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Original Research

Development of poly ADP-ribose polymerase-1 inhibitor with anti-cervical carcinoma activity

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Abstract: This research aimed to discover and identify new poly ADP-ribose polymerase-1 (PARP) inhibitors with potent anti-cervical carcinoma activity, and then explore their potential biological roles on cervical carcinoma cell. For this purpose, we identified a new PARP inhibitor from a high-throughput virtual screening method and found that the compound strongly inhibited cervical carcinoma HeLa cell. Cell proliferation was evaluated by an MTT assay, and the cell apoptosis was assessed by flow cytometry. Results showed that PARP1 is a poly ADP-ribose catalyzing enzyme in eukaryotic cells, which is activated during DNA damage and repair, and plays an important role in DNA repair and cell apoptosis. Herein we report the first discovery of a new PARP inhibitor from a high-throughput virtual screening method, then the compound was measured its anti-cervical carcinoma activity by using an MTT assay, which suggested that the compound strongly inhibited HeLa cell proliferation, the IC_{50} value is 0.65 μM. In addition, the compound induced HeLa cell apoptosis in a dose-response manner. All these data suggested that the compound is a promising lead compound, which deserves further investigation. It is concluded that the compound discover herein is a promising PARP-1 inhibitor with potent anti-cervical carcinoma activity, which deserves further investigation.

Key words: Cervical carcinoma; PARP inhibitor; Proliferation; Apoptosis.

Introduction

Cervical cancer is the second-largest malignancy in women worldwide. It is a relatively common cancer and in many cases has very dangerous complications for women. This type of cancer occurs in cells in the lower part of the uterus that attach to the vagina. It occurs when cervical cells grow abnormally and multiply rapidly. For example, one of the triggers for cervical cancer is exposure to the human papillomavirus, which causes mutations in cervical cells. This mutation causes normal cells to become abnormal cells. Abnormal cells shrink to form a tumor. A tumor in the cervix can be benign or malignant (1-3). The incidence rate of cervical cancer has been increasing in recent years. To date, the main treatment strategy of cervical cancer includes surgery, radiotherapy and drug therapy (2, 3). The initial introduction of drug therapy in the field of cervical cancer treatment is aimed at patients with relapse and metastasis who failed in the first treatment (4). With the application of new molecular targeted drugs and immune checkpoint inhibitors, the quality of life of cervical cancer patients has been greatly improved (5).

Poly ADP ribose polymerase-1 (PARP-1) is a kind of cellular ribozyme that catalyzes the ribosylation of poly (ADP) in eukaryotic cells. It is a kind of protease with many important functions in cells (6, 7). At present, it has been found that PARP plays an important role in DNA repair and replication, transcriptional regulation of cell cycle and regulation of centromere function, as well as in cell apoptosis and other physiological processes (8). PARP is involved in the process of DNA repair and replication and plays an important role in repairing and maintaining gene integrity after DNA damage (9). In all types of DNA damage, DNA double-strand damage is one of the most serious types of human cell genome damage (10). If it could not be repaired or wrongly repaired, it will lead to cell apoptosis, chromosome translocation or deletion, gene mutation, and the formation of tumor genes, and eventually lead to the occurrence of the tumor (11). PARP includes 18 sub-types, such as PARP1, PARP2, PARP 3, vault-parp and tankyrases. Among them, PARP1 is the first protein found to have par modifying activity. In the whole PARP population, PARP1 accounts for the largest proportion (12-15). PARP1 plays a key role in maintaining DNA repair, gene transcription and expression, cell apoptosis and other biological functions. In recent years, research results show that PARP is also related to the occurrence and development of a variety of diseases, including many inflammatory diseases such as ischemia-reperfusion injury, inflammation, shock, diabetes, autoimmune diseases and so on. In addition, PARP is also involved in the occurrence and development of tumors (16-20).

In this study, a new compound namely TM1 was identified from a high-throughput virtual screening method, which was then measured anti-cervical carcinoma activity by using an MTT assay, suggesting that TM1 strongly inhibited PARP-1 protein and HeLa cell proliferation, the EC50 value is 79 nM for PARP-1, and

the IC50 value is 0.65 μM for TM1 against HeLa. In addition, the compound induced HeLa cell apoptosis in a dose-response manner.

Materials and Methods

Materials

TM1 was purchased from local agents with the purity is more than 95% confirmed by HPLC. DMEM (Dulbecco's modified eagle medium) cell culture medium was purchased from Abcam (Cambridge, MA, USA). TM1 was dissolved in 10 mM DMSO as a stock solution and diluted with culture medium. The maximum concentration of DMSO was used in the bioassay in the culture medium <0.1% (v/v). The HeLa cell line was achieved from the Cell Bank of the Shanghai Institute of Biochemistry & Cell Biology (Chinese Academy of Sciences, China). The cells were cultured with DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured at 37°C and 5% CO_2.

