Modulatory role of resveratrol on cytotoxic activity of cisplatin, sensitization and modification of cisplatin resistance in colorectal cancer cells

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Abstract. Colorectal cancer (CRC) is a leading cause of cancer-associated mortality worldwide. Cisplatin (CIS) is one of the most active cytotoxic agents in current use and it has proven efficacy against various human malignancies. However, its clinical usefulness has been restricted by detrimental side effects, including nephrotoxicity and myelosuppression. The aim of the present study was to attempt to decrease the required dose of CIS, in order to minimize its side effects, and increase its capability to arrest, delay or reverse carcinogenesis. In addition, the present study aimed to ameliorate CIS-resistance in CRC cells, using the natural compound resveratrol (RSVL). RSVL (3,4', 5-trihydroxy-trans-stilbene) is a naturally occurring polyphenol present in the roots of white hellebore (*Veratrum grandiflorum* O. Loes) and extracted from >70 other plant species. RSVL can exert antioxidant and anti-inflammatory activities, and it has been shown to be active in the regulation of numerous cellular events associated with carcinogenesis. The present study evaluated the effects of RSVL on sensitization of both parent and CIS-resistant HCT-116 CRC cells to the action of cisplatin. The CIS was administered at a dose of 5 and 20 µg/ml, and CIS cytotoxicity, apoptosis, cell cycle and cisplatin cellular uptake were examined in the presence and absence of RSVL (15 µg/ml). RSVL treatment showed anti-proliferative effects and enhanced the cytotoxic effects of CIS against the growth of both parent and CIS-resistant HCT-116 CRC cells, with a half maximal inhibitory concentration of 4.20 µg/ml and 4.72 µg/ml respectively. RSVL also induced a significant increase in the early apoptosis fraction and enhanced the subsequent apoptotic effects of CIS. The cellular uptake of CIS was significantly increased in the presence of RSVL, as compared with CIS treatment alone, and RSVL treatment sensitized the CIS-resistant HCT-116 cells. In conclusion, RSVL treatment increased the cytotoxic activity of CIS against the growth of both parent and CIS-resistant HCT-116 CRC cells.

Introduction

The Kingdom of Saudi Arabia is considered to have a low incidence of colorectal cancer (CRC); however, the incidence of CRC and related mortalities has been steadily increasing over the past 20 years. It currently ranks first among all cancers in males and third among females in Saudi Arabia (1,2). Chemotherapy is often used as the main treatment option in the overall therapy of most cancers. Cisplatin (CIS) is one of the most active cytotoxic agents in current use that has a proven efficacy against numerous human malignancies, either alone or in combination with other cytocidal drugs. However, its clinical usefulness has been restricted by detrimental side effects, including nephrotoxicity (3,4). The development of tumor resistance to chemotherapy presents a major complication in cancer therapy. Chemosensitization is one strategy that may be used to overcome chemoresistance. It is based on the use of one drug to enhance the activity of another, by modulating one or more mechanisms of resistance. In an attempt to minimize the side effects of CIS, and at the same time increase its anticancer efficacy, various approaches have been studied (5-8). One approach is the search for natural compounds with chemopreventative or anticancer properties, that may be used in combination with CIS. A potential natural chemosensitizer is resveratrol (RSVL). RSVL (3,4', 5-trihydroxy-trans-stilbene) was first isolated in 1940 as a component of the roots of white hellebore (*Veratrum*

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grandiflorum O. Loes), since then it has been identified in extracts from >70 different species of plant (3,9). The aim of the present study was to explore whether RSVL may enhance the cytotoxic effects of CIS against the growth of CRC cells. The possible mechanisms of drug interactions between CIS and RSVL were investigated, regarding CIS cytotoxicity, apoptosis induction, cellular uptake and cell cycle distribution of CRC cells in the presence and absence of RSVL.

