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Effect of Quercetin on Cell Cycle and Cyclin Expression in Ovarian Carcinoma and Osteosarcoma Cell Lines

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Resistance to chemotherapeutic drugs is a major problem in cancer treatment. The search for new interventions able to overcome this resistance may involve compounds of natural origin, such as flavonoids, ubiquitously present in many foods. In the present study, the cytotoxic effects and cell cycle modulation of the flavonoid quercetin were investigated in ovarian carcinoma (SKOV3) and osteosarcoma (U2OS) human cell lines and in their cisplatin (CDDP)-resistant counterparts (SKOV3/CDDP and U2OSPt cells, respectively). Quercetin (10-50 μM) caused evident changes in the distribution of cell cycle phases in the CDDP-resistant SKOV3/CDDP ovarian cell line. The levels of cyclin D1 and cyclin B1 were determined by means of Western blot in all cell lines incubated with quercetin (50 μM) for 48 hours. The cyclin D1 expression was significantly decreased following the treatment with quercetin in SKOV3 and U2OSPt cells, but not in SKOV3/CDDP and U2OS cells. The reduction of cyclin D1 level could be linked to the G1/S phase alteration found in quercetin-treated cells. Although cyclin B1 is required for G2/M phase, and despite our observation that quercetin influenced the G2/M phase of cell cycle, the flavonoid did not affect cyclin B1 levels in all cell lines, indicating the involvement of other possible mechanisms. These results suggest that quercetin, exceeding the resistance to CDDP, might become an interesting tool to evaluate cytotoxic activity in combination with chemotherapy drugs.

Keywords: Flavonoids, SKOV3 cells, U2OS cells, Ovarian carcinoma, Osteosarcoma, Cisplatin, Cell cycle, Cyclin D1.

Epidemiological studies have shown that a diet rich in fruit and vegetables reduces the risk of several types of cancer [1-3]. Numerous dietary phytochemicals have been observed to inhibit the initiation phase of carcinogenesis, and therefore they could be useful in primary chemoprevention. The cancer preventing properties of fruit and vegetables have been ascribed, at least in part, to their high content of polyphenols. This group of widely distributed plant metabolites, commonly found in human diet, has been extensively studied in the last decade as chemo-preventive agents [1-3]. The majority of polyphenols present in food are flavonoids and phenolic acids [1].

Quercetin (3,3′,4′,5,7-pentahydroxylflavone) is a typical flavonol-type flavonoid ubiquitously present in fruits and vegetables, such as onion, tea, apples and berries. It exhibits anti-oxidative, anti-inflammatory and vasodilating effects, and has been proposed as a potential anti-cancer agent [3]. However, the effect of quercetin in human drug-resistant cancer cells and the mechanism of action are still not well investigated. Previous studies conducted in our laboratory suggest that certain chemopreventive agents can trigger apoptosis in transformed cells *in vitro*, and this effect appears to be associated with the effectiveness in modulating the proliferation process in cisplatin-resistant cell lines [4, 5]. Cisplatin (CDDP) is a widely used chemotherapeutic drug in the treatment of several cancer types. Despite its cytotoxic effect, clinical efficacy is impaired by the development of drug resistance, which occurs through a number of different mechanisms [6]. Circumventing cisplatin resistance remains therefore a critical goal for anticancer therapy and considerable efforts have been undertaken to solve this problem throughout the past three decades.

Uncontrolled cell proliferation is one of the main hallmarks of cancer, and tumor cells have acquired damage to genes that are directly involved in regulating the cell cycle [7]. It is now believed that anticancer agents that target cell cycle may significantly determine the success of anticancer chemotherapy, as well as provide clues to finding a complete cure for many tumors; hence, targeting the different stages of cell cycle could play a pivotal role in the discovery of new anticancer drugs [8]. In the present study, we investigated the cytotoxic effects and cell cycle modulation of quercetin in wild type and CDDP-resistant ovarian cancer and osteosarcoma human cell lines.

