

Wild-type p53-induced phosphatase sensitizes acute myeloid leukemia cells to conventional chemotherapy

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Abstract

Recently wild-type p53-induced phosphatase was implicated in the pathogenesis of acute myeloid leukemia (AML) and “pre-leukemia” myeloproliferative conditions. Here we decided to check how the strategy directed to phosphatase inhibition affected sensitivity to conventional chemotherapy. All experiments were conducted on AML cell lines cultivated *in vitro*. The levels of wild-type p53-induced phosphatase vary in different AML cell lines. The chemical compound GSK2830371 reduced levels of phosphatase and diminished its activity. GSK2830371 did not significantly change the cell cycle distribution of AML cells when used alone or in combination with the anti-cancer chemotherapeutic drug Cytosar but increased caspase-dependent PARP1 cleavage. In contrast with previous studies, we did not observe the negative effect of phosphatase activity inhibition and depletion on cells when a chemical inhibitor was used as monotherapy. Using a combination of GSK2830371 with Cytosar we were able to reduce the threshold of chemotherapy-dependent cytotoxicity and more efficiently eliminate leukemic cells. We propose considering inhibition of wild-type p53-induced phosphatase as a prospective strategy in improving anti-AML therapy.

Keywords: leukemia, AML, chemotherapy, cytarabine

Introduction

Acute myeloid leukemia (AML) is a frequent hematological malignancy that can develop spontaneously or be induced by chemotherapy or ionizing radiation. It is characterized by the clonal expansion of myeloid progenitors in the bone marrow and peripheral blood. AML has a multigenic nature with many genes involved in the pathogenesis of the disease, mainly genes involved in cell signaling, transcriptional and epigenetic regulation (Cai and Levine, 2019). Many other myeloid proliferative or dysplastic pathologies with time may be transformed into AML. Currently, researchers and physicians count three steps in AML evolution from single mutations in progenitor cells in the bone marrow to fully developed leukemic cells with multiple mutations in various genes.

The first step when the hematopoietic progenitor acquires a mutation that gives it a competitive advantage in more rapid proliferation or resistance to stress is called Clonal Hematopoiesis of Indeterminate Potential, or CHIP. It is characterized by the appearance and expansion of hematopoietic cells with certain mutations detected in at least 1% of blood cells. It can lead to changes in the functions of the gene and its products, but does not significantly affect the morphology of cells and does not cause any pathological condition in the hemato-

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poietic system immediately, has indeterminate potential in contrast with the clonal detection of cells already associated with morphological changes (Myelodysplastic syndrome, MDS) or disease (Acute myeloid leukemia, AML). CHIP may predispose to the development of MDS, a condition with the appearance of atypical cells in the blood. Myelodysplastic syndrome includes several oncohematological conditions characterized by a defect in the maturation of cells in bone marrow, but usually at the beginning there are no other symptoms except atypical blood formula. In many cases, MDS cells acquire additional mutations and transform into fully malignant leukemic cells, AML.

Wild-type p53-induced phosphatase, WIP1, is one of the major regulators of DNA damage response and the p53 pathway that is frequently compromised in AML by mutations in the *TP53* gene or the members of the DNA damage signaling pathway (Uyanik et al., 2017). Despite the recent addition of targeted therapy and the use of epigenetic drugs, the main drug of choice for AML treatment remains cytarabine. Here we investigated the effect of WIP1 inhibition on AML cells' sensitivity to cytarabine treatment.

Recently, Yu et al. showed that without any relation to mutation status in the fifth and sixth exons of the *PPM1D* gene (WIP1 protein), this gene was overexpressed in bone marrow cells of AML patients in comparison with control bone marrow cells from healthy donors (2020). Other researchers used RNA interference to deplete WIP1 in several AML cell lines. They showed that reducing WIP1 levels with siRNA to protein phosphatase, Mg²⁺/Mn²⁺ dependent 1D suppressed cell proliferation and promoted cell apoptosis in AML-193 cells and KG-1 cells (Li et al., 2020).

Here we decided to analyze an approach more relevant to clinical practice, by blocking WIP1 activity with a specific chemical inhibitor, GSK2830371. Moreover, we decided to test how WIP1 inhibition affects AML sensitivity to anti-cancer chemotherapy.

Material and methods

AML cell cultivation

Cells were obtained from the shared research facility “Vertebrate cell culture collection” supported by the Ministry of Science and Higher Education of the Russian Federation (Agreement # 075-15-2021-683).

