Hsp90 inhibitor Geldanamycin increases the sensitivity of resistant ovarian adenocarcinoma cell line A2780cis to cisplatin

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Ovarian carcinoma is the leading cause of death among gynecological neoplasms in the world. The chemoresistance is a major obstacle in the effective treatment of ovarian and other cancers. We evaluated the effects of Hsp90 inhibitor geldanamycin (GEL) alone and in combination with cisplatin in cisplatin resistant ovarian adenocarcinoma cell line. Our results showed Akt depletion and S-phase arrest of A2780cis cells after GEL treatment. Combined exposure of A2780cis cells to GEL and cisplatin resulted in greater than additive cytotoxic effect.

Key words: chemoresistance, cisplatin, A2780cis, Hsp90, geldanamycin

Cis-dichlorodiammine platinum (II) (cisplatin) a platinum containing compound, is widely used in the treatment of ovarian, testicular, head and neck, lung and bladder cancers. In spite considerable anticancer activity of cisplatin, long term application is limited because of rapid resistance development. Cisplatin resistance can operate by a number of mechanisms [1,2,3,4] which appears to fall into four major categories: 1) decreased intracellular accumulation of the drug; 2) increased sulfur-containing macromolecules (glutathione and metallothionein); 3) increased repair of platinum-induced DNA lesions and enhanced ability to remove cisplatin-DNA adducts; 4) apoptosis regulation and cellular survival signals. One of the most important mechanisms of cisplatin resistance is the ability of resistant cells to repair platinum-induced DNA damage. The association between excision repair cross complementing-group 1 (ERCC1) gene and platinum resistance in ovarian cancer cells is well known from the literature published so far [5,6]. Parker et al. [7] showed that a specific increase in the ability to repair cisplatin-DNA lesions is a major component, but not the only one of acquired cisplatin resistance in human ovarian cancer cells. Reduced DNA platination only accounts for twofold resistance in A2780/CP70 vs. A2780 cells, whereas A2780/CP70 cell line was 13-fold more resistant to cisplatin than A2780 cells [7]. Understanding the molecular mechanism of cisplatin cell resistance many molecular markers involved in cell survival and/or apoptosis have been identified. In this number significant role could play Akt2 [8] overexpressed and/or activated in human ovarian and breast cancers. The results of Asselin et al. [1] demonstrate a novel mechanism of chemoresistance by which X-linked inhibitor of apoptosis protein (XIAP) regulates apoptosis through a phosphatidylinositol 3-kinase (PI3K)/Akt dependent inhibition of the caspase cascade. Furthermore, heat shock protein 90 (Hsp90) binding to Akt was responsible for protection of Akt from protein phosphatase 2A-mediated dephosphorylation [9]. Therefore, Akt-Hsp90 complex might play an important role in stabilization of Akt kinase activity [3]. The results mentioned above indicate that Hsp90 might be a promising target for developing new chemotherapeutic drugs to suppress the Akt-mediated survival-signalling pathway. In our study, we demonstrate the influence of Hsp90 inhibitor – geldanamycin (GEL) on cisplatin chemoresistance of A2780cis cells.

Materials and methods

Cell Lines. The A2780cis human ovarian adenocarcinoma cell line was obtained from the ECAC (European Collection

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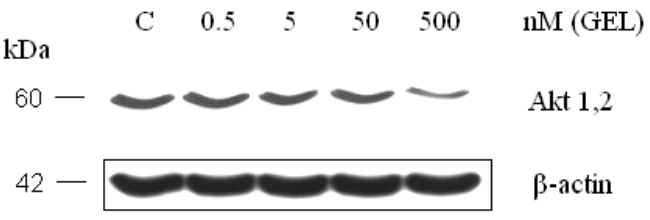

Figure 1. Western blot analysis of Akt protein in whole A2780cis cell lysates. Cell lysates were prepared 24 h after geldanamycin (GEL) treatment (5 h). β-actin serves as a control for loading. The experiment was repeated 3 times and representative result is shown.

