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Research Paper

The timing of caffeic acid treatment with cisplatin determines sensitization or resistance of ovarian carcinoma cell lines

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A B S T R A C T

Cisplatin is a widely used chemotherapeutic drug showing high efficiency in the treatment of primary tumors such as ovarian, testicular and cervical cancers. The major drawback of cisplatin is tumor resistance either acquired or intrinsic. Many mechanisms are involved in the resistance, among them is the Nrf2 pathway which regulates glutathione related enzymes. Caffeic acid, a non-toxic polyphenol which is abundant in many foods modulates glutathione S-transferase (GST) and glutathione reductase (GSR) activity, these enzymes were shown to be involved in resistance of cells towards cisplatin. Caffeic acid induces the Nrf2 pathway and can also inhibit the activity of GST and GSR.

Our findings demonstrate that the co-treatment of cancer cells with cisplatin and caffeic acid can enhance cisplatin cytotoxicity and increases the amount of platinum bound to nuclear DNA. However, 6 h of pre incubation with caffeic acid prior to cisplatin treatment led to acquired resistance to cisplatin and reduced DNA binding.

In conclusion, the enzyme inhibitory action of caffeic acid is dominant when the two agents are co-administered leading to increased cytotoxicity, and the Nrf2 induction is dominant when the cells are treated with caffeic acid prior to cisplatin treatment leading to resistance.

The use of caffeic acid as adjuvant for cisplatin should be carefully examined due to different pharmacokinetic profiles of caffeic acid and cisplatin. Thus, it is questionable if the two agents can reach the tumors at the right time frame *in vivo*.

1. Introduction

Since its discovery in 1965 and entrance to the clinic in 1978, cisplatin became one of the most important and efficient chemotherapeutic drugs [1–3].

Cisplatin [*cis*-diamminedichloroplatinum(II)] is administered in the clinic with other drugs to treat ovarian, testicular, cervical cancer and additional more cancer types [4–6]. Cisplatin binds DNA preferably to adjacent guanines on the same strand, leading to DNA lesions, distortion of the DNA structure and consequently to cell death *via* apoptosis [7]. The efficacy of cisplatin in the clinic is limited by severe side effects in some cases but more prominently by tumor resistance [8]. The side effects of cisplatin include nephrotoxicity, ototoxicity, neurotoxicity and other side effects common to chemotherapy [9–11].

Cisplatin resistance can be either intrinsic (*e.g.* as observed in patients with colorectal, lung and prostate cancer) [12–14] or acquired following cisplatin chemotherapy (as often seen in patients with ovarian cancer) [15]. The mechanisms of cisplatin resistance had been studied in several types of cisplatin resistant cell lines and appear to be multifactorial. It has been shown that cancer cells can develop cisplatin resistance through (1) decreasing cisplatin concentration within the cells by reducing its influx (*via* CRT1 copper transporters) [16] and increasing its efflux (*via* ATP7A transporters) [17], (2) changing the balance of pro-apoptotic and anti-apoptotic factors [18], (3) inducing changes in DNA repair system that results in increased nucleotide excision repair [19], interstrand crosslink repair or loss of mismatch repair [20,21], (4) affecting the DNA damage tolerance mechanisms [22] and finally (5) enhancing the drug detoxification system by elevating the levels of intracellular scavengers such as glutathione (GSH) [23].

While the detoxification of cisplatin by its interaction with glutathione may be due to spontaneous binding [24], it is probably catalyzed in the cells by glutathione S-transferases (GSTs) [25]. Indeed, high GST levels are found to correlate to cisplatin resistance in the clinic [26].

Other GSH related enzymes may play a role in this resistance pathway. GSH is synthesized by γ-glutamate cysteine ligase and GSH

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synthase. GSH reductase (GSR) is recycling the oxidized GSSG back to its reduced form. Those enzymes have been also linked to cisplatin resistance [27,28]. GSH related enzymes are part of the phase II enzymes family, which are under the control of the Nrf2/Keap1 pathway.

Nrf2/Keap1 pathway is one of the key cellular pathways regulating cell defenses against oxidative stress and reactive electrophilic xenobiotics [29]. Nrf2 (NF-E2 p45-related Factor 2) is a bZip transcription factor and a member of the Cap 'n' Collar family which is bound to the suppressor Keap1 homodimer in the cytoplasm. The association between Nrf2 and Keap1 facilitates ubiquitination of the Nrf2 protein and its degradation in the proteasome as part of the Nrf2 basal activity control [30]. Nrf2 is activated upon changes in the redox state in the cell or in response to electrophiles. Thiol groups of the cysteine residues of Keap1 are oxidized to form disulphide bonds in response to oxidative stress or modified by electrophiles, these modifications lead to conformational change of the protein and release of Nrf2 [31,32].

Free Nrf2 undergoes kinase mediated phosphorylations and translocates into the nucleus. In the nucleus Nrf2 binds together with small Maf proteins to the antioxidant response element (ARE) in the regulatory regions of target genes and promotes the induction of the phase II enzymes [33].

Caffeic acid is a polyphenol from the hydrocinnamic acid family which is found in many foods including coffee, fruits, cereals and more [34].

