Oxibendazole inhibits prostate cancer cell growth

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Abstract. Prostate cancer (PCa) is one of the most common malignancies among men and is the second leading cause of cancer-associated mortality in the developed world. Androgen deprivation therapy (ADT) is the most common treatment for PCa. However, the majority of androgen-sensitive PCa patients will eventually develop resistance to ADT and the disease will become androgen-independent. There is, therefore, an immediate requirement to develop effective therapeutic techniques towards the treatment of recurrent PCa. Oxibendazole (OBZ) is an anthelmintic drug that has also shown promise in the treatment of malignancies. In the present study, the capability of OBZ to repress the growth of PCa cells was assessed in human androgen-independent PCa 22Rv1 and PC-3 cell lines. The growth of the 22Rv1 and PC-3 cell lines, as assessed with a trypan blue exclusion assay, was markedly inhibited by OBZ treatment *in vitro*, with half-maximal inhibitory concentration values of 0.25 and 0.64 μM, respectively. The mean size of 22Rv1 tumors in nude mice treated with OBZ (25 mg/kg/day) was 47.96% smaller than that of the control mice. Treatment with OBZ increased the expression of microRNA-204 (miR-204), as determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and the level of p53 as determined with western blotting, two well-characterized tumor suppressor genes. When miR-204 expression was knocked down by introduction of an miR-204 inhibitor, the inhibitory effect of OBZ was markedly reduced; however, when it was overexpressed, the inhibitory efficiency of OBZ was markedly higher, indicating that upregulation of miR-204 is key for the efficacy of OBZ. Additionally, OBZ was demonstrated with RT-qPCR to repress the expression of the androgen receptor, and by western blotting to reduce prostate-specific androgen in 22Rv1 cells. The results suggest that OBZ has potential for clinical use in the treatment of recurrent PCa.

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Abbreviations: ADT, androgen deprivation therapy; AR, androgen receptor; ASP, androgen signaling pathway; NEPC, neuroendocrine-like prostate cancer; OBZ, oxibendazole; PCa, prostate cancer; PSA, prostate-specific antigen

Key words: 22Rv1, microRNA-204, oxibendazole, PC-3, prostate cancer

Introduction

Prostate cancer (PCa) is the most common malignancy of the male genitourinary system and primarily occurs in aging men (1). PCa is the second leading cause of cancer-associated mortality in developed countries (2). Conventional treatments for PCa include surgery, chemotherapy, hormonal therapy, radiation therapy, radiofrequency ablation, high-intensity focused ultrasound and cryosurgery (3-5). Currently, androgen-deprivation therapy (ADT) is widely used for PCa treatment; however, the majority of PCa patients relapse within 1 to 3 years and develop androgen-independent disease, which is unresponsive to ADT (6-8). At present, there is no effective therapy for recurrent PCa (9). Therefore, a novel therapeutic method for PCa is required. Recently, the US Food and Drugs Administration approved novel compounds for the treatment of PCa, including abiraterone acetate (10), enzalutamide (11), sipuleucel-T (12), and Alpharadin (radium-233) (13). One study proposed that certain non-antitumor chemicals may have an antitumor effect (14). Sulfur, which is widely used for detoxifying the body and the treatment of scabies in traditional Chinese medicine (15,16), has been shown to suppress the growth of PCa *in vivo* (17). Itraconzole, a common triazole antifungal drug in widespread clinical use, has shown evidence of clinical anticancer effects, including against PCa (18).

Oxibendazole (methyl-5-n-propoxy-2-benzimidazole-carbamate; OBZ), was first synthesized in 1973 (19). OBZ is a benzimidazole drug that is used to treat infection by roundworms, strongyles, pinworms, threadworms and lungworm infestation in horses and other animals (20-22). The benzimidazole derivatives exhibit, among others, anti-ulcerative (23), anti-inflammatory (24), antibacterial (25), and anti-carcinogenic (26) bioactivities. These drugs are widely available for veterinary use and a number of them, such as thiabendazole, albendazole and mebendazole, have been used in human medicine for several years (27). A previous study found that benzimidazoles could have potential cytostatic effects, through the inhibition of microtubule formation and glucose uptake (28). Notably, albendazole, mebendazole and

flubendazole, which are all benzimidazole drugs, have been observed to inhibit tumor growth (29).

The ability of OBZ to suppress growth in PCa was assessed in the present study using the PCa 22Rv1 and PC-3 cell lines. Previous studies have shown that growth of recurrent PCa cells (also termed androgen-independent PCa cells) depends on the androgen receptor (AR) or the AR signaling pathway (ASP), although it is independent of androgens themselves (30,31). The 22Rv1 cell line is an androgen-independent PCa epithelial cell line that is representative of clinical recurrent PCa (32). This cell line expresses AR and prostate-specific antigen (PSA) (33,34), which has been widely used as a clinically diagnostic biomarker and a key prognostic factor for PCa (35). However, the androgen-independent PC-3 cells do not express AR or PSA (36). The two markers are frequently used to evaluate the anti-PCa effects of diverse chemicals (37-39).

In the present study, 22Rv1 cells were studied *in vitro* and *in vivo*, and PC-3 cells were studied *in vitro*. The aim of the study was to investigate the inhibitory effect of oxibendazole on prostate cancer cells.