Materials and methods

Drugs and chemicals. The CIS "Ebewe" and RSVL were purchased from Sigma-Aldrich (St Louis, MO, USA). The CIS was stored at room temperature (not exceeding 25°C), protected from light and diluted in phosphate-buffered saline (PBS). RSVL was supplied as a white powder, with a yellow cast and stored at -20°C. Media and other tissue culture reagents used in the present study were all purchased Sigma-Aldrich. The cell cycle determination kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Cells and cell cultures. The HCT-116 human CRC cells were acquired from Professor Abdel-Moneim M. Osman, from the National Cancer Institute, Cairo University (Egypt). The cells were grown as monolayer cultures and maintained in RPMI-1640 medium, supplemented with penicillin G and streptomycin antibiotics and 10% fetal bovine serum (FBS), and were cultured at 37°C in a humidified incubator containing 5% CO_2. The cells were collected by trypsinization and passaged every 4-6 days. The passage level was in the range of 5-10, following receipt of the cell line from the source.

Establishment of CIS-resistant HCT-116 colorectal cancer cells. An isogenic model of cisplatin resistance was generated in a panel of parent HCT-116 cells. The resistant cell line was established using dose-dependent cytotoxicity curves where the cells were exposed to stepwise increasing concentrations of CIS. The parental HCT-116 cell line was exposed to a concentration of CIS between 0.05 and 0.50 μg/ml, over a period of five months. The exposure to each concentration was continuous for 72 h, the drug-containing media was then removed and replaced with fresh media containing the same concentration of CIS for a further 72 h. Typically, the concentration was increased by 0.05 μg/ml at each step, with four exposures to each concentration, allowing for growth recovery between the cycles. The cells were then exposed to the next concentration, until the final concentration was reached by the same manner. Following the exposure to the final concentration, the half maximal inhibitory concentrations (IC_{50}) were re-assessed in the CIS-resistant cell line. Once the resistance had been developed, the cell line was maintained by a continuous exposure to 0.5 μg/ml CIS. The cells were grown as a monolayer culture in RPMI-1640, supplemented with antibiotics [penicillin (100 IU/ml) and streptomycin (100 μg/ml)] and 10% FBS. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere, collected by trypsinization and passaged every 4-6 days. The doubling time of the CIS-resistant cells was 37 h, as compared with 28 h in the parent cell line.

Assessment of cytotoxic activity. Cytotoxicity was determined using the sulforhodamine B (SRB) method, as previously described by Skehan *et al* (10). The cells were seeded in 96 well microtiter plates, at a concentration of 40 x 10^3 cells/well in RPMI-1640 medium. Following a 24 h incubation the cells were incubated for an additional 48 h with various concentrations of CIS (1.25, 2.5-20 μg/ml) and RSVL (15 μg/ml) supplied simultaneously (two wells were used for each concentration). The incubation was followed by the addition of 50 μl of 50% cold trichloroacetic acid for 1 h at 4°C, and the supernatant was discarded. The plates were washed five times with water and air dried, and then stained for 30 min with 100 μl 0.4% (w/v) SRB solution in 1% acetic acid. The unbound dye was removed by five washes with 1% acetic acid and the plates were air dried. The bound stain was solubilized using 100 μl/well 10 mM Tris base (pH 10.5) for 10 min. The optical density (OD) was measured using an ELx808 absorbance microplate reader (BioTek Instruments Inc., Winooski, VT, USA) at a wavelength of 490-530 nm. The surviving fraction = OD of treated cells/OD of control cells. The IC_{50} of CIS (necessary to produce 50% inhibition of cell growth) was calculated from the following linear equation of the survival fraction curve:

$$Y = mX + b$$

Y = 0.5 (the surviving fraction when there is a 50% inhibition of cell growth); m = the slope; X = dose of CIS that induces 50% inhibition; b = the y-intercept.

Flow-cytometric assay of apoptosis. The percentage of apoptotic cells was quantified by Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI) double staining, using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, Saint Louis, MO, USA), according to the method of Kuypers *et al* (11). The cells were seeded in 6 well plates, at a cell density of 5-8 x 10^5 cells/well, in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine, the plate was then incubated for 24 h. The cells were incubated with CIS (5 and 20 μg/ml), either alone or in combination with 15 μg/ml RSVL for 48 h. Following the incubation, the medium was removed and the cells were washed with PBS. The cells were detached using trypsin/EDTA, washed once with cold PBS and 10^5-10^6 cells were resuspended in 100 μl Annexin V incubation reagent. The solution was incubated in the dark for 15 min at room temperature. A total of 400 μl 1X binding buffer was added to each 100 μl sample and measured using a Navios flow cytometer (Beckman Coulter, Brea, CA, USA) within one hour, in order to obtain the maximal signal.

