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Itraconazole Increases Resolvin E3 Concentration and 12/15-lipoxygenase Inhibitor Attenuates Itraconazole Cytotoxicity in Cervical Cancer Cells

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Abstract. *Background/Aim: The anticancer mechanism of itraconazole remains unsolved; therefore, we studied itraconazole-induced alterations in specialized pro-resolving mediators (SPMs) in cancer cells. Materials and Methods: The human cervical squamous carcinoma cell line CaSki was cultured with or without 1 μM itraconazole. Liquid chromatography/mass spectrometry analysis was conducted to identify SPMs that were influenced by itraconazole. Cell growth experiments were conducted using itraconazole and inhibitors targeting the metabolic pathways of candidate SPMs. Results: Resolvin E3, resolvin E2, prostaglandin J2 (PGJ2), delta-12-PGJ2, and maresin 2 were identified as candidate SPMs. The 12/15-lipoxygenase inhibitor, which is involved in the conversion of 18-hydroxy-eicosapentaenoic acid to resolvin E3, attenuated the inhibitory effect of itraconazole. Inhibition of the PGJ2 metabolic pathway did not interfere with itraconazole treatment. Conclusion: The metabolic pathway of SPMs, including resolving E3, could be proposed as an anticancer target of itraconazole.*

Itraconazole, an antifungal agent, has shown anticancer activities in clinical and preclinical studies. The reversal effect of itraconazole on chemoresistance *via* inhibition of P-gp of cancer cells was reported in 1999 (1). Since 2008, itraconazole has been used in experimental studies to treat cancer patients in combination with chemotherapy. Patients with ovarian, triple-negative breast, biliary tract, pancreatic, and gastric cancers showed better survival with the addition of itraconazole administration (2, 3). In 2007 and 2010, Liu *et al*. identified itraconazole as an anti-angiogenic agent and as an inhibitor of hedgehog signalling, respectively (4). Itraconazole inhibits intracellular signal transduction (Akt/mechanistic target of rapamycin, hedgehog, Wnt/β-catenin), voltage-dependent anion-selective channel 1 in mitochondria, and lipid transportation (sterol carrier protein-2 and Niemann-Pick disease type C1). The mechanisms of growth inhibition vary among cancer cell types and require further elucidation (2).

Interim analysis of an ongoing window of opportunity trial (jRCTs051190006) revealed a clinical response to itraconazole in a patient with vaginal melanoma and several patients with cervical cancer. The patient with vaginal melanoma and other responders experienced effective pain relief within 1 week of oral administration of 400 mg/day itraconazole (5). Inflammation-associated bioactive lipid mediators (LMs) can play a crucial role in regulatory networks affecting cancer cell biology and the tumour microenvironment (6).

In this study, we identified a novel therapeutic target using itraconazole as an anticancer agent. A human cervical squamous carcinoma cell line (CaSki) was used, as it is the most extensively affected by itraconazole in comparison to other cancer cell lines (7).

Materials and Methods

Cell cultures. The CaSki HPV-16+ cell line was obtained from the RIKEN BioResource Center (Tsukuba, Japan). Cells were cultured according to the instructions of the manufacturer.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Itraconazole (Sigma-Aldrich, Tokyo, Japan) was dissolved in N,N-

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dimethylformamide (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions. Thereafter, cancer cells were cultured for 6 h in 6 ml of RPMI 1640 Medium (Thermo Fisher Scientific K.K., Tokyo, Japan) containing itraconazole at a concentration of 10^{-6} M. As a control, cancer cells were cultured in RPMI 1640 and vehicle N,N-dimethylformamide. Methanol at –30˚C was added, and the cells were scraped and stored at –30˚C. Lipidomics studies on both itraconazole-treated and control cells were conducted as previously described (8). Briefly, deuterated internal standards (500 pg of d4–leukotriene B4 (LTB4), d8–5-hydroxy-eicosatetraenoic acid, d4–prostaglandin E2, and d5–resolvin D2) representing each chromatographic region of identified LMs were added for facilitating sample quantification. The samples were extracted using an automated SPE system with C18 columns and were subjected to LC-MS/MS analysis using a Qtrap 6500 system (Sciex) connected to a Shimadzu LC-30AD HPLC system. A ZORBAX Eclipse Plus C18 column (100×4.6 mm, 3.5 μm; Agilent Technologies) was used to elute LMs at a flow rate of 0.4 ml/min. To monitor and quantify the target, a multiple reaction monitoring method was developed with signature ion pairs–Q1 (parent ion) or Q3 (characteristic fragment ion)–for each molecule. The experiments were repeated twice. Molecules whose levels increased more than two-fold either 30 min or 60 min after incubation with 10^{-6} M itraconazole were considered as LMs that were affected by itraconazole, and their biochemical pathways were subjected to further inhibitory assays under itraconazole-induced growth inhibition.

