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Synergism between arsenic trioxide and cyclopamine in the inhibition of PC3 cell survival via the Hedgehog signaling pathway

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Previous studies have shown that Hh signaling is overexpressed in the development and progression of prostate cancer (PCa), suggesting that Hh pathway inhibitors might be an effective strategy in the treatment of PCa. The combination of chemotherapeutic agents is one of the main approaches in cancer treatment, with the objective of improving efficacy. In the present study, we examined the effect of combing arsenic trioxide (ATO), a useful agent for Hedgehog-driven cancers, and cyclopamine (CYA), a classic Hh pathway inhibitor, on the suppression of PC3 cells (i.e., an androgen-independent PCa cell line). The combination of ATO and CYA more effectively inhibited the proliferation of PC3 cells than either single agent alone. In a xenograft mouse model, the combination of ATO and CYA significantly reduced tumor weight and volume in nude mice that were implanted with PC3 cells. The combination of ATO and CYA in PC3 cells resulted in a more distinct mode of Hh pathway inhibition and strengthened the S phase arrest. The present results indicate that a combination of ATO and CYA may be a rational strategy for treating PCa.

Key words: prostate cancer, arsenic trioxide, cyclopamine, hedgehog pathway, cell cycle

Among deaths related to various forms of cancer, prostate cancer (PCa) ranks second in Western males [1], the incidence of which is rapidly increasing in Asia. Definitive therapy (surgery or radiation) is highly effective in the early stage of PCa. Most prostate tumors are sensitive to androgens in the early stages of the disease, and androgen-deprivation therapy is the first-line treatment. However, over time, almost all of them will develop to castration-resistant PCa. Till now, the prognosis of patient with prostate cancer remains dismal [2-5]. Chemotherapies aiming at efficiently killing PCa cells via interfering cellular signal pathways remain as important treatment choices.

Hedgehog (Hh) pathway is important in tissue patterning and morphogenesis during embryonic development and functions postnatally in tissue homeostasis through its action on stem or progenitor cells [6-9]. Canonical Hh signaling is initiated by binding of the ligands: Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh (Dhh) to their receptors Patched (Ptch). Ptch release the protooncogene Smoothened (Smo). Subsequent activation of the intracellular signaling cascade leads to the transcriptional regulation of Shh target genes by the Gli family of transcription factors, inducing the expression of numerous target genes that regulate proliferation and differentiation [10-13]. Ectopic activation of the Hh signaling pathway has recently been shown to be involved in several cancers, such as cancer of pancrease, stomach, colon, lung, and prostate [14-17].

Previous studies have shown that Hh signaling is overexpressed in the development and progression of PCa [18, 19] and the Hh signaling seems to be more significantly up-regulated in advanced PCa [17]. In some preclinical experiments, inhibition of Hh signaling has shown the potential effect of reducing the invasiveness and metastasis in prostate cancer [12, 20]. These studies indicate that Hh pathway plays a crucial

role in the pathogenesis of Pca and Hh pathway inhibitors might be an effective strategy in treatment of PCa.

Arsenic trioxide (ATO) has long been used as anticancer agent in traditional Chinese medicine and has been approved by the US Food and Drug Administration (FDA) for treatment of relapsed or refractory acute promyelocytic leukemia (APL) with only mild adverse effects [21]. The successful application of ATO in the treatment of APL results in the widely exploration of its anticancer effect in other malignant tumors. Recent studies provided evidence that ATO is highly effective *in vitro* and *in vivo* in a variety of solid tumor cells, including human hepatocellular carcinoma, osteosarcoma and breast cancer [22-24]. These evidences strongly raise the possibility of the application of ATO in teatment of PCa. A few studies have reported that ATO could inhibit tumor cell growth by blocking Hh pathway in rhabdoid tumor, osteosarcoma, Ewing sarcoma, pancreatic cancer stem cells and APL patients [24-29]. Furthermore, ATO has been established as a Hh pathway inhibitor [29] and a promising anti-tumor agent for Hedgehog-driven cancers [30]. However, the limited efficacy of ATO used as a single therapeutic agent on treating solid tumors rather than APL is reported from 2 clinical trials on patients with metastatic melanoma[31]. The combination of ATO with other agents may be a good strategy to enhance therapeutic efficacy.

Cyclopamine (CYA), a naturally occurring steroidal alkaloid, is an available FDA approved drug that inhibits Hh signaling by targeting Smoothened (Smo) receptor [14, 20, 32]. Several studies suggest drugs like CYA that interfere with Hh signaling could be beneficial in preventing androgen resistance and progression in prostate cancer cells [33-35]. A previous study showed that a combination of ATO and CYA appeared to permit greater Hh pathway inhibition at lower drug concentrations in NIH3T3 fibroblasts cells (transfected with Gli-luciferase) [36]. Therefore, CYA, as a classical Hh pathway inhibitor, was combined with ATO and used in the present experiment for increasing the anti-tumor effects.

In order to verify our hypothesis, PC3 cells (androgen-independent human prostate cancer cell line) was used in the present study and the effect of the combination of ATO and CYA on the proliferation of PC3 cells *in vitro* and *in vivo* were detected. Furthermore, the possible mechanism of ATO and CYA on Hh signaling pathway was explored as well.

