Role of endoplasmic reticulum stress in size-dependent inhibition of P-glycoprotein by silver nanoparticles in multidrug-resistant breast cancer cells

Summary of Ph.D. Thesis

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INTRODUCTION

Uncontrolled cell proliferation leads to cancer. Benign tumours pose little risk to the host whereas, malignant tumours pose immoderate risk due to their rapid proliferative capacity and tendency to metastasize (migration to other parts from their primary origin). To remove cancerous outgrowth surgery is used as the first line of therapy and can be curative for early stage cancers. In localised setting and in conjunction with surgery, radiotherapy is also a frequently applied treatment modality. Other conventional and modern types of cancer therapy comprise chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell therapy and personalized medicine. Chemotherapy involves the application of cytotoxic drugs to kill cancer cells and is generally the preferred line of cancer management owing to its effectiveness in adjuvant therapy and in the treatment of overtly disseminated cancers. Despite of responding to chemotherapy initially, many cancers develop resistance to drugs over prolonged exposure. Although single compound resistance is relatively easier to deal with, multidrug resistance (MDR) in cancers, when cells lose their sensitivity not only to that particular drug but also to other structurally and functionally dissimilar drugs, creates a severe impediment to successful chemotherapy, since these cells are mostly unresponsive to drug-induced cytotoxicity. The principal component of MDR is linked to P-glycoprotein (Pgp) overexpression. Pgp is a 170 kDa N-glycosylated transmembrane protein that binds and transports several drugs and molecules out of the cytoplasm and is evolutionarily conserved. Pgp efflux is fuelled by ATP hydrolysis, catalysed by one of its structural motifs called ATP Binding Cassette (thus Pgp is classified as an ABC transporter). Therefore, inhibition of Pgp activity could be the ultimate solution to improve the success rate of conventional chemotherapy. Although significant scientific effort focused on the development of Pgp inhibitors, most of them were dismissed on the grounds of safety, efficacy and of disappointing performance in clinical trials.

Silver nanoparticles (AgNPs) have been extensively studied in recent years, thus their unique physicochemical, antibacterial, antifungal and antiviral features are already characterized in detail. However, it was also suggested that AgNPs might have a potential in cancer therapy owing to their prominent anti-proliferative, cytotoxic and pro-apoptotic features. Exposure to AgNPs can lead to the accumulation and aggregation of misfolded proteins, activation of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in cells. Previously published reports from our lab and by others suggest that AgNPs are able to target the MDR-related biological profile of tumor cells, namely modulate the expression and the efflux activity of Pgp, but the molecular background of the reduced transport activity

and its dependence on the nanoparticle diameters remain elusive. Therefore, the main goal of our present study was to investigate whether the actual AgNP size would influence the AgNP-induced molecular mechanisms and the inhibitory actions on P-glycoprotein in multidrug-resistant breast cancer cells. To verify this, quasi-spherical citrate-coated silver nanoparticles of two different sizes (5 nm and 75 nm diameter) were synthetized and were applied on drug-sensitive MCF-7 and multidrug-resistant MCF-7/KCR breast adenocarcinoma cells.

AIMS OF THE STUDY

Specific goal of the present thesis was to study the size-dependent effect of silver nanoparticles in inhibiting P-glycoprotein efflux function in multidrug-resistant breast cancer cells *in vitro*. Our special focus was to unravel the molecular and cellular mechanisms that have been influenced by AgNPs to realize the inhibitory action on P-glycoprotein efflux activity. For this, we synthesized quasi-spherical citrate coated silver nanoparticles of two different sizes and used them to treat P-glycoprotein overexpressing MCF-7/KCR cells that were developed from MCF-7 breast cancer cells as a multidrug-resistant cancer model.

Thus, the aims of the present thesis are:

1. To examine which silver nanoparticles, those of smaller or larger diameter, are more efficient in inhibiting drug efflux via Pgp in MDR breast cancer cells.
2. To study the capacity of AgNPs to sensitize multidrug resistant breast adenocarcinoma cells to drug-induced cell death.
3. To verify if transcriptional or translational modulation of Pgp levels are involved in the AgNP-induced molecular mechanisms in MDR cancer cells
4. To examine if there is a connection between the Pgp inhibitory activity of differently sized AgNPs and mitochondrial damage associated with ROS generation.
5. To relate ER stress inducing capacity of AgNPs to their Pgp inhibitory potential.

