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Itraconazole Inhibits Intracellular Cholesterol Trafficking and Decreases Phosphatidylserine Level in Cervical Cancer Cells

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Abstract. *Background/Aim: Itraconazole shows anticancer activity in various types of cancer but its underlying mechanism is unclear. We investigated the effect of itraconazole on membrane-associated lipids. Materials and Methods: To investigate the influences of itraconazole on cholesterol trafficking, cervical cancer CaSki cells were cultured with itraconazole and analyzed by Filipin staining followed by confocal microscopy. Effect on the glycerophospholipid profiles was analyzed by liquid chromatography/mass spectrometry (LC/MS). Results: After itraconazole treatment, Filipin staining revealed cholesterol accumulation in the intracellular compartments, which was similar to the distribution after treatment of U18666A (cholesterol transport inhibitor). LC/MS analysis showed a significant decrease in phosphatidylserine levels and an increase in lysophosphatidylcholine levels in CaSki cells. Conclusion: Itraconazole inhibited cholesterol trafficking and altered the phospholipid composition. Alterations in the cell membrane can potentiate the anticancer activity of itraconazole.*

Itraconazole is used as an antifungal agent and inhibits ergosterol synthesis in the fungal cell membrane by blocking the enzyme lanosterol 14a-demethylase. Since its ability to reverse chemoresistance was reported in 1999 (1), experimental studies have revealed the underlying anticancer mechanisms and that itraconazole down-regulates different signaling pathways in cancer cells and surrounding stromal cells (2). Twelve clinical studies of various types of cancer reported promising results for repurposing itraconazole as an anticancer agent (3-6). Interim analysis of our ongoing WOO trial (jRCTs051190006) revealed a rapid clinical response to itraconazole among enrolled patients with vaginal melanoma and cervical cancer; however, the specific target of itraconazole was not identified in transcriptome analysis (7).

In the 1970s, antifungal drugs were found to exhibit synergistic effects with certain chemotherapeutic drugs by altering the membrane lipid composition of cancer cells (8). Cholesterol and phospholipids are major components of the cell membrane. Previous studies demonstrated that itraconazole inhibited intracellular cholesterol transport in human umbilical vein endothelial cells, glioblastoma cells, and endometrial cancer cells (2). There has been no report on phospholipids associated with itraconazole. In this study, we investigated itraconazole-induced inhibition of cholesterol trafficking and alterations in the membrane lipid composition in CaSki cervical cancer cells.

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Key Words: Itraconazole, drug repurposing, cholesterol trafficking, phosphatidylserine, anticancer activity.

Materials and Methods

Cell culture. The human cervical squamous cell carcinoma cell line, CaSki, was purchased from the RIKEN BioResource Center (Tsukuba, Japan). The cells were cultured according to the manufacturer's instructions.

Filipin staining. Filipin staining was performed as described by Xu *et al*. (9). Briefly, CaSki cells were cultured with 1 μM itraconazole or 1.25 μM U18666A (Sigma-Aldrich, St. Louis, MO, USA) for 24 h, fixed with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, and stained with 50 μg/ml filipin (Sigma-Aldrich) in PBS at room temperature for 2 h. The cells were then washed with PBS three times and mounted for microscopic examination. Images were captured using a Zeiss confocal microscope (Jena, Germany).

Liquid chromatography/mass spectrometry analysis. CaSki cells were cultured with or without 10 μM itraconazole for 48 h, placed on ice, washed with ice-cold PBS, and then detached using a cell scraper. After cell counting, the collected cells were homogenized in 150 μl of methanol containing lysophospha tidylcholine (LPC) (17:0),

