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Avinash Kumar
University of Mississippi Medical Center, akumar@umc.edu

Shih-Yun Lin
University of Mississippi Medical Center

Swati Dhar
University of Mississippi Medical Center, sdhar@umc.edu

Agnes M. Rimando
USDA,Agriculture Research Service, Natural Products Utilization Research Unit, arimando@olemiss.edu

Anait S. Levenson
University of Mississippi Medical Center, alevenson@umc.edu

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Stilbenes Inhibit Androgen Receptor Expression in 22Rv1 Castrate-Resistant Prostate Cancer Cells

Avinash Kumar[1], Shih-Yun Lin[1], Swati Dhar[1], Agnes M. Rimando[2] and Anait S. Levenson[*1, 3]

[1]Cancer Institute and [3]Department of Pathology, University of Mississippi Medical Center, Jackson, MS 39216, USA

[2]Unites States Department of Agriculture, Agriculture Research Service, Natural Products Utilization Research Unit, University, MS, USA

[*]**Corresponding author:** alevenson@umc.edu

ABSTRACT

Androgen receptor (AR) signaling plays an important role in the development and progression of prostate cancer (PCa). Importantly, AR continues to be expressed in advanced castrate-resistant PCa (CRPC), where the AR can have ligand-independent activity. Identification of naturally occurring substances that can inhibit AR expression holds promise for PCa chemoprevention and therapy. Earlier research demonstrated that resveratrol (Res) inhibited androgen-promoted growth, AR expression, and transactivation in androgen-responsive non-metastatic LNCaP PCa cells. In the current study, the effects of Res and three natural analogs [trimethoxy-resveratrol (3M-Res), pterostilbene (Pter), and piceatannol (Pic)] were investigated for effects on the growth of 22Rv1 castrate-resistant cells that express full-length (AR114/110) and truncated form (AR80) of AR. Although all the stilbenes tested inhibited the proliferation of 22Rv1 cells in a dose-dependent manner, 3M-Res was the most potent inhibitor. While AR114/110 responded to the syn-thetic androgen agonist methyltrienolone (R1881) as well as to antiandrogen flutamide, AR80, which lacks a ligand-binding domain, did not respond to R1881, but was inhibited by flutamide. Interestingly, Res, Pter, and Pic, but not 3M-Res, similar to flutamide, inhibited both AR114/110 and AR80 with the effect on AR80 being more prominent with the use of high concentrations of stilbenes. Collectively, the data indicate that both AR-independent (3M-Res) and possibly AR-dependent (Res, Pter, and Pic) mechanisms of cell growth inhibition occurs via these stilbenes. These findings provide evidence for plant-derived stilbenes as attractive and promising pharmacologically safe compounds to be used for diminishing progression and curb worsening of CRPC.

INTRODUCTION

Plant-derived polyphenols are attractive clinical candidates for cancer prevention and treatment. Resveratrol (Res) (*trans*-3,5,4'-trihydroxystilbene) is a polyphenol, a stilbene phytoalexin that is synthesized by a wide variety of plants in response to environmental stress and other stressors (Bhat and Pezzuto, 2002). *Polygonum cuspidatum* (common name Kojokon) is one such plant that produces Res (Burns et al., 2002). Res is also produced by grape

vines (*Vitis vinifera*), primarily in the grape berry skin (Fremont, 2000).

