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Calcium homeostasis in cisplatin resistant epithelial ovarian cancer

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Abstract. Intracellular calcium concentration ($[Ca^{2+}]_i$) may have an important role in the development of chemoresistance, which is an essential problem in cancer chemotherapy. Cisplatin (DDP), which modulates the intracellular calcium concentration by different mechanisms, is an antineoplastic agent with high success rate in cancer therapies. We investigated the regulatory role of $[Ca^{2+}]$ in cisplatin resistance in epithelial ovarian cancer cell line, in MDAH-2774, and its chemoresistant subclone MDAH-2774/DDP. The measurement of $[Ca^{2+}]_i$ using fluorescence microscope, and flow cytometry revealed that the amount of intracellular calcium decreased in cisplatin resistant cells compared to the amounts in parental cells. mRNA expression profiles of calcium homeostasis-associated major genes (*$IP_3R1/2/3$, RYR1/2, SERCA1/2/3, NCX1/2/3, PMCA1/2/3*, and *PMCA4*) decreased in cisplatin resistant cell line in comparison to the expression profiles in parental cells. Owing to the changes in the expression of genes involved in calcium regulation, these results show, drug resistance may be prevented by introducing a new perspective on the use of inhibitors and activators of these genes, and thus of cytostatic treatment strategies, due to changes in the expression of genes involved in calcium regulation.

Key words: Drug resistance — MDAH-2774 — Cisplatin — Calcium

Introduction

Cytotoxic drugs such as doxorubicin, paclitaxel, or cisplatin (cis-diamine-dichloroplatinum (II) or DDP) damage the 'genetic material' of cells by interfering with the proliferation of cancer cells. That might be caused by cell cycle arrest and/or apoptosis (Florea and Büsselberg 2011a, 2011b; Kim et al. 2011; Kutanzi et al. 2011; Galluzzi et al. 2012). Cisplatin induces concentration-dependent cytotoxicity in diverse types of cancer cells due to targeting at the DNA level, in addition with the transcription and replication mechanisms (Shen et al. 2012). Furthermore, cisplatin targets tumor cells due to its feature to trigger "on" signal transduction pathways (e.g., death receptor, calcium signaling, mitochndrial-dependent apoptosis) that results in tumor cell death (Florea and Büsselberg 2011a, 2011b; Galluzzi et al. 2012).

Ovarian cancer is the most fatal gynecologic malignancy worldwide. Ovarian cancer cell lines can potentially overcome the experimental limitations inherent both in animal models of ovarian cancer, and the primary cloning of human ovarian cancer specimens (Karabulut et al. 2010). Ovarian cancer is a model disease to investigate chemotherapeutic resistance owing to its intrinsic, and acquired resistance to drugs. Cisplatin is the first globally approved platinum drug which has been used in cancer chemotherapy for more than 30 years(Achkar et al. 2018). Significant challenges regarding cisplatin resistance, and cisplatin- induced toxicity still remain despite the widely use of cisplatin as an anticancer drug. Cisplatin resistance is mostly a secondary effect, and its mechanism is unclear. Cisplatin resistance may be associated with the altered regulation of multiple signaling pathways, including downregulation of apoptotic signals, and activation of pro-survival signals (Ali et al. 2012; Gal-

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luzzi et al. 2012; Davis et al. 2014). Recent studies have shown that endoplasmic reticulum (ER) stress-associated apoptosis can be induced by cisplatin, and that ER stress tolerance may be involved in cisplatin resistance (Xu et al. 2012, 2014, 2015a).

The second most abundant messenger, intracellular calcium ions $[Ca^{2+}]_i$, in the human body, have substantial diversity of roles in fundamental cellular physiology including gene expression, cell cycle control, cell motility, autophagy, and apoptosis. Since the cytosolic calcium is maintained in very small concentration($\sim 10^{-7}$ mol/l), a small fraction of Ca^{2+} either through release from intracellular organelles ($\sim 10^{-5}$ mol/l) or through influx from extracellular reservoir ($\sim 10^{-3}$ mol/l) can generate marked signals to activate downstream signaling cascade. Some agents such as glucocorticoids, ER Ca^{2+}ATPase inhibitor thapsigargin and various cancer therapeutic drugs mobilize Ca^{2+} stores, and trigger apoptosis by early transient elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$). In this respect, the cytotoxic effects of some drugs used in cancer treatment are implicated to occur due to increase in intracellular Ca^{2+} levels(Flore and Büsselberg 2009). Hence, an intracellular calcium concentration ($[Ca^{2+}]_i$) overload as well as a disturbance in calcium homeostasis might cause cytotoxicity, and trigger either apoptotic or necrotic cell death (Orrenius et al. 2003). Interestingly, some metal-based anti-cancer drugs (As_2O_3, and DDP, etc.) have the potential to modulate $[Ca^{2+}]_i$ (Florea and Büsselberg 2006).

The intracellular Ca^{2+} homeostasis is controlled by a network composed of various Ca^{2+} channels, transporters, and exchangers. To overcome drug resistance, determining the deregulating Ca^{2+} channels/transporters/exchangers may provide a promising chemotherapy for cancer resistance. In the present study, the expression of major channels, pumps, and exchangers involved in the calcium flux in MDAH (epithelial ovarian cancer cell line MDAH-2774) and MDAH/DDP (cisplatin-treated MDAH-2774) cells was examined. In addition, the calcium content in these cells was determined using flow cytometry and fluorescence microscopy experiments using Fura-2 calcium stain, and the relationship between altered calcium homeostasis related to cisplatin resistance and anti-apoptotic Bcl-2 protein was studied.