phosphatidylcholine (37:4), lysophosphatidylethanolamine (17:1), phosphatidylethanolamine (37:4), lysophosphatidylglycerol (17:1), phosphatidylglycerol (37:4), lysophosphatidylserine (17:1), phosphatidylserine (PS) (37:4), lysophosphatidylinositol (17:1), and phosphatidylinositol (37:4) (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) as internal standards. The mixture was then incubated on ice for 10 min. The homogenates were centrifuged at 20,600 × *g* for 5 min at 4˚C, and the supernatants were collected for liquid chromatography/mass spectrometry (LC/MS) analysis. LC/MS analysis was performed using LCMS-8060 (Shimadzu Co., Kyoto, Japan) accompanied by LC/MS/MS multiple reaction monitoring (MRM) library for phospholipid profiling (Shimadzu Co.), which contains method information on the LC analytical conditions and MRM parameters used for phospholipid analysis. MRM parameters for some lipids and the LC analytical conditions, including the column, which were designed in the LC/MS/MS MRM library for Phospholipid Profiling, were changed by adding information on *sn-1* and *sn-2* lyso-types of glycerophospholipids. Additionally, rather than the column designated in the LC/MS/MS MRM library for phospholipid profiling, L-column2 ODS (2.0×150 mm, 3 μm, metal free: CERI, Tokyo, Japan) was used. The mobile phases were changed into water/acetonitrile=1:2 with 0.1% ammonium formate and methanol/isopropanol=1:19 with 0.1% ammonium formate. The peak area values for each lipid were normalized to the corresponding internal standards, and the relative values were calculated based on the number of collected cells.

Statistical analysis. Statistical comparisons were performed using the Mann–Whitney *U*-test or Wilcoxon signed-rank test. In all cases, a *p*-value of <0.05 was considered as statistically significant. Statistical analysis was performed using the default conditions of JMP9 (SAS Institute, Inc., Cary, NC, USA).

Results

Filipin staining revealed the cholesterol distribution in cells treated with itraconazole, U18666A, and vehicle. Cholesterol was visible on the plasma membrane and nuclear membrane of the control (Figure 1A). In cells treated with U18666A or itraconazole, cholesterol accumulated in the intracellular compartment (Figure 1B and C).

To investigate the influences of itraconazole on the glycerophospholipid profiles, CaSki cells were treated with 10 μM itraconazole for 48 h. Next, glycerophospholipids in the cells were analyzed, and a volcano plot [X-axis: fold-change with *vs*. without itraconazole treatment; Y-axis: -log10 (*p*-values)] was drawn based on the results of lipid analysis. As a result, lipid analysis of the cell membrane showed that itraconazole significantly decreased PS (blue-colored cross) levels, whereas LPC (orange-colored circle) levels were increased (Figure 2).

Discussion

We found that itraconazole altered the lipid composition of cell membranes, decreased PS levels, and inhibited cholesterol trafficking in cervical cancer cells. Previous reports showed that inhibition of cholesterol transportation by itraconazole was mediated by Niemann-Pick C1 (NPC1) protein in human umbilical vein endothelial cells and by sterol carrier protein 2 (SCP2) in glioblastoma cells, respectively (10, 11). Cholesterol depletion appeared to be recovered by downregulation of ATP-binding cassette transporter B1 in endometrial cancer cells (12). In cervical cancer CaSki cells, neither transcriptome nor phosphoproteome analysis revealed the itraconazole-mediated cholesterol redistribution (data not shown).

PS is the major component of the plasma membrane, accounting for up to 15% of the total lipids (13). Under physiological conditions, healthy cells contain low levels of PS. In apoptotic cells, PS is translocated from the inner surface of the plasma membrane to the outer external surface. Exposed PS is recognized by phagocytes, and the removal of immunogenic debris prevents autoimmunity. Thus, externalized PS functions in local and systemic immunosuppression. In cancer cells, PS is expressed at high levels on the outer leaflet of the plasma membrane, and the immunosuppressive function of PS protects cancer cells from immune detection (14). The high level of apoptosis in tumor tissue and difference in PS expression on the cell surface between cancer cells and healthy cells presents a selective therapeutic target and may be useful for overcoming the immunosuppressive microenvironment. Recently, a series of PS-targeting monoclonal antibodies and PS-targeting biomolecules were developed. Bavituximab, a PS-targeting monoclonal antibody, repolarizes myeloid-derived suppressor cells and M2 macrophages to M1, as well as activates antitumor immunity (15). PS-targeting monoclonal antibodies can activate T cell-mediated immunity, and combining these antibodies with immune checkpoint inhibitors is a potential therapeutic strategy. In post-hoc analysis of a phase III lung cancer study (16), bavituximab improved the overall survival of patients administered immune checkpoint inhibitor therapy after study therapy (hazard ratio=0.46, $p=0.006$) (17). Interim analysis of bavituximab plus pembrolizumab in patients with gastric cancer also showed promising results (ESMO, 2020, #1446P).

