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PARP1 inhibitors attenuate AKT phosphorylation via the upregulation of PHLPP1

Shuai Wang^{a,b,1}, Huibo Wang^{b,1}, Ben C. Davis^b, Jiyong Liang^c, Rutao Cui^d, Sai-Juan Chen^{a,*}, and Zhi-Xiang Xu^{b,*}

^aState Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China.

^bDivision of Hematology and Oncology, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35205, USA.

^cDepartment of Systems Biology, University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA

^dDepartment of Dermatology, Boston University School of Medicine, Boston, MA 02118, USA

Abstract

Poly (ADP-ribose) polymerase-1 (PARP1) inhibitors are emerging as an important class of drugs for treating BRCA-deficient cancers. Recent discoveries have shown that PARP1 inhibitors may treat other cancer patients in addition to the relatively small proportion of patients carrying BRCA mutations. However, the additional targets by which PARP1 inhibitor-mediated tumor suppression remain poorly understood. In this study, we show that two PARP1 inhibitors, PJ-34 and 3-AB, attenuate AKT phosphorylation at serine 473 (S473) independent of DNA repair impairment. These inhibitors decrease the AKT-associated phosphorylation of FOXO3A, enhance the nuclear retention of FOXO3A, and activate its transcriptional activity. We further demonstrate that treatment with PJ-34 or 3-AB dramatically increases the level of PHLPP1. Overexpression of PHLPP1 enhances the PARP1 inhibitor-induced downregulation of AKT phosphorylation and increases tumor cell death. In contrast, knockdown of PHLPP1 abrogates the PARP1 inhibitor-mediated AKT inhibition and desensitizes cells to its treatment. Therefore, our findings not only show the robust role of PARP1 inhibitors in AKT inhibition but also develop a novel strategy to increase the effectiveness of cancer treatment via PARP1 inhibitor-induced PHLPP1 upregulation.

Keywords

PARP1 inhibitor; AKT; FOXO3A; PHLPP1

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*Corresponding author: Zhi-Xiang Xu, Division of Hematology and Oncology, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35205, USA. zhi-xiang.xu@ccc.uab.edu. Tel: 205-934-1868 or Sai-Juan Chen, State Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China. sjchen@stn.sh.cn. Tel: 86-21-64377859.

¹These authors contributed equally to this work.

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1. Introduction

PARP1 is a highly conserved DNA-binding protein and is the most abundant member of the PARP family. PARP family proteins are essential in the repair of single-stranded DNA breaks via the base excision repair pathway [1-2]. Numerous studies suggest that PARP1 is involved in maintaining genomic stability as well as regulating DNA repair and transcriptional processes. PARP1 is now considered to be a central regulatory hub of cell survival and cell death as well as a key component of a number of transcription factors involved in tumor development and inflammation [3-5]. Therefore, pharmacological modulation of PARP1 activity may represent an attractive therapeutic strategy for human disorders. PARP1 inhibitors, a promising new class of drug for the treatment of cancers, are being tested as a single agent and in combination with other chemotherapeutic drugs or ionizing radiation [6].

Notably, BRCA1- or BRCA2-mutant breast and ovarian cancer cells, which are defective in homologous recombination (HR), are incredibly sensitive to PARP1 inhibitors [7-8]. Indeed, recent clinical trials have shown that PARP1 inhibitors show considerable promise in treating women with hereditary breast and ovarian cancers associated with BRCA deficiency [9-10]. Taking advantage of siRNA screening and cytotoxic assays, several recent investigations have indicated that PARP1 inhibitors may be effective against other cancers as well [11-12], suggesting that PARP1 inhibitor-mediated tumor suppression may involve additional targets or mechanisms that are independent of BRCA-associated HR function.

In this study, we reveal a novel function of two PARP1 inhibitors, PJ-34 and 3-AB. Treatment with these inhibitors attenuated AKT-FOXO3A signaling by simultaneously activating PHLPP1, leading to apoptosis in cancer cells. Our findings highlight the potential benefit of PARP1 inhibitors for cancer patients with high AKT expression.

