Itraconazole Alters the Stem Cell Characteristics of A549 and NCI-H460 Human Lung Cancer Cells by Suppressing Wnt Signaling

Chuanhui Chen
Wei Zhang

Background: Cancer stem cells (CSCs) behave as their malignant counterparts, but persist after treatment, and possess properties that allow them to interact with their environment. Itraconazole, an antifungal agent, also has a role in suppressing tumor progression, but its effects in regulating tumor cell stemness remain unclear. This study aimed to evaluate the effects of itraconazole on A549 and NCI-H460 human lung cancer cell stemness in vitro.

Material/Methods: A549 and NCI-H460 human lung cancer cells and BEAS-2B normal bronchial epithelial cells were cultured with and without itraconazole. Cell viability was evaluated. The expression of stem cell markers, CD133, ATP binding cassette subfamily G member 2 (ABCG2), and aldehyde dehydrogenase 1 (ALDH1), were measured by Western blot and quantitative real-time polymerase chain reaction (qRT-PCR). Sphere-forming cells were evaluated in vitro.

Results: Itraconazole reduced the expression of stemness molecules CD133, ABCG2, and ALDH1 in A549 and NCI-H460 human lung cancer cells, and the numbers of sphere-forming cells were reduced. However, itraconazole had little effect on cell viability but enhanced the chemosensitivity of A549 and NCI-H460 cells. Itraconazole inhibited Wnt signaling. Re-activation of Wnt signaling restored itraconazole-mediated inhibition on A549 and NCI-H460 cell stemness.

Conclusions: Itraconazole altered the stemness characteristics of A549 and NCI-H460 human lung cancer cells by suppressing Wnt signaling but did not affect cell viability.

MeSH Keywords: Itraconazole • Lung Neoplasms • Neoplastic Stem Cells

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Corresponding Author: Wei Zhang, e-mail: wfeng_shen@163.com
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**Background**

Worldwide, lung cancer is a leading cause of cancer death, and clinical treatment includes surgery, radiotherapy, and chemotherapy [1]. However, many patients present with advanced-stage lung cancer, and the prognosis is poor, despite treatment [2]. There have recently been developments in targeted therapy for non-small cell lung cancer (NSCLC), including immunotherapy, which has resulted in the availability of precision or personalized treatments for patients with advanced-stage lung cancer [3]. However, the high rate of metastasis from lung cancer and drug resistance are reasons for the continued high mortality rates from lung cancer. Continued research is needed to identify new therapeutic targets to improve clinical outcome for patients with lung cancer.

Lung cancer stem cells (CSCs) are a cell subset with properties of persistence or immortalization, self-renewal, and the potential to differentiate phenotypically in lung cancer or supporting tissues [4]. Lung CSCs may be involved in the occurrence and development of lung cancer and can provide novel therapeutic targets for treatment [4]. For example, Wu et al. recently showed that depletion of the oncogene, prostate tumor overexpressed gene 1 (PTOV1), sensitized lung cancer cells to chemotherapy by reducing lung CSC-like traits [5]. Also, Wang et al. recently showed that Nutlin-3 could induce apoptosis in lung cancer cells treated with axitinib through inhibition of Akt1/Wnt signaling to reduce lung cancer cell stemness [6]. Hung et al. have recently shown that overexpression of Aiolos in A549 human lung cancer cells promoted the development of lung CSC-like properties [7].

Itraconazole is an antifungal agent that is approved by the US Food and Drug Administration (FDA) that has anti-cancer effects in several tumor types, including inhibiting pancreatic carcinoma cell proliferation through the activation of BAK1-mediated apoptosis [8]. Itraconazole has been shown to inhibit gastric cancer cell proliferation and may have a role in improving prognosis in patients with cancer [9]. Itraconazole also inhibited the progression of human esophageal cancer cells by activating the AMPK signaling pathway [10]. Using organoid cultures of colorectal cancer cells, drug screening showed that itraconazole perturbed cell dormancy through hedgehog signaling effects on the Wnt signaling pathway [11], and the combined use of micellar paclitaxel combined with itraconazole had greater effects [12]. Since most of the lung CSCs are in a dormant state and contribute to chemoresistance, itraconazole may also reduce lung cancer cell stemness.

Therefore, this study aimed to evaluate the effects of itraconazole on A549 and NCI-H460 human lung cancer cell line stemness in vitro by measuring the expression of CSC markers, including CD133, ATP binding cassette subfamily G member 2 (ABCG2), aldehyde dehydrogenase 1 (ALDH1), and sphere-forming cells.

