RESEARCH ARTICLE

Journal of
Applied Toxicology WILEY

Fructose-1,6-bisphosphate induces generation of reactive oxygen species and activation of p53-dependent cell death in human endometrial cancer cells

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Abstract

Fructose-1,6-bisphosphate (F1,6BP), an intermediate of the glycolytic pathway, has been found to play a promising anticancer effect; nevertheless, the mechanisms involved remain poorly understood. The present study aimed to evaluate the effect and mechanisms of F1,6BP in a human endometrial cancer cell line (Ishikawa). F1,6BP showed an antiproliferative and non-cytotoxic effect on endometrial cancer cells. These effects are related to the increase in reactive oxygen species (ROS) levels and mitochondrial membrane potential (ΔΨm). These harmful stimuli trigger the upregulation of the expression of pro-apoptotic genes (p53 and Bax), leading to the reduction of cell proliferation through inducing programmed cell death by apoptosis. Furthermore, F1,6BP-treated cells had the formation of autophagosomes induced, as well as a decrease in their proliferative capacity after withdrawing the treatment. Our results demonstrate that F1,6BP acts as an anticancer agent through the generation of mitochondrial instability, loss of cell function, and p53-dependent cell death. Thus, F1,6BP proves to be a potential molecule for use in the treatment against endometrial cancer.

KEYWORDS
endometrial cancer, fructose-1,6-bisphosphate, mitochondrial dysfunction, p53-dependent cell death, reactive oxygen species

1 | INTRODUCTION

Uterine corpus cancer is the sixth most common type of cancer in the female population and the 15th most commonly occurring cancer overall (International Agency for Research on Cancer [IARC], 2018; Siegel, Miller, & Jemal, 2019), with over than 380,000 new cases (33.9 cases per 100,000) and approximately 90,000 deaths in 2018 (IARC, 2018). Most uterine cancers are usually referred to as endometrial cancer (EC), originated from the epithelial lining of the uterine cavity. EC is classified into two types: (1) endometrioid endometrial carcinoma (EEC), which corresponds to more than 80% of the cases,

and (2) the non-EEC subtype (Morice, Leary, Creutzberg, Abu-Rustum, & Darai, 2016). Although non-EECs are generally more clinically aggressive, the worse prognosis is associated with high-grade, recurrent, or metastatic ECCs (Urick & Bell, 2019).

The degree of tumor differentiation (preoperative staging) is the most important factor on treatment selection. According to the International Federation of Gynecology and Obstetrics (FIGO) (Pecorelli, 2009) and the American Joint Committee on Cancer TNM staging system (Edge & Compton, 2010), the stages of EC vary from I to IV. The lower the stage, the lower the grade and the less cancer has spread. The higher the stage, the higher the grade and the higher

the chances that cancer has spread to other parts of the body. Surgery (hysterectomy with bilateral salpingo-oophorectomy) is the first treatment for almost all women with EC (Morice, Leary, Creutzberg, Abu-Rustum, & Darai, 2016). Depending on the stage of the cancer, adjuvant radiation and/or chemotherapy may be recommended. In the case of patients who wish to preserve fertility or with metastatic/recurrent disease, a hormonal therapy is an alternative treatment (Rodolakis et al., 2015). Hormone therapy for EC uses hormones or hormone-blocking drugs, such as progestins, luteinizing hormone-releasing hormone agonists, and aromatase inhibitors (De Haydu, Black, Schwab, English, & Santin, 2016). Targeted therapy is a type of treatment with drugs that have been developed to affect a specific target, such as an enzyme or receptor to treat a given disease and can be used by itself or in combination with other treatments. It is still fairly new in the treatment of EC and includes angiogenesis and mTOR inhibitors (Barra et al., 2019; Chellappan et al., 2018). Considering the restricted non-hormonal therapeutic options, the search for new anticancer drugs is increasingly necessary.

Fructose-1,6-bisphosphate (F1,6BP), an endogenous intermediate of the glycolytic pathway, is produced through phosphorylation of fructose 6-phosphate by the phosphofructokinase-1 activity (Kirtley & McKay, 1977). Its therapeutic effects have been documented in a wide range of pathological situations, including sepsis (Catarina et al., 2018), hepatic diseases (De Mesquita et al., 2013), and nephrotoxicity (Azambuja et al., 2011). Also, it was demonstrated that F1,6BP has antiproliferative effects against hepatocellular carcinoma (Lima et al., 2018; Lu, Yu, & Zhu, 2014) and papillary carcinoma (Li, Wei, Shen, & Hu, 2014). The underlying mechanisms of F1,6BP to exert its protective and/or antiproliferative effects are complex and involve different signaling pathways. Therefore, the present study aimed to evaluate the antitumoral effects and mechanisms triggered by treatment with F1,6BP in EC using a human endometrial adenocarcinoma cell line (Ishikawa).

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human endometrial adenocarcinoma Ishikawa cell line (Banco de Células do Rio de Janeiro, BCRJ, code: 0364) and African green monkey kidney (Vero) cells (ATCC®; CCL-81™) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco™, USA), supplemented with 10% fetal bovine serum (FBS; Gibco™, USA) and 1% penicillin/streptomycin antibiotics (Gibco™, USA) in a humidified incubator at 37°C and 5% CO_2.

2.2 | Cell proliferation and cell viability assays

Ishikawa cells were seeded in 24-well plates (1×10^4 cells per well) and incubated for 24 h before treatment. Cells were treated with F1,6BP (Sigma-Aldrich, USA) dissolved in fresh medium at

concentrations of 150, 300, 600, and 1,250 μM (De Mesquita et al., 2013; Jost et al., 2018; Krause et al., 2017). As a positive control, cells were treated with 10 μM of cisplatin (CPPD; Fauldcispla®, Libbs Farmacêutica Ltda) (Thigpen et al., 2004). Three replicate wells were used at each F1,6BP concentration tested, including CPPD-treated group and control cells that received no treatment. After 72 h of treatment, cell proliferation was assessed using the trypan blue exclusion assay and cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The non-tumoral Vero cells were also submitted to F1,6BP treatment. Vero cells were seeded at a density of 2×10^3 cells per well in 96-well plates, and after 72 h, MTT assay was performed.

The number of viable cells was quantified by mixing 25 μl of cell suspension and 25 μl of 0.4% trypan blue (Gibco™, USA) using a Neubauer hemocytometer and optical microscope (Nikon Optiphot, Japan). To determine cell viability, the culture medium was replaced by MTT solution (5 mg/ml) (Sigma-Aldrich, USA) and incubated at 37°C in a CO_2 incubator for 3 h. Formazan crystals were dissolved with dimethyl sulfoxide (DMSO; Merck, USA), and the optical density (OD) was measured at 570/620 nm in an enzyme-linked immunosorbent assay (ELISA) microplate reader (EZ Read 400; Biochrom, UK).

2.3 | LDH assay

The lactate dehydrogenase (LDH) assay was performed to evaluate the cytotoxicity of the F1,6BP treatment. LDH leakage to extracellular medium evidences the cell membrane disruption. Ishikawa cells were seeded into 24-well plates (1×10^4 cells per well), cultured and treated as mentioned before. Untreated cells represent the control group. Enzyme activity was measured in both supernatants and cell lysate using the colorimetric assay LDH kit (Labtest, Minas Gerais, Brazil). For the cell lysis control, DMEM was supplemented with 5% Tween® 20 (Sigma-Aldrich, USA). LDH released was measured by an ELISA microplate reader using an absorbance of 492 nm. The percentage released (i.e., present in the supernatant) was calculated based on the total enzyme content. Results are expressed as percent of LDH release in the cell supernatant.

2.4 | Nuclear morphometric analysis

To investigate the phenotype parameters related to the treatments, the analysis of the nuclear size and irregularity was performed. Ishikawa cells were seeded into 24-well plates (1×10^4 cells per well), treated with F1,6BP (300 μM) or CPPD (10 μM) and compared with untreated cells (control group). The concentration of 300 μM was chosen based on the results obtained in the assays described above. After 48 h of treatment, culture medium was discarded, and three steps were performed: (1) cells were fixed with 4% paraformaldehyde for 2 h and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 30 min; (2) cells were marked with a fluorescent dye solution containing 300 nM of 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, USA) for 2 min; and (3) acquisition of cell images

by inverted fluorescence microscope (Eclipse TE2000-S, Nikon). The nuclear morphometric analysis was performed using the Image-Pro Plus 6.0 software (IPP6–Media Cybernetics, Silver Spring, MD). Nuclear phenotypes were separated in an area versus nuclear irregularity index (NII) plot. Control cell nuclei were used to set the normal parameters (Filippi-Chiela et al., 2012).

2.5 | Cell death assay

To quantify cell death by apoptosis or necrosis, the double staining Annexin V fluorescein (FITC) and propidium iodide (PI) assay was performed using Anexina V-FITC flow cytometry kit (QuatroG, Brazil). Cells were seeded into 24-well plates (1×10^4 cells per well), treated with F1,6BP (300 μM) or CPPD (10 μM) for 48 h. Untreated cells represent the control group. Cells were harvested, washed with ice-cold PBS, resuspended in 100 μl of binding buffer at a cell density of 1×10^6 cells/ml and incubated with 4 μl of Annexin V-FITC and 4 μl of PI for 15 min at room temperature protected from light. Fluorescence analysis was performed by FACSCanto II Flow Cytometer (BD, Becton-Dickinson, USA) discriminating between live cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), late apoptotic cells (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+). Data were analyzed using FlowJo 7.6.5 software (Tree Star Inc., Ashland, OR).