METHODS

Cell culture and treatments

The MCF-7 human breast adenocarcinoma cell line was purchased from ATCC and were maintained in RPMI-1640 (LONZA) complete medium. The drug-resistant MCF-7/KCR cell line was developed from MCF-7 under doxorubicin selection pressure. To maintain the drug-resistant phenotype, MCF-7/KCR cells were cultured in media with and without 1 μM doxorubicin for 1 week each. Before experiments, MCF-7/KCR cells were grown in doxorubicin-free medium.

Synthesis and characterization of AgNPs

Citrate-capped silver nanoparticles of 5 nm and 75 nm mean size were synthesized using a standard method. Morphology and size distribution of the synthetized nanoparticles was characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS).

Rhodamine 123 accumulation assay

This assay was performed to measure the efflux activity of the Pgp pump. Briefly, cells were subjected to AgNPs treatment and were subsequently loaded with the Pgp substrate dye Rhodamine 123 (RH123, Sigma-Aldrich). RH123 accumulation was analysed using flow cytometry.

Preparation of plasma membrane and cytoplasmic fractions

MCF-7/KCR cells treated with 75 nm AgNPs were homogenized and the homogenate was subjected to differential centrifugation to collect cytoplasmic and plasma membrane fractions.

Immunoblotting

MCF-7/KCR cells treated with AgNPs were lyzed using RIPA buffer. From the lysate total protein was collected and protein quantity was measured using the Bradford method. The total protein preparations were used for western blot analysis of cytochrome c, LC3, Pgp, Grp94, Grp78, GADD153, EDEM, α-tubulin and Na/K-ATPase.

Cell viability assay

This assay was performed to quantify the cytotoxicity caused by the treatments. Briefly, MCF-7/KCR cells were exposed to AgNPs for 24 hours and were then incubated with MTT reagent. The developed formazan crystals were solubilized in DMSO and the absorbance of the solutions was measured using Synergy HTX microplate reader (BIOTEK®).

Apoptosis detection assay

To quantify the percentage of apoptotic/necrotic MCF-7/KCR cells upon the treatments with AgNPs, AnnexinV-FITC and propidium iodide (Dead Cell Apoptosis Kit, Life Technologies) was applied according to the manufacturer's recommendation. Fluorescence intensities of cells were measured by FACSCalibur™ flow cytometer.

JC-1 staining

Possible mitochondrial damage triggered by AgNPs was assessed by JC-1 staining. JC-1 is a ratiometric fluorochrome that aggregates in mitochondria with active membrane potential and emits red fluorescence, whereas monomeric JC-1 emits green fluorescence. Aggregated to monomeric JC-1 ratio gives information about mitochondrial membrane integrity. Fluorescence intensities of cells were visualized by OLYMPUS BX51 microscope equipped with Olympus DP70 camera. Image analysis was performed by ImageJ software.

ROS detection

2′,7′-dichlorofluorescein diacetate (DCFDA) staining method was used to detect ROS production upon AgNPs treatments. Cells were seeded onto 2% gelatine coated coverslips placed in 24-well plates. On the next day cells were treated with AgNPs then with DCFDA (Sigma-Aldrich) in the dark for 20 min. Cover slips were mounted on glass slides, and DCF fluorescence was visualized by OLYMPUS BX51 microscope equipped with Olympus DP70 camera. Fluorescence intensity measurements were performed using ImageJ software.

Reverse transcription and real-time RT-PCR

Total cellular RNA was prepared using RNeasy® Mini Kit (QIAGEN) according to the manufacturer's recommendation and reverse transcription of total isolated RNA was performed using the TaqMan® Reverse Transcription kit (Applied Biosystems). Quantitative real time PCR reactions were executed on PicoReal™ Real-time PCR (Thermo Scientific) using SYBR Green qPCR Master Mix (Thermo Scientific) with an input of 1 µL cDNA. Each primer was

used at 200 nM concentration. Relative transcript levels were determined by the ΔΔCt analysis using GAPDH as reference gene. Experiments were repeated three times using three biological replicates.