Cell viability assay. Cells (5×10^3/well) were seeded in 96-well culture plates and were allowed to adhere overnight. The attached cells were cultured for 48 h with 10-6 M itraconazole and molecules inhibiting candidate metabolic pathways. For the inhibitory assay of the candidate resolvin E3, a downstream metabolite of 18-hydroxy-eicosapentaenoic acid (18-HEPE) through the omega 3-hydroxylation pathway (9), the 12/15-lipoxygenase (LOX) inhibitor ML351 (Sigma-Aldrich Japan K.K. Tokyo Japan), and the 5-LOX inhibitor zileuton (Sigma-Aldrich Japan K.K. Tokyo Japan) were used. Prostaglandin J2 (PGJ2) and its derivative delta-12-PGJ2 are non-enzymatic metabolites of prostaglandin D2 (PGD2), which is produced by lipocalin-type prostaglandin D synthase (L-PGDS) from prostaglandin H2. To block PGJ2 synthesis, AT56 (Cayman Chemical, Ann Arbor, MI, USA), an L-PGDS inhibitor, was used. In addition, the culture medium containing resolvin E3 with or without itraconazole was used for the cell viability assay. Resolvin E3 was provided by Yuichi Kobayashi, Tokyo Institute of Technology, Japan (10). Cell viability was evaluated using the Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan), according to the instructions of the manufacturer. The cells were incubated with WST-1 for an additional 2 h, and the formazan products were evaluated by measuring their absorbance at 450 nm on a microplate reader. Each experiment was repeated at least thrice.

Statistical analysis. The Mann–Whitney *U*-test was used to evaluate differences between the two groups using the XLSTAT 2014 software (Addinsoft, Paris, France). Statistical significance was set at $p<0.05$.

Results

Itraconazole induced alterations in bioactive LM concentrations in cervical cancer cells. After incubation with 10^{-6} M itraconazole, the concentrations of resolvin E3 and resolvin E2, downstream metabolites of eicosapentaenoic acid (EPA), increased two-fold at 30 min and three-fold at 1 h, respectively (Figure 1). The sum of the concentrations of the arachidonic acid (AA) derivatives PGJ2 and delta-12-PGJ2 increased after 30 min as well as that of their precursor PGD2 (Figure 2). The levels of the downstream metabolite of docosahexaenoic acid (DHA) maresin 2 increased three-fold after 1 h incubation with itraconazole (Figure 3). Moreover, itraconazole is a well-known inhibitor of cytochrome P-450, which converts EPA to epoxyeicosatetraenoic acids (EpETEs), AA to epoxyeicosatrienoic acids (EETs), and DHA to epoxydocosapentaenoic acids (EpDPEs). The concentrations of 14,15-EpETE and 5,6-EET decreased by less than half after 1 h incubation with itraconazole.

An increase in the concentration of resolvin E3 preceded that of resolvin E2. The association between maresin 2 and cell growth is not reported in the literature, according to a search on PubMed. Therefore, the metabolic pathways of resolvin E3 and PGJ2 were subjected to further study.

WST-1 assay. Culture medium containing ML351 (12/15-LOX inhibitor) alone did not affect the proliferation of CaSki cells. Co-treatment with ML351 and itraconazole showed that ML351 negatively interfered with itraconazole (Figure 4). The 5-LOX inhibitor zileuton neither affected cell proliferation nor interfered with itraconazole (data not shown). Cell growth and itraconazole-induced inhibition remain unaffected after the addition of resolvin E3 to the culture medium (Figure 5). The L-PGDS inhibitor AT-56 alone promoted cell proliferation and increased the cytotoxicity of itraconazole (Figure 6).