Materials and Methods

Cell line and chemicals

Human epithelial ovarian cancer (EOC) cell line, MDAH cells were obtained from the Department of Biophysics in Istanbul Faculty of Medicine, and maintained in Dulbecco's Modified Eagles medium (DMEM) F-12 supplemented with 10% heat-inactivated fetal calf serum (FCS), and 100 IU/ml penicillin–streptomycin. Incubation conditions at 37°C in a humidified atmosphere of 5% CO_2 were maintained in a Heraeus incubator (Hanau, Germany). Cisplatin (Sigma) was applied to the MDAH cell line with dose increments. The subline resistant to cisplatin (MDAH/DDP) from parental MDAH cell line was developed by increasing the 5 μM dose, stepwise, 5 μM to 70 μM for cisplatin. Before the dose increments, adapted cells became confluent in respective drug concentrations and maintained at least for three weeks (approximately 3–4 weeks). Cells selected in 25 μM cisplatin were maintained in medium with 25 μM cisplatin for assays.

3-(4,5)-dimethyl thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay, cell viability assay

Cell viability was assessed using the MTT cell staining as previously described (Prencipe et al. 2009). MDAH and MDAH/DDP groups were seeded in 96-well plates in triplicate at a density of 2×10^5 cells *per* well, and cultured for 24 hours in DMEM containing 10% FCS. MDAH/DDP group was treated using various concentrations of cisplatin (0 to 70 μM) for 24 hours at 37°C. After drug exposure, we added MTT working solution into wells being assayed at 37°C for 30 min to 4 hours. We removed medium with a needle, added 100 μl of DMSO to each well, and pipetted up and down to dissolve crystals. Absorbance of the converted dye was measured at a wavelength of 570 nm by microplate ELISA reader. Data were presented as the percentage cell viability calculated using the following formula: Cell viability (%) = (sample OD/control OD) × 100.

RNA isolation and Real-Time Quantitative RT-PCR

For the isolation of the total RNA, MDAH and MDAH/DDP cells were cultured in four wells for each treatment, and the total RNA was extracted using total RNA extraction kit (Thermo). The concentration of the extracted RNA was determined by spectrophotometric analysis (Multiskan GO, Thermo). Purified RNA (500 ng) was used for cDNA synthesis using RevertAid First Strand cDNA synthesis kit (Thermo Scientific) in accordance with the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using primers specific for *IP_3R 1/2/3, RyR 1/2, SERCA 1/2/3, NCX 1/2/3* and *PMCA 1/2/3/4* genes (Table 1). Specific PCR primers were designed using primer3 software (http://primer3plus.com/). The primer sequences of the genes are given in Table 1. Quantitative analysis was performed using the Fast SYBR Green Master Mix (Thermo). Reaction mixture contained a total volume of 20 μl. The PCR conditions were 95°C for 3 minutes, then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The relative amount of target mRNA in test sample was calculated, and normalized to the corresponding β-actin

Table 1. The primer sequences of the genes

General Name	Primer Pairs (5'-3')	Product Size (bp)
IP$_3$R1	Forward: TGACGAGAACCTGCCCTAT Reverse: TCCTTTCGCCATCTTGCT	304
IP$_3$R2	Forward: GCAATCGTGTCTGTTCCA Reverse: TCTTCAAGTCTCAGCATCG	332
IP$_3$R3	Forward: GCCTACTATGAGAACCACACG Reverse: CAGAAGAGCAATGAGATGAGAG	389
RYR1	Forward: TGACTACCATCAGCACGACA Reverse: ACGAAGACGGCAGGAAATA	297
RYR2	Forward: AACAAGTACATGCCTGGTTTGC Reverse: TTGTTCTCATCAGGGAACAGGG	348
SERCA1	Forward: GTGATCCGCCAGCTAATG Reverse: CGAATGTCAGGTCCGTCT	361
SERCA2	Forward: CGCTACCTCATCTCGTCCA Reverse: TCGGGTATGGGGATTCAA	406
SERCA3	Forward: GATGGAGTGAACGACGCA Reverse: CCAGGTATCGGAAGAAGAG	409
NCX1	Forward: GTCGCACTTGGAACATCA Reverse: CCAGGGAGGAGAAGAAAA	373
NCX2	Forward: CGGTGGATAAACTCATCAAGAA Reverse: CAGGGCAACGAAGACAACA	359
NCX3	Forward: GAGATGGGAAAGCCAGTAT Reverse: ATGCCACGAAAACAACAG	430
PMCA1	Forward: GAGATGGGAAAGCCAGTAT Reverse: ATGCCACGAAAACAACAG	418
PMCA2	Forward: GAGATGGGAAAGCCAGTAT Reverse: ATGCCACGAAAACAACAG	214
PMCA3	Forward: TGGTCCTCTACTTTGTGATTG Reverse: TGGTGGTATAGGCACTGTTG	417
PMCA4	Forward: CTGTGCGTAATGAAGTGC Reverse: AGTCCCGGTAAGCTATG	279

mRNA transcript level as a housekeeping gene. All reactions were performed three times, and the mean values were used. Transcriptional gene expression, which is subsequently referred to as gene expression, was defined as fold change (FC) *versus* ethanol-treated controls and it was determined with the ΔΔCt method (Schmittgen and Livak 2008).

