

# A novel all-*trans* retinoic acid derivative 4-amino-2-trifluoromethyl-phenyl retinate inhibits the proliferation of human hepatocellular carcinoma HepG2 cells by inducing G0/G1 cell cycle arrest and apoptosis via upregulation of p53 and ASPP1 and downregulation of iASPP

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**Abstract.** 4-Amino-2-trifluoromethyl-phenyl retinate (ATPR), a novel all-*trans* retinoic acid (ATRA) derivative, was reported to function as a tumor inhibitor in various types of cancer cells *in vitro*. However, little is known concerning its antitumor effect on human hepatocellular carcinoma (HCC) HepG2 cells. The aims of the present study were to investigate the effects of ATPR on the proliferation of HepG2 cells and to explore the probable mechanisms. A series of experiments were performed following the treatment of HepG2 cells with ATRA and ATPR. MTT and plate colony formation assays were used to measure the cell viability. To confirm the influence on proliferation, flow cytometry was used to detect the distribution of the cell cycle. Apoptosis was observed by Hoechst staining and flow cytometry. In addition, to characterize the underlying molecular mechanisms, immunofluorescence was applied to observe the distribution of p53. The transcription and translation levels of p53 were analyzed by real-time quantitative RT-PCR (qRT-PCR) and western blotting. The expression levels of murine double minute 2 (MDM2), apoptosis stimulating proteins of p53 (ASPP), cell cycle- and apoptosis-associated proteins were detected by western blotting. After HepG2 cells were incubated with ATRA and ATPR, the viability of the HepG2 cells

was inhibited in a dose- and time-dependent manner. As well, ATPR significantly suppressed HepG2 cell colony formation and arrested cells at the G0/G1 phase, while ATRA had no obvious effects. Both Hoechst staining and flow cytometry unveiled the apoptosis of HepG2 cells. Moreover, the fluorescent density of p53 was higher in the nuclei after exposure to ATPR than that in the ATRA group. HepG2 cells treated with ATPR showed elevated mRNA and protein levels of p53 when compared with these levels in the ATRA-treated cells. Western blotting showed that ATPR increased ASPP1, p21 and Bax expression and decreased MDM2, iASPP, cyclin D and E, cyclin-dependent kinase 6 (CDK6) and Bcl-2 expression, while CDK4 and ASPP2 expression were scarcely altered. Consequently, ATPR exerted a better inhibitory effect on the proliferation of HepG2 cells than ATRA through increased expression of p53 and ASPP1 and downregulation of iASPP, thereby resulting in G0/G1 cell cycle arrest and apoptosis.

## Introduction

Hepatocellular carcinoma (HCC), one of the most aggressive malignancies, is the third leading cause of cancer-related death worldwide (1,2). Annually, ~750,000 new cases are diagnosed and more than 75% of cases occur in the Asia-Pacific region, and the incidence of HCC is still increasing in the US and Europe (3-5). To date, the treatment modality is largely reliant on surgical resection or liver transplantation as HCC is notoriously resistant to chemotherapy and other systemic treatment options. Moreover, the prognosis of HCC is extremely poor due to hepatic metastasis (6). Therefore, it is urgent to develop an effective therapeutic method for HCC treatment.

Disturbance of the balance between proliferation and apoptosis is a fundamental hallmark of cancer development (7,8). The p53 tumor-suppressor protein, also known as the 'guardian of the genome', plays a pivotal role in inducing apoptosis

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or cell cycle arrest to prevent cell proliferation in response to stress such as DNA damage, hypoxia and activation of oncogenes. Conversely, inactivation or mutation of the *p53* gene contributes to abnormal growth due to loss of control in cell cycle regulation or failure to trigger apoptosis, which could in turn, leads to carcinogenesis (9-11). In the absence of stress, *p53* is a short-lived protein of extremely low levels, and is negatively regulated by murine double minute 2 protein (MDM2) (12,13). On the contrary, MDM2 expression is induced by *p53*. Hence, MDM2 and *p53* can interact to form an autoregulatory loop in which *p53* activates expression of its own inhibitor (13,14). Upon receiving the upstream stress signals, *p53* is elevated to mediate an array of apoptosis and cell cycle genes (12,15). Commonly, *p53* usually remains in its wild-type configuration in at least 10% of human cancers displaying unusual MDM2 overexpression (16). Furthermore, it is well documented that inhibitors or knockdown of MDM2 can enhance the activity and expression of *p53*, by which to recover its tumor-suppressing effects (17-20).

Various elements are reported to be involved in cell cycle transition, including cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). CDKs are a family of serine/threonine protein kinases playing pivotal roles in such a process in combination with various endogenous cyclins (21,22). *P21*, a *p53* downstream gene, is known as a CKI to stop the replication of damaged DNA (22,23). However, it should be noted that arrested cells are not necessarily protected from apoptosis (10). It has been reported that *p53* activates the apoptotic machinery by regulating Bax function and mitochondrial integrity (24). In addition, restoration of *p53* in murine myeloid leukemia cell line M1 was associated with Bax protein elevation accompanied by a simultaneous decrease in Bcl-2 (25). Furthermore, the apoptotic function of *p53* was found to be regulated by apoptosis-stimulating proteins of the *p53* (ASPP) family consisting of ASPP1, ASPP2 and iASPP. The similar sequences at the C-terminus of the three members which is the preferred binding site for *p53* determines that ASPP1, ASPP2 and iASPP could compete with each other to bind to *p53* (26). In regards to the roles of these proteins in apoptosis, ASPP1 and ASPP2 enhance *p53*-mediated apoptosis, while iASPP inhibits the process (26-28). The abnormal expression of ASPP members may explain the failure to induce apoptosis of some tumors expressing wild-type *p53* (26,29-31). To date, few studies have been carried out to detect the expression and possible roles of the ASPP family in the pathogenesis of HCC.

