ORIGINAL ARTICLE

Mechanism of apoptosis induced by Mcl-1 inhibitor UMI-77 on gallbladder carcinoma GBC-SD cells

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ABSTRACT

Objective: To investigate the mechanism of apoptosis induced by myeloid cell leukemia-1 (Mcl-1) inhibitor UMI-77 on gallbladder carcinoma GBC-SD cells.

Methods: GBC-SD cells were treated with different concentrations of UMI-77. GBC-SD cell proliferation and apoptosis were detected by MTT assay and Annexin V/PI. The expressions of Mcl-1, Bcl-2, Bcl-xL, Bax, Bak, cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP proteins in GBC-SD cells treated with UMI-77 were detected by Western blotting.

Results: The results of MTT showed that different concentrations of UMI-77 had different inhibitory effects on cell proliferation of GBC-SD cells in a dose-dependent and time-dependent manner. Annexin V/PI results showed that the apoptosis rate was increasing gradually with the increase of UMI-77 concentration in a dose-dependent manner. Western blotting results showed that the expression of anti-apoptotic protein Mcl-1 was significantly decreased ($p < .05$), and the expressions of Bax and Bak proteins were significantly increased respectively ($p < .05$), but there were no significant changes in the expressions of Bcl-2 and Bcl-xL proteins, and the expression levels of cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP proteins were significantly increased ($p < .05$) in 24 h after GBC-SD cells were treated with 10 μmol/L of UMI-77.

Conclusions: Mcl-1 inhibitor UMI-77 can induce the apoptosis of GBC-SD cells in a dose-dependent manner through the caspase-mediated endogenous apoptosis pathway. Therefore, Mcl-1 may become a new therapeutic target in the research on gallbladder cancer.

Key Words: Mcl-1 inhibitor, UMI-77, Gallbladder carcinoma, Apoptosis

1. INTRODUCTION

Gallbladder carcinoma is one of the most common malignant tumors which occur in the biliary system, accounting for 2/3 of the biliary tumors. Nevertheless, most of the patients are found to be in the middle or the terminal stage of cancer with local infiltration or tissue transfer when they are diagnosed as gallbladder carcinoma due to unclear pathogenesis, the lack of specific clinical manifestations and concealed onset.[1,2] One of important symbols for the occurrence and development of tumors is the disorder of cell apoptosis, which is also one of factors that tumors are not sensitive to radiotherapy and chemotherapy. Therefore, to induce the apoptosis of tumor cells is a type of mechanism for the treatment of tumors.[3,4] The apoptosis pathway can be subdivided into the exogenous pathway and the endogenous pathway. B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia-1 (Mcl-1) participate in the endogenous apoptosis pathway as anti-apoptotic proteins.[5] In the aspect of cytology, the devel-

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opment of gallbladder carcinoma results from the disorder of the expression of cell proliferation- and apoptosis-associated factors. Gallbladder carcinoma cells show overexpression of anti-apoptotic factors Bcl-2, Mcl-1 and B-cell lymphoma extra large (Bcl-xL), with a low expression of pro-apoptotic factors Bcl-2 assaciated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak). With the help of its BH3 domain, anti-apoptotic factors can bind to pro-apoptotic factors to form into heterodimers, protecting cancer cells from entering into apoptosis programme to inhibit the apoptosis of cancer cells and promote the development of gallbladder carcinoma.[6, 7] In recent years, researchers have synthesized various small-molecule inhibitors targeting anti-apoptotic proteins from the research of Bcl-2 and Mcl-1. The application of inhibitors alone or in combination with chemotherapy drugs has a good therapeutic effect.[8] In 2014, with the application of structural biology and high throughput screening, Abulwerdi et al.[9] identified a type of small-molecule inhibitor of Mcl-1, called UMI-77. It can selectively bind to Mcl-1 to rival the anti-apoptotic function of Mcl-1 and play a role in inducing apoptosis and inhibiting cell growth. This research applies UMI-77 to gallbladder carcinoma GBC-SD cells to explore the mechanism of UMI-77 on inducing the apoptosis of gallbladder carcinoma cells, with the aim of providing a theoretical basis for clinical application.