Materials and methods

Reagents, antibodies and cell culture. ATO was purchased from Beijing SL Pharmaceutical Co, Ltd (Beijing, China) and Cyclopamine was purchased from LC Laboratories. (USA). The antibody against Ptch, Gli1, Smo, Prostate specific membrane antigen (PSAM), Ki67 and GAPDH were from Abcam Inc. (UK). The PC3 cell line was obtained directly from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai Institute of Cell Biology, China) for fewer than 6 months and maintained in Rose well Park Memorial Institute (HAM'F12 (GIBCO) supplemented with 10 % fetal calf serum (FCS; Hyclone, Logan, UT, USA), 50 mg/mL streptomycin and 50 IU/mL penicillin , and cultured at 37 °C in a humidified atmosphere with 5 % CO_2.

Cell proliferation assay. Cell viability was examined by methlthiazolyl tetrazolium (MTT) assay. After cells were cultured in the 96-well plate and treated with different doses of ATO (0, 2.5, 5, 10,20 μM) and CYA (0, 4, 8, 16, 32 μM), then 0.5 mg/mL MTT (Sigma-Aldrich, USA) was added to each well, and the mixture was incubated at 37 °C for 4 h. Then after culture medium was replaced with 200 μL of dimethyl sulfoxide to dissolve formazan crystals, the 96-well plate was shaken at room temperature for 10 min, absorbance of each well was determined at 490 nm using a scanning multi-well spectrophotometer (Multiskan MK3; Thermo Labsystems, China). Five replicate wells were examined for each cell sample.

Immunofluorescence assay. Cells were grown on sterile glass coverslips, washed three times in cold phosphate buffered saline (PBS), and fixed with 4 % paraformaldehyde for 30 min at room temperature. After fixation, cells were permeabilized with 0.1 % Triton X-100 (Sigma) for 30 min, blocked with 2 % bovine serum albumin (BSA) in PBS, followed by 1h incubation with anti-Ptch, Gli1 or Smoothened antibody for overnight at 4 °C. After incubation, the cells were washed several times with PBS and incubated for 30 min with corresponding secondary antibody conjugated with the appropriate fluorochrome together with DAPI (Sigma-Aldrich, USA) for nuclear staining. Images were collected using a spinning disk confocal microscope (IX81; Olympus) equipped with a camera (Cascade 512; Photometrics).

Real-time PCR analysis. Total RNA was isolated from PC3 cells using Trizol (Invitrogen), purified using the RNeasy Mini Kit (QIAGEN). The QuantiTect SYBR Green PCR Kit (QIAGEN) along with QuantiTect Reverse Transcription Kit (QIAGEN) was used for cDNA synthesis. PCR primers for genes of interest and housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were as follows: Ptch (F: 5'- TGCTGCTAGTCCTCGTCTCCT -3', R: 5'-TTTTGGGGTGCCTCCTCTT-3'); Smo (F:5'-GGGAGGCTACTTCCTCATCC-3', R:5'-GGCAGCTGAAGGTAATGAGC-3') Gli-1 (F:5'-CCCAACTCCACAGGCATACA-3', R: 5'-GCTCACGCTTCTCCTCT CTCTC -3'); GAPDH (F:5'-CGCTCTCT GCTCCTCCTGTT -3', R:5'-AAATCCGTTGACTCCGACCTT -3'). mRNA expression was measured in triplicate per sample using 40 cycles (5 seconds at 95 °C and 30 seconds at 55 °C) of amplification in the 7500 fast real-time PCR system (Applied Biosystems). The results are presented as transcript levels relative to the level in control group by using the Ct (ΔΔCt) analysis, and GAPDH mRNA levels were used as the normalization control.

Western blot assay. Cells were extracted with lysis buffer supplemented with protease inhibitors, phosphatase inhibitors and PMSF. The protein concentration was determined using the BCA Assay kit (Keygen Biotech. Co., Ltd., China). The proteins were separated in 12 % sodium dodecyl sulfate

polyacrylamide gels then transferred to nitrocellulose filter membranes (Bio-Rad, USA). The membranes were blocked with 5 % BSA for 2 h at room temperature and probed with antibodies to Ptch, Gli1, Smoothened and GAPDH overnight at 4 °C, then incubated with horseradish peroxidase conjugated secondary antibodies for 2 h at 37 °C. The bands were detected with the ECL system (Amersham, Sweden) and scanned by Image Quant 5.2 software (Amersham).

Xenograft treatment in nude mice. The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (SYXK) (Approval No: 2013-0006). A total of 24 male athymic nude mice (8 weeks old) were purchased from the Animal Facility of Dalian Medical University (China) and housed in a specific pathogen free facility. Tumors were generated by implanting PC3 cells. Briefly, PC3 cells (1×10^6) were resuspended in 50 μL of Matrigel (BD Biosciences) in a 1:1 volume, and were inoculated subcutaneously into the right flank of each nude mouse. Three days later, the average tumor size was 120 mm^3, and the mice were randomly divided into the four groups of six mice each. Tumor-bearing mice were injected i.p. with 5 mg/kg of ATO, 16 mg/kg of CYA, or in combination every other day. Tumor volume was determined weekly by external caliper using the volume formula (length ×width× width × 0.5) [37]. All mice were sacrificed after 5 weeks of treatment. Tumors were isolated, weighed, and photographed, then immediately fixed with 4 % paraformaldehyde for immunohistochemical staining. Tumor growth inhibition rate was calculated as follows: Inhibition (%) = (mean tumor volume of untreated control Mice – that of tumor volume of treated mice) / mean tumor volume of untreated control mice ×100.

