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Original Research Article

Anticancer effect of 7-hydroxycoumarin in cisplatin-resistant ovarian cancer cell is mediated via apoptosis induction, caspase activation and cell cycle arrest at G2M phase

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Abstract

Purpose: *To investigate the anticancer effects of 7-hydroxycoumarin against cisplatin-resistant ovarian cancer cell line, and the underlying mechanism(s).*
Methods: *Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The 4',6-diamidino-2-phenylindole (DAPI) and acridine orange/ethidium bromide (AO/EB) dual staining methods were used for measuring cell apoptosis in terms of DNA damage. Flow cytometry was used for analysis of mitosis of cancer cells, while protein expression levels were assayed with western blotting.*
Results: *The 7-hydroxycoumarin preferentially inhibited the proliferation of the ovarian cancer cells, but had significantly less prominent effects on normal cells ($p < 0.05$). The decrease in cell proliferation was due to induction of cell apoptosis via caspase-linked apoptotic pathway. Treatment with 7-hdoxycoumarin further led to the arrest of cancer cell cycle at G2/M stage ($p < 0.05$) via down-regulation of the expressions of regulatory proteins that promote mitotic entry.*
Conclusion: *7-Hydroxycoumarin exerts significant anticancer effect against cisplatin-resistant ovarian cancer cells via decrease in cell proliferation, induction of apoptosis and mitotic cell cycle arrest. Thus, the compound could emerge as a vital lead molecule in the treatment of cisplatin-resistant type of human ovarian cancer.*

Keywords: *7-Hydroxycoumarin, Cisplatin-resistant ovarian cancer, Anticancer, Apoptosis, Cell cycle arrest, DNA damage*

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INTRODUCTION

Ovarian cancer is one of the most dominant malignancies in women [1]. The disease has a prevalence of approximately 2.5 % among women worldwide [2]. It accounts for 5 % of the total cancer-related mortalities among women at the global level [3]. Some of the major hindrances in the management of this disorder are lack of early diagnostic procedures and effective treatment measures [4]. Furthermore, studies have shown that treatment of ovarian

cancer is complicated due to instances of development of resistance to the chemotherapeutic agents by the ovarian cancer cells [5].

Cisplatin is among the most widely used chemical compounds in anticancer chemotherapy [6]. However, studies have reported that in some instances, ovarian cancer cells developed resistance to cisplatin [7]. The acquisition of drug resistance by ovarian cancer cells poses a major hurdle in treating ovarian cancer [8]. Thus, it is necessary to evolve anticancer agents that will be effective against cisplatin-resistant ovarian cancer cells. In order to achieve this goal, investigations have been carried out on the anticancer properties of 7-hydroxycoumarin or coumarin against cisplatin-resistant ovarian cancer cells. 7-Hyroxycoumarin has been shown to act as effective anticancer compound against a number of human cancers [9].

In this study, 7-hydroxycoumarin was shown to negatively affect the proliferation of cisplatin-resistant ovarian cancer cells, with no effect on normal ovarian cells. The anti-proliferative effects of 7-hydroxycoumarin were evident in terms of its potential to induce caspase-driven apoptosis of cisplatin-resistant ovarian cancer cells. Moreover, 7-hydroxycoumarin restricted the growth of the cancer cells by induction of arrest of mitotic cell cycle through modulation of expression levels of regulatory proteins involved in mitotic phase entry. Together, the results showed that 7-hydroxycoumarin restricted the growth of cisplatin-resistant ovarian cancer cells through induction of apoptosis and cell cycle arrest.

EXPERIMENTAL

Culture of cell lines

Cisplatin-resistant ovarian cancer cell line (SKVCR) and normal ovarian cell line (SV40) were obtained from the ATCC, USA. The cells were cultured in RPMI 1640 (Thermo Scientific) medium supplemented with ampicillin and streptomycin (100 U/mL each) and 10 % FBS at 37^{o}C in a humidified incubator containing 5 % CO_2.

