A Novel PARP Inhibitor YHP-836 For the Treatment of BRCA-Deficiency Cancers

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PARP inhibitors have clinically demonstrated good antitumor activity in patients with BRCA mutations. Here, we described YHP-836, a novel PARP inhibitor, YHP-836 demonstrated excellent inhibitory activity for both PARP1 and PARP2 enzymes. It also allosterically regulated PARP1 and PARP2 via DNA trapping. YHP-836 showed cytotoxicity in tumor cell lines with BRCA mutations and induced cell cycle arrest in the G2/M phase. YHP-836 also sensitized tumor cells to chemotherapy agents in vitro. Oral administration of YHP-836 elicited remarkable antitumor activity either as a single agent or in combination with chemotherapy agents in vivo. These results indicated that YHP-836 is a well-defined PARP inhibitor.

Keywords: PARP inhibitor, BRCA, chemotherapy agent, PARP (poly(ADP-ribose) polymerase, cancer therapy

INTRODUCTION

The poly-adenosyl-ribose polymerases (PARPs) are a family of enzymes that regulate protein post-translational modification by transferring the ADP-ribose group to target proteins (Gibson and Kraus, 2012; Bai, 2015). PARP1 and PARP2 are the main PARP enzymes involved in base-excision repair of DNA single-strand breaks. PARP1 also plays roles in other DNA damage repair including nucleotide excision repair, nonhomologous end-joining repair, and microhomology-mediated end-joining repair (Couto et al., 2011; Patel et al., 2011). Targeting PARP is an attractive oncologic therapy as genomic instability is a hallmark of cancer that drives tumorigenesis and progression (Hanahan and Weinberg, 2011; Do and Chen, 2013). Indeed, inhibition of PARP1/2 is synthetically lethal with homologous recombination deficiency (HRD) including germline BRCA1 or BRCA2 (gBRCA) mutations or non-germline HRD-enriched tumors (Bryant et al., 2005; Farmer et al., 2005; Underhill et al., 2011).

PARP inhibitors, including olaparib, rucaparib, niraparib, talazoparib, and pamiparib, have clinically demonstrated significant and sustained antitumor responses as a single agent in patients with gBRCA mutation tumors with a favorable toxicity profile (Brown et al., 2016; Spriggs and Longo, 2016; Yuan et al., 2017; Mateo et al., 2019; Markham, 2021; Paluch-Shimon and Cardoso, 2021). PARP inhibitors have also been shown to sensitize tumors cells with chemotherapy drugs such as alkylating agents, topoisomerase I inhibitors, and anti-angiogenesis agents (Plummer et al., 2013; Norris et al., 2014; Ivy et al., 2016; Matulonis and Monk, 2017; Lu et al., 2018; Bizzaro et al., 2021; Chatterjee et al., 2021). Recently, PARP1/2 inhibitors have been reported to be involved in cancer
immunity via various mechanisms (Lee and Konstantinopoulos, 2019; Lampert et al., 2020; Lee and Konstantinopoulos, 2020). In ovarian cancer, PARP1/2 inhibitors exhibited antitumor immunity via a stimulator of interferon genes (STING) in a dependent manner (Ding et al., 2018). PARP1/2 inhibitors yielded encouraging results in combination with immune checkpoint inhibitors by promoting neoantigen release, increasing tumor mutational burden, and enhancing PD-L1 expression (Ding et al., 2019; Lampert et al., 2020). These promising data in preclinical and early clinical studies provide a wide clinical application of PARP1/2 inhibitors in the future.

Here, we reported a novel PARP1/2 inhibitor, YHP-836. YHP-836 showed the inhibitory effect of both enzymes and DNA trapping against PARP1 and PARP2. YHP-836 exhibited cytotoxicity and induced cell cycle arrest in the G2/M phase in BRCA-deficient tumor cells. The antitumor roles of YHP-836 alone or in combination with chemotherapy agents were evaluated in vitro and in vivo. Oral administration of YHP-836 elicited good antitumor activity in vivo.

