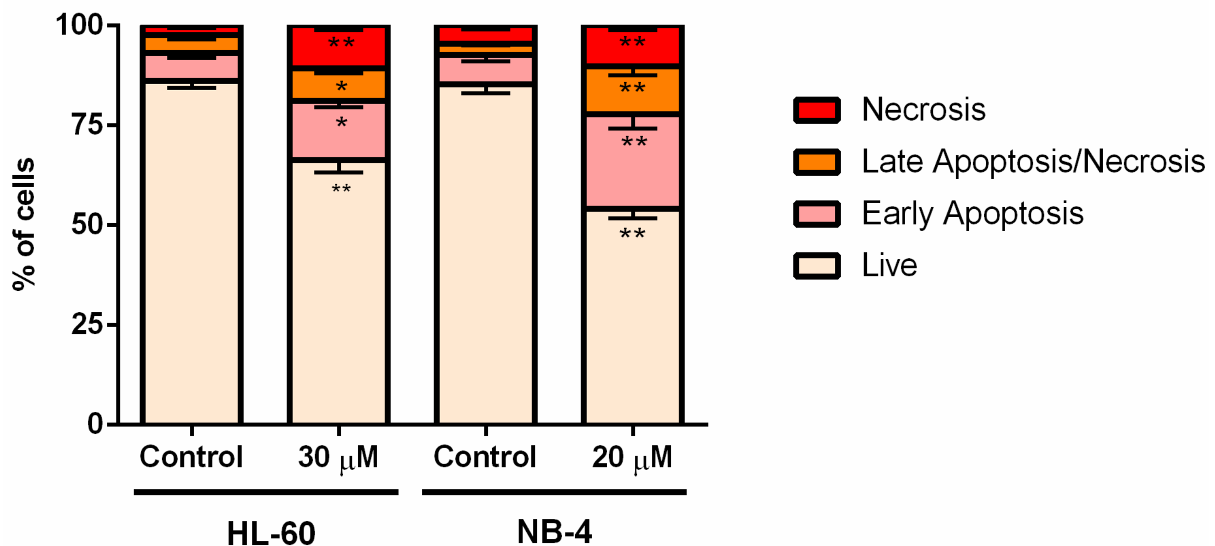

Figure 6 – Effect of IWR-1 on cell death of HL-60 and NB-4 cells by flow cytometry. Cells were incubated during 48h with IWR-1 30µM for HL-60 cells and 20µM for NB-4 cells. Cell death was assessed by flow cytometry, using annexin V/propidium iodide staining, as described in the methods section. Data are expressed as percentage (%) of cells and represent mean ± SEM obtained from 5 independent experiments. *$p<0.05$, ** $p<0.01$

Cell death assessment by morphological analysis

We also evaluated the cell death by optical microscopy, as referred, using May-Grünwald-Giemsa staining protocol.

After IWR-1 incubation, both cell lines presented morphological features that suggested apoptosis (Fig. 7). Blebbing, cell shrinkage, chromatin condensation and the presence of apoptotic bodies were some of the alterations observed. Besides the majority of apoptotic cells, it was also observed some cells dying by necrosis. This supports the results obtained with AV/IP staining.