Cell cycle analysis. The cells were seeded in 6 well plates at a density of 10^5-10^6 cells/well in RPMI-1640 supplemented medium. Following a 24 h incubation, the cells were maintained in CO_2 incubator at 37°C for an additional 48 h with CIS (5 and 20 μg/ml) and RSVL (15 μg/ml). The medium was then removed and the cells were washed with PBS and harvested with trypsin/EDTA, followed by a further two washes with PBS. The cell cycle analysis was performed according to the previously described method of Pozarowski and Darzynkiewicz (12), using a flow cytometer (FACSCA; BD Biosciences, Franklin Lakes, NJ, USA).

Assessment of CIS cellular uptake. The cells were seeded in 6 well plates, at a density of 10^5-10^6 cells/well in RPMI-1640

Table I. Effects of cisplatin and/or resveratrol on the growth of parent and cisplatin-resistant HCT-116 colorectal cancer cells.

	IC_{50} (μg/ml)	
Treatment	Parent HCT-116 cells	Resistant HCT-116 cells
CIS	7.9±0.66	20.7±3.8[b]
CIS and RSVL (15 μg/ml)	4.2±0.14[a]	6.15±0.63[a]

The data are expressed as the means ± standard error of the mean of two experiments, each in duplicate. IC_{50}, the concentration of CIS necessary to produce 50% inhibition in the growth of cells. [a]P<0.01, as compared with the corresponding CIS treatment alone; [b]P<0.05, as compared with the parent cells. CIS, cisplatin; RSVL, resveratrol.

Table II. Cisplatin uptake by parent and resistant HCT-116 colorectal cancer cells in the presence of resveratrol.

	CIS concentration (ng/10^6 cells)			
	Parent HCT-116 cells		Resistant HCT-116 cells	
Concentration (μg/ml)	CIS	CIS+RSVL (15 μg/ml)	CIS	CIS+RSV (15 μg/ml)
5	4.36±0.78	16.2±1.93[a]	1.57±0.08	4.33±0.6
20	72.7±7.5	110.8±13.9[b]	14.3±10.11	73.6±6.1[b]

The growing HCT-116 colorectal cancer cells were treated with cisplatin (CIS) and/or resveratrol (RSVL) (15 μg/ml) in combination for 48 h. The cells were then washed once with phosphate-buffered saline and harvested, counted and digested using 1% nitric acid. Cellular uptake of CIS was determined by inductively coupled plasma mass spectrophotometry. The data are expressed as the means ± standard error of the mean, of each experiment (n=3). [a]P<0.05, [b]P<0.01, as compared with the corresponding CIS treatment alone.

supplemented medium. The cells were cultured in a CO_2 incubator at 37°C for ≥24 h prior to treatment, then the cells were incubated with CIS (5 and 20 μg/ml) either alone or with RSVL (15 μg/ml), for 48 h. Following the treatment, the medium was removed and the cells were washed with PBS, then harvested with trypsin/EDTA. The cells were washed with PBS again and counted, prior to being resuspended (1 x 10^6 cells/well) in 1% HNO_3 for 24 h at 70°C. The lysed cells were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS provides a quantitative analysis of the concentration of an element in aqueous solution, and has a sensitivity of 5 ppt.