Materials and methods

Drugs and animals. OBZ was purchased from Selleck Chemicals, Inc. (Houston, TX, USA) and was prepared as homogeneous suspensions in corn oil and administered to the nude mice by gavage. A total of 20 specific pathogen-free (SPF) male BALB/c nude mice aged between 6 and 8 weeks (weight range, 18-25 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), maintained under SPF conditions with a 12 h light-dark cycle and provided with food and water *ad libitum*. Mice in the experiment were randomly divided into two groups, the control and the OBZ-treated groups, with 10 mice in each group. Ethical approval for the present study was obtained from the Animal Ethics Committee of Shanghai Institute of Planned Parenthood Research (Shanghai, China).

Cell culture and transfection. Human PCa 22Rv1 and PC-3 cell lines were purchased from Shanghai Institute of Cell Life Science Resource Center (Shanghai, China). The cells were maintained in RPMI-1640 medium (Hyclone; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin, at 37˚C with an atmosphere of 5% CO_2. Transfection with 100 nM microRNA-204 (miR-204) inhibitor (cat. no. miR20000265; RiboBio Co., Ltd., Guangzhou, China) was performed with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in 22Rv1 cells according to the manufacturer's instructions. An GFP-expressing miR-204-expressing recombinant lentivirus and GFP-expressing control were purchased from Kangchen Bio-tech (Shanghai, China). The delivery of miR-204 to the 22Rv1 and PC-3 cells was performed by $1x10^8$ TU/ml viral infection according to the manufacturer's instructions.

Cell proliferation assays. Cells were seeded in 96-well plates at a cell density of $1x10^4$ cells per well in 100 μl RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin, and incubated at 37˚C with an atmosphere of 5% CO_2 overnight. 22Rv1 and PC-3 cells were treated with 0.12, 0.25, 0.50, 1.00, 2.00 and 3.00 μM OBZ for 48 h, or with 0.25 and 1.00 μM OBZ for 96 h. In order to assess the role of miR-204 in mediating the effect of OBZ, 22Rv1 and PC-3 cells were transfected with the miR-204 or miR-204 inhibitor, followed by treatment with 1 μM OBZ or dimethyl sulfoxide (DMSO; as a control) for 48 h. Cells were trypsinized and live cell numbers were counted in four areas under an inverted microscope (magnification, x40) using a hemocytometer and the trypan blue exclusion assay.

Flow cytometry. Apoptosis was determined using a double-staining Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences Inc., Franklin Lakes, NJ, USA). The 22Rv1 and PC-3 cells were treated with DMSO control or 0.25 μM OBZ. After 48 h, the cells were collected, washed in phosphate-buffered saline (PBS) and suspended in binding buffer. The cells were then stained using annexin V and propidium iodide (PI) (5 μl). Following incubation for 15 min at room temperature in the dark, the cells were diluted and analyzed using a flow cytometer (Beckman Coulter, Inc., Atlanta, GA, USA). When green fluorescence (FITC) was plotted against red fluorescence (PI), the cell populations could be detected in a dot-plot that indicated the following conditions: Viable cells ($FITC^-/PI^-$), early apoptotic cells ($FITC^+/PI^-$) and late apoptotic cells ($FITC^+/PI^+$). The data were reported as the percentage of early apoptotic cells ($FITC^+/PI^-$) and late apoptotic cells ($FITC^+/PI^+$).

Xenograft tumor development in nude mice. At the exponential growth stage, 22Rv1 cells were harvested, washed and suspended in PBS. A trypan blue exclusion assay was performed to ensure cell viability (>99%) prior to inoculation. The cells were counted and $2x10^6$ cells suspended in 0.1 ml PBS were subcutaneously injected into the right flank of each mouse. At 10 days after tumor cell inoculation, each mouse in the OBZ-treated group was provided with 25 mg/kg homogeneous suspension of OBZ by intragastric gavage. The treatment was administered once a day for 14 days; mice in the control group was provided with the same amount of corn oil. Tumor size was measured in two dimensions every other day. Tumor volume (measured in cm^3) was calculated using the following formula: Tumor volume=$axb^2x0.5$ (a, length; b, width).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on the total RNAs using Quant Reverse Transcriptase (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions with a PCR machine (cat. no. 580BR6819; RiboBio Co., Ltd, Guangzhou, China). Gene amplifications were performed with a Eco Real time PCR System (Model Ec-100-1001; Illumina, Inc., San Diego, CA, USA) using SYBR-Green (Thermo Fisher Scientific, Inc.). Gene expression in cells and tumors was measured using qPCR. The cycling conditions of GAPDH, AR, ARN1 and CD44 were as followed: 95˚C for 3 min, 95˚C for 20 sec and 58˚C for 20 sec, for 40 cycles. The cycling conditions of U6,

miR-204 and miR-34a were as follows: 95˚C for 1 min, 95˚C for 15 sec and 60˚C for 15 sec, for 40 cycles. Bulge-loop™ miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for miR-34a were designed by RiboBio. Primers for β-actin, AR and 5'-3' exoribonuclease 1 (XRN1) were purchased from Sangon Biotech, Inc. (Shanghai, China). The sequences of the primers are included in Table I. The relative expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ method (40). The mean Cq was calculated from triplicate PCRs.