MATERIALS AND METHODS

Reagents and Antibodies

YHP-836 was synthesized in-house. PARP1/2 inhibitor olaparib was purchased from TargetMol, United States. Temozolomide (TMZ), topotecan, cisplatin, and adriamycin were purchased from J&K Scientific (Beijing, China). Anti-γH2AX and anti-RAD51 were obtained from Cell Signaling Technology (Danvers, MA, United States). An anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Anti-PARP1 and anti-PARP2 antibodies were from Abcam (Cambridge, United Kingdom). Anti-PARP antibody and HT PARP pharmacodynamic assay kit were purchased from Trevigen (Gaithersburg, MD, United States). The subcellular protein fractionation kit was purchased from Thermo Scientific (Rockford, IL, United States).

Cell Culture

The cell lines MCF-7, MDA-MB-436, MDA-MB-231, MDA-MB-453, MDA-MB-468, SUM149PT, Capan-1, and OVCA8 were obtained from the Cell Resource Centre at the Institute of Medical Sciences, Peking Union Medical College. UWB1.289 and UWB1.289 + BRCA cells were obtained from ATCC. MX-1 was available in our lab. All cell lines were cultured in a humidified atmosphere of 5% CO2 at 37°C. MDA-MB-436, MDA-MB-231, MDA-MB-453, MDA-MB-468, and OVCA8 cells were in RPMI1640 medium (Gibico, TX, United States) with 10% FBS and 1 × penicillin–streptomycin. MCF-7 and MX-1 cells were in Dulbecco’s modified eagle medium (Gibico) with 10% FBS and 1 × penicillin–streptomycin. The SUM149PT cell was cultured in Ham’s F-12 medium containing 5% FBS, 10 μg/ml insulin, 1 × penicillin–streptomycin and supplemented with 0.5 μg/ml hydrocortisone. The Capan-1 cell was in Iscove’s modified Dulbecco’s medium (Gibico) with 10% FBS and 1 × penicillin–streptomycin. According to ATCC handling information, UWB1.289 was cultured in a medium containing 50% RPMI1640 medium and 50% MEGM (MEBM basal medium and SingleQuot additives) (Lonza, Basel, Switzerland) with a final concentration of 3% FBS and 1 × penicillin–streptomycin. UWB1.289 + BRCA1 was in the same medium condition as UWB1.289 with 200 μg/ml G418.

PARP1/2 Enzymatic Assay

The enzymatic assay of PARP1 and PARP2 was measured as described before (Zhu et al., 2014; Yao et al., 2015). Briefly, 100 μl of histone (10 μg/ml) in assay buffer was coated in a clear flat-bottom 96-well plate at 4°C overnight. After a washing step, 35 μl of NAD+ (25 pmol NAD+), 10 μl of PARP1 or PARP2 (0.05 unit), and 5 μl of YHP-836 or olaparib (3-fold dilution from 100 nM) were added and incubated at room temperature for 1 h. Then, the PAR product was determined. IC50 values of compounds were calculated (Zhu et al., 2014).

Cell Viability Assay

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, Darmstadt, Germany). Briefly, 2000 cells/well were seeded into a 96-well plate. After incubation overnight, the cells were treated with different concentrations (1.5625, 3.125, 6.25, 12.5, 25, and 50 μM) of YHP-836 or olaparib with three replicates for 72 h. Then, MTT solution was added and incubated for 4 h. Then, MTT solution was gently removed and 100 μl DMSO was added. Absorbance values were measured at the wavelength of 570 nm using a microplate reader (Biotek Instruments, Inc., United States). The half maximal inhibitory concentration (IC50) was calculated using GraphPad Prism v8.0.1 (La Jolla, CA). For the combination assay, diluted concentrations of chemotherapy agents were added with 2.5 or 5 μM YHP-836.

PARP-DNA Trapping Analysis

MX-1 cells were treated with various concentrations of YHP-836 (1, 5, and 10 μM) for 24 h. Then, the cells were harvested. The nuclear soluble and chromatin sections were collected following the protocol of the subcellular protein fractionation kit. Then, the subcellular fractions were tested by immunoblotting.

