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Original Article

Anti-proliferation of breast cancer cells with itraconazole: Hedgehog pathway inhibition induces apoptosis and autophagic cell death

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ABSTRACT

Itraconazole is a common antifungal which may have promise for treating various human cancers. We report that itraconazole was cytotoxic to MCF-7 and SKBR-3 breast cancer cell lines via apoptosis by altering mitochondria membrane potential, reducing BCL-2 expression and elevating caspase-3 activity. Itraconazole also induced autophagic cell death via LC3-II expression upregulation, P62/SQSTM1 degradation, autophagosome formation and increases in autophagic puncta. Itraconazole treatment inhibited hedgehog pathway key molecular expression, such as SHH and Gli1, resulting in promotion of apoptosis and autophagy. The anti-proliferation effect of itraconazole-induced apoptosis and autophagy via hedgehog pathway inhibition was confirmed with Gli1 inhibitor GANT61 and SHH siRNA, GANT61 and SHH siRNA synergistically enhanced cytotoxicity induced by itraconazole. A human xenograft nude mouse model corroborated the anti-breast cancer activity as evidenced by reduced tumor size, and increased tumor tissue apoptosis and autophagy. Thus, itraconazole has a potent anti-breast cancer activity that may be improved when combined with hedgehog pathway inhibitors.

Introduction

Breast cancer is a malignant mammary disease representing a significant health burden that is increasing: 14.1 million newly-diagnosed cancer cases and 8.02 million deaths occurred in 2012 [1]. Thus breast cancer is a chief cause of cancer death for females. Chemoprevention is often used to treat cancer, particularly in early tumor stages. Chemotherapy inhibits cancer cell proliferation, enhances surgery and radiation therapy and suppresses metastasis. Taxol, cyclophosphamide, adriacin, fluorouracil and other anti-estrogens are traditional agents for preventing breast cancer [2], but due to resistance and side effects [3], better drugs are needed.

As a broad-spectrum antifungal, itraconazole prevents the synthesis of ergosterol in the fungal cell membrane, suppressing growth [4], but data suggest that it may also have antitumor activity against non-small lung cancer, medulloblastoma, basal cell carcinoma and prostate cancer via inhibiting endothelial cell proliferation, tube formation and tumor vascularity [5–8]. Itraconazole has been studied in phase II trial of non-small-cell lung cancer, basal cell carcinoma, and prostate cancer [9–12]; also, an on-going phase I trial for glioblastoma is carrying on (NCT02770378). Itraconazole extends median progression-free survival to 5.5 months for advanced lung cancer patients, and reduced tumor size by 24% for basal cell carcinoma patients. High-dose itraconazole (600 mg/kg) inhibited metastasis of castration-resistant prostate cancer Thus, itraconazole has potential as a chemotherapeutic agent.

Programmed cell death is essential for maintenance of homeostasis, and protection against disease by removing cells destroyed by cancer, aging, and infection. Therefore, cell death is a valid strategy for controlling cancer progression and several chemotherapeutics use this mechanism of action to treat cancer [13]. Three types of programmed cell death exist: apoptosis (type I),

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autophagy (type II) and necrosis (type III) [14]. Apoptosis is critical to cancer regression as cells initiate it in response to stress or chemotherapeutic agents. Intrinsic and extrinsic signal transduction pathways trigger apoptosis, and apoptotic proteins target mitochondria to alter the membrane potential, releasing cytochrome C. Then, caspase-9 and -3 are activated to initiate intrinsic apoptosis and cause cell death [15]. Autophagy is activated by nutrient deprivation and pathological processes or treatment with pharmacological agents [16]. Activated autophagy promotes autophagosome formation, increase in LC3-II expression, autophagic puncta elevation and P62/SQSTM1 degradation, which contribute to autophagic cell death [17]. PI3k/Akt/mTOR and MAPK signaling pathways are vital regulators in autophagy activation [18]. Also, inhibition of the hedgehog (Hh) induces autophagy [19], indicating that the Hh pathway suppression is a better strategy for cancer cell elimination via autophagic cell death.

