



