2. MATERIALS AND METHODS

2.1 Materials

Human gallbladder carcinoma cell line GBC-SD was provided by Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Mcl-1 inhibitor UMI-77 was purchased from Selleck (USA); Annexin V/PI double staining kits were merchandised from Beijing Jingmei Biotechnology Co., Ltd.; RPMI-1640 medium and fetal calf serum (FBS) were purchased from Invitrogen (USA); cell proliferation assay kits were acquired from Sigma (USA). Rabbit anti-human polyclonal antibodies Mcl-1 (ab28147), Bcl-2 (ab59348), Bcl-xL (ab2568), Bax (ab53154), Bak (ab69404), cleaved cysteinyl aspartate specific proteinase (cleaved-caspase 9) (ab2324), cleaved-caspase 3 (ab2302), rabbit anti-human monoclonal antibodies cleaved poly ADP-ribose polymerase (cleaved-PARP) (ab32064), and goat anti-rabbit secondary antibody (ab6721) were purchased from Abcam (UK).

2.2 Cell culture

Conventionally, human gallbladder carcinoma GBC-SD cells were cultured in RPMI-1640 medium containing 10% FBS (fetal bovine serum) and placed into the incubator (37°C, 5% CO_2). Cells in the logarithmic phase were taken and used in the experiment.

2.3 MTT assay of cell proliferation

Cells in the logarithmic phase were inoculated in 96-well cell culture plates with a seeding density of 1×10^6 cells/well, the volume of medium in each well was 100 μl. UMI-77 at gradient concentrations of 0, 5, 10, 15, 20 and 30 μmol/L were added into each well respectively. Each group was made in quintuple. Each group of cells were cultured for 12 h, 24 h and 48 h, and then each well was added into 20 μl of MTT solution. The supernatant was removed carefully after another 3-6 h cell culture, with 100 μl of formazan solution added into each well for incubation. OD value of each well was measured by the microplate reader, with the average of OD values at the same time point taken from that of OD values from 5 parallel holes. Cell survival rate (%) = (OD value of each concentration group/OD value of 0 μmol/L group) $\times$ 100%.

2.4 The detection of cell apoptosis by flow cytometry (FCM)

Cells in the logarithmic phase were inoculated in 24-well cell culture plates with a seeding density of 1×10^5 cells/well. Up to the next day, if the proportion of cell fusion exceeded 80%, the cells were treated with 0, 5, 10, 15, 20 and 30 μmol/L of UMI-77 respectively. Each group was made in triplicate. Cells in each group were cultured respectively for 24 h, collected and added with PBS for resuspension and centrifugation. Besides, these cells were resuspended in 300 μl 1$\times$ binding buffer, added by 5 μl of Annexin V-FITC and labeled by 5 μl of propodium iodide (PI). The mixture was fully mixed and then incubated in a light-proof place for 20 min. The FCM was used to detect cell apoptosis.

2.5 The detection of the expression of proteins by Western blotting

Western blotting can be used to detect the expressions of Mcl-1, Bcl-2, Bcl-xL, Bax, Bak, cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP in each group of GBC-SD cells at the optimal concentration and the optimal time point. Each group of cells were collected, washed with PBS and added by lysis buffer. After cell lysis, the supernatant was collected by means of centrifugation. Bicinchoninic acid (BCA) was used to measure the concentration of proteins. Each well was added into 20 μl of sample proteins. After 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to PVDF, which was blocked for 1 h at room temperature in 5% skim milk, added by primary antibodies (diluted under the ratio of 1:1,000) and incubated overnight at 4°C. After washing with TBST for 3 times, human specific secondary antibodies (diluted under the ratio of 1:5,000) were added, with the incubation continued for 1 h at room temperature. PVDF was fully rinsed out and

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developed for photomicrograph. Each group was made in triplicate, and Image J software was used to make a quantitative gray-degree analysis of the bands, with the average value taken.

2.6 Statistical treatment

The experimental data were represented by mean ± standard deviation, t-test was applied to the comparison between two groups and one-way ANOVA was applied to the comparison among groups. SPSS 19.0 statistical software was used in the statistical treatment, and $p < .05$ indicated the difference was of statistical significance.

