

# Reversine, a substituted purine, exerts an inhibitive effect on human renal carcinoma cells via induction of cell apoptosis and polyploidy

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**Background:** Human renal cell carcinoma (RCC) is the most common type of kidney cancer that arises from the renal epithelium. Up to 33.3% of RCC patients treated with local tumor resections will subsequently develop recurrence or metastases. Thus, optimized therapeutic regimes are urgently needed to improve the prognosis of RCC. Reversine was recently reported to exert critical roles in cancer therapy.

**Materials and methods:** This study evaluated the anti-tumor effects of reversine on cell viability, colony formation, apoptosis, and cell cycle in 786-O and ACHN cell lines.

**Results:** It was demonstrated that reversine significantly inhibited the proliferation of both cell lines in time- and dose-dependent manners. Polyploidy formation was observed under high-concentration reversine treatment. In addition, reversine induced cell death via caspase-dependent apoptotic pathways, which could be partially inhibited by Z-VAD-FMK, a pan-caspase inhibitor.

**Conclusion:** Reversine could effectively suppress the proliferation of human RCC cells, and may serve as a novel therapeutic regimen for RCC in clinical practice.

**Keywords:** anti-cancer, apoptosis, Aurora kinase, human renal cell carcinoma, polyploidy, reversine

## Introduction

Renal cell carcinoma (RCC), which is the most common form of adult kidney cancer, arises from the renal epithelium, and is comprised of three main histologic subtypes, namely, clear-cell RCC, papillary carcinoma, and chromophobe tumors.<sup>1,2</sup> RCC is estimated to be the seventh most frequently diagnosed cancer in men, and the ninth in women, representing approximately 2%–3% of adult malignancies worldwide.<sup>3</sup> In 2012, about 84,400 new cases of RCC were diagnosed in the European Union, and approximately 34,700 tumor-related deaths were recorded. Moreover, the incidence of RCC worldwide is steadily increasing, with an approximate annual rate of 2%.<sup>4,5</sup> For patients with localized RCC, surgery is currently the golden standard of curative treatment with favorable prognosis. However, after surgery, up to 33.3% of the patients will subsequently develop metastases.<sup>6</sup> Moreover, about 25%–30% of patients demonstrate locally advanced or metastatic stage by the first diagnosis with RCC.<sup>7</sup> Thus, optimized diagnostic and therapeutic regimes are urgently needed to improve the prognosis of RCC.

Reversine, a substituted purine, was initially identified by Chen et al<sup>8</sup> in 2004. This molecule induces the dedifferentiation of C2C12 cells, a murine myoblast cell line, into multipotent progenitor cells.<sup>8</sup> Hence, various studies have focused on the roles of reversine in cell reprogramming and regenerative medicine.<sup>9–12</sup> Reversine is supposed

to reprogram somatic cells into a multipotent state, which could then be converted into different cell types. The impact of reversine on cell differentiation is also applicable to cancer therapy. Numerous studies have demonstrated the tumor-suppression activity of reversine against human cancer cells by induction of apoptosis, cell cycle arrest, polyploidy, and (or) autophagy.<sup>13–16</sup> D'Alise et al<sup>17</sup> reported that reversine could exert the same inhibitive influence in the colony formation of leukemic cells, such as VX-680, a potent Aurora kinase inhibitor which has entered phase II clinical trials. Reversine is superior to VX-680, because of its lower toxicity in this study. This derivative could cause a failure in cytokinesis and induce polyploidization by inhibiting the bioactivity of Aurora kinases A and B, which are involved in multi-aspects of mitosis. Moreover, using a tumor cell-specificity in vitro bioluminescence imaging assay, McMillin et al<sup>18</sup> showed that reversine could inhibit multiple myeloma cells in vitro by suppressing the bioactivity of Aurora kinases, JAK2, and SRC.

Aurora kinases have been reported to be frequently overexpressed in human tumors. These kinases have been deemed as valuable targets for cancer therapy because of their prominent role in mitotic regulation.<sup>19</sup> Therefore, reversine, as a novel Aurora kinase inhibitor, might serve as an available agent in clinical cancer chemotherapy.<sup>17</sup>

To our knowledge, the anti-tumor mechanism of reversine against human RCC has not been elucidated yet. Here, we demonstrated the cytotoxic effect of reversine on two human RCC cells. This molecule could suppress cell viability and colony formation by inducing caspase-dependent apoptosis in 786-O (p53 mutant) and ACHN (p53 wide-type) cell lines. Moreover, polyploidy cells also formed after the cells were incubated with high-concentration reversine (0.8–1.6  $\mu$ M). The data suggested that reversine might serve as a candidate chemotherapeutic agent against RCC.

## Materials and methods

### Cell lines and culture

Two human RCC cell lines, namely 786-O (p53 mutant) and ACHN (p53 wide-type), were used in this study. Both cell lines were purchased from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Waltham, CA, USA) with 10% fetal bovine serum (Corning Incorporated, Corning, NY, USA) and incubated in a 5% CO<sub>2</sub> humidified atmosphere at 37°C.

### Reagents

Reversine and Z-VAD-FMK were purchased from MCE (Middlesex County, NJ, USA) and dissolved in dimethyl sulfoxide

(DMSO) according to the manufacturer's recommendations. The 10 mmol/L stock solutions were stored at –80°C. Antibodies against cyclin A2, cyclin B1, cyclin D1, phosphor-Rb, p21, Bcl-2, Bcl-xL, and Bax were from Abcam (Cambridge, UK). Antibodies against cleaved-caspase-3 and cleaved-PARP were from Cell Signaling Technology (Danvers, MA, USA).

### Cell proliferation assay

A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed to evaluate cell proliferation. Briefly, 786-O and ACHN cells were plated in quadruplicate in a 96-well plate at  $1 \times 10^4$ /well density in a final volume of 100  $\mu$ L of Dulbecco's Modified Eagle's Medium (DMEM) medium/well in a 5% CO<sub>2</sub> environment at 37°C. After overnight attachment, the cells were incubated with DMEM (with 0.01% DMSO) or DMEM containing varied dosages of reversine at 0.1, 0.2, 0.4, 0.8, and 1.6  $\mu$ M. After incubation for 24, 48, 72, and 96 h, MTS labeling reagent (Promega Corporation, Fitchburg, WI, USA) was mixed with DMEM and incubated with cells for 2 h. Absorbance at 490 nm and 690 nm was examined using a VARIOSCAN FLASH microplate reader (Thermo Fisher Scientific). Cell viability was represented by percentage values compared to a control.