Discussion

Through LC-MS/MS analysis, this study revealed that itraconazole induced rapid production of resolvin E3. Inhibiting the key enzymes that convert 18-HEPE to resolvin E3 negatively interfered with the anticancer activities of itraconazole. This is the first report on the association of resolvin E3 and cancer. Biosynthesis of specialized pro-resolving mediators (SPMs) has been proposed as an anticancer target of itraconazole.

Cancer development and progression require local chronic inflammation, where crosstalk, including lipid signal communication, between cancer cells and the surrounding stromal cells is necessary (11). Inflammation resolution is highly programmed by SPMs, including resolvins, maresins, protectins, and lipoxins. Resolvin E and D series were classified according to their precursors EPA and DHA, respectively (12). Chemotherapy or radiation therapy resulted in the accumulation of cancer debris, which induces inflammation of the tumour microenvironment and promotes

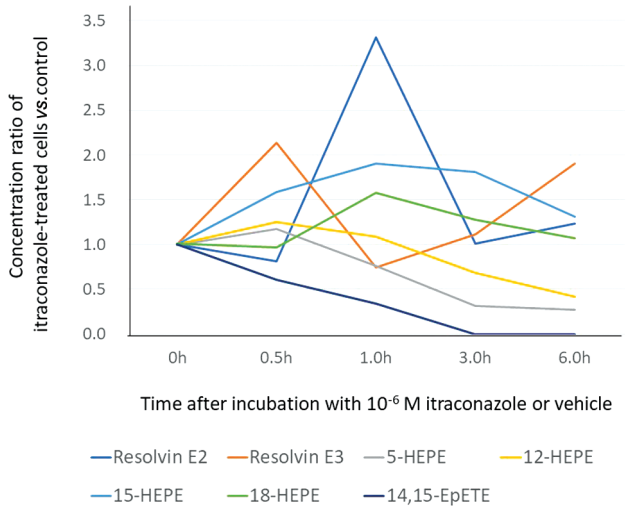

Figure 1. *Downstream metabolites of eicosapentaenoic acid (EPA). Resolvin E3 concentration increased over two-fold and was followed by a three-fold increase in resolvin E2 concentration. 5-HEPE: 5-Hydroxyeicosapentaenoic acid; 12-HEPE: 12- hydroxyeicosapentaenoic acid; 15-HEPE: 15- hydroxyeicosapentaenoic acid; 18-HEPE: 18-hydroxyeicosapentaenoic acid; 14,15-EpETE: 14,15-epoxy eicosatetraenoic acid.*

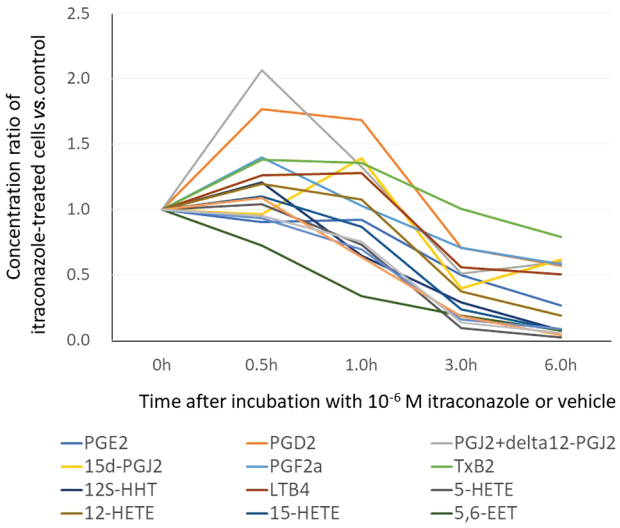

Figure 2. *Downstream metabolites of arachidonic acid (AA). Delta-12-PGJ2 concentration increased over two-fold. PGE2: Prostaglandin E2; PGD2: prostaglandin D2; PGJ2: prostaglandin J2; 15d-PGJ2: 15-deoxy-delta (12,14)-prostaglandin J2; PGF2α: prostaglandin F2α; TxB2: thromboxane B2; 12S-HHT: 12(S)-hydroxyheptadecatrienoic acid; LTB4, leukotriene B4; 5-HETE, 5-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 5,6-EET: 5,6-epoxyeicosatrienoic acid.*

tumour progression. Sulciner *et al*. reported that resolvin D1, resolvin D2, and resolvin E1 terminated the debris-promoting inflammation, thus inhibiting cancer progression (13). Mattosico *et al*. reported that resolvin D1 reprogramed tumour-associated neutrophils and stimulated intratumoural recruitment of anticancer monocytes that inhibit tumour growth (14). Shan *et al*. reported that resolvins D1 and D2 did not affect the proliferation of prostate cancer cells (in vitro) but exerted anticancer activity by affecting tumour-associated macrophages (TAMs) (15). They proposed the interaction between resolvin D series and TAMs as a potential therapeutic target.