3. RESULTS

3.1 The inhibition of UMI-77 on GBC-SD cell proliferation

According to Figure 1, MTT results showed that different concentrations of UMI-77 acted on GBC-SD cells for different time durations, the survival rate of cells in each group was significantly decreased in a dose-dependent and time-dependent manner. Compared to cells with no UMI-77 addition of the corresponding period, cells treated with no less than 10 μmol/L of UMI-77 for 12 h, 24 h and 48 h, showed a significant decrease in the survival rate ($p < .05$), i.e., UMI-77 can significantly inhibit cell proliferation. UMI-77 at the concentration of 10 μmol/L was applied to the follow-up study, and the treatment time was 24 h.

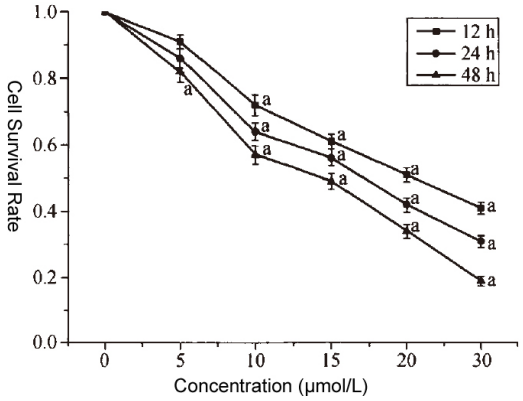

Figure 1. Inhibitory effects of UMI-77 at different concentrations on the proliferation of GBC-SD cells (Compared with 0 μmol/L group, a $p < .05$)

3.2 The effect of UMI-77 on promoting GBC-SD cell apoptosis

After GBC-SD cells were treated with UMI-77 at the concentration of 0, 5, 10, 15, 20 and 30 μmol/L respectively for 24 h, FCM was applied to the detection of cell apoptosis. The results (see Figure 2) showed that, the apoptosis rate was gradually increased with the increase of drug concentration in a dose-dependent manner. Compared to cells with no UMI-77 addition of the corresponding period, cells treated with UMI-77 at different concentrations for 24 h, showed a significant increase in the apoptosis rate ($p < .05$), i.e., UMI-77 at different concentrations can significantly promote cell apoptosis (see Figure 3).

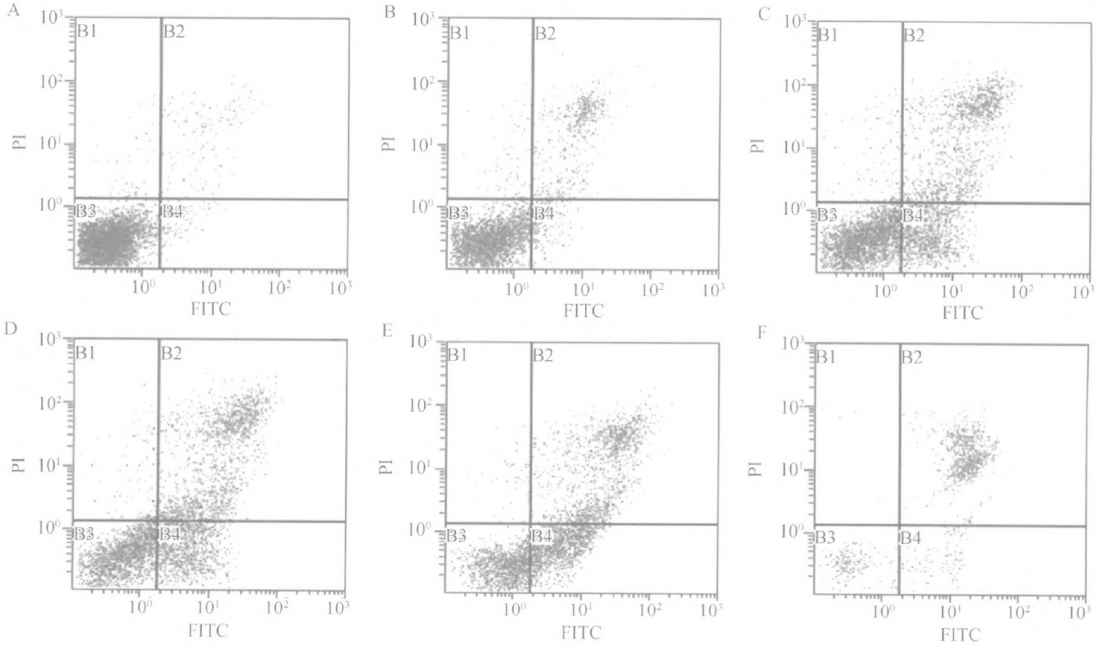

Figure 2. Apoptosis rates of GBC-SD cells treated with UMI-77 at different concentrations for 24 h
A. 0 μmol/L group; B. 5 μmol/L group; C. 10 μmol/L group; D. 15 μmol/L group; E. 20 μmol/L group; F. 30 μmol/L group