Cytotoxicity: Cytotoxic effects of quercetin (0.01-100 μM) were evaluated in human ovarian carcinoma (SKOV3) and human osteosarcoma (U2OS) cells and in their CDDP-resistant variants (SKOV3/CDDP3 and U2OSPt, respectively). The activity was evaluated with the MTT assay [9]. When SKOV3 and U2OS cells were treated with quercetin for 48 hours, the relative cell proliferation decreased at 10 μM, as shown in Figure 1A and 1B. The relative cell survival rate was similar for their CDDP-resistant variants. Quercetin was almost equi-active in wild type and CDDP-resistant cells, indicating lack of cross-resistance with cisplatin. Quercetin IC_{50} values were approximately 100 μM for SKOV3 cells and 50 μM for U2OS cells.

Cell cycle and apoptosis: Propidium iodide-stained SKOV3 and U2OS cells and their CDDP-resistant variants treated with quercetin were analyzed by flow cytometry. Cells were incubated for 48 hours with 10 or 50 μM drug concentrations; results are reported in Figure 2. In the ovarian cancer cell line, quercetin changed the overall profile of the cell cycle (Figure 2), particularly at 50 μM concentration. In SKOV3, as well as SKOV3/CDDP3 cells, quercetin at 50 μM concentration caused a decrease in the G0/G1 phase and a modest increase in the S and G2/M phases. In the U2OS cell line, the G0/G1 phase was augmented by quercetin, compared with the control, and the other phases reduced (Figure 2). In U2OSPt cells the overall cytogram was different from that of the other three cell lines; the G0/G1 phase was lower than the M phase. In this cell line, quercetin only marginally affected the distribution profile.

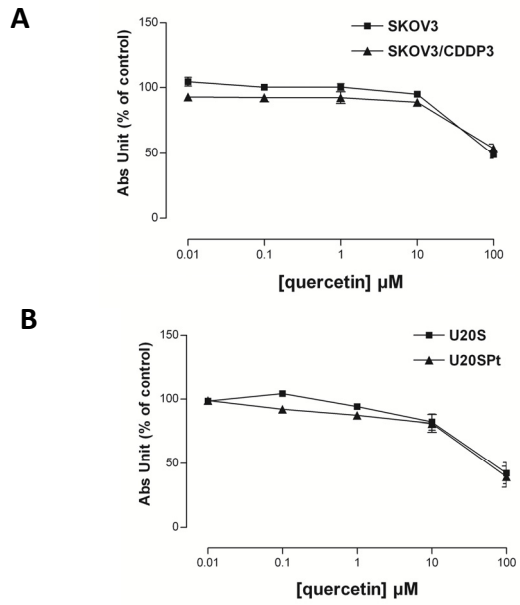

Figure 1: Effect of quercetin (0.01-100 µM) on cell growth after 48 h of drug treatment. A) wild type SKOV3 cell line (SKOV3), and its CDDP-resistant counterpart (SKOV3/CDDP3); B) wild type U2OS cell line and its CDDP-resistant counterpart (U2OSPt). Data are the mean ±SD of at least three determinations.

Effect of quercetin on cyclin D1 and B1: Since flow cytometry revealed a change in the various cell phases following quercetin treatment, the levels of cyclin D1 and cyclin B1 were determined by means of Western blot in all cell lines incubated with quercetin (50 µM) for 48 hours (Figure 3). The cyclin D1 expression (Figure 3A-B) was significantly decreased following the treatment with quercetin (50 µM) in SKOV3 and U2OSPt cells, but not in SKOV3/CDDP and U2OS cells. In all the four cancer cell lines, incubation with quercetin (50 µM) did not significantly affect cyclin B1 levels (Figure 3A-B).

In this work we analyzed the proliferation and the cell cycle profile of human sensitive and CDDP-resistant cancer cells treated with quercetin. Quercetin is a typical flavonoid ubiquitously contained in vegetables and fruits with several biological effects demonstrated *in vitro* and *in vivo*, including anti-oxidative, anti-inflammatory, anticancer, and antidiabetic activities [10].

