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Evaluation of nitric oxide donors impact on cisplatin resistance in various ovarian cancer cell lines

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ABSTRACT

Ovarian cancer chemoresistance, both intrinsic and acquired, is the main obstacle in improving the outcome of anticancer therapies. Therefore the development of new treatment strategies, including the use of new compounds that can support the standard therapeutics is required. Among many candidates, nitric oxide (NO) donors, agents with multivalent targeted activities in cancer cells, are worth considering. The aim of this study was evaluation of SPER/NO and DETA/NO ability to enhance cisplatin cytotoxicity against different ovarian cancer cell lines. Obtained data indicate that NO donors action varies between different cancer cell lines and is strongest in low aggressive and cisplatin sensitive cells. While statistically significant, the enhancement of cisplatin cytotoxicity by NO donors is of low magnitude. The rise in the percentage of late apoptotic/necrotic ovarian cancer cells may suggest that NO donors enhancement action might be based on the cellular ATP depletion. Nevertheless, no significant impact of the NO donors, cisplatin or their combination on the expressions of *ABCB1*, *BIRC5* and *PTEN* genes has been found. Although our data puts the therapeutical potential of NO donors to aid cisplatin action in question it may also point out at the further approach to utilize these compounds in therapies.

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1. Introduction

Ovarian cancer is the leading cause of morbidity and mortality from the gynecologic malignancies among women worldwide (Bray et al., 2005; Siegel et al., 2014). Conventional therapy is based on the cytoreductive surgery followed by the administration of chemotherapy (Al Rawahi et al., 2013; Goff et al., 2007). Platinum-based compounds (such as cisplatin or carboplatin) are applied alone or in combination with other drugs (such as taxanes or anthracyclines) in the first line treatment of advanced stage ovarian cancer (Ahmad et al., 2006; Desoize and Madoulet, 2002). Although the initial response for the platinum-based therapy is very promising, the emerging resistance to this cytostatic drug is a limitation in a great deal of ovarian cancer therapies (Cao et al., 2008; Morgan et al., 2014).

Ovarian cancer chemoresistance is multifactorial in nature and involves overexpression of key genes responsible for decreased intracellular drug accumulation, as well as insensitivity to the induction of apoptosis (Al-Dimassi et al., 2014; Gottesman, 2002). The mechanism of decreased drug accumulation is based mainly on the activity of ABC (ATP binding cassette) transporters - proteins, which utilize the energy from ATP to translocate various substrates (including chemotherapeutic drugs) across the membrane (Zhang et al., 2015). One of the most important ABC proteins is glycoprotein P (P-gp), encoded by *ABCB1* (multidrug resistance protein 1 - MDR1) gene (Breier et al., 2013). The resistance to drug-dependent apoptosis involves the enhanced activity of the inhibitors of apoptosis (IAPs) (Singh et al., 2015). Enhanced activity of a key IAP - survivin (SVV), encoded by *BIRC5* (baculoviral inhibitor of apoptosis repeat-containing 5) gene, is very often observed in numerous types of tumors (Zaffaroni and Daidone, 2002). Finally, increased cancer survival and chemoresistance are often caused by the deregulation of key oncogenic cellular signaling pathways, such as the PI3K/AKT/mTOR(McCubrey et al., 2015). Its hyperactivation is very often connected with the loss of tumor suppressor phosphatase and tensin homolog (PTEN) activity. It has been reported that the overexpression of both P-gp and survivin, as well as deletion of PTEN leads to enhanced growth and development of drug resistance in various cancer types (Cassinelli et al., 2013; Cheung et al., 2013; Jiang et al., 2013; Ozben, 2006; Xu et al., 2014).