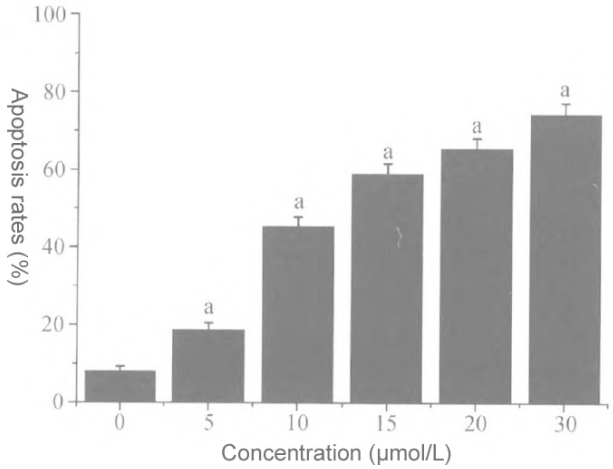

Figure 3. Apoptosis rates of GBC-SD cells treated with UMI-77 at different concentrations for 24 h
Compared with 0 μmol/L group, a $p < .05$

3.3 The effect of UMI-77 on the expressions of Mcl-1, Bcl-2, Bcl-xL, Bax and Bak in GBC-SD cells

Western blotting was used to detect the expressions of anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1, pro-apoptotic proteins Bax and Bak before and after GBC-SD cells were treated with UMI-77 at the concentration of 10 μmol/L (see Figure 4). Compared with the untreated group, after GBC-SD cells were treated with UMI-77 at the concentration of 10 μmol/L for 24 h, the expression of Mcl-1 was significantly decreased ($p < .05$), and the expressions of Bax and Bak were significantly increased ($p < .05$), but the expressions of Bcl-2 and Bcl-xL showed no obvious changes.

3.4 The effect of UMI-77 on the expressions of cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP in GBC-SD cells

Western blotting was applied to the detection of the expressions of cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP proteins before and after GBC-SD cells were treated with UMI-77 at the concentration of 10 μmol/L (see Figure 5). Compared with the untreated group, after GBC-SD cells were treated with UMI-77 at the concentration of 10 μmol/L for 24 h, the expressions of active fragments cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP were significantly increased, and the difference was of statistical significance ($p < .05$).