### Colony formation assay

Colony formation assay was performed to determine the long-range impact of reversine exposure on RCC cells. Briefly, the cells were seeded in a 6-well culture plate ( $1 \times 10^3$ /well). After cell attachment for 6 h, DMEM with varied dosages of reversine or 0.01% DMSO was added into each well. Then, all experimental samples were incubated in a 5% CO<sub>2</sub> environment at 37°C, until appropriately large colonies have formed. After approximately 10–14 days, the medium was removed. The samples were stained with crystal violet for 45 min and then washed three times with PBS. Three independent experiments were performed.

### Analysis of apoptosis and cell death

Apoptosis and cell death were analyzed by flow cytometry using propidium iodide (PI) and Annexin-V-FITC staining kit (Nanjing Keygen Biotech. Co., Ltd., Nanjing China). The cells were washed with phosphate buffer saline (PBS) and then centrifuged at  $2,000 \times g$  for 5 min, according to the manufacturer's instructions. Next, the cell pellets were resuspended in 500  $\mu$ L of binding buffer. Then, 5  $\mu$ L of Annexin-V-FITC and 5  $\mu$ L of PI were successively added. After incubating in the dark at room temperature for 15 min, Annexin-V or PI fluorescent intensities were analyzed by FACSscan (Becton–Dickinson, Franklin Lakes, NJ, USA), 20,000 cells were evaluated in each sample.

## DNA content analysis

The Cellular DNA content of each well was determined by flow cytometry according to the protocol (KeyGEN Biotech). After harvesting and washing twice with ice-cold PBS, the cells were resuspended in 1 mL of 70% ethanol and fixed at 4°C overnight. Then, the fixed cells were washed twice with ice-cold PBS and treated with 100  $\mu$ L RNase A for 30 min. Afterward, 400  $\mu$ L of PI was added to the cells. Samples were acquired by flow cytometry using Millipore (Becton–Dickinson).

## Western blot analysis

Briefly, RCC cell extracts were washed three times in cold PBS and lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 (Beyotime, Jiangsu, China), supplemented with Protease Inhibitor Cocktail, and Phosphatase Inhibitor Cocktail (MCE) for 30 min on ice. The homogenates were centrifuged at 12,000 $\times$ g for 10 min, and the extracted protein concentrations were determined by the Bio-Rad protein assay. The protein lysates (~20  $\mu$ g) were electrophoresed in SDS-PAGE gels and transferred onto polyvinylidene fluoride membrane (Merck Millipore, Burlington, MA, USA). The membranes were blocked with TBST (tris-buffered saline with Tween® 20) containing 5% dried milk/BSA for 1 h at room temperature. After incubating in appropriate primary and fluorescent dye-labeled secondary antibodies, the blots were visualized using the ECL Western blot detection system (Syngene, Bangalore, India).

## Statistical analysis

All data are presented as mean  $\pm$  standard deviation for the indicated number of separate experiments. Statistical significance of the differences between the mean values was measured by the Student's *t*-test with a paired, 2-tailed

distribution. The data were considered significant when the *P*-value was less than 0.05 (\*) or 0.01 (\*\*).

## Results

### Reversine reduced cellular viability and inhibited cell colony formation of human RCC cells

Human RCC cells 786-O and ACHN were used to evaluate the proliferation inhibitive effect of reversine. DMSO was used as a negative control. Microscopy results showed the inferior condition of both cell lines after reversine treatment for 48 h. Cell abundance of both cell lines decreased significantly compared with the negative control (Figure 1A and C). Moreover, we further verified the inhibitive effect of reversine on the cellular viability by MTS analysis. Figure 1B and D shows that 786-O and ACHN cells decreased in time- and dose-dependent manners after reversine treatment.

In addition, the growth modulatory effect of reversine in 786-O and ACHN cells was determined using a colony formation assay. Figure 2 shows that reversine could significantly inhibit the numbers of colony formations in both cell lines. Furthermore, cloning was virtually impossible under high-concentration reversine treatment (0.4–1.6  $\mu$ M). Thus, these data indicated that reversine could reduce cell viability and inhibit cell colony formation of human RCC cells.

### Reversine induced polyploidization in human RCC cells

Reversine could therapeutically cause a failure in cytokinesis and induce polyploidization by suppressing Aurora kinases, which are prominent enzymes in mitotic regulation. This phenomenon has also been reported in multiple human cancer cells.<sup>14,20</sup> Here, we explored whether reversine could cause an impaired mitosis in human RCC cell lines. Microscopy