**Material and Methods**

**Cell culture and reagents**

A549 and NCI-H460 human lung cancer cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The normal pulmonary epithelial cell line, BEAS-2B, was purchased from Cobioint (Nanjing, China). The cell lines were maintained in RPMI-1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C with 5% CO₂. The Wnt signaling agonist, SKL2001 (40 μM) was purchased from MedChem Express (Monmouth Junction, NJ, USA). Itraconazole was purchased from Selleck (Houston, TX, USA).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

The complementary DNA (cDNA) was generated with EpiNext Hi-Fi cDNA Synthesis Kit (EpiGentek, Farmingdale, NY, USA) using the total RNA extracted with TRIzol reagent (Sigma-Aldrich, St. Louis MO, USA). The expression levels of transcripts were determined with SYBR Green qPCR Master Mix (MedChem Express, Monmouth Junction, NJ, USA) on the qPCR Detection System ( HealForce, CG-05, Shanghai, China). The relative mRNA levels were analyzed and normalized to GAPDH expression using the 2⁻ΔΔct method [13].

**Western blot**

A549 and NCI-H460 human lung cancer cells and BEAS-2B normal bronchial epithelial cells were gently washed with pre-cooled phosphate-buffered saline (PBS) three times, lysed with protein lystate for 15 min on ice and centrifuged for 15 minutes at 12,000 rpm to collect the supernatant. The protein concentration was quantified by the BCA method (Beyotime, Beijing, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was used to separate the protein samples, which were transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millpore, Billerica, MA, USA) using a 200 mA constant current electrophoresis for 90 min. Then PVDF membranes were blocked using 5% dried skimmed milk powder in PBST (PBS with 0.1% Tween-20), and incubated with primary antibodies overnight at 4°C (1: 1000). After washing three times with PBST, the membranes were incubated with the secondary antibodies and washed three times with PBST. The membranes were developed using the ECL chemiluminescent reagent.

**Analysis of cell spheroid formation**

A549 and NCI-H460 human lung cancer cells and BEAS-2B normal bronchial epithelial cells were inoculated in 12-well
low-adherent culture plates at a cell density of 1000 cell/mL. Three parallel cell groups were set up and 1 mL of serum-free Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium with B27 reagent. Epidermal growth factor (EGF) (20 ng/mL), basic fibroblast growth factor (bFGF) (20 ng/mL), insulin (5–20 g/mL), and hydrocortisone (1 g/mL) were added to the medium every three days. After nine days of culture, the formation of cell spheres was observed under a microscope. The cell spheres in each culture pore (>50 μm) were counted and photographed.

Aldehyde dehydrogenase 1 (ALDH1) activity

The micro acetaldehyde dehydrogenase (ALDH) assay kit (Cat # BC0755) (Solarbio, Beijing, China) was used to measure the activity of aldehyde dehydrogenase 1 (ALDH1) in A549 and NCI-H460 human lung cancer cells with different treatments, according to the manufacturer’s instructions.

Cell viability

A549 and NCI-H460 human lung cancer cells and BEAS-2B normal bronchial epithelial cells were seeded into 96-well plates at the density of 3000 cells/well. After cell adherence, cells were treated with different concentrations of itraconazole and further cultured for another 24 h, 48 h, and 72 h. Cell viability was measured using the cell counting kit-8 (CCK-8) assay (Yi Fei Xue Biotechnology, Nanjing, China).

T-cell factor/lymphoid enhancer factor (TCF/LEF) Wnt reporter plasmid assay

The transcriptional activity of T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) was assayed by firefly luciferase (TOPFlash). Cells were transfected with TOPFlash reporter or FOPFlash luciferase reporter and β-gal. β-gal activity was used for normalization. Then, 40 ng/mL Wnt3a (PeproTech, Rocky Hill, NJ, USA) was used for Wnt3a activation. After 72 h, the measurement of luciferase activity was performed, as previously described [14].

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**Figure 1.** Itraconazole reduced the expression of lung cancer stem cell (CSC) biomarkers and aldehyde dehydrogenase 1 (ALDH1) activity in A549 and NCI-H460 human lung cancer cells. **(A–C)** Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis for the expression levels of CD133 and ABCG2 in A549 and NCI-H460 human lung cancer cells treated with itraconazole. **(D)** Aldehyde dehydrogenase 1 (ALDH1) activity in A549 and NCI-H460 human lung cancer cells treated with itraconazole. * P<0.05 and ** P<0.01.
Statistical analysis

Data were presented as the mean±standard deviation (SD). Student’s t-test or the Tukey-Kramer post hoc test were used to compare data using GraphPad Prism software version X (GraphPad, La Jolla, CA, USA). P <0.05 was assumed to represent statistical significance.