2. Materials and methods

2.1. Cell culture and reagents

Human osteosarcoma cell line U2OS and ovarian cancer cell line SKOV3 were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific). Non-small cell lung cancer cell line H358 was cultured in RPMI 1640 media supplemented with 10% FBS. PARP1 inhibitors 3-AB and PJ-34 were purchased from Sigma-Aldrich. Phospho-AKT S473, phospho-AKT-threonine 308 (T308), total AKT, and cleaved caspase-3 antibodies were obtained from Cell Signaling. Phospho-FOXO3A-S253 and total FOXO3A antibody were obtained from Upstate. p27^{kip} and PTEN antibodies were obtained from Santa Cruz Biotechnology. PHLPP1 and β -actin antibodies were obtained from Bethyl Laboratories.

2.2 Cell viability assay

Cells were seeded into 96-well plates at 3×10^3 cells per well and allowed to adhere overnight. Cell viability was determined after 24 to 72 h in the presence or absence of 3-AB or PJ-34 using the MTT assay. Plates were read with a SpectraMax 190 microplate spectrophotometer (Beckman Coulter, Inc.) at a wavelength of 540/570 nm.

2.3. FOXO3A-enhanced green fluorescent protein (EGFP) translocation assay

U2OS cells were transfected with the pEGFP-N1-FOXO3A plasmid and selected with 200 μ g/ml G418 to make the stable cell line U2OS-FOXO3A-EGFP. The stable cells (2×10^5) were treated with 3-AB or PJ-34 for 6 h and 24 h. Cellular DNA was counterstained with an

anti-fade reagent containing DAPI (Vector Labs), and the cells were analyzed by fluorescence microscopy (Carl Zeiss, Inc.).

2.4. Transfection

Cells were plated at 2×10^5 in 6-well plates and transfected with 2 μ g of pcDNA3.1-GFP or pcDNA3.1-HA-PHLPP1 plasmid (generously provided by Dr. Alexandra Newton, University of California at San Diego) using the FuGENE 6 Transfection Reagent (Roche Applied Science). Twenty-four hours after transfection, cells were split and seeded into 6-well plates for further experiments. For the siRNA knockdown experiments, cells were plated at 2×10^5 in 6-well plates and transfected with 800 pmol of PHLPP1 siRNA (Dharmacon) or control siRNA (Santa Cruz Biotechnology) using the Dharmacon transfection reagent.

2.5. Dual-luciferase reporter assay

Cells were transfected with 100 ng of FOXO3A-FHRE-Luc plasmid (generously provided by Dr. Valsala Haridas, University of Texas, M. D. Anderson Cancer Center) or pGL3 vector (Promega). Twenty-five ng of the *Renilla* luciferase reporter construct (Promega) was included in each transfection to monitor the transfection efficiency. After 24 h, cells were treated with PARP inhibitors for 6 h and then lysed with passive lysis buffer (Promega). Luciferase assays were performed using the Dual-Luciferase Reporter System (Promega) on a Flash'n Glow LB 955 luminometer (EG&G Berthold). Normalized relative luciferase units (RLUs) were calculated by dividing firefly luciferase units by *Renilla* luciferase units.

2.6. Colony formation assay

Cells (0.5×10^3) were cultured in 60-mm dish after 24 h transfection and allowed to attach overnight. The cells were then treated with 15 μ M PJ-34 for 6 h and allowed to grow for 12-14 days in regular cell culture medium. The colonies were then fixed with ice-cold methanol and stained with 0.05% crystal violet for 15 min. Colonies containing more than 50 cells were counted. The experiments were repeated at least 3 times.

2.7. Apoptosis analysis

Cells (2×10^5) were exposed to 15 μ M PJ-34 or 10 μ M 3-AB for 24 h. Both adherent and detached cells were collected and subjected to the apoptosis assay. The percentage of apoptotic cells was determined by Annexin V-PI staining followed by flow cytometry analysis using the FACScan flow cytometer (BD Pharmingen) with CellQuest software (BD Pharmingen).