Because itraconazole is a multifunctional anti-fungal and can be used to treat carcinomas, repurposing this drug for inhibiting tumorigenesis is a novel approach. Recent work confirms the advantages of itraconazole for preventing heavily pre-treated recurrent triple-negative breast cancer (TNBC) combined with other chemotherapy [20]. However, these pilot data were collected in a small sample size. Therefore, more study is warranted to understand the underlying mechanism in breast cancer prevention. To address this, we investigated the cytotoxicity of itraconazole and attempted to elucidate the mechanism of action in MCF-7 and SKBR-3 human breast cancer cell lines.

Materials and methods

Cell culture

MCF-7 and SKBR-3 cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high glucose DMEM or RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37 °C respectively.

Chemical reagents and antibodies

Itraconazole powder, purmorphamine, z-VAD-fmk and 3-methyladenine (3-MA) were provided by Selleckchem (Huston, TX), and itraconazole injection was purchased from Jassen Pharmaceutical Co. (Xi'an, China). Itraconazole powder was dissolved (50 mg/ml) in DMSO as a stock solution, stored at 4 °C, and diluted with medium before each experiment. GANT61 was purchased from MedChem Express (Guangzhou, China) and dissolved in DMSO. Recombinant human sonic hedgehog/SHH protein (N-terminus, rSHH) was from R&D Systems China Co. Ltd (Shanghai, China). Anti-LC3 (1:800) and anti-P62/SQSTM1 (1:1000) antibodies were provided from MBL (Woburn, MA), anti-sonic hedgehog antibody (1:1000) and Gli1 antibody (1:1000) were purchased from Abcam (Cambridge, MA). BCL-2 (1:1000) and caspase-3 (1:500) antibodies were provided by Biolegend (San Diego, CA). CCK-8 and annexin V-FITC/PI apoptosis kits were purchased from 7sea Biotech (Shanghai, China). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Ig) G (1:1000) and anti-mouse IgG (1:1000) were purchased from CWBiotech company (Beijing, China). A JC-1 mitochondrial membrane potential assay kit was from Beyotime biotechnology (Hangzhou, China). A TUNEL apoptosis kit was provided by Promega (Madison, WI).

Cell viability and cell death assay

MCF-7 and SKBR-3 cells were seeded into 96-well plates (5×10^3 cells/well), and cells were incubated for 24 h and then treated with itraconazole for different duration. After 48 h later, 10 μl per well of CCK-8 was added and incubated for additional 3 h, and optical density was measured under 450 nm with spectrophotometer (Biotek, VT, USA). Cell viability was calculated according to the manufacture's protocol (7sea Biotech). Cell viability = $(OD_{experiment} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$. Treated cells were harvested and suspended with PI buffer. After PI staining at 4 °C for 20 min, samples were assayed with flow cytometry to measure cell death.

Colony formation assay

Cells were plated in 6-well plates (1 × 10 cells/well), and after cells were adherent they were treated with itraconazole for 24 h, and then incubated for 2 weeks. Cells were washed twice with PBS, fixed with methanol, and stained with Giemsa. Colonies were counted under an inverted microscope.

Mitochondrial membrane potential assay

MCF-7 and SKBR-3 were cultured in confocal petri dishes for 24 h and then treated with 20 or 5 μg/ml itraconazole for 12 or 24 h. Subsequently, cells were washed with cold PBS three times. Mitochondrial membrane potential variation was measured using a JC-1 kit under confocal microscopy according to the manufacture's protocol.