All-*trans* retinoic acid (ATRA), a major active metabolite of vitamin A, is reported to induce cell differentiation of acute promyelocytic leukemia (APL) and achieve complete remission (32,33). Subsequently, increasing studies have been conducted to determine the efficiency of ATRA on solid tumors. These studies revealed that it exerted anticancer effects on liver, breast and lung cancer (34-36). To the best of our knowledge, the activities of ATRA mainly rely on two nuclear receptor families including retinoid receptors (RARs) and retinoid X receptors (RXRs). RARs act as retinoid-inducible transcriptional factors and form heterodimers with RXRs, which regulate the expression of genes involved in cell growth, differentiation, migration and apoptosis (37,38). However, in clinical practice, the application of ATRA is

far from satisfactory as it may upregulate gene transcription of vascular endothelial growth factor (VEGF), resulting in angiogenesis and subsequent cancer growth (39). In addition, ATRA resistance and high toxicity also hinder its application in cancer treatment (40-42). Although As<sub>2</sub>O<sub>3</sub> was reported to rescue most relapsed/refractory patients treated with ATRA, its severe side-effects have limited its long-term use (43). Hence, it is essential to develop a novel all-*trans* retinoid acid derivative with higher sensitivity and efficiency.

4-Amino-2-trifluoromethyl-phenyl retinate (ATPR), designed and synthesized by the College of Pharmacy, Anhui Medical University, is a novel retinoid derivative with lower toxicity than ATRA. Our previous studies revealed that ATPR was superior to ATRA in terms of inhibiting cellular migration as well as promoting apoptosis and differentiation in lung, breast or gastric cancer cell lines (44-46). However, the effects of ATPR on HepG2 cell proliferation remain unknown.

Based on the evidence described above, in the present study, we aimed to verify the potential effects of ATPR on the proliferation of HCC cell line HepG2 for the first time and elucidate the involvement of *p53* and the ASPP family in cell proliferation through analysis of cell cycle progression and apoptosis.

## Materials and methods

**Cell lines and reagents.** Human HCC cell line HepG2 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). ATRA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ATPR was synthesized and kindly provided by the School of Pharmacy, Anhui Medical University (China) and the purity was 99.66%. Dulbecco's modified Eagle's medium (DMEM; low glucose) was purchased from Gibco Life Technologies (Carlsbad, CA, USA). Newborn calf serum was obtained from Zhejiang Tianhang Biological Technology Co., Ltd. (China). Primary antibodies: mouse anti-*p21*, -MDM2 and - $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-*p53*, -ASPP1, -ASPP2, -iASPP, -CDK4, mouse anti-cyclin D, -cyclin E and -CDK6 were purchased from Lianshi Biological Technology Co., Ltd. (China). Mouse anti-Bcl-2 and rabbit anti-Bax and all secondary antibodies were purchased from Millipore (Billerica, MA, USA). Fluorescein (FITC)-conjugated affinitive goat anti-rabbit IgG and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from ZSGB-BIO (Beijing, China). Coulter DNA detection kit was purchased from Beckman Coulter (Miami, FL, USA). Annexin V apoptosis detection kit FITC was purchased from eBioscience (San Diego, CA, USA). The BCA protein determination kit and ECL reagent were purchased from the Beyotime Institute of Biotechnology (China). ATRA and ATPR were dissolved in DMSO at a concentration of 20 mM, and were stored at -20°C.

**Cell culture.** HepG2 cells were maintained in DMEM supplemented with 10% newborn calf serum and antibiotics containing penicillin (100 U/ml) and streptomycin (100 U/ml) in a humidified 5% CO<sub>2</sub> incubator at 37°C. The medium was

changed every 2 or 3 days and cells were passaged when their density reached 80-90% confluency. To assess the cytotoxicity of ATRA and ATPR, HepG2 cells were incubated in medium with the indicated concentrations of ATRA and ATPR. In order to exclude a potential bias of the solvent DMSO, the DMSO group operated as the vehicle control, in which the volume of DMSO in the culture medium equaled that contained in the medium with 25  $\mu$ M ATRA or ATPR. The cells exposed to normal medium were used as the cell control.

**Cell proliferation assessment.** MTT assay was used to analyze the cell viability. HepG2 cells ( $5 \times 10^3$  cells/well) were seeded into 96-well plates and treated with a desired dose of ATRA or ATPR (25  $\mu$ M) for different times (1-4 days) or treated with a dose range of 5-100  $\mu$ M of ATRA or ATPR for 48 h. At each time point, 20  $\mu$ l of 5 mg/ml MTT was added into each well and the cells were incubated for an additional 4-6 h. Culture medium was then removed and 100  $\mu$ l DMSO was further added into each well to solubilize the formazan crystals for 15 min. The optical density (OD) value at a wavelength of 570 nm was then measured by an absorbance microplate reader (ELX800; BioTek, Winooski, VT, USA). Cell inhibition rate was calculated by the following formula: Inhibition rate (%) = (OD<sub>570</sub> of the cell control group - OD<sub>570</sub> of the experimental group)/OD<sub>570</sub> of the cell control group  $\times$  100%. The experiment was repeated at least three times to detect the cell growth.

**Plate colony formation assay.** The colony formation ability of the HepG2 cells *in vitro* was measured by plate colony formation assay. HepG2 cells at logarithmic growth were plated in 6-well plates. Twenty-four hours after plating, the cells were treated with various concentrations (5 and 25  $\mu$ M) of ATRA and ATPR, and then harvested as single cell suspensions. Approximately 2,000 cells/well were seeded in a new 6-well plate, and the plate was incubated for 7-10 days until the colonies were clearly visible to the naked eye. Then, the medium in the plate was discarded and cells were washed with phosphate-buffered saline (PBS) two times, fixed by 4% paraformaldehyde and stained with 1% crystal violet. The colonies ( $\geq 50$  cells) were counted in five random visual fields and photographed.