Statistical analyses. Statistical analyses were performed using SPSS version 16 (SPSS Inc., Chicago, IL, USA). A one way analysis of variance, followed by a least significant difference *post hoc* analysis, was used for multiple comparisons. A P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of RSVL treatment on the cytotoxic activity of CIS. The cytotoxicity of CIS in the parent and CIS-resistant HCT-116 cells was expressed as the surviving fraction following treatment, as compared with the untreated control (Table I and Fig. 1). The parent CRC cells treated with various concentrations of CIS, had an IC_{50} value of 7.9 μg/ml, as compared with the CIS-resistant cells which had an IC_{50} value of 20.7 μg/ml.
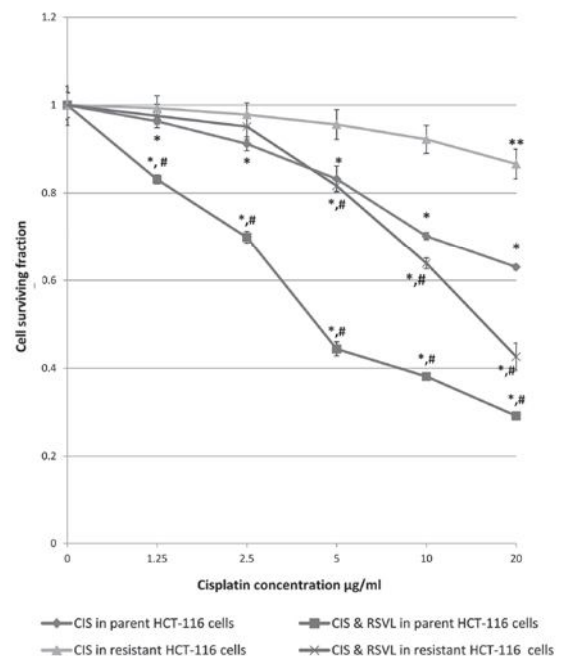

Figure 1. Effects of cisplatin (CIS) and/or resveratrol (RSVL) treatment on the growth of HCT-116 human colorectal cancer cells.