MTT assay

The SKVCR cisplatin-resistant ovarian cancer cells and normal ovarian cells were cultured with 6, 12, 18, 50 or 100 µM 7-hydroxycoumarin for 24 h at 37^{o}C in a 96-well plate. Untreated cells served as control. Following 24-h cell culturing, each well of the plate was inoculated with 10 µl of MTT reagent. The plate was again incubated for 4 h at 37^{o}C. The resultant formazan crystals were dissolved in DMSO. The absorbance of the formazan solution in each well was read in a spectrophotometer at 570 nm and the values were used for determination of percentage cell proliferation.

Determination of cell apoptosis

To investigate the effects of 7-hydroxycoumarin on apoptosis in SKVCR cancer cells, the cells were co-cultured with 6, 12 or 18 µM 7-hydroxycoumarin for 24 h in 12-well plates, after which they were washed with cold PBS buffer and fixed using ethyl alcohol. Then, the cells were stained using DAPI or AO/EB dual staining mix. The stained cells were examined under a fluorescent microscope for changes in nuclear morphology so as to assess the levels of apoptosis.

Flow cytometry

The effect of 7- hydroxycoumarin on the cell cycle of cisplatin-resistant ovarian cancer cells was determined flow cytometrically by culturing the cells with 6, 12 or 18 µM 7-hydroxycoumarin in 6-well plates for 24 h at 37 ^{o}C. Following harvesting and rinsing in PBS, the cells were fixed in methanol and stained with propidium iodide (PI) solution. The stained cells were then subjected to cell cycle analysis using a flow cytometer.

Western blotting assay

Cisplatin-resistant ovarian cancer cells treated with 7-hydroxycoumarin for 24 h were digested in RIPA lysis buffer containing protease inhibitors. The cell lysates were centrifuged, and the total proteins content of the supernatants were determined using the Lowry method. From all samples, equal amounts of proteins were loaded and subjected to SDS-polyacrylamide gel electrophoresis, followed by blotting to polyvinylidene membranes. After transferring the gel contents to the membranes, the membranes were serially incubated with primary and secondary antibodies. Finally, high performance chemiluminescence reagent was used for obtaining the protein bands. Actin was used as the control in the western blotting studies.

Statistical analysis

Data are presented as mean ± SD. Student's *t*-test and ANOVA were used for determination of the level of statistical difference, between and

amongst data points, respectively. Values of $p < 0.05$ were taken as indicative of statistically significant difference.

RESULTS

Effect of 7-hydroxycoumarin on proliferation of cisplatin-resistant ovarian cancer cells

Figure 1 shows the molecular structure of 7-hydroxycoumarin. The SKVCR cisplatin-resistant ovarian cancer cells and normal ovarian cells were treated with different concentrations of 7-hydroxycoumarin, and MTT assay was performed to assess the effects of 7-hydroxycoumarin treatment on the proliferation of the cancerous and normal ovarian cells. The proliferation decreased in a concentration-dependent manner, with more prominent proliferation inhibition in the cancer cells than in normal cells (Figure 2). The IC_{50} values of 7-hydroxycoumarin were 12 and 95 µM, for ovarian cancer cells and normal cells, respectively. Thus, the results showed selective inhibitory effect of 7-hydroxycoumarin on the proliferation of cisplatin-resistant ovarian cancer cells.