Tab. 1. The effect of geldanamycin on the viability, cell number and the cell cycle distribution of A2780cis cells

	C	*0.5 nM*	*5 nM* (IC_{10})	*50 nM* (IC_{50})
Viability (%)	95.8±1.4	94.1±1.2	93.3±1.5	92.4±1.66
Cell number (x10^3)	462±30.3	463±46.7	453±26.5	357±10.4**xx**
G0/G1	68.6±0.4	66.2±1.1	66.4±1.2	38.0±2.9
S	20.8±0.9	20.6±1.1	20.1±0.5	46.0±2.3**xx**
G2	10.5±0.5	13.1±0.2	13.5±0.7	16.0±0.5

Control (C) and geldanamycin (GEL) treated cells were harvested and fixed 24 h after GEL treatment (5 h). The values are evaluated as means ± standard deviations (S.D.) of at least 3 independent experiments and the significance in GEL vs. C is signed as (xx) ($p<0.01$).

of Animal Culture, Salisbury, UK). Cells were grown as monolayers in RPMI 1640 medium with L-glutamine (Gibco BRL, Paisley, UK) supplemented with 10 % fetal calf serum (Gibco BRL) and antibiotic/antimycotic solution (100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B) (Gibco BRL) and were maintained under standard tissue culture conditions of 37°C and 5 % atmosphere of CO_2. The acquired resistance of of A2780cis cells was maintained by supplementation of media with 1 µM of cisplatin (Sigma-Aldrich Cor., St. Louis, MI, USA) every second passage.

The number of cells was determined using Coulter Counter (Beckman-Coulter) and the total cell viability was analysed by staining of cells with 0.15 % eosin via light microscopy.

*Cytotoxicity assay***.** A2780cis cells were seeded into 96-well cell culture plates at a density of $1x10^4$cells/well. After overnight incubation, the cells were treated with geldanamycin (Sigma-Aldrich Cor.) for 5 h followed by cisplatin treatment in geldanamycin free media for 72 h. Then 3-[4,5-dimetyltiazolyl]-2,5-difenyltetrazolium bromid (MTT; Sigma-Aldrich Cor.) was added to the final concentration of 0.2 mg/ml and after 4 h incubation (37°C) the MTT-formazan product was solubilized using 10% SDS (Sigma-Aldrich Cor.). The absorbance measurements were carried out using a universal microplate reader Fluostar Optima (BMG Labtech GmbH, Offenburg, Germany) at 584 nm.

Western blotting. The cells were washed twice with ice-cold PBS and scraped into the RIPA buffer (1xPBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS). Protease inhibitor cocktail "Complete" (Roche Diagnostics, Penzberg, Germany) was freshly added to RIPA buffer. Scraped lysate was transferred into the microcentrifuge tube and passed through a 21 gauge needle to shear the DNA. After the incubation of lysate on ice for 45 min and after centrifugation at 10,000x g for 10 min at 4°C, the supernatant was transferred into a new microcentrifuge tube. Protein sample was separated on 10 % SDS-polyacrylamide gel, electroblotted onto Immobilon-P transfer membrane (Millipore Co., Billerica, MA, USA) and detected using anti-Akt (#9272, 1:1,000; Cell Signaling Technology Inc., Beverly, MA, USA) and anti-β-actin (clone AC-74, 1:10,000; Sigma-Aldrich) primary antibodies. Then the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies (Goat anti-Rabbit IgG F(AB') 2, 1:10,000, PI-31461 and Goat anti-Mouse IgG F(AB') 2, 1:10,000, PI-31436, Pierce, Rockford, IL, USA) for 1 h and the antibody reactivity was visualized with ECL Western blotting substrate (PI-32106, Pierce) using Kodak Biomax film (#1788207, Sigma – Aldrich).

Cell cycle analysis. The cells were washed twice with ice-cold PBS and fixed in 70% cold ethanol overnight at 4°C. The PBS washed cells subsequently rinsed with 0.2 M phosphate-citrate buffer (pH 7.8) were incubated with propidium iodide solution (20 µg/ml of propidium iodide and 5 U/ml of RNAse A) for 30 min at 37°C in the dark. The DNA content (fluorescence) was measured by flow cytometry using FACSCalibur (Becton Dickinson, Mountain View, CA) equipped with an argon ion laser (excitation at 488 nm). The percentage of cells in the individual cell cycle phases were analyzed using ModFit 2.0 software (Verity Software House).

Statistical analysis. Data were processed by GraphPadPrism (GraphPad Software Inc., San Diego, CA) and statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test.