PARP-1 inhibition assay

To measures PARP-1 inhibitory activity, the previously described method with minor modification was used. Purified PARP (0.3 units, Gaithersburg) was added to 96-well plates containing assay buffer (50 mM Tris, pH 8.0, 25 mM $MgCl_2$, 1 mM DTT, 0.05 mM NAD, 1 μg/mL activated DNA) and various concentration of TM1 (1% DMSO). 15 min after mixing the plate at room temperature, the reaction was transferred to the UniFilter™-96, GF/B using the harvester for a 96-well plate. The filter was washed with 10% trichloroacetic acid once before and 2-times after this transfer. After drying for at least 2 h, 50 μL of liquid scintillator was added to each well. EC50 values for inhibition of PARP activity were calculated using GraphPad software.

Cell viability assay

The cell viability was evaluated by MTT analysis according to the manufacturer's instructions. HeLa cells (5000/well) were seeded into 96-well plates overnight, and then incubated with 20, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06 μM compound TM1 for 72 hours. Then remove the medium, add 100 μL of fresh medium containing 0.5 mg/mL MTT, and incubate for 4 hours. Then the supernatant was removed, and 100 μL of DMSO was added to each well. The absorbance of each well was measured at 570nm in the Microplate Reader. Then follow the above steps to determine the IC50 value.

Annexin V-FITC/PI double-staining assay

The death of apoptotic cells was determined by Annexin V fluorescein isothiocyanate (FITC). HeLa cell was seeded in 6-well plates at 2.0×10^5 cells per well and cultured for 24 h, and then treated with 4, 1, 1 and 0.5 μM compound TM1 for 12 hours. The cells were then collected and washed twice with cold PBS, and then stained with the kit according to the manufacturer's instructions. Lastly, the prepared cells were analyzed by the BD FACSCanto II flow cytometer (BD Biosciences, CA).

Molecular docking

The PDB code 1UK1 was selected as our binding model, compounds were docked to the binding pocket of 1UK1, and analyzed their binding behaviors. First, the crystal structure PARP-1 (1UK1) was prepared by a software preparation program, for example, H atoms were added to the amino acids, and charged by the AMBER7 FF99 method. Small molecules were prepared by hydrogenation and the use of three gravitational fields to optimize energy. The total binding score was obtained from the docking of the prepared molecule and the protein.

Statistics

In this investigation, all experiments used SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA) for statistical analysis. Tukey's HSD (Honesty Significant Difference) test was used in conjunction with ANOVA to find means that are significantly different from each other. $P<0.05$ was considered statistically significant.

Results

Identification of PARP-1 inhibitor through a docking-based virtual screening method

First, the crystal file of PARP-1-ligand complex (PDB entry 1UK1) was selected as a template for molecular docking. The complex was downloaded from the protein data bank (http://www.rcsb.org/pdb), then was analyzed and optimized using Sybyl-X 2.1 software package. Secondly, a commercially available small molecules library with 5000 compounds was downloaded from the ZINC database. Then, small molecules were prepared by the Ligand Structure Preparation Procedure in the software. Thirdly, the optimized compounds were docked into the binding pocket of the ligand in PARP-1. From this workflow, we successfully identified one compound, namely TM1, which strongly inhibited PARP-1, the EC_{50} value is 79.3 nM, as shown in Figure 1.

The identified inhibitors reduced HeLa cancer cells proliferation

With TM1 showing promising PARP-1 inhibitory

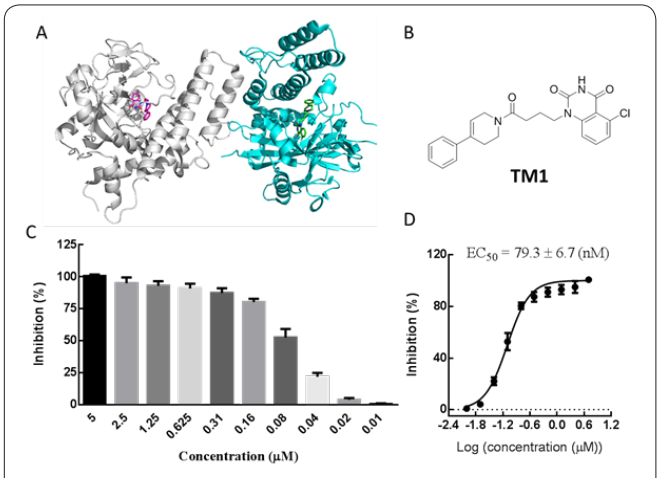

Figure 1. TM1 inhibited PARP-1 protein. (A) Structures of PARP-1. PARP-1 is a dimer in solution. (B) Chemical structures of the identified compound TM1. (C) The bar graph of TM1 inhibited PARP-1. (D) EC_{50} curve of **TM1** dose-response inhibited PARP-1.