2.6 | Acidic vesicular organelle quantification

Acridine orange (AO; Sigma-Aldrich, EUA) is a cell-permeant dye that emits green fluorescence in the whole cell except in the acidic compartments (mainly late autophagosomes), where it fluoresces red, indicating that the cells are in an autophagic process. Cells were seeded into 24-well plates (1×10^4 cells per well) and treated with F1,6BP (300 μM). For positive control, cells were treated with rapamycin (RAPA; 1 ng/ml), a potent inducer of autophagy (Li, Kim, & Blenis, 2014). Untreated cells represent the control group. Cells were treated for 48 h, harvested, and incubated with AO (1 μg/ml) in culture medium for 15 min at room temperature protected from light. Intensity of fluorescence was performed by FACSCanto II Flow Cytometer and quantified by FlowJo 7.6.5 software. The images of the fluorescent cells were captured using an inverted fluorescence microscope.

2.7 | mRNA expression of p53/Bax pathway

The relative expression of genes responsible for the synthesis of pro-apoptotic proteins Bcl-2-associated X protein (Bax) and tumor protein TP53 (p53) was evaluated by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Ishikawa cells were seeded into six-well plates (1×10^5 cells per well) and treated with F1,6BP (300 μM) or CPPD (10 μM). Untreated cells represent the control group. Total RNA was extracted using the TRIzol™ reagent (Invitrogen, USA) and reverse transcribed into complementary DNA (cDNA) using the GoScript™ Reverse Transcription System kit (Promega, USA), both following the manufacturer's protocol. The RT-qPCR was performed with PowerUp™ SYBR™ Green Master Mix kit (Applied Biosystems™, USA) in StepOne™ Real-Time PCR System (Applied Biosystems™, USA). To calculate the expression of the target genes (p53 and Bax), the $2^{-\Delta\Delta Ct}$ formula was performed using the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in Table 1.

2.8 | DPPH assay

2,2-Diphenyl-1-picryl-hydrazyl-hydrate (DPPH) is composed of stable, free radical molecules and has a deep violet color in solution. Based on electron transfer, in the presence of an antioxidant molecule, it becomes colorless. The antioxidant activity of F1,6BP (300 μM) was tested. Ascorbic acid (AsA, 550 μg/ml) (Sigma-Aldrich, USA) was used as a positive control. The free radical scavenging activity was followed by preparing DPPH solution (60 μM; Sigma-Aldrich, USA) in methanol. The OD was measured at 515 nm in an ELISA microplate reader.

2.9 | DCFH-DA assay

To determine the intracellular reactive oxygen species (ROS) levels, the DCFH-DA assay was performed. Through oxidation by the presence of ROS, the DCFH-DA compound becomes fluorescent. Ishikawa cells were seeded into 24-well plates (1×10^4 cells per well), treated with F1,6BP (300 μM) or with the positive control hydrogen peroxide (H_2O_2; 500 μM) (Wang & Roper, 2014) for 48 h. Untreated cells represent the control group. Cells were harvested, washed with PBS, and incubated with DCFH-DA (10 μM) for 30 min at 37°C. Intensity of fluorescence was measured at 485/520 nm in a

TABLE 1 Primer sequences for real-time quantitative reverse transcription polymerase chain reaction

Genes	Primer sequences (5′-3′)	Product length (bp)
GAPDH	Forward CTT TGT CAA GCT CAT TTC CTG G	133
	Reverse TCT TCC TCT TGT GCT CTT GC	
p53	Forward GTA CCA CCA TCC ACT ACA ACT AC	142
	Reverse CAC AAA CAC GCA ACC TCA AAG	
Bax	Forward GTT GTC GCC CTT TTC TAC TTTG	98
	Reverse TCC AAT GTC AGC CCC ATG	

microplate reader (VICTOR®, PerkinElmer, USA). Data are expressed as fluorescence/10^4 cells.

2.10 | Mitochondrial membrane potential

To evaluate the mitochondrial membrane potential ($\Delta\Psi$m), cells were seeded into 24-well plates (1×10^4 cells per well) and treated with F1,6BP (300 μM) or CPPD (10 μM) for 48 h. Untreated cells represent the control group. Cells were harvested, washed with PBS, and incubated with 100 nM of MitoTracker™ Red CMXRos (Invitrogen™, USA) for 30 min at 37°C protected for the light. According to the manufacturer's instructions, in viable cells, MitoTracker™ Red CMXRos stains mitochondria and its accumulation is directly dependent upon membrane potential. To quantify the intensity of fluorescence, samples were analyzed by flow cytometry, using the FACSCanto II Flow Cytometer and FlowJo 7.6.5 software.

2.11 | Clonogenic cell survival assay

Clonogenic cell survival assay assesses the ability of a single cell to proliferate and form a colony after removing the action of an external stressor. Ishikawa cells were seeded into 24-well plates (1×10^4 cells per well), treated with F1,6BP (300 μM) or CPPD (10 μM) for 48 h. Untreated cells represent the control group. Subsequently to cell treatment, viable cells were counted by trypan blue exclusion assay and seeded into 12-well plates (400 cells per well). Cells were cultivated in a humidified incubator at 37°C and 5% CO_2 for 7 days. DMEM was discarded and colonies were washed with PBS and fixed with 4% paraformaldehyde for 20 min. Gentian violet solution (Sigma-Aldrich, USA) was used to stain the fixed colonies. Data are expressed as the number of colonies per well.

2.12 | Statistical analysis

All results were presented through descriptive statistics (mean ± standard deviation [SD]). One-way analysis of variance (ANOVA) followed by the post hoc test of Tukey for multiple comparisons were used for the comparison of means between groups. A significance level was statistically accepted when $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

3 | RESULTS

3.1 | F1,6BP decreases cell proliferation and cell viability in Ishikawa cells

The treatment of Ishikawa cells with different concentrations of F1,6BP decreased the cell number (Figure 1A) and cell viability
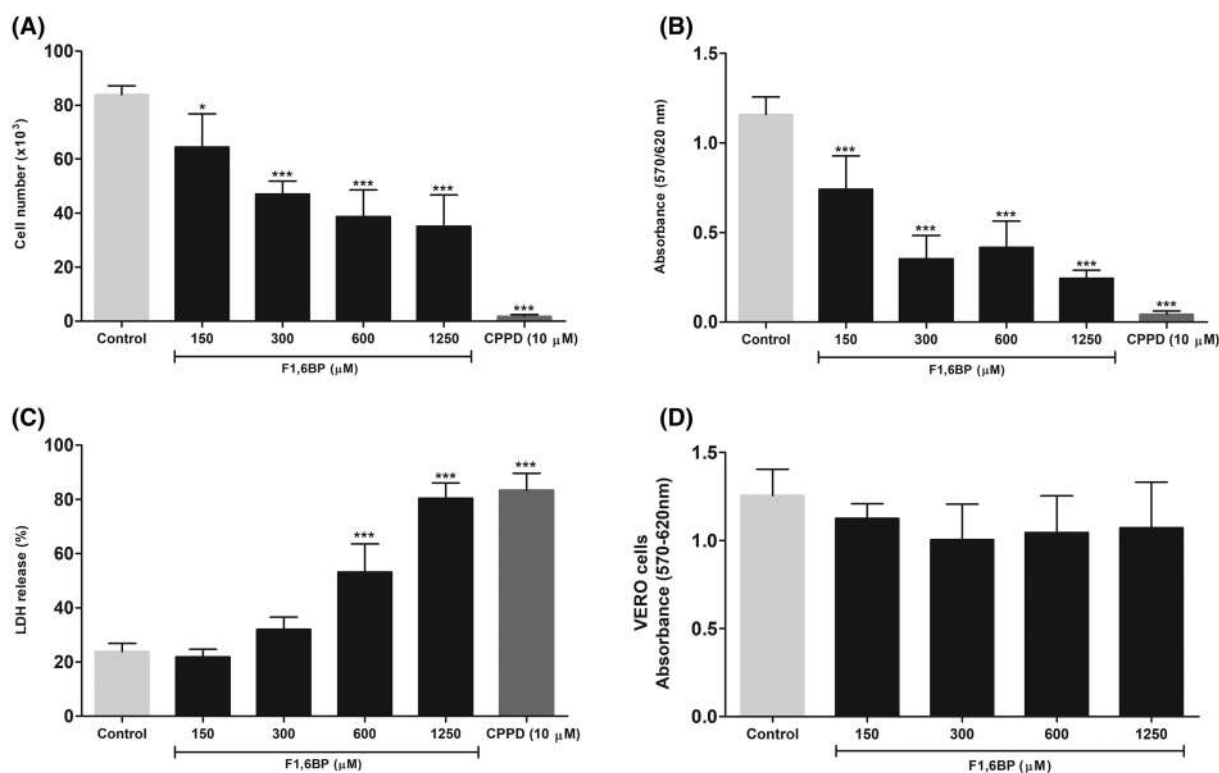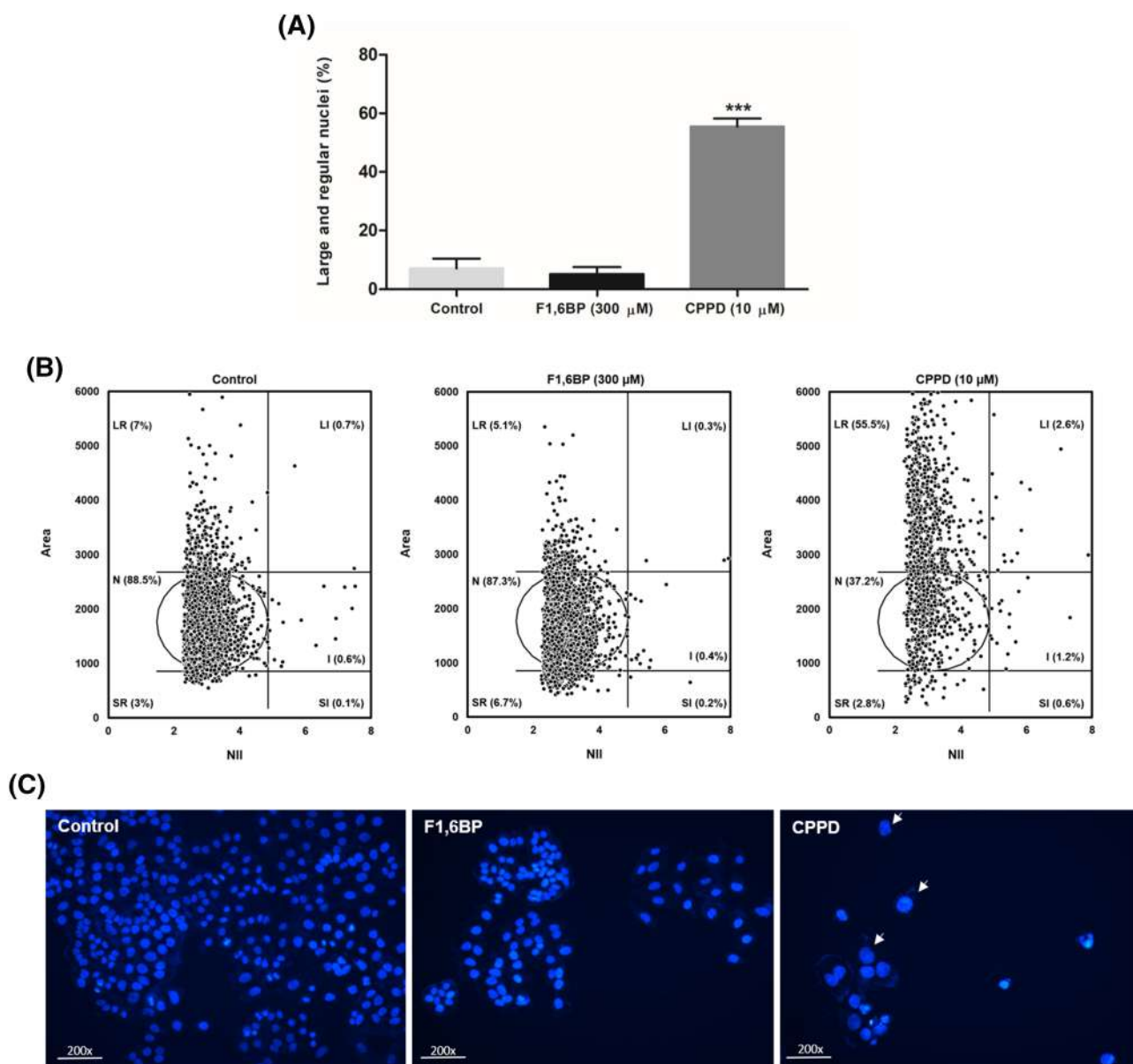