The OCI-AML-2 cell line (DSMZ no.: ACC 99) was cultured using 80–90% alpha-MEM (with riboxyribonucleosides and deoxyribonucleosides) + 20% h.i. FBS. The MONO-MAC-1 cell line (DSMZ ACC 252) was cultured in 90% RPMI 1640 + 10% h.i. FBS + 2 mM L-glutamine + 1x non-essential amino acids + 1 mM sodium pyruvate.

Cell viability assay

The CyQUANT™ XTT Cell Viability Assay kit was used to analyze viability. Cells at a concentration of 1,000,000 per mL were seeded with the test compounds (GSK2830371 (GSK) (Selleckchem) and Cytosar (Cytarabine) (Astellas Pharma Inc)) in a 96-well plate and cultured for 48 hours at 37°C (5% CO₂). The readings were carried out according to the kit's manual.

Western blotting

A polyacrylamide gel (10%) was used for protein electrophoresis according to the standard technique. A nitrocellulose membrane (Bio-Rad) with a pore diameter of 0.2 μm was used. The transfer of proteins from the gel to the membrane was carried out in a chamber (Bio-Rad) at 0.3A for 2 hours. After blocking with 5% milk in PBS-T (+ 0.1% Tween-20), membranes were incubated with specific antibodies (WIP1 F-10 (SantaCruz), PARP (Invitrogen), p-p38 MAPK (Cell Signaling), p-P53 (Ser15) (Cell Signaling)) overnight. After three washes with PBS-T (+ 0.1% Tween-20), secondary antibodies were applied accordingly and membranes were revealed with ECL reagents.

Flow cytometry

CELL CYCLE

The cells were washed in PBS buffer, then fixed by the addition of cold ethanol to a final concentration of 50% and subsequent incubation for 20 minutes at -20°C. Cells were stained at 37°C in PBS buffer containing RNase and propidium iodide. Stained cells were then analyzed on a CytoFLEX Beckman Counter in the PE channel.

APOPTOSIS

All of the procedures were performed according to kit instructions with Dead Cell Apoptosis Kit with Annexin V PE and SYTOX™ Green. Stained cells were then analyzed on a CytoFLEX Beckman Coulter in the APC and FITC channels.

Results

First, we performed data mining and found that mutation frequency in the *PPM1D* gene is different in various subsets of AML and AML-related diseases. The data presented in Table 1 indicate that despite a low general frequency of the *PPM1D* mutations in AML in general, about 1% of the cases, up to one-fifth of patients with a therapy-related AML or therapy-related pre-AML condition, MDS, with a reported previous history of DNA

Table 1. *PPM1D* mutation frequency in CHIP, MDS, AML

Disease	Frequency of <i>PPM1D</i> mutations	References
Healthy individuals	0.42 %	Motomura et al., 2019
CHIP	4.6 %	Genovese et al., 2014
Primary MDS	3 %	Lindsley et al., 2017
<i>De novo</i> AML	1.2 %	Kartal-Kaess et al., 2018
Therapy related MDS	15–20 %	Hsu et al., 2018; Lindsley et al., 2017
Therapy related AML	19.5 %	Hsu et al., 2018

damaging agent treatment, had mutations in the regulatory domain of WIP1 phosphatase.

As discussed above, such mutations lead to the expression of a more stable and active enzyme in AML cells that may affect the leukemogenesis and leukemic cells' responsiveness to the therapy. Therefore, we concluded that WIP1 could play a role in the pathogenesis of AML, and the presence of mutations in the *PPM1D* gene could influence the choice of therapeutic strategy.

Our own Sanger sequencing of the *PPM1D* gene for more than 20 patient samples from the archives of the Institute of Oncology and Hematology, Almazov National Medical Research Centre, did not reveal any samples with mutations in *PPM1D*. The absence of mutations may not only indicate the low frequency of mutated *PPM1D*, but also can point to the necessity of using next generation sequencing with a reading depth of at least 500 copies to detect clones of AML cells with mutations in the *PPM1D* gene.

In the next step, we decided to analyze WIP1 inhibition effects on AML cells and changes in AML sensitivity to anti-cancer chemotherapy.

For our studies, we selected two AML cell lines that are well-described in the literature: OCI-AML2 and Mono-Mac-1. According to French-British-American classification, they belong to FAB M4 (Acute myelo-

monocytic leukemia) and FAB M5 (Acute monocytic leukemia) classes of AML, correspondingly.