Results

Itraconazole reduced the expression of lung cancer stem cell (CSC) biomarkers and the activity of aldehyde dehydrogenase 1 (ALDH1) in A549 and NCI-H460 human lung cancer cells

To investigate whether itraconazole could reduce the stem cell-like traits of A549 and NCI-H460 human lung cancer cells, we firstly detected the effects of itraconazole on the expression of lung cancer stem cell (CSC) markers, CD133 and ATP binding cassette subfamily G member 2 (ABCG2). Itraconazole reduced the expression of CD133 and ABCG2 in a concentration-dependent manner (Figure 1A–1C). Also, as lung CSCs had a higher level of aldehyde dehydrogenase 1 (ALDH1) activity compared with lung cancer cells, we further examined the effects of itraconazole on ALDH1 activity in A549 and NCI-H460 human lung cancer cells. As shown in Figure 1D, ALDH1 activity was significantly reduced in A549 and NCI-H460 human lung cancer cells treated with itraconazole.

Itraconazole reduced cell spheroid formation of A549 and NCI-H460 human lung cancer cells

Since the spheroid forming ability reflects the self-renewal capacity of lung CSCs, we further detected the effects of itraconazole on the spheroid forming ability of A549 and NCI-H460 human lung cancer cells. Itraconazole reduced the cell sphere size of lung CSCs (Figure 2A). The cell sphere number was also reduced in A549 and NCI-H460 human lung cancer cells treated with itraconazole (Figure 2B). Itraconazole had little effects on the viability of A549 and NCI-H460 human lung cancer cells and BEAS-2B normal bronchial epithelial cells (Figure 2C–2E).
Itraconazole inhibited Wnt signaling in A549 and NCI-H460 human lung cancer cells

We explored the mechanisms by which itraconazole exerts its effects on lung cancer cell stemness. Since Wnt signaling contributed to lung CSC progression, and a previous study showed that itraconazole could inhibit Wnt signaling in cervical cancer, melanoma, and colorectal cancer [11,15,16], we investigated whether itraconazole could suppress Wnt signaling in A549 and NCI-H460 human lung cancer cells. The expression of β-catenin and Wnt3a was reduced in A549 and NCI-H460 human lung cancer cells treated with itraconazole (Figure 3A–3C). Also, the activity of TCF/LEF reporter plasmid, a Wnt reporter plasmid, was reduced in A549 and NCI-H460 human lung cancer cells treated with itraconazole (Figure 3D, 3E). These results showed that itraconazole inhibited Wnt signaling in A549 and NCI-H460 human lung cancer cells.
Reactivation of Wnt signaling recovered itraconazole-mediated inhibition of A549 and NCI-H460 human lung cancer cell stemness

Finally, we investigated whether itraconazole exerts its effects on lung cancer cell stemness in a Wnt signaling-dependent manner. The Wnt signaling agonist, SKL2001, was added to A549 and NCI-H460 human lung cancer cells treated with itraconazole and Western blot analysis for CD133 and ABCG2 expression in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. (C–E) A549 and NCI-H460 human lung cancer cells and Western blot analysis for CD133 and ABCG2 expression in A549 and NCI-H460 human lung cancer cells treated with itraconazole and Western blot analysis for CD133 and ABCG2 expression in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. (F) Evaluation of aldehyde dehydrogenase 1 (ALDH1) activity in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. (G) The cell sphere size in A549 and NCI-H460 human lung cancer cells treated with itraconazole and Western blot analysis for CD133 and ABCG2 expression in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. (H) The sphere-forming cell number was evaluated in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. ** P<0.01.

Figure 4. Reactivation of Wnt signaling reversed itraconazole-mediated inhibition of A549 and NCI-H460 human lung cancer cell stemness. (A, B) The luciferase activity of TOP\textsuperscript{Flash} and FOP\textsuperscript{Flash} was detected in A549 and NCI-H460 human lung cancer cells treated with or without the Wnt signaling agonist, SKL2001. (C–E) A549 and NCI-H460 human lung cancer cells and Western blot analysis for CD133 and ABCG2 expression in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. (F) Evaluation of aldehyde dehydrogenase 1 (ALDH1) activity in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. (G) The cell sphere size in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. (H) The sphere-forming cell number was evaluated in A549 and NCI-H460 human lung cancer cells treated with itraconazole with and without the Wnt signaling agonist, SKL2001. ** P<0.01.