In the present investigation, cytotoxicity tests showed that quercetin inhibits proliferation of human ovarian cancer and osteosarcoma cell lines, sensitive and resistant to cisplatin. The subsequent step involved testing if quercetin could affect the cell cycle of the cancer cells, since it is known that cell cycle alteration is often associated with cancer development and progression [11]. Progression through the cell cycle is promoted by a number of cyclin-dependent kinases (Cdks), an essential part of the cell cycle machinery which, when complexed with specific regulatory proteins called cyclins, drives the cell forward through the cell cycle (G1 to S or G2 to M; see Figure 3C). In several studies with human cancer cells, including human leukemia cells [12], human esophageal adenocarcinoma cells [13], and breast cancer cells [14], quercetin induces G2/M phase block. In human breast cancer [15] and osteosarcoma cells [16], an alteration of G0/G1 phase has been detected. In all cell lines we observed that, at concentrations close to or lower than IC_{50}, quercetin induced alterations in cell cycle distributions, under our experimental conditions. Depending on the cancer cell type, quercetin was able to block the cell cycle at either G2/M or at the G1/S transition. The observed differences in the cell lines investigated point out the complexity of the steps possibly linked to chemosensitivity. Interestingly, quercetin was able to interfere with the cell cycle of the ovarian CDDP-resistant cell line. Human cancers often develop CDDP resistance, which is a major cause of chemotherapy failure; therefore, the possibility to reverse resistance is of peculiar interest also in a clinical perspective. Cisplatin is employed for the treatment of a wide spectrum of solid malignancies, including testicular, ovarian, head and neck, colorectal, bladder and lung cancer [17]. In the past several years, a number of different drug resistance mechanisms have been identified. As in some clinical settings cisplatin constitutes the major therapeutic option, the development of chemosensitization strategies constitutes a goal with important clinical implications.

Increased knowledge of the molecular mechanisms of cell cycle transition involved in tumor formation suggests that modulators of Cdks and cyclins are potential therapeutic targets in cancer therapy [18], and several agents are already under clinical development [19]. The relevance of cyclin D1 in cancer suggests it may be an appealing target for anti-cancer therapy, and ablative agents are under investigation [20]. In fact, cyclin D1 has been recently found to be involved in the regulation of apoptosis in some kinds of tumors. To analyze further the molecular mechanisms by which quercetin influences the cell cycle in cells, we evaluated the protein expression levels in cell cycle regulatory cyclins. Cyclin D1 is one of the cyclins required for advance from G_1 to S, and it is consistently deregulated in most human cancers [21]. The reduction of cyclin D1 level found in our experiments could be one of the possible causes for the G1/S change that appeared in quercetin-treated cells. Cyclin B1 is required for the G2/M phase (Figure 3C); despite our observation that quercetin influenced the G2/M phase of the cell cycle, the flavonoid did not affect cyclin B1 levels in all cell lines used (Figure 3B), suggesting the involvement of other possible mechanisms.

Increased cyclin D1 expression is correlated with increased nuclear factor-κB (NF-κB) activity and maintenance of Bcl-2 and Bcl-xL expression following cell exposure to either cisplatin or gemcitabine. Overexpression of cyclin D1 in pancreatic cancer cells has been reported to contribute to chemoresistance to cisplatin therapy [22]. Following these considerations, the data from our study suggest that quercetin may be a promising anti-proliferative substance against cancer cells, particularly for the CDDP-resistant phenotype in which cell cycle arrest is induced. The safety of the flavonoid has been demonstrated both *in vitro* (see for instance data in McCoy mouse fibroblast cells [23]) and *in vivo* studies [24]. These results indicate that this flavonoid, exceeding the resistance to CDDP (as shown by the concentration-response curves in this work, in agreement with our previous results [5]), might become an interesting tool to evaluate any chemosensitizing activity. From a pharmacokinetic point of view, following intravenous administration quercetin presents an elimination half-life of about 2 hours and a wide volume of distribution [25], compatible with a systemic treatment; however, the drug is extensively metabolized by the liver into conjugates, and, therefore, the possible cytostatic activity of the metabolites still remains to be elucidated in order to assess any therapeutic role. Furthermore, our results suggest that the flavonoid molecular structure might represent the basis to design more selective compounds active as cell cycle inhibitors in CDDP-resistant cells.