Western blotting. Total protein was extracted from cells and tumors using radioimmunoprecipitation assay buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, 5% sodium deoxycholate and 0.1% SDS] supplemented with inhibitors of proteinase and phosphatase. The proteins (20 μg) were separated by 12% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk for 1 h at room temperature, washed with Tris-buffered saline with Tween-20 (TBST) buffer three times, and incubated overnight at 4˚C with anti-glyceraldehyde 3-phosphate (GAPDH; cat. no. AG019; 1:1,000 dilution), anti-tumor protein 53 (p53; cat. no. AP062; 1:1,000 dilution), anti-p21 (cat. no. AP021; 1:1,000 dilution) and anti-PSA (cat. no. ab53774; 1:1,000 dilution). Next, the membrane was washed 3x15 min with TBST and incubated with the horseradish peroxidase-conjugated secondary antibodies (anti-mouse, cat. no. A0216; 1:3,000 dilution; anti-rabbit; cat. no. A0208; 1:3,000 dilution) at room temperature. All primary antibodies were purchased from Beyotime Institute of Biotechnology (Haimen, China) except anti-PSA (Abcam, Cambridge, UK). The antibody-reactive bands were visualized using enhanced chemiluminescence detection reagents and a gel imaging system (Tanon Science & Technology, Co., Ltd., Shanghai, China).

Statistical analysis. Data were analyzed using SPSS software, version 11.5 (SPSS, Inc., Chicago, IL, USA). Differences in the continuous variable 'tumor volume' were compared using a one-way analysis of variance followed by a Student-Newman-Keuls test. Densitometry analysis of western blots was performed using Image J software (version 1.37, National Institute of Health, Bethesda, MD, USA). Data are presented as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

Results

OBZ inhibits growth of 22Rv1 and PC-3 cells. The ability of OBZ to inhibit the growth of 22Rv1 and PC-3 cells was determined by counting cell number. OBZ markedly inhibited the cell viability of 22Rv1 and PC-3 cells in a dose-dependent manner (Fig. 1A). As little as 0.12 μM of OBZ was observed to significantly inhibit the growth of the 22Rv1 and PC-3 cells, respectively (both $P<0.05$). The 22Rv1 cells were more sensitive to OBZ treatment, with a half-maximal inhibitory concentration (IC_{50}) value of 0.25 μM, compared with 0.64 μM in PC-3 cells. OBZ inhibited the cell viability of 22Rv1 and PC-3 cells in a time-dependent manner (Fig. 1B and C). These results demonstrated that OBZ inhibited the growth of PCa cells *in vitro* with varied efficiency.

Table I. Primer sequences for target genes.

Gene name	Primer sequence
GAPDH	Forward: 5'-CCTGTACGCCAACACAGTGC-3'
	Reverse: 5'-ATACTCCTGCTTGCTGATCC-3'
	Forward: 5'-TTCCCTCCCTATCTAACCCTC-3'
AR	Reverse: 5'-TCTAAACTTCCCGTGGCATAA-3'
	Forward: 5'-GGAAACAACAGGAATGGGA AGC-3'
XRN1	Reverse: 5'-ACCAGCACATTAGGCACTCAC-3'
	Forward: 5'-AGCAACCAAGAGGCAAGAAA-3'
CD44	Reverse: 5'-GTGTGGTTGAAATGGTGCTG-3'
	Reverse transcription: 5'-CGCTTCACGAATTTG CGTGTCAT-3'
U6	Forward: 5'-GCTTCGGCAGCACATATACTAA AAT-3'
	Reverse: 5'-CGCTTCACGAATTTGCGTGT CAT-3'
miR-204	Reverse transcription: 5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGAT ACGACAGGCATA-3'
	Forward: 5'-GGTTCCCTTTGTCATCC-3'
	Reverse: 5'-TGCGTGTCGTGGAGTC-3'

miR-204, microRNA-204.

OBZ causes apoptosis of 22Rv1 and PC-3 cells. The apoptosis-inducing capability of OBZ in 22Rv1 and PC-3 cells was evaluated by Annexin V-FITC and PI double staining. Provided that the IC_{50} value was 0.25 μM in 22Rv1 cells, 0.25 μM OBZ was used to treat 22Rv1 and PC-3 cells for 48 h. A notable increase in the number of apoptotic cells was observed in the OBZ-treated group compared with DMSO-treated cells (the negative control) (Fig. 2A and B). The apoptotic rate of 22Rv1 cells was 1.41% in DMSO-treated cells and 9.45% in OBZ-treated cells. The apoptotic rate in PC-3 cells was 0.92 and 4.58% in DMSO- and OBZ-treated cells, respectively (Fig. 2C). These results indicated that treatment with OBZ resulted in an increased apoptotic rate in PCa cells, and the apoptosis-inducing capability of OBZ was more marked in 22Rv1 compared with PC-3 cells.

To investigate the mechanism of the inhibitory effect of OBZ in PCa cells, the effect of OBZ on the expression of p53 and p21 (41,42), which are known to be the key regulators of apoptosis, was measured via western blot analysis in the OBZ-treated cells. An OBZ dose of ≥0.25 μM markedly increased p53 and p21 expression in 22Rv1 cells (Fig. 2D). These results suggested that upregulation of p53 and p21 served an important role in the OBZ-induced apoptosis of 22Rv1 cells.