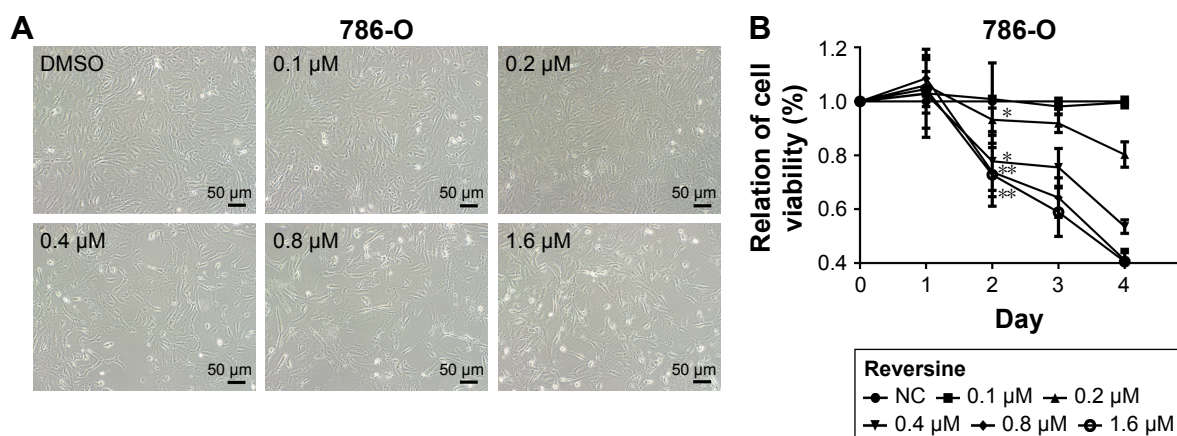
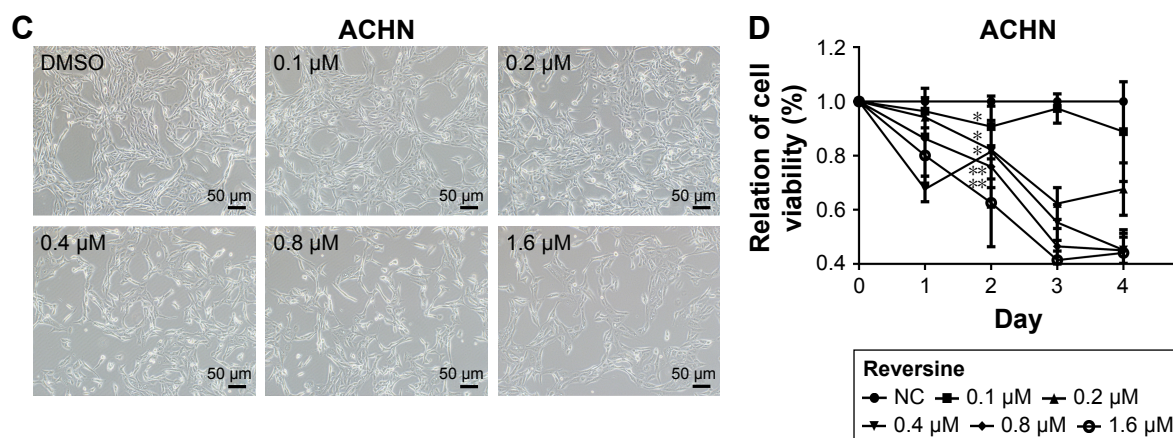


Figure 1 (Continued)

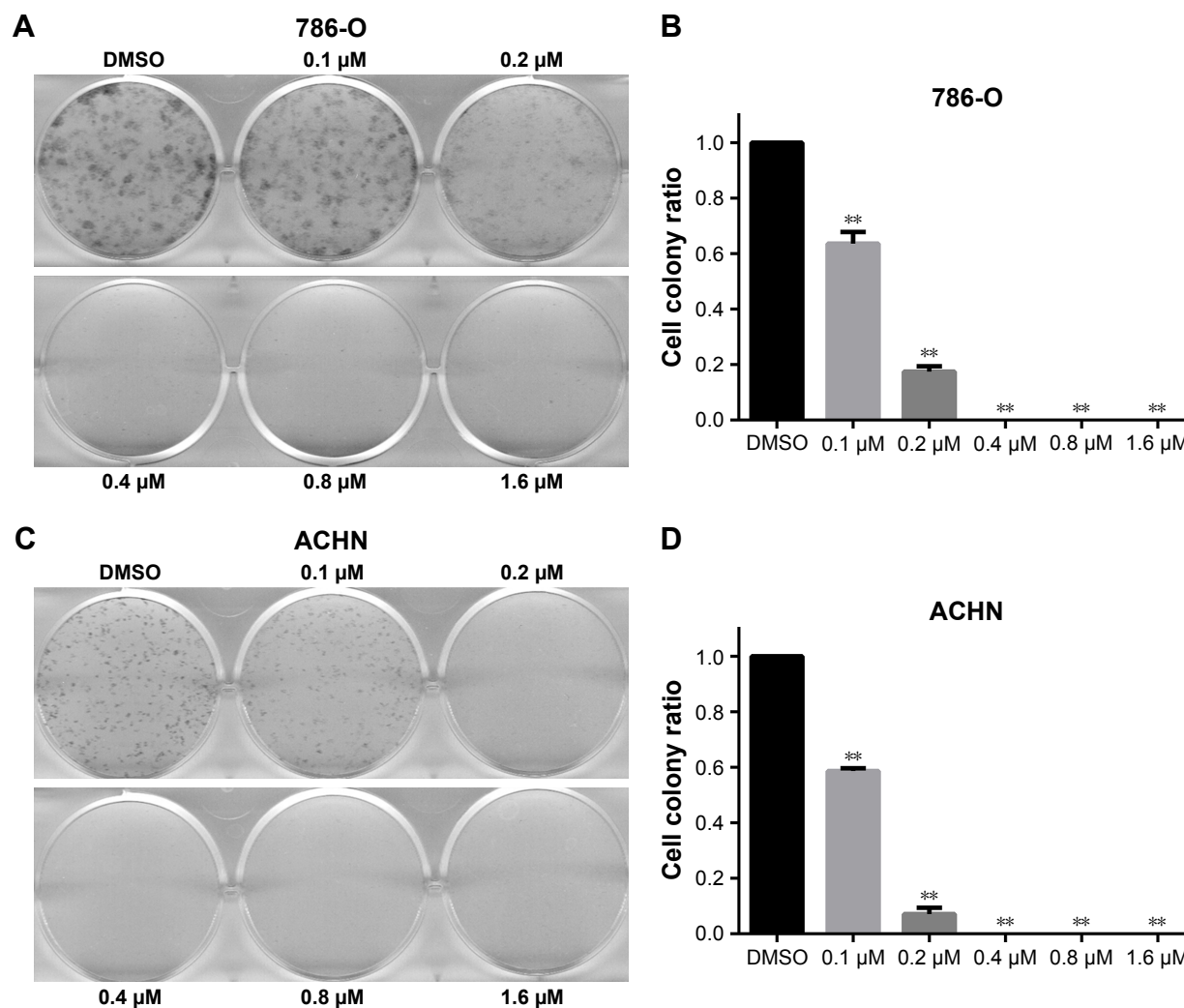




**Figure 1** Reversine suppressed cell growth in human RCC cells.

**Notes:** 786-O and ACHN cells were incubated with DMSO or various dosages of reversine (0.1–1.6  $\mu\text{M}$ ) for 48 h, and the effects on the growth inhibition of 786-O (A, B) and ACHN (C, D) cells were observed by microscopy (A, C) and MTS assay (B, D). The data (B, D) represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; magnification details: 100 $\times$ .