Experimental

Cells: Human ovarian carcinoma cell lines (SKOV3 wild type and cisplatin-resistant cells: SKOV3 and SKOV3/CDDP3 kindly provided by Prof. Toffoli, CRO Aviano, Italy) were grown in

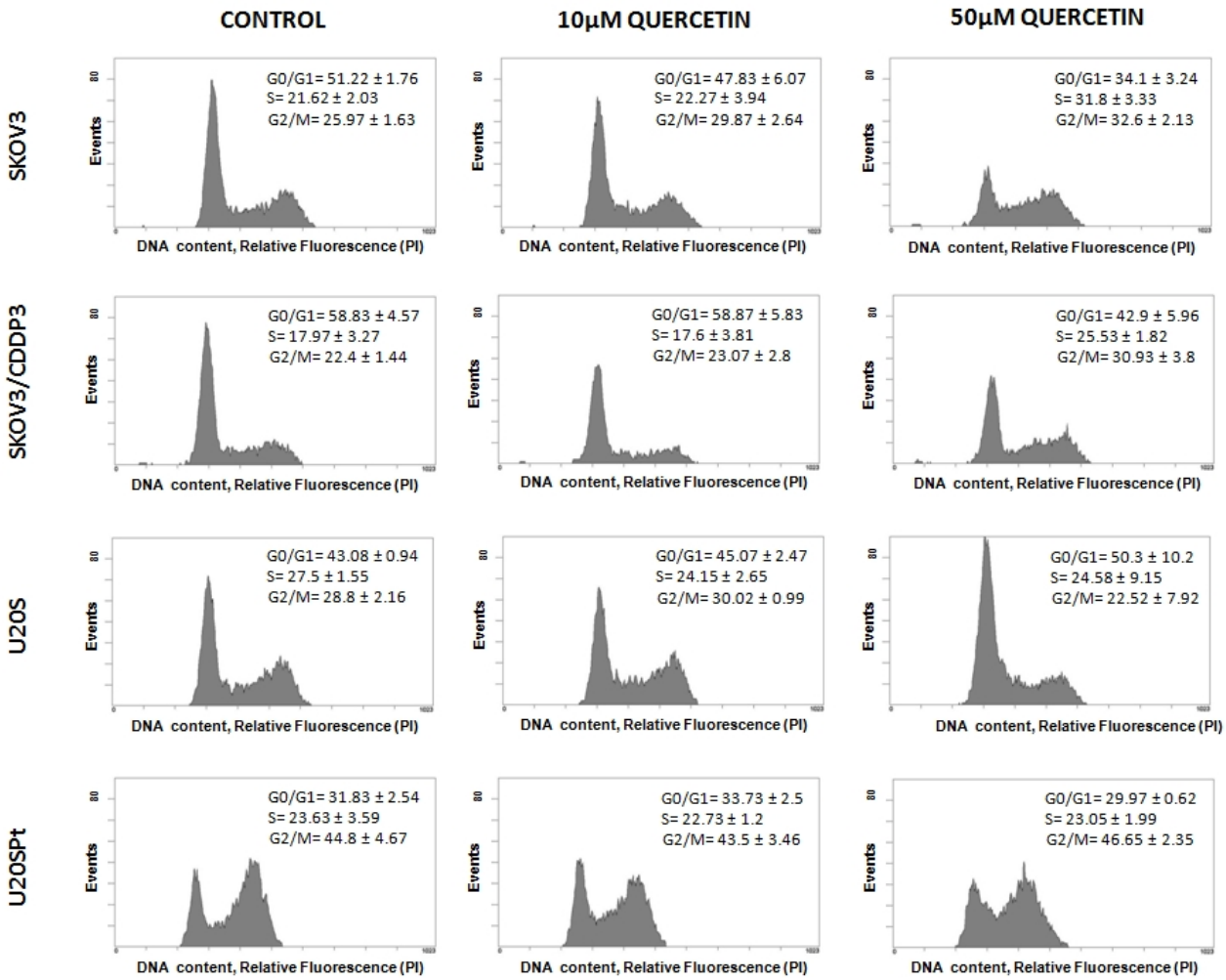

Figure 2: Cell-cycle distribution of the four cell lines treated for 48 h with quercetin at two concentrations (10 and 50 µM) and analyzed by flow cytometry. Percentage of cells in G0/G1, S and G2-M phase was calculated using *Multicycle* software. The cytograms are representative of three-five independent experiments. PI: propidium iodide.