Endoplasmic reticulum calcium release measurements

To measure in real-time the calcium releasing potential of the endoplasmic reticulum, cells were seeded on coverslips. This was mounted on the stage of a Zeiss LSM880 confocal laser scanning microscope and cells were bathed with standard HEPES solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES acid, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose) for 5 minutes and then in HEPES containing 100 μM carbachol (Sigma) for 2 minutes at 37°C at 5–6 mL/min perfusion rate. In each experiment real-time calcium release was measured from a pre-set 6–10 region of interests (ROIs)/ cell. Fluorescence signals were normalized to initial fluorescence intensity (F/F0) and expressed as relative fluorescence (ΔF/F0).

Transmission Electron microscopy

Cells were grown on 0.4 μm pore size polyester membrane inserts (Corning) for transmission electron microscopy (TEM) analysis. Cells were fixed for 2 hours in 4% glutaraldehyde and then in 2% gelatin, which were embedded into epoxy resin (Epon 812, EMS, PA 19440) and sliced into 70 nm sections. Samples were then stained with 25% uranyl acetate and 1% lead acetate. These TEM preparations were examined on Philips CM10 electron microscope equipped with Megaview G2 digital camera (ITEM, Olympus Soft Imaging Solution GmbH, Münster) located at University of Szeged Institute of Pathology.

SUMMARY OF FINDINGS

Silver nanoparticles induce size-dependent cytotoxicity in breast cancer cells

In order to verify the cytotoxic potential of both sized AgNPs on MCF-7 and MCF-7/KCR cells, we performed MTT viability assay with various concentrations of AgNPs for 24 and 48h. The results indicated that the AgNP-induced cytotoxicity depended on nanoparticle size, as 5 nm AgNPs were more toxic than 75 nm AgNPs. AgNPs effectively killed MCF-7/KCR cells, but as expected, these cells were more resistant to the cytotoxic effects of both 5 nm and 75 nm AgNPs compared to MCF-7 cells.

Treatment with 75 nm AgNPs inhibits Pgp efflux activity but does not affect protein levels in drug-resistant cells.

We exposed MCF-7/KCR cells to 5 nm or to 75 nm AgNPs and performed TEM analysis to verify the uptake of AgNPs by MCF-7/KCR cells. The results show that AgNPs were successfully taken up by MCF-7/KCR cells and were mainly observed in membrane-coated bodies. To evaluate Pgp efflux activity, we detected the intracellular accumulation of RH123 by flow cytometry on cells exposed to AgNPs. Results also showed that inhibition of Pgp efflux activity proved to be dependent on nanoparticle size, since 75 nm AgNPs, but not 5 nm counterparts, were capable to reduce Pgp transport activity in drug-resistant MCF-7/KCR cells, despite manifesting no change in Pgp protein expression.

Inhibition of Pgp by 75 nm AgNPs sensitizes drug-resistant cells to doxorubicin-induced apoptosis

MCF-7/KCR is an *in vitro* drug resistance model, which was developed to resist cytotoxic effects of doxorubicin at high doses. As 75 nm AgNPs inhibited Pgp efflux activity, this treatment should also sensitize MCF-7/KCR cells to doxorubicin-induced killing. Therefore, we tested and proved that the cytotoxic and apoptosis-inducing potency of doxorubicin, (also a Pgp substrate) has been significantly raised in co-treatments with 75 nm AgNPs in multidrug-resistant MCF-7/KCR cells, meaning that exposing MDR cancer cells to 75 nm AgNPs sensitizes them for doxorubicin-induced apoptosis.

AgNPs induce oxidative stress and mitochondrial damage

Inhibition of Pgp efflux activity by 75 nm AgNP treatment, without compromising the Pgp expression was quite intriguing, which prompted us to investigate the possibility of AgNP-induced mitochondrial damage and ROS production, both of which can ultimately influence drug transport activity, mainly by diminishing cellular ATP levels to fuel Pgp efflux. However, our results indicated that 75 nm AgNPs were less potent than 5 nm AgNPs in triggering ROS generation and damaging mitochondria. Therefore, oxidative stress-related mitochondrial dysfunction cannot be the fundamental reason behind the reduced Pgp activity in 75 nm AgNP-treated drug-resistant MCF-7/KCR cells.