The study findings will be helpful for potentiating the existing anticancer activity of itraconazole against cancer cells or for studying the synergistic effect of itraconazole with other anticancer drugs.

Conflicts of Interest

SN belongs to a department funded by Shimadzu Co. The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

HT and SN conceived and designed the study. All Authors performed the experiments. HT and RI analyzed and interpreted the data. RI wrote the draft and made critical revisions. All Authors approved the final version of the manuscript.

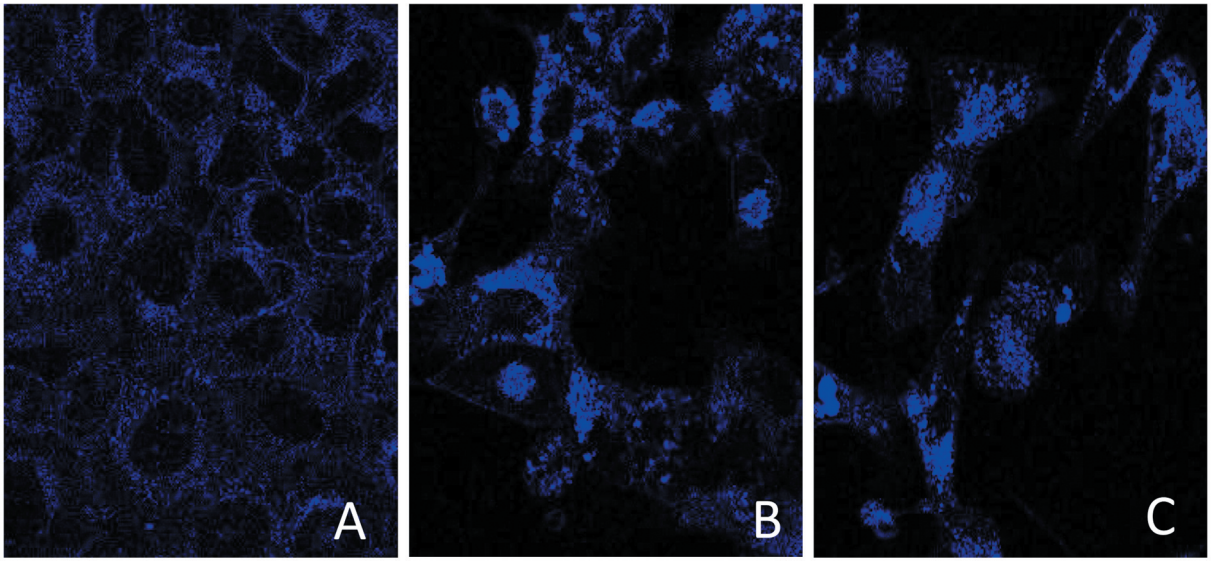

Figure 1. *Confocal microscopy images of CaSki cells treated with vehicle (A), 1.25 μM U18666A (B), and 1 μM itraconazole (C) for 24 h followed by Filipin staining.*