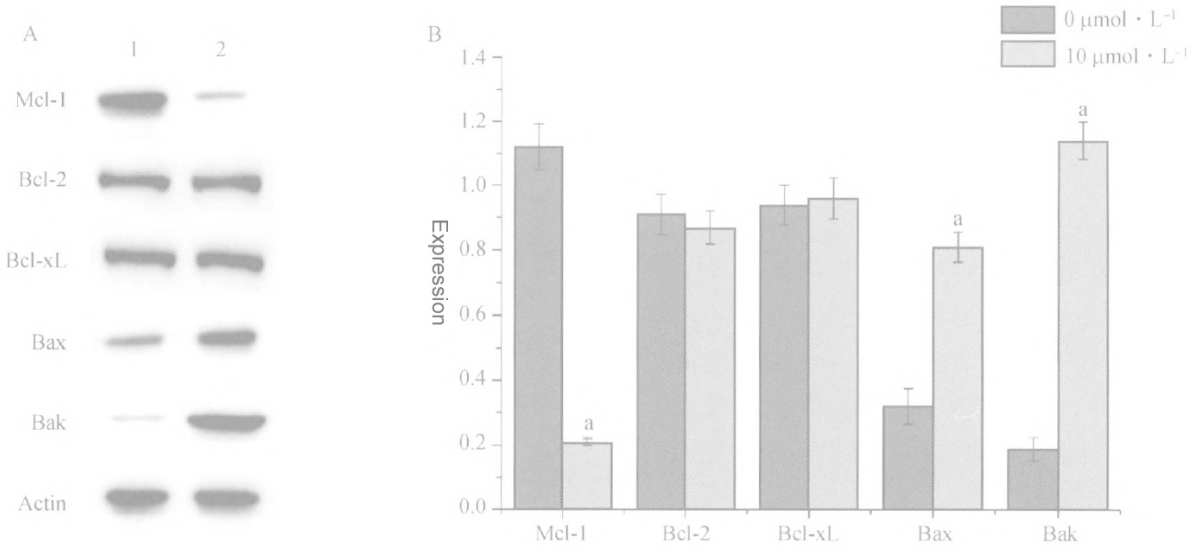

Figure 4. The effect of UMI-77 on the expressions of Mcl-1, Bcl-2, Bcl-xL, Bax and Bak proteins in GBC-SD cells
1 stands for 0 μmol/L group; 2 represents 10 μmol/L group; In comparison with 0 μmol/L group, a $p < .05$; A. The detection of Mcl-1, Bcl-2, Bcl-xL, Bax and Bak proteins by Western blotting; B. The expressions of Mcl-1, Bcl-2, Bcl-xL, Bax and Bak proteins

4. Discussion

As a type of the most common malignant tumor in the biliary system, gallbladder carcinoma accounts for 2/3 of the biliary tumors. Nevertheless, most of the patients are found to be in the middle or the terminal stage of cancer with local infiltration and tissue transfer when they are diagnosed as gallbladder carcinoma due to unclear pathogenesis, the lack of specific clinical index and conceal onset.[1] Meanwhile, gallbladder carcinoma is prone to drug resistance, and is not sensitive to radiotherapy and chemotherapy. Tumor prognosis is not promising.[10] Therefore, it is crucial to make a research to explore the therapeutic target associated with gallbladder carcinoma. Mcl-1 is a type of anti-apoptotic factors in Bcl-2 family, with a powerful and unique function and low

half-life period. It is highly expressed in various tumor tissues and cells.[11,12] When Mcl-1 is over-expressed in tumor tissues, it can significantly suppress the anti-tumor effect of inhibitors towards Bcl-2.[13] Starting with Mcl-1, researchers have synthesized various small-molecule inhibitors targeting anti-apoptotic proteins. The application of inhibitors alone or in combination with chemotherapy drugs has a good therapeutic effect.[8] These researches indicate that Mcl-1 is probably a new target in the treatment of tumors.

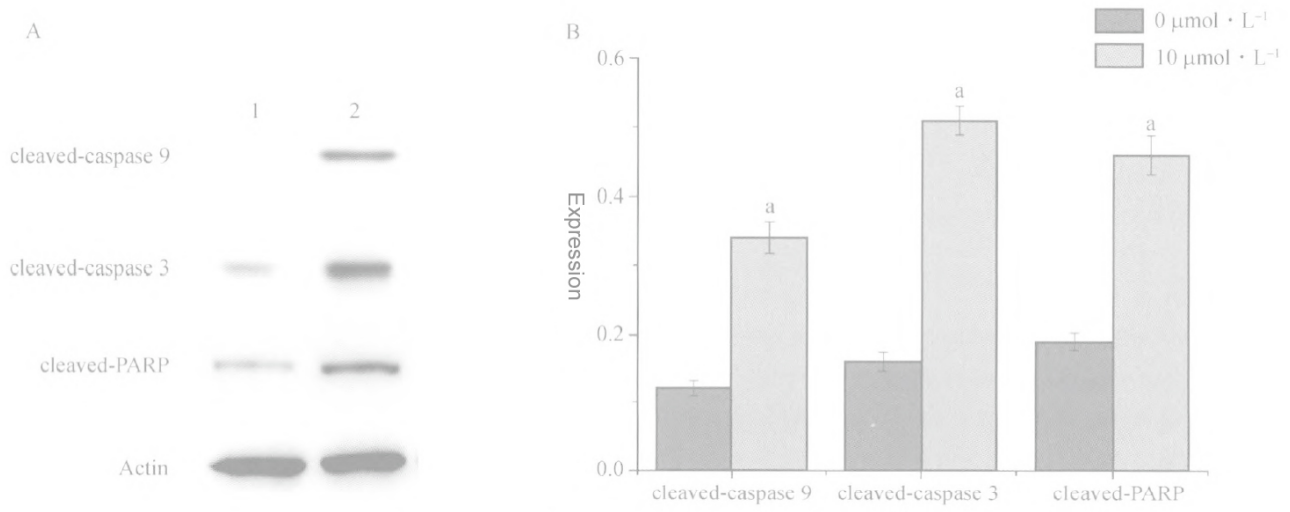

Figure 5. The effect of UMI-77 on the expressions of cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP proteins in GBC-SD cells