2.8. Western blotting

Cell lysates were prepared by suspending cell pellets in lysis buffer. The proteins were separated by SDS-PAGE, and immunoblotting was performed as described previously [13]. A total of 50 μ g protein was used for the immunoblotting unless otherwise indicated. β -actin was used for the loading control.

2.9. Alkaline comet assay analysis

The extent of DNA damage was analyzed using a commercial comet assay (Sigma Aldrich); the assay was performed following the manufacturer's protocol. Cells were treated with 15 μ M PJ-34 or 10 μ M 3-AB for 6 h. After treatment and before apoptosis could be detected by Annexin V-PI analysis, the cells were washed, trypsinized, and collected for comet analysis. The DNA damage was quantified by Comet Assay IV software (Perceptive Instruments, Ltd) as a tail moment (tail length multiplied by the relative tail DNA content).

2.10. Statistical analysis

All comparisons between groups were performed using a 2-tailed paired student's t-test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. PARP1 inhibitors cause a cytotoxic effect independent of DNA repair impairment

To investigate the cytotoxic effects of the PARP1 inhibitors, 3 human cancer cell lines (U2OS, H358, and SKOV3) were exposed to different doses of 3-AB or PJ-34 for varying amounts of time and assayed for cell viability. We found that all 3 cell lines responded to 3-AB or PJ-34 treatment efficiently (Supplementary Fig. S1 and Fig. 1A). Because the damage due to PARP1 inhibition is generally associated with the impairment of DNA break repair, we next examined the presence of double-strand breaks (DSBs) by comet assay. However, there was no significant difference in the occurrence of DSBs in response to 3-AB or PJ-34 when compared with untreated cells (Fig. 1B and C). These results suggest that PARP1 inhibitors exert a cytotoxic effect on cancer cells that is independent of DNA repair impairment.

3.2. PARP1 inhibitors attenuate the AKT-FOXO3A pathway

Previous studies have reported that several PARP1 inhibitors may activate the PI3K-AKT pathway [14-16]. To determine whether PARP inhibitor-induced cytotoxicity is associated with the AKT pathway in our model, we exposed H358, U2OS, and SKOV3 cells to 15 μ M PJ-34 or 10 μ M 3-AB for different amounts of time and looked for changes in phospho-AKT S473. We found that phosphorylation of AKT on S473 was only slightly upregulated at the early time points (1 h and 2 h) and then downregulated 3 h after PJ-34 or 3-AB treatment, whereas total AKT protein levels showed no significant change (Fig. 2A). Consistent with this observation, PJ-34 or 3-AB treatment led to the downregulation of phospho-FOXO3A S253 and the upregulation of p27^{kip} (Fig. 2B), two important downstream targets of AKT. Thus, these results suggest that PARP1 inhibitors may transiently upregulate AKT S473 phosphorylation at the early time points and significantly attenuate AKT-FOXO3A signaling later.

Under stress conditions, unphosphorylated FOXO3A translocates from the cytoplasm to the nucleus to execute its transcriptional functions. To investigate whether PARP1 inhibitors contribute to FOXO3A translocation in cancer cells, we established a stable cell line expressing FOXO3A-EGFP in U2OS cells (U2OS-FOXO3A-EGFP). As shown in Fig. 2C, FOXO3A resided almost exclusively in the cytosol of untreated U2OS-FOXO3A-EGFP cells, and its nuclear signal was negligible. However, after being exposed to 10 μ M 3-AB for 6 h, FOXO3A entered into nucleus increasingly, although some perinuclear and cytoplasmic FOXO3A-EGFP signal remained apparent. After 24 h of exposure, most of the FOXO3A relocated into the nucleus. Similar results were seen in U2OS cells treated with PJ-34 (data not shown). Taken together, these data suggest that PARP1 inhibitors could enhance the nuclear translocation of FOXO3A, which may potentially elevate its transcriptional activity.

