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Pimozide suppresses colorectal cancer via inhibition of Wnt/β-catenin signaling pathway

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ABSTRACT

Objective: Wnt/β-catenin signaling pathway plays important role in colorectal cancer (CRC) and acts as a potential therapeutic target. Pimozide is a FDA-approved clinical drug used to treat psychotic diseases and it has shown anticancer effect in some tumors partially via inhibition of Wnt/β-catenin signaling pathway. This study aimed to investigate whether pimozide exerts anticancer effect on CRC and explore underlying mechanism.
Methods and results: Pimozide was administrated to treat HCT116 and SW480 cells. Quantitative real-time polymerase chain reaction and western blot were used to detect the expression of epithelial-to-mesenchymal transition markers and Wnt/β-catenin signaling pathway-related proteins. Cell proliferation and migration were measured by Cell Counting Kit-8 and Transwell assays respectively. HCT116 and SW480 cells were subcutaneously injected into nude mice and when the volume of tumor grown measureable (approximately 100 mm^3) animals were treated with vehicle saline or pimozide at a dose of 25 mg/kg·d by oral gavage and then tumor size was measured at 7, 14, 21 and 28 days post treatment. Pimozide dose-dependently inhibited cell proliferation and migration in both HCT116 and SW480 cells, increased expression of E-cadherin and decreased expression of N-cadherin, vimentin and Snail. In addition, tumor growth was inhibited by pimozide in both HCT116 and SW480 xenografts in vivo. Expression of β-catenin and Wnt target genes c-Myc, cyclin D1, Axin 2 and survivin was reduced by pimozide treatment in both HCT 116 and SW480 cells.
Conclusion: Pimozide exerts anticancer effect in CRC via inhibition of wnt/β-catenin signaling pathway, suggesting it as a potential therapeutic drug for CRC.

1. Introduction

Colorectal cancer, with high morbidity and mortality, is the third common cause of cancer-related deaths worldwide [1]. In addition, incidence and mortality rate of CRC is still increasing in some countries, making it a growing public health problem [2]. Currently, it affects 1.23 million patients worldwide each year and approximately accounts for almost 10% of all cancers [3]. In the past decades, considerable effort has been made to elucidate the mechanism of CRC and to improve the therapy for it and advances have been accomplished [4–6]. However, therapeutic utility remains limited and survival rate is still not remarkably improved. Therefore, it is urgent to development new drug with better efficacy and safety.

The mechanism of pathogenesis and progress of CRC is complex and remains not fully understood. It has been evidenced that dysregulation of many cellular signaling pathways including Wnt/β-catenin, PI3K/AKT/mTOR, RAS/RAF/MEK/ERK are involved in it [7–9]. Notably, Wnt/β-catenin signaling pathway is highly focused by many studies and its aberrant activation in CRC plays essential role in tumor initiation, tumor growth and metastasis. In addition, much evidence revealed that activation of Wnt/β-catenin signaling pathway can lead to epithelial-to-mesenchymal transition (EMT) in many cancers, especially CRC [10,11]. As EMT acts as one of the most important events in cancer metastasis, disruption of it through targeting Wnt/β-catenin has become a promising strategy to treat CRC [12]. In fact, many researchers have made great attempt to treat CRC by inhibiting Wnt/β-catenin signaling pathway. For example, miR-490-3p and SMAR1 were found to inhibit development of CRC through inactivation of Wnt/β-catenin signaling pathway [13,14]. However, these strategies are far from application of clinical therapy in CRC. Therefore, it is of great significance to develop or discovery therapeutic agent targeting the Wnt/β-catenin pathway which is available for clinical application.

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Pimozide, a FDA-approved clinical drug used to clinically treat psychotic diseases, Tourette syndrome and resistant tics, has showed anticancer effect on some tumors including breast cancer [15], prostate cancer [16] and hepatocellular carcinoma [17]. Pimozide also has been demonstrated inhibitive for proliferation and maintenance of cancer stem cell and osteosarcoma cell [18,19]. Mechanismly, pimozide exerts its anti-cancer effect partially through inhibition of Wnt/β-catenin signaling pathway [17]. However, whether pimozide can suppress CRC is unknown. In this study we aimed to explore whether pimozide suppresses CRC and investigate potential mechanism.

2. Materials and methods

2.1. Cell culture

Two human colon carcinoma cell lines HCT116 and SW480 were supplied by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum (Gibco) and 100 Units of Penicillin Streptomycin antibiotics (Beyotime Biotechnology, China) and were incubated in humidified atmosphere with 5% CO^2 at 37 °C.

2.2. Cell proliferation assay

The proliferation of HCT116 and SW480 cells was measured by Cell Counting Kit-8 (CCK-8, Tongren, Shanghai, China) following manufacturer's instructions. Briefly, cells were seeded in 96-well plate 12 h before treatment, and then cells were treated with pimozide (Sigma) at various concentrations (0 μM, 5 μM, 10 μM, 20 μM, 50 μM). Then CCK-8 was conducted at the following time points: 0 h, 12 h, 24 h and 48 h. CCK-8 reagent was added into the wells and incubated for 2 h followed by measurement of the absorbance at a wavelength of 450 nm. All experiments were repeated at least three times.