OBZ inhibits 22Rv1 tumor growth in nude mice. The antitumor effect of OBZ was next evaluated *in vivo*. First, 22Rv1 cells were injected into the right flank of nude mice. Approximately

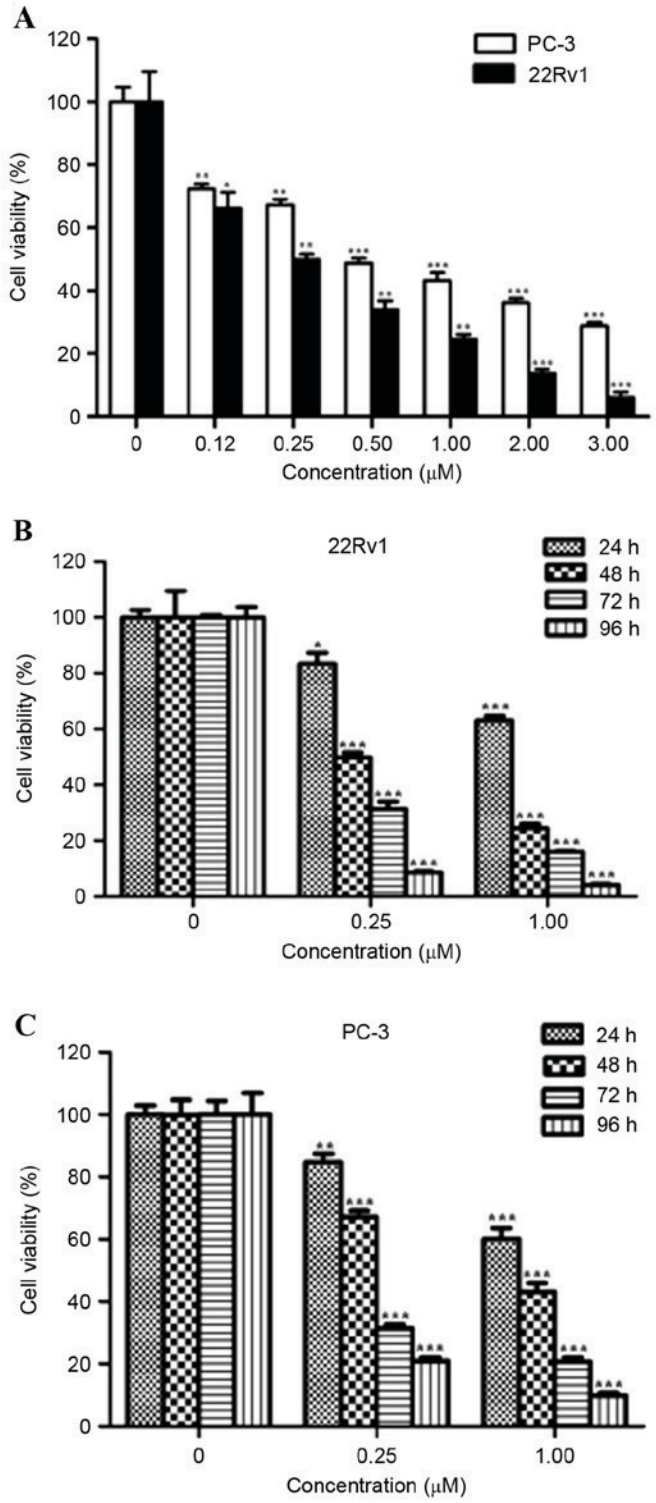

Figure 1. OBZ inhibits the proliferation of 22Rv1 and PC-3 cells. Cells were seeded in 96-well plates at a cell density of $1x10^4$ cells per well in 100 μl complete medium and incubated under standard cell culture conditions overnight. (A) 22Rv1 and PC-3 cells were treated with the indicated doses of OBZ for 48 h. (B) 22Rv1 and (C) PC-3 cells were treated with 0.25 and 1.00 μM OBZ, and harvested at the indicated times for cell counting. Each reported value represents the mean ± standard error of the mean from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μM OBZ. OBZ, oxibendazole.