FIGURE 1 Effects of fructose-1,6-bisphosphate (F1,6BP) in Ishikawa cells after 72 h of treatment. A, Antiproliferative effect of F1,6BP on the total Ishikawa cell number count. B, Reduction of cell viability in response to treatment with F1,6BP in Ishikawa cells. C, Measurement of cytotoxic potential of F1,6BP in Ishikawa cells through the release of the lactate dehydrogenase (LDH) enzyme. Cisplatin (CPPD; 10 μM) was used as positive control. D, Effect of F1,6BP on the cell viability of non-tumoral Vero cells. Data are represented as the mean ± standard deviation from four (A and B) and three (C and D) independent experiments (*$p < 0.05$, ***$p < 0.001$ vs. control)

(Figure 1B) in a dose-dependent manner. The possibility of the antip- roliferative effect found being mediated by cellular toxicity was assessed to rule out any membrane damage that may be linked to the targeting of cell death by necrosis. Ishikawa cells treated with F1,6BP (150 and 300 μM) did not present significant released levels of LDH, demonstrating that there is no membrane damage in these cells in response to the treatment (Figure 1C). On the other side, F1,6BP (600 and 1,250 μM) showed a significant rise in LDH levels in the extracellular medium, evidencing the cell membrane disruption (Figure 1C). Nevertheless, no concentration of F1,6BP tested showed

to affect the viability of the non-tumoral Vero cells (Figure 1D). Based on the results described, 300 μM of F1,6BP was selected to continue the experiments in this study.

3.2 | Senescence is not involved in the antiproliferative effects of F1,6BP

Once the treatment of Ishikawa cells with F1,6BP (300 μM) decreased the cell number without causing cytotoxicity, we evaluated if
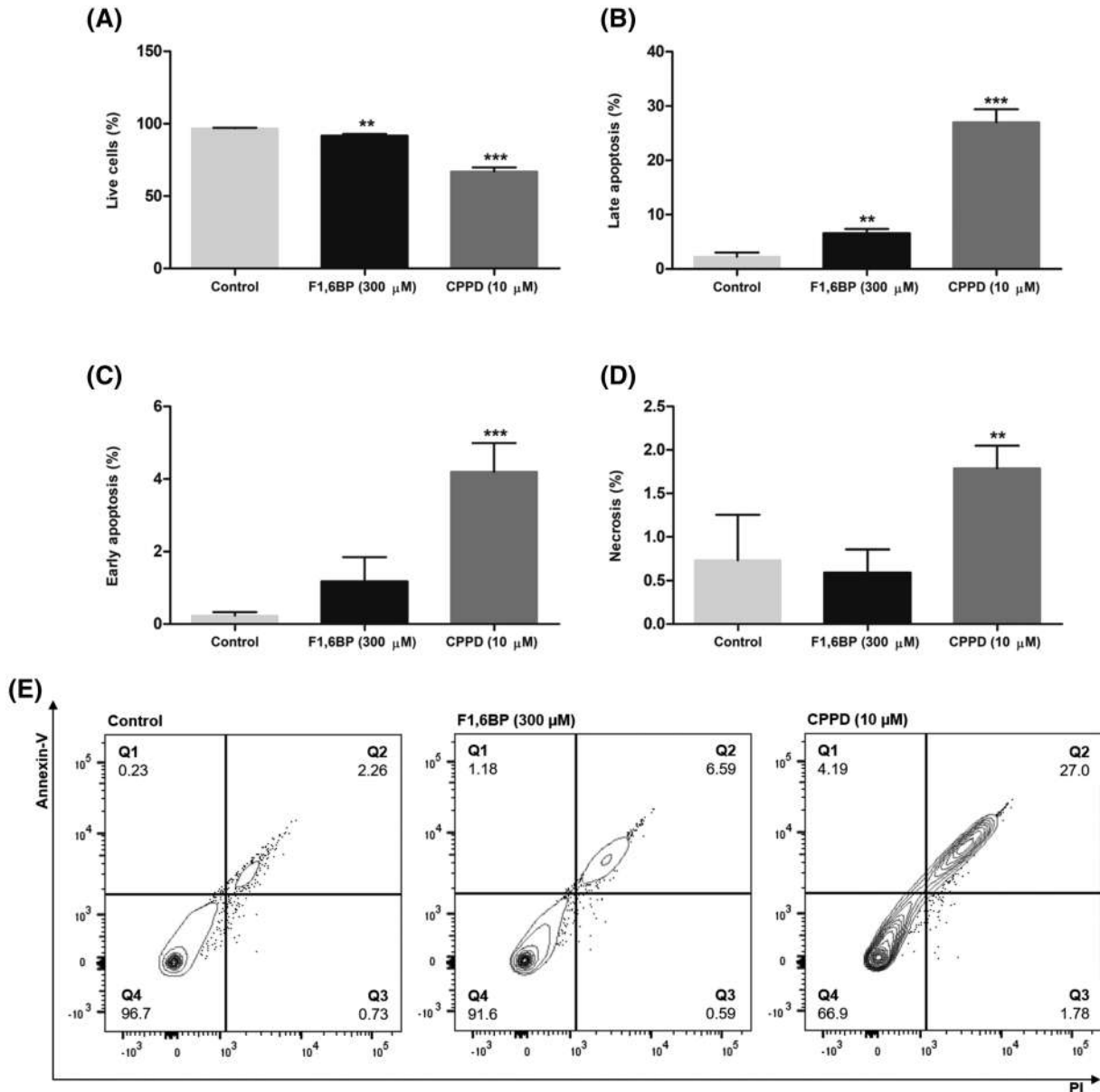

FIGURE 2 Effect of fructose-1,6-bisphosphate (F1,6BP) on the nuclear morphometry of Ishikawa cells after 48 h of treatment. A, Percentage of large and regular (LR) nuclei (senescence nuclei). Data are represented as the mean ± standard deviation from three independent experiments (***p < 0.001 vs. control). B, DAPI-stained nuclei distribution analyses for size and irregularity and the percentages of normal (N), LR, small and regular (SR), large and irregular (LI), small and irregular (SI), and irregular (I) nuclei. C, Representative images of nuclei from control and treated cells with F1,6BP (300 μM) or cisplatin (CPPD; 10 μM). At least 500 nuclei in three independent experiments were analyzed. The white arrows show the LR nuclei of senescent cells in CPPD treatment

senescence could be one of the antiproliferative mechanisms involved. The percentage of large and regular (LR) nuclei is shown in Figure 2A, and the distribution of nuclei size and irregularity are shown in Figure 2B. Representative images of nuclei are shown in Figure 2C according to the treatment received. As shown in Figure 2A, there was no significant difference in the percentage of LR nuclei in the F1,6BP-treated group compared with the control ($p > 0.05$). CPPD showed a raised number of LR nuclei compared with the control ($p < 0.001$).