potential, the next question we have to solve is whether the identified compounds will reduce the growth of cancer cells. Therefore, we tested the anticancer activity of TM1 on HeLa cells using MTT analysis. As shown in Figure 2, TM1 displayed good antiproliferative activity from 20 to 1 μM, the IC_{50} value was 0.65 μM, as shown in Figure 2.

The identified inhibitor-induced apoptosis of HeLa

In order to explore the cell death mode of HeLa cancer cells, TM1 was used to induce apoptosis of HeLa cancer cells, and then they were examined using Annexin V-FITC/PI FACS analysis. As shown in Figures 3 and 4, the percentage of apoptosis of LaLa cells treated with TM1 at 0.5, 1 and 4 μM for 24 hours was 6.43%, 16.1% and 43.4%, respectively. This indicates that TM1 induces apoptosis of HeLa cancer cells in a dose-dependent manner.

Discussion

PARP1 is a kind of protease with polyadenyldiphosphate ribosyl (PAR) catalytic activity in eukaryotic cells. It is activated when DNA breaks and participates in DNA repair and plays an important role in DNA repair and apoptosis (21). The loss of PARP1 can make DNA susceptible to cell damage and induce tumors. The current results show that the overexpression of PARP1 in tumor cells suggests that the use of PARP1 inhibitors in clinical practice can inhibit the repair activity of PARP1 after DNA damage, so as to inhibit the participation of PARP1 in the mediated repair of cell DNA damage, which has a certain effect on tumor treatment (22). Although the research of PARP in the occurrence and development of tumor has a certain foundation at present, especially in the treatment of BRCA gene mutation tumor has a good effect. However, there are still many problems. We should further study the role of PARP in DNA damage repair and tumor development.

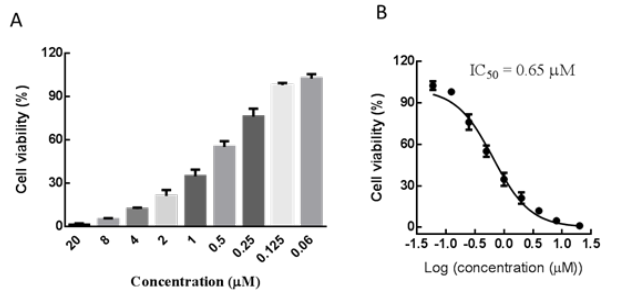

Figure 2. TM1 inhibited HeLa proliferation. (A) TM1 dose-response inhibited the growth of HeLa. (B) The IC_{50} curve of TM1 inhibited HeLa proliferation, which was measured by using an MTT assay.

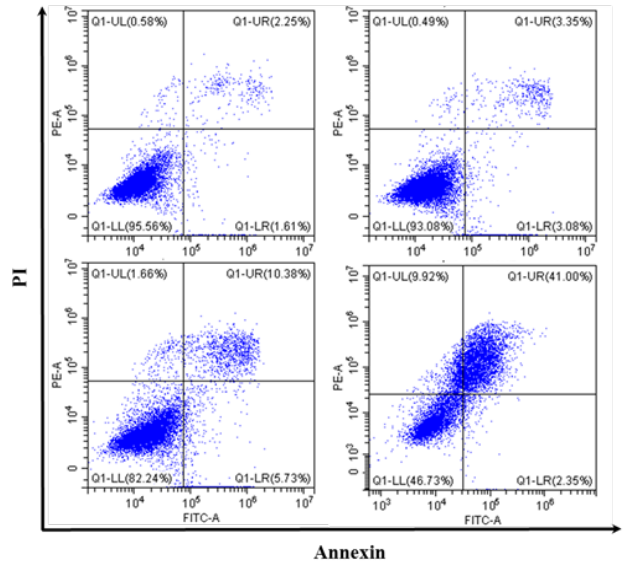

Figure 3. TM1 induced HeLa apoptosis in a dose-response manner. The cells were treated with TM1 for 24 h, then stained with FITC Annexin V/ PI. Cells in the lower right quadrant indicate PI-positive/Annexin V negative, late apoptotic, or necrotic cells. The cells in the upper right quadrant indicate Annexin V-positive/PI positive, early apoptotic cells.