Simultaneous treatment with RSVL (15 μg/ml) resulted in IC_{50} values of 4.2 and 6.15 μg/ml in the parent and CIS-resistant cells, respectively.
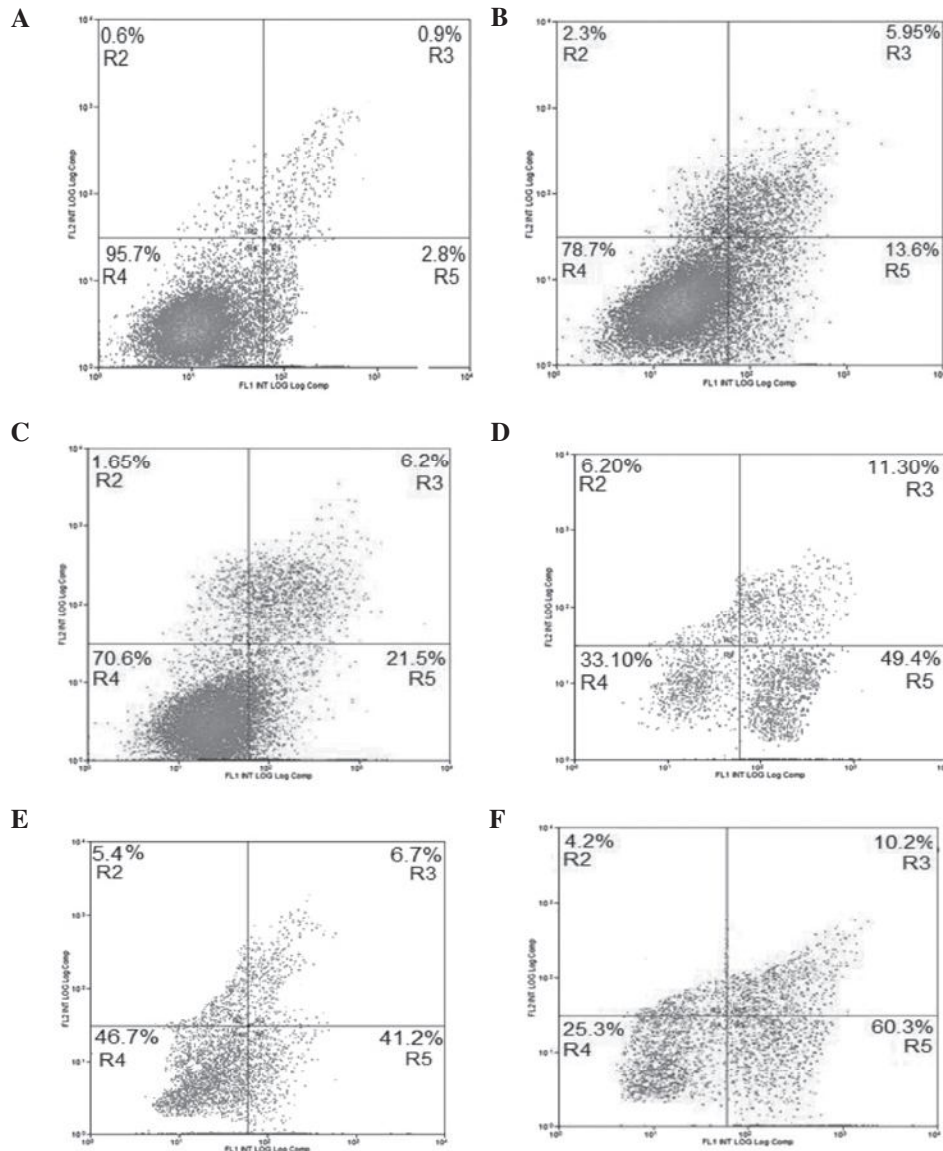
Figure 2. Effects of cisplatin (CIS) and /or resveratrol (RSVL) on the induction of apoptosis in CIS-sensitive HCT-116 colorectal cancer cell. Apoptosis was analyzed following cell exposure to the drugs for 48 h by staining with Annexin-fluorescein isothiocyanate (FITC) and propodium iodide (PI). (A) Control. (B Cells treated with 15 μg/ml RSVL. (C) Cells treated with 5 μg/ml CIS. (D) Cells treated with 5 μg/ml CIS and 15 μg/ml RSVL. (E) Cells treated with 20 μg /ml CIS. (F) Cells treated with 20 μg /ml CIS and 15 μg /ml RSVL. The cell percentage in each quadrant are indicated. R2, necrosis; R3, late apoptosis; R4, viable cell; R5, early apoptosis.
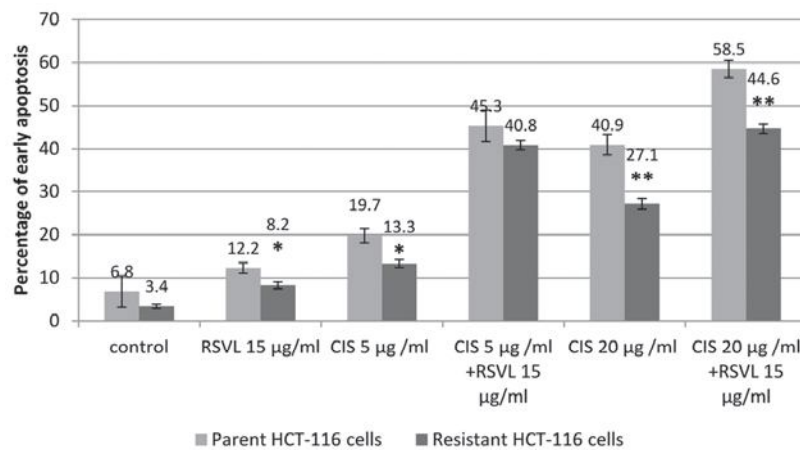