Cell Cycle Analysis

Flow cytometry assays were used to analyze the cell cycle distribution as previously reported (Ji et al., 2018). In brief, MX-1 and MCF-7 cells were dispensed into six-well plates at a density of 50,000 cells/well. After growing overnight in a humidified atmosphere of 5% CO2 at 37°C, the cells were treated with indicated concentrations of YHP-836 (5 and 10 μM) or olaparib (10 μM) for 24 h. Then, the cells were harvested and fixed with ice cold 70% ethanol overnight at −20°C, washed with PBS, and stained with propidium iodide (PI) solution containing 20 mg/ml PI and 20 mg/ml RNaseA in PBS for 30 min. DNA contents were measured using the BD fluorescence-activated cell sorting (FACS) verse flow cytometer (BD Biosciences, NJ, United States), and the cell cycle distribution was analyzed.

Immunoblotting Analysis

Cells or mice tumor tissues were collected and lysed in RIPA lysis buffer supplemented with 1% protease inhibitor cocktail
and 1% phosphatase inhibitor cocktail (TargetMol, United States). Lysates were then centrifuged at 12,000 g for 30 min. Proteins were quantified using a bicinchoninic acid (BCA) assay kit (Solarbio, Beijing, China). Resultant samples containing equal amounts of proteins were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Millipore, Darmstadt, Germany). The membrane was blocked with TBST buffer containing 5% non-fat milk for 30 min and incubated with appropriate primary antibodies (1:1000 dilution) in TBST at 4°C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 dilution; Cell Signaling Technologies, Boston, MA) for 1 h at room temperature. Bound proteins were visualized using enhanced chemiluminescence and detected using ImageQuant LAS 4000 software.

### Immunofluorescent Staining

Cells at the appropriate density were cultured in the confocal culture dishes and treated with YHP-836 alone or in combination with TMZ for 24 h. The cells were then washed in PBS and fixed with 4% PFA at 4°C for 30 min. The permeabilization was carried out with 0.1% TritonX-100 for 10 min. Anti-RAD51 and anti-γH2AX antibodies at 1:200 dilution were dissolved in 1% bovine serum albumin (BSA). The cells were incubated with primary antibody solutions for 2 h at room temperature. Secondary Alexa Fluor 594 or 488 antibodies were used to bind and visualize the primary antibody. The culture dishes were then mounted using Origene ZLI-9556 mounting medium with DAPI. The photographs were taken using the Olympus FV1000MPE Confocal microscope.

### Animal Study

Female Balb/c athymic nude mice (8–10 weeks old) were subcutaneously implanted with $1 \times 10^7$ MDA-MB-436, MX-1, or MCF-7 cells in 0.1 ml matrigel solution in the right flank of nude mice. After 2 weeks, the tumor tissue was harvested aseptically, and tumor cells were extracted from tissue homogenate. Then, the mice were implanted with $5 \times 10^6$ tumor cells each. Seven days later, when the average tumor volumes reached $100–300 \text{ mm}^3$, the mice were randomized and received treatment (Day 0). For the MDA-MB-436 xenograft model, mice were orally administered vehicle or
YHP-836 at a dose of 50, 100, or 150 mg/kg dissolved in 0.5% CMC twice daily for 25 days. In the MX-1 xenograft model, mice were orally administered vehicle (once per day for 5 days), TMZ at the dose of 50 mg/kg/day (once per day for 5 days), or YHP-836 at the dose of 25 mg/kg/day alone (once per day for 5 days) or in combination with TMZ (once per day for 5 days). For other chemotherapy agents, CDDP at a dose of 6 mg/kg was intraperitoneally injected once per week and ADM at a dose of 5 mg/kg was intraperitoneally administrated every 3 days. YHP-836 was orally administered once per day alone or combined with chemotherapy agents for 9 days. In the MCF-7 xenograft model, YHP-836 at the dose of 25 mg/kg/day was administered alone (once per day daily for 24 days) or combined with TMZ (once per day for 5 days). Tumor volumes and body weights were monitored twice a week. Tumor volume was calculated as V = 1/2 × L × W^2, where L is the maximum length of the tumor, and W is the maximum width of the tumor. The mice were euthanized, and the tumor tissues were collected for immunoblotting or ELISA assay.

