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Modulation of cisplatin sensitivity in human ovarian carcinoma A2780 and SKOV3 cell lines by sulforaphane

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HIGHLIGHTS

- Synergic (A2780) and antagonistic (SKOV3) interactions between SFN and cisPt.
- Potentiation (A2780) and protection (SKOV3) against cisPt-induced DNA damage.
- Greater genomic stability of A2780 cells in comparison with SKOV3.
- Different activation of Nrf-2 pathway by SFN in A2780 compared to SKOV3 cells.

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ABSTRACT

Cisplatin resistance is one of the major obstacles in the treatment of ovarian cancer. In an effort to look for new possibilities of how to overcome this difficulty, we studied the mechanisms of the interactions between sulforaphane (SFN) and cisplatin (cisPt) in combined treatment of human ovarian carcinoma A2780 and SKOV3 cell lines. Synergy (A2780) and antagonism (SKOV3) found in MTT assay was confirmed by apoptosis. While SFN significantly potentiated cisPt-induced DNA damage in A2780 cells, it protected SKOV3 cells against cisPt-crosslinking. We revealed a less efficient Nrf-2 pathway inducibility by SFN in A2780 compared to SKOV3 cells, when activation of the Nrf-2 pathway incites its protectivity against cisPt. Thus, different activation of the Nrf-2 pathway may explain the dual effects of SFN.

1. Introduction

Cisplatin is a common chemotherapeutic agent used in the treatment of gynecologic malignancies, including cervical, ovarian, and endometrial cancers. Its use is restricted by significant variability in tumor response, which can influence the clinical outcome. Cytotoxicity of cisPt is mediated by its interaction with DNA and the formation of DNA adducts (mainly intrastrand crosslinks), thus activating several signal transduction pathways and culminating in the activation of apoptosis (Siddik, 2003). Cellular resistance to platinum can arise by multiple mechanisms related to glutathione (GSH)-based detoxification and metallothionein content, cisPt interactions with the cellular proteins (e.g., copper transport protein Ctr1 and ATP7A/B) prior to reaching the nucleus and the DNA target, ABC transporters overexpression, increased DNA adduct repair, loss of damage recognition, but also because of aberrant properties of the signal transduction pathways involved in cell cycle control and apoptosis (activation of the PI3-K/Akt pathway, loss of p53 functions, the overexpression of the antiapoptotic gene *Bcl-2*, interference in caspase activation) (Wernyj and Morin, 2004).

Combinations of drugs in specific sequence, aimed at bringing tumor cell populations into a state more susceptible to the cytotoxic effects of chemotherapeutic agents, can be of particular interest (Sampath and Plunkett, 2001) in cancer research. The combination of natural compounds with anticancer drugs aimed at increasing antitumour responses is a new strategy for cancer chemotherapy (Duraj et al., 2013; Huq et al., 2014).

Isothiocyanates (ITCs) are known as potential chemopreventive agents (Steinmetz and Potter, 1996), although their cytotoxic potential has also been described (Fimognari et al., 2005). The naturally occurring ITC sulforaphane, present in cruciferous

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vegetable as the major hydrolysis product of glucosinolates, has frequently been said to exert anticarcinogenic properties, mainly due to the induction of the Nrf-2/Keap1/ARE-signaling pathway, the major regulator of cytoprotective responses to oxidative and electrophilic stress (Piberger et al., 2014).

In this study, we assess the type of interactions between SFN and cisPt in the combined treatment of human ovarian carcinoma A2780 and SKOV3 cell lines and the molecular mechanisms underlying the observed modulation of cisPt sensitivity.

2. Material and methods

2.1. Reagents

Cisplatin was acquired from Lachema (Czech Republic). SFN was purchased from Calbiochem (Germany). Fluorescein diacetate (FDA), propidium iodide (PI), monochlorobimane (MCB) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Cells and treatment

Human ovarian carcinoma cells A2780 and cisplatin resistant SKOV3 were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 μg/ml penicillin and 50 μg/ml streptomycin. 0.5×10^6 cells/ml were cultured in 96-, 24- or six-well plates (Greiner, Germany). The cultures were passaged twice a week after reaching a cell density of $0.8–1.0 \times 10^6$ cells/ml. Cells were plated at $3–6 \times 10^4$ cells/cm^2 density on the day before treatment and exposed to various concentrations of SFN or cisPt for the respective time indicated. In combination, SFN was added 1 h before the cisPt treatment, if not indicated otherwise. The stock solution of SFN was originally dissolved in DMSO, and an equal volume of DMSO (final concentration <0.02%) was added to the control cells.

2.3. Cytotoxicity assay

The effect of SFN, cisPt or their combination on the survival of cells was determined by MTT assay (Mosmann, 1983). The cells were seeded at $1–2 \times 10^3$ cell density in 96-well culture plates. Each dose of tested compounds (added in the volume of 50 μl) was tested in triplicate or quadruplicate. After 72 h, the cells were incubated with 50 μl of MTT (1 mg/ml) and left in the dark at 37 °C for an additional 4 h. Thereafter, the medium was removed, the formazan crystals were dissolved in 200 μl of DMSO, and the absorbance was measured at 540 and 690 nm in xMark™ Microplate Spectrophotometer (Bio-Rad Laboratories, Inc.). The concentration of drug that inhibited cell survival to 50% (IC_{50}) and the combination index (CI) were determined by Calcusyn software (version 1.1, Biosoft).