2.3. Cell migration assay

Cell migration of HCT116 and SW480 cells in vitro was measured by Transwell assay (Corning Incorporated, Corning, NY, USA) with 24-well uncoated transwell cell culture chambers. Briefly, cells were re-suspended in serum-free medium and seeded into the top chamber with the lower chamber containing media with 10% FBS and then treated with pimozide (0 μM, 5 μM, 10 μM, 20 μM, 50 μM) for 24 h. Cells on the lower chamber were then fixed with 4% paraformaldehyde in PBS buffer followed by staining with 0.1% crystal violet. The migrated cells were observed by inverted microscopy and were counted in six randomly selected fields to evaluate the cell migration. In order to exclude the interference from the effect of pimozide on proliferation, migrated cell numbers was normalized to total surviving cell numbers.

2.4. Xenograft studies

All experiments using animals were conducted in accordance with the regulations of the Animal Care and Use Committee of North Sichuan Medical College, China. Male Balb/c nude mice (5–6 weeks old, 18–24 g) were purchased from the Laboratory Animal Center of North Sichuan Medical College (Nanchong, China) and kept at 27 ± 2 °C with proper food and water. About 5×10^6 HCT116 or SW480 cells were subcutaneously injected into the right flank of the animals. The animals were then treated with pimozide at dose of 25 mg/kg·d by oral gavage or with saline (control) when the volume of tumor grown measureable (approximately 100 mm^3). According to previous study, the oral LD50 of pimozide is 228 mg/kg in mice and pimozide at 25 mg/kg·d is well tolerated in a mouse model with no significant effects on body weight, indicating this dose is of safety 19. In addition, dose of pimozide at about 25 mg/kg·d showed anticancer effect in hepatocellular carcinoma and other tumors in mice, indicating this dose is effective for treatment of cancer [16,17]. Therefore the dose of 25 mg/kg·d was used to explore the in-vivo effect of pimozide on CRC. Xenografts were collected at the following time points: 7 days, 14 days, 21 days and 28 days and vernier caliper was used to measure diameter. Tumor volume was calculated using the formula: $V = L \times S \times S/2$ (V is tumor volume, L means long diameter, S refers to short diameter).

2.5. Quantitative real-time-PCR (qRT-PCR)

HCT116 and SW480 cells were treated with pimozide (20 μM) for 24 h. Then Total RNA was extracted using TRIzol (ThermoFisher, Waltham, MA, USA) following manufacturers' protocols.

Reverse transcription was performed using 1 μg mRNA with reverse transcriptase enzyme (Bangalore Genei). Then SYBR Select Master Mix (ThermoFisher) was used with ABI 7500 Real-Time PCR System for qPCR. GAPDH was used as internal control and relative expression of mRNA was calculated using the double delta Cq method.

2.6. Western blot

HCT116 and SW480 cells were seeded in 6-well plate and treated with pimozide (20 μM) for 24 h. Then cells were lysed with RIPA buffer (150 mM NaCl,50 mM Tris-HCl [pH 7.4], 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM PMSF and 1% NP-40, 1 mM EDTA). The lysates were incubated on ice for 30 min and centrifuged at 12000*g*/min for 30 min. Then the supernatants were collected and protein concentration was measured with Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). 50 μg of protein was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidine difluoride membranes. The membranes were then blocked with 5% non-fat milk for 1 h at room temperature followed by incubation over night at 4 °C with primary antibodies. After being washed with TBS, the membranes were incubated with corresponding secondary antibodies at room temperature for 1 h. The membranes were then washed and bounds were visualized by the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). GAPDH was used as internal control. The antibodies used were as following: anti-E-cadherin (1:500 dilution, Sigma), anti-N-cadherin (1:500 dilution, Sigma), anti-Vimentin (1:800 dilution, Abcam), anti-Snail (1:800 dilution, Abcam), anti-β-catenin (1:500 dilution, Sigma), anti-c-Myc (1:500 dilution, Sigma), anti-cyclin D1 (1:800 dilution, Abcam), anti-Axin2 (1:800 dilution, Abcam), anti-survivin (1:800 dilution, Abcam), anti-GAPDH (1:1000 dilution, Abcam).

2.7. Statistical analysis

All data are expressed as Means ± SD and analyzed using SPSS 18.0 statistical package. Comparison of means between two groups was conducted using two-tailed Student's *t*-test. Comparison of means among more than two groups was conducted using one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) post hoc test. $P < 0.05$ was defined as statistically significant.

3. Results

3.1. Pimozide inhibits proliferation and migration in colorectal carcinoma cell lines at a dose dependent manner

To characterize the effect of pimozide on CRC cell proliferation, we used CCK-8. Briefly, two human colorectal carcinoma cell lines HCT116 and SW480 were treated with various concentrations (0 μM, 5 μM, 10 μM, 20 μM, 50 μM) of pimozide for 12, 24 and 48 h and then CCK-8 was performed to detect cell proliferation. As shown in Fig. 1A, pimozide at the dose of 5 μM didn't exerted inhibitive effect on proliferation in HCT116 cells. However, pimozide significantly reduced the