**Flow cytometry for cell cycle analysis.** Flow cytometry was performed to analyze the distribution of the cell cycle. HepG2 cells were seeded into 6-well plates equally and incubated for 24 h in serum-free medium to synchronize the cell cycle progression. Then, the cells were treated with 25  $\mu$ M ATRA and ATPR for 48 h. After treatment, the adherent HepG2 cells were harvested, washed twice with ice-cold PBS and resuspended with 100  $\mu$ l PBS, and then counted and diluted to  $1 \times 10^6$  cells/ml. Cells were stained with 1 ml of hypotonic propidium iodide (PI) solution (50 mg/ml PI, 0.1% Triton X-100, 0.1% sodium citrate) in the presence of 1% RNase A for 30 min at 37°C in the dark. Then, flow cytometry (Becton-Dickinson, San Diego, CA, USA) was used to detect cell cycle distribution. Finally, G0/G1, S and G2/M phase cells were analyzed using ModFIT software.

**Hoechst 33258 staining.** Hoechst 33258 staining was used to visualize nuclear changes to indicate apoptosis. HepG2 cells

were cultured on sterile circle coverslips in 6-well plates and allowed to adhere overnight and then incubated with ATRA and ATPR (5 and 25  $\mu$ M) for 48 h. Cells were sequentially washed with PBS three times, fixed with 0.5 ml/well of 4% paraformaldehyde for 10 min, and washed again with PBS two times, finally followed by the addition of 0.1 ml of a DNA-specific fluorescent dye, Hoechst 33258 for 5 min. After being washed with PBS, the slides were mounted with aqueous-based anti-fade mounting agent and then examined and photographed under an upright fluorescence microscope.

**Flow cytometric analysis of apoptosis.** Flow cytometry was used to further confirm the occurrence of apoptosis. After treatment for 48 h, the cells were collected, washed twice with PBS, and resuspended in 100  $\mu$ l of Annexin V binding buffer at  $1 \times 10^6$  cells/ml. Then, cells were stained with 5  $\mu$ l of Annexin V-FITC and incubated for 15 min in the dark. Next, 400  $\mu$ l of Annexin V binding buffer and 5  $\mu$ l of PI were added, and the cells were immediately analyzed by flow cytometry.

**Immunofluorescence assay.** The cellular fluorescent density of p53 was observed by immunofluorescence assay. HepG2 cells were cultured in 12-well plates with sterile circle coverslips and allowed to adhere overnight, followed by the treatments (ATRA and ATPR, 25  $\mu$ M) for 48 h. Cells were washed with PBS three times, fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100. To minimize the non-specific binding of the antibody, cells were blocked with 5% bovine serum albumin (BSA) for 2 h at room temperature, then incubated with p53 primary antibody (1:50 dilution in 5% BSA) overnight at 4°C, washed and incubated with FITC-conjugated secondary antibody (1:100 dilution in PBS) for 2 h at room temperature away from light. DAPI was used to stain nuclei for 5 min and finally the coverslips with cells were mounted with aqueous-based anti-fade mounting medium. Images of the stained cells were visualized by a fluorescence microscope (Leica DMI4000 B).

**Western blot analysis.** HepG2 cells exposed to ATRA and ATPR (5 and 25  $\mu$ M) for 48 h were washed with cold PBS three times and lysed in lysis buffer (Tris-HCl, pH 7.14, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM leupeptin, 1 mM PMSF) on ice for 30 min. The protein extracts were quantified with a BCA kit and resolved in SDS sample buffer. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with blocking buffer [TBST buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.05% Tween-20), 5% skimmed milk] for 2 h at room temperature and incubated with diluted primary antibodies overnight at 4°C with the indicated primary antibody against p53 (1:500), MDM2 (1:250), ASPP1 (1:500), ASPP2 (1:500), iASPP (1:500), p21 (1:250), cyclin D (1:1,000), cyclin E (1:1,000), CDK4 (1:1,000), CDK6 (1:1,000), Bcl-2 (1:500), Bax (1:1,000) and  $\beta$ -actin (1:1,000) respectively, then exerted to incubation with the correspondingly diluted horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. The protein bands were visualized with enhanced chemiluminescence (ECL) reagent. Finally,

blots were exposed to Kodak film. Negatives were scanned using a ScanPrisa1240OUT (Acer, China). The data were quantified from three independent experiments using Quantity One software.

**Real-time quantitative PCR (qRT-PCR).** Total RNA was extracted using the TRIzol procedure (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 4  $\mu$ g RNA using the reverse transcription system (Takara, China). The resulting 4  $\mu$ g cDNA was amplified by UltraSYBR Mixture (CWBiotech, China) using a Real-Time PCR system (Thermo, USA) with the following primer sets: p53, 5'-gttcc-gagagctgaatgagg-3' (forward) and 5'-ttatggcgggaggtagactg-3' (reverse); GAPDH, 5'-aggctcgagagtgcaacggatttg-3' (forward) and 5'-cctggaagatggtgatgggat-3' (reverse). All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). GAPDH served as a reference gene. Real-time PCR cycle program was as follows: an initial denaturation at 95°C for 30 sec, followed by 40 cycles of a 5-sec extension step at 95°C, and annealing for 30 sec at 60°C. Results are shown as the mean normalized values of cDNA levels among each group to the reference gene.

**Statistical analysis.** All data are expressed as mean  $\pm$  standard deviation (SD). SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Data from multiple groups were analyzed by one-way ANOVA, followed by Dunnett's or LSD t-tests. A value of  $P < 0.05$  was considered statistically significant.