Pterostilbene (Pter) (*trans*-3,5-dimethoxy-4'-hydroxystilbene), a natural analog of Res, is a constituent in blueberries and grapes (Rimando et al., 2004), and trimethoxy-resveratrol (3M-Res) (*trans*-3,5,4'-trimethoxystilbene) is a constituent in *Pterobolium hexapatallum* (common name Indian Redwing) (Wang et al., 2010). Another naturally occurring analog, piceatannol (Pic) (*trans*-3,4,3',5'-tetrahydroxystilbene), which is a constituent in grapes and red wine, contains one extra hydroxy group (Kang et al., 2011). Resveratrol has been widely studied because of various health benefits associated with the compound, such as cardiovascular, neuroprotective, anti-inflammatory, antioxidant, and anticancer effects (Athar et al., 2007; Harikumar and Aggarwal, 2008; Markus and Morris, 2008; Pirola and Frojdo, 2008). In recent years natural analogs of Res have attracted increasing attention as a number of studies have suggested that modifications to the chemical structure, such as methoxylation-hydroxylation (Fig. 1) could enhance bioactivity (Huang et al., 2007; Kondratyuk et al., 2011; Wilson et al., 2008; Gosslau et al., 2008).
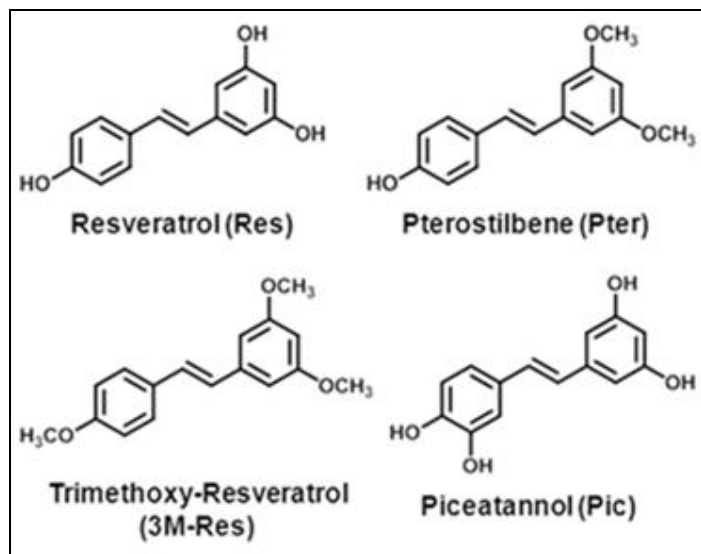

Figure 1. Chemical structures of natural stilbenes used in this study.
Resveratrol (Res), *trans*-3,4,5'-trihydroxystilbene; Pterostilbene (Pter), *trans*-3,5-dimethoxystilbene; Trimethoxy-Resveratrol (3M-Res), *trans*—3,5,4''-trimethoxystilbene; Piceatannol (Pic), *trans*-3,5,3'4'-tetrahydroxystilbene.

Prostate carcinoma, the most commonly diagnosed cancer in men in the Western countries, represents a public health problem with unmet therapy. Signaling through the androgen receptor (AR) plays an essential role in the initiation and progression of prostate cancer. Androgen-deprivation therapy (ADT) is used as a first-line treatment for metastatic prostate cancer, in which AR continues to be expressed and active. After significant clinical response, however, patients with advanced prostate cancer consistently relapse with a more aggressive form of PCa known as castration-resistant PCa (CRPC).

Several mechanisms have been suggested to mediate AR reactivation during CRPC progression, including AR gene amplification or overexpression, expression of splice variants, AR mutations – all of these conferring ligand promiscuity and/or ligand-independent activity that lead to cancer cell pro-liferation. Since the available anti-androgens have low affinity for AR and cannot completely block androgen action, especially in the presence of increased AR levels and AR variants, the discovery of novel potent and antagonistic blockers of AR is very important.

Kai and Levenson (2011) have previously demonstrated that Res inhibits the activity and decreases levels of AR in the androgen-dependent cell line LNCaP. The AR in these cells bear a mutation T877A in the ligand binding domain that affects the response to androgens and anti-androgens (Mahmoud et al., 2013; Otsuka et al., 2011). Although initially isolated from lymph node metastasis, LNCaP cells in tissue culture represent a relatively "non-aggressive" stage of PCa as these cells form tumors with much difficulty and do not form metastasis in xenografts.

In contrast, 22Rv1 cells are castrate-resistant cells derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft (Sramkoski et al., 1999). In pathophysiology, this represents a late, aggressive stage of PCa expressing 114/110 kDa full-length and truncated 80 kDa AR variants (Guo et al., 2009).

The goal of the current study was to examine the effects of Res and the natural analogs of Res (3M-Res, Pic, and Pter) as potential new AR blockers in 22Rv1 cells. In this study, Res and Res analogs inhibited cell proliferation and full-length as well as truncated AR expression in 22Rv1 cells. The inhibitory effect on truncated AR levels was most prominent at the higher concentrations of the tested compounds. The study results provide substantial evidence for plant-derived stilbenes as promising compounds for use, probably in combination with other anti-androgens, as inhibitors of AR signaling and thereby diminishing progression and worsening of CRPC.