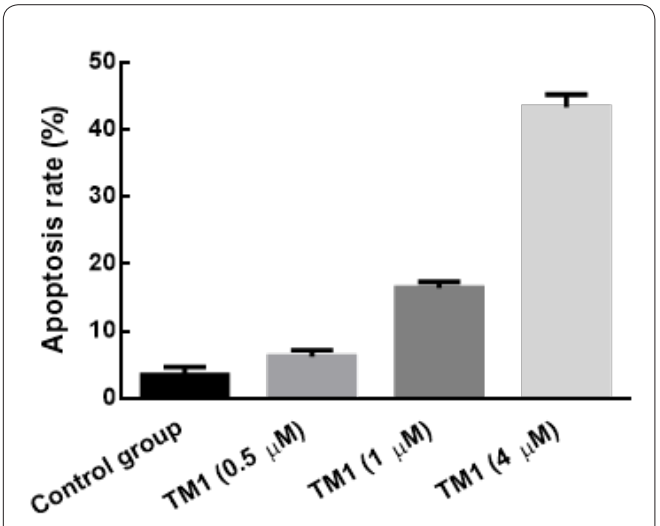

Figure 4. The apoptosis rates induced by TM1.

The clinical development of PARP-1 inhibitors was not smooth but was ultimately successful advance into clinical use. Iniparib was the first PARP-1 inhibitor tested in phase III clinical trials for triple-negative breast cancer (TNBC) (23). Unfortunately, the expanded phase III clinical trial of the compound was declared a failure in 2011. Phase II clinical trials of non-small cell lung cancer (NSCLC) also did not show an improved objective response. Subsequent studies on the reasons for these failures proved that iniparib is not a true PARP-1 inhibitor because it has a poor inhibitory effect on PARP-1 (IC50 value is approximately equal to 100μM) and cannot selectively kill homologous recombination repair (HRR)-insufficient cells (24). Later in the same year, AstraZeneca announced that it would discontinue its planned Phase III clinical trial of PARP-1 olaparib because of its poor efficacy in the Phase II study of ovarian cancer. However, positive results were obtained from the subsequent retrospective analysis of phase II clinical trial data. When reassigning patients based on BRCA status, the median progression-free survival (PFS) of patients with BRCA mutations in the two treatment groups was significantly longer than that of the placebo group. However, the differences between the wild-type BRCA patient groups were smaller. The results indicated that patients with BRCA mutations may benefit from olaparib treatment, and demonstrated that olaparib monotherapy is an effective targeted therapy for platinum-sensitive recurrent ovarian cancer

with BRCA mutations.

We downloaded the crystal file of PARP-1-ligand complex (PDB entry 1UK1) from protein data bank (http://www.rcsb.org/pdb), which was then analyzed and optimized by Sybyl-X 2.1 software package. Then a commercially available small molecules library with 5000 compounds was downloaded from the ZINC database, and prepared by the Ligand Structure Preparation Procedure in the software. The optimized compounds were docked into the binding pocket of the ligand in PARP-1. From this workflow, we successfully identified one compound, namely TM1, which dose-response inhibited PARP-1, the EC50 value is 79.3 nM, as shown in Figure 1.

Since the TM1 displaying a promising PARP-1 inhibitory potency, we have the interest to test whether the compound inhibited the HeLa cell proliferation. So HeLa cells were treated with TM1 at the various concentrations (20, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06 μM) for 72 h, then the cell viability was measured by using an MTT assay. As shown in Figure 2, the TM1 dose-response inhibited the growth of HeLa, the IC50 value was 0.65 μM.

To explore the mode of cell death for HeLa cells, TM1 was used to induce HeLa cancer cell apoptosis, which then was examined by using Annexin V-FITC/PI FACS assay. As shown in Figures 3 and 4, the percentages of apoptosis for HeLa cells treated with TM1 in 0.5, 1 and 4 μM for 24 h were 6.43, 16.1, and 43.4 %, respectively. This indicated that TM1 induced the apoptosis of HeLa cancer cells in a dose-dependent manner. Collectively, our data shown here indicate that TM1 is a promising anti- cervical carcinoma agent, and targeting PARP-1 with a potent enzymatic inhibitory activity.

In this study, we have discovered a PARP-1 inhibitor through docking-based virtual screening, which was then confirmed by an enzymatic assay. The compound inhibited PARP-1 with an EC_{50} value of 79.3 nM, and reduced the HeLa cell growth with an IC_{50} value of 0.65 μM, indicating the compound is a promising PARP-1 inhibitor, which deserves further study. Further investigation is ongoing and the result will report in due course.

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