Reactivation of Wnt signaling recovered itraconazole-mediated inhibition of A549 and NCI-H460 human lung cancer cell stemness

Finally, we investigated whether itraconazole exerts its effects on lung cancer cell stemness in a Wnt signaling-dependent manner. The Wnt signaling agonist, SKL2001, was added to A549 and NCI-H460 human lung cancer cells treated with itraconazole. The efficacy of the Wnt signaling agonist, SKL2001, was confirmed by luciferase reporter analysis (Figure 4A, 4B). SKL2001 reversed the inhibition of itraconazole on the expression of lung CSC biomarkers (Figure 4C–4E). Also, the reduction of ALDH1 activity mediated by itraconazole was reversed by SKL2001 treatment of A549 and NCI-H460 human...
lungs cancer cells (Figure 4F). Although itraconazole suppressed spheroid formation, which was partially reversed by the Wnt signaling agonist, SKL2001, which was shown by the reduction of the cell sphere number and size (Figure 4G, 4H). SKL2001 enhanced the sphere-forming cells of A549 and NCI-H460 human lung cancer cells (Figure 4G, 4H). These findings indicated that itraconazole inhibited the stem cell-like characteristics of A549 and NCI-H460 human lung cancer cells by suppressing Wnt signaling.

Discussion

The activity of lung cancer stem cells (CSCs) explains the occurrence and progression of lung cancer and may have significance for the development of more effective treatments for lung cancer [17]. Importantly, the discovery of lung CSC markers facilitates the early diagnosis and differentiation of lung cancer. In the present study, itraconazole reduced the stemness of A549 and NCI-H460 human lung cancer cells, which was demonstrated by the reduced expression of aldehyde dehydrogenase 1 (ALDH1) and reduced sphere-forming cells. Itraconazole had little effect on the cell viability of A549 and NCI-H460 human lung cancer cells and BEAS-2B normal bronchial epithelial cells. To our knowledge, this is the first study on the effects of itraconazole on A549 and NCI-H460 human lung cancer cell stemness.

Previous studies have shown that human lung cancer contains CD133-positive CSCs, which have the ability for self-renewal and have high tumorigenicity [18]. The expression of CD133 in non-small cell lung cancer (NSCLC) is associated with the degree of differentiation, lymph node metastasis, and prognosis [19]. The expression of CD133 is negatively correlated with the prognosis of patients with lung cancer, as lung tumors containing CD133-positive cells are resistant to cisplatin [20]. Therefore, CD133 expression is a marker for lung CSCs.

In 2014, Tang et al. showed that ATP binding cassette subfamily G member 2 (ABCG2) was a regulator of adult stem cell differentiation and was involved in cell division in drug-resistant cancer cells [21]. ABCG2 is widely expressed in normal cells and highly expressed in a subset of stem cells, the side population (SP) cells [22]. A recent study showed that SP cells isolated from multiple lung cancer cell lines exhibited invasive properties and resistance to chemotherapeutic drugs [23]. Lung CSCs have been identified in A549 lung cancer cells, including populations of SP cells. In the present study, itraconazole significantly reduced the expression of CD133 and ABCG2 in A549 and NCI-H460 human lung cancer cells but had little effect on cell viability. These results suggest that itraconazole might specifically lyse lung CSCs.

Several signaling pathways contribute to the progression of lung CSCs, including Wnt signaling, Notch signaling, and Hedgehog signaling. Wnt signaling includes the classical Wnt pathway, and non-classical Wnt/Ca²⁺ pathway, and the Wnt/stress-activated protein kinase (SAPK) or JNK pathway. The classical Wnt signaling pathway induces the proliferation and differentiation of tumors. Dephosphorylated β-catenin accumulates in the cytoplasm, then enters the nucleus and interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) to activate the transcription of downstream targets. In 2014, Tang et al. showed that upregulation of β-catenin in A549 cells could inhibit the proliferation, migration, and drug resistance of A549 cells [21]. Another study found that Wnt5a, a ligand in atypical Wnt pathway, could promote the proliferation, invasion, and migration of A549 cells and cisplatin-resistant A549/DDP cells, and increase the proportion of ALDH-positive cells in the A549/DDP cell population [23], indicating that inhibition of Wnt signaling pathway may be a new strategy for the treatment of lung cancer.

The findings from the present study showed that itraconazole inhibited the expression of β-catenin and Wnt3 in A549 and NCI-H460 human lung cancer cells and suppressed the activity of TOPflash reporter. Importantly, treatment with the Wnt signaling agonist, SKL2001, reversed the itraconazole-mediated suppression of A549 and NCI-H460 human lung cancer cell stemness. Therefore, itraconazole could reduce the CSC characteristics of lung cancer cells by suppressing Wnt signaling. However, other signaling pathways, such as Notch signaling and Hedgehog signaling, are likely to be involved in itraconazole-induced effects on lung CSC, which should also be investigated in future studies.

Conclusions

This study aimed to evaluate the effects of itraconazole on A549 and NCI-H460 human lung cancer cell stemness in vitro and showed that itraconazole altered the stemness characteristics by suppressing Wnt signaling but did not affect cell viability. These preliminary in vitro findings support the need for future in vivo studies to investigate possible therapeutic mechanisms for itraconazole in lung cancer.
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