3.3 | F1,6BP induces late apoptosis in Ishikawa cells

Aimed at finding the mechanism involved in the antiproliferative effect of F1,6BP, cell death by apoptosis was evaluated. As shown in Figure 3A, F1,6BP (300 μM) treatment decreased viable cells ($p < 0.01$) and increased cells in late apoptosis (Figure 3B; $p < 0.01$). The treatment with F1,6BP did not significantly raise the percentage of early apoptosis (Figure 3C; $p > 0.05$). Furthermore, F1,6BP (300 μM) does not induce necrosis (Figure 3D; $p > 0.05$), confirming

FIGURE 3 Effect of fructose-1,6-bisphosphate (F1,6BP) on the induction of apoptosis in Ishikawa cells after 48 h of treatment. Results are expressed as percentage of A, live cells, B, late apoptosis, C, early apoptosis, and D, necrosis. A total of 10,000 events were collected, and data are represented as the mean ± standard deviation from four independent experiments (**$p < 0.01$, ***$p < 0.001$ vs. control). E, Representative flow cytometric plots of control and treatments group (F1,6BP and CPPD). The lower left quadrant (Q4) represents a negative cell cluster (Annexin V−/PI−), the upper left quadrant (Q1) shows the cell population positive for one parameter (Annexin V+/PI−), the lower right quadrant (Q3) depicts cells positive for the second parameter (Annexin V−/PI+), and the upper right quadrant (Q2) represents cells that expressed both parameters (Annexin V+/PI+). Cisplatin (CPPD; 10 μM) was used as positive control

the non-toxicity of the treatment found by the LDH assay. The positive control CPPD (10 μM) increased all death parameters, reducing the percentage of the live cells.

3.4 | F1,6BP induces autophagosome formation in Ishikawa cells

Baseline levels of autophagy do not only contribute to the maintenance of biological functions and cell survival but also play a role in tumor suppression. As shown in Figure 4A, the percentage of autophagic cells increased in response to the treatment with F1,6BP

(300 μM; $p < 0.001$). As positive control, RAPA (1 ng/ml) confirmed the autophagy induction ($p < 0.001$). Figure 4B shows the representative flow cytometric plots. Representative images of nuclei are shown in Figure 4C according to the treatment received.

## 3.5 | F1,6BP increases p53 and Bax expression in Ishikawa cells

The transcription factor p53 is an essential tumor suppressor and plays a complex role in the regulation of autophagy and apoptosis (Crighton et al., 2006). As shown in Figure 5A, the expression of p53
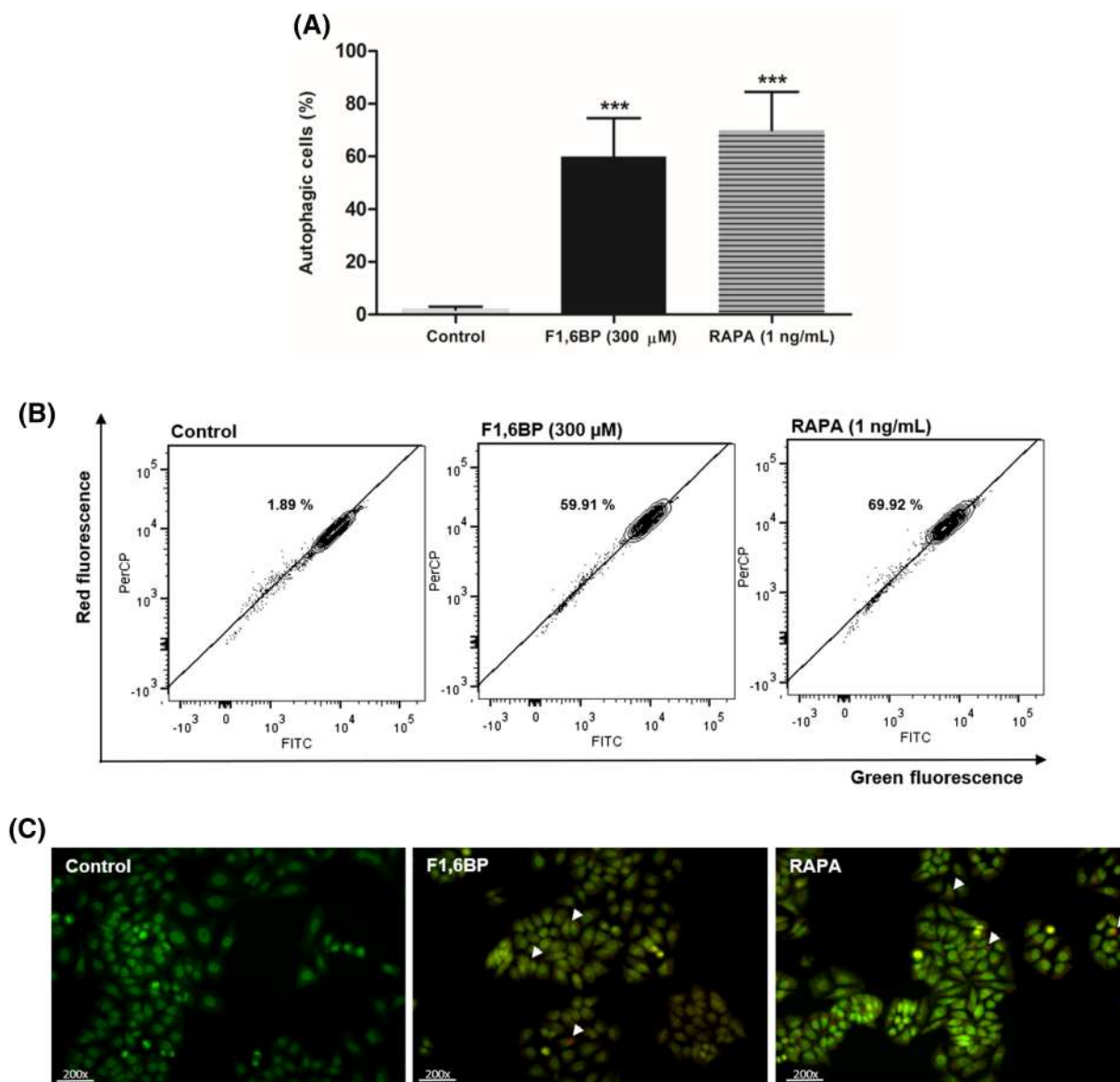
FIGURE 4 Effect of fructose-1,6-bisphosphate (F1,6BP) on the induction of autophagy in Ishikawa cells after 48 h of treatment. A, Percentage of autophagic cells. A total of 10,000 events were collected, and data are represented as the mean ± standard deviation from four independent experiments (***$p < 0.001$ vs. control). B, Representative flow cytometric plots of control and treatments group. C, Representative images of nuclei from control and treated cells with F1,6BP (300 μM), or with rapamycin (RAPA; 1 ng/ml). The white arrows show the lysosome stain in orange and the nuclei stain in green

(A)

(B)
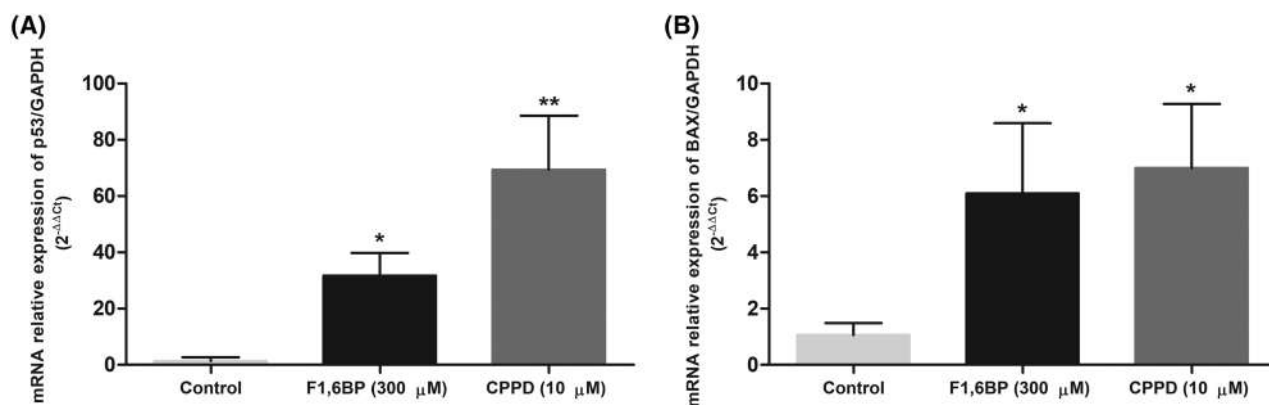
FIGURE 5 Effect of fructose-1,6-bisphosphate (F1,6BP) on the expression levels of p53 and Bax in Ishikawa cells after 48 h of treatment. Relative mRNA expression of A, p53 and B, Bax. GAPDH was used as an internal control. Cisplatin (CPPD; 10 μM) was used as positive control. Results are expressed as target gene/GAPDH, and data are represented as the mean ± standard deviation from three independent experiments (*$p < 0.05$, **$p < 0.01$ vs. control)