First, we analyzed how these two cell lines respond to the basic anti-AML chemotherapeutic drug Cytosar. Figure 1A shows that OCI-AML 2 cells are more sensitive to the first-line treatment drug Cytosar, than Mono-Mac-1 cells (Fig. 1A). Then we tested the toxicity of GSK2830371 (GSK), a highly specific WIP1 inhibitor, on those two AML cell lines in a biologically relevant range of concentrations from 0.1 μ M to 5 μ M. In contrast with previously published studies on the toxicity of WIP1 RNA interference in AML cells (Li et al., 2020), we did not observe the negative effect of WIP1 phosphatase activity inhibition on cell viability when a chemical inhibitor was used as monotherapy. At the same time, the combination of GSK2830371 with Cytosar significantly potentiated the efficiency of chemotherapy in OCI-AML2 cells but not in Mono-Mac-1 cells, which are resistant to Cytosar (Fig. 1B).

As expected, treatment with Cytosar induced intra-S-phase checkpoint and delayed cell cycle progression and proliferation of tumor cells (Fig. 1C). Cytarabine activates the major regulator of the intra-S-phase checkpoint, ATR-Chk1 signaling. This signaling was reported as WIP1 phosphatase target previously (Lu et al., 2005; Mesa et al., 2005). GSK2830371 did not change the cell cycle profile either in control cells or in AML cells treated with Cytosar (Fig. 1C).

The caspase-dependent cleavage of PARP1 protein serves as a marker of apoptotic cell death. We observed the maximum intensity of cleaved product (89 kDa) in OCI-AML2 cells treated with a combination of WIP1 inhibitor and chemotherapeutic agent (Fig. 1D). In Mono-Mac-1 cells, there was no obvious difference between cells treated only with Cytosar or with a combination of Cytosar and GSK2830371, but both samples had an extra band of 92 kDa that was not present in the control and in cells treated only with GSK.

Another marker of apoptosis, the binding of Annexin V to the outer leaflet of the plasma membrane, was analyzed by flow cytometry. The results showed that,

Fig. 1. WIP1 inhibition sensitizes AML cells to chemotherapy

A — Influence of Cytosar (Cytarabine) on the viability of OCI-AML-2 and MONO-MAC-1 Cell lines.

Cell viability XTT assay was performed after 48 h of cultivation. The exponential concentration curve was built with Origin PRO (2019) software.

B — The effect of GSK2830371 (GSK) WIP1 inhibitor and chemotherapeutic drug Cytosar on AML cell viability.

Two AML cell lines, OCI-AML-2 and MONO-MAC-1, were cultivated in the presence of GSK2830371 (GSK) and Cytosar for 48h before XTT assay.

— — no drug added; + — 1 μ M each of the compounds; ++ — 2 μ M each of the compounds; * - $p < 0.05$ ($n = 3$).

C — The effect of GSK2830371 (GSK) and Cytosar on cell cycle

The cell cycle distribution of treated and non-treated OCI-AML-2 cells were analyzed using PI staining and FACS