MATERIALS AND METHODS

Chemicals. Resveratrol (Res) and piceatannol (Pic) were purchased from Sigma-Aldrich (St. Louis, MO) and Calbiochem-Novabiochem (San Diego, CA), respectively. Trimethoxy-resveratrol (3M-Res) and pterostilbene (Pter) were chemically synthesized at the USDA, ARS, National Products Utilization Research Unit in Oxford, MS, as previously described (Paul et al., 2010; Rimando et al., 2002). Structures of the synthesized compounds were confirmed by nuclear magnetic resonance spectroscopy and mass spectrometry. All compounds had $\geq 99\%$ purity and were dissolved in high purity DMSO and stored in the dark at -20^0C.

Cell culture. The 22Rv1 cells were grown in RPMI 1640 media (GIBCO, Grand Island, NY) containing 10% Fetal Bovine Serum (GIBCO, Grand Island, NY) and 1% antibiotic-antimycotic (GIBCO, Grand Island, NY) at 37^oC and 5% CO_2 as previously described for other prostate cancer cells (Dias et al., 2013). For experiments involving treatment with Res and analogs, media was replaced with phenol red-free RPMI-1640 (GIBCO, Grand Island, NY) containing 5% charcoal-stripped serum (GIBCO, Grand Island, NY) at least 8-10 h prior to treatment to eliminate hormonal background.

Cell proliferation assay. Cell viability was measured by the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 22Rv1 cells were seeded at 3×10^3 cells/well density in 96-well plates using usual media. The media was changed to phenol red-free with 5% charcoal-stripped serum 8-10 h prior to treatment. Fresh media (100 μL), containing serial concentrations of Res, 3M-Res, Pter, and Pic, was added each day for 72 h, after which 20 μL of 5 mg/mL MTT (Sigma-Aldrich, St. Louis, MO) was added to the treatment media in the cells. After a 4 h incubation at 37^0 C, the media containing MTT was removed and the formazan crystals were dissolved in 100 μLof solvent (4 mM HCl and 0.1% TritonX-100 in isopropanol). The absorbance of the formazan at 590 nm was measured using Synergy-4 plate reader (BioTek Instruments Inc., Winooski, VT). IC_{50} values were calculated by the linear interpolation method, using MS Excel software (Microsoft, Redmond, WA).

Western blot analysis. Western blot analysis was done as described previously (Dias et al., 2013; Li et al., 2013). Briefly, 22Rv1 cells at 50-60% confluency were treated with selected concentrations of Res and Res analogs for 24 h. The cells were subsequently lysed in RIPA buffer (ThermoFisher, Waltham, MA) containing protease and phosphatase inhibitor cocktail (ThermoFisher, Waltham, MA). The protein concentration in the lysates was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (50 μg) were resolved in 10% SDS-PAGE gel and transferred to a PVDF membrane by Mini-Trans-Blot Electrophoresis Transfer System. The membranes were subsequently probed with AR (N20) antibody (Santa Cruz Biotechnology, Dallas, TX). β-actin (Santa Cruz Biotechnology, Dallas, TX) was used as a loading control. Signals were visualized using the chemiluminescent substrate Super-signal West Dura (ThermoFisher, Waltham, MA). Densitometry was done using Image J software.

Statistical analysis. The differences between the values of experimental and control treatments were analyzed for statistical significance by two-tail Student t test. The p values ≤ 0.05 were considered to be significant.

RESULTS

Prostate cancer inhibition. An examination on the growth of 22Rv1 castrate resistant prostate cancer cells treated with Res and the three natural analogs of Res (3M-Res, Pter, and Pic) was performed. The cells were treated with various