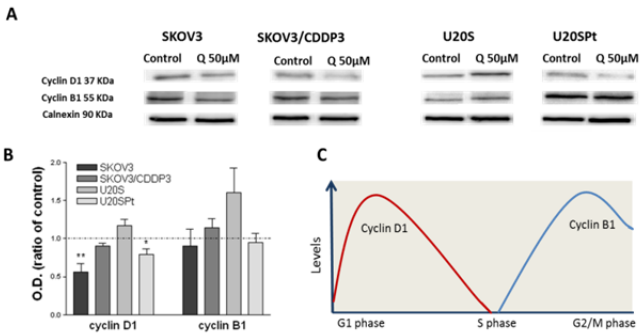

Figure 3: Levels of cyclin D1 and cyclin B1 (panel A) analyzed by Western blot in all cells upon incubation with quercetin (50 µM) for 48 hours. The graph (panel B) shows the relative levels of cyclin D1 and B1 in the four cell lines (mean ±SD of 3-4 determinations; ** $p<0.01$; * $p<0.05$ *vs* control). Panel C is a sketch of the cyclin expression through the cell cycle phases.

Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Human osteosarcoma cell lines (U20S wild type and cisplatin-resistant cells: U20S-U20Spt) were grown in McCoy's 5A supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were routinely grown in humidified conditions with 5% CO_2 at 37°C, and collected every 2 days with a minimum amount of 0.05% trypsin-0.02% EDTA. All reagents for cell culture were from Cambrex-Lonza (NY, USA) and FBS from Gibco, Invitrogen (Carlsbad, CA, USA).

MTT assay: Cell viability was determined using the 3-(4,5-dimethyl-thizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [5, 9]. Briefly, cells were plated on 96-multiwell plates. Following overnight incubation, cells were exposed to a range of different concentrations of quercetin according to the experimental protocol. After 48 h, 20 µL/well of a 5 mg/mL MTT solution (Sigma-Aldrich, St Louis, MO, USA) was added and the cells were incubated 4 h at 37°C. Formazan crystals were dissolved by adding 200 µL of acidic isopropanol and the absorbance (Abs) was measured at 570 nm using a Victor3X multilabel plate counter (Wallac Instruments, Turku, Finland).

Cell cycle and apoptosis analysis by flow cytometry: Cells were seeded on 6-well plates and, following overnight incubation, were exposed to different treatments according to experimental protocols. At the end of the treatments, samples were prepared as previously described [26]. Briefly, cells were washed, detached with 0.25% trypsin-0.2% EDTA and fixed with cold ethanol at 70% for 30 min on ice. After washing, cells were resuspended in a PBS solution added to 0.03 mg/mL propidium iodide (Molecular Probes, Invitrogen, UK) and 0.03 mg/mL RNAase-DNAase free (Sigma, St.

Louis, MO, USA) and incubated for about 30 min at room temperature in the dark. About $3x10^4$ cells/sample were analyzed on an Epics XL-flow cytometer (Beckman Coulter, USA), with an Argon Laser λexc = 488 nm and a λem= 575 ± 20 nm. The percentages of cells in the different phases of the cell cycle were calculated with *Multicycle* software provided by the manufacturer, considering diploid cycle and correcting for cell clusters.

Western blotting: Cells were plated in a 100 mm cell culture dish and allowed to attach overnight. After 48 h of treatments, cells were lysed with ice-cold lysis buffer supplemented with protease inhibitor cocktails (Roche Molecular Biochemicals, Mannheim, Germany). The protein content was determined by Lowry procedure (Bio-rad DC Protein Assay, MA, USA) and equal amounts of protein (40 µg) were loaded on a polyacrylamide gel and electrophoretically separated in running buffer. After electrophoresis, the proteins were blotted onto a Hybond-P PVDF membrane (Amersham Biosciences, Bucks, UK). The membrane was exposed to the elected primary antibodies: anti-cyclin D1 (1:250; Santa Cruz Biotechnology Inc., Heidelberg, Germany) and anti-cyclin B1 (1:1000; Cell Signaling, MA, USA). After washing, the membrane was incubated with either HRP-conjugated anti-mouse secondary antibody (1:10000; DakoCytomation, Glostrup, Denmark) or anti-rabbit secondary antibody (1:3500; PerkinElmer, MA, USA). The signal was visualized with an enhanced chemoluminescent kit (Amersham Biosciences) according to the manufacturer's instructions and analyzed by Molecular Imager VersaDoc MP 4000 (Bio-rad). Proteins were normalized to calnexin (1:1000; AbCam, Cambridge, UK).

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