Hematoxylin and eosin (H&E) and Immunohistochemistry (IHC). The fixed tumor tissues were routinely prepared for paraffin sections (4 μm per section). H&E and IHC stainings were performed as previously described with minor modifications [38]. The images were taken under a spinning disk confocal microscope (IX81; Olympus) equipped with a camera (Cascade 512; Photometrics). In IHC staining, the primary antibodies of Ki67 (1:100) and PSAM (1:200) were used to evaluate the expressions of cellular proliferation and prostate specific membrane antigen, respectively.

Flow cytometric analysis of cell cycle. A total of 1×10^6 control cells and cells treated with 2.5 μmol/L ATO, 8 μmol/L CYA or combination were harvested by trypsinization, washed twice with PBS, fixed in cold ethanol (70 %) overnight at 4 °C. Cells were stained with propidium iodide (PI, Sigma Chemical,

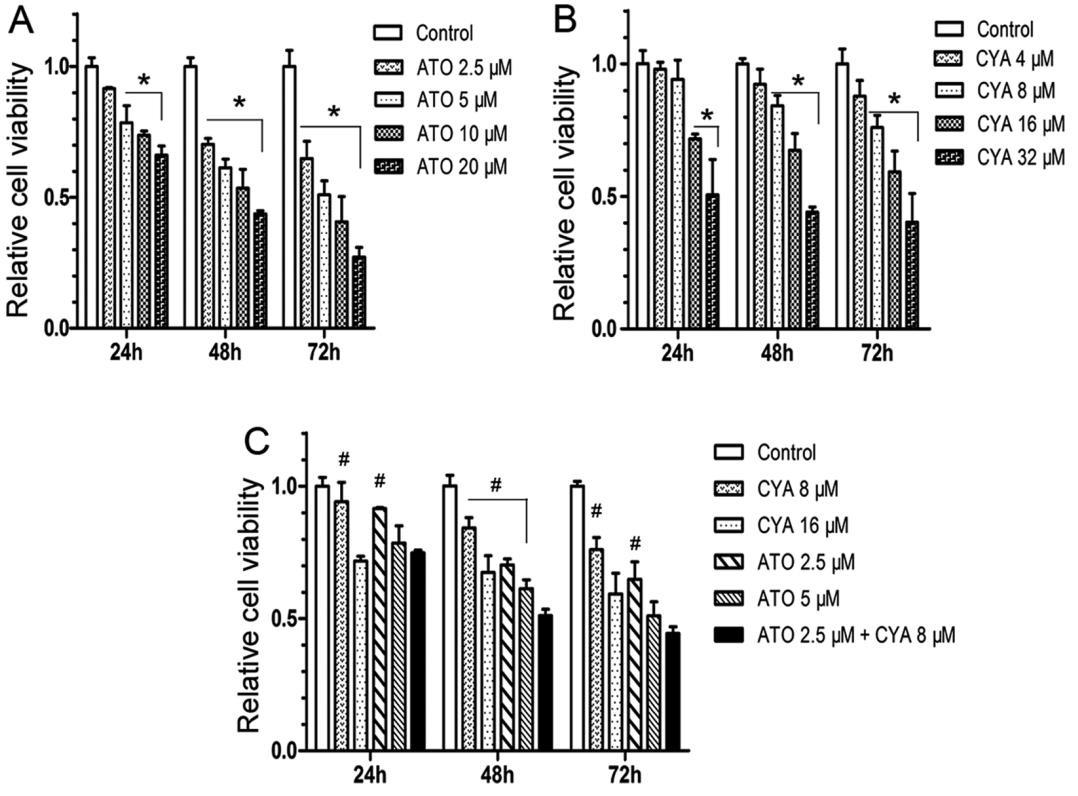

Figure 1. Antiproliferative effects of arsenic trioxide and cyclopamine in PC3 cell line. (A) PC3 cells were treated with arsenic trioxide at different concentration (0, 2.5, 5, 10,20 μM) for 24 h, 48 h and 72 h, and then cell viability was determined by MTT assay. (B) PC3 cells were treated with cyclopamine at different concentration (0, 4, 8, 16, 32 μM) for 24 h, 48 h and 72 h, and then cell viability was determined by MTT assay. (*p<0.05 versus the control.) (C) Combined arsenic trioxide and cyclopamine significantly inhibits PC3 cell proliferation at different time points (# p <0.05 versus combination treatment with arsenic trioxide and cyclopamine). Each error bar represents the SEM of three independent experiments by one way ANOVA.

St. Louis, MO, USA) after incubating in 0.2 mg mL RNAase (Promega, Madison, WI, USA) at 37 °C for 30 minutes. Finally, cell samples were analyzed by a FACS Calibur (Becton-Dickinson, USA). Cell cycle phase distributions were determined using CellQuest Pro software (Becton-Dickinson) and ModFit LT software (Verity Software House, USA).

Statistical analysis. All statistical analysis was performed by Microsoft Office Excel and SPSS 17.0 software. All data were expressed as mean ± SEM and analyzed using one way ANOVA to evaluate the differences between groups. Each sample was performed at least three times with values of $p < 0.05$ considered statically significant. The GraphPad Prism 5 software was used to perform all data analysis.