**Abbreviations:** RCC, renal cell carcinoma; DMSO, dimethyl sulfoxide; NC, negative control; SD, standard deviation; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

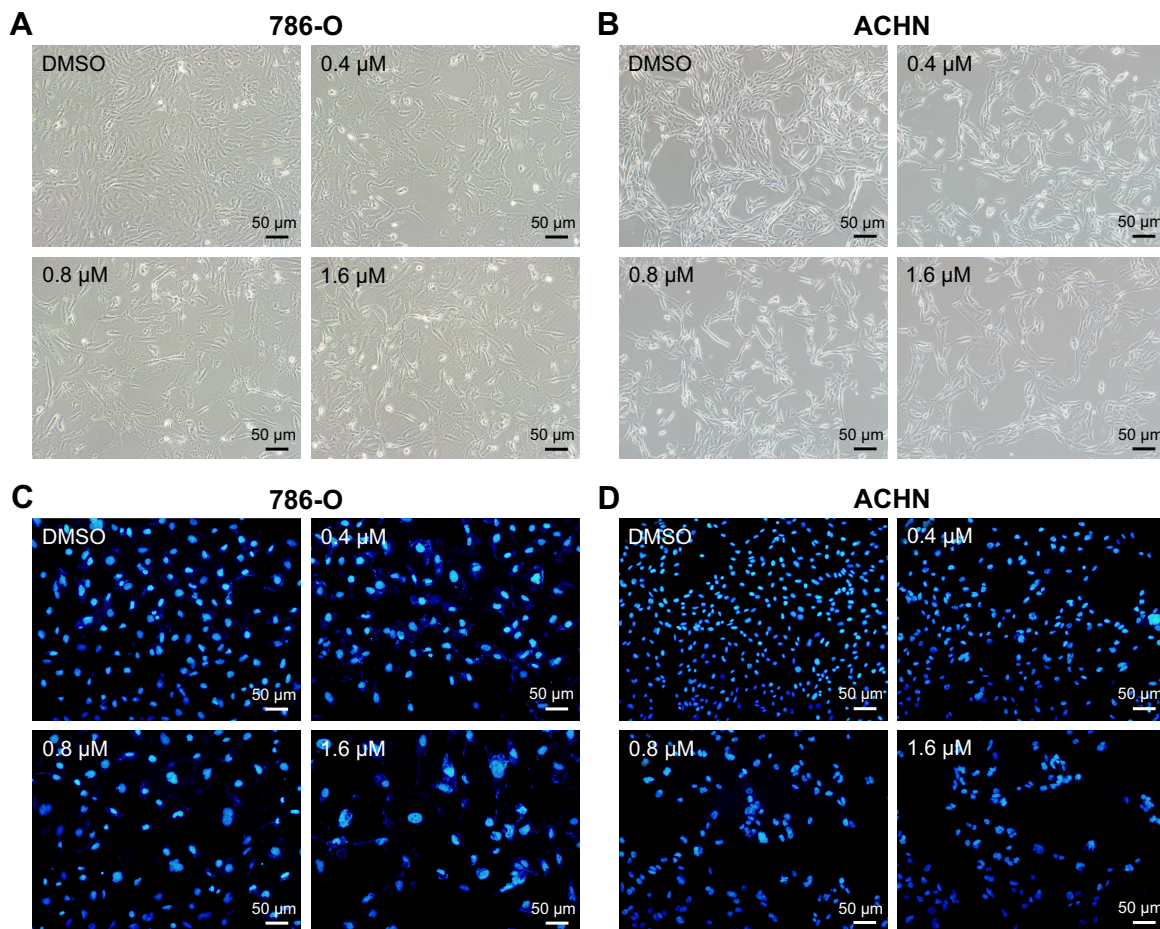


**Figure 2** Reversine suppressed colony formation in human RCC cells.

**Notes:** 786-O (A, B) and ACHN (C, D) cells were incubated with DMSO or various dosages of reversine (0.1–1.6  $\mu\text{M}$ ), and the cell colonies were stained with crystal violet. The data (B, D) represent the mean  $\pm$  SD from three independent experiments. \*\* $P < 0.01$ .

**Abbreviations:** RCC, renal cell carcinoma; DMSO, dimethyl sulfoxide; SD, standard deviation.





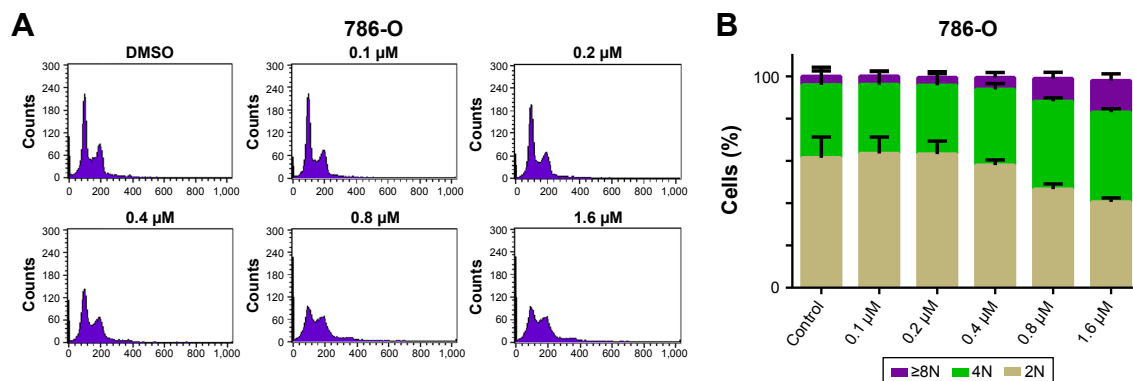
**Figure 3** Reversine induced polyploidy in human RCC cells.

**Notes:** 786-O (**A**) and ACHN (**B**) cells were treated with reversine for 48 h. Microscopy results show the enlarged cellular morphology of groups treated with high-concentration reversine (0.8–1.6  $\mu$ M). The 786-O (**C**) and ACHN (**D**) cellular nuclei were stained with DAPI, and enlarged nuclei of both cells were observed under fluorescence microscopy. Magnification details: 100 $\times$ .