(A)

(B)

(C)

(D)
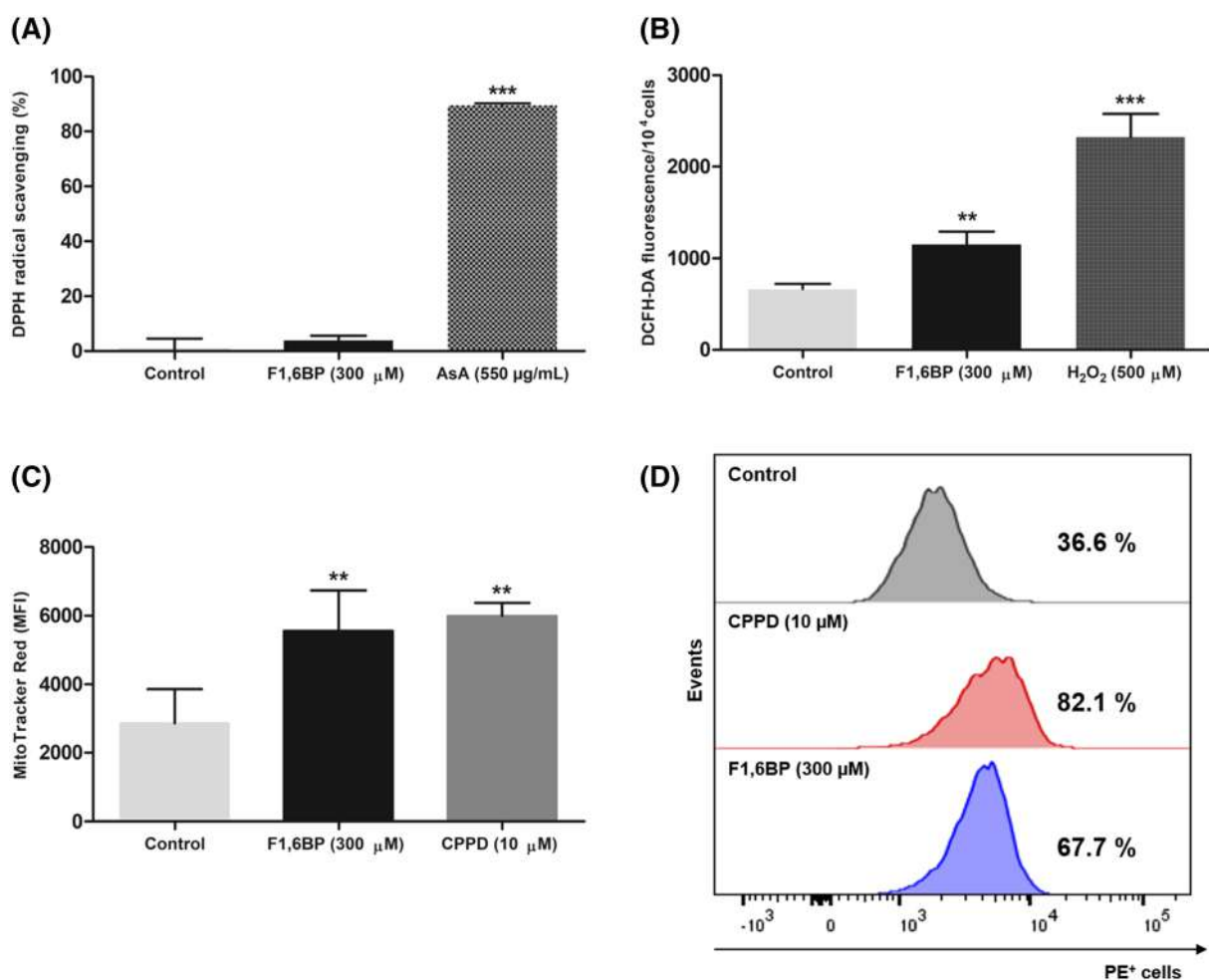
FIGURE 6 Fructose-1,6-bisphosphate (F1,6BP) antioxidant activity, reactive oxygen species (ROS) production, and ΔΨm in Ishikawa cells after 48 h of treatment. A, Antioxidant activity of the F1,6BP (300 μM). Ascorbic acid (AsA; 500 μg/ml) was used as positive control. Results are presented as percentage of DPPH reduction. B, Effect of F1,6BP (300 μM) treatment on intracellular ROS levels. Hydrogen peroxide (H$_2$O$_2$; 500 μM) was used as positive control. Results are expressed as DCFH-DA fluorescence/10^4 cells. C, Median fluorescence intensity (MFI) of ΔΨm-sensitive fluorochrome MitoTracker™ Red. A total of 10,000 events were collected in each replicate. D, Representative flow cytometric plots of the percentage of PE-positive cells in control and treated groups. Cisplatin (CPPD; 10 μM) was used as positive control. Data are represented as the mean ± standard deviation from three (A) and four (B, C, and D) independent experiments (**$p < 0.01$, ***$p < 0.001$ vs. control) [Colour figure can be viewed at wileyonlinelibrary.com]

raised in response to the treatments with F1,6BP (300 μM; $p < 0.05$) and CPPD (10 μM; $p < 0.01$). Bax is related to p53-mediated apoptosis and is involved in several cellular activities (Su, Mei, & Sinha, 2013). The treatment with F1,6BP (300 μM) also increased the expression levels of Bax (Figure 5B; $p < 0.05$) as well as the CPPD (10 μM) treatment ($p < 0.05$).

3.6 | F1,6BP induces ROS production in Ishikawa cells

Molecules can present antioxidant or pro-oxidant properties, which can influence the balance of ROS levels. F1,6BP (300 μM) showed no antioxidant effect (Figure 6A; $p > 0.05$), unlike AsA (550 μg/ml), which has a high antioxidant power ($p < 0.001$). As shown in Figure 6B, the ROS levels in Ishikawa cells significantly increased in response to the treatments with F1,6BP (300 μM; $p < 0.01$) and positive control H_2O_2 (500 μM; $p < 0.001$).

3.7 | F1,6BP increases mitochondrial membrane potential

Once treatment with F1,6BP (300 μM) raises the intracellular ROS levels, the mitochondrial membrane potential ($\Delta\Psi m$) was evaluated due to a strong positive correlation between ROS production and

$\Delta\Psi m$ (Miwa & Brand, 2003). Interestingly, after 48 h, the treatments with F1,6BP (300 μM) or CPPD (10 μM) increased the median fluorescence intensity (Figure 6C; $p < 0.01$) and the percentage of PE-positive cells (Figure 6D), suggesting a positive relationship between the high ROS levels and the increased $\Delta\Psi m$.

3.8 | F1,6BP reduces the colony-forming capability of Ishikawa cells

Clonogenic survival assay plays an essential role in evaluating the effects of agents with potential applications in the clinic because it measures the growth inhibition effects. A reduced ability to form colonies was seen by those cells that received treatments with F1,6BP (300 μM; Figure 7A, $p < 0.05$) or CPPD (10 μM; Figure 7A, $p < 0.001$) before the clonogenic survival assays. Representative images of the wells containing the colonies marked according to the treatment are shown in Figure 7B.

4 | DISCUSSION

Uncontrolled cell proliferation is a key feature and a target of many anticancer strategies primarily aimed at stopping cellular divisions. These strategies can be triggered by programmed cell death (PCD) (apoptosis, autophagy, or programmed necrosis) (Ouyang et al., 2012)
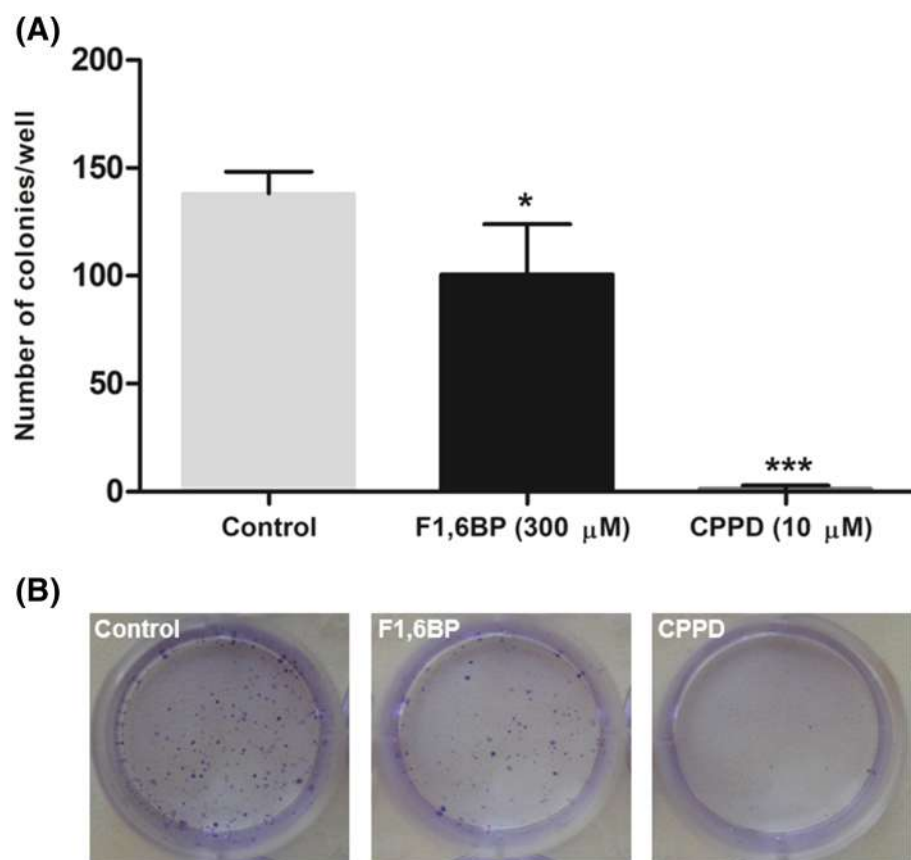
FIGURE 7 Effect of fructose-1,6-bisphosphate (F1,6BP) on the colony-forming capability of Ishikawa cells. A, Total of number of colonies per well. Data are represented as the mean ± standard deviation from three independent experiments (*$p < 0.05$, ***$p < 0.001$ vs. control). B, Representative images of colonies from control and treated cells with F1,6BP (300 μM) and cisplatin (CPPD; 10 μM) [Colour figure can be viewed at wileyonlinelibrary.com]

or through senescence induction (Schmitt, 2017). In this study, F1,6BP (300 μM) proved to be able to reduce cell proliferation and cell viability in EC cells. These effects are unrelated to cytotoxicity or necrosis induction in response to the treatment. Also, F1,6BP does not modify the viability of non-tumoral Vero cells, indicating a possible specificity against cancer cells.