## Results

**ATPR significantly inhibits HepG2 cell proliferation in vitro.** The effects of ATRA and ATPR on the proliferation of HepG2 cells were determined by MTT assay, plate colony formation and flow cytometric analysis, respectively. As shown in Fig. 1A and B, ATPR significantly inhibited the cell viability of the HepG2 cells in a dose- and time-dependent manner. The inhibition rate of ATPR was much higher than that of ATRA at the same dose. According to the data, the  $IC_{50}$  value of ATPR was 25.723  $\mu$ M. Finally, two concentrations (5 and 25  $\mu$ M) were chosen for the following assays. In addition, plate colony formation assay was carried out to evaluate the colony formation of the HepG2 cells *in vitro*. The results indicated the ATPR induced a decrease in the density and size of the colonies in a dose-dependent manner (Fig. 1C). Taken together, it is reasonable to conclude that ATPR induced marked inhibition of HepG2 cell proliferation.

Cell cycle analysis was used to further confirm the growth inhibitory effects of ATPR on HepG2 cells. As shown in Fig. 1D ATPR (25  $\mu$ M) caused a significant accumulation of G0/G1 phase cells. The percentage of cells arrested in the G0/G1 phase increased from  $35.37 \pm 1.59$  to  $67.11 \pm 2.54\%$  and cells in the S phase decreased from  $34.52 \pm 2$  to  $25.73 \pm 1.53\%$  after ATPR interference (25  $\mu$ M) compared with those of the vehicle group. No significant difference was observed in these percentages in the ATRA group (25  $\mu$ M) compared with the vehicle group (Fig. 1D). Therefore, we speculated that ATPR may suppress HepG2 proliferation by arresting cells in the G0/G1 phase.

**ATPR induces apoptosis in HepG2 cells.** The apoptotic morphology of the cells was assessed with Hoechst staining. The nuclei were homogeneous in the control group, while in the ATPR groups (5 and 25  $\mu$ M), the nuclei were condensed with enhanced fraction, and appeared as obvious apoptotic bodies (Fig. 2A). Moreover, HepG2 cells were double-stained with Annexin V and PI and analyzed through flow cytometry. The fourth- and the first-quadrant cells represented early apoptotic cells (Annexin V<sup>+</sup>/PI<sup>-</sup>) and late apoptotic cells (Annexin V<sup>+</sup>/PI<sup>+</sup>), respectively. As shown in Fig. 2B, the apoptosis rate in the ATPR group (25  $\mu$ M) was significantly increased when compared with the vehicle group. These results demonstrated that HepG2 cells underwent apoptosis following ATPR treatment.

**ATPR increases expression of p53 in the HepG2 cells.** To illuminate the participation of p53 in ATPR-inhibited proliferation, the level of p53 was examined. In the immunofluorescence assay, p53 was labeled with green fluorescent protein and the fluorescence was evenly distributed in the nuclei in the control group (Fig. 3A). After treatment with ATPR (25  $\mu$ M), the fluorescent density of p53 was higher in the nuclei compared with that noted in the control and ATRA (25  $\mu$ M) groups (Fig. 3A). Meanwhile, western blotting and qRT-PCR were applied to detect the protein and mRNA expression of p53. As shown in Fig. 3B, p53 protein expression was significantly activated in a dose-dependent manner after exposure to ATRA and ATPR for 48 h, particularly in the ATPR groups. qRT-PCR analysis displayed that the mRNA level of p53 was markedly elevated in the ATRA (25  $\mu$ M) and ATPR (5 and 25  $\mu$ M) groups (Fig. 3C). In contrast, no statistical significance was noted in regards to the mRNA level in the ATRA group (5  $\mu$ M) compared to the vehicle group. These outcomes demonstrated that ATPR increased p53 accumulation in the HepG2 cells.

**ATPR alters the expression of MDM2 and ASPP family proteins in the HepG2 cells.** We next determined the expression of MDM2, one of the main factors causing p53 degradation, and the ASPP family, specific regulators of p53, in HepG2 cells. As shown in Fig. 4A, ATPR inhibited MDM2 expression in a dose-dependent manner compared with control group. In addition, ATPR induced elevation of ASPP1 expression and downregulation of iASPP expression in the HepG2 cells. Nevertheless, no statistical difference was noted in regards to the ASPP2 expression in the HepG2 cells subject to ATPR compared with the control group (Fig. 4B). Therefore, MDM2, ASPP1 and iASPP proteins may participate in the mechanisms mediating ATPR-inhibited proliferation.

**Effects of ATPR on cell cycle-related proteins in HepG2 cells.** To further examine the mechanisms of G0/G1 cell cycle arrest induced by ATPR in HepG2 cells, western blotting was conducted to determine the expression of cycle-related proteins p21, cyclin E and D, CDK4 and CDK6 that are critical for G1-S progression. The results revealed that the expression of p21 was increased, while that of cyclin E and D, and CDK6 was decreased in the ATPR groups (Fig. 5A and B). No statistical difference was observed in the expression of CDK4 in any treatment groups compared with the control group. This indicated that induction of G0/G1 phase arrest by ATPR may