Results

Synergism of ATO with CYA in inhibiting the proliferation of PC3 cells *in vitro*. To assess the effect of ATO and CYA on PC3 cell growth, the cells were treated with different doses of ATO (2.5-20 μM) or CYA (4-32 μM) for different durations (24-72 h). Then, a MTT assay was performed to determine cell viability. CYA or ATO alone reduced the viability of PC3 cells in both a dose-dependent and a time-dependent manner (*$p<0.05$) (Fig. 1a and b). The viability of PC3 cells treated with 2.5 μM ATO was decreased to 91.4 %, 70.1 % and 64.7 % of control at 24 h, 48 h and 72 h, respectively. The viability of PC3 cells treated with 8 μM CYA was decreased to 94 % (24 h), 86 % (48 h) and 76 % (72 h), respectively. Since a low concentration of ATO (2.5 μM) or CYA (8 μM) barely affect the viability of PC3 cells at 24 h and not significantly at 48 h and 72 h, either, combination treatment with 2.5 μM ATO and 8 μM CYA dramatically reduced the viability of PC3 cells to 74.7 % (24 h), 51.1 % (48 h) and 44.3 % (72 h). Especially for the duration of 48 h and 72 h, the inhibiting effect of combination of the two agents showed more significant trend than not only single agent alone (2.5 μM ATO or 8 μM CYA), but also two times of single agents (5 μM ATO or 16 μM CYA) (Fig. 1c).

To determine whether the effects of ATO and CYA were additive or synergistic, we calculated the combination index value (CI) according to Chou's method, where CI value < 1,

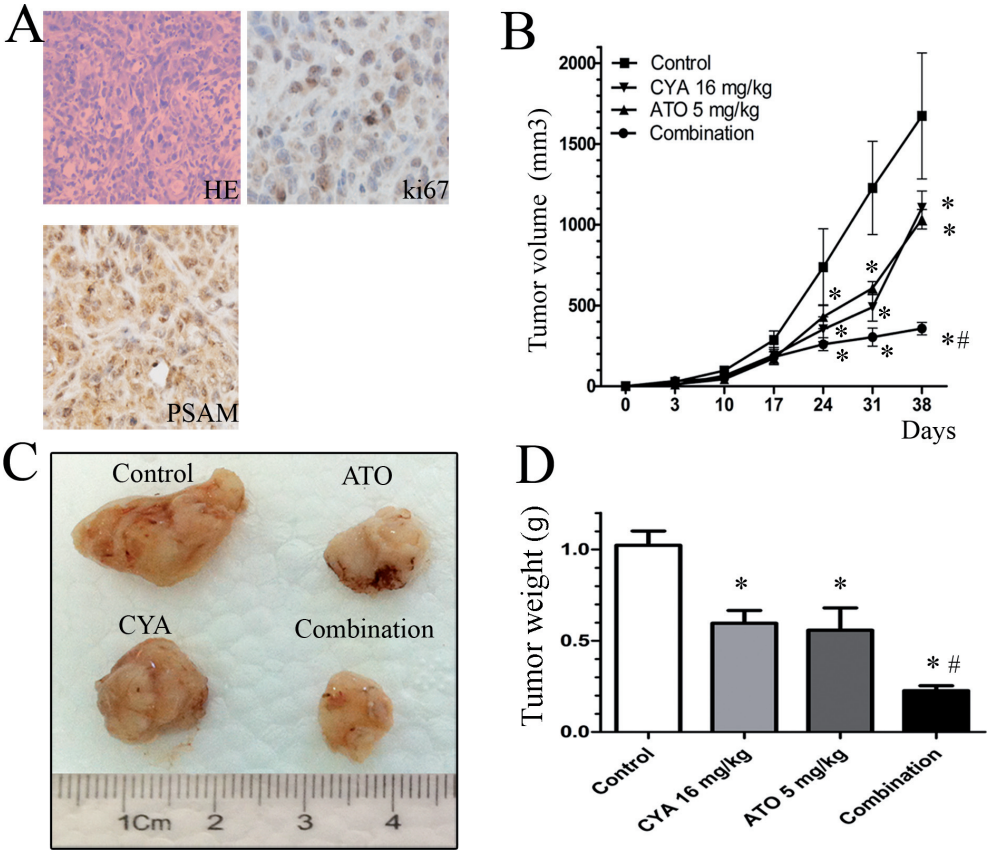

Figure 2. The *in vivo* arsenic trioxide, cyclopamine or the combination of two agent suppressed PC3 xenograft growth. (A) Representative H&E staining picture (200×) and the expressions of Prostate specific membrane antigen (PSAM) and cell proliferation markers (ki-67) (400×). (B) The tumor volumes were measured with calipers every 7 days (*$p<0.05$ compared with Control, #$p<0.05$ for the combination compared to single agents). (C) Combined treatment suppressed the growth of the tumors compared with the single agent treatment and the control. (D) Measurement of tumor weight in the nude mice after sacrifice (*$p<0.05$ compared with Control, #$p<0.05$ for the combination compared to single agents).