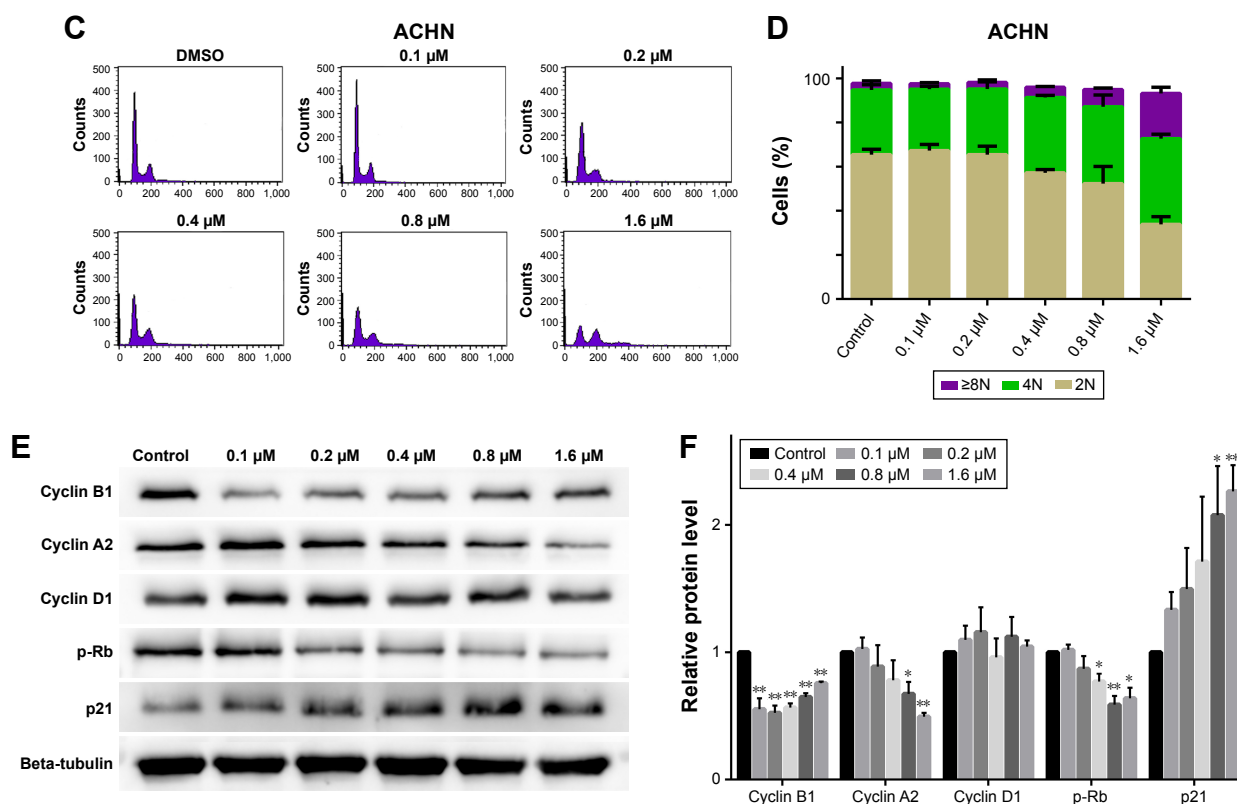
**Abbreviations:** RCC, renal cell carcinoma; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

results showed that reversine treatment caused enlarged cell phenotypes (Figure 3A and B). We further confirmed this phenomenon by nucleo-staining with DAPI (4',6-diamidino-2-phenylindole), and multinucleated cells were observed after reversine treatment in both cell lines (Figure 3C and D). In addition, flow cytometry was used to detect the DNA

content of 786-O and ACHN cells after reversine treatment. The percentages of 2N, 4N, and  $\geq 8$ N distribution of all cells were measured with CellQuest Pro software (BD Biosciences, San Jose, CA, USA), with DMSO as a negative control. Figure 4A–D indicates that polyploidization occurred and increased in a dosage-dependent manner in



**Figure 4** (Continued)



**Figure 4** DNA contents of the 786-O (A, B) and ACHN (C, D) cells were determined by flow cytometry with PI labeling after reversine incubation.

**Notes:** Three independent experiments were confirmed. The percentages of 2N, 4N, and  $\geq$ 8N distribution of all cells were measured with CellQuest Pro software and shown as (B, D). DMSO was used as a negative control. (E, F). Western blot was used to measure the expression of cell-cycle regulating proteins in 786-O cells. Beta-tubulin was used as control. The results (B, D, F) represent the mean  $\pm$  SD from three independent experiments. \*P<0.05; \*\*P<0.01.