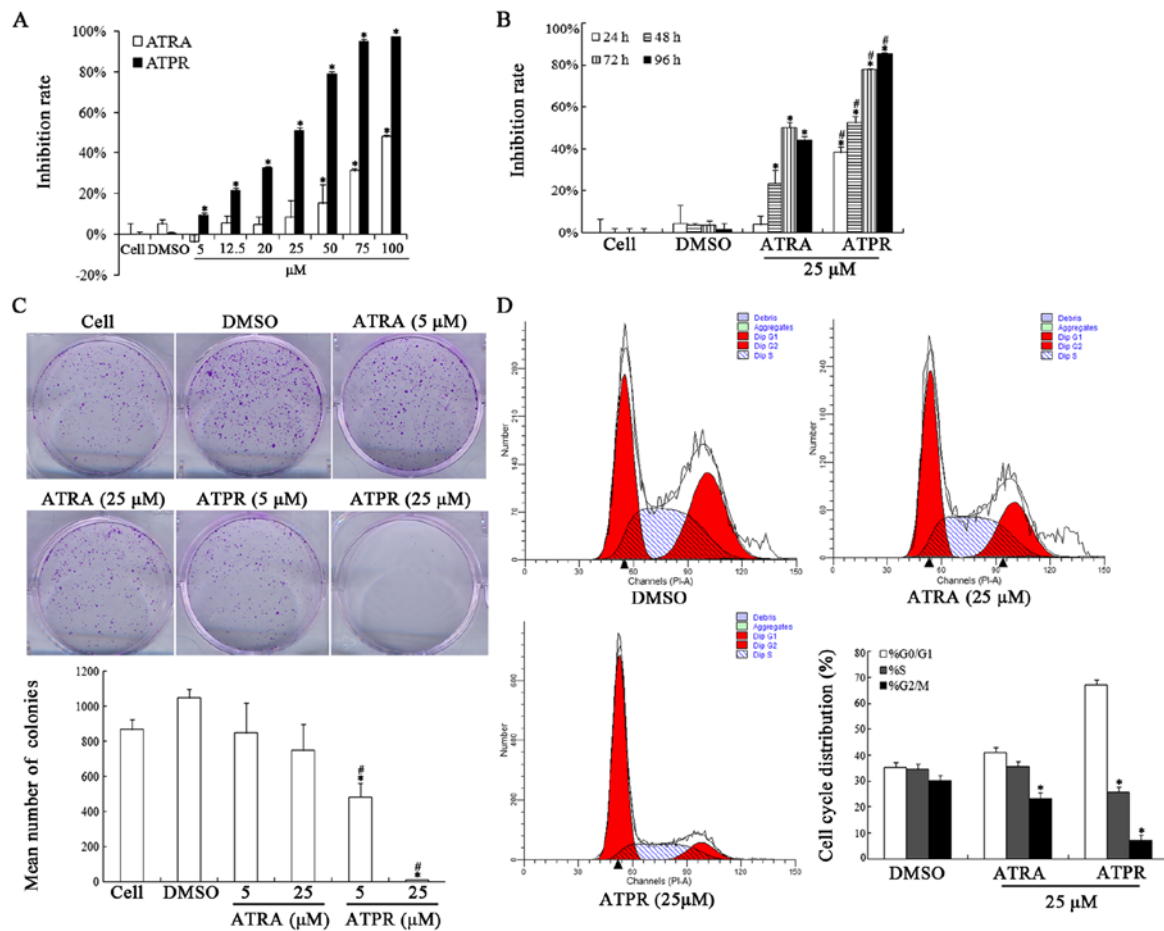


Figure 1. Effects of ATPR on the proliferation of HepG2 cells *in vitro*. Cells were incubated with the indicated concentrations of ATRA and ATPR for the indicated times. (A) The dose-effect of ATPR on HepG2 cell viability was measured using MTT assay. (B) The time-effect of ATPR on HepG2 cell viability was determined using MTT assay. (C) Colony formation map and mean numbers of colonies. (D) Cell cycle map and the distribution of the cell cycle. Cell cycle distribution was analyzed by PI staining and quantified by ModFit. ATPR markedly arrested HepG2 cells at the G0/G1 phase. All values are presented as mean  $\pm$  SD. \* $p$ <0.05 vs. the control group. # $p$ <0.05 vs. the ATRA groups.

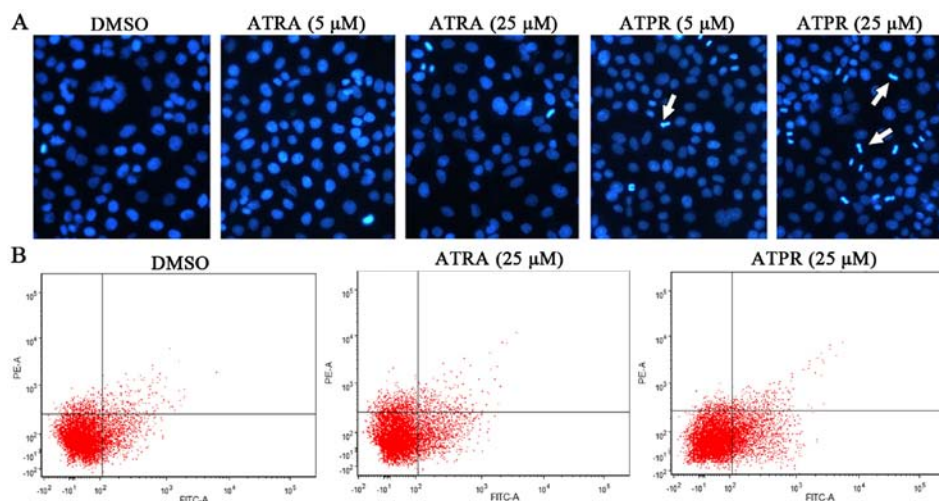


Figure 2. Effect of ATPR on the apoptosis of HepG2 cells. HepG2 cells were treated with ATRA and ATPR for 48 h. (A) Apoptotic morphologic observation. Apoptosis was examined under an inverted microscope at a magnification of x100. Typical apoptotic changes of the nucleus (chromatin condensation, nuclear fragmentation and appearance of apoptotic bodies) are indicated by arrows. (B) Cytometric analysis with Annexin V and PI double staining. Apoptosis was detected by flow cytometry in HepG2 cells treated with 25  $\mu$ M ATPR for 48 h.

be associated with upregulation of p21 and downregulation of cyclin E and D, and CDK6.