All procedures were approved by the Ethics Committee for Animal Experiments of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, and conducted following the Guidelines for Animal Experiments of Peking Union Medical College.
Statistical Analysis
Most statistical analyses were performed utilizing GraphPad Prism 8.0.1 (La Jolla, CA), and significance levels were evaluated using analysis of variance (ANOVA) or t-tests, as appropriate. Here, we distinguish between three \( p \) values of significance (*** \( p < 0.001 \), ** \( p < 0.01 \), and * \( p < 0.05 \), respectively).

RESULTS

YHP-836 Inhibited PARP1/2 Activity In Vitro
YHP-836 (Figure 1A) is a novel PARP inhibitor with PARP1 and PARP2 enzymatic IC\(_{50}\) values of 6.328 and 3.621 nmol/L, respectively (Figure 1B). The IC\(_{50}\) values of olaparib for PARP1 and PARP2 were 1.832 and 7.773 nmol/L. The ELISA assay showed that YHP-836 dose-dependently reduced intracellular PAR levels in MX-1 breast cancer cells (Figure 1C), which reflected the catalytic activity of PARP1 and PARP2. Immunoblotting results also showed the PAR levels were decreased in MX-1 exposed to various concentrations of YHP-836 for 24 h (Figure 1D). Exposed to YHP-836 at the concentration of 1 \( \mu \)M, intracellular PAR levels in cells were notably reduced. PARP inhibitor olaparib was used as a positive control. It was reported that PARP inhibitors can not only inhibit PARP1 and PARP2 catalytic domain, but also allosterically regulate them to bind to damaged single-strand DNA continually and impede the recruitment of DNA damage-related proteins. Thus, we tested the function of YHP-836 on the PARP-DNA complex via DNA trapping in MX-1 cells. As shown in Figure 1E, PARP1 accumulated in a dose-dependent manner in the chromatin section after YHP-836 treatment. At the concentration of 5 \( \mu \)M, YHP-836 strongly increased the level of PARP1 binding to chromatin. Similar results were also observed for PARP2. These results indicated that YHP-836 is a definitive PARP1/2 inhibitor.

The Cytotoxicity of YHP-836 In Vitro
As YHP-836 showed enzymatic inhibitory activity against PARP1 and PARP2, we investigated if YHP-836 could suppress cancer cell proliferation with BRCA mutations via
synthetic lethality. Cell cytotoxicity was detected in a panel of cancer cells exposed to YHP-836 or olaparib at 72 h. As shown in Figure 2A, YHP-836 inhibited cancer cell growth with BRCA1/2 mutation more effectively than those with wild type. YHP-836 in UWB1.289, a BRCA1-null human ovarian cancer cell, was much more sensitive than this cell-restored wildtype BRCA1 (UWB1.289 + BRCA1). Consistently, in MX-1 cells with the BRCA1 mutation and BRCA2 null and in MDA-MB-436 with the BRCA1 mutation, YHP-836 downregulated the levels of PAR, which was catalyzed by PARP1 and PARP2, consequently increasing the levels of γH2A, a DNA damage marker, and RAD51 protein, which is essential for homologous recombination (Figure 2B). In MCF-7 cells without BRCA mutation, YHP-836 could downregulate the PAR level with increased γH2A, but could not elevate the level of RAD51. Immunofluorescence results indicated that γH2A staining increased in MDA-MB-436 at concentrations of 1 and 5 μM (Figure 2C). RAD51 foci also accumulated after treatment. Consistent with immunoblotting results, RAD51 foci did not significantly increase after treatment in MCF-7 cells. Taken together, YHP-836 showed cytotoxicity in cancer cells with BRCA mutations.