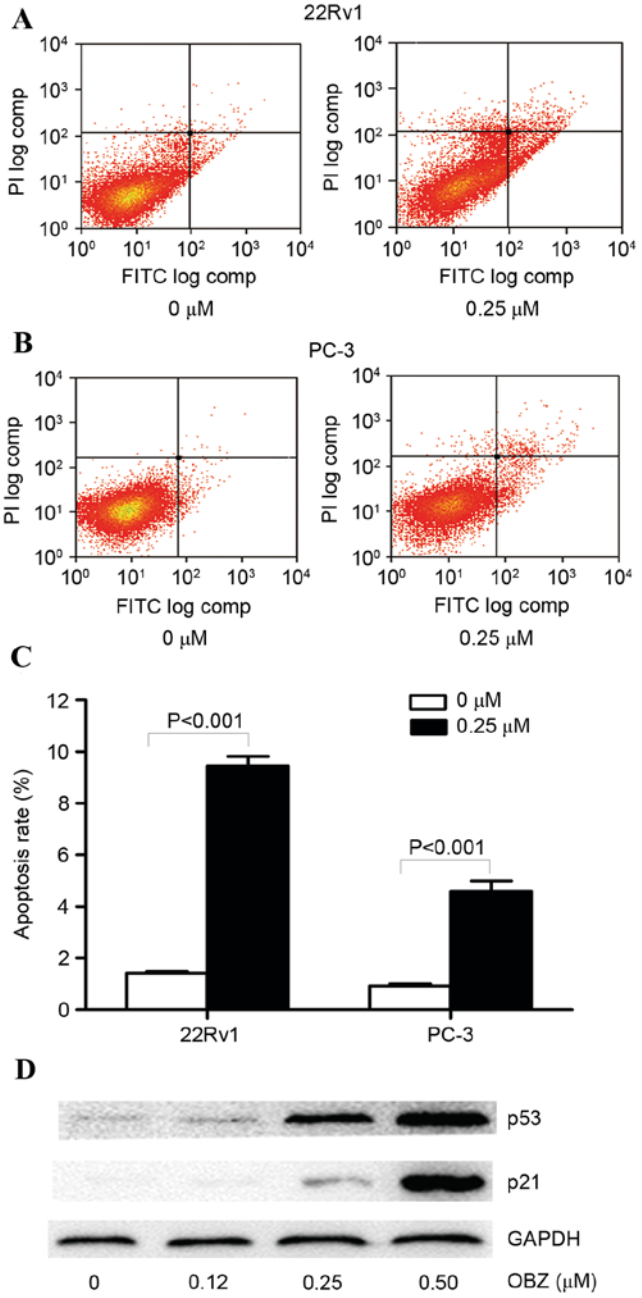

Figure 2. OBZ increases apoptosis in 22Rv1 and PC-3 cells. (A) 22Rv1 and (B) PC-3 cells were treated with 0.25 μM OBZ for 48 h, and then subjected to analysis by flow cytometry. (C) The data were reported as the percentage of early apoptotic cells ($FITC^+/PI^-$) and late apoptotic cells ($FITC^+/PI^+$). (D) p53 and p21 protein levels were determined by western blotting following OBZ treatment for 48 h in 22Rv1 cells. Each reported value represents the mean ± standard error of the mean from three independent experiments. OBZ, oxibendazole; PI, propidium iodide; FITC, fluorescein isothiocyanate; p53, tumor protein p53; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

10 days after injection of the cells, the tumor sizes were measurable. On day 10, the mice were treated with OBZ (25 mg/kg) by intragastric gavage. The treatment was administered once a day for 14 days. The control group of mice was treated in the same way, but OBX was substituted with corn oil. OBZ significantly repressed tumor growth in a time-dependent manner, with a significant difference identified at 20 days after cancer cell inoculation (P<0.05; Fig. 3A). The mean tumor volume of the OBZ-treated group was 0.63 cm^3, whereas in the control group it was 1.20 cm^3; OBZ inhibited growth of the tumor by ~47.96%. Additionally, the mean body weight of the tumor-bearing mice was 24.06±1.28 and 23.10±3.39 g

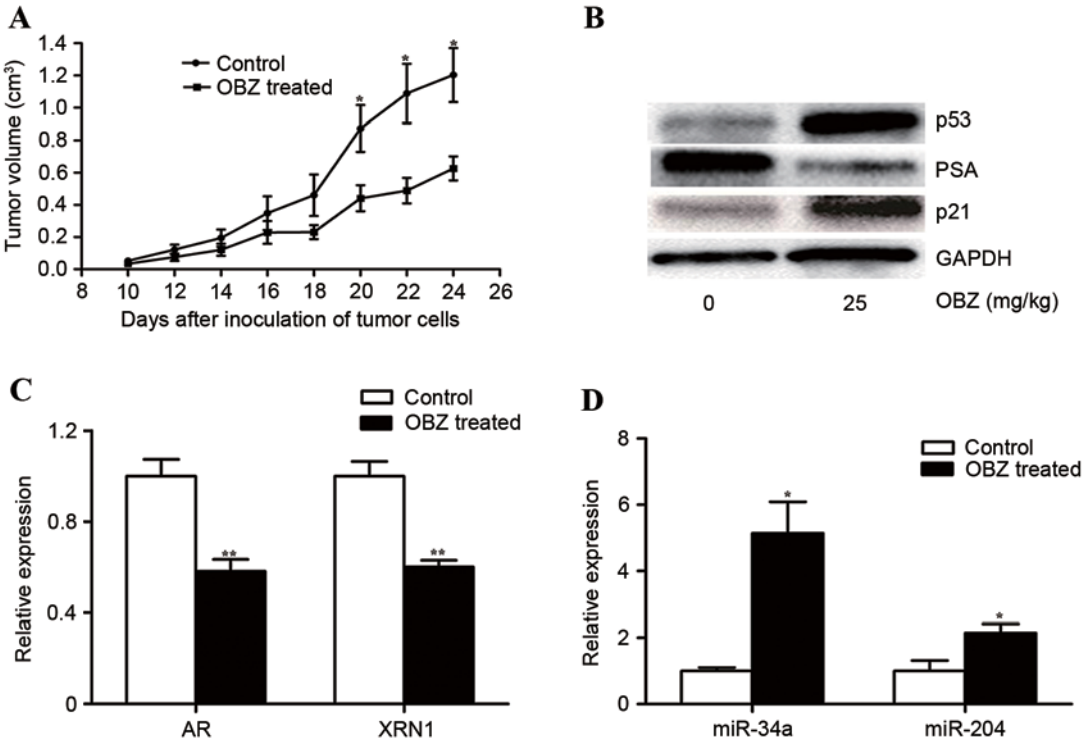

Figure 3. OBZ inhibits 22Rv1 tumor growth *in vivo*. A total of $2x10^6$ 22Rv1 cells were inoculated in the flank of each nude mouse. Tumor volumes were measured at the indicated times. (A) The volume of 22Rv1 tumors was detected every other day in nude mice treated with 25 mg/kg OBZ or vehicle. (B) Protein levels of p53, PSA and p21 were detected by western blotting in the OBZ-treated and control groups. (C and D) Total RNA was prepared from tumors. The expression of (C) AR and XRN1, and (D) miR-204 and miR-34a were measured by reverse transcription-quantitative polymerase chain reaction. *P<0.05, **P<0.01 vs. control. OBZ, oxibendazole; PSA, prostate-specific antigen; p53, tumor protein p53; AR, androgen receptor; XRN1, 5'-3'exoribonuclease 1; miR, microRNA.

in the OBZ-treated and control groups, respectively. However, this difference was not statistically significant, demonstrating that OBZ did not exert a significant general toxic effect *in vivo*, consistent with the results of a previous study (43).