D — PARP cleavage assay

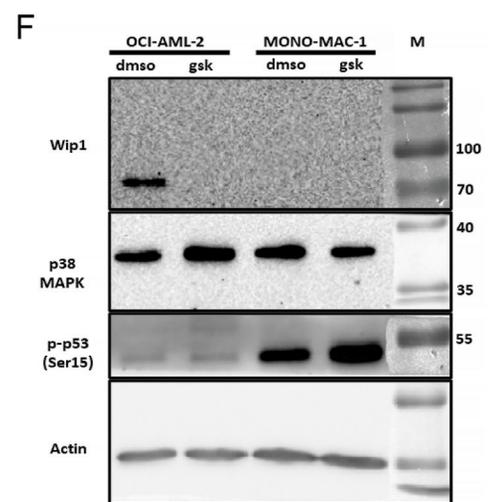
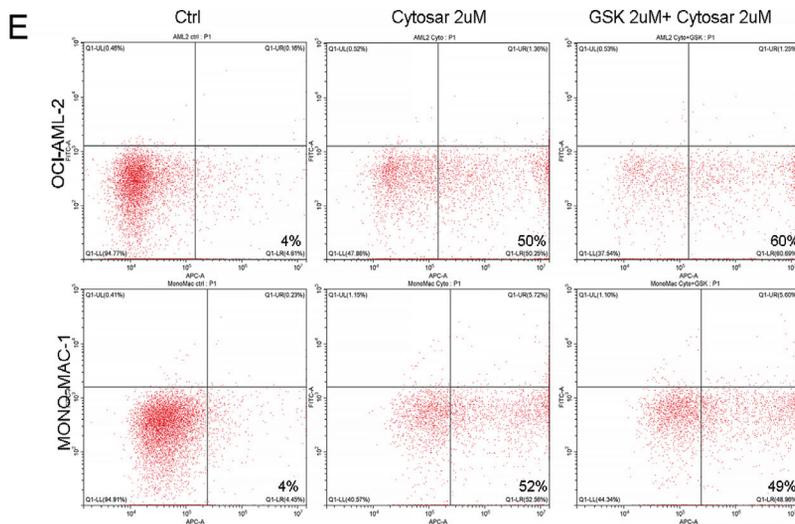
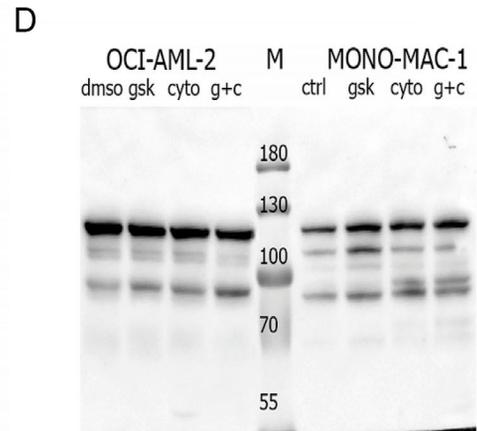
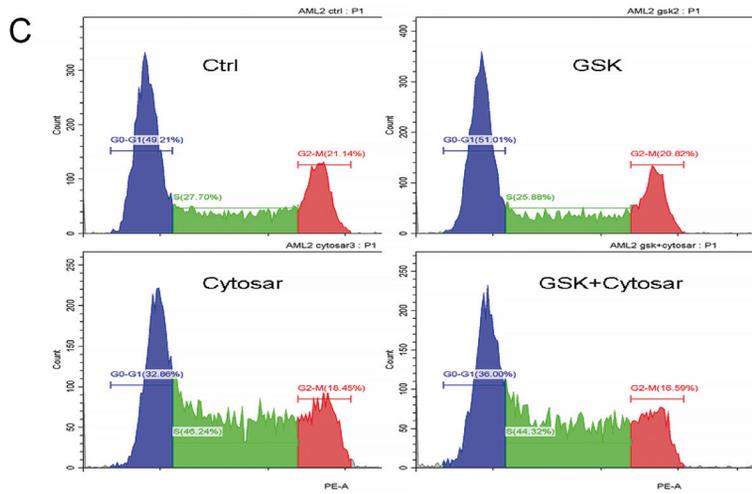
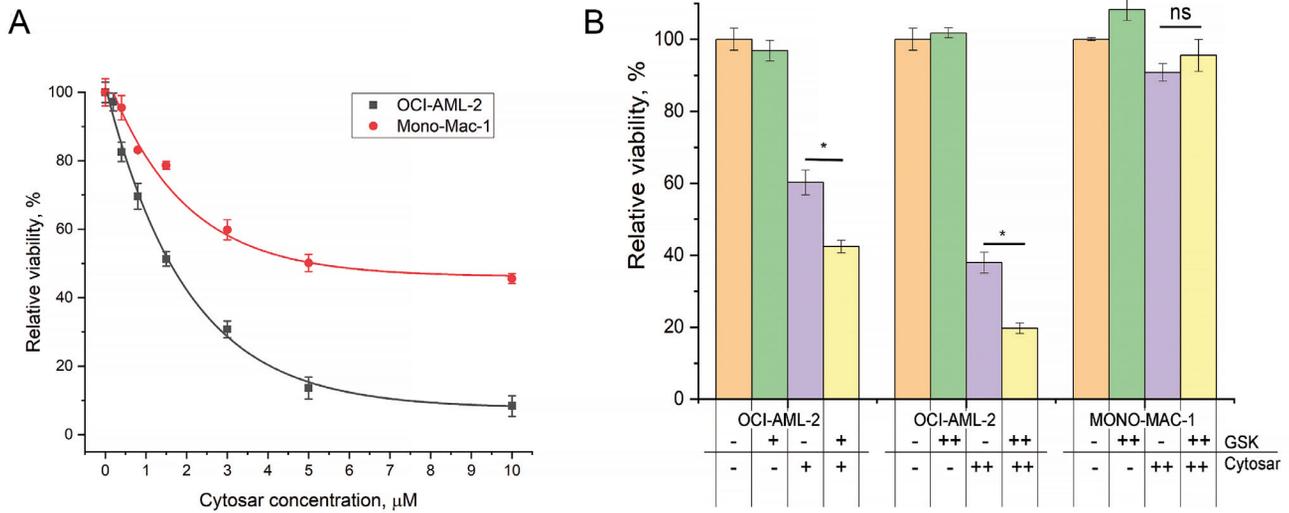
The PARP cleavage by caspases was analyzed by Western blot with anti- PARP antibodies (Invitrogen).