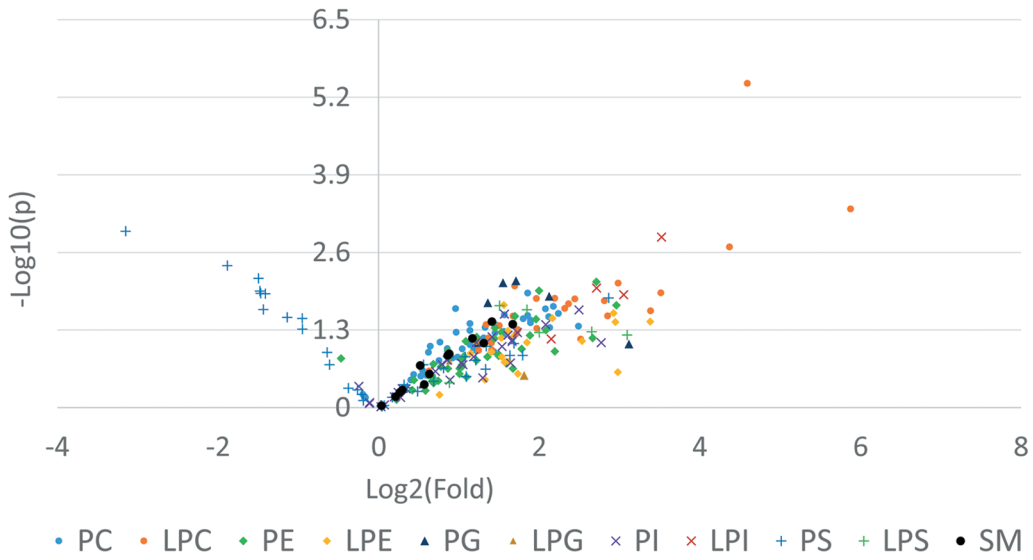

Figure 2. *Volcano plot showing changes in lipid levels after 10 μM itraconazole treatment for 48 h. The phosphatidylserine levels decreased, whereas lysophosphatidylcholine levels increased. X-axis: with vs. without itraconazole treatment. Y-axis: -log10 (p-values). PC: Phosphatidylcholine; LPC: lysophosphatidylcholine; PE: phosphatidylethanolamine; LPE: lysophosphatidylethanolamine; PG: phosphatidylglycerol; LPG: lysophosphatidylglycerol; PI: phosphatidylinositol; LPI: lysophosphatidylinositol; PS: phosphatidylserine; LPS: lysophosphatidylserine; SM: sphingomyelin.*

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References

1 Takara K, Tanigawara Y, Komada F, Nishiguchi K, Sakaeda T and Okumura K: Cellular pharmacokinetic aspects of reversal effect of itraconazole on P-glycoprotein-mediated resistance of anticancer drugs. Biol Pharm Bull *22(12)*: 1355-1359, 1999. PMID: 10746169. DOI: 10.1248/bpb.22.1355

2 Tsubamoto H, Ueda T, Inoue K, Sakata K, Shibahara H and Sonoda T: Repurposing itraconazole as an anticancer agent. Oncol Lett *14(2)*: 1240-1246, 2017. PMID: 28789339. DOI: 10.3892/ol.2017.6325

3 Lan K, Yan R, Zhu K, Li W, Xu Z, Dang C and Li K: Itraconazole inhibits the proliferation of gastric cancer cells *in vitro* and improves patient survival. Oncol Lett *16(3)*: 3651-3657, 2018. PMID: 30127974. DOI: 10.3892/ol.2018.9072

4 Sawasaki M, Tsubamoto H, Nakamoto Y, Kakuno A and Sonoda T: S-1, oxaliplatin, nab-paclitaxel and itraconazole for conversion surgery for advanced or recurrent gastric cancer. Anticancer Res *40(2)*: 991-997, 2020. PMID: 32014944. DOI: 10.21873/anticanres.14033

5 Mohamed AW, Elbassiouny M, Elkhodary DA, Shawki MA and Saad AS: The effect of itraconazole on the clinical outcomes of patients with advanced non-small cell lung cancer receiving platinum-based chemotherapy: a randomized controlled study. Med Oncol *38(3)*: 23, 2021. PMID: 33559053. DOI: 10.1007/s12032-021-01475-0

6 Gerber DE, Putnam WC, Fattah FJ, Kernstine KH, Brekken RA, Pedrosa I, Skelton R, Saltarski JM, Lenkinski RE, Leff RD, Ahn C, Padmanabhan C, Chembukar V, Kasiri S, Kallem RR, Subramaniyan I, Yuan Q, Do QN, Xi Y, Reznik SI, Pelosof L, Faubert B, DeBerardinis RJ and Kim J: Concentration-dependent early antivascular and antitumor effects of itraconazole in non-small cell lung cancer. Clin Cancer Res *26(22)*: 6017-6027, 2020. PMID: 32847935. DOI: 10.1158/1078-0432.CCR-20-1916