The mechanism for the tumor-suppressive effect of OBZ *in vivo* was also studied. First, the expression of p53 and p21 in the tumor tissues of OBZ-treated mice and their negative control was measured using western blot analysis. The density of the two proteins increased by 821.34 and 75.18%, respectively, in the OBZ-treated tumor compared with the control samples (Fig. 3B). Therefore, the *in vivo* results are consistent with those observed *in vitro*.

Given that PSA is a clinically diagnostic biomarker and key prognostic factor for PCa (35), anti-PCa chemicals have also been assessed for their effect on reducing the level of PSA either in PCa cells or in the blood (44). The level of PSA in OBZ-treated 22Rv1 tumors was measured via western blotting in the present study. The concentration of PSA decreased by 78.16% more in OBZ-treated tumors compared with the control, indicating that OBZ repressed PSA expression.

The AR serves a critical role in the progression and recurrence of PCa (45), and is also the transcriptional activator of PSA expression (46). Therefore, downregulation of PSA by OBZ indicates that OBZ affects the AR or ASP. Recently, a study reported the existence of the AR-miR-204-XRN1 axis in PCa cells, which has a dual regulatory function in mediating the growth of different PCa cells (47). In this axis, androgen/AR raises the level of XRN1, a target of miR-204, by inhibiting expression of miR-204. Accordingly, whether OBZ affects the AR-miR-204-XRN1 axis in 22Rv1 tumors was investigated. Levels of AR, miR-204 and XRN1 in the mouse tumors were measured by qPCR. Expression of AR and XRN1 was significantly lower in OBZ-treated tumors than in the control (both P<0.01; Fig. 3C). By contrast, OBZ raised the expression of miR-204 ~115% (Fig. 3D). Together, these results indicated that OBZ interfered with the AR-miR-204-XRN1 axis in PCa tumors.

miR-34a has been reported to be a tumor suppressive miRNA (48). miR-34a represses the growth of PCa cells by targeting the AR (49,50). As such, the level of miR-34a in OBZ-treated 22Rv1 tumors was measured in the present study. OBZ treatment raised the expression of miR-34a by ~5-fold (Fig. 3D). These results, therefore, were consistent with the hypothesis that OBZ suppresses AR expression by raising the level of miR-34a in 22Rv1 cells.

Manipulated expression of miR-204 affects the sensitivity of 22Rv1 cells to OBZ. To evaluate the role of a disturbed AR-miR-204-XRN1 axis in the inhibition of growth of 22Rv1 tumors using OBZ, the effect of OBZ on miR-204 expression was studied in cultured 22Rv1 cells and PC-3 cells. miR-204 has been widely shown to be a tumor suppressive gene in multiple types of cancer, including AR-expressing PCa cells (47,51-53). However, miR-204 has been reported to be an oncomiR in the PC-3 cell line (47), which is AR-negative and represents a neuroendocrine-like PCa (NEPC) cell (54). The present results, based on RT-qPCR data, showed that 1.00 μM OBZ significantly raised the expression of miR-34a