Flow cytometry analysis

To determine $[Ca^{2+}]_i$, 2×10^4 cells were rinsed with HBSS (20 mM Hepes, 10 mM glucose, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, pH 7.4). They were then loaded with 0.5 ml of 5 μM Furo-2AM, dissolved in HBSS from stock solution of 1.5 mM in DMSO containing 20% pluronic acid F-127 at room temperature for 30 min, and subsequently rinsed twice with HBSS. Cells were then analyzed using a BD Aria flow cytometer (BD Bioscience, San Jose, CA, USA).

Confocal fluorescence microscope analysis

Cells were seeded in 6 well plates on glass coverslips at a density of 2×10^4 for 24 hours. After washing with PBS, they were then loaded with 0.5 ml of 5 μM Furo-2AM, dissolved in HBSS from stock solution of 1.5 mM in DMSO containing 20% pluronic acid F-127 at room temperature for 30 min, and subsequently rinsed twice with HBSS. Cells were then analyzed using a Zeiss axio observer Z1 inverted fluorescence microscope (Germany). The fluorescence intensity was calculated as Grynkiewicz's equation (Grynkiewicz et al. 1985).

Western blotting

MDAH, and MDAH/DDP cells were harvested, and cells in equal numbers were analyzed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; acrylamide, Sigma-Aldrich; bisacrylamide, Amresco, Solon, OH, USA; Tris, Sigma-Aldrich; TEMED, Sigma-Aldrich; ammonium persulfate, Sigma-Aldrich), as previously described (Laemmli 1970). Briefly, equal numbers(2×10^5 cells) of cell lysate were loaded onto SDS-PAGE gels using the Mini-PROTEAN Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Prestained molecular weight standars (Precision Plus Protein Standards, Bio Rad) were used for size estimation. Proteins were transferred electrophoretically onto nitro-cellulose membranes (Towbin et al. 1970). The membranes were blocked with Tris-Buffered Saline (TBS) containing 3% bovine serum albumin (BSA), and 0.05% Tween-20 at room temperature and incubated overnight at 4°C with the relevant primary antibody. Anti-Bcl-2 monoclonal antibody(used at 1:1000 dilution, catalog number, ABM40041) and anti-GAPDH monoclonal antibody (used at 1:1000 dilution, catalog number, ABM40029) were obtained from Abbkine. Primary antibodies were detected by incubation with secondary alkaline phophatase-conjugated anti-mouse antibody for 1 h at room temperature (dilution; 1:3000 in TBS), followed by 3 washes with TBST. Alkaline phosphatase activity was detected using 5-bromo-4-chloro-3'-indolyl phosphate/nitro-blue tetrazolium as substrate (Promega Corp., Madison, WI, USA) for colorimetric detection.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). The non-parametric Kruskal-Wallis test was applied followed by Dunn'spost-test for multiple comparisons to test the significance of the various data collected during the study. Statistical comparisons of fluorescence microscopy images were performed utilizing a two-tail Welch's corrected t-test for parametric data or a two-tail Mann-Whitney test for non-parametric data.

Results

Growth inhibition of cisplatin resistant cells

Drug resistant subclone MDAH/DDP established by stepwise treatment with increasing cisplatin concentration, is resistant to up to 24 μM cisplatin. The effect of anti-cancer drug cisplatin on MDAH cell line viability was determined using MTT colorimetric assay. The MDAH and MDAH/DDP cells were treated with cisplatin for 24 hours (Fig. 1). Cisplatin decreased cell viability both in MDAH and MDAH/DDP cells in time and dose-dependent manner. As shown in Figure 1, IC50 value of MDAH cells was 8.6 μM and IC50 value of MDAH/DDP cells was 47.64 μM. A 5.5-fold difference in IC50 value was found between the cell lines ($p < 0.01$, $n = 3$).

Expression levels of Ca^{2+}-related genes

To assess the effects of cisplatin resistance on mRNA expression, relative gene expression differences of fifteen selected genes (*IP_3R 1/2/3, RYR 1/2, SERCA 1/2/3, NCX 1/2/3, PMCA 1/2/3/4*) between MDAH and MDAH/DDP were examined by quantitative RT-PCR (Fig. 2). Comparison of the $2^{-\Delta\Delta Ct}$ values were performed by the measurement of the MDAH and MDAH/DDP cells. A two-fold expression difference ($p < 0.05$) was taken as a cut-off level.

We tested the gene expression of endo/sarcoplasmic reticulum calcium channels, IP_3Rs and RYRs. We showed that *IP_3R2* expression level of MDAH/DDP cells significantly decreased (103-fold) and also observed a decrease in the expression of *IP_3R1* and *IP_3R3* gene. An increase was observed in the comparison of *RYR1* expression in MDAH/DDP with *RyR1* expression in MDAH cells, while *RYR2* of gene expression significantly decreased in cisplatin resistant cells.