Nuclear morphology changes occur in several processes associated with normal physiology and cell death. Senescence cells in general show an increase in nuclear size (Filippi-Chiela et al., 2012). Our results suggest that the reduction in the number of viable cells is not related to the nuclear phenotype of cellular senescence induction. However, the present study evidences that F1,6BP is able to inhibit the proliferative ability of human endometrial adenocarcinoma cell line (Ishikawa) through the induction of cell death by apoptosis. Apoptosis, or type I PCD, was first described by Kerr, Wyllie, and Currie (1972) and involves pathways and signaling proteins, such as the nuclear transcription factor p53 and Bcl-2 family proteins (Ouyang et al., 2012). p53 promotes apoptotic cell death by activating many positive regulators such as Bax, a pro-apoptotic member of Bcl-2 (Benchimol, 2001). Depending on the apoptosis-inducing signal, apoptosis can be triggered by extrinsic or intrinsic pathways. The extrinsic pathway starts from outside the cell through pro-apoptotic receptors (Taylor, Cullen, & Martin, 2008). The intrinsic pathway initiates within the cell by the permeabilization of the external mitochondrial membrane due to the formation of transmembrane channels in the mitochondrial membrane. These channels permit the release of cytochrome c, as well as other apoptosis effectors, that can associate with apoptotic protease-activating factor-1 and caspase-9 to form a complex called the apoptosome, leading to cell death (Giansanti, Torriglia, & Scovassi, 2011).

Suggestive of autophagy induction, the formation of the autophagosomes induced by F1,6BP treatment in EC cells corroborates with previous studies in cancer cells treated with the same molecule (Da Silva et al., 2016). This is the first report of F1,6BP-mediated autophagy in Ishikawa cells as far as we know. Under normal conditions, autophagy is a pro-survival pathway involved in cell homeostasis through selective degradation of cellular components resulting in the recycling of nutrients and the generation of energy (Levine & Klionsky, 2004). In tumor cells, autophagy has been reported not only to act as a facilitator of tumorigenesis but also to play a role in tumor suppression, by inhibiting cell survival, inducing death, and containing tumor metastases (Yun & Lee, 2018). On the other hand, the induction of autophagy by the drug can reduce the effectiveness of the antitumor response. Therefore, to establish a cross-talking between autophagy, cell death, and treatment is complex, in addition to further studies. As reported, resveratrol (RSV) is capable of reducing proliferation, inducing apoptosis, and promoting autophagy in Ishikawa cells. However, in this tumor type, the use of an autophagy inhibitor promoted an improvement in the effectiveness of RSV's antitumor activity in an in vitro study model (Fukuda et al., 2016). Nevertheless, the relationship between autophagy and cell death in an in vivo model is more positive for modulating the response of interest. Increased autophagy is reported to be accompanied by the induction of

apoptotic cell death in Ishikawa cells (De et al., 2018). The results show the complexity of the role autophagy plays in cancer, which makes evident the need for a greater understanding of the properties of the cancer cell, tumor microenvironment, and other signaling pathways.

Although autophagy is usually activated when apoptosis is suppressed, studies have shown that apoptosis and autophagy can have synergistic effects (Giansanti, Torriglia, & Scovassi, 2011; Su, Mei, & Sinha, 2013). p53 is involved in both pro-apoptotic pathway induction and autophagy facilitation, mainly by binding to the promoter region of multiple genes that code for pro-autophagic modulators, including damage-regulated autophagy modulator (DRAM), which promotes the formation of autophagosomes (Crighton et al., 2006). Our results showed an upregulation in gene expression of p53 and Bax after F1,6BP treatment, evidencing that the induction of death by apoptosis was due to the activation of a p53-dependent pathway with consequent activation of a pro-apoptotic protein Bax. In addition, our results indicate, when compared with a known inducer of autophagy (rapamycin), that F1,6BP may be inducing autophagy in Ishikawa cells evidenced by an increase in the number of autophagic vesicles, indicating a synergistic action with cell death due to apoptosis. These results showed that F1,6BP can also trigger antiproliferative effects through the forced autophagy that turns the physiological protective effect into cell death. Classic chemotherapy drugs aim to compromise cellular integrity by damaging nuclear DNA and to eliminate cancer cells via apoptosis. In contrast, studies have shown that some of these classic drugs, as well as new molecular target drugs, are also involved in modulating the autophagic death pathway (Janku, McConkey, Hong, & Kurzrock, 2011; Sakamoto et al., 2011).

Chemotherapy drugs like doxorubicin and cisplatin could induce apoptosis in parallel with the upregulation of ROS levels, which contributes to their genotoxicity (Conklin, 2004). The mitochondrion is a major source of ROS, and the production of ROS is derived from molecular oxygen by electron transfer reactions resulting in the formation of superoxide anion radical (O_2^-), and subsequently, H_2O_2, spontaneously or by the action of superoxide dismutase (Ježek & Hlavatá, 2005). A tight regulation of ROS levels is crucial for cellular life. Moderate ROS levels contribute to the control of cell proliferation and differentiation, whereas moderate increases of ROS contribute to several pathologic conditions, including tumor promotion and progression (Perillo et al., 2020). Oxidative stress arises from a significant increase in concentrations of ROS to toxic levels to biomolecules, including lipids, DNA, and proteins (Hamanaka & Chandel, 2010).

However, ROS are also able to trigger PCD (Perillo et al., 2020). Interestingly, there is an increasing evidence that in addition to the detrimental effects of high ROS levels exceeding the cellular antioxidant capacity, increased ROS-induced apoptosis has been reported in cancer cells after manipulation of glycolytic enzymes, chemotherapy, or radiation therapy (Kim, Kim, & Bae, 2016; Panieri & Santoro, 2016). The balance of ROS is among the effector mechanisms involved in the control and regulation of cell death pathways, including autophagy and apoptosis. DNA damage induced by ROS and various chemotherapeutic agents promotes the activation of p53, a transcription factor

known to regulate apoptotic cell death (Liu, Chen, & St. Clair, 2008). ROS is also known to trigger autophagy-mediated cell death in cancer cells (Azad, Chen, & Gibson, 2009). These data highlight the potential eminent role of ROS modulation as an anticancer treatment pathway. Our results showed an increase of ROS levels generated by treatment with F1,6BP in human EC cells (Ishikawa). Studies have also identified that cancer cells may become vulnerable to PCD in response to exogenous F1,6BP administration due to the induction of increased ROS levels (Da Silva et al., 2016; Li, Wei, Shen, & Hu, 2014; Lu, Yu, & Zhu, 2014).

F1,6BP can be considered a bioactive and highly dynamic molecule. Both the protective action of F1,6BP against oxidative stress, by decreasing lipid peroxidation (Alva, Carbonell, Roig, Bermúdez, & Palomeque, 2011), and its pro-oxidative action, by inducing the production of intracellular ROS, were reported (Calafell et al., 2009). DPPH assay demonstrated that F1,6BP is not an antioxidant molecule. However, a rise in ROS levels in response to treatment was observed in Ishikawa cells. Although the deregulation of redox homeostasis is considered a hallmark of cancer, the increase of intracellular ROS levels far above the toxicity threshold can be used as the therapeutic strategy. Thereby, the dependence of cancer cells on their antioxidant systems represents a specific vulnerability that has been exploited to induce cell death (Panieri & Santoro, 2016).

The opposite effects of F1,6BP's action on redox status can be explained based on the different endogenous levels of antioxidant enzymes present in distinct types of tissue, which act to protect cells against damage from oxidative stress. Considering that most types of tumor cells have low intrinsic levels of antioxidant enzymes compared with non-tumor cells, tumor cells may not be able to efficiently detoxify higher concentrations of H_2O_2 (Marklund, Westman, Lundgren, & Roos, 1982; Oberley & Oberley, 1997). A study evaluated the expression of antioxidant enzymes in gynecological disorders and identified that endometrial adenocarcinoma cells have low levels of catalase (CAT) enzyme, responsible for the decomposition of H_2O_2 in water and oxygen (Todorović et al., 2019). Cancer cells that have a limited amount of CAT are sensitive to a high level of H_2O_2 and end up being attacked by the generated hydroxyl radicals and induced to cell death (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). Therefore, even though the tumor cells support higher levels of ROS, treatment with F1,6BP demonstrates to be involved in the elevation of ROS to levels higher than those that are tolerable and compatible with the survival of Ishikawa cells, leading to cell death.