Major emphasis is put on finding the new cancer treatment strategies, based on the use of novel compounds/therapeutics with anti-tumoral activity to aid the effectiveness of standard chemotherapeutic drugs. Among many agents that have been studied for their potential anti-cancer activity, the nitric oxide (NO) donors are very promising. These pharmacologically active synthetic compounds are able to release NO in vivo and/or in vitro (Ignarro et al., 2002). One of the most interesting class of nitric oxide donors is diazoniumdiolates (also known as

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'NONOates'), where NO adduct is combined with nucleophiles. NONOates decompose spontaneously in aqueous environment at physiological pH and temperature, and generate up to 2 M equivalents of NO on the stable rate (Huerta et al., 2008; Megson and Webb, 2002).

In our previous research we have proved that the members of diazoniumdiolates present direct anti-cancer activity by reducing ovarian cancer cell line viability, inhibiting phosphorylation of their key signaling proteins and reducing their invasiveness (Kielbik et al., 2014, 2013). However, the capacity of NONOates to aid the effectiveness of standard cytostatic drugs has not been investigated yet. Therefore, in this study, the NO donors impact on the cytotoxic activity of cisplatin on four ovarian cancer cell lines: cisplatin sensitive and resistant - A2780 and A2780cis, as well as two cell lines differing in the degree of aggressiveness - SK-OV-3 (highly aggressive) and OVCAR-3 (low aggressive), both resistant to cisplatin. What is more, apart from the viability assays, the expression of drug resistance regulating factors: glycoprotein P, survivin and PTEN has been determined in ovarian cancer cell lines after their treatment with NO donors and cisplatin. Two members of NONOates family with different half-life times: spermine nitric oxide complex hydrate (SPER/NO, $t_{1/2} = 39$ min) and diethylenetriamine nitric oxide adduct (DETA/NO, $t_{1/2} = 20$ h) were used in this study.

2. Materials and methods

2.1. Reagents

Trypsin 0.05% EDTA solution, RPMI 1640 (Roswell Park Memorial Institute) medium with 2 mM L-glutamine and 1 mM sodium pyruvate, Dulbecco's phosphate buffered saline (D-PBS) were purchased from Gibco (Inchinnan, Scotland). Fetal bovine serum (FBS) was obtained from PAA The Cell Culture Company (Pasching, Austria). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cis-diamineplatinum(II) dichloride, NaCl, propidium iodide (PI), spermine – nitric oxide complex hydrate (SPER/NO), diethylenetriamine/nitric oxide adduct (DETA/NO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). FITC AnnexinV Apoptosis Detection Kit II was obtained from Becton Dickinson (San Jose, CA, USA). TRIzol®Reagent, TaqMan® Universal PCR Master Mix and TaqMan® Gene Expression Assays for *β-actin*, *GAPDH*, *ABCB1*, *BIRC5*, *PTEN* were bought in Life Technologies (Carlsbad, CA, USA).

2.2. Cell lines

SK-OV-3, OVCAR-3, A2780 and A2780cis human ovarian cancer cell lines were obtained from ATCC (Manassas, VA, USA). SK-OV-3, A2780 and A2780cis were cultured in growth medium (GM), which consisted of RPMI 1640 medium and 10% FBS, while OVCAR-3 cells were cultured in GM composed with RPMI 1640 medium and 20% of FBS. Additionally, the 1 μM of cisplatin was added to the A2780cis cell line every 2–3 passages in order to sustain their cisplatin resistance. All cell lines were passaged every 2–3 days by trypsinization (Trypsin 0.05% EDTA solution) for 10–15 min at 37 °C with a 5% CO_2 atmosphere. Afterwards, to remove all traces of trypsin, cells were washed once with GM. Finally, cells were resuspended in fresh GM or used to experiments. Each time, the viability of cells was assessed by trypan blue exclusion (>95%).