Figure 3. Comparison of the percentage of cells in early apoptosis in parent and CIS-resistant HCT-116 colorectal cancer cells. Apoptosis was analyzed following cell exposure to the drugs for 48 h by staining with annexin-fluorescein isothiocyanate and propidium iodide. The data are expressed as the means ± standard error of the mean of two experiments, each in duplicate. *P<0.05, **P<0.01, as compared with the parent cells.
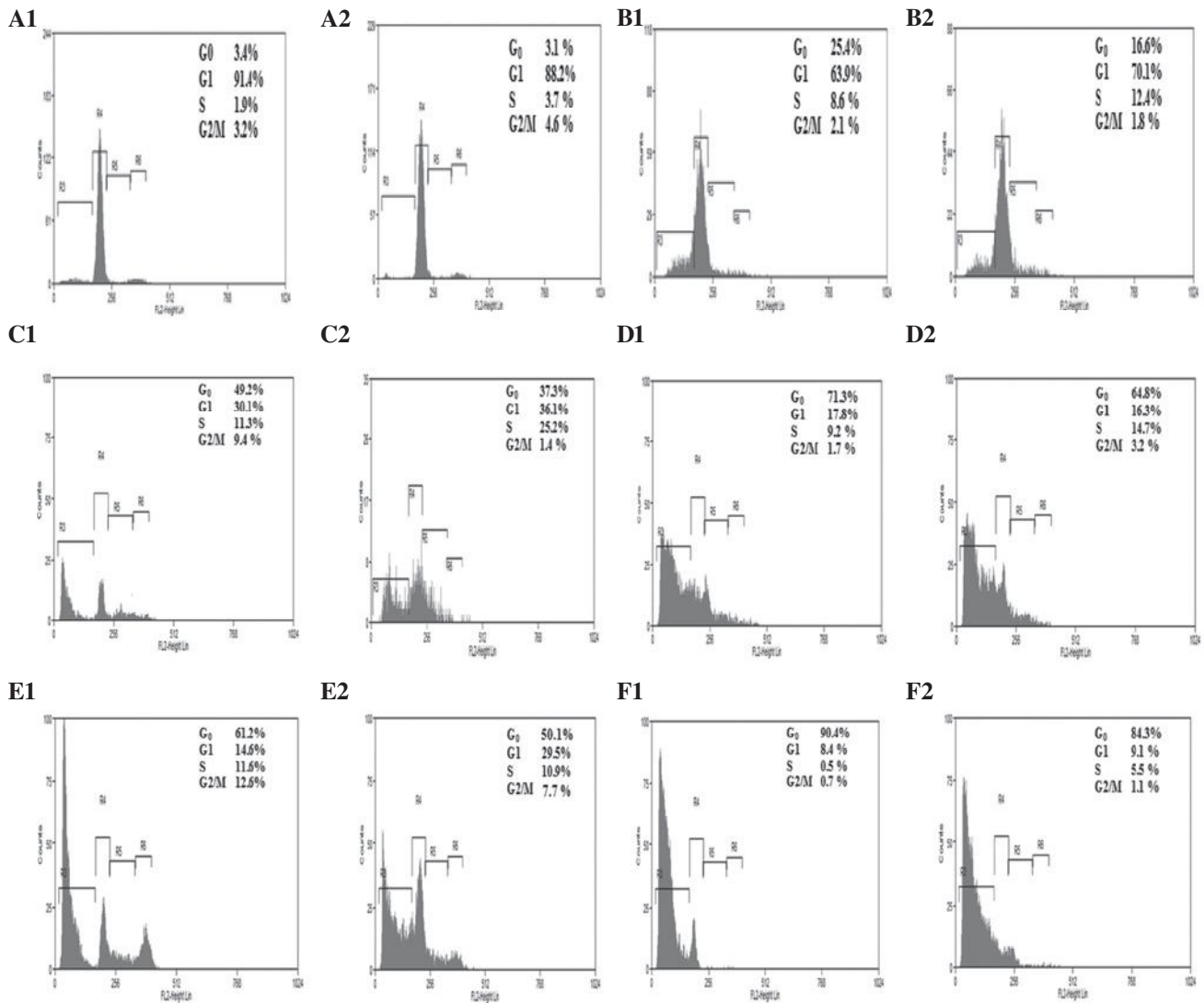
Figure 4. Effects of cisplatin (CIS) and/or resveratrol (RSVL) on cell cycle phase distribution of the parent and CIS-resistant HCT-116 colorectal cancer cells. The cell cycle distribution was analyzed following cell exposure to CIS and RSVL for 48 h by staining with propidium iodide. (A) Control. (B) Cells treated with 15 μg/ml RSVL. (C) Cells treated with 5 μg/ml CIS. (D) Cells treated with 5 μg/ml CIS and 15 μg/ml RSVL. (E) Cells treated with 20 μg/ml CIS. (F) Cells treated with 20 μg/ml CIS and 15 μg/ml RSVL. A1-F1, parent HCT-116 cells; A2-F2, resistant HCT-116 cells.
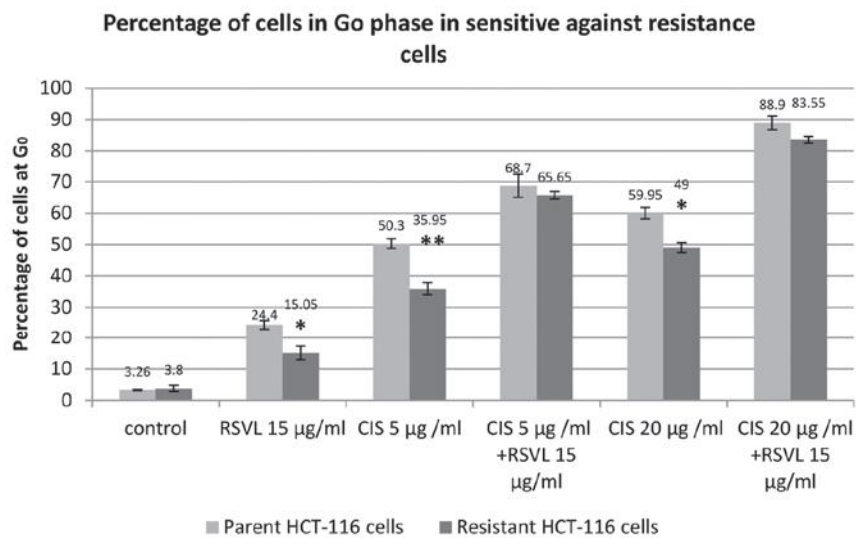