*Effects of ATPR on expression of Bcl-2 family proteins in the HepG2 cells.* To evaluate the involvement of the Bcl-2 family in

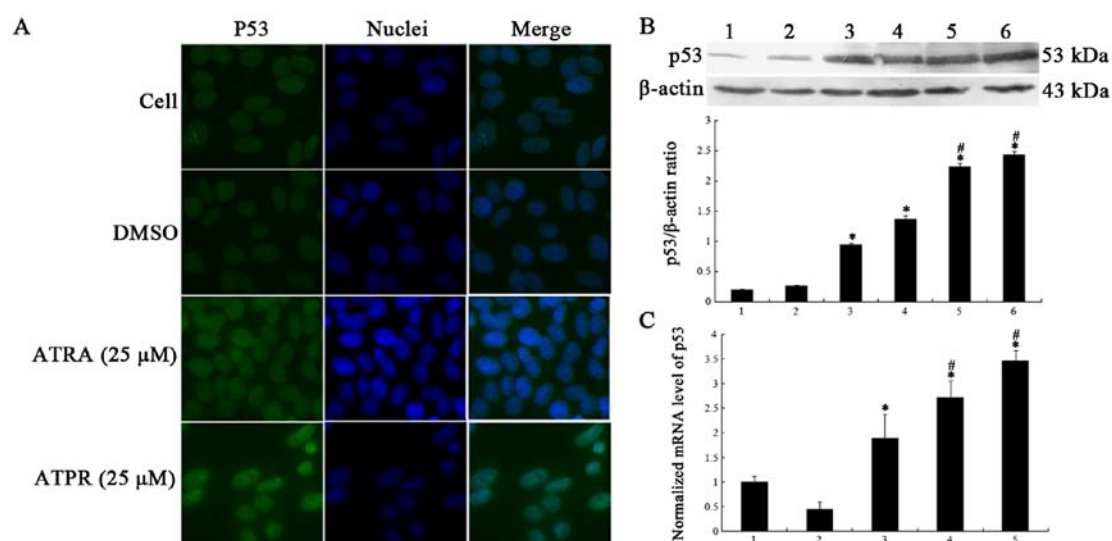


Figure 3. Effects of ATPR on p53 expression in HepG2 cells. Cells were treated with 5 or 25 μM ATRA and ATPR for 48 h. (A) ATPR elevated p53 accumulation in the nuclei. Immunofluorescence microscopy showed that p53 was accumulated in the nuclei in the ATPR-treated group. (B) Western blot analyses revealed that ATPR increased more protein expression of p53 than ATRA. Lane 1, cells; lane 2, DMSO; lane 3, 5 μM ATRA; lane 4, 25 μM ATRA; lane 5, 5 μM ATPR; and lane 6, 25 μM ATPR. (C) ATPR markedly upregulated the mRNA levels of p53 in the HepG2 cells. Lane 1, DMSO; lane 2, 5 μM ATRA; lane 3, 25 μM ATRA; lane 4, 5 μM ATPR; and lane 5, 25 μM ATPR. All numerical data are presented as mean ± SD. \*p<0.05 vs. the control group. #p<0.05 vs. the ATRA groups.

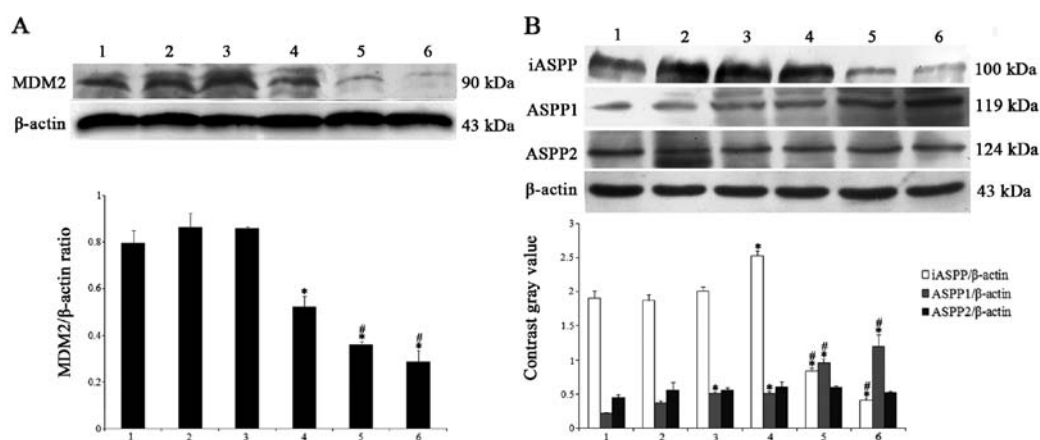


Figure 4. Effects of ATPR on the expression of MDM2 and ASPP family proteins in the HepG2 cells. Cells were treated with 5 and 25 μM ATRA and ATPR for 48 h. (A) ATPR significantly suppressed MDM2 expression in the HepG2 cells. (B) ATPR markedly upregulated ASPP1 expression and downregulated iASPP expression in the HepG2 cells, while ASPP2 expression was constant. Lane 1, cells; lane 2, DMSO; lane 3, 5 μM ATRA; lane 4, 25 μM ATRA; lane 5, 5 μM ATPR; and lane 6, 25 μM ATPR. β-actin was used as a loading control. All values are presented as mean ± SD. \*p<0.05 vs. the control group. #p<0.05 vs. the ATRA groups.