E — The effect of GSK2830371 (GSK) and Cytosar on apoptosis

Flow cytometry dot plots for annexin V and CytotoxGreen staining of the two cell lines after 48 h of incubation with drugs

F — WIP1 signaling in AML cells after inhibition

Cells were treated with GSK2830371 (GSK) and Western blot was performed for WIP1, p-p38 MAPK and p-p53, and actin.



similar to the viability assay, WIP1 inhibitor potentiated efficacy of Cytosar and increased the number of apoptotic cells in treated OCI-AML2 cells. The same concentration of Cytosar induced cell death in Mono-Mac-1 cells to a lesser extent, and there was no difference between the treatment with Cytosar alone and the treatment with a combination of Cytosar and GSK2830371 (Fig. 1E).

Finally, we checked WIP1 downstream signaling and verified the levels of WIP1 in both cell lines. We found that OCI-AML2 had high levels of WIP1 protein. In contrast, we were not able to detect the expression of the *PPM1D* gene in Mono-Mac1 AML cells with low-affinity anti-WIP1 SCBT antibodies. As expected from the previous reports, GSK2830371 affected not only phosphatase activity but also reduced protein levels of WIP1 in OCI-AML2 cells (Fig. 1F). One of the main WIP1 targets, p38MAPK kinase was hyperphosphorylated on WIP1-targeted threonine 180 when dephosphorylation activity was inhibited by GSK2830371 in OCI-AML2 cells, but not in WIP1-deficient Mono-Mac-1 AML cells. p53 transcriptional factor is another WIP1 target linked to apoptosis induction was detected at very low levels in OCI-AML2 cells. The levels of phosphorylated p53 at serine-15 were very high in Mono-Mac-1 AML cells but in this cell line, p53 was inactivated by mutations in the DNA binding domain (p.Arg273His).

Discussion

In our study, monotherapy with WIP1 inhibitor GSK2830371 did not reduce the number of leukemic cells in comparison with the report published earlier this year, in which researchers used a different shRNA approach to deplete WIP1 in AML cells. WIP1 inhibition strategy in the treatment of AML was inefficient as monotherapy, but it shifted AML cells' response to the widely used chemotherapeutic agent Cytosar towards apoptotic cell death. The difference in the response to WIP1 inhibitor treatment between the two AML cell lines we used in our study can be explained by differences in *PPM1D* expression. The undetectable levels of WIP1 in Mono-Mac-1 can be linked to the inactive mutant p53. In our previous work, we showed that transcriptional factor p53 could bind to the promoter of the *PPM1D* gene and could activate its transcription (Rossi et al., 2008). The mutation in *TP53* in Mono-Mac-1 cells is located in the DNA binding domain and severely compromised p53 functions as a transcriptional regulator. P53 is a major tumor suppressor and a key protein in DNA damage response. P53 activation by a DNA-damaging chemotherapeutic agent turns on apoptotic cell death. Therefore, the higher resistance of Mono-Mac-1 cells to Cytosar can also be explained by inactivating *TP53* mutations in this cell line.

Increasing the efficiency of current protocols for the treatment of AML is one of the major tasks in oncohematology. We conclude that WIP1 phosphatase could be a new target in the treatment of AML, and its inhibition potentiates the cytarabine efficiency in inducing cell death of AML cells by favoring apoptosis. At the same time, WIP1 levels in AML cells could be a prognostic marker determining the efficiency of the proposed anti-tumor strategy.

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