To evaluate the transcriptional activity of FOXO3A during PARP1 inhibitor treatment, we transiently transfected U2OS cells with pGL3/FHRE-Luciferase, which possesses 3 copies of a Forkhead responsive element. The cells were then treated with 15 μ M PJ-34 or 10 μ M 3-AB for 6 h, and FOXO3A transcription function was assessed by a dual-luciferase assay. Pretreatment of cells with PJ-34 or 3-AB resulted in a significant increase in FHRE-luciferase activity (Supplementary Fig. S2A), suggesting an increase in the transcriptional potential of FOXO3A. Taken together, these data show that PARP1 inhibitor treatment

suppresses AKT activity, thereby leading to the nuclear retention of FOXO3A and an elevation of its transcription factor activity.

A direct outcome of AKT inhibition is reduced cell growth and increased programmed cell death. To investigate whether PARP1 inhibitor-induced AKT suppression leads to apoptosis, we exposed U2OS cells to 15 μ M PJ-34 or 10 μ M 3-AB for 24 h and stained cells with Annexin V-FITC. Indeed, treatment with these inhibitors dramatically induced apoptosis (Supplementary Fig. S2B). Moreover, the apoptotic effect was supported by the elevated cleavage of caspase-3 (Fig. 3C and Fig. 4A). Taken together, our data suggest that PARP1 inhibitor-mediated cytotoxicity is associated with the activation of the apoptotic pathway.

3.3. PARP1 inhibitors upregulate the expression of PHLPP1, but not PTEN

AKT activity is negatively regulated by PTEN [17-18] and PHLPP1 [19]. To determine whether these 2 phosphatases are involved in the PARP1 inhibitor-induced reduction in phospho-AKT, we looked at PTEN and PHLPP1 expression in U2OS and H358 cells in response to PJ-34 or 3-AB treatment. We found that PTEN levels were not affected by PJ-34 or 3-AB treatment (Fig. 3A and B). However, these inhibitors triggered a dramatic increase in PHLPP1 levels (Fig. 3A, B). In addition, we observed that the alteration in PHLPP1 expression happened as early as 2 h after treatment (data not shown). Consistent with the previous report that PHLPP1 dephosphorylates the hydrophobic motif of AKT S473, our results showed that PARP1 inhibitor-induced inactivation of AKT was due, in large part, to the decrease of AKT S473 phosphorylation, not AKT T308 phosphorylation (Fig. 2B). Collectively, our data indicate that the inhibitory effect of the PARP inhibitors on AKT phosphorylation is partially due to PHLPP upregulation.

3.4. PHLPP1 regulates the sensitivity of cancer cells to PARP1 inhibitors

To gain insight into the functional significance of PHLPP1 in PARP1 inhibitor-induced cell death, we transfected U2OS cells with pcDNA3.1-HA-PHLPP1 and determined the cytotoxic effects of PJ-34 treatment. Colony formation assays showed that PJ-34 treatment reduced colony formation by around 50%. Overexpression of PHLPP1 further decreased the colony formation rate to approximately 10% of the control cells (Fig. 3C, Supplementary Fig. S3A). To evaluate whether this effect was associated with the termination of AKT signaling and increased levels of apoptosis, we further monitored AKT S473 phosphorylation and cleaved caspase-3 levels in PHLPP1-overexpressing cells after PJ-34 treatment. We found that PHLPP1 overexpression further reduced the phosphorylation of AKT (Fig. 3C). Simultaneously, cleavage of caspase-3 was dramatically increased in PJ-34-treated PHLPP1-overexpressing cells (Fig. 3C). PHLPP1-overexpressing cells showed a marked increase in the number of apoptotic cells compared to control cells in response to PJ-34, as judged by increased Annexin V-FITC-positive staining (Supplementary Fig. S3B). Taken together, our results demonstrate that PHLPP1 enhances PARP1 inhibitor-induced apoptotic cell death by the attenuation of AKT phosphorylation.