Mitochondria play a critical and central role in bioenergetic interactions that control a large part of cellular functions, such as adenosine triphosphate (ATP) generation, Ca^{2+} uptake and storage, and generation and sequestration of potentially damaging metabolic byproducts such as ROS (Herst, Rowe, Carson, & Berridge, 2017). Driven by the $\Delta\Psi m$, mitochondria play a key role in cellular metabolism and ion homeostasis and produce precursors for macromolecules (DNA, proteins, and lipids). Although not fully understood, $\Delta\Psi m$ directly or secondarily affects several cellular processes, such as the control of redox and pH microenvironments, as well as cell proliferation and death (Zorova et al., 2018). The $\Delta\Psi m$ magnitude can be significantly compromised in the event of ion leakage through the inner membrane, and this leakage might be exponentially dependent on $\Delta\Psi m$ (Brand, Chien, Ainscow, Rolfe, & Porter, 1994). Thus, low values of $\Delta\Psi m$ are related to an insufficient capacity to produce ATP, potentially related to a low level of mitochondrial ROS production that can lead to a consequent cell death (Perillo et al., 2020).

Also, in response to a high $\Delta\Psi m$, the mitochondrial respiratory chain becomes a significant producer of ROS. The generation of ROS also depends exponentially on $\Delta\Psi m$ (Starkov & Fiskum, 2003), mitochondrial hyperpolarization leads to an exponential increase in the generation of ROS, and the maintenance of excessively high $\Delta\Psi m$ is potentially harmful to mitochondria and, consequently, to cell (Gergely et al., 2002). Interestingly, an increase in the mitochondrial membrane potential of Ishikawa cells was found in response to treatment with F1,6BP. Kleih et al. (2019) recently showed that cisplatin induces an increase in ROS production and in the overall potential of the mitochondrial membrane in ovarian cancer cells sensitive to cisplatin OVCAR-3 and OVCAR-4 in comparison with resistant OVCAR-8 cells. Cisplatin binds to DNA and induces DNA damage, culminating in mitochondrial-mediated apoptosis (Kleih et al., 2019).

5 | CONCLUSION

Based on our results, F1,6BP (300 μM) is likely to induce a reduction in the cell proliferation of human EC cells (Ishikawa) by increasing the levels of ROS and generating mitochondrial damage. The increase of ROS can act as an activator factor for p53 expression, which culminates in the death pathway activation by p53-dependent apoptosis mediated by Bax expression. Even though autophagy may initially occur as a defense mechanism, the extrapolation of this mechanism seems to act synergistically with PCD by apoptosis. In conclusion, this was the first study to evaluate the antitumor effect of F1,6BP in EC cells. Although it has not provided critical information about the inducing of autophagy and mitochondrial hyperpolarization, F1,6BP treatment showed effectiveness in significantly raising ROS levels and triggering cell death by apoptosis, without cytotoxic effects. Also, the concentration of F1,6BP used in this study was effective in decreasing the proliferative capacity of cancer cells, even after withdrawing treatment, which demonstrates a promising antitumor effect against EC.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES

Alva, N., Carbonell, T., Roig, T., Bermúdez, J., & Palomeque, J. (2011). Fructose 1,6 biphosphate administration to rats prevents metabolic acidosis and oxidative stress induced by deep hypothermia and rewarming. *European Journal of Pharmacology*, 659(2–3), 259–264. https://doi.org/10.1016/j.ejphar.2011.03.034

Azad, M. B., Chen, Y., & Gibson, S. B. (2009). Regulation of autophagy by reactive oxygen species (ROS): Implications for cancer progression and treatment. *Antioxidants & Redox Signaling*, 11, 777–790. https://doi.org/10.1089/ars.2008.2270

Azambuja, A. A., Lunardelli, A., Nunes, F. B., Gaspareto, P. B., Donadio, M. V. F., De Figueiredo, C. E. P., & De Oliveira, J. R. (2011). Effect of fructose-1,6-bisphosphate on the nephrotoxicity induced by cisplatin in rats. *Inflammation*, 34, 67–71. https://doi.org/10.1007/s10753-010-9212-5

Barra, F., Evangelisti, G., Ferro Desideri, L., Di Domenico, S., Ferraioli, D., Vellone, V. G., ... Ferrero, S. (2019). Investigational PI3K/AKT/mTOR inhibitors in development for endometrial cancer. *Expert Opinion on Investigational Drugs*, 28(2), 131–142. https://doi.org/10.1080/13543784.2018.1558202

Benchimol, S. (2001). p53-dependent pathways of apoptosis. *Cell Death and Differentiation*, 8, 1049–1051. https://doi.org/10.1038/sj.cdd.4400918

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal*, 5, 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Brand, M. D., Chien, L. F., Ainscow, E. K., Rolfe, D. F. S., & Porter, R. K. (1994). The causes and functions of mitochondrial proton leak. *BBA - Bioenergetics.*, 1187, 132–139. https://doi.org/10.1016/0005-2728(94)90099-X

Calafell, R., Boada, J., Santidrian, A. F., Gil, J., Roig, T., Perales, J. C., & Bermudez, J. (2009). Fructose 1,6-bisphosphate reduced TNF-α-induced apoptosis in galactosamine sensitized rat hepatocytes through activation of nitric oxide and cGMP production. *European Journal of Pharmacology*, 610(1–3), 128–133. https://doi.org/10.1016/j.ejphar.2009.03.044

Catarina, A. V., Luft, C., Greggio, S., Venturin, G. T., Ferreira, F., Marques, E. P., ... Nunes, F. B. (2018). Fructose-1,6-bisphosphate preserves glucose metabolism integrity and reduces reactive oxygen species in the brain during experimental sepsis. *Brain Research*, 1698, 54–61. https://doi.org/10.1016/j.brainres.2018.06.024

Chellappan, D. K., Leng, K. H., Jia, L. J., Aziz, N. A. B. A., Hoong, W. C., Qian, Y. C., ... Dua, K. (2018). The role of bevacizumab on tumour angiogenesis and in the management of gynaecological cancers: A review. *Biomedicine & Pharmacotherapy*, 102, 1127–1144. https://doi.org/10.1016/j.biopha.2018.03.061

Conklin, K. A. (2004). Chemotherapy-associated oxidative stress: Impact on chemotherapeutic effectiveness. *Integrative Cancer Therapies*, 3, 294–300. https://doi.org/10.1177/1534735404270335

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P. R., ... Ryan, K. M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell*, 126, 121–134. https://doi.org/10.1016/j.cell.2006.05.034

Da Silva, E. F. G., Le Catyana Krause, G., Lima, K. G., Haute, G. V., Pedrazza, L., Mesquita, F. C., ... De Oliveira, J. R. (2016). Rapamycin and fructose-1,6-bisphosphate reduce the HEPG2 cell proliferation via increase of free radicals and apoptosis. *Oncology Reports*, 36, 2647–2652. https://doi.org/10.3892/or.2016.5111

De Haydu, C., Black, J. D., Schwab, C. L., English, D. P., & Santin, A. D. (2016). An update on the current pharmacotherapy for endometrial cancer. *Expert Opinion on Pharmacotherapy*, 17, 489–499. https://doi.org/10.1517/14656566.2016.1127351

De Mesquita, F. C., Bitencourt, S., Caberlon, E., Da Silva, G. V., Basso, B. S., Schmid, J., ... De Oliveira, J. R. (2013). Fructose-1,6-bisphosphate induces phenotypic reversion of activated hepatic stellate cell. *European Journal of Pharmacology*, 720(1–3), 320–325. https://doi.org/10.1016/j.ejphar.2013.09.067

De, U., Son, J., Sachan, R., Park, Y., Kang, D., Yoon, K., ... Kim, H. (2018). A new synthetic histone deacetylase inhibitor, MHY2256, induces apoptosis and autophagy cell death in endometrial cancer cells via p53 acetylation. *International Journal of Molecular Sciences*, 19(9), 2743. https://doi.org/10.3390/ijms19092743

Edge, S. B., & Compton, C. C. (2010). The American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. *Annals of Surgical Oncology*, 17, 1471–1474. https://doi.org/10.1245/s10434-010-0985-4

Filippi-Chiela, E. C., Oliveira, M. M., Jurkovski, B., Callegari-Jacques, S. M., da Silva, V. D., & Lenz, G. (2012). Nuclear morphometric analysis (NMA): Screening of senescence, apoptosis and nuclear irregularities. *PLoS ONE*, 7(8), e42522. https://doi.org/10.1371/journal.pone.0042522

Fukuda, T., Oda, K., Wada-Hiraike, O., Sone, K., Inaba, K., Ikeda, Y., ... Fujii, T. (2016). Autophagy inhibition augments resveratrol-induced apoptosis in Ishikawa endometrial cancer cells. *Oncology Letters*, 12(4), 2560–2566. https://doi.org/10.3892/ol.2016.4978

Gergely, P., Niland, B., Gonchoroff, N., Pullmann, R., Phillips, P. E., & Perl, A. (2002). Persistent mitochondrial hyperpolarization, increased reactive oxygen intermediate production, and cytoplasmic alkalinization characterize altered IL-10 signaling in patients with systemic lupus erythematosus. *The Journal of Immunology.*, 169, 1092–1101. https://doi.org/10.4049/jimmunol.169.2.1092