75 nm AgNP treatments cause depletion of ER calcium stores and ER stress

Endoplasmic reticulum (ER) is a major assembly site of secretory and integral membrane proteins. ER stress disturbs the homeostasis of protein folding machinery. Hence, we

hypothesized that 75 nm AgNPs, by inducing ER stress in drug-resistant cells, decrease the number of properly folded Pgp reaching the plasma membrane, where these transporters should manifest their function. Our results showed that 75 nm AgNPs, but not 5 nm AgNPs induce ER stress in MCF-7/KCR cells which was coupled with the depletion of ER calcium levels. ER stress activates autophagy in order to clear out aberrantly folded protein burden. Therefore, we examined autophagy activation and our results showed that 75 nm AgNPs trigger autophagy, whereas 5 nm AgNPs do not.

75 nm AgNPs disrupt cellular Pgp distribution

We hypothesized that ER stress caused by 75 nm AgNPs might constrain Pgp targeting to plasma membrane. Therefore, we quantified Pgp protein levels in the plasma membrane and in cytosolic fractions of MCF-7/KCR cells treated with 75 nm AgNPs. Our results demonstrated that plasma membrane Pgp levels were significantly decreased, whereas cytoplasmic Pgp levels increased in MCF-7/KCR cells treated with 75 nm AgNPs compared to untreated control.

CONCLUSIONS

- 75 nm AgNPs inhibit Pgp efflux activity and sensitize multidrug resistant MCF-7/KCR cells to doxorubicin-induced apoptosis without causing significant changes in Pgp expression.
- AgNPs size-dependently induce oxidative stress, autophagy and mitochondrial damage in multidrug resitant MCF-7/KCR cells.
- 75 nm AgNPs deplete ER calcium stores and induce ER stress, which eventually reduce the plasma membrane targeting of Pgp, thereby it ultimately leads to MDR reversal.

PUBLICATIONS

Publications related to thesis

- **Gopisetty, M.K**.; Kovács, D.; Igaz, N.; Rónavári, A.; Bélteky, P.; Rázga, Z.; Venglovecz, V.; Csoboz, B.; Boros, I.M.; Kónya, Z.; Kiricsi M. Endoplasmic reticulum stress: major player in size-dependent inhibition of P-glycoprotein by silver nanoparticles in multidrug-resistant breast cancer cells. *J. Nanobiotechnology* **2019**, *17*, 9 **IF-6.518, D1**

Considerable publications for thesis submission

- **Gopisetty, M.K**.; Kovács, D.; Igaz, N.; Rónavári, A.; Bélteky, P.; Rázga, Z.; Venglovecz, V.; Csoboz, B.; Boros, I.M.; Kónya, Z.; Kiricsi M. Endoplasmic reticulum stress: major player in size-dependent inhibition of P-glycoprotein by silver nanoparticles in multidrug-resistant breast cancer cells. *J. Nanobiotechnology* **2019**, *17*, 9 - **IF-6.518, D1**

- **Gopisetty, M.K**.; Adamecz, D.I.; Nagy, F.I.; Baji, Á.; Lathira, V.; Szabó, M.; Gáspár, R.; Csont, T.; Frank, É.; Kiricsi, M. Androstano-arylpyrimidines: novel small molecule inhibitors of MDR1for sensitizing multidrug-resistant breast cancer cells. *Eur. J. Pharm. Sci* **2020,** 156 (2021), 105587 **- IF-3.616, Q1**

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- Rónavári, A.; Igaz, N.; **Gopisetty, M.K**.; Szerencsés, B.; Kovács, D.; Papp, C.; Vágvölgyi, C.; Boros, I.M.; Kónya, Z.; Kiricsi, M.; Pfeiffer, I. Biosynthesized silver and gold nanoparticles are potent antimycotics against opportunistic pathogenic yeasts and dermatophytes. *Int. J. Nanomedicine* **2018**, *13* **- IF-4.471, Q1**
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