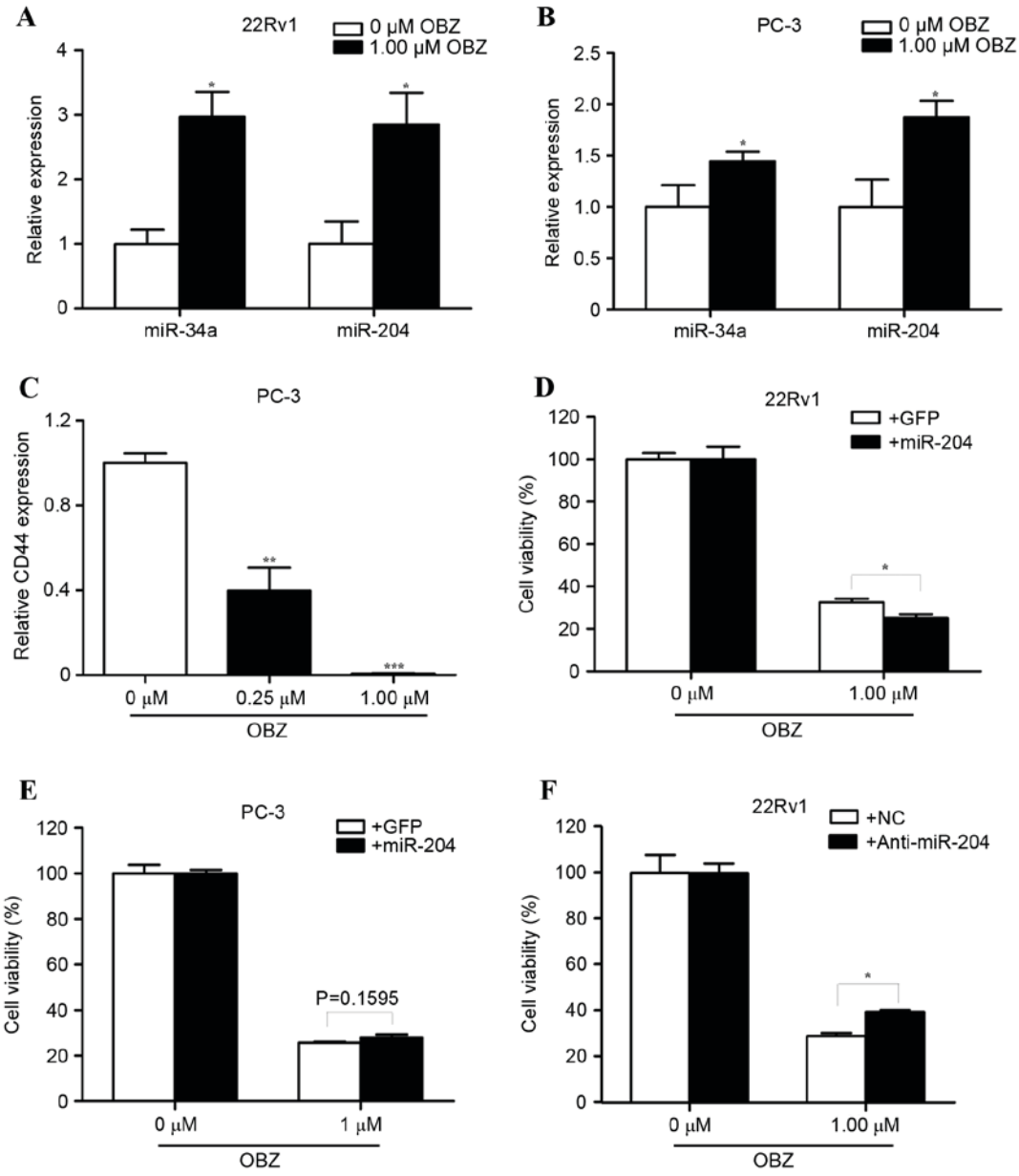

Figure 4. Altered expression of miR-204 affects the sensitivity of 22Rv1 cells to OBZ treatment. Following treatment with 1.00 μM OBZ for 48 h, the total RNA was prepared from (A) 22Rv1 and (B) PC-3 cells. The relative expression level of miR-34a and miR-204 was determined with RT-qPCR. (C) Following treatment with 0.25 or 1.00 μM OBZ for 48 h, the relative level of CD44 was determined with RT-qPCR in PC-3 cells. (D) 22Rv1 and (E) PC-3 cells were stably transfected with an miR-204 or control (GFP) virus. At 24 h after transfection, 1.00 μM OBZ was added for a further 48 h. The cell viability was determined by a trypan blue exclusion assay. (F) 22Rv1 cells were transiently transfected with an miR-204 inhibitor or NC nucleotide. At 8 h after transfection, 1.00 μM OBZ was added for a further 48 h. The cell viability was determined by a trypan blue exclusion assay. All values are the mean ± standard error of the mean from three independent experiments. *P<0.05, **P<0.01, ***P<0.001. miR-204, microRNA 204; OBZ, oxibendazole; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CD44, cluster of differentiation 44; GFP, green fluorescent protein; NC, negative control.

and miR-204 in 22Rv1 cells by 197.07 and 185.11%, respectively (both P<0.05; Fig. 4A). Additionally, 1.00 μM OBZ significantly raised the expression of miR-34a and miR-204 in PC-3 cells by 44.77 and 87.46%, respectively (both P<0.05; Fig. 4B).

Cluster of differentiation 44 (CD44) is a PCa stem-cell marker that is expressed in NEPC cells, including PC-3 cells, but not in AR-expressing PCa cells (54-56). A previous report demonstrated that CD44 is the target gene of miR-34a (57). Considering that OBZ raised the expression of miR-34a in PC-3 cells in the present study, it was speculated that OBZ may suppress the expression of CD44. Notably, 0.25 μM OBZ significantly reduced the expression of CD44 compared with the control (by 60.22%; P<0.01). CD44 expression was almost completely repressed in PC-3 cells that were treated with 1.00 μM OBZ (Fig. 4C). These results supported the hypothesis that OBZ represses CD44 expression by raising the level of miR-34a in PC-3 cells.

If the upregulation of miR-204 is essential for the growth inhibition mediated by OBZ, then ectopic expression of miR-204 should enhance its inhibitory efficiency. To test the hypothesis, 22Rv1 cells and PC-3 cells were stably infected with a recombinant lentivirus expressing miR-204 and green fluorescent protein (GFP). As a control, the cells were stably

infected with a recombinant lentivirus expressing only GFP. Overexpression of miR-204 significantly raised the inhibitory effect of OBZ (by 22.77%; P<0.05; Fig. 4D), suggesting that miR-204 is important to the tumor-suppressive effect of OBZ in 22Rv1 cells. However, the ectopic expression of miR-204 did not significantly change the effect of OBZ in PC-3 cells (P=0.1595; Fig. 4E). To exclude further any potential artificial effect exerted by the overexpression of miR-204 in 22Rv1 cells, the study also assessed whether knockdown of miR-204 could change effect of OBZ. 22Rv1 cells were transiently transfected with a miR-204 inhibitor or a non-targeting control prior to treatment with OBZ. OBZ exerted its inhibitory effect with a lower (by 36.11%) efficiency in cells that were transfected with the miR-204 inhibitor than in those transfected with the control (Fig. 4F). Taken together, these results indicated that upregulation of miR-204 is key for the tumor-suppressive effect of OBZ in 22Rv1 cells.