Figure 5. Effects of cisplatin (CIS) and/or resveratrol (RSVL) on cell cycle phase distribution between parent and resistant HCT-116 colorectal cancer cells. Cell cycle distribution was analyzed following cell exposure to the drugs for 48 h, by staining with propidium iodide. The data are expressed as the means ± standard error of the mean of two experiments, each in duplicate. *$P<0.05$, **$P<0.01$, as compared with the parent cells.

Effects of RSVL and CIS treatment on the induction of apoptosis. The HCT-116 cells were stained with Annexin V-FITC and PI and the rate of apoptosis was analyzed using flow cytometry. The percentage of the parent HCT-116 cells in early apoptosis, when treated with CIS at 5 and 20 μg/ml, was 19.7 and 40.9%, respectively. The addition of RSVL (15 μg/ml), in combination with the same doses of CIS, resulted in a significant increase in the percentage of cells in early apoptosis (45.3 and 58.8%, respectively; Fig. 2 and 3).

Effects of RSVL and CIS treatment on the cell cycle phase progression of HCT-116 cells. The treatment of the cells with RSVL (15 μg/ml) resulted in a preferential accumulation of the cells in G_0 and S phases, at the expense of G_1 phase cells (Fig. 4). The percentage of accumulation was 24.4 and 8.6% in the G_0 and S phases, respectively.

Simultaneous treatment of CIS (5 and 20 μg/ml) with RSVL (15 μg/ml) resulted in a significant increase in the percentage of cells in the G_0 phase of parent cells, to 68.7 and 88.9% respectively (Fig. 4 and 5).

Effects of RSVL on uptake of CIS in HCT-116 cells. The cellular uptake of 5 μg/ml CIS in parent and CIS-resistant cells was 4.36 and 1.57 ng/10^6 cells, respectively. The uptake of CIS was significantly increased in the presence of 15 μg/ml RSVL, to 16.2 and 4.33 ng/10^6 cells respectively (Table II).

Similarly, 20 μg/ml CIS treatment resulted in a cellular uptake of 72.7 and 14.3 ng/10^6 in the parent and CIS-resistant cells, whereas in the presence of RSVL (15 μg/ml) the uptake of CIS increased to 110.8 and 73.6 ng/10^6 cells, respectively.