Results

Presented study showed a significant cytotoxic effect of GEL on cisplatin resistant ovarian adenocarcinoma cell line A2780cis (derivative of cisplatin sensitive parental A2780 cells). In this regard the concentration of GEL inhibiting 50 % of metabolic activity (IC_{50}) of A2780cis cells was 50 nM (not shown). This concentration reduced significantly the number of A2780cis cells without the changes in the cell viability (Tab. 1). Furthermore, IC_{50} concentration of GEL induced the S-phase arrest of A2780cis cells, which correlated very well with the cell number decline mentioned above (Tab. 1). On the contrary, we have not found Akt depletion when IC_{50} concentration of GEL was used. The only reduction of Akt protein level was observed when cells were treated with IC_{90} concen-

tration of GEL (500 nM) (Fig. 1). We have also studied the effect of combined treatment of GEL and cisplatin. Our results of combined treatment demonstrate significant potentiation effect of Hsp90 inhibitor – GEL on the cytotoxicity induced by cisplatin in A2780cis. In fact, pre-treatment of A2780cis cells with IC_{10} of GEL (5 nM) for 5 h followed by cisplatin treatment of cells for another 72 h reduced the IC_{50} concentration of cisplatin from 20 μM to 9 μM (Fig. 2).

Discussion

Hsp90 is one of the most abundant molecular chaperones and highly conserved protein whose association is required for the stability and function of multiple mutated, chimeric and overexpressed signalling proteins that promote the growth and/or survival of cancer cells [10]. Akt kinase is one from the wide variety of "Hsp90 client proteins" associated with Hsp90 [9]. GEL, a natural protein isolated from Streptomyces hygroscopicus, bound specifically to Hsp90 and inhibited the association of the chaperone with oncogenic protein kinases via its proteasomal degradation [11]. In our case, the Akt protein depletion appeared only after the treatment of A2780cis cells with 500 nM of GEL (10 times higher concentration of GEL than IC50). The IC50 concentration of GEL reduced the cell number but did not have any affect on the Akt protein level. On the other hand, we have not observed any effect of IC_{10} concentration of GEL on the viability, cell number and Akt depletion of A2780cis cells. However, IC_{10} concentration of GEL significantly increased the cytotoxic effect of cisplatin, when combined together. In HCT116 human colon adenocarcinoma cell line, the effects of GEL and 17-allylamino-17-demethoxygeldanamycin (17-AAG), clinically relevant analogue of GEL, with cisplatin were additive and schedule dependent. In the contrary, in HT-29 cells GEL antagonized cisplatin effects resulting in less cytotoxicity values than expected. The antagonism in HT-29 cells might be a consequence of altered p53 function in this cell line [12]. Interestingly, Rakitina et al. [13] found that the additive effect of oxaliplatin and 17-AAG resulted from the inhibition of NF-κB in colon cancer cell lines but modulation of extracellular signal-regulated kinase and Akt signalling had no impact on oxaliplatin toxicity in these cells. In our case, additional studies are needed for to be able to explain, whether the same or different mechanism of GEL impact on cisplatin treatment of A2780cis cells is present.

McIlwrath et al. [14] demonstrated a dose-dependent G2 arrest and reversible inhibition of entry into the S-phase of the cell cycle in parental A2780 cells. This is in opposite to our results, where GEL exposure (IC_{50}) resulted in accumulation of cisplatin resistant A2780cis cells in the S-phase of the cell cycle. Kozubik et al. [15] showed the accumulation of A2780cis cells in the S-phase fraction after the treatment with cisplatin alone. In this regard, it is reasonable to study more detailed the effect of combined treatment of GEL and cisplatin on the A2780cis cell cycle distribution. In the future, except of studying the mechanism of GEL impact during combined treatment, different cisplatin resistant cell lines in similar experimental model will be also included.

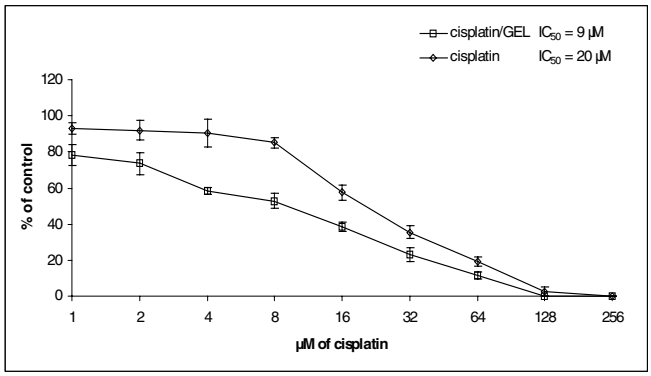

Figure 2. The effect of cisplatin treatment and its combination with 5nM of geldanamycin (GEL) (5 h pre-treatment) on the proliferation and/or survival of A2780cis cells. The concentrations inhibiting metabolic activity of cells by 50% are displayed for both treatments. The experiment was performed in quadruplicate and repeated 3 times. The values are evaluated as means ± standard deviations (S.D.) and the significance in GEL + cisplatin vs. cisplatin except of two highest concentrations of cisplatin (128 μM and 256 μM) was observed ($p<0.01$).

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