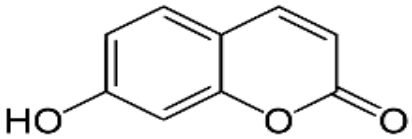

Figure 1: Molecular structure of 7-hyroxycoumarin

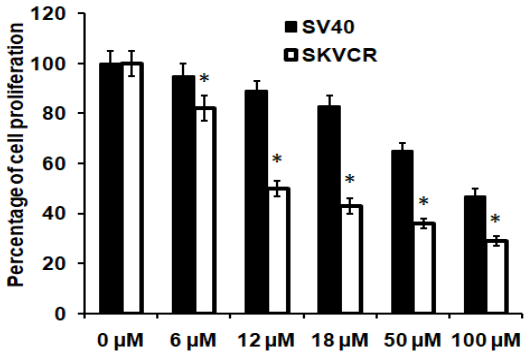

Figure 2: Effect of 7-hyroxycoumarin on the proliferation of cisplatin-resistant ovarian cancer cells. MTT assay was used for determination of proliferation of normal ovarian cells (SV40) and cisplatin-resistant ovarian cancer cells (SKVCR) treated with 6, 12, 18, 50 or 100 µM 7-hydroxycoumarin

Induction of apoptosis of cancer cells by 7-hydroxycoumarin

Results from DAPI staining of SKVCR cancer cells treated with different concentrations of 7-hydroxycoumarin showed that 7-hydroxycoumarin induced apoptosis of the cancer cells (Figure 3). Similar results were obtained with AO/EB staining method (Figure 4). Apoptosis of the cancer cells was evident as deformation of nuclear morphology, with the effects being comparatively greater at high concentrations of 7-hydroxycoumarin.

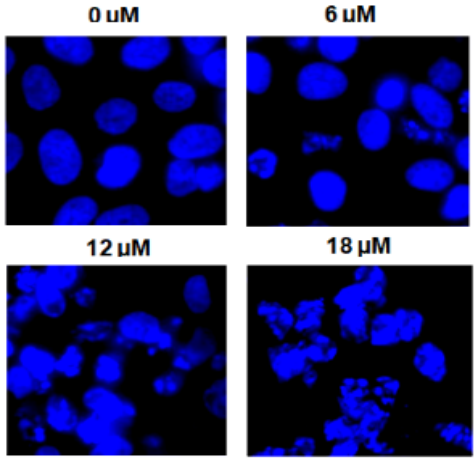

Figure 3: Effect of 7-hyroxycoumarin on apoptosis of cisplatin-resistant ovarian cancer cells. DAPI staining was used for nuclear morphology analysis of SKVCR treated with 6, 12 or 18 µM 7-hydroxycoumarin

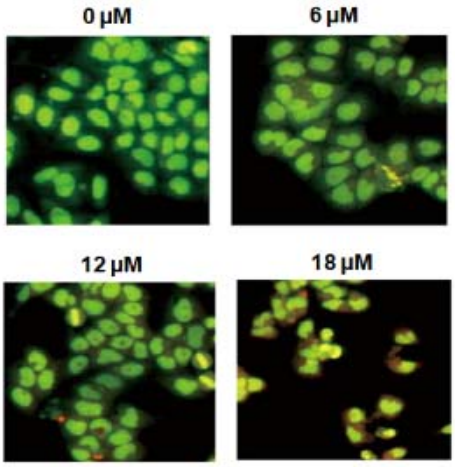

Figure 4: Effect of 7-hydroxycoumarin on apoptosis of cisplatin-resistant ovarian cancer cells. AO/EB dual staining was used for nuclear morphology analysis of SKVCR treated with 6, 12 or 18 µM 7-hydroxycoumarin

7-Hydroxycoumarin induced apoptosis of cancer cells via activation of caspase proteins

To investigate the mechanism underlying 7-hydroxycoumarin-induced apoptosis of cisplatin-resistant ovarian cancer cells, western blotting

assay was used. The results showed that 7-hydroxycoumarin-treated cancer cells had higher protein expression levels of activated caspase i.e., cleaved products of caspase 3, caspase 7 and caspase 8 (Figure 5). The levels of proactive forms of caspase-3, caspase-7 and caspase-8 decreased with increase in concentration of 7-hydroxycoumarin. These results indicate that 7-hydroxycoumarin treatment led to activation of the caspase signaling pathway, thereby resulting in induction of apoptosis of cancer cells.