Resolvin E3 and resolvin E1/E2 were generated by 12/15-LOX and 5-LOX, respectively, from the EPA metabolite 18-HEPE (9). In 2012, Arita *et al*. found that resolvin E3 blocked neutrophil migration 102-fold more than resolvin E2 and 103-fold more than dexamethasone (16). Furthermore, endometriosis is a common gynaecological disease that affects up to 10% of women of reproductive age and is characterized by exaggerated inflammation around the ectopic endometrial tissues. Tomio *et al*. reported that 12/15-LOX knockout mice had reduced levels of resolvin E3 and increased endometriosis tissue compared with the control (17). In this study, cell growth inhibition by itraconazole was prevented by a 12/15-LOX inhibitor and was uninfluenced by five LOX inhibitors. The culture medium containing resolvin E3 neither affected the cell growth nor interfered

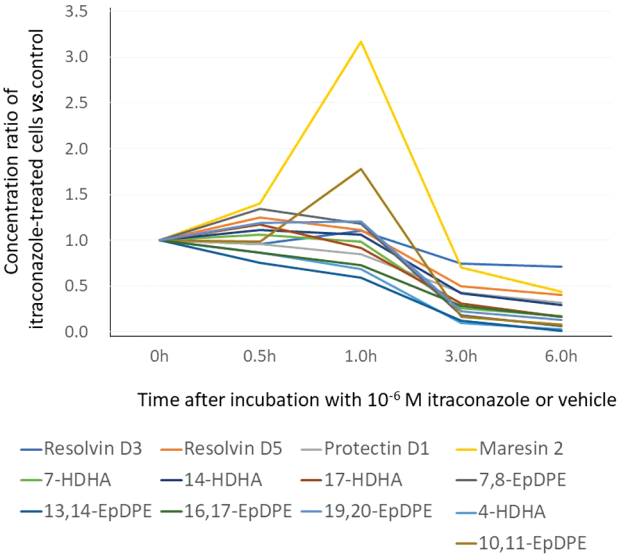

Figure 3. *Downstream metabolites of docosahexaenoic acid (DHA). Maresin 2 concentration increased over two-fold at 1 h. 4-HDHA: 4-Hydroxy docosahexaenoic acid; 7-HDHA: 7-hydroxy docosahexaenoic acid; 14-HDHA: 14-hydroxy docosahexaenoic acid; 17-HDHA: 17-hydroxy docosahexaenoic acid; 7,8-EpDPE: 7,8-epoxy docosapentaenoic acid; 10,11-EpDPE: 10,11-epoxy docosapentaenoic acid; 13,14-EpDPE: 13,14-epoxy docosapentaenoic acid; 16,17-EpDPE: 16,17-epoxy docosapentaenoic acid; 19,20-EpDPE: 19,20-epoxy docosapentaenoic acid.*