We investigated whether there was a change in the expression of *SERCA1/2/3*, which is responsible for the regulation of cytoplasmic Ca^{2+}, in cisplatin resistant MDAH cells and MDAH cells. We found that *SERCA1/2/3* expressions levels in MDAH/DDP cells were lower than MDAH cell in the comparison of the *SERCA1/2/3* mRNA expression levels in MDAH and the MDAH/DDP cells. We then examined gene expressions of Na^+/Ca^{2+} exchanger 1/2/3 (*NCX*) genes. We found an increase in NCX3 although a decrease in mRNA expression of *NCX1* and *NCX2* was detected in cisplatin resistant cells. In addition, we examined the plasma membrane Ca^{2+}-ATPase (PMCA), which is a transport protein in the plasma membrane of cells and which functions to remove Ca^{2+} from the cell. A slight decrease was detected in PMCA2/3, and 4 expressions in the comparison of MDAH/DDP cells with MDAH cells. PMCA1 expression level in MDAH/DDP was higher than the expression level in MDAH cells.

Flow cytometry

Measurement of cytosolic calcium levels in MDAH, and cisplatin resistant MDAH cells were detected by Fura-2-acetoxymethyl ester staining, and flow cytometry. Histograms Fig. 3A and 3B, and bar graph in Fig. 3C showed that MDAH cells had a significantly higher concentration of calcium ions in the cytosol than MDAH/DDP cells.

Fluorescence microscope analysis

MDAH, and cisplatin resistant MDAH cells were treated with 5 μM Fura-2 for 30 min, and the intracellular Ca^{2+} changes were detected by fluorescence microscopy. The intracellular Ca^{2+} concentration was expressed as the 340/380 ratio (MDAH; $n = 16$, cisplatin resistance MDAH/DDP; $n = 10$). Inverted fluorescence microscopy images revealed that MDAH

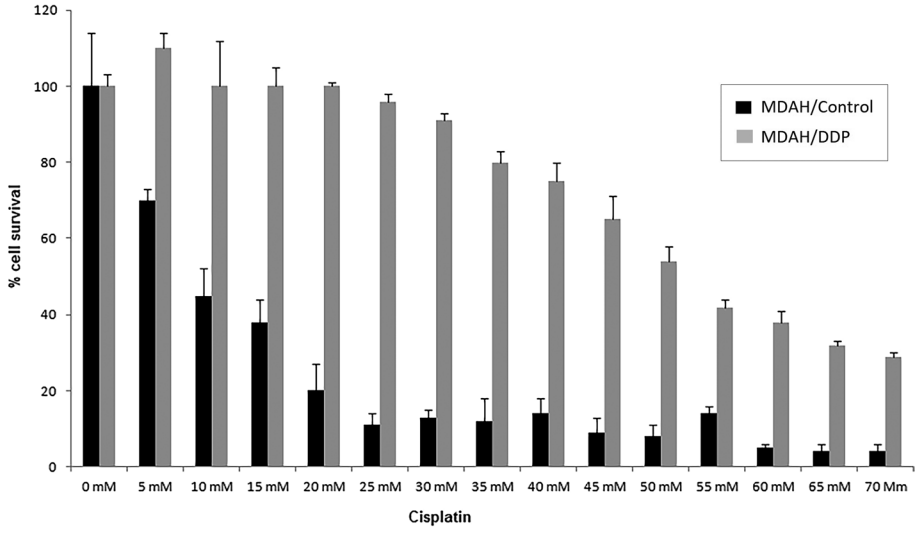

Figure 1. Dose response of cisplatin after 24 hours treatment in MDAH cell line. Cell viability was measured using MTT assay as described in Methods section. The values represent the mean cell survival ± standard deviation (SD) from three independent experiments compared with the untreated control cells (100% survival).

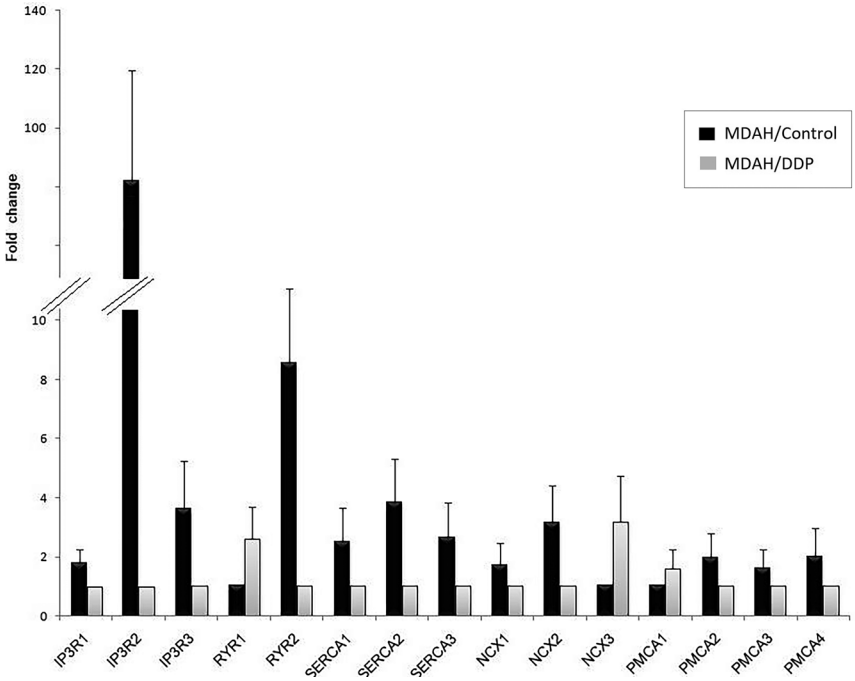

Figure 2. Gene expression of Ca^{2+} channels, pumps, and exchangers in MDAH and MDAH/DDP. mRNA expression profiles of major genes; *$IP_3R1/2/3$, RYR1/2, SERCA1/2/3, NCX1/2/3, and PMCA1/2/3/4* associated to calcium homeostasis in MDAH/Control and MDAH/DDP cells were examined by quantitative RT-PCR. Expression levels were adjusted to β-actin for each sample and graphed as fold-changes relative to MDAH cells.