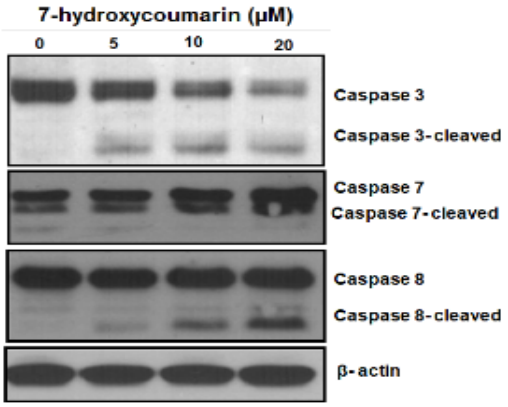

Figure 5: Effect of 7-hyroxycoumarin on the expressions of caspase apoptotic signal proteins in cisplatin-resistant ovarian cancer cells. Western blotting was used for assay of the protein expressions of caspase-3, caspase-7 and caspase-8 in SKVCR treated with 6, 12 or 18 μM 7-hydroxycoumarin

Effect of 7-hydroxycoumarin on cell cycle of cancer cells

A study of the cell cycle of SKVCR cisplatin-resistant ovarian cancer cells treated with 6, 12 or 18 μM 7-hydroxycoumarin revealed that the population of cells at G2 phase of the cell cycle increased with increases in 7-hydroxycoumarin concentration (Figure 6). The percentages of G2/M phase cells were 13.7, 39 and 51 at 6, 12 and 18 μM 7-hydroxycoumarin, respectively, relative to 12.5 % for untreated cells. These results show that 7-hydroxycoumarin treatment induced G2/M cell cycle arrest in cisplatin-resistant ovarian cancer cells.

7-Hydroxycoumarin induced G2/M cell cycle arrest in cancer cells

To unravel the mechanism involved in induction of mitotic cell cycle arrest in 7-hydroxycoumarin-treated cancer cells, cells incubated with 6, 12 or 18 μM 7-hydroxycoumarin were analyzed for the expression levels of different regulatory proteins of the cell cycle involved in controlling entry of cells from the G2 to M phase. The results revealed that the protein expressions of cyclin-dependent kinases (Cdk1 and Cdk4) and cyclins (cyclin D1 and cyclin E) were decreased by 7-hydroxycoumarin (Figure 7). However, the concentration of cyclin B1 protein was unchanged. Thus, 7-hydroxycoumarin downregulated the protein expressions of the cell cycle regulators which positively regulate mitotic entry, thereby inducing arrest of the cancer cells at G2/M phase of the cell cycle. This is also evidence of the anticancer potential of 7-hydroxycoumarin against cisplatin-resistant human ovarian cancer.

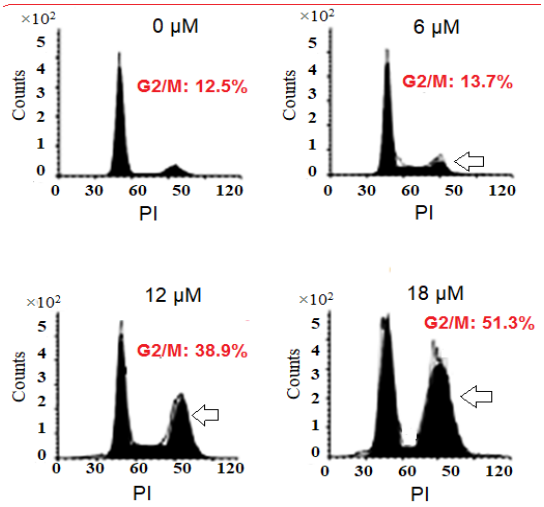

Figure 6: Effect of **7**-hyroxycoumarin on the cell cycle in cisplatin-resistant ovarian cancer cells. Flow cytometry was used for analysis of cell cycle phase distribution of SKVCR cells treated with 6, 12 or 18 μM 7-hydroxycoumarin.