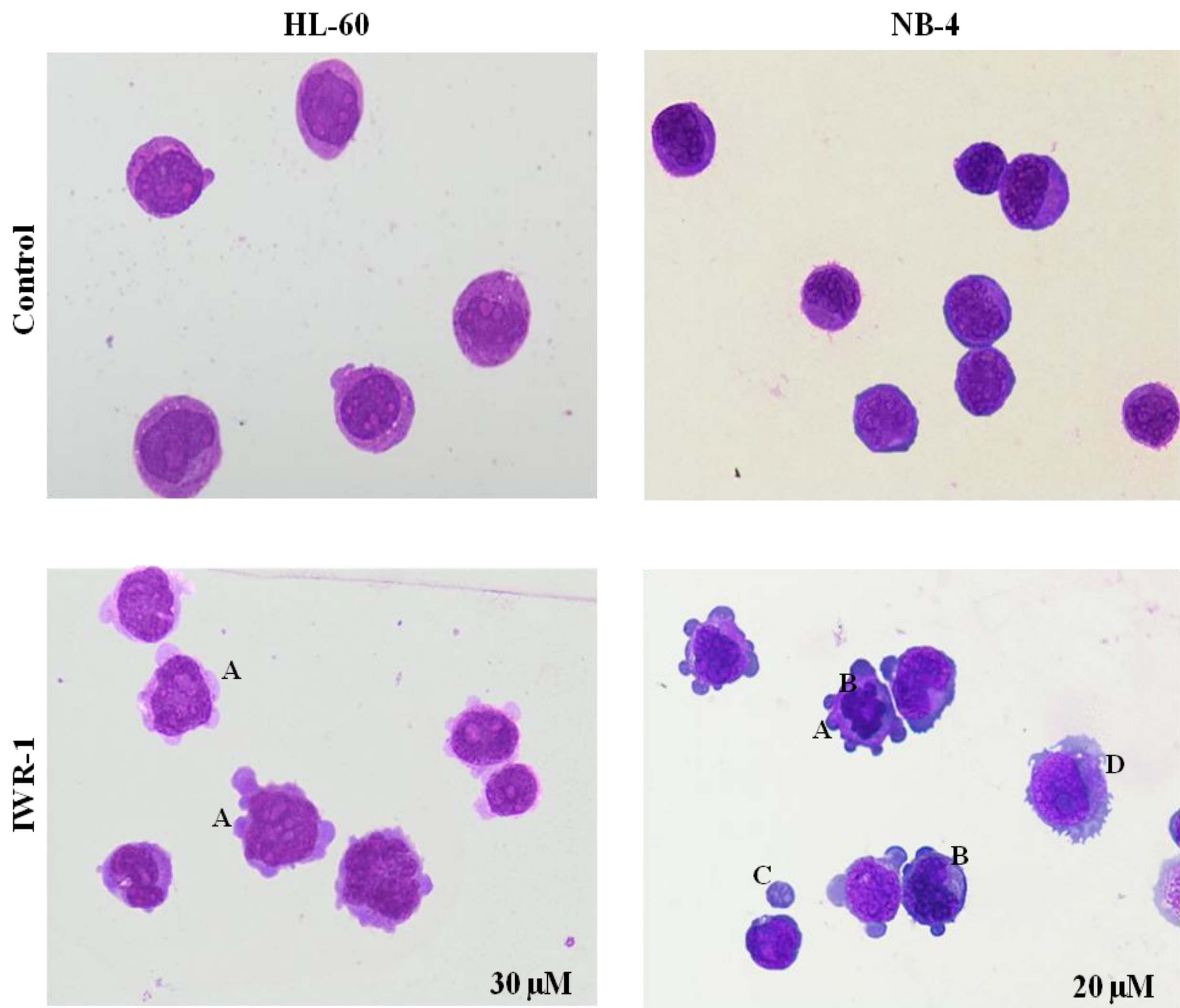

Figure 7 – Morphological analysis of HL-60 and NB-4 cells by optical microscopy. Cells were incubated during 48h with IWR-1 30µM for HL-60 cells and 20µM for NB-4 cells. Then, cells were stained using May-Grünwald-Giemsa, as described in the methods section. The cells were analyzed by light microscopy (amplification of 500x). In the figure we can see examples of characteristics of apoptotic cells, as blebbing (A), chromatin condensation (B), apoptotic body (C), and also a necrotic cell (D).

Discussion

The role of WNT signaling in carcinogenesis has most significantly been described for colorectal cancer, but aberrant WNT signaling is observed in many more cancer entities, like leukemia, breast cancer, melanoma and lung cancer.[27,] It was found that AML cells express higher β-catenin levels than their normal hematopoietic precursors[28] and in AML mouse models, β-catenin appeared to be essential for self-renewal of leukemic cells.[29] Although few studies in the literature described the treatment of AML with WNT inhibitors as a possibility, we expected that these drugs would have efficacy in this disease.