**YHP-836 Induced Cell Cycle Arrest**

Moreover, cell cycle analysis was performed to evaluate the function of YHP-836 in tumor cells. Both MX-1 and MCF-7 cells were treated with YHP-836 or olaparib. In MX-1 cells, YHP-836 at the concentrations of 5 and 10 μM induced cell cycle arrest in the G2/M phase (Figures 3A,B and Supplementary Figure S1). Olaparib had a similar result. The cell cycle was slightly arrested in MCF-7 cells exposed to YHP-836 or olaparib at the same concentrations. Immunoblotting results showed that cyclin B1 and phosphorylation levels of Cdc2 and Cdc25c dose-dependently increased after YHP-836 treatment in both MX-1 cells and MCF-7 cells (Figure 3C). The levels of Cdc2 dramatically reduced in MX-1 cells exposed to YHP-836 at the concentration of 10 μM. These data indicated that YHP-836 induced cell cycle arrest at the G2/M phase.
YHP-836 Enhanced Chemotherapy Reagents Cytotoxicity In Vitro

It is reported that PARP inhibitors can potentiate the antitumor effect of chemotherapy agents such as temozolomide (TMZ) and topotecan (TPT). Thus, we detected the combination effect of YHP-836 with chemotherapy agents in MX-1 cells. As shown in Figure 4A and Supplementary Table S1, YHP-836 at the concentration of 2.5 μM enhanced the cytotoxicity of chemotherapy agents including TMZ, TPT, cisplatin (CDDP) and adriamycin (ADM) in MX-1 cells. The synergistic effects were similar to those of olaparib. We also explored these effects on other cells with or without BRCA mutation. At the concentration of 5 μM, YHP-836 exhibited good potential effects with TMZ on serial tumor cells (Figure 4B and Supplementary Table S2). Sequentially, we detected γH2AX levels combined with TMZ in MX-1 cells and MCF-7 cells. YHP-836 also increased the levels of γH2AX together with TMZ (Figure 4C), indicating the enhanced cytotoxicity of TMZ. Confocal analysis also demonstrated that DNA damage loci were accumulated in the nucleus as γH2AX and RAD51 were increased in the combination treatment (Figure 4D and Supplementary Figure S2).
Antitumor Activity of YHP-836 In Vivo

To confirm the antitumor activity of YHP-836 in vivo, we first characterized the pharmacokinetic (PK) properties in mice. As shown in Figure 5A, the maximum plasma concentration of YHP-836 reached about 2500 ng/ml after single oral administration at the dose of 25 mg/kg. However, it was rapidly eliminated, and the shelf-life was not long in mice. Based on the PK properties in mice, we used the MDA-MB-436 xenograft mice model to assess its antitumor activity and the mice were orally administered YHP-836 twice per day or olaparib once per day. As shown in Figures 5B,C, YHP-836 significantly repressed tumor growth in a dose-dependent manner with tumor growth inhibition (TGI) of 41.0, 74.6, and 94.0% for 50 mg/kg, 100 mg/kg, and 150 mg/kg, respectively. Olaparib at the dose of 50 mg/kg on single daily also exhibited good antitumor activity with 89.0% TGI. The tumor samples were collected to test the PAR level using ELISA assay. It was observed that YHP-836 dramatically inhibited the PAR synthesis in tumor tissues compared with the vehicle group (Figure 5D). During the experiment, YHP-836 did not cause significant reduction in body weight (Figure 5E).

As YHP-836 could enhance chemotherapy reagents’ cytotoxicity in vitro, we explored the combined antitumor activity of YHP-836 with TMZ in vivo as well. YHP-836 at the dose of 25 mg/kg once daily and TMZ at the dose of 50 mg/kg once daily or in combination were orally administered for 5 consecutive days, and the mice were under continuous observation. As shown in Figure 6A, the antitumor activity in the combination group was significantly better than that in the TMZ or YHP-836 group. This effect lasted until the end of the experiment. During the experiment, the body weight in the combination group decreased from day
1 to day 6 and recovered after compound withdrawal (Figure 6B). The tumors were collected for immunoblotting. The results showed that the levels of γH2AX increased in the combination group compared to the TMZ or YHP-836 group (Figure 6C). Next, we investigated the antitumor activity of YHP-836 in combination with MCF-7 mice xenograft models. As shown in Figure 6D, TMZ combined with YHP-836 showed better antitumor activity compared with the groups that received either TMZ or YHP-836 alone. The combination effects of YHP-836 with other chemotherapy agents including cisplatin (CDDP) or adriamycin (ADM) were evaluated in MX-1 mice xenograft models as well. As expected, YHP-836 also enhanced the antitumor activities of these chemotherapy agents (Figure 6E).