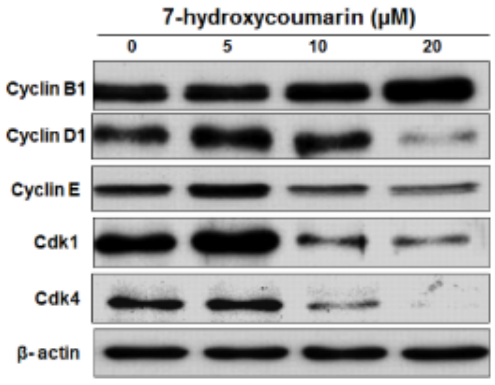

Figure 7: 7-hydroxycoumarin arrested cell cycle at G2/M phase via down-regulation of cell cycle regulatory proteins. Western blotting was used to assay the protein expressions of cyclins B1, D1 and E, as well as cdks (Cdk1 and Cdk4) in cisplatin-resistant ovarian cancer cells (SKVCR) treated with 6, 12 or 18 μM 7-hydroxycoumarin

DISCUSSION

Treatment strategies employed against human ovarian cancer have not yet achieved satisfactory outcomes. Mortality from ovarian cancer still remains high, and the degree of survival is not encouraging, despite advancements in anticancer approaches and curative strategies. The development of chemo-resistance is a major hurdle in the management of ovarian cancer [10]. Cisplatin is one of the most-commonly used chemo-therapeutic agents [11]. Studies have shown that human ovarian cancer cells resistant to cisplatin may emerge when the drug is used for chemotherapy [12]. Thus, there is considerable interest in the search for chemical entities which are effective against cisplatin-resistant type of ovarian cancer cells. Interestingly, 7-oydroxycoumarin has been shown to possess significant anticancer potential against different human cancers [13,14].

In the present study, when the cisplatin-resistant ovarian cancer cells were cultured with different concentrations of 7-hydroxycoumarin, their percentage proliferation was seen to significantly decrease. The decrease in proliferation was not seen in the normal ovarian cells, indicating that the inhibitory effect of 7-hydroxycoumarin on cell proliferation of cancer cells was selective. Similar findings have been obtained in previous research studies [15]. The 7-hydroxycoumarin-treated cancer cells showed deformed nuclear morphology, indicating induction of apoptosis. The pro-apoptotic effect of 7-hydroxycoumarin has already been demonstrated in a previous investigation [16].

The induction of apoptosis in cisplatin-resistant ovarian cancer cells was due to activation of the caspase signaling pathway. Caspases are vital mediators of apoptosis in eukaryotic cells [17,18]. Moreover, 7-hydroxycoumarin was effective in causing arrest of cell cycle of cisplatin-resistant ovarian cancer cells by suppressing their mitotic entry. The arrest of cell cycle of ovarian cancer cells was modulated through down-regulation of regulators of cell cycle which positively regulate the entry of cells from G2 to mitotic phase of the cell cycle. Treatment with 7-hydroxycoumarin reduced the concentrations of cyclin-dependent kinases (CDKS) and cyclin proteins. The CDKS and cyclins operate at the G2/M check-point of the cell cycle in eukaryotes, and their coordinated interplay mediates the progression of eukaryotic cells from G2 to M phase of the cell cycle [19,20].

The results of the current study reveal that 7-hydroxycoumarin restricted the growth and proliferation of cisplatin-resistant ovarian cancer cells via induction of the caspase apoptotic pathway and cell cycle arrest through down-regulation of cell cycle regulators.

CONCLUSION

The results of the present study indicate that 7-hydroxycoumarin exhibits anti-proliferative and pro-apoptotic effects against human cisplatin-resistant ovarian cancer cells, and also induces cell cycle arrest in the cancer cells at the G2/M phase. Thus, the compound has promise as a vital lead molecule in the design of therapeutic agents against cisplatin-resistant human ovarian cancer.

DECLARATIONS

Acknowledgement

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Conflict of interest

None.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The manuscript was drafted and all experiments were performed by Hongmei Wang supervised by Yina Wang.

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