cells treated with cisplatin for 24 h showed a significant decrease in green fluorescence (Fig. 4A2). Also, quantification of the area and the total intensity fluorescence of intracellular calcium in cisplatin resistant MDAH cells were decreased when compared with the values in MDAH cells (Fig. 4B and C). These findings showed that cisplatin appears to induce decrease in Ca^{2+} concentrations in the cytosol in MDAH cells.

Western blotting

We examined the expression of Bcl-2 (26 kDa), an anti-apoptotic protein, to demonstrate apoptosis in cells treated with cisplatin, and in the cells not treated with cisplatin (Fig. 5). We showed that Bcl-2 protein expression was lost in cisplatin-resistant cells.

Discussion

Ovarian cancer is a common cancer of the female reproductive organs, and is associated with the highest death rates among all gynecological cancers (Chen et al. 2013). Cisplatin chemotherapy. and adjuvant therapy are common in ovarian cancer and drug resistance is a major cause of death (Book-

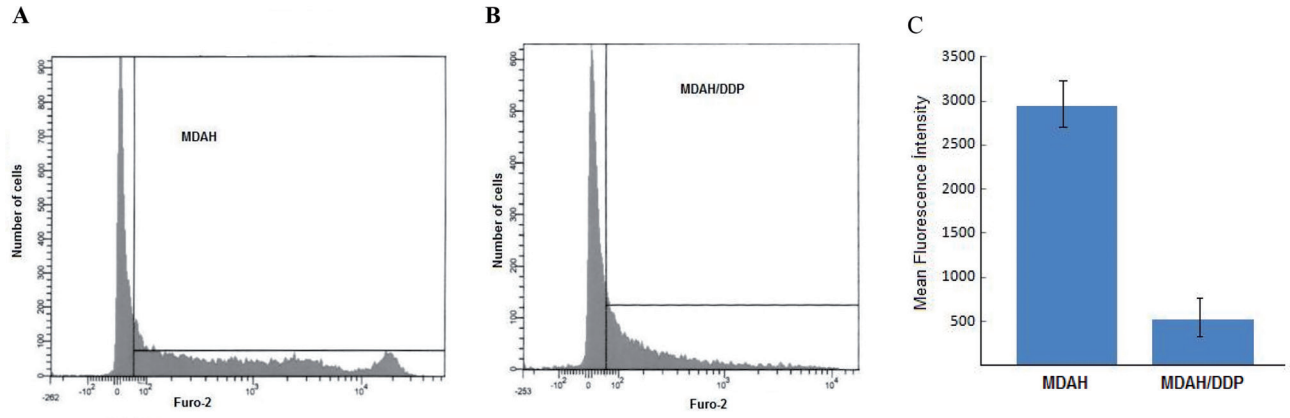

Figure 3. Measurement of cytosolic calcium levels in MDAH/Control and MDAH/DDP cells by Fura-2-acetoxymethyl ester staining and flow cytometry. Histograms **(A)** and **(B),** and bar graph **(C)** showing that MDAH cells had a significantly higher concentration of calcium ions in the cytosol than MDAH/DDP cells. $p < 0.05$ compared to MDAH/Control cells.