1 stands for 0 μmol/L group; 2 represents 10 μmol/L group; In comparison with 0 μmol/L group, a $p < .05$; A. The detection of cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP proteins by Western blotting; B. The expressions of cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP proteins

One of important symbols for the occurrence and development of tumors is the disorder of cell apoptosis, which is also one of factors that tumors are not sensitive to radiotherapy and chemotherapy. Therefore, to induce the apoptosis of tumor cells is a type of mechanism for the treatment of tumors.[3,4] In recent years, there have apppeared an increasing number of researches on inhibitors of anti-apoptotic proteins. Most of the researches focus on ABT-737 and its oral derivative ABT-263, the small-molecule inhibitors of Bcl-2 protein. This type of inhibitors has an inhibitory effect on the activity of most anti-apoptotic proteins, but poorly binds to Mcl-1, so that tumor cells with a high expression of Mcl-1 are prone to drug resistance to ABT-737. Therefore, it is of critical significance for the treatment of tumors to make a research on the inhibitor of Mcl-1 protein.[14,15] UMI-77 is a small-molecule inhibitor targeting Mcl-1. It can selectively bind to Mcl-l to rival the anti-apoptotic function of Mcl-1 and play a role in promoting apoptosis.[9] Studies by Xueping Zhu et al.[16] showed that the mechanism of inducing the apoptosis of gastric carcinoma cell MGC-803 by UMI-77 is probably to activate endogenous apoptosis pathway. In this research, UMI-77 with different concentrations was applied to the treatment of gallbladder carcinoma GBC-SD cells. The results showed that UMI-77 can significantly inhibit the proliferation of GBC-SD cells and promote the apoptosis of GBC-SD cells in a dose-dependent and time-dependent manner. After gallbladder carcinoma GBC-SD cells were treated with UMI-77 for 24 h, the expressions of anti-apoptotic proteins Bcl-2 and Bcl-xL in Bcl-2 family showed no significant changes. Nevertheless, the expression of Mcl-1 was significantly reduced and the expressions of pro-apoptotic proteins Bax and Bak were obviously up-regulated. All these results futher identified that UMI-77 can selectively bind to Mcl-1, reduce the expression of Mcl-1 in the cells and prevent the heterodimerization of Mcl-1/Bax and Mcl-1/Bak to increase the exoressions of Bax and Bak proteins and promote the apoptosis of tumor cells. In this research, after GBC-SD cells were treated with UMI-77 at the concentration of 10 μmol/L for 24 h, the expressions of active cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP were significantly higher than those in the untreated cells. The difference ($p < .05$) was of statistical significance. Endogenous apoptosis pathway, also called mitochondrial pathway, starts at mitochondria. It can recruit and activate caspase 9 by releasing apoptosis-associated factor, and then activate caspase 2, caspase 3, caspase 6 and other factors to initiate cascade reactions of caspases, eventualy lead to cell apoptosis.[17] When the apoptosis of tumor cells is inhibited, caspase 3,

caspase 9 and PARP stays in an unactivated condition. After cells are treated with UMI-77, a series of stress responses will appear so that caspase (e.g., cleaved-caspase 3, cleaved-caspase 9) and PARP can be cleaved and activated by means of cascade reactions of caspases. Eventually, cell apoptosis will be induced through endogenous apoptosis pathway.

5. Conclusions

In summary, this research indicates that the small-molecule inhibitor UMI-77 targeting Mcl-1 can inhibit the proliferation of gallbladder carcinoma GBC-SD cells in a dose-dependent and time-dependent manner. Meanwhile, it can also induce the apoptosis of GBC-SD cells through caspase-mediated endogenous apoptosis pathway in a dose-dependent manner, the mechanism is probably realized by reducing the expression of Mcl-1. It is indicated that Mcl-1 can be considered as a new therapeutic target in the research on gallbladder carcinoma. Therefore, the specific mechanism on how the small-molecule inhibitor UMI-77 targeting Mcl-1 has a good anti-tumor effect needs to be further studied, which provides a new choice for anti-tumor treatment.

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Conflicts of Interest Disclosure

The authors declare they have no conflicts of interest.

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