**Abbreviations:** PI, propidium iodide; DMSO, dimethyl sulfoxide; SD, standard deviation.

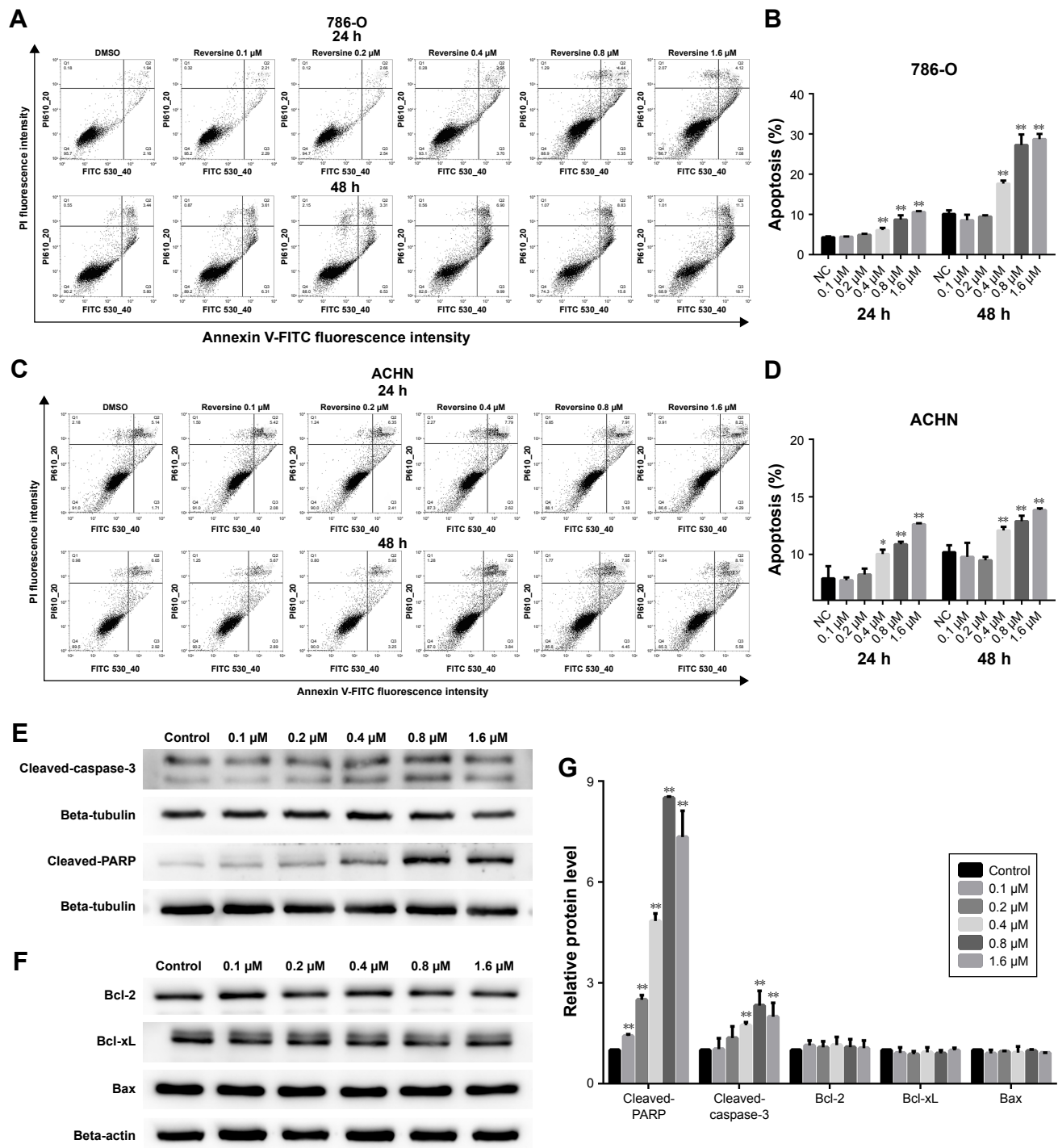
both cells with a higher drug concentration (0.8–1.6  $\mu$ M). Furthermore, the failure of cell mitosis caused by reversine treatment was confirmed by determining the cell-cycle regulating proteins, namely, cyclin A2, cyclin B1, cyclin D1, p21, and p-Rb by Western blot in reversine-treated 786-O cells. The results indicated that reversine inhibited the expression levels of Cyclin A2, Cyclin B1, and p-Rb, but promoted p21 expression (Figure 4E and F). These data demonstrated that reversine-treated cells could exit from uncomplicated cytokinesis and re-enter into new rounds of cycle.

## Reversine induced caspase-dependent apoptosis in human RCC cells

We also explored whether reversine could mediate apoptosis induction in human RCC cells. In this study, 786-O and ACHN were treated with reversine or DMSO, and flow cytometry analysis with AnnexinV and PI double staining were performed to determine apoptosis. The data showed that reversine could induce apoptosis in both human RCC cells in time- and dosage-dependent manners (Figure 5A–D). The p53-deficient 786-O cells were more sensitive to exposure to reversine than the p53-proficient

ACHN cells. Furthermore, Western blot was performed to detect the activation of caspase-3 and PARP to explore the mechanism of reversine-mediated apoptosis in human RCC cells. As expected, the expression of cleaved-caspase-3 was significantly upregulated in the 786-O cells in a dosage-dependent manner after incubation with reversine for 48 h (Figure 5E and G). Moreover, the expression of cleaved PARP was also determined by Western blot to further confirm the increased apoptosis, and the result was consistent with cleaved-caspase-3 (Figure 5E and G). The expression levels of Bcl-2, Bcl-xL, and Bax after reversine incubation showed no significant difference compared with the control group (Figure 5F and G). Generally, our results demonstrated that reversine could induce apoptosis in human RCC cells through an extrinsic pathway, and this result is consistent with those of previous studies.<sup>13,21</sup>

In addition, Z-VAD-FMK, a pan-caspase inhibitor, was used to further elucidate the mechanism of apoptosis induced by reversine in RCC cells. After blocking the activation of caspase with Z-VAD-FMK, reversine-mediated activation of cleaved caspase-3 was partially suppressed (Figure 6A and B), and the cell number of 786-O cells was restored



**Figure 5** Reversine induced caspase-dependent apoptosis in human RCC cells.

**Notes:** Cells were treated with various concentrations of reversine (0.1–1.6  $\mu$ M) or DMSO, and the apoptotic cells of 786-O (**A, B**) and ACHN (**C, D**) cells were evaluated by flow cytometry analysis with PI/Annexin-V double staining. (**E–G**). Western blot was performed to detect the expression of cleaved-caspase-3 and cleaved-PARP in 786-O cells. Beta-tubulin was used as control. The results (**B, D, G**) represent the mean  $\pm$  SD from three independent experiments. \*P < 0.05; \*\*P < 0.01.