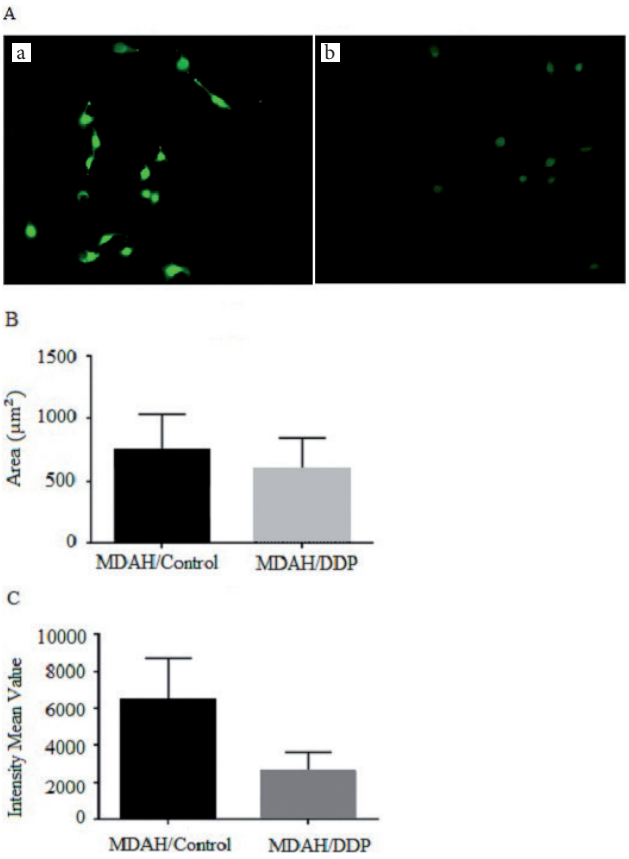

Figure 4. Representative fluorescence intensity and the area variations between MDAH/Control and MDAH/DDP cells. **A.** Fluorescence microscopy images of MDAH/Control (**a**) and MDAH/DDP (**b**) cells stained by Fura-2. **B.** Quantification of the area of MDAH/Control and MDAH/DDP cells. The mean area value of MDAH cells was 741.3218 ± 292.7147 and the area of the cisplatin resistant cells was 595.8555 ± 248.1282. Data are mean ± SD. Statistical comparisons were performed utilizing unpaired t-test with Welch correction for parametric data by Graphpad Prism and $p > 0.05$. **C.** Quantification of the total intensity fluorescence of intracellular calcium. Data are expressed as mean calcium fluorescence intensity ± SD. Statistical comparisons were performed utilizing unpaired t-test Welch corrected for parametric data; $p < 0.0001$ compared to MDAH/Control cells.

man 2012; Chen et al. 2013; Davis et al. 2014). Identifying the resistance mechanism is therefore very important. Mostly, cisplatin resistance is a secondary effect, and its mechanisms are unclear. Cisplatin resistance may be associated with the altered regulation of multiple signaling pathways, including downregulation of apoptotic signals, and activation of pro-survival signals (Ali et al. 2012; Galluzzi et al. 2012; Davis et al. 2014). Recent studies have shown that the calcium signal is implicated in a variety of important processes in tumor progression (e.g. proliferation and invasiveness). Calcium signal was also shown to be important in other processes in cancer progression including the development of resistance to current cancer therapies (Xu et al. 2015b). Therefore, we hypothesized that cancer drug resistance alters intracellular Ca^{2+} signaling through an IP_3Rs, RyRs, SERCAs, NCXs and PMCAs controlled pathway. We used MDAH cells and MDAH/DDP cells as an *in vitro* model of cisplatin resistance. We selected cisplatin for testing because cisplatin induces cytoplasmic Ca^{2+} elevation by an IP_3-dependent mechanism (Splettstoesser et al. 2007). We first showed the dose response in MDAH and MDAH/DDP cells after 24 h with cisplatin treatment. Our cytotoxicity studies revealed that MDAH/DDP cells were resistant to cisplatin-induced apoptosis, the IC50 of cisplatin was 5.5 times higher in these cells compared to their parent cell line.

In the present study, we demonstrated that cisplatin resistance has the ability to modulate calcium homeostasis in MDAH cells. In fluorescence microscopy images, cytosolic $[Ca^{2+}]$ was reduced in MDAH/DDP cells compared to levels in MDAH cells. Fluorescence microscopy results were confirmed by flow cytometer analysis. Cytosolic calcium levels were shown to decrease in cisplatin resistant cells compared with MDAH cells by flow cytometry. This finding emphasized that calcium signaling might play a major role in acquired drug resistance and failure in triggering apoptosis by cisplatin.

Various studies have described changes in different cancer cell types and models in intracellular Ca^{2+} signals induced by some anti-cancer agents. Drug treatments that appeared to increase Ca^{2+} influx as a result of short-term (0 to 8 h) exposure included 5-Fluorouracil (5FU) (Can et al. 2013), dexamethasone (Abdul-Azize et al. 2017), Tipifarnib (Cuevas 2011; Yanamandra et al. 2011) or ionizing radiation (Klumpp et al. 2016). Drugs such as arsenic trioxide (As_2O_3) (Gunes et al. 2009) and gamitrinib (Park et al. 2014) were suggested to promote ER Ca^{2+} release during short-term exposure. Short-term exposure of cancer cells to cisplatin (Shen et al. 2016; Chakraborty et al. 2017), doxorubicin (Giorgi et al. 2015a), or photodynamic therapy (PDT) (Giorgi et al. 2015b), may enhance ER-mitochondria Ca^{2+} transfer in some models. Examples of the remodeling of Ca^{2+} signaling with longer term drug treatments include altered store-operated Ca^{2+} entry (SOCE) as a result of treatment with 5-Fluorouracil (Kondratska et al. 2014; Tang et al. 2017), gemcitabine (Kondratska et al. 2014) or cisplatin (Schmidt et al. 2014). Long-term treatment with vemurafenib (Hegedus et al. 2017) can enhance cytosolic Ca^{2+} removal after store-operated Ca^{2+} entry. Altered ER calcium release mediated by SERCA inhibition is observed in cisplatin-resistant cancer cells (generated from prolonged exposure of cancer cells to cisplatin), i.e. reduced in resistant lung cancer cell lines(Schrodl et al. 2009), and increased in resistant ovarian cancer cell lines (Schmidt et al. 2014). Our

results confirm that the overall effect of drug treatment is to alter the [Ca^{2+}], effect that is likely to be linked to its ability to trigger apoptosis (Muscella et al. 2011).