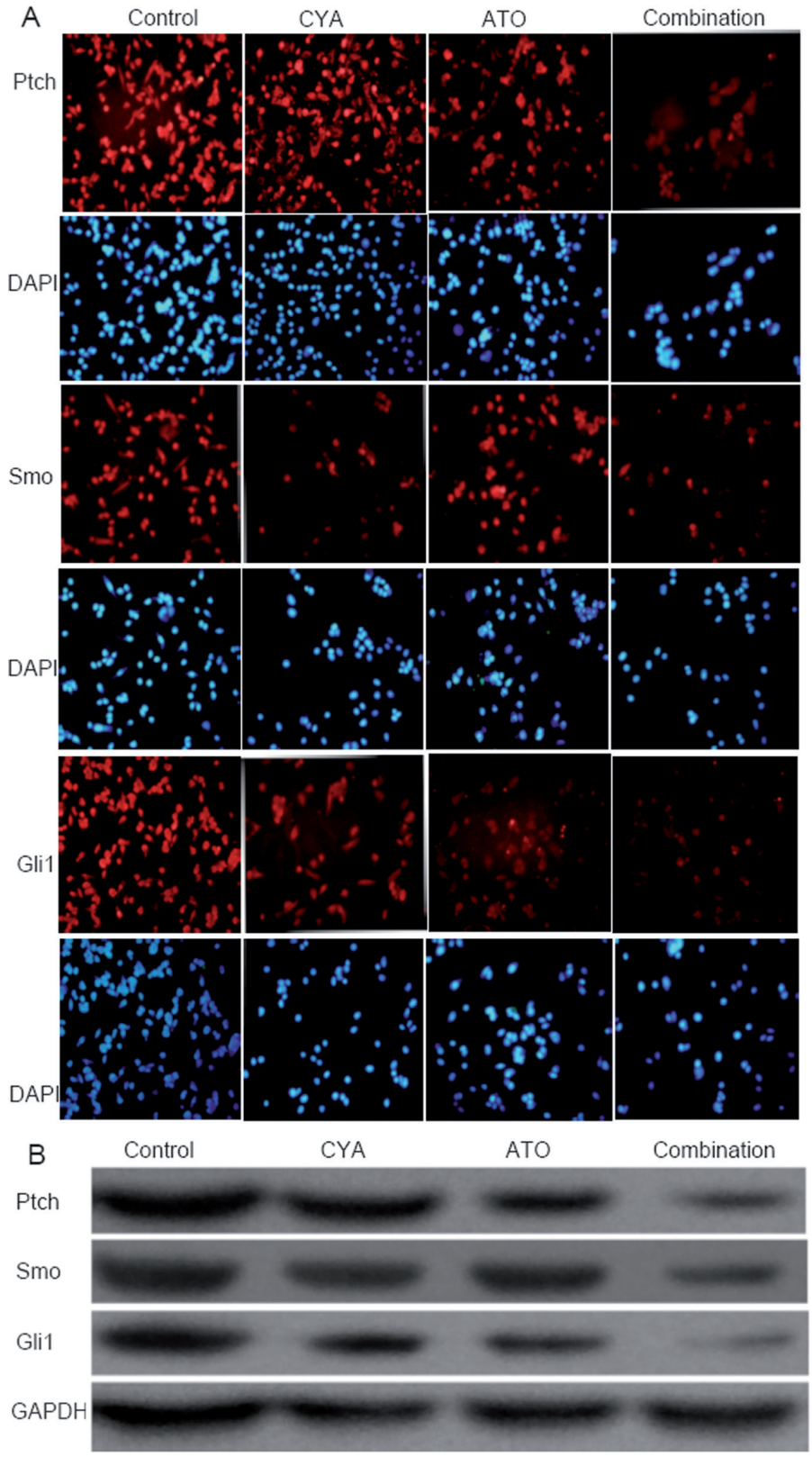

Figure 3. Immunofluorescence and Western blot analysis of Hh target genes Ptch, Smo, and Gli1 expression in PC3 cells treated with 2.5 μM arsenic trioxide, 8 μM cyclopamine or combination. (A) Ptch, Smo, and Gli1 expressions decreased significantly compared with the control and single agent treatment (original magnification 200×). (B) Ptch, Smo, and Gli1 protein expression levels decreased most significantly in combination treatment.

=1, and >1, indicate synergism, additive effect, or antagonism, respectively [39, 40]. The CI for PC3 cells treated with combination of 8 μM CYA and 2.5 μM ATO for 24 h, 48 h and 72 h was 0.722, 0.521 and 0.548, respectively. The calculated result indicated that ATO and CYA showed significant synergistic effect on inhibiting PC3 cell growth.

The suppressed effect of the combination of ATO and CYA on the growth of prostate xenograft *in vivo*. To confirm synergetic antitumor effect of the ATO and CYA *in vivo*, we turned to a xenograft model of prostate tumors in nude mice implanted with PC3 cells. The treatment was initiated three days after tumor inoculation. The nude mice with established tumors were injected intraperitoneally with 5 mg/kg ATO, 16 mg/kg CYA, or a combination of both agents every other day for 35 days. As compared to the control group on day 38 after tumor inoculation, treatment with ATO, CYA alone or combination of both agents significantly inhibited tumor growth and the combination group showed more significantly inhibitory effect than any of single agent group (ATO:35.4 %, CYA:39.5 %, ATO+CYA:79.1 %, Fig. 2c). The tumor volume of each group was calculated and showed that there was no significant difference among four groups between day 0 to 10 after tumor inoculation. However, the tumor volume of three agent treatment groups began to decline on day 17, and an obvious difference among groups could be observed on day 24 and the difference was expanding on day 31. A significant difference could be seen on day 38 (Fig. 2b), which was the combination treatment significantly suppressed tumor volume in nude mice, compared with ATO or CYA treatments alone ($p < 0.05$, Fig. 2b). In addition, the tumor weight of the four groups showed similar trend with tumor volume (Fig. 2d).