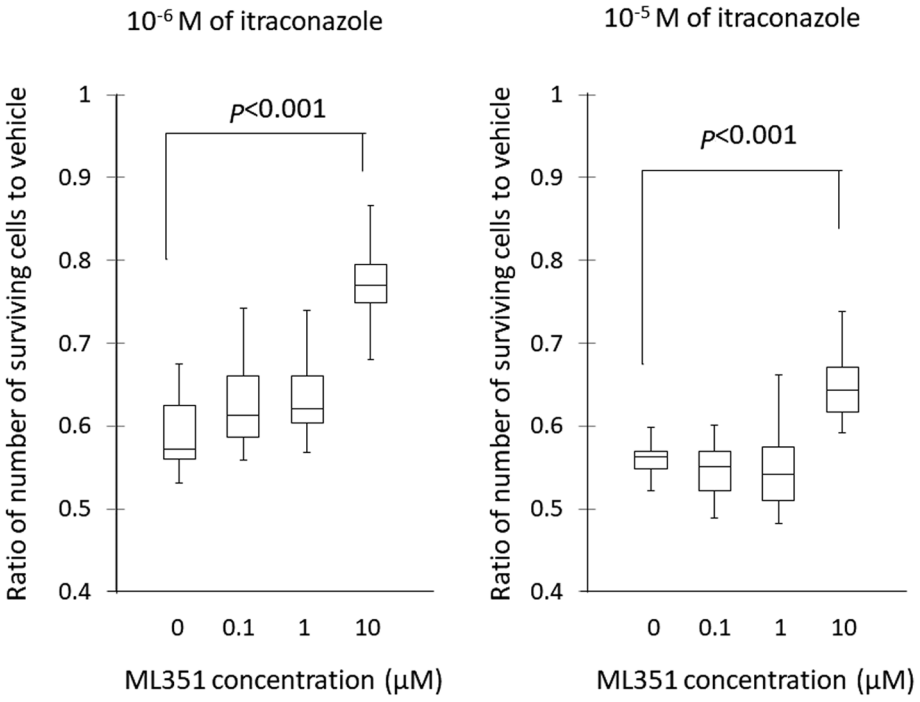

Figure 4. *Co-treatment with ML351 (12/15-lipoxygenase inhibitor) and itraconazole. ML351 interfered with the itraconazole-induced cell growth inhibition.*

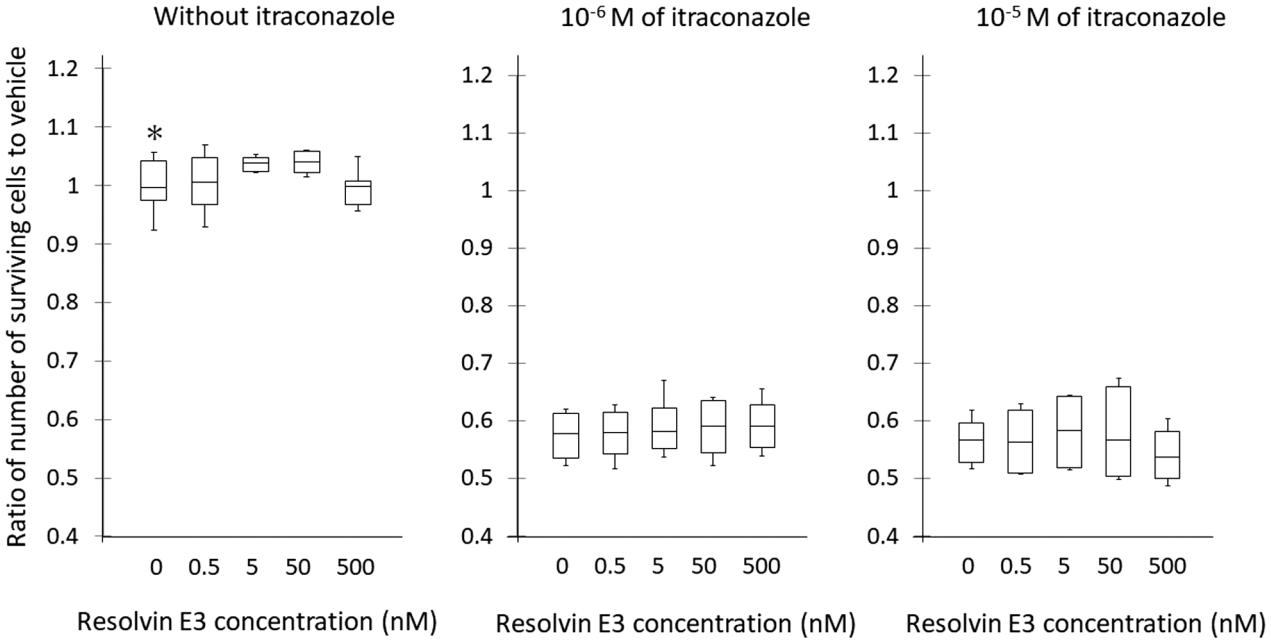

Figure 5. *Effect of resolvin E3 on cell proliferation in the absence or presence of itraconazole. Addition of resolvin E3 to the culture medium did not influence CaSki cell proliferation. The asterisk indicates the vehicle.*