To further assess the PHLPP1-mediated cytotoxic effect of the PARP1 inhibitors, U2OS cells were transfected with control siRNA or siRNA against PHLPP1. A significant knockdown of PHLPP1 was achieved by siRNA transfection (Fig. 4A). Colony formation assays showed that silencing PHLPP1 conferred cellular resistance to PJ-34 (Fig. 4B and C). To evaluate the functional effect of PHLPP1 knockdown, levels of AKT S473 phosphorylation were examined. siRNA-mediated reduction of PHLPP1 caused an increase in AKT S473 phosphorylation. The PJ-34-induced downregulation of AKT S473 phosphorylation was reversed by knocking down PHLPP1 (Fig. 4A). We observed a significant decrease in cleaved caspase-3 levels and apoptosis in the PHLPP1-depleted cells when compared with control cells in response to PJ-34 (Fig. 4A and D). These results

indicate that PHLPP1 knockdown reduces the apoptotic effect of PJ-34 through the upregulation of phospho-AKT. All together, our results provide further evidence that the attenuation of AKT phosphorylation and activity by PHLPP1 is functionally important for PARP1 inhibitor-induced apoptotic cell death.

4. Discussion

In most cell lines, treatment with current PARP inhibitors at doses that successfully inhibit PARP activity does not cause cell death, especially in cells with an intact DSB DNA repair pathway. Although PARP inhibitors are currently being tested in clinical trials for cancers that are not associated with DNA repair deficiency, such as triple-negative breast cancers [20], the agents are still limited to patients with abnormal DNA repair function, called BRCAness [21]. In this study, we show that PARP1 inhibitors exhibit an anti-tumor effect independent of the classic DNA damage sensitizer and that this effect is through the attenuation of AKT-FOXO3A signaling. Although several publications have reported that several PARP1 inhibitors facilitate AKT activation, in our model, we consistently observed only transient upregulation of AKT S473 phosphorylation after brief treatment (1-2 h) and a significant reduction of AKT S473 phosphorylation 3 h after treatment with PJ-34 or 3-AB, indicating that PARP1 inhibitors have an inhibitory effect on AKT signaling.

It has been reported that AKT phosphorylates and maintains FOXO3A in the cytoplasm under nutrient redundant condition. In contrast, when the cells are subjected to nutrient deficiency, FOXO3A is dephosphorylated and retained in nucleus, where it acts as a transcript factor to activate cell cycle arrest, DNA repair, and apoptosis [17,22-24]. In our study, we observed a strong increase in nuclear translocation of FOXO3A after PARP1 inhibitor treatment. In addition, we found that both PJ-34 and 3-AB were able to increase FOXO3A transcription activity. FOXO3A also plays a critical role in the cellular response to stresses [18,25]. Thus, PARP1 inhibitors may function in alleviating stresses through their action on FOXO3A.

Physiologically, the most important negative regulator of AKT signaling is PTEN, which maintains a certain level of AKT phosphorylation [26]. In our study, we observed that the level of PTEN expression was not affected in response to PJ-34 or 3-AB, indicating that the inhibitory effect of PARP1 inhibitors on AKT phosphorylation is not due to PTEN alteration.

PHLPP represents a novel family of protein phosphatases that serve as tumor suppressors; they exert their tumor-suppressing functions by the dephosphorylation and attenuation of AKT. It has been reported that PHLPP levels are markedly reduced in colon cancer and glioblastoma cell lines that exhibit elevated AKT phosphorylation [19,21]. A recent study showed that loss of PHLPP induced by rapamycin is a contributing factor to rapamycin resistance in cancer cells [27]. Hirano *et al.* reported that downregulation of PHLPP is correlated with activated BCR-ABL in chronic myelogenous leukemia (CML) cells and that further depletion of PHLPP in CML cells confers resistance to ABL kinase inhibitors [28], suggesting that enhancing PHLPP function may represent a novel and reasonable strategy to improve the effectiveness of cancer therapy. In the current study, we report that the PARP1 inhibitors, PJ-34 or 3-AB enhanced PHLPP1 phosphatase activity, resulting in the inactivation of AKT and its downstream signaling. Depletion of PHLPP1 led to a remarkable increase in AKT activity and diminished PARP1 inhibitor-associated cytotoxicity, suggesting that PHLPP1 plays a critical role in mediating PARP1 inhibitor-induced cell death. To our knowledge, no PHLPP1 activator has been previously reported. Our study not only shows the robust role of the PARP1 inhibitors in the inhibition of AKT