One xenograft tissue from the control group was used for morphological examination. The morphological features of the tissue were tesed by H&E staining and the expressions of Ki-67 and PSAM were tested by IHC. H&E staining showed obvious cell polymorphism in tumor tissues and typical morphological features of PC3 cell. IHC staining showed apparent strong staining of Ki-67 in the nuclei and PSAM in the cytoplasm (Fig. 2a).

The suppressed effect of combined administration of ATO and CYA on Hh activity in PC3 cells. To demonstrate the involvement of Hh-Gli pathway in the anti-tumor effect of the combination of CYA and ATO, the expressions of Hh target genes Ptch, Smo, and Gli1 were firstly determined by immunofluorescence assay and Western blot, then by quantitative real time RT-PCR (qPCR). Given the results of MTT, 2.5 μM ATO, 8 μM CYA or the combination of the two agents (2.5 μM ATO + 8 μM CYA) was chosen to be used in all the following mechanism tests. As shown in Fig 3, ATO decreased the expression of Ptch more than Smo, while CYA decreased the expression of Smo more than Ptch. The combination treatment of CYA and ATO was much more effective than that with CYA or ATO alone in terms of down-regulating the Gli1 (the final component of Hh pathway) expressions in PC3 cells. To further confirm this results, the change of the three proteins in Hh-Gli pathway was quantitatively tested by qPCR.

qRT-PCR data revealed that, after receiving single-agent ATO or CYA and combination treatment, Ptch mRNA expression was down-regulated to 0.55, 0.69, or 0.54, respectively; Smo mRNA expression was decreased to 0.97, 0.79 and 0.75, respectively; Gli1 mRNA expression was decreased to 0.56, 0.87 and 0.3, respectively (Fig. 4).

Strengthening effect of CYA on the S phase arrest induced by ATO. The selective Smo inhibitor CYA can inhibit the proliferation of PCa cells via accumulation of cells in stage G1, [41] implying that some cell cycle proteins may be the potential targets of Hh signaling pathway. To determine whether the cell cycle will be changed by the combination treatment of ATO and CYA, the cell cycle progression was detected by flow cytometry. Compared with the control, 8 μM

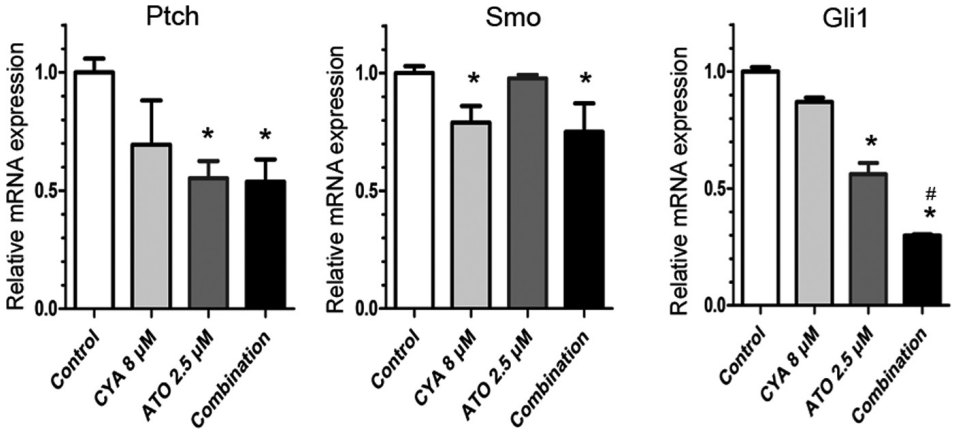

Figure 4. Real-time RT-PCR anaysis of Hh target genes Ptch, Smo, and Gli1 expression in PC3 cells treated with 2.5 μM arsenic trioxide, 8 μM cyclopamine or combination. Ptch, Smo, and Gli1 mRNA expression levels decreased most significantly in combination treatment (*p<0.05 compared with Control, #p<0.05 for the combination compared to single agents).

CYA alone didn't induce any significant change in cell cycle distribution, whereas 2.5 μM ATO alone treatment resulted in the accumulation of cells in the S phase and G2/M phase ($p< 0.01$). When ATO-treated PC3 cells were post-treated with CYA, more significant S arrests were noticeable ($p < 0.01$), which indicates that CYA could strengthen the S phase arrest induced by ATO in PC3 cells (Fig. 5).

Discussion

Androgen-independent PCa, as a Hedgehog-driven cancer, remains high metastasis and poor prognosis due to lack of effective intervention strategies [42-47]. Recent discoveries highlight the importance of the Hh signaling pathway in prostate growth regulation [12, 48] and Hh signaling pathway inhibitors therefore would be promising agents for prostate cancer therapy [12]. However, major issues with current Hedgehog pathway inhibitors include rapid acquired resistance, severe side effects and potential developmental toxicities to use in children [49]. Furthermore, most of Hh antagonists target Smo receptor and little target the signaling components down-stream of Smo, such as fused homolog (Sufu) or GLI [50, 51]. A few recent studies have demonstrated that ATO interferes with GLI proteins in the context of a dysregulated Hh signaling pathway in many human cancers, including malignant rhabdoid tumors, pancreatic cancer stem cells, acute promyelocytic leukemia, osteosarcoma and medulloblastoma [24-27, 52]. Importantly, the study of Beauchamp has shown that the underlying mechanism of the anti-tumor effect of ATO is likely through direct binding and inhibition of GLI1 and/or GLI2 transcriptional activity [29]. These data suggests ATO may provide an alternative option when alone or in combination with Hh signaling inhibitors for treating Hedgehog-driven cancer.

