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Intracellular ATP depletion leads to reduced platinum accumulation in ovarian cancer cells*

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Key words

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Introduction

Cisplatin is one of the most effective drugs in the treatment of ovarian cancer. However, its therapeutic potential is limited by intrinsic or acquired resistance. Several molecular mechanisms of cisplatin resistance have been described including increased repair of DNA adducts, increased tolerance to DNA damage, elevated intracellular levels of glutathione or metallothioneines, increased efflux and decreased cellular uptake of platinum. The most frequently observed feature in cisplatin-resistant cell lines is reduced accumulation of the drug [1]. The exact mechanisms involved have not been completely identified yet. Although some findings suggest that passive diffusion across the membrane is a major mechanism of cisplatin uptake, other studies indicate that various energy-dependent cellular proteins, which utilize ATP hydrolysis as energy source, may be involved in intracellular trafficking of cisplatin [1].

Extracellular ATP is known to enhance cellular membrane permeability, which may lead to increased cellular accumulation of anticancer drugs due to enhanced passive diffusion. Therefore, extracellular ATP may be used to sensitize ovarian carcinoma cells [2]. However, the exposure to ATP does not increase the accumulation of cisplatin in A2780 and A2780cis cells [3]. One possible explanation is a simultaneous increase in cisplatin efflux by activating ATP-dependent efflux transporters (e.g., ATP7A, ATP7B and MRP2). Therefore, treatment of cancer cells with ATP may increase both cellular uptake and efflux of cisplatin at the same time resulting in unchanged net drug accumulation.

To further assess the role of ATP in cisplatin uptake and efflux, we investigated the accumulation of cisplatin under ATP-depleting conditions. ATP depletion can be achieved by using inhibitors of oxidative phosphorylation or mitochondrial electron transport chain (ETC) complex I – IV inhibitors. The latter are known to induce the production of reactive oxygen species leading to apoptosis [4]. For that reason, we chose an in-

hibitor of the oxidative phosphorylation, oligomycin, which depletes intracellular ATP levels by specific inhibition of the F₀F₁ ATP synthase.

Material and methods

Cell culture

The human ovarian carcinoma cell line A2780 and the cisplatin-resistant variant A2780cis were cultivated as monolayer in RPMI-1640™ medium with 10% fetal calf serum, 0.6 mM L-glutamine, 100 I.E./ml penicillin and 0.1 mg/ml streptomycin at 37 °C and 5% CO₂.

Measurement of intracellular ATP

Cellular ATP levels were determined using a bioluminescence-based assay [5]. 2 × 10⁴ cells were allowed to attach in white 96-well plates overnight. In some experiments the cells were subsequently treated with various concentrations of oligomycin in glucose-free medium for up to 60 minutes.

The solvent was then removed and the cells were lysed with 1% triton and incubated with D-luciferin and recombinant firefly luciferase in reaction buffer (ATP determination Kit™, Invitrogen, Germany) for 10 minutes. The luminescence signal was measured using the microtiter plate reader Lumistar™ (BMG-Lab Technologies, Offenburg, Germany) at 560 nm [5].

ATP concentrations were related to the protein content determined by the bicinchoninic acid protein assay (BCA™ Protein Assay Kit, Pierce, Rockford, USA). The between-day precision for the determination of ATP/protein was in the range of 18.9 – 24.2%.

Cytotoxicity

The cytotoxicity of cisplatin and oligomycin were determined using a MTT-based

assay (MTT = 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) [6]. Cells were allowed to attach in 96-well plates overnight and were subsequently incubated with various concentrations of the above-mentioned substances for 72 hours. The EC₅₀ values (drug concentration which produces 50% of the maximum possible response) were estimated using the GraphPad Prism™ analysis software package (GraphPad Software, San Diego, USA) by means of non-linear regression (sigmoidal dose response).

Intracellular platinum accumulation

In order to characterize the influence of oligomycin on the uptake of cisplatin, 2×10^6 cells were allowed to attach in 6-well plates overnight and were co-incubated with oligomycin (1 μ M) and cisplatin (100 μ M) for up to 120 minutes after pretreatment with 1 μ M oligomycin for 30 minutes. The cells were then trypsinized and centrifuged.

After lysis with concentrated nitric acid for 60 minutes at 80 °C, the intracellular platinum concentration was measured by flameless atomic absorption spectrometry (SpectrAA™ Zeeman 220 System, Varian, Darmstadt, Germany). Platinum concentrations were related to the protein content determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). All experiments were conducted in triplicate and were repeated 3 – 5 times.