7 Inoue K, Tsubamoto H, Isono-Nakata R, Sakata K and Nakagomi N: Itraconazole treatment of primary malignant melanoma of the vagina evaluated using positron emission tomography and tissue cDNA microarray: a case report. BMC Cancer *18(1)*: 630, 2018. PMID: 29866134. DOI: 10.1186/s12885-018-4520-5

8 Kuwano M, Kamiya T, Endo H and Komiyama S: Potentiation of 5-fluorouracil, chromomycin A3, and bleomycin by amphotericin B or polymyxin B in transformed fibroblastic cells. Antimicrob Agents Chemother *3(5)*: 580-584, 1973. PMID: 4133497. DOI: 10.1128/AAC.3.5.580

9 Xu J, Dang Y, Ren YR and Liu JO: Cholesterol trafficking is required for mTOR activation in endothelial cells. Proc Natl Acad Sci USA *107(10)*: 4764-4769, 2010. PMID: 20176935. DOI: 10.1073/pnas.0910872107

10 Head SA, Shi WQ, Yang EJ, Nacev BA, Hong SY, Pasunooti KK, Li RJ, Shim JS and Liu JO: Simultaneous targeting of NPC1 and VDAC1 by itraconazole leads to synergistic inhibition of mTOR signaling and angiogenesis. ACS Chem Biol *12(1)*: 174-182, 2017. PMID: 28103683. DOI: 10.1021/acschembio.6b00849

11 Liu R, Li J, Zhang T, Zou L, Chen Y, Wang K, Lei Y, Yuan K, Li Y, Lan J, Cheng L, Xie N, Xiang R, Nice EC, Huang C and Wei Y: Itraconazole suppresses the growth of glioblastoma through induction of autophagy: involvement of abnormal cholesterol trafficking. Autophagy *10(7)*: 1241-1255, 2014. PMID: 24905460. DOI: 10.4161/auto.28912

12 Tsubamoto H, Inoue K, Sakata K, Ueda T, Takeyama R, Shibahara H and Sonoda T: Itraconazole inhibits AKT/mTOR signaling and proliferation in endometrial cancer cells. Anticancer Res *37(2)*: 515-519, 2017. PMID: 28179296. DOI: 10.21873/anticanres.11343

13 Kay JG and Fairn GD: Distribution, dynamics and functional roles of phosphatidylserine within the cell. Cell Commun Signal *17(1)*: 126, 2019. PMID: 31615534. DOI: 10.1186/s12964-019-0438-z

14 Birge RB, Boeltz S, Kumar S, Carlson J, Wanderley J, Calianese D, Barcinski M, Brekken RA, Huang X, Hutchins JT, Freimark B, Empig C, Mercer J, Schroit AJ, Schett G and Herrmann M: Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. Cell Death Differ *23(6)*: 962-978, 2016. PMID: 26915293. DOI: 10.1038/cdd.2016.11

15 Yin Y, Huang X, Lynn KD and Thorpe PE: Phosphatidylserine-targeting antibody induces M1 macrophage polarization and promotes myeloid-derived suppressor cell differentiation. Cancer Immunol Res *1(4)*: 256-268, 2013. PMID: 24777853. DOI: 10.1158/2326-6066.CIR-13-0073

16 Chau I, Park H, Lee J, Rege J, Culm-Merdek K and Bendell J: Initial safety run-in findings with bavituximab plus pembrolizumab in patients with advanced gastric or gastroesophageal cancer. Journal of Clinical Oncology *38(15_suppl)*: e16537-e16537, 2020. DOI: 10.1200/JCO.2020.38.15_suppl.e16537

17 Gerber DE, Horn L, Boyer M, Sanborn R, Natale R, Palmero R, Bidoli P, Bondarenko I, Germonpre P, Ghizdavescu D, Kotsakis A, Lena H, Losonczy G, Park K, Su WC, Tang M, Lai J, Kallinteris NL, Shan JS, Reck M and Spigel DR: Randomized phase III study of docetaxel plus bavituximab in previously treated advanced non-squamous non-small-cell lung cancer. Ann Oncol *29(7)*: 1548-1553, 2018. PMID: 29767677. DOI: 10.1093/annonc/mdy177

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