The intracellular Ca^{2+} homeostasis is governed by a network composed of various Ca^{2+} channels, pumps, and exchangers. Intracellular Ca^{2+} alterations may be generated by an interaction of the drugs with IP_3 receptors, ryanodine receptors, NCX exchangers, SERCA and PMCA pumps resulting in a depletion of intracellular calcium stores. Therefore, we investigated and compared the possible presence of aberrations in multiple Ca^{2+} channels, pumps, and exchangers in MDAH and MDAH/DDP cells. The IP_3Rs are a ubiquitous family of Ca^{2+} release channels composed of three isoforms (1, 2 and 3) which present primarily in the ER. IP_3, produced by phospholipase C after cell activation by hormones, growth factors or neurotansmitters, diffuses into the cytosol, binds to and activates the IP_3R leading to Ca^{2+} release from the endoplasmic reticulum. ER, a major intracellular Ca^{2+} storage organelle, is involved in several biological processes (Foskett et al. 2007). Altered IP_3R expression level profiles were found in colon, glioma, gastric, head and neck cancer (Sakakura et al. 2003; Kang et al. 2010; Shibao et al. 2010; Hedberg et al. 2016). Over the last 20 years, IP_3R channels have emerged as key regulators that control cell death and survival in a variety of cellular systems (Joseph et al. 2007; Decuypere et al. 2011; Ivanova et al. 2014). Also, the role of IP_3R2 in cell death has been elucidated in different studies using different approaches. IP_3R2 channels are not only implicated in apoptosis but also play a role in cellular senescence (Vervloessem et al. 2015). There is evidence that cell death triggered by cellular exposure to cytotoxic compounds and agents that induce oxidative stress were associated with an increase in *IP_3R2* levels, and activity. Increasing oxidative stress in a neuronal cell line exposed to sublethal concentrations of tert-butyl hydroperoxide-mediated oxidative stress led to prominent upregulation of *IP_3R2* mRNA and protein levels, while *IP_3R1* and *IP_3R3*-expression levels remained unaltered (Kaja et al. 2011). Carboplatin (CARB) treatment can increase the activity of RYR Ca^{2+} release channels on the ER *via* GSTO1 (Lu et al. 2017). In our study, *IP_3R1*, and *IP_3R3* gene expression levels were relatively decreased in MDAH/DDP cells compared to MDAH cells, but *IP_3R2* gene expression level is decreased significantly in MDAH/DDP cells. This can mean two things: i) *IP_3R2*, similar to other *IP_3R* isoforms, is regulated in a biphasic way by the cytosolic [Ca^{2+}], meaning that a relatively low [Ca^{2+}] potentiate IP_3-induced Ca^{2+} release, while higher [Ca^{2+}] leads to an inhibition of the *IP_3R2* (Vervloessem et al. 2015) and, ii) dampening the IP_3R-mediated [Ca^{2+}], either by lowering *IP_3R* levels or altering the *IP_3R*-expression profile, by inhibiting the Ca^{2+}-flux properties of *IP_3R*s, or by lowering the ER Ca^{2+} content, which decreases the driving force for Ca^{2+} release into the cytosol upon *IP_3R* activation, will be cytoprotective (Akl and Bultynck 2013a).

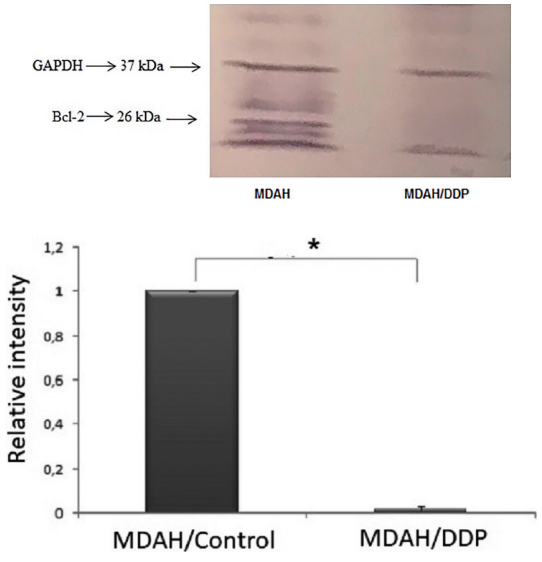

Figure 5. The expression of Bcl-2 (26 kDa) and GAPDH (37 kDa) were analyzed using Western blot analysis. GAPDH was used for normalization. The graph shows the quantification of Bcl-2 expression. Each column represents the mean ± SEM ($n = 3$). * $p < 0.00015$ compared to MDAH/Control.

Furthermore, it is important to emphasize that a complex interaction exists between IP_3Rs, and proteins from the B-cell lymphoma (Bcl-2) family involved in the control of apoptosis, whereby several interaction sites for such proteins have already been identified on the IP_3R (Distelhorst and Bootman 2011). Bcl-2 was shown to interact with IP_3R, and that this interaction involves binding of the BH4 domain of Bcl-2 to a region located within the regulatory and coupling domain of the IP_3R (Rong et al. 2009a). Bcl-2 may also regulate ER Ca^{2+} release through other mechanisms in addition to its interaction with IP_3R. One proposed mechanism involves Bcl-2 interaction with SERCA. These proteins pump Ca^{2+} ions from cytoplasm into the ER lumen, maintaining large ER luminal Ca^{2+} stores. This steep Ca^{2+} concentration gradient from ER lumen to cytoplasm facilitates Ca^{2+} efflux from the ER lumen *via* IP_3R channel opening, leading to cytoplasmic Ca^{2+} elevation. The interaction of Bcl-2 with SERCA attenuates ER Ca^{2+} filling, indirectly diminishing IP_3R-mediated Ca^{2+} release and Ca^{2+}-mediated apoptosis (Volpe et al. 1990; Dremina et al. 2004). We found that *SERCA1/2/3* gene expression levels considerably decreased in MDAH/DDP cells. Mutations, and altered expression levels of *SERCA* isoforms were determined in many cancers, including colon, prostate, and in lung cancers. To give an example, *SERCA3* expression was induced in colon adenocarcinoma cell lines during differentiation, and was reported to be lost during multistage process of colon tumorigenesis

(Brouland et al. 2005). Our results suggested that the down regulation of *SERCA* might be one of the reason of the decrease in intracellular calcium concentration.