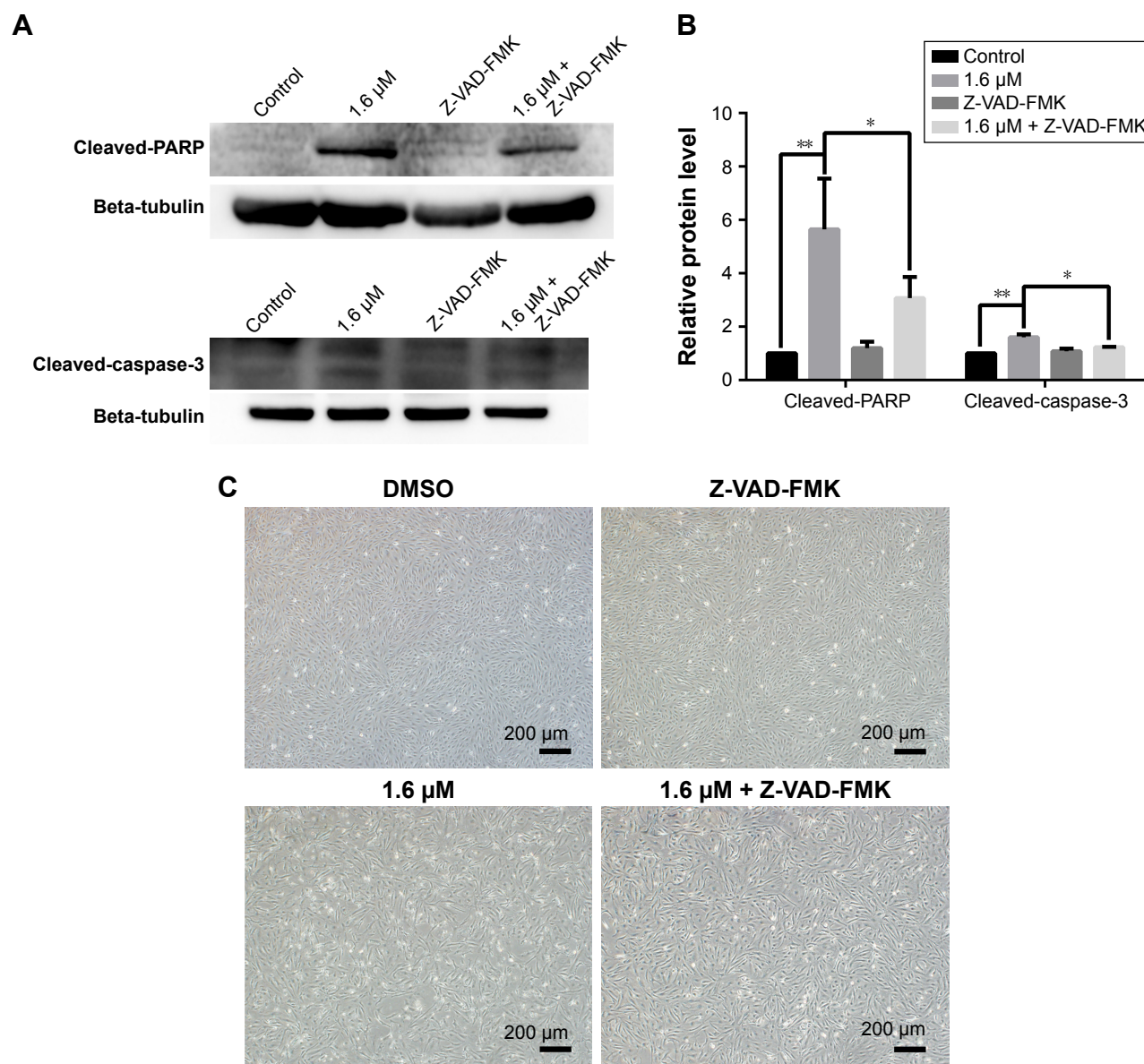
**Abbreviations:** RCC, renal cell carcinoma; DMSO, dimethyl sulfoxide; PI, propidium iodide; SD, standard deviation; NC, negative control.

(Figure 6C). Moreover, the expression of cleaved-PARP was reduced (Figure 6A and B). These data indicated that reversine could reduce cell viability by inducing apoptosis in human RCC cells partially through a caspase-dependent pathway.

## Discussion

Reversine, a 2,6-disubstituted purine, has been originally reported to induce dedifferentiation of C2C12 cells.<sup>8</sup> Several studies have demonstrated that reversine could inhibit Aurora kinases and various kinds of cellular enzymatic (such as





**Figure 6 (A, B)** Cells were incubated with reversine (1.6  $\mu$ M) or Z-VAD-FMK (pan-caspase inhibitor, 20  $\mu$ M) for 48 h.

**Notes:** The expression of cleaved-caspase-3 and cleaved-PARP was determined by Western blot. Beta-tubulin was used as control. The data (B) represent the mean  $\pm$  SD from three independent experiments. \* $P$  < 0.05; \*\* $P$  < 0.01. (C) The restored cell numbers and morphology were observed in the group with Z-VAD-FMK treatment, compared with reversine treatment. DMSO was used as a control. Magnification details: 40 $\times$ .

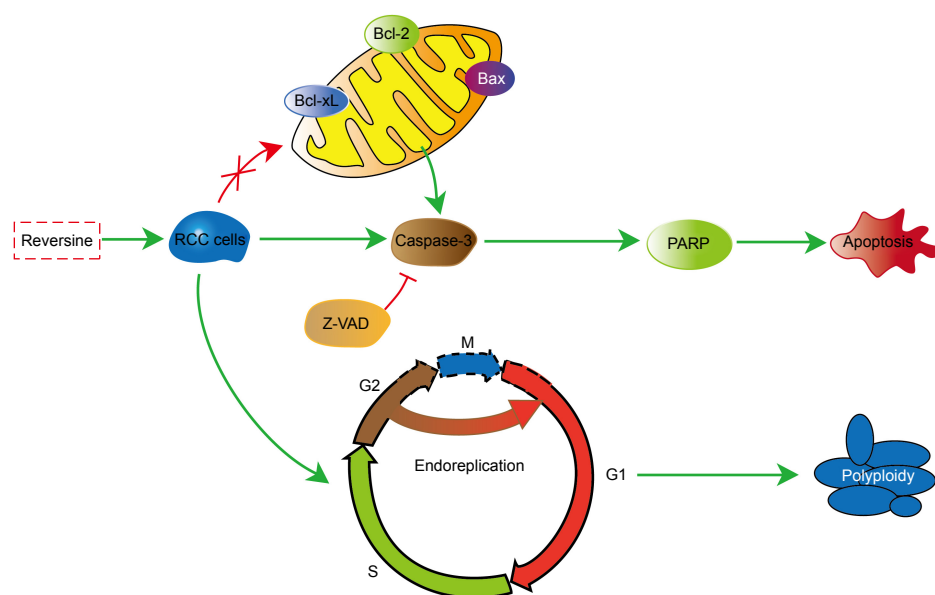
**Abbreviations:** DMSO, dimethyl sulfoxide; SD, standard deviation.

AKT, SRC, and JAK2) activities in vitro.<sup>9,18</sup> The Aurora kinases comprised Aurora-A, Aurora-B, and Aurora-C, which play a vital role in regulating mitosis.<sup>22</sup> The anti-tumor capability of reversine has been confirmed in numerous cancer cells.<sup>15–18,23</sup> Thus, we investigated the growth modulatory effects of reversine in human RCC cells.