The anti-tumor effect of ATO or CYA for PCa has been known for a dozen years. CYA has been reported to be effective in inhibiting the growth of some type of PCa cells [12]. Recently, to minimize the serious side effects of CYA, two novel peptide-cyclopamine conjugates as prostate-specific antigen (PSA)-activated prodrugs have been designed and synthesized for use against prostate cancer [53]. The study indicates that although Hh pathway inhibitor is a promising approach against PCa, application of CYA alone could not show a satisfactory therapeutic effect. Thus, CYA derivatives

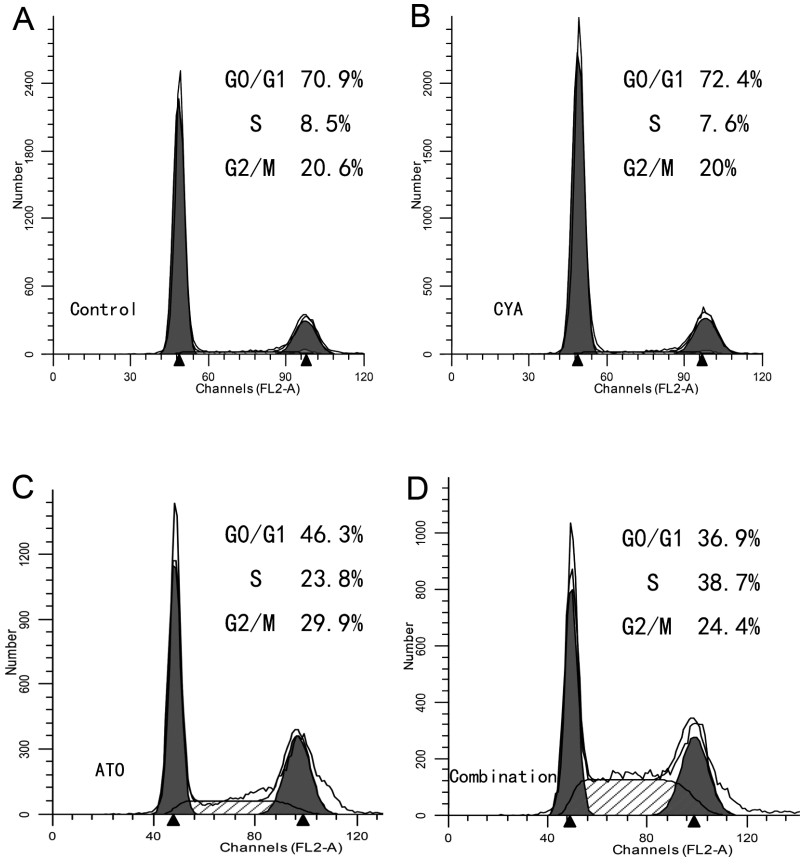

Figure 5. CYA enhanced S cell-cycle arrest induced by ATO in PC3 Cells *in vitro*. PC3 cells treated with 2.5 μM ATO, 8 μM CYA alone or in combination for 48 h, and then cell cycle progression was determined by flow cytometry. S cell-cycle arrest was significantly propelled by co-treatment compared with the control and single agent.

or other compounds that mimic or facilitate CYA action would be necessary. In 2000, Uslu R et al found that ATO has significant cytotoxic effect on DU145 and PC-3 PCa cell lines and the mechanism underlying cytotoxicity of ATO was shown to be apoptosis [54]. In 2001, Maeda H et al. further found ATO induced apoptosis at high doses and inhibited cell growth at low doses in all three of androgen-independent PCa cell lines (PC-3, DU-145, and TSU-PR1). Furthermore, a vivo study revealed that ATO (5mg/kg, i.p.) significantly inhibited the orthotopic tumor growth and retroperitoneal lymph node metastases in an orthotopic mouse model of PC-3 cells [55]. According to the above studies, ATO or CYA has been regarded as a promising agent for use in prostate cancer therapy.

One of the main approaches in cancer therapy is to utilize a combination of chemotherapeutic agents with the objective of improving efficacy. There is an increasing body of evidence on the synergistic effects of combined anti-cancer agents for androgen-independent PCa. The combination of NVP-BEZ235 (a phosphoinositide 3-kinase/mTOR dual inhibitor) and sunitinib (tyrosine kinase inhibitor) caused a significant synergistic antitumor effect over a wide range of doses in docetaxel-resistant castration-resistant PCa cells [56]. The efficacy somatostatin analogue octreotide (OCT) combined with a low dose of docetaxel (DTX) caused a more marked anti-proliferative effects on castration resistant prostate cancer cells than either individual agent [57]. These data indicate that combined therapy may be an effective modality for the treatment of androgen-independent PCa.