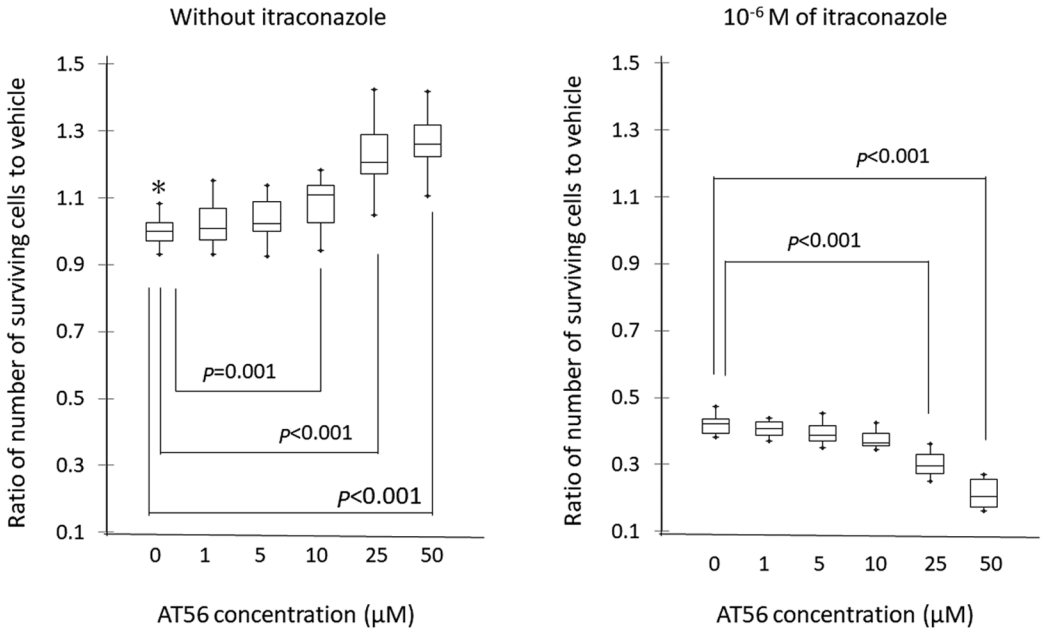

Figure 6. *Co-treatment experiments with AT-56 (lipocalin-type prostaglandin D synthase inhibitor) and itraconazole. AT-56 alone promoted cell proliferation and increased cytotoxicity of itraconazole. The asterisk indicates the vehicle.*

with itraconazole. Unlike resolvin D series (13), resolvin E3 might not directly affect cancer cells.

The limitation of this study is that the 12/15-LOX inhibitor suppresses pathways other than the omega 3-hydroxylation pathway, where resolvins are biosynthesized from EPA. Studies using 12/15-LOX knockout animals demonstrated its multimodal involvement in the pathogenesis of human diseases (18). AA is metabolized by 12/15-LOX to generate 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 15-HPETE, which can be further converted to SPMs, including lipoxins. DHA is converted to 14-hydroxy docosahexaenoic acid (14-HDHA) and 17-HDHA, which is a resolvin D1 precursor.

Spontaneous release of a water molecule from PGD2 forms PGJ2. Delta-12-PGJ2 and 15-deoxy-delta (12,14)-prostaglandin J2 (15d-PGJ2) are generated from PGJ2 *via* albumin-independent and albumin-dependent reactions, respectively (19). Both delta-12-PGJ2 and 15d-PGJ2 showed antitumour activities, and the latter is the most extensively studied natural agonist of peroxisome proliferator-activated receptor-γ (PPAR-γ). PPARs are ligand-activated transcription factors belonging to the nuclear receptor family. Depending on the cell type and concentration used, 15d-PGJ2 exerts both pro-inflammatory and anti-inflammatory effects (20). This study investigated the inhibitory effect of itraconazole on cell proliferation using an L-PGDS inhibitor, which suppressed the synthesis of PGD2–a precursor of PGJ2, delta 12-PGJ2, and 15d-PGJ2. The proliferation of CaSki cells was stimulated in a dose-dependent manner by AT-56. However, when used in combination with itraconazole, a higher concentration of AT-56 showed an additive effect on itraconazole-induced growth inhibition. These results suggest that the anticancer activity of itraconazole is not mediated *via* the metabolic pathways of PGJ2 and delta-12-PGJ2.

Conclusion

Resolvins and the majority of SPMs are originally synthesized by inflammation-associated stromal cells, and their crosstalk with cancer cells is important for cancer progression and metastasis. Further studies are needed to unveil the relationship between CaSki cells and TAMs.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

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

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