In the present study, we first demonstrated the anti-tumor capability of reversine in 786-O and ACHN RCC cells. Cell viability and colony formation were significantly inhibited in both cell lines. Moreover, reversine-mediated polyploidy formation and apoptosis were induced in a dose-dependent manner.

In our study, polyploidy formation was induced in both cell lines under reversine treatment. The formation was characterized by an enlargement of cell nuclei and an enrichment of 4N and 8N interphase populations. This finding is consistent with previous studies.<sup>13,14</sup> Notably, polyploidy cancer cells show increased sensitivity to anti-tumor agents or gamma irradiation by inducing late-stage cell apoptosis and/or mitotic cell death.<sup>24,25</sup>

Endoreplication mechanisms may contribute to the induction of polyploidy formation in RCC cells in response to reversine treatment. We observed changes in the expression of cell cycle regulating proteins. The cell cycle is controlled by



**Figure 7** Schematic diagrams depicting the anti-tumor effects of reversine on RCC cells.

**Notes:** Reversene is proposed to induce caspase-dependent apoptosis, yet the mitochondrion-dependent apoptotic pathway is not involved. Moreover, RCC cells treated with reversene exit from impaired cytokinesis followed by re-entering of G1 phase and continuous DNA replication, resulting in induction of polyploidy.

**Abbreviation:** RCC, renal cell carcinoma.

a complex bio-chemical-molecular system including cyclins, cdks, and other factors. Activation of CDK1-cyclin B1 is necessary for mitotic entry.<sup>26</sup> We discovered that the down-regulation of cyclin B1 was coordinated with an increased level of p21 in the 786-O cell line under reversine treatment. These changes may account for the exit from impaired cytokinesis followed by re-entering of the G1 phase and continuous DNA replication, and these processes subsequently increased polyploidy formation. Our results are in agreement with the results from other studies.<sup>16,20,27,28</sup> Hence, the accumulation of polyploid cells may be one of the mechanisms of cytotoxicity in the treatment of RCC cells. Whether reversine-mediated polyploidy formation was through the inhibition of Aurora kinases and/or other factors in RCC cells deserves to be verified.

Moreover, we first demonstrated that apoptosis was triggered by reversine in 786-O and ACHN RCC cells. Reversine has been reported to induce apoptosis in several human cancer cells. Consistent with previous studies,<sup>13,21</sup> we exhibited that reversine induced apoptosis by activating caspase-dependent apoptotic pathways. Moreover, the mitochondrion-dependent apoptotic pathway was not involved in reversine-mediated RCC cell apoptosis. This phenomenon was confirmed by detection of Bcl-2, Bcl-xL, and Bax.

Previous studies have demonstrated that reversine could induce autophagosome formation in numerous cancer cells *in vitro*.<sup>15,16</sup> However, no change of autophagy was observed in our study (Figure S1), which indicates that the

induction of autophagosome formation by reversine was cell type-dependent.

Several cancer cells have been reported to present TP53 mutations which lead to resistance against anti-cancer agents.<sup>29–33</sup> Jemaà et al<sup>34</sup> demonstrated that reversine killed p53-proficient cells more efficiently at low-concentration (~0.5  $\mu\text{M}$ ). Conversely, p53-deficient cells were more susceptible under high-concentrations (~5  $\mu\text{M}$ ) of reversine.<sup>34</sup> In our study, we evaluated the cytotoxic profile of reversine (from 0.1–1.6  $\mu\text{M}$ ) in 786-O (p53 mutation) and ACHN (p53 wild-type) cell lines. Reversine exerted antiproliferative activity with the IC<sub>50</sub> value of 1.61  $\mu\text{M}$  in 786-O and 0.74  $\mu\text{M}$  in ACHN (72 h), showing that ACHN cells were more susceptible than 786-O cells. Colony formations were completely inhibited in both cell lines under reversine treatment ( $\geq 0.4 \mu\text{M}$ ). Moreover, at a concentration of 1.6  $\mu\text{M}$ , reversine killed 786-O cells more efficiently than ACHN cells (28.70% $\pm$ 1.30%, compared with 13.84% $\pm$ 0.16%), while ACHN cells showed a higher distribution of polyploid (20.5% $\pm$ 2.7%, compared with 15% $\pm$ 3.1%). In general, the mechanism by which reversine exerts anti-tumor effects on RCC cells is shown in Figure 7.

## Conclusion

We demonstrated that reversine is a potential therapeutic drug that can reduce cell viability by inducing apoptosis and polyploidy formation. Our study suggests that reversine may serve as a novel therapeutic drug for RCC patients.

## Acknowledgments

This study was funded by the Natural Science Foundation of China (No. 81670617) and the Beijing Nature Science Foundation (No. 7142158).

## Disclosure

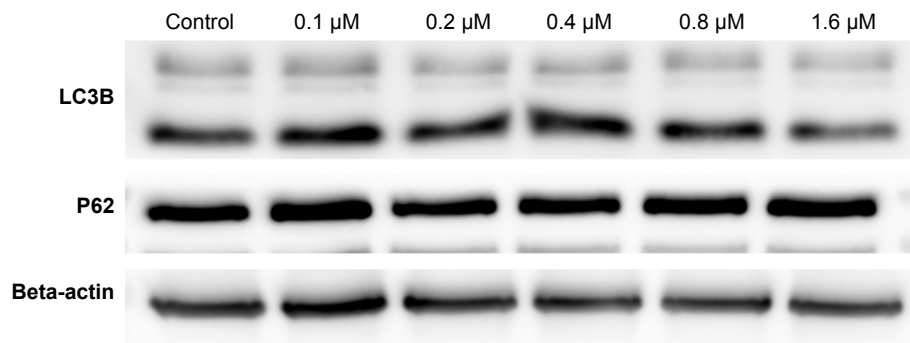
The authors report no conflicts of interest in this work.

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## Supplementary material



**Figure S1** The expression of autophagy-related proteins in 786-O cells after reversine incubation. Cells were treated with various concentrations of reversine (0.1–1.6  $\mu$ M) or DMSO for 48 h, and the expression of LC3B and P62 was determined by Western blotting. Beta-actin was used as a control.

**Abbreviation:** DMSO, dimethyl sulfoxide.

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