concentrations of the analogs (1-100 µM) in phenol red-free RPMI 1640 media supplemented with 5% charcoal-stripped FBS for 3 days. After treatment, a cell proliferation assay was done to assess the growth inhibitory activity of Res and analogs on 22Rv1 cells. All the tested compounds inhibited the growth of 22Rv1 cells in a dose dependent manner, but with different potencies (Fig. 2).
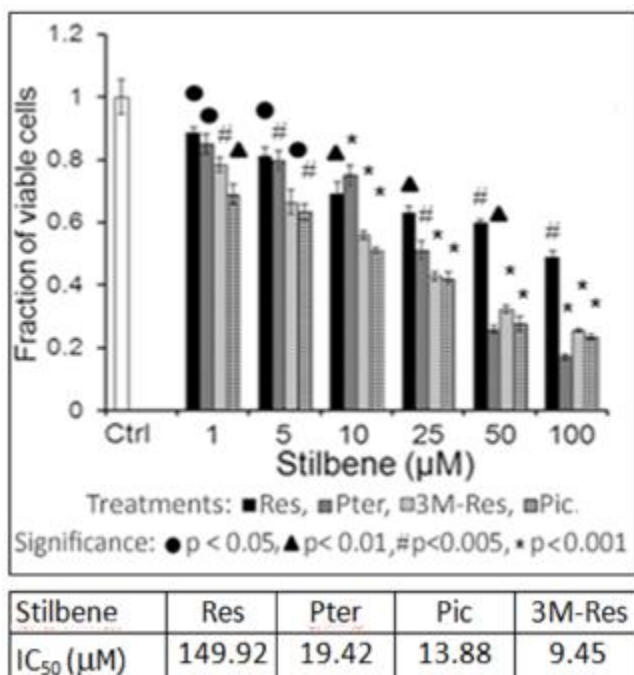

Figure 2. The effect of resveratrol and analogs on inhibition of cell growth.

Supplemental: Resveratrol and analogs inhibited growth of 22Rv1 castrate-resistant prostate cancer cells. Cells were grown in media containing selected doses of resveratrol (Res), trimethoxy-resveratrol (3M-Res), pterostilbene (Pter), and piceatannol (Pic). MTT assay was done with formazan measurements at 72 h. Viable cells were plotted as a fraction of untreated cells (Ctrl) (which was set to 1). Data represent the mean ± SE of three independent experiments in which each treatment (data point) was done in triplicates. Statistical significance was determined by two-tail Student t test. The p value ≤ 0.05 was considered significant. The IC_{50} values were calculated using the linear interpolation method with MS Excel software.

The cell growth inhibitory effect of Res and analog was determined by the IC_{50} values [50% inhibitory effect cell proliferation], using the liner interpolation method in MS Excel software. IC_{50} of Res 149.92 µM, whereas the other three analogs exhibited a stronger effect on cell proliferation as indicated by lower IC_{50} values of 19.42, 13.88, and 9.45 µM for Pter, Pic, and 3M-Res, respectively. The 3M-Res was the most potent among the Res analogs

in inhibiting the growth of 22Rv1 cells, supporting the fact that replacement of all three hydroxy (OH) substituents with methoxy (OCH_3) groups results in a greater growth inhibitory activity (Dias et al., 2013, Gosslau et al., 2008).

Resveratrol and analog inhibition of AR.

Since the growth inhibition of the castrate-resistant prostate cancer cells (22Rv1) by resveratrol and analogs may be related to modulation of AR, the AR protein levels were assessed by Western blot analysis (Fig. 3). The 22Rv1 cells are known to express full-length 114/110 kDa and truncated (80 kDa) AR variants (Guo et al., 2009). The 80 kDa AR variant, which is truncated at the C-terminal, contains N-terminal domain (NTD) and a DNA-binding domain (DBD), but lacks the ligand-binding domain (LBD) (Fig. 4A) (Dehm et al., 2011).
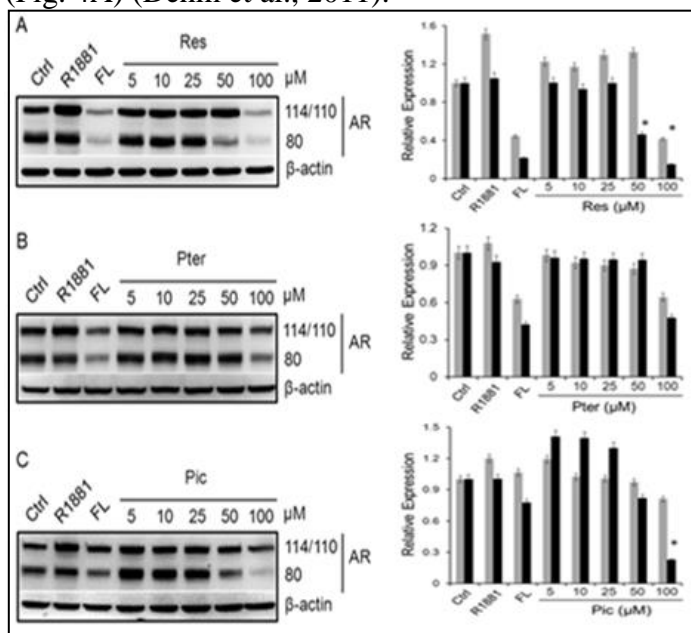