2.3. Treatment of ovarian cancer cells

Cancer cell lines in the culture medium (CM): RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate and 10% FBS, were seeded in 96-well or 24-well plates at the concentration of 10^5 cells/well or 10^6 cells/well, respectively. A 24-hour incubation has been performed at 37 °C with a 5% CO_2 atmosphere to allow the attachment of cells to the surface of the wells. The CM was removed and further treatment was conducted in two different ways. First way (pre-treatment model): SPER/NO or DETA/NO at the concentrations of 10 μM and 100 μM (as indicated in figures) were added or not (control) for 24 or 48 h (37 °C, 5% CO_2). Afterwards, the CM was replaced and two different concentrations of cisplatin (low and high), based on the half maximal effective concentration (EC_{50}) value (10 μM and 50 μM for SK-OV-3; 5 μM and 10 μM for OVCAR-3; 5 μM and 25 μM for A2780 and A2780cis),were added and all cell lines were incubated for another 24 or 48 h, as indicated in figures. Second way (tandem compound model): the cell lines were treated simultaneously with SPER/NO or DETA/NO and cisplatin in the same concentrations as indicated above and incubated for 48 h. Cells prepared in those ways were then used for MTT assay, annexin V apoptosis assay or mRNA expression.

2.4. MTT assay

After the treatment of all cell lines with NO donors and cisplatin, supernatants were discarded, 100 μl of MTT solution (2 mg/ml) was added and cells were incubated for 3 h at 37 °C with a 5% CO_2 atmosphere in order to reduce tetrazolium salt into formazan. Afterwards, formazan was dissolved in 200 μl of 2-propanol. The absorbance was measured on Multiskan RC plate reader (Labsystem, Helsinki, Finland) with dual wavelength of 595 nm and 630 nm, using Genesis Lite software. The optical density (OD) of background was subtracted from all samples automatically via software during sample measure. The data were not presented as OD values but as the percentage of cytotoxicity, calculated according to the formula:

$$\text{Cytotoxicity\%} = \left(1 - \left(\frac{\text{optical density of cells treated with NO donors or ciplatin}}{\text{optical density of untreated cells [control]}}\right)\right) \text{x}100$$

2.5. PI exclusion assay

Propidium iodide (PI) is a dye used to quantify dead cells with damaged (interrupted) cell membrane. After the treatment with NO donors and cisplatin, cancer cells were washed, suspended in 350 μl of D-PBS and stained with 5 μl of PI (2 μg/ml) for 30 min, in the dark, at room temperature. The intensity of PI fluorescence of control and treated cells was measured the same day on Becton Dickinson (San Jose, CA, USA) LSR II flow cytometer with BD FACS (fluorescence-activated cell sorting) Diva software. The results were analyzed by WinMDI software. The data were presented as the percentage of dead cells.

2.6. FITC annexin V apoptosis detection assay

To evaluate the number of early and late apoptotic cancer cells treated with NO donors and cisplatin we used FITC Annexin V apoptosis detection kit II. Briefly, treated cells at the density of 1×10^6 were washed twice with PBS and resuspended in 1 ml of 1 × binding buffer. After that, 100 μl of the cell suspension was transferred to a 5 ml culture tube and 5 μl of FITC Annexin V and 5 μl of PI was added to each tube for 15 min (room temperature). The apoptosis was measured within an hour, using Becton Dickinson (San Jose, CA, USA) LSR II flow cytometer with BD FACS (fluorescence-activated cell sorting) Diva software. Viable cells were defined as PI negative, FITC Annexin V negative, early apoptotic as PI negative, FITC Annexin V positive and late apoptotic/necrotic as PI positive, FITC Annexin V positive. The obtained data was analyzed by FlowJo software (Tree Star Inc., Ashland, USA) and presented as the percentage of apoptotic cells.

2.7. Total RNA isolation and qRT-PCR analysis

Total RNA was isolated from cells using TRIzol®Reagent (Life Technologies) according to the manufacturer procedure. Next, the quality control of isolated RNA was performed on the Nanodrop with ND 1000 Software (Thermo Scientific, Fremont, CA, USA) and the 2100

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