but also provides a novel strategy for increasing the effectiveness of cancer treatment by the PARP1 inhibitor-induced PHLPP1 upregulation.

In summary, this study sheds new light on the anti-tumor effects of PARP1 inhibitors, which attenuate AKT-FOXO3A signaling through the activation of PHLPP1, leading to apoptosis in cancer cells. Our findings indicate that the use of PARP inhibitors should be extended beyond those with BRCA mutations to include a larger group of cancer patients with hyperactivated AKT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- ▶ PARP1 inhibitors cause a cytotoxic effect independent of DNA repair impairment
- ▶ PARP1 inhibitors attenuated AKT-FOXO3A signaling by activating PHLPP1
- ▶ PHLPP1 regulates the sensitivity of cancer cells to PARP1 inhibitors

Fig. 1. PARP1 inhibitors cause a cytotoxic effect independent of DNA repair impairment. (A) Microscopic analysis of untreated U2OS cells or cells treated with 15 μ M PJ-34. (B) Representative images of untreated or PJ-34-treated U2OS cells after comet assay. (C) Quantitative analysis of comet assay in untreated or PJ-34-treated cells for 24-72 h.

Fig. 2.

PARP1 inhibitors attenuate AKT phosphorylation and its function. (A) Western blot analysis of phospho-AKT S473 and total AKT in U2OS cells in the absence or presence of 15 μ M PJ-34 or 10 μ M 3-AB for the indicated time. (B) Western blot analysis of phospho-AKT S473, phospho-AKT T308, total AKT, phospho-FOXO3A S253, total FOXO3A, and p27 in U2OS and H358 cells before or after 24 h treatment with 15 μ M PJ-34 or 10 μ M 3-AB. β -actin was used as a loading control. (C) FOXO3A distribution in U2OS-FOXO3A-EGFP cells treated with 10 μ M 3-AB.

Fig. 3. PARP1 inhibitors upregulate the expression of PHLPP1, which enhances their cytotoxic effects through the inhibition of phospho-AKT. (A) Western blot analysis of PTEN and PHLPP1 in U2OS and H358 cells before or after treatment with 15 μM PJ-34 for 24 h. (B) Expression of PTEN and PHLPP1 in U2OS and H358 cells before or after treatment with 10 μM 3-AB for 24 h. (C) Western blot analysis of PHLPP1, phospho-AKT S473, and cleaved caspase-3 in control vector- and PHLPP1-expressing U2OS cells without or with 15 μM PJ-34 treatment for 24 h. (D) Colony formation assay of U2OS cells expressing control vector or PHLPP1 in the presence or absence of PJ-34 treatment.

Fig. 4. PHLPP1 knockdown decreases the cytotoxic effect of PJ-34 through the upregulation of phospho-AKT. (A) Western blot analysis of PHLPP1, phospho-AKT S473, and cleaved caspase-3 in control and PHLPP1-knockdown cells without or with 15 μ M PJ-34 treatment for 24 h. (B) Colony formation assay of control siRNA and PHLPP1-depleted U2OS cells in the presence or absence of PJ-34 treatment. (C) The colony-forming results of 3 independent experiments. Columns, mean of 3 determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$. (D) Percentage of apoptotic cells detected by Annexin V-PI staining in control and PHLPP1-depleted U2OS without or with PJ-34 treatment. Columns, mean of 3 determinations; bars, SD. *, $P < 0.05$.