Statistics

Since intracellular ATP levels, expressed as cellular ATP content related to cellular protein content, could not be assumed to have a Gaussian distribution, we used the median as a measure of central tendency. Consequently, the differences were analyzed using the non-parametric Mann-Whitney U-test.

Two-way ANOVA was used to analyze the effect of two independent variables (presence/absence of oligomycin and time) on intracellular cisplatin accumulation.

P values < 0.05 were considered significant.

Results and discussion

Firstly, we determined the baseline ATP levels (related to cellular protein content) in the A2780 human ovarian carcinoma cell line and its cisplatin-resistant variant A2780cis. Significant differences in ATP levels between sensitive (3.4 pmol ATP/ μ g protein) and re-

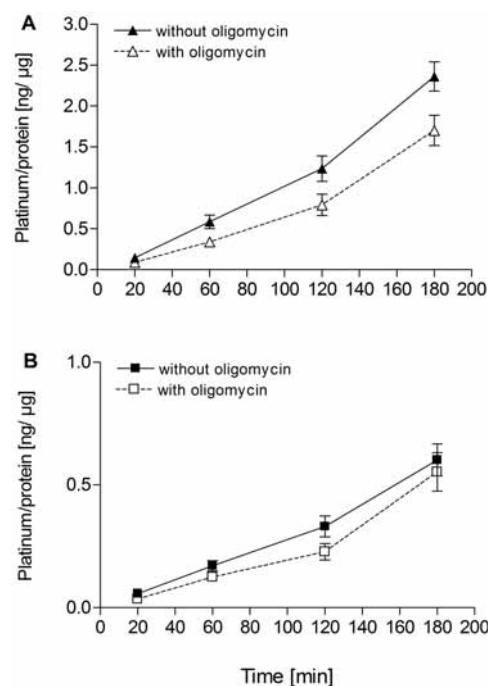


Figure 1. Influence of oligomycin on cellular platinum accumulation in A2780 (A) cells and A2780cis (B) cells (mean \pm SEM, n = 9 – 15).

sistant (4.1 pmol ATP/ μ g protein) cells were found (n = 28, p < 0.0001, Mann-Whitney U-test) suggesting that ATP may play a role in cisplatin resistance.

To investigate whether the accumulation of platinum is energy-dependent, we pre- and co-incubated the cells with oligomycin, an inhibitor of the ATP synthesis. The cytotoxicity tests showed that the EC₅₀ values for oligomycin were 5.5 μ M in the sensitive ovarian carcinoma cell line and 5.3 μ M in the resistant cell line, respectively (n = 5).

In order to find the appropriate concentration for the reduction of intracellular ATP levels, we treated the cells with various concentrations of oligomycin (1.5 nM – 10 μ M) in glucose-free medium. The lowest effective oligomycin concentration was determined as 1 μ M reducing the intracellular ATP levels to $36.7 \pm 5.6\%$ (mean \pm SEM, n = 3: conducted in triplicates) in the sensitive and to $41.4 \pm 5.3\%$ in the resistant cell line. The maximum effect was achieved within 30 minutes. Replacement of the oligomycin-containing medium with drug-free medium led to restoration of the initial ATP levels within 10 minutes. Therefore, we investigated the accumulation of cisplatin in our cell system in the presence of 1 μ M oligomycin after 30 minutes pre-incubation with the same concentration of the inhibitor.

Earlier studies showed that on exposure to cisplatin for 2 hours, the cisplatin-resistant cell line accumulated 2.5-fold less platinum compared to the sensitive A2780 cells [7].

Under the conditions of ATP deficiency, the intracellular accumulation of cisplatin was reduced in A2780 cells compared to standard culture conditions ($p < 0.0001$) (Figure 1A). In the A2780cis cell line, cisplatin accumulation was also reduced, however, to a smaller extent (Figure 1B) ($p = 0.02$).

Conclusions

Our results indicate that cisplatin is taken up by energy-dependent processes in the sensitive A2780 cells, whereas cisplatin uptake in the resistant A2780cis cells is less dependent on intracellular energy sources. This may, however, be the result of the higher basal ATP levels in A2780cis cells compared to the sensitive A2780 cells. For that reason, further studies should focus on the contribution of increased intracellular ATP concentrations to cisplatin resistance in the A2780cis cell line.

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