Discussion

CIS is one of the most active cytotoxic agents in current use, with a proven efficacy in numerous human malignancies, either alone or in combination with other cytocidal drugs. However, its clinical usefulness has been restricted by detrimental side effects, including nephrotoxicity (3,4). In an attempt to minimize the side effects of cisplatin, and at the same time increase its anticancer efficacy, various approaches have been attempted. One approach is the search for natural compounds with chemopreventive or anticancer properties, that may be used in combination with CIS. A potential natural chemosensitizer is RSVL; therefore, in the present study, the modulatory effects of RSVL on the cytotoxic activity of CIS were determined against the growth of parent and CIS-resistant CRC cells. The treatment of HCT-116 cells with different CIS doses was shown to be cytotoxic to the cells. There was a 2.6 fold increase in the IC_{50} in the CIS-resistant cells, as compared with the parent cells. It is known that CIS-induced DNA damage occurs through an initial transient S phase arrest of the cancer cells, followed by inhibition of the Cdc2-cyclin A or B kinases, which results in a persistent G_2/M phase arrest (13,14). In the present study an induction of early apoptosis was observed in the treated cells, with an accumulation of the cells in G_0 and S phases. The precise mechanism of action of platinum analogs remains unclear. It is currently known that platinum analogs destroy tumor cells in all stages of the cell cycle and bind DNA through the formation of intra- and inter-strand cross-links, thereby leading to inhibition of DNA synthesis and function (15-17). Tumor cell resistance to CIS and its analogs is still not completely understood, and various mechanisms are likely to be involved, which may be multifactorial and lead to severe limitations in the medical efficacy of CIS (18,19). Decreasing intracellular CIS accumulation has been identified as a major cause of acquired resistance, and may be due to either inhibited drug uptake or increased drug efflux (19,20). The present study showed a 5 fold decrease in the cellular uptake of CIS (20 μg/ml) into the CIS-resistant cells, as compared with the parent cells. This may be due to overexpression of P-glycoprotein (Pgp), which is a representative membrane efflux pump encoded for by the multidrug resistance 1 (MDR1) gene, which may reduce the intracellular levels of drug accumulation (21). In the human intestine, Pgp has been shown to be strongly expressed on the apical surface of the superficial columnar epithelial cells of the ileum and colon, and its expression levels were shown to reduce in a gradual manner in the jejunum, duodenum and stomach (22-24).

In the present study, RSVL was tested as a candidate for sensitizing CRC cells to the action of CIS, in both CIS-resistant and parent cells. RSVL treatment increased the cytotoxic activity of CIS against the growth of both parent and CIS-resistant cells, with an IC_{50} 3.2 fold less in the CIS-resistant cells, as compared to 1.8 fold in the parent cells (Fig. 1 and Table I). This result was accompanied by an increase in the percentage of apoptotic cells, an increased accumulation of cells in G_0 phase and an increased cellular uptake of CIS in the CIS-resistant and parent cells. There was a 5.1 fold increase in the uptake of CIS in the CIS-resistant cells in response to RSVL treatment, as compared with the parent cells. These results imply that RSVL not only exposed higher proportions of CRC cells to CIS, by inducing cell cycle arrest in the G_0 and S phases, but also increased the concentration of CIS available inside the parent and CIS-resistant cells, with an increased accumulation in the CIS-resistant cells. The increase in the cellular uptake of CIS in the HCT-116 cells may be due to the inhibition of Pgp (25), which has an important role in the absorption, distribution and elimination of CIS; therefore, determining its efficiency and toxicity (26). Conversely, Wang *et al* (27), demonstrated that RSVL enhanced the activity of Pgp. These conflicting findings may be explained on the basis that multi-drug resistance may be acquired following an initial exposure to anticancer drugs (28). In addition, previous studies have shown that some Pgp antagonists, such as verapamil and cyclosporine A, may induce Pgp expression in colon carcinoma cells (29). However, the time needed for expression and inhibition of Pgp by their antagonists remains controversial. One way to enhance the cytotoxicity of CIS in sensitive and resistant CRC cells may be to target the FOXO3a signaling pathway (30). Further findings from the authors of the present study showed that induction of FOXOa expression in the presence of RSVL in HTC-116 cells (data not shown).

In conclusion, RSVL treatment increased the cytotoxic activity of CIS against the growth of CRC cells, with pronounced effects in CIS-resistant cells, through alteration of the cell cycle and an increased cellular uptake of CIS.

Acknowledgements

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