The primary function of Bcl-2 is to inhibit apoptosis. Morphological and biochemical studies demonstrated that Bcl-2 has two major intracellular localizations as mitochondria, and the ER (Hockenbery et al. 1990; Monaghan et al. 1992; Jacobson et al. 1993; Krajewski et al. 1993). An increase in Bcl-2 was suggested to cause resistance to chemotherapeutic agents, and the down-regulation of Bcl-2 enhanced chemosensitivity (Reed et al. 1998). The other result was contradictory because the decrease of Bcl-2 protein expression was also shown in cisplatin resistance in SCLC H69 cells (Kumar Biswas et al. 2004). Bcl-2 was shown to constitutively be phosphorylated at Ser70 in H69 cells but not in H69/DDP cells. They suggested that a decrease in constitutively phosphorylated Bcl-2 in H69/CP cells appeared to be associated with cisplatin resistance (Kumar Biswas et al. 2004), and also with the phosphorylation of Bcl-2 regulated ER Ca^{2+} homeostasis (Bassik 2004). The other article consistent with the previous report suggested that primary SCLC patients with Bcl-2-positive tumors had a complete remission rate of 40% *versus* 27% complete remissions in patients with Bcl-2-negative tumors after initial chemotherapy (Kaiser et al. 1996). We found that the band of Bcl-2 protein expression disappeared on blotting membrane. These results suggest that the decrease in Bcl-2 protein expression may be due to the rearrangement of calcium homeostasis in resistant cells and due to the resistance to cisplatin-induced apoptosis.

IP_3Rs located at the ER membrane play an essential role in ER to mitochondria Ca^{2+} trafficking, and Bcl-2 could also regulate calcium homeostasis at the ER. Taking all the results together, the first is the reduction of *IP$_3$R* expression levels, especially *IP$_3$R2* gene expression level, may inhibit (or suppress) the transfer of Ca^{2+} from the ER to the mitochondria, which increases the apoptotic resistance of cells and may decrease the cellular responsiveness to apoptotic signaling in response to cellular damage or alterations (Akl et al. 2013a); the second is Bcl-2 inhibits IP_3-induced Ca^{2+} release by interacting *via* its BH4 domain of Bcl-2 with the modulatory and transducing domain of the *IP$_3$R*s (Chen et al. 2004; Rong et al. 2008, 2009). The binding site for the BH4 domain of Bcl-2 has been identified (Rong et al. 2008), and is conserved between the three *IP$_3$R* isoforms (Monaco et al. 2012). Importantly, a peptide tool designed to disrupt *IP$_3$R*/Bcl-2-complexes by targeting Bcl-2's BH4 domain (Rong et al. 2008, 2009a, 2009b; Monaco et al. 2012) was very effective in inducing intracellular Ca^{2+} overload, and in provoking cell death in DL-BCL cells that express high levels of *IP$_3$R2*, such as SU-DHL-4 cells (Akl et al. 2013b). Therefore, our results suggested that the interaction of Bcl-2, and *IP$_3$R2* on ER may inhibit Ca^{2+} transfer from ER to cytosol, protect the rise in Ca^{2+} increase in cytosolic, and prevents apoptosis.

PMCA is an important calcium transporter in all cells, and plays an essential role in maintaining low intracellular Ca^{2+} levels through the active transport of calcium across the plasma membrane. We showed that *PMCA1* expression levels increased, and *PMCA2/3/4* expression level decreased compared to parental MDAH cells. Furthermore, we found that Na^+/Ca^{2+} exchanger expression profiles differ between cisplatin resistant and parental cells. *NCX1* and *NCX2* expression levels were lower in MDAH/DDP cells. *NCX3* expression level was higher in resistant cells and this result was consistent with the result in the study of Pelzl et al. (2015).

In the light of these data, the induction of RyRs, IP_3Rs and SERCAs receptor and pumps on ER and NCX and PMCA channels on cell membrane, and also Bcl-2 protein expression were a result of cisplatin resistance, and cisplatin resistance must be further investigated.

Conclusion

Ca^{2+} signaling seems to play an important role from cancer initiations to drug resistance. It becomes evident that drug resistance is associated with major changes in the expression of specific Ca^{2+} channels/transporters/pumps. It is important to identify the dysregulated Ca^{2+} channels/transporters/pumps that contribute to chemoresistance, which may provide a promising chemotherapy for cancer patients. Therefore, evaluation of the blockers, inhibitors or regulators for Ca^{2+} channels/transporters/pumps as anti-cancer drugs seem to provide promising leads for novel cancer treatment in the future years.

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