Figure 3. Inhibition of AR expression in 22Rv1 castrate resistant prostate cancer cells.

Supplemental: Western blots (left) indicate the effect of (A) Res, (B) Pter, and (C) Pic on AR protein levels at selected concentrations along with 10 nM R1881 (synthetic androgen); and 100 µM FL (flutamide - anti-androgen) for 24 h. β-actin was used as a loading agent. Graphical representation (right) is from quantitative analysis by Image J software. Control (Ctrl) was set as 1 and AR (☐ AR 114/110, ■ AR 80) levels are expressed relative to the control. Data represent the mean ± SE of three independent trials. *Significant at p < 0.05.

The 114/110 kDa AR variant was recognized as a single band at 110 kDa (AR114/110) while the 80

kDa AR variant (AR80) was recognized separately. The AR114/110, which has ligand-binding domain, responded to the ligand, synthetic androgen (R1881), and the nonsteroidal anti-androgen flutamide (FL), as indicated by the increase and decrease in AR levels upon treatment with R1881 and FL, respectively. AR80, however, did not respond to R1881, but responded to FL, suggesting different mechanisms of FL action than binding to the ligand-binding domain of AR.

Resveratrol treatment decreased AR114/110 levels in these cells, but at a high concentration of 100 μM, whereas AR80 levels were decreased in a dose dependent manner. Pterostilbene treatment initiated reduction of both AR114/110 and AR80 levels, but only at the 100 μM concentration. In contrast, treatment with Pic showed dose dependent lowering of both AR114/110 and AR80 levels with the effect being more prominent in the AR80 levels.

The 3M-Res did not produce a reliable dose-dependent inhibition of either AR114/110 or AR80 (data not shown). The differential effects of Res and analogs on AR114/110 and AR80 may be due to difference in the interaction of these compounds with specific domains of the AR variants (Fig. 4). Cumulative analysis of changes in AR levels by R1881, FL and stilbenes at 50 and 100 μM concentrations is shown in Fig. 4B.

DISCUSSION

A mainstay therapy of prostate cancer depends on androgen deprivation to which most patients do respond, but relapse with a castrate-resistant clinical outcome. The AR remains constitutively active in several cases of CRPC, however, several cellular and molecular alterations are related to this post-castration activation of the AR, including in-complete blockade of AR-ligand signaling, AR ampli-fications, AR mutations, aberrant AR co-regulator activities and AR splice-variant expression (Karantanos et al., 2013). Accumulating data suggests that both AR-dependent and AR-independent mechanisms are active and contribute to castrate-resistance (Kobayashi et al., 2013).
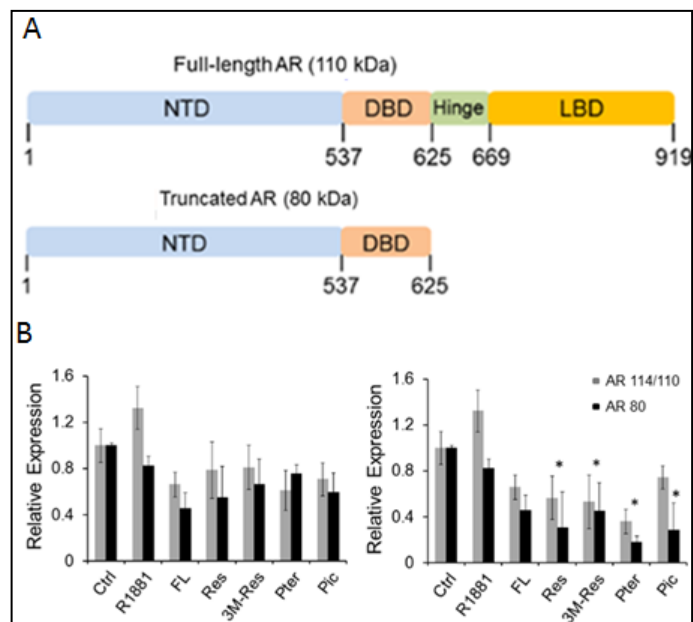