In our previous work we found that caffeic acid acts in a dual way as an inducer of the Nrf2 pathway and as an inhibitor of GST and GSR [35].

We demonstrated that GST and GSR activity in cisplatin sensitive cell line (A2780) and in cisplatin resistant cell line (A2780cisR) is affected in a different way following caffeic acid treatment. While A2780 cells demonstrate bell-shaped activity of GST following caffeic acid treatment, in A2780cisR cells the GST activity is U-shaped.

These results suggest that there is a competition between the induction and the inhibitory effects of caffeic acid.

Therefore, we hypothesize that co-administration of cisplatin with caffeic acid may affect the cells sensitivity to cisplatin. Particularly, the Nrf2 induction by caffeic acid might contribute to the acquired resistance through the induction of protective phase II enzymes. While the inhibition of GST and GSR can partly circumvent the resistance and allow cisplatin reach the nucleus in higher amounts and therfore be more potent.

2. Materials and methods

2.1. Materials

Caffeic acid (CA), trigonelline, potassium iodide, potassium tetrachloroplatinate, silver nitrate,70% nitric acid (redistilled), 3-(4,5-dimethylthiazole-2-yl)−2,5-diphenyl tetrazolium bromide(MTT) and other common reagents were purchased from Sigma Chemical Co., St. Louis, MO. Ammonium hydroxide was purchased from Bio-Lab Jerusalem, Israel. Cell culture medium, L-glutamine, gentamycin and fetal calf serum were purchased from BioInd Bet Dagan, Israel.

2.2. Cell culture

Human ovarian carcinoma A2780 and daughter line A2780cisR were obtained from ATCC, USA. The cells were cultured in RPMI 1640 medium supplemented with10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/mL gentamycin. The cultures were maintained in a humidified 5% CO2 incubator at 37 °C. Cells were subcultured every 3–4 days to maintain logarithmic growth and were allowed to grow for 24 h in the experiment wells before use.

2.3. Cytotoxicity MTT assay

The cytotoxic effects of cisplatin and caffeic acid against the A2780 and A2780cisR tumor cells were assessed *via* MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cells were seeded into 96- wells at a density of 6×10^3 cells per well. Cells were permitted to adhere for 24 h, and then treated with the various concentrations of cisplatin and caffeic acid for 24 and 48 h. The cultured medium was removed and replaced with 150 μL MTT (0.5 mg/mL) per well before termination at 2 h. After removal of the MTT solution, 200 μL DMSO was added to each well. The absorbance was recorded on a Biotek microplate reader (Biotek Instruments, Inc., Winooski, VT) at the wavelength of 540 nm.

All experiments were performed independently in triplicate and data were presented as mean ± S.E.M.

2.4. Caspase 3 activity assay

Treated cells were incubated for 1 h in PBS containing 2.5 μM Ac-DEVD-AMC, a fluorogenic substrate specific of caspase 3 (Calbiochem, Darmstadt, Germany), with 0.02% Triton X-100, 10 mM dithiothreitol, and 20 mM Tris pH 7.4, at 37 °C. Fluorescence was measured at 355 nm/460 nm on a Citation 3 fluorometer (BioTek Instruments, Inc., VT, USA) for 40 min, and the activity was calculated in the linear range of the slope and normalized to cell count in each well.

2.5. Pt-DNA adducts quantification

Cells were seeded in 6-well 24 h prior treatment at a density of 3×10^5 cells per well. Cells were treated for 24 h with cisplatin and caffeic acid then the medium was removed and the cell trypsinizid. Trypsin was removed after centrifugation and the DNA was extracted using DNeasy Blood & Tissue kit (QIAGEN, Germany). DNA concentration in each sample was quantified by absorbance measuring using ND-1000 UV–Vis Spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA).

The samples were diluted with 1% redistilled nitric acid (without metal traces) to the amounts of 5-20 ng per sample.

^{195}Pt content in the DNA samples was measured by ICP-MS (Agilent 7500cx, Santa Carla, CA) and normalized to μg of DNA for comparing.

All experiments were performed independently in triplicate and data were presented as mean ± SD.

2.6. Statistical analysis

Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. Difference of $P < 0.05$ was considered statistically significant compared to the untreated control group, or as defined in the figure legends.

3. Results

3.1. Cell viability after cisplatin and caffeic acid treatment

In order to establish the effect of caffeic acid treatment in combination with cisplatin, we treated both cisplatin sensitive and resistant cells and measured the viability by the MTT assay. Following the treatments for 48 h the IC_{50} of cisplatin was 6.5 and 10 μM in sensitive and resistant cells, respectively (Fig. 1). Caffeic acid alone was not toxic to the cells. At 100 μM it reduced sensitive cells viability only by 20% (Fig. 1a).

In the sensitive cells (A2780) the addition of caffeic acid resulted in the decrease in the IC_{50} values from 6.5 μM to 6, 4 and 2.5 μM when cisplatin was combined with 10, 50 and 100 μM of caffeic acid, respectively (Fig. 1a). A more prominent effect was observed in the