ATPR-induced apoptosis in HepG2 cells, the expression of Bcl-2 and Bax was measured. As shown in Fig. 6, Bax expression was enhanced, while the expression of Bcl-2 was reduced in a dose-dependent manner, which clearly demonstrated that ATPR may contribute to the apoptosis in HepG2 cells.

## Discussion

Generally, aberrant proliferation of cancer cells mainly relies on the activation of oncogenes and inactivation of tumor-suppressor genes (47). p53, commonly regarded as a cell transcription factor, shows anti-carcinogenic activities by inducing cell cycle arrest and apoptosis in malignant cells. In contrast, malfunction of p53 has been observed in up to 50% of HCC for carcinogenesis and chemotherapeutic

resistance (48). In the present study, we demonstrated that ATPR more effectively inhibited the proliferation of HepG2 cells than ATRA by inducing G0/G1 arrest and apoptosis by increasing expression of p53 and ASPP1, and reducing iASPP expression, which may provide a promising option to restore functional p53 expression in HCC therapy.

Currently, numerous studies have been carried out to explore the mechanisms of anticancer drugs through therapeutic targeting of p53-mediated apoptosis in cancers (24,49-51). For example, Mu *et al* reported that oroxylin A promotes apoptosis in cancer cells by modulating the expression of p53 (49). In addition, chrysin and cisplatin promote the apoptosis of HepG2 cells by upregulating p53 (24). Yet, certain factors may be responsible for resisting ATRA-induced apoptosis in HCC (52). Therefore, in the present study, we investigated the effects of a



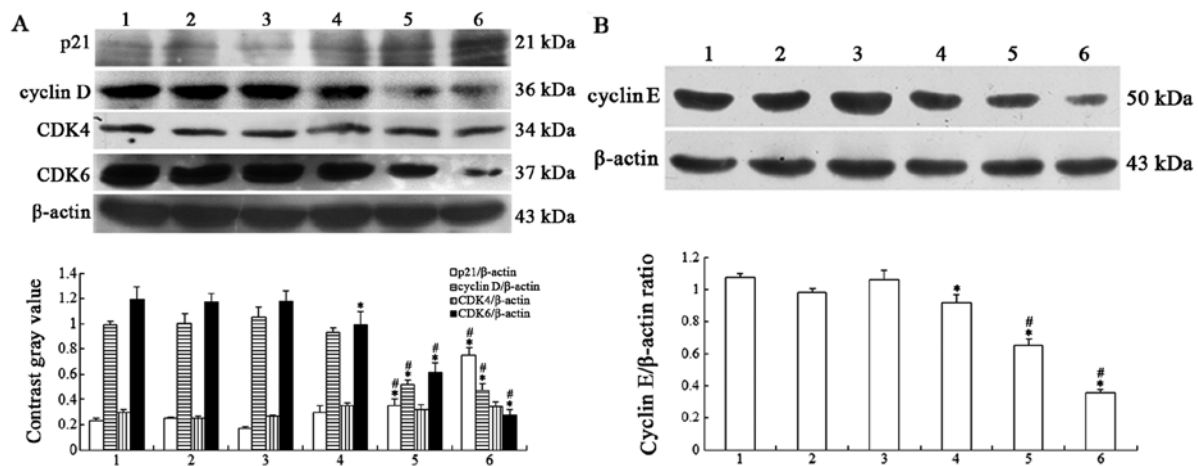


Figure 5. Effects of ATPR on cell cycle-related proteins in HepG2 cells. Cells were treated with 5 and 25  $\mu$ M ATRA and ATPR for 48 h. (A) The expression of p21 was markedly increased, cyclin D and CDK6 were decreased in HepG2 cells treated with ATPR, while the level of CDK4 was not obviously changed. (B) Meanwhile, cyclin E expression was reduced in the ATPR-treated cells. Lane 1, cells; lane 2, DMSO; lane 3, 5  $\mu$ M ATRA; lane 4, 25  $\mu$ M ATRA; lane 5, 5  $\mu$ M ATPR; and lane 6, 25  $\mu$ M ATPR.  $\beta$ -actin expression was detected to ascertain equal protein loading. All values are presented as mean  $\pm$  SD. \* $p$ <0.05 vs. the control group. # $p$ <0.05 vs. the ATRA groups.

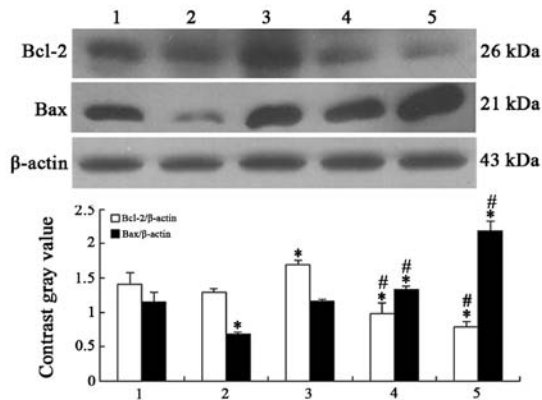


Figure 6. Effects of ATPR on expression of Bcl-2 and Bax in HepG2 cells. Cells were treated with 5 and 25  $\mu$ M ATRA and ATPR for 48 h. Lane 1, DMSO; lane 2, 5  $\mu$ M ATRA; lane 3, 25  $\mu$ M ATRA; lane 4, 5  $\mu$ M ATPR; and lane 5, 25  $\mu$ M ATPR.  $\beta$ -actin was used as a loading control. All values are presented as mean  $\pm$  SD. \* $p$ <0.05 vs. the vehicle control group. # $p$ <0.05 vs. the ATRA groups.

novel ATRA derivative, ATPR, on the proliferation, functional recovery of p53 and its downstream targets in HepG2 cells. Our results indicated that ATPR inhibited the proliferation of HepG2 cells in a dose- and time-dependent manner compared to ATRA. To address the preliminary mechanism, immunofluorescence assay, western blot and qRT-PCR analyses were performed to detect p53 expression in the HepG2 cells in the presence of ATRA or ATPR. The accumulation of fluorescence in the nuclei, the protein and mRNA expression of p53 were obviously elevated in the ATPR groups, whereas, in the ATRA groups, no significant changes were observed in these aspects. Based on this, it is reasonable to speculate that p53 may be involved in ATPR-inhibited proliferation in HepG2 cells, while the high ATRA resistance of HepG2 cells may be related to the loss of regulation in p53 expression. In future studies, knock-down or overexpression of the p53 gene could be used to better understand the role of p53 in the ATPR-mediated inhibition of proliferation in the HepG2 cells.