Evaluation of *AXIN2* gene expression levels revealed, as expected, that both AML cells lines, HL-60 and NB4 cells, express this gene, which indicated that IWR-1 could be an appropriate drug to study the influence of the WNT pathway in AML, although we did not evaluate the expression levels of the protein Axin, that could not be proportional to RNA expression.

Our results showed that IWR-1 induces a decrease in metabolic activity in a dose, time and cell line dependent manner. The IC_{50} for NB-4 cells (23.0μM) is approximately 1.4x lower than in HL-60 cells (32.8μM). Some genetic features of the used cell lines may explain the efficacy of the administration of IWR-1, as well the differences observed between both cell lines. High levels of β-catenin, Cyclin D1 and c-MYC are associated with the presence of PML-RARα fusion protein[30], which is present in NB-4 cells. This fusion protein behaves as an altered retinoic acid receptor with the ability to transmit oncogenic signaling, leading to accumulation of undifferentiated promyelocytes.[31] c-MYC and Cyclin D1 control the transition from the G_1 phase to S phase of the cell cycle. So, the cell cycle arrest in G_0/G_1 that is observed in NB-4 cells might be due to decrease in the expression levels of these proteins, which might be one of the mechanisms that explain the sensibility of NB-4 cells. On the other hand, the only alteration described for HL-60 cells is the overexpression of *C-MYC*

oncogene. In a study, it was observed that in *in vitro* (human cell lines) and *in vivo* (rats) models of AML, knockdown of MYC (shRNAs) prolonged survival.[32] Another study wit HL-60 cells showed the differentiation of this cell line being activated by a multi-drug treatment, and this resulted in a decrease in the canonical WNT pathway, which is in agreement with our results.[33]

In spite of HL-60 cells presenting higher expression levels of *AXIN2* gene, NB-4 cells revealed more sensitivity to IWR-1, and by that reason, the efficacy of the inhibitor IWR-1 seems not dependent on the expression levels of the target *AXIN2*.

No clinical trials are being conducted with IWR-1. Speaking of solid tumors, several studies involving cells have been developed. In a study using lung cancer cells, it was demonstrated that human and murine cell lines from lung neoplasms treated with IWR-1 (using doses that ranged from 100nM to 50µM) had a decrease in cell viability in a dose-dependent manner. This treatment increased intracellular levels of Axin and Tankyrase (measured by qPCR and microarrays).[21] Another study performed in colorectal human cancer cells demonstrated that IWR-1 (doses from 5 to 50µM) decreased the proliferation of the cells in a dose- and time-dependent manner. Western blot analysis showed that IWR-1 decreased the expression of β-catenin.[22]

As far as hematological malignancies are concerned, a study performed in an acute lymphoblastic leukemia cell line, treated with IWR-1 (10-50µM), showed suppression of cell growth/proliferation (with trypan blue assay) and induced a decrease in cell viability in a time and dose dependent manner, as we observed in our study. The IC_{50} was 30-40µM, which is a similar value to the ones we obtained for our cell lines. Flow cytometry revealed that cell death occurred mainly by apoptosis, what also happened with AML cell lines. The concentration of Cyclin D1 in that study was diminished. It was not observed a notable influence in cell cycle arrest, what is also in agreement with our study.[23]

Our results are in line with the studies presented above, showing that IWR-1 can also be explored as a potential therapeutic approach in the treatment of hematological malignancies, in particular AML. However, further studies are needed to validate this compound as an effective and secure treatment for AML. We propose the utilization of other *in vitro* models, as well as *in vivo* animal models since other factors, not represented on this cell cultures, like the micro- and macroenvironment, also play a central role in the pathogenesis of these malignancies. Other interesting aspects that could be studied would be the effect of a scheme of a daily dose administration, and also the effect of a combined therapy with association of conventional therapeutic agents, like ATRA or ATO.

We expect that, in a near future, the therapeutic approach in malignancies will potentially benefit from a more personalized Medicine, with a wider range of therapeutic options directed to specific targets of the cells of the patient, that might overcome the problem of drug resistance and relapse that affects AML, among other diseases.

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