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Itraconazole Modulates Hedgehog, WNT/β-catenin, as well as Akt Signalling, and Inhibits Proliferation of Cervical Cancer Cells

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Abstract. *Background/Aim: Repurposing itraconazole as an anticancer agent has been evaluated in several studies. The present study investigated whether itraconazole exerts an anticancer effect on cervical cancer cells. Materials and Methods: CaSki and HeLa cells were cultured in itraconazole and vehicle after which colony-forming and cell viability assays were performed. Transcription and protein expression were assessed by cDNA microarray analysis and immunoblotting, respectively. Results: Itraconazole suppressed proliferation of CaSki and HeLa cells in a dose- and time-dependent manner. Furthermore, CaSki cells were more significantly affected by itraconazole than HeLa cells. The microarray analysis showed an 8-fold down-regulation in the expression of GLI1, WNT4 and WNT10A among itraconazole-treated CaSki cells. Moreover, the transcription of sterol carrier protein-2 and ATP-binding cassette transporter-1 was unaffected by itraconazole. Immunoblots showed suppression in β-catenin expression and Akt phosphorylation. Conclusion: Itraconazole is a multi-targeting anticancer agent and a promising therapeutic agent for cervical cancer.*

Cervical cancer is caused by persistent infection with human papillomavirus (HPV), particularly the oncogenic subtypes, such as HPV16 and 18. Screening for cervical cancer *via* Papanicolaou smears or HPV DNA testing and vaccination against the disease were introduced in developed countries, that have resulted in reduced mortality. However, cervical cancer is still a major gynaecologic cancer especially in developing countries. In 2013, 485,000 new cases were diagnosed and 236,000 mortalities occurred worldwide. In addition, more than 85% of the morbidities and mortalities were recorded in low- and middle-income countries (1). In a randomised trial, bevacizumab, a recombinant humanized monoclonal antibody directed against circulating vascular endothelial growth factor, was found to improve overall survival (OS) among patients with metastatic or recurrent disease. The median OS in the trial was 17 months (2); however, the enrolled patients were in good physical condition. Six percent of the patients who were administered bevacizumab had fistulas of grade 3 or higher, whereas 8% of the patients had thromboembolism of grade 3 or higher, which might have deteriorated their quality of life. There is, therefore, an urgent need for the development of new treatments for cervical cancer.

Drug repurposing meets the economic and time demands of developing new and safe anticancer agents. Itraconazole is a common antifungal agent that has demonstrated reversal effects on P-glycoprotein-mediated chemoresistance and the suppression of Akt/mechanistic target of rapamycin (mTOR), Hedgehog (Hh) and the WNT/β-catenin pathway in various cancer cells. In addition, it has shown effects in the inhibition of angiogenesis and lymphangiogenesis. Survival benefit in cancer patients treated with itraconazole has been proposed in non-small cell lung, ovarian, triple-negative breast, pancreatic and biliary tract cancers (3). Furthermore, itraconazole was found to reverse P-glycoprotein-mediated taxane resistance in HeLa-derived cells (4). It has also been shown that itraconazole alone inhibits proliferation of HeLa cells at a 50% growth inhibitory concentration of 5 μM (5).

In light of the anti-cancerous properties of itraconazole, we investigated the effects of itraconazole on cervical cancer cells and explored molecular mechanisms underlying the effects. We found that itraconazole suppressed Hh, WNT/β-

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catenin, as well as Akt signalling pathways, and inhibited the proliferation of cervical cancer cells in a dose- and time-dependent manner.

Materials and Methods

Cell culture. Human cervical squamous cell carcinoma cell lines (HeLa, HPV-18+; CaSki, HPV-16+) were obtained from RIKEN BioResource Center (Tsukuba, Japan). The cells were cultured according to the supplier's instructions.

Colony-forming assay. The cells were seeded at a density of 1×10^3 cells/well, cultured in a medium containing a specific concentration of itraconazole for 2 weeks and stained with Giemsa's solution.

Cell viability assay (WST-1). Cells (5×10^3/well) were seeded in 96-well culture plates and allowed to adhere to the plates overnight. Attached cells were treated with up to 10 μM itraconazole (Sigma-Aldrich, Tokyo, Japan) for 48, 72 or 96 h. Cell viability was evaluated using Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The cells were incubated with WST-1 for an additional 4 h. Dissolved formazan product was evaluated by measuring the absorbances of the samples at 440 and 630 nm on a microplate reader. Each experiment was repeated at least three times.

Microarray analysis of gene expression. Cells were cultured with 10 μM of itraconazole or vehicle for 48 or 96 h and harvested. RNA was extracted from five paired samples and analysed using SurePrint G3 Human Gene Expression 8×60K v2 Microarray kit (Agilent Technologies, Tokyo, Japan). Altered gene expression was calculated as log2 (mRNA level in itraconazole-treated cells *vs*. mRNA level in vehicle-treated cells), with a difference of more than 4-fold ($\log_2$ ratio >2 or <-2) being considered as a change at the level of transcription.

Immunoblotting. Cell samples were lysed with CelLytic M lysis buffer containing Phosphatase Inhibitor Cocktail 2 and Protease Inhibitor Cocktail. All the reagents were purchased from Sigma-Aldrich. Proteins (2 μg) were size-fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 15% or 5-20% gel. The proteins were then transferred onto a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan), which was blocked with EzBlock Chemi (ATTO) and incubated with primary antibodies against the following proteins: mTOR, phospho-mTOR, p70 S6 kinase (S6k), phospho-p70S6K (p-S6k), β-catenin, C-MYC and Akt (each at 1:1,000 dilution; Cell Signalling Technology, Danvers, MA, USA); Wnt4 and Wnt10A (each at 1:1,000 from Abcam, Cambridge, UK); and β-actin (at 1:1,000 from MBL, Nagoya, Japan). After washing with phosphate-buffered saline containing 0.01% Tween 20 (Wako Pure Chemical Industries, Osaka, Japan), the blots were probed with secondary antibodies for 1 h at room temperature. Immunoreactivity was visualised using an ECL Prime Western Blot Detection kit (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. The Mann-Whitney *U*-test was used to evaluate differences between two groups using XLSTAT 2014 software (Addinsoft, Paris, France). All *p*-values <0.05 were considered statistically significant.

Results

Itraconazole inhibits cervical cancer cell proliferation. Itraconazole inhibited the proliferation of CaSki and HeLa cells in a dose- and time-dependent manner (Figures 1 and 2). However, after incubation with itraconazole at a concentration of 1 μM for 96 h, CaSki cells were more significantly affected than HeLa cells were ($p<0.001$ and $p=0.01$, respectively).

Itraconazole alters the gene expression of the Hh and WNT/β-catenin pathway proteins. In the absence of itraconazole, the transcription of *GLI1* in CaSki cells was 18-fold higher than that in HeLa cells. However, after incubation with 10 μM itraconazole for 48 h, there was down-regulation in *GLI1* transcription by 20-fold (Table I). Moreover, itraconazole caused an 8-fold down-regulation in the transcription of *WNT4* and *WNT10A* and more than a 6-fold up-regulation in the expression of WNT inhibitory factor-1 (*WIF1*) in the CaSki cells (Table II). Sterol carrier protein-2 (*SCP2*) and ATP-binding cassette transporter-1 (*ABCA1*) were unaffected at the transcriptional level by itraconazole in the CaSki or HeLa cells.

Immunoblotting of proteins extracted from CaSki cells after treatment with itraconazole. The expression levels of β-catenin and C-MYC proteins were suppressed after CaSki cells were incubated with 10 μM itraconazole; however, the expression of WNT4 and WNT10A were unchanged. The phosphorylation of Akt was decreased by itraconazole at a concentration of 10 μM; however, that of mTOR or S6k was not affected (Figure 3).

Discussion

The Hh signaling pathway involves three ligands: Indian Hh, desert Hh and sonic Hh (sHh). Transmembrane protein patched 1 (PTCH1) inhibits the activity of smoothened (SMO). When Hh ligands binds to PTCH1, SMO is activated, which leads to the activation of the translational modulators GLI1, GLI2, and GLI3 (6). Hh signaling is activated at advanced stages of cervical cancer. Moreover, it is associated with carcinogenesis, invasion, metastasis, recurrence and chemoresistance (7-10). It has been reported that, in cervical cancer cells, including HeLa and CaSki cells, there is overexpression of GLI1, PTCH1, SMO and SHh. Moreover, it has been noted that Hh inhibitors reduce the proliferation and survival of cervical cancer cells (11).

Furthermore, it has been reported that the Hh signaling pathway is up-regulated in basal cell carcinomas (BCCs) of the skin. Additionally, SMO antagonists, such as vismodegib and sonidegib, are recommended for the treatment of advanced and recurrent disease. Itraconazole reduces sHh-

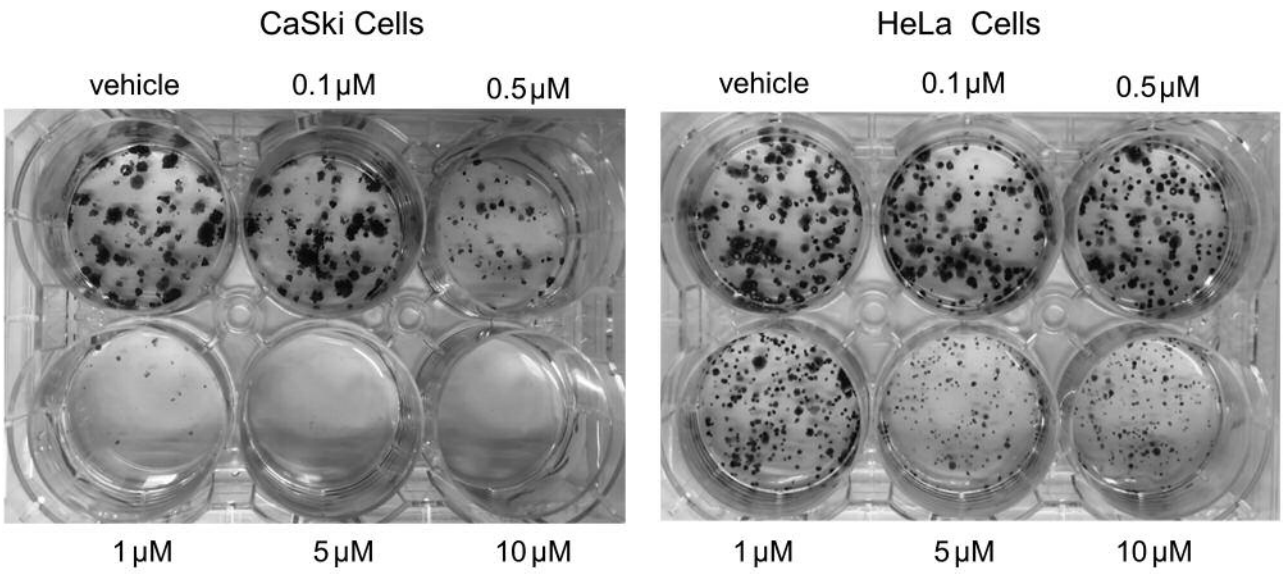

Figure 1. *Representative images showing colony formation after incubation with itraconazole for 14 days. CaSki and HeLa cells were seeded at 1×10^3 cells/well, cultured with a medium containing a specific concentration of itraconazole for 2 weeks and stained with Giemsa's solution.*

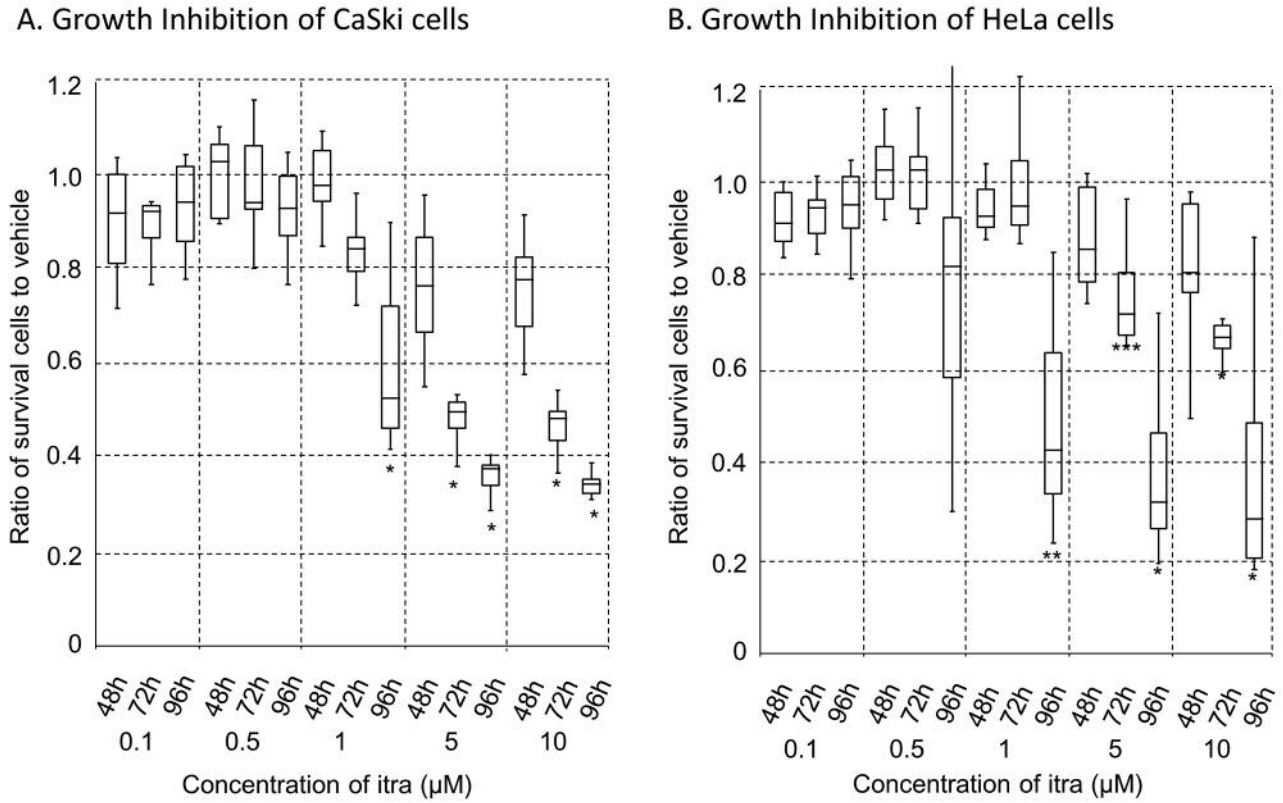

Figure 2. *Inhibition of cell growth by itraconazole. A cell proliferation WST assay of cervical cancer cells was conducted and the number of surviving cells in each medium containing itraconazole was compared to that in the vehicle. Itra, Itraconazole. Statistical analysis was conducted using the Mann-Whitney U-test. *p<0.001, **p<0.01, ***p<0.04.*

Table I. *cDNA microarray analysis of Hh signalling after treatment with itraconazole.*

Log_2ratio	CaSki/HeLa			HeLa		CaSki	
	Vehicle	48 h	96 h	48 h/vehicle	96 h/vehicle	48 h/vehicle	96 h/vehicle
GLI1	4.3	–0.08	1.94	–0.07	–0.18	–4.44	–2.54
GLI2	–3	–2.56	–1.9	–0.16	–0.44	0.04	0.36
GLI3	–1.98	–1.23	–1.84	–0.46	–0.39	0.3	–0.25
sHh	0.01	–0.8	0.32	0.39	0.34	–0.41	0.65
dHh	3.34	–0.79	2.17	–0.68	–0.82	–4.8	–1.99
Ihh	0.31	0.9	1.91	–1.45	–0.71	–0.85	0.89
PTCH2	0.21	0.3	0.21	0.1	0.63	0.26	0.23
SMO	–1.13	0.2	–0.85	–1.18	0.24	0.15	0.52

GLI1 expression was higher in CaSki cells than in HeLa cells. In addition, it was significantly down-regulated by itraconazole in the CaSki cells. The coloured cells indicate >4-fold changes in transcription. dHh, Desert hedgehog; GLI, glioma-associated oncogene homolog; iHh, Indian hedgehog; PTCH, patched family hedgehog receptor; sHh, sonic hedgehog; SMO, smoothened.

induced accumulation of SMO in the primary cilium and suppresses the signals (12). Furthermore, treatment with itraconazole was noted to produce clinical response to BCC in a phase II clinical trial (13). CaSki cells show mutations in sHh (D154E) and PIK3CA (E545K) (14). In the present study, the transcription of *GLI1* was higher in CaSki cells than it was in HeLa cells; however, after treatment with itraconazole, it was at similar levels in the two cell lines.

The phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway has a critical role in tumour progression. The downstream targets of mTORC1 include eIF4E binding protein 1 and S6K, which regulate mRNA translation (15). Abnormal activation of mTOR signalling occurs in various human cancers, including cervical cancer (16, 17). The Cancer Genome Atlas Research Network has revealed that 26% and 8% of squamous cell carcinomas show mutations in in *PIK3CA* and *PTEN*, respectively (18). There is only one reported clinical trial that investigated targeting of the PI3K/AKT/mTOR signaling pathway in cervical cancer. It was a phase II clinical trial in which 38 patients with advanced or recurrent cervical cancer were treated with temsirolimus. The 6-month progression-free survival rate of the patients was 28%. Additionally, the statuses of *PTEN* and *PIK3CA* were studied; however, no correlation between molecular expression signatures and clinical activity of temsirolimus was observed (19). CaSki cells show mutation in *PIK3CA* (E545E) (14). Moreover, phosphorylation of Akt was decreased by itraconazole; however, the downstream pathway involving mTOR and S6k was not affected by the treatment.

The WNT/β-catenin signalling pathway includes canonical and non-canonical pathways, which are activated by the Frizzled family of receptors and a co-receptor (LRP5/6). The canonical signal activates gene transcription and is associated with survival, invasion, metastases and stemness of several cancers. In the absence of WNT ligands, phosphorylated β-catenin undergoes ubiquitination and proteasomal degradation. WNT activation leads to inhibition of the formation of the destruction complex, resulting in β-catenin stabilisation and translocation to the nucleus, where it interacts with transcription factors, such as the T cell factor/lymphoid enhancer-binding factor family. This leads to increased expression of oncogenic genes, such as c-MYC and cyclin D1 (*CCND1*).

Table II. *cDNA microarray analysis of Wnt/β-catenin signalling after treatment with itraconazole.*

Log2ratio	HeLa 48h		CaSki	
	48 h	96 h	48 h	96 h
WNT3A	–0.01	0.47	–0.13	0.21
WNT4	–1.86	–0.56	–3.52	–2.56
WNT10A	–0.08	1.03	–3.11	–2.07
WNT inhibitor 1	–1.99	–2.06	2.56	2.67
WNT inhibitory factor 1	–1.84	–0.19	0.69	0.04
Axin-1	0.44	0.22	–0.8	–0.1
β-catenin	–0.02	0.1	0.27	–0.02
TCF4	–0.06	0.7	0.14	–1.76
TCF7	–0.48	–1.25	1.01	0.91
LEF1	0.24	0.7	–0.41	0.02
C-MYC	0.42	0.89	–1.54	–0.48
Cyclin D1	–0.02	0.38	0.84	0.05

In CaSki cells, itraconazole down-regulated the expression of *WNT3* and *WNT10A*, and up-regulated that of WNT inhibitory factor-1 (*WIF1*). The coloured cells indicate >4-fold changes in transcription. LEF, Lymphoid enhancer-binding factor; TCF, T-cell factor.

It has been reported that WNT4 is up-regulated and associated with tumour progression in gastric cancer (20, 21). Moreover, in oesophageal squamous cell carcinoma, tumour invasion is associated with up-regulation of WNT10A and

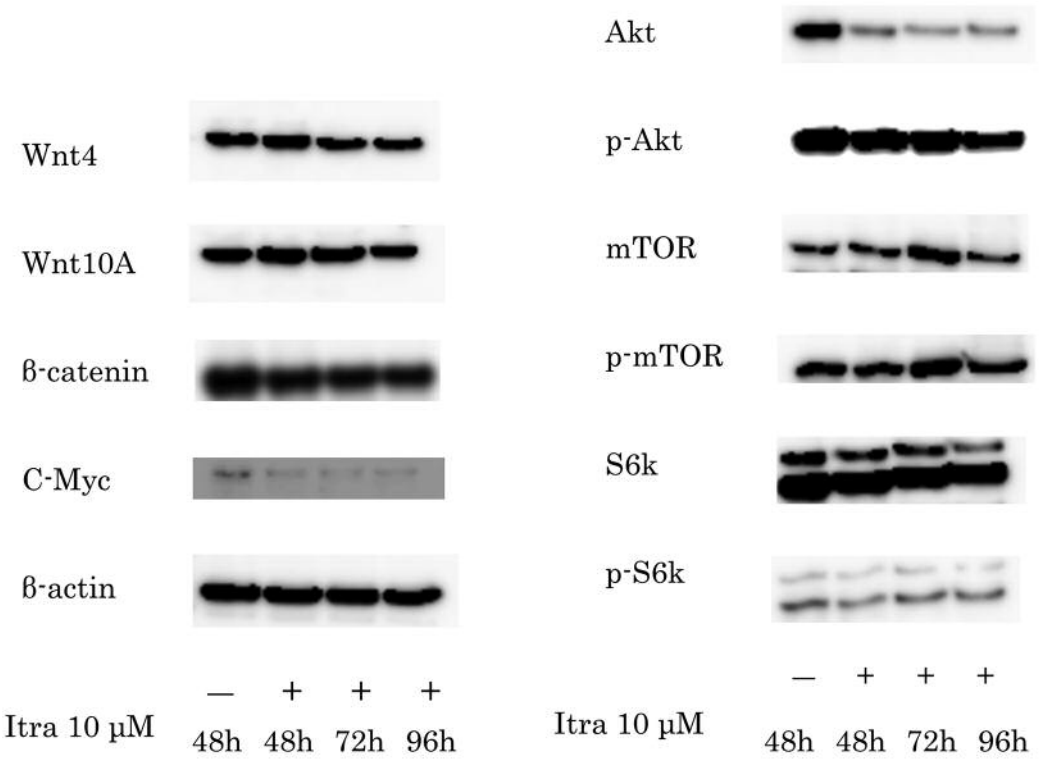

Figure 3. *Western blotting of CaSki cells after treatment with itraconazole. After incubating CaSki cells with 10 μM itraconazole, the expression levels of β-catenin and C-MYC proteins decreased. In addition, the phosphorylation of Akt was decreased, whereas neither that of mTOR or S6k was affected. Itra, itraconazole; mTOR, mechanistic target of rapamycin; p-S6k, phosphorylation of p70 S6 kinase; S6k, p70 S6 kinase.*

down-regulation of WNT inhibitory factor-1. In addition, WNT10A induces stem-cell-like properties of self-renewal (22).

In cervical cancer, HPV E6 and E7 oncoproteins are involved in β-catenin nuclear accumulation and induce the activation of the WNT/β-catenin signalling pathway (23, 24). β-Catenin expression is associated with impaired survival (25).

Furthermore, in human umbilical vein endothelial cells, itraconazole inhibits intracellular cholesterol trafficking by binding to Niemann-Pick C1 protein (26, 27). In U87 glioblastoma cells, itraconazole blocked the trafficking of cholesterol by reducing the transcription of *SCP2* (28). In another study, the transcription of *ABCA1*, which promotes cholesterol efflux across the plasma membrane, was down-regulated in endometrial cancer cells, whose proliferation was unaffected by itraconazole (29). Similarly, in the present study, neither *SCP2* nor *ABCA1* was affected at the transcriptional level by itraconazole in CaSki or HeLa cells.

Conclusion

We have previously reported that itraconazole exhibited anticancer effect against endometrial cancer cells (29). Cervical and endometrial cancers are diagnosed by outpatient tissue sampling in daily practice. We are currently carrying-out a window-of-opportunity clinical trial to assess the anticancer activity of itraconazole. We aim to identify relevant biomarkers in responders by obtaining tissue samples, as well as peripheral blood samples, before and after oral administration of itraconazole (UMIN000018388).

Conflicts of Interest

The Authors have no conflict of interest to declare.

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