In this study, we combined ATO and CYA in treating PC3 cells (androgen-independent human prostate cancer cell line) and an athymic nude mouse model bearing subcutaneous xenografts of PC3 cells with the objective of improving efficacy, as a separate application requires higher dose of a drug. We found that low doses of ATO (2.5μM)/CYA (8μM) combination strategy significantly suppressed the growth of PC3 cell and exhibited a synergism with CI < 1. The *in vitro* finding was confirmed by a tumor xenograft model using a PC3 cell line in nude mice (16 mg/kg CYA + 5 mg/kg ATO, i.p. every other day, for 35 days). Compared with the effect of 4 μM CYA *in vitro* (there was no significant effect for CYA), it seems that the inhibited effect of CYA on the xenograft *in vivo* (16 mg/kg) more obvious, since 16 mg/kg CYA *in vivo* actually is close to 4 μM CYA *in vitro* by calculation. It may be because the treatment for PC3 cell xenograft mice lasted 35 days and the accumulative increase of blood concentration of CYA in those animals may contribute to strengthen the effect of CYA.

The potential benefit of combining ATO/CYA and other chemotherapeutic drugs for applying in PCa has been reported. Mimeault, M et al. found that a lower concentration of CYA in combination with gefitinib, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, can inhibit the growth of androgen-independent LNCaP-C81, DU145 and PC3 cells [58]. Recently, Chiu HW reported that ionizing radiation combined with ATO increased the therapeutic efficacy compared to individual treatments in LNCaP and PC-3 PCa cells [37]. Noteworthy, Kim J et al has reported that the combination of ATO and itraconazole which also is a Hedgehog signaling inhibitor by mechanisms distinct from that of current Smoothened antagonists inhibited the growth of medulloblastoma and basal cell carcinoma *in vivo* [28]. This study supports our finding that the use of ATO in combination with CYA can synergistically inhibit the growth of PCa cells, which provides a preclinical rationale for the therapeutic strategies to improve the treatment in androgen-independent PCa.

We further explore the possible mechanism of combination of ATO and CYA in the treatment of PCa. Given the important role of Hh pathway in PCa and the close relationship between Hh pathway and ATO and CYA, three critical components in Hh pathway, Ptch, Smo, and Gli1, were tested by immunofluorescence and Western blot assay, then by qPCR. Compared with the control, the immunofluorescence and western blot assays consistently showed that the expressions of all the three proteins were decreased to different degree by ATO, CYA alone or in combination of the two agents. ATO decreased the expression of Ptch more than Smo, while CYA decreased the expression of Smo more than Ptch and both ATO and CYA alone apparently decreased the expression of GLi1 (the final component of Hh pathway). When the two agents were combined, more distinct decreases were observed in Gli1 than any single agent. The similar change was quantitatively confirmed by qPCR. These results suggest that the synergism interaction between ATO and CYA is mediated through a significant greater degree of Hh pathway inhibitory in PCa cells. In addition, our results demonstrated for the first time the inhibitory effect of ATO on Ptch. In the Hh pathway, Ptch is not only a receptor as a pathway inhibitor but also a target gene of this pathway, which form a positive feedback mechanism to maintain the pathway activity at an appropriate level [59]. So far, there has not been any direct evidence to show the effect of ATO on the expression of Ptch protein. Hence, the decrease of Ptch induced by ATO may be the consequence of down-regulation in Hh pathway. Taken together, multifocal targeting in the Hh pathway inhibition may contribute to the synergistic anticancer effects of ATO and CYA.

Hh signaling induces the expression of principal cell cycle genes including CyclinD1, c-MYC and MYCN, providing insight into the mechanism by which deregulated Hh signaling promotes tumor formation [60]. In our study, ATO alone treatment resulted in the accumulation of cells in the S phase and G2/M phase, and CYA could strengthen the S phase arrest in PC3 cells. The change of S phase induced by ATO is supported by a previous study which showed that sodium arsenite induced a dose-dependent increase in the proportion of bladder cancer cells in S-phase in bladder cancer [61]. Combining the above results of cell proliferation and xenograft nude mouse assays, we infer that ATO may induce the S phase arrest and block tumor cells to enter into G2-phase and M-phase in PC3 cells. It is noteworthy that CYA alone didn't affect the S phase, but significantly increased the ratio of

the cells in the S phase induced by ATO, which demonstrates a synergistic effect in S phase due to the combination of ATO and CYA. The two synergistic effects between S phase arrest of cell cycle and the inhibition of Hh pathway induced by the combination of ATO and CYA suggest that there may be some connection between them. A few previous studies have demonstrated that Hh pathway can regulate cell cycle. Yu FY et al found that the molecular mechanisms regulated by the non-canonical Hh pathway mediated through ptch1 and cyclin B1 is involved in the pathogenesis of nevoid basal cell carcinoma syndrome (NBCCS)-associated keratocystic odontogenic tumors (KCOTs) [62]. Tripathi K et al. demonstrated that a novel and tumor-specific role for aberrant Gli1 in the regulation of the S-phase checkpoint that suppresses replication stress and resistance to chemotherapy [63]. Therefore, the S phase arrest may be caused by a more significant inhibition in Hh pathway induced by the combination of ATO and CYA. Further study will be needed to elucidate this issue.

In conclusion, our results demonstrated that ATO combined with CYA exhibited a synergistic effect in inhibiting the proliferation of PC3 cells both *in vitro* and *in vivo* and the potential underlying mechanism of it may relate to multifocal targeting inhibition in the Hh pathway and S-phase arrest of cell cycle induced by the combination of ATO and CYA. The present results indicate that combinational treatment may be a potential therapeutic agent against androgen-independent prostate cancer.

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