As is known, MDM2 negatively regulates p53 by binding to the N-terminus transactivation domain, and forming the MDM2-p53 complex to export p53 from the nucleus towards proteasomal degradation (53). Accordingly, several strategies have been explored to disrupt the p53-MDM2 interaction for the functional recovery of p53, including the use of small peptides, antisense oligonucleotides, and inhibitors of MDM2 (54). Based on this, MDM2 inhibition may represent an appealing therapeutic strategy for the treatment of cancer. Intriguingly, in the present study, ATPR induced a decrease in MDM2 in the HepG2 cells. It blocked the entry of MDM2 into the nucleus, and thereby allowed p53 to escape from the negative feedback loop. Further experiments on their interactions are needed.

ASPP family proteins have been reported to be involved in regulating the function of p53 (26,27). In cancer cells, upregulation of the iASPP protein and downregulation of ASPP1 and ASPP2 were found, and may play important roles in the pathogenesis of cancer (31,55). Similarly, in the present study, the levels of ASPP1 and ASPP2 were low, while iASPP expression was comparatively high in the HepG2 cells, which may have weakened p53-induced apoptosis. Notably, the expression of ASPP1 and iASPP was reversed after interference of ATPR in the HepG2 cells, however, the expression of ASPP2 showed no change after ATPR interference. Thus, it was plausible to demonstrate that ATPR could recover the function of p53 by inducing the elevation of ASPP1 and the decrease of iASPP. In line with our results, some studies have reported that downregulation of iASPP inhibited the proliferation and promoted apoptosis in cells expressing wild-type p53 (31,56,57). Although studies have reported that ASPP2 promoted apoptosis in cancers (55,58,59), ASPP2 was not involved in the apoptosis process after ATPR treatment, indicating that iASPP and ASPP1 compete with ASPP2 to bind to p53. Taken together, ATPR induced a decline in MDM2 and iASPP and an elevation in ASPP1 in the HepG2 cells, which may partly enhance the function of p53, leading to an inhibitory effect on proliferation.

Upon activation of p53, downstream target genes were found to be upregulated, including Bax, p53-upregulated modulator of apoptosis (PUMA), and cell cycle regulator p21 (60). The dysfunction of the cell cycle causes malignant cell proliferation (61). The early G1 stage of the cell cycle is coordinated by cyclin D/CDK4 and cyclin D/CDK6 complexes that result in phosphorylation and inactivation of the retinoblastoma (Rb) protein. Subsequently, this process leads to the derepression and activation of E2F target genes such as cyclin E gene facilitating G1 and S phase progression (62). p21 serves as a specific inhibitor of the cyclin/CDK complexes and its overexpression can inactivate them to promote cell cycle arrest in the G0/G1 or G2/M phase. Growth arrest through overexpression of p21 is the normal response to p53 activation in cancer cells (60). In the present study, the expression of p21 was increased by the elevation of ATPR. Unlike previous drugs (e.g. melatonin and oridonin) causing HepG2 cell cycle arrest in the G2/M phase through increasing expression of p53 and p21 (63,64), ATPR markedly arrested HepG2 cells in the G0/G1 phase, and induced a marked reduction in G1 and S transition-related proteins, including cyclin D and E, and CDK6. However, CDK4 expression was constant in each group. Collectively, our results demonstrated that ATPR may induce G0/G1 arrest through increasing the expression of p21 and decreasing the expression of cyclin D and E, and CDK6 via accumulation of p53. The results are in agreement with previous studies in which MDM2-mediated p53 activation promoted cell cycle arrest by increasing p21 expression (65,66). In addition, ATPR-induced apoptosis in HepG2 cells was observed by Hoechst staining featured by nuclear condensation and fragmentation and flow cytometry. It has been reported that the Bcl-2/Bax ratio could reflect the release of cytochrome *c* and the initiation of apoptosis (67). Several studies have revealed that accumulation and activation of p53 induced apoptosis by regulating the expression of Bax. In addition, Bcl-2 blocked apoptosis by binding to Bax to prevent the release of the pro-apoptotic proteins (24,49,51). Similarly, in the present study, the subsequent elevation in Bax and the reduction in Bcl-2 may be associated with accumulation of p53 and modulation of the ASPP family in HepG2 cells treated with ATPR. These downstream molecular events perhaps support the critical roles of p53 in the apoptosis of HepG2 cells induced by ATPR. However, the detailed mechanism of p53 in apoptosis progression should be further studied.

In summary, ATPR was superior to ATRA in inhibiting the proliferation of HepG2 cells *in vitro*. Upregulation of p53 and ASPP1 as well as downregulation of iASPP may be involved in the ATPR-mediated inhibition of proliferation by inducing G0/G1 cell cycle arrest and apoptosis. Therefore, ATPR may be a novel and efficient solution to restore the function of p53 in HCC therapy. In the future, other cell lines with different phenotypes (e.g. mutated or null p53) should be used to further confirm the roles of p53 and the ASPP family in cancer cell proliferation.

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