2.4. Flow cytometry

The apoptotic cell enumeration was done according to the amount of cells with cell membrane impermeable for PI and low FDA fluorescence. As for the FDA/PI staining (Bartkowiak et al., 1999), both cell lines were collected as described above. Pooled cells were washed twice with cold PBS. Approximately 5×10^5 cells were resuspended in 400 μl of PBS/0.2% BSA, containing 10 nM of FDA (from a 5 mM stock in DMSO) for 30 min at room temperature. Then the cells were cooled and 4 μl of PI (1 mg/ml) was added. Finally, the cells were measured using CANTO II, Becton Dickinson flow cytometer and analysed by FCS Express 4.0 (De Novo Software, CA, USA).

Intracellular total glutathione was measured by flow cytometry, using MCB staining for iGSH. $1–2 \times 10^6$ cells were stained with 40 μM MCB at room temperature for 20 min. The cells were chilled by the addition of ice-cold PBS at 0 °C to stop any enzyme-dependent staining reaction. The cells were then washed, maintained at 4 °C, and 5 μl of PI (1 mg/ml) was added. PI fluorescence was used to exclude dead cells from the analysis. Fluorescence of MCB–GSH conjugate was detected using a 405 nm excitation laser and a 450/50 emission bandpass filter. Viable cells were analysed by FCS Express 4.0 software.

2.5. Analysis of gene expression

SKOV3 and A2780 cells were treated for 1, 3, 8, and 24 h with different concentrations of SFN. Total RNA was isolated using TRIzol® Reagent (Ambion®). Two micrograms of total RNA was reverse transcribed with RevertAid™ H minus First Strand cDNA Synthesis Kit (Fermentas, Germany), using BioRad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, USA). cDNA (20× diluted) was used as a template for a qRT-PCR analysis, performed using the Maxima SYBR Green qPCR Master Mix (2×) (Fermentas, Germany) and the Bio-Rad CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories) in a 20 μl PCR reaction mix. The PCR protocol consisted of 10 min 95 °C initial denaturation, followed by 39 repeats of 30 s 95 °C denaturation, 30 s 58 °C annealing, 30 s 72 °C extension and 5 s 73 °C or 76 °C and 80 °C plate reading. Gene expression analysis was calculated by Bio-Rad Software Manager, Version 1.6 provided by the manufacturer (Bio-Rad Laboratories). A normalized fold expression was compared with the $\Delta\Delta C_q$ method. The target genes *NRF-2* (*NFE2L2*: nuclear factor, erythroid 2-like 2) and *GCLC* (glutamate-cysteine ligase, catalytic subunit) were normalized to the reference gene *β actin* from each treated sample. Analysis was performed twice in triplicates. The obtained results were analysed by *t*-test.

The following primers were used: human *β actin*: 5′-CCAACCGCGAGAAGATGACC-3′ (forward) and 5′-AGGATCTTCATGAGGTAGTCAGTC-3′ (reverse); human *NRF-2*: 5′-AGCAGGACATGGATTTGATTG-3′ (forward) and 5′-TGGGAGAAATTCACCTGTCTC-3′ (reverse); human *GCLC* 5′-GGCGATGAGGTGGAATACAT-3′ (forward) and 5′-GCATGTTGGCCTCAACTGTA-3′ (reverse).

2.6. Alkaline single cell gel electrophoresis (alkaline comet assay)

The procedure of Singh et al. (1988) was used with minor modifications suggested by Slamenova et al. (1997). Briefly, in the comet assay aliquots of 2.5×10^4, control or appropriately treated A2780 and SKOV3 cells embedded in 0.75% low-melting-point agarose were spread on a base layer of 1% normal-melting-point agarose in PBS on microscopic slides and covered with cover slips. After the solidification of the gel, the cover slips were removed and incubation of the cells with 600 μM styrene oxide (StO; 30 min at 4 °C in the dark) in PBS was carried out to study and distinguish between frank DNA breaks and DNA damage induced with DNA-crosslinking agent cisPt. StO induces frank DNA breaks, which lead in untreated cells to the formation of comets containing about 80–90% of DNA in the tail. When crosslinks are alkali-induced unwinding is blocked (due to covalent binding to DNA) and the percentage of DNA in the tail is reduced accordingly (Fikrova et al., 2013). The slides without StO-incubation were put into a lysis solution for 30 min longer. The slides containing A2780 and SKOV3 cells treated with StO were then placed in the lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH 10 and 1% Triton X-100, at 4 °C) for 1 h. After lysis, all the slides were transferred to an electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 40 min, unwinding at 4 °C, and then subjected to electrophoresis at 25 V