Apoptosis assay

Cells were seeded in 6-well plates and treated with 10 μg/ml itraconazole for 12, 24, or 30 h. Apoptosis was quantified with an annexin V-FITC/PI kit according to the manufacturer's protocol. Briefly, control and itraconazole-treated tumor cells were collected via centrifugation and washed once with PBS. Cells were subsequently stained with 5 μl FITC annexin V and 10 μl PI for 15 min at room temperature and analyzed with flow cytometry.

Caspase-3 activity

Caspase-3 protease activity was measured according to the manufacturer's protocol (Beyotime Institute of Biotechnology). In brief, cells were lysed at different time points after treatment with itraconazole. Cell lysates were clarified by centrifugation before incubation with *Ac-DEVD-pNA* for 60 min. Absorbance at 405 nm was measured with a Biotek Eon microplate spectrophotometer (Winooski, VT) and caspase-3 activity was calculated according to a *pNA* standard curve.

Western blot

MCF-7 and SKBR-3 cells were incubated with itraconazole and harvested. Protein was extracted with RIPA lysis buffer after washing with PBS 3 times. Proteins was separated with SDS-PAGE and transferred to a PVDF membrane, which was blocked and washed. Membranes were incubated overnight at 4 °C with primary antibodies (see above for dilutions). After washing, membranes were incubated with secondary antibodies for 1 h, ECL was used to visualize protein bands and protein band quantification was measured with the ImageJ software (version 1.38).

Confocal immunofluorescence

MCF-7 and SKBR-3 cells in the logarithmic growth phase were seeded in 6-well plates. Transient transfection was then performed according to the manufacturer protocol. Briefly, 4 μg GFP-LC3 plasmids and 10 μl Lipofectamine 2000 were suspended with Opti-MEM and mixed and incubated for 20 min at room template. Mixtures were added into cells for transfection, and 24 h later, transfected cells were incubated with 10 μg/ml itraconazole for 30 h. Treated cells were fixed with 4% paraformaldehyde and permeablized with 1% Triton X-100. Then, cells were stained with anti-LC3 primary antibody and FITC-labeled goat anti-rabbit secondary antibody, autophagic puncta were quantified with confocal microscopy.

Transmission electronic microscope (TEM)

MCF-7 and SKBR-3 cells were exposed to 10 μg/ml itraconazole for 30 h. Cells were harvested and fixed with 2.5% glutaraldehyde in PBS (pH 7.8) for 2 h at room temperature and then in 0.1% osmium tetroxide for 1 h at 4 °C. After dehydration with a series of graded ethyl alcohols, cells were embedded in epoxy resin and sectioned. The 50–60 nm ultrathin sections were loaded on copper grids and stained with uranyl acetate and lead citrate. Autophagosome morphology was observed with TEM (JEM-1230, Japan Electron Optics Laboratory, Japan).

Real-time quantitative RT-PCR

Total mRNA was extracted from the treated cells. cDNA was then synthesized, and real-time PCR was performed using a SYBR Green PCR kit (TakaRa, Dalian, China). Each sample was run in triplicate in a final volume of 25 μl containing 1 μl cDNA template, 10 pmol of each primer (Supplementary Table 1), and 12.5 μl of SYBR Green PCR master mix. Reaction conditions were 1 cycle of 95 °C 30 s, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. Data was analyzed with the $2^{-\Delta\Delta CT}$ method.

Human breast cancer xenograft mice model

Six-week-old female nude mice (18 ± 22 g) were inoculated with SKBR-3 cells (1×10^7 cells/each, sc). All experiments were performed in accordance with the protocols approved by the Fourth Military Medical University of Institutional Animal Care and Use Committee (IACUC). Mice were maintained under specific pathogen-free conditions. When tumors reached 100 mm^3, mice were randomized into three groups (n = 5) and treated with itraconazole (30 mg/kg, iv) or with itraconazole and GANT61 (20 mg/kg) per day. Tumors were measured with calipers every other day and tumor volume was calculated as follows: tumor volume = (length × $width^2$) × 1/2. After 14 day's treatment, mice were sacrificed,