DISCUSSION

PARPs are attractive targets for cancer therapy. PARP inhibitors such as olaparib and pamiparib have been demonstrated to be indicative of monotherapy in patients with ovarian tumor harboring BRCA1 or BRCA2 mutations (Ledermann, 2016; Markham, 2021). The indicators also extended to breast cancers, prostate cancers, and pancreatic cancers with HR deficiency (Kamel et al., 2018; Litton et al., 2018; Charkes, 2019; Aschenbrenner, 2020; de Bono et al., 2020; de Bono et al., 2021). In this report, we presented a novel PARP inhibitor, YHP-836. The compound exhibited good cytotoxicity in cells harboring BRCA mutations. Oral administration of YHP-836 demonstrated remarkable antitumor activity in the MDA-MD-436 breast cancer xenograft model.

Enzymatic inhibition and DNA trapping are important parameters to evaluate the activity of PARP inhibitors. YHP-836 exhibited strong enzymatic inhibitory activity against PARP1 and PARP2, and dose-dependently suppressed the PAR levels in MX-1 cells. YHP-836 also strongly induced DNA trapping in MX-1 cells. Additionally, YHP-836 increased the DNA damage markers γH2A and RAD51 foci in vitro. These data characterize YHP-836 as a defined PARP inhibitor. In the MDA-MB-436 mice xenograft model with the BRCA1 mutation, YHP-836 indeed exhibited good antitumor activity by synthetic lethality.

In addition to monotherapy for cancers with HRD, PARP inhibitors are under clinical assessment in combination with other antitumor agents referred for chemotherapy, targeted therapy, and immunotherapy (Plummer et al., 2013; Norris et al., 2014; Matulonis and Monk, 2017; Tomao et al., 2017; Friedlander et al., 2019; Lee and Konstantinopoulos, 2019; Lampert et al., 2020; Palaia et al., 2020; Bizzaro et al., 2021; Waddington et al., 2021). Similar to other PARP inhibitors, YHP-836 also potentiates chemotherapy agents against various tumor cells. In MX-1 and MCF-7 breast cancer xenograft models, YHP-836 could enhance the antitumor activity of TMZ, CDDP, and ADM.

There are several limitations to using YHP-836. First, the selectivity of YHP-836 for PARP1 and PARP2 is not satisfactory. Although clinical benefits of PARP inhibitors have been proved, safety issues such as hematological toxicity need to be addressed (Farres et al., 2013; LaFargue et al., 2019). The next generation of PARP inhibitors is under development, targeting selective PARP1, to remedy the adverse events caused by inhibition of PARP2 (Curtin and Szabo, 2020; Dias et al., 2021; Johannes et al., 2021; Ngoi et al., 2021). Secondly, the PK characteristics of YHP-836 did not support its further development. The shelf-life is very short and maximum plasma concentration is not high, leading to poor bioavailability. Thus, the compound should be further modified.

In conclusion, we reported a novel PARP inhibitor YHP-836 with acceptable antitumor activity in vitro and in vivo.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethics Committee for Animal Experiments of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College.

AUTHOR CONTRIBUTIONS

TD designed the experiments. TD, ZZ, and LS conducted all the experiments. JZ and HY synthesized the compound. MJ drafted the manuscript. BX and XC reviewed and edited the manuscript and supervised the whole study as well. All authors approved the final version of the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 82073891).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.865085/full#supplementary-material
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