Discussion

The present study demonstrated that OBZ markedly inhibited the growth of androgen-independent PCa cells in cultured cells and xenograft models (Figs. 1 and 3), at least partially by inducing the apoptosis of the PCa cells (Fig. 2).

AR serves a key role in PCa progression (45), and is the target of drugs used in PCa therapy. AR-regulated genes have been extensively studied in primary and recurrent PCa, but the key genes under the control of AR have remained elusive. Activation of the AR in the PCa AR/miR-204/XRN1/miR-34a positive feedback loop (47) upregulates XRN1 expression by repressing miR-204 expression, whereas XRN1 selectively degrades miR-34a, eventually resulting in the raised expression of AR, since AR is a target gene of miR-34a.

The regulation of the AR/miR-204/XRN1/miR-34a positive feedback loop is severely disturbed in OBZ-treated 22Rv1 tumors, given that OBZ upregulated the expression of miR-204 and miR-34a, but inhibited the expression of AR and XRN1 in the present study (Fig. 3B and C). miR-204 has been reported to act as a tumor suppressor, and is markedly downregulated in various types of solid malignant tumors (51-53) and AR-positive PCa tumors (47). The tumor suppressor activity of miR-204 is mediated not only by its capability to reduce apoptosis and inhibit the epithelial-to-mesenchymal transition (EMT), but also by its power to increase the efficiency of chemotherapy of cancer (58-60). For example, overexpression of miR-204 increased the responsiveness of gastric cancer cells to 5-fluorouracil and oxaliplatin treatment in gastric cancer (52). Similarly, ectopic expression of miR-204 markedly raised the sensitivity of 22Rv1 cells to OBZ in the present study (Fig. 4D). Consistent with these findings, knockdown of miR-204 promoted OBZ resistance (Fig. 4F). These results, together with the observation that OBZ treatment upregulated miR-204, indicates that miR-204 serves a key role in mediating the anticancer effect of OBZ.

Besides interfering with the AR/miR-204/XRN1/miR-34a regulatory loop, OBZ also upregulated the expression of p53 and p21. The upstream event induced by OBZ in 22Rv1 cells is currently unknown. It has been noted that the effect of OBZ on the expression of miR-34a is markedly stronger in 22Rv1 cells, which express wild-type p53, than in the p53-null PC3 cells (Fig. 4A and B) (61). The discrepancy is consistent with the hypothesis that OBZ raises miR-34a expression by upregulating p53 in 22Rv1 cells. However, a further study approach is warranted.

ADT has been used clinically to treat PCa for >70 years. However, ADT can only induce apoptosis in AR-positive cells (or prostatic adenocarcinoma cells) in primary cancer, but does not have a marked effect on AR-negative PCa cells, such as those of NEPC (62). Although NEPC cells only represent a small population (~1%) of PCa cells, they are distributed randomly within prostatic adenocarcinomas and secrete a variety of growth factors that can promote the proliferation of adjacent prostatic adenocarcinoma cells via a paracrine mechanism in an androgen-ablated environment (63,64). Therefore, it has been proposed that NEPC cells should be targeted by treatment in order to prevent the recurrence of PCa (65). PC-3 cells have previously been characterized as small cell neuroendocrine carcinoma cells, a subtype of NEPC cells (54). Notably, OBZ repressed the growth of PC-3 cells *in vitro*, an observation that requires further study *in vivo*. CD44 is a marker of NEPC cells and is highly expressed in PC-3 cells (54-56). Recent research found that CD44 is important to the tumorigenicity of PC-3 cells (66). CD44 expression is markedly repressed in PC-3 cells treated with 1.00 μM OBZ (Fig. 4C), indicating that CD44 serves an indispensable role in the anticancer effect of OBZ.

It should be noted that the capability of OBZ to cause apoptosis and inhibit growth is markedly higher in 22Rv1 cells than PC-3 cells. This is likely to be caused by the two following mechanisms, one associated with p53 and the other with miR-204. Apoptosis can be induced by different stimuli, including chemotherapy, which allows p53 to regulate cellular fate by activating the transcription of several pro-apoptotic BCL2 family members (67). As aforementioned, upregulation of p53 is likely to be the pivotal step mediating the anticancer effect of OBZ in 22Rv1 cells. However, the p53 gene is not present in PC-3 cells (61). As reported previously, miR-204 is a tumor-suppressive gene in 22Rv1 cells, but acts as an oncomiR in PC-3 cells (47). Accordingly, the OBZ-dependent upregulation of miR-204 should, in theory, partially neutralize the growth-inhibitory effect of OBZ in PC-3 cells. However, the method by which OBZ raises expression of miR-204 in PC-3 cells is currently unknown.

Previous studies have shown that OBZ is safe for use in ruminants, in laboratory animals and in humans at concentrations up to 30 mg/kg (43,68), evidence that supports the further study of OBZ as a novel anti-PCa drug.

The present study demonstrated that OBZ markedly inhibited the growth of androgen-independent tumors by the upregulation of miR-204 *in vitro* and *in vivo*. These findings support the potential application of OBZ alone or in combination with other drugs, such as enzalutamide, in the clinical treatment of PCa, particularly recurrent PCa.

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