Giansanti, V., Torriglia, A., & Scovassi, A. I. (2011). Conversation between apoptosis and autophagy: Is it your turn or mine? *Apoptosis*, 16, 321–333. https://doi.org/10.1007/s10495-011-0589-x

Hamanaka, R. B., & Chandel, N. S. (2010). Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends in Biochemical Sciences*, 35, 505–513. https://doi.org/10.1016/j.tibs.2010.04.002

Herst, P. M., Rowe, M. R., Carson, G. M., & Berridge, M. V. (2017). Functional mitochondria in health and disease. *Frontiers in Endocrinology*, 8, 296. https://doi.org/10.3389/fendo.2017.00296

IARC. (2018). World Health Organization International Agency for Research on Cancer (IARC)—Global Cancer Observatory (GLOBOCAN). Retrieved from http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

Janku, F., McConkey, D. J., Hong, D. S., & Kurzrock, R. (2011). Autophagy as a target for anticancer therapy. *Nature Reviews. Clinical Oncology*, 8, 528–539. https://doi.org/10.1038/nrclinonc.2011.71

Ježek, P., & Hlavatá, L. (2005). Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *The International Journal of Biochemistry & Cell Biology*, 37, 2478–2503. https://doi.org/10.1016/j.biocel.2005.05.013

Jost, R. T., Dias, H. B., Krause, G. C., de Souza, R. G., de Souza, T. R., Nuñez, N. K., ... de Oliveira, J. R. (2018). Fructose-1,6-bisphosphate prevents bleomycin-induced pulmonary fibrosis in mice and inhibits the proliferation of lung fibroblasts. *Inflammation*, 41(5), 1987–2001. https://doi.org/10.1007/s10753-018-0842-3

Kerr, J. F. R., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, 26, 239–257. https://doi.org/10.1038/bjc.1972.33

Kim, J., Kim, J., & Bae, J. S. (2016). ROS homeostasis and metabolism: A critical liaison for cancer therapy. *Experimental & Molecular Medicine*, 48, e269. https://doi.org/10.1038/emm.2016.119

Kirtley, M. E., & McKay, M. (1977). Fructose-1,6-bisphosphate, a regulator of metabolism. *Molecular and Cellular Biochemistry*, 18(2–3), 141–149. https://doi.org/10.1007/BF00280279

Kleih, M., Böpple, K., Dong, M., Gaißler, A., Heine, S., Olayioye, M. A., ... Essmann, F. (2019). Direct impact of cisplatin on mitochondria induces ROS production that dictates cell fate of ovarian cancer cells. *Cell*

Death & Disease, 10, 1–12. https://doi.org/10.1038/s41419-019-2081-4

Krause, G. C., Lima, K. G., Haute, G. V., Schuster, A. D., Dias, H. B., Mesquita, F. C., ... de Oliveira, J. R. (2017). Fructose-1,6-bisphosphate decreases IL-8 levels and increases the activity of pro-apoptotic proteins in HepG2 cells. *Biomedicine & Pharmacotherapy*, 89, 358–365. https://doi.org/10.1016/j.biopha.2017.01.178

Levine, B., & Klionsky, D. J. (2004). Development by self-digestion: Molecular mechanisms and biological functions of autophagy. *Developmental Cell*, 6, 463–477. https://doi.org/10.1016/S1534-5807(04)00099-1

Li, J., Kim, S. G., & Blenis, J. (2014). Rapamycin: One drug, many effects. *Cell Metabolism*, 19, 373–379. https://doi.org/10.1016/j.cmet.2014.01.001

Li, Y., Wei, W., Shen, H. W., & Hu, W. Q. (2014). The study of inducing apoptosis effect of fructose 1,6-bisphosphateon the papillary thyroid carcinoma cell and its related mechanism. *Tumor Biology*, 35, 4539–4544. https://doi.org/10.1007/s13277-013-1597-y

Lima, K. G., Krause, G. C., da Silva, E. F. G., Xavier, L. L., Martins, L. A. M., Alice, L. M., ... Garcia, M. C. (2018). Octyl gallate reduces ATP levels and Ki67 expression leading HepG2 cells to cell cycle arrest and mitochondria-mediated apoptosis. *Toxicology In Vitro*, 48, 11–25. https://doi.org/10.1016/j.tiv.2017.12.017

Liu, B., Chen, Y., & St. Clair, D. K. (2008). ROS and p53: A versatile partnership. *Free Radical Biology & Medicine*, 44, 1529–1535. https://doi.org/10.1016/j.freeradbiomed.2008.01.011

Lu, Y. X., Yu, X. C., & Zhu, M. Y. (2014). Antitumor effect of fructose 1, 6-bisphosphate and its mechanism in hepatocellular carcinoma cells. *Tumor Biology*, 35, 1679–1685. https://doi.org/10.1007/s13277-013-1231-z

Marklund, S. L., Westman, N. G., Lundgren, E., & Roos, G. (1982). Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. *Cancer Research*, 42(5), 1955–1961. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/7066906

Miwa, S., & Brand, M. D. (2003). Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *In Biochemical Society Transactions.*, 31, 1300–1301. https://doi.org/10.1042/bst0311300

Morice, P., Leary, A., Creutzberg, C., Abu-Rustum, N., & Darai, E. (2016). Endometrial cancer. *In The Lancet.*, 387, 1094–1108. https://doi.org/10.1016/S0140-6736(15)00130-0

Oberley, T. D., & Oberley, L. W. (1997). Antioxidant enzyme levels in cancer. *Histology and Histopathology*, 12(2), 525–535. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/9151141

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: A review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation*, 45, 487–498. https://doi.org/10.1111/j.1365-2184.2012.00845.x

Panieri, E., & Santoro, M. M. (2016). Ros homeostasis and metabolism: A dangerous liason in cancer cells. *Cell Death & Disease*, 7, e2253. https://doi.org/10.1038/cddis.2016.105

Pecorelli, S. (2009). Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. *International Journal of Gynaecology and Obstetrics: The Official Organ of the International Federation of Gynaecology and Obstetrics.*, 105, 103–104. https://doi.org/10.1016/j.ijgo.2009.02.012

Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., ... Migliaccio, A. (2020). ROS in cancer therapy: The bright side of the moon. *Experimental & Molecular Medicine*, 52, 192–203. https://doi.org/10.1038/s12276-020-0384-2

Rodolakis, A., Biliatis, I., Morice, P., Reed, N., Mangler, M., Kesic, V., & Denschlag, D. (2015). European society of gynecological oncology task force for fertility preservation: Clinical recommendations for fertility-sparing management in young endometrial cancer patients. *International Journal of Gynecological Cancer*, 25, 1258–1265. https://doi.org/10.1097/IGC.0000000000000493

Sakamoto, Y., Kato, S., Takahashi, M., Okada, Y., Yasuda, K., Watanabe, G., ... Ishioka, C. (2011). Contribution of autophagic cell death to p53-dependent cell death in human glioblastoma cell line SF126. *Cancer Science*, 102, 799–807. https://doi.org/10.1111/j.1349-7006.2011.01857.x

Schmitt, R. (2017). Senotherapy: Growing old and staying young? *Pflügers Archiv - European Journal of Physiology*, 469(9), 1051–1059. https://doi.org/10.1007/s00424-017-1972-4

Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians*, 69, 7–34. https://doi.org/10.3322/caac.21551

Starkov, A. A., & Fiskum, G. (2003). Regulation of brain mitochondrial H2O2 production by membrane potential and NAD(P)H redox state. *Journal of Neurochemistry*, 86, 1101–1107. https://doi.org/10.1046/j.1471-4159.2003.01908.x

Su, M., Mei, Y., & Sinha, S. (2013). Role of the crosstalk between autophagy and apoptosis in cancer. *Journal of Oncology*, 2013, 1–14. https://doi.org/10.1155/2013/102735

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: Controlled demolition at the cellular level. *Nature Reviews. Molecular Cell Biology*, 9, 231–241. https://doi.org/10.1038/nrm2312

Thigpen, J. T., Brady, M. F., Homesley, H. D., Malfetano, J., DuBeshter, B., Burger, R. A., & Liao, S. (2004). Phase III trial of doxorubicin with or without cisplatin in advanced endometrial carcinoma: A gynecologic oncology group study. *Journal of Clinical Oncology*, 22(19), 3902–3908. https://doi.org/10.1200/JCO.2004.02.088

Todorović, A., Pejić, S., Gavrilović, L., Pavlović, I., Stojiljković, V., Popović, N., & Pajović, S. B. (2019). Expression of antioxidant enzymes in patients with uterine polyp, myoma, hyperplasia, and adenocarcinoma. *Antioxidants*, 8(4), 97. https://doi.org/10.3390/antiox8040097

Urick, M. E., & Bell, D. W. (2019). Clinical actionability of molecular targets in endometrial cancer. *Nature Reviews. Cancer*, 19, 510–521. https://doi.org/10.1038/s41568-019-0177-x

Wang, X., & Roper, M. G. (2014). Measurement of DCF fluorescence as a measure of reactive oxygen species in murine islets of Langerhans. *Analytical Methods*, 6, 3019–3024. https://doi.org/10.1039/c4ay00288a

Yun, C. W., & Lee, S. H. (2018). The roles of autophagy in cancer. *International Journal of Molecular Sciences*, 19. https://doi.org/10.3390/ijms19113466

Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., ... Zorov, D. B. (2018). Mitochondrial membrane potential. *Analytical Biochemistry*, 552, 50–59. https://doi.org/10.1016/j.ab.2017.07.009