Figure 4. Regulation of full- length and truncated AR levels in 22Rv1 cells by resveratrol and analogs.

Supplemental: **A**. Domain structure of full-length AR (110 kDa) and truncated AR (80 kDa) protein in 22Rv1 cells. **B**. Graphical representa-tion of western blots quantitated by Image J software. Res, 3M-Res, Pter, and Pic reduced the full-length AR (AR114/110) and truncated AR (AR80) levels at 50 μM (left) and 100 μM (right) concentrations. R1881 is a synthetic androgen agonist, and Fl (flutamide is an anti-androgen. Data represent the mean ± SE of three independent replicate trials, *P<0.05.

In the present study, the effect of Res and natural Res analogs on full-length and truncated AR in 22Rv1 cells were investigated. The 22Rv1 cells are unique in harboring splice variants of AR lacking the ligand-binding domain (Tepper et al., 2002) that is frequently expressed (Libertini et al., 2007). This AR is up-regulated in prostate cancer progression and promotes androgen-independent growth resistance (Guo et al., 2009). In earlier studies, Res was shown to induce down-regulation of AR in LNCaP cells (harboring a point mutation in AR protein) by inducing protein degradation through the caspase-3 proteolytic pathway (Kai and Levenson, 2011). From our earlier observations that Res can inhibit AR expression in LNCaP cells led to consideration whether Res and Res natural analogs would have a similar effect on the AR expression in castrate-resistant 22Rv1 cells.

Our data revealed that 3M-Res was

approximately 16-fold the efficiency of Res in inhibiting proliferation of 22Rv1 cells as indicated by their IC_{50} values (Fig. 2). It has been reported that the truncated isoform of AR (AR80) mediates the ligand-independent AR activity responsible for cell proliferation (Marcias et al., 2010). Interestingly, all three analogs were almost equally effective in inducing growth-inhibitory activity at the higher concentrations, and were much more potent than Res. The current data support our earlier observation that replacement of all three hydroxy (OH) substituents of Res with methoxy (OCH_3) groups results in greater growth inhibitory activity (Dias et al., 2013).

Thinking that the growth inhibition by Res and analogs in these cells may be related to modulation of AR, and to understand the effect of Res and Res analogs on the dynamics of the AR protein in 22Rv1 cells, the cells were treated with the test compounds in increasing doses from 5-100 μM and subjected to a western blot analysis to determine the AR protein levels. For controls, the cells were also treated with the synthetic androgen agonist R1881 and the anti-androgen, FL. R1881 enhanced and FL reduced the expression of AR114/110, while AR80 responded only to FL. This action could be expected due to the lack of LBD in AR80. Only Pic demonstrated a dose-dependent down-regulation in AR levels, whereas Res and Pter were effective only at the higher concentrations. Interestingly, 3M-Res did not produce any down-regulation of AR (data not shown) although the 3M-Res was more efficient than the other stilbenes in inhibiting the proliferation of 22Rv1 cells.

An AR-independent inhibition mechanism mediated by 3M-Res may be active in these cells. Of note, the truncated isoform of AR (AR80) was down-regulated more efficiently, as compared to the full-length counterpart, by FL or the stilbenes, suggesting involvement of mechanisms other than AR binding. These observations were corroborated by a test comparison of the relative expression of the full-length vis-à-vis the truncated AR with all the stilbenes at 50 and 100 μM concentrations.

Since the truncated AR can be down-regulated more effectively by the stilbenes than the full-length AR, this may possibly provide a target for therapeutic intervention using combinations of Res and Res ana-logs in patients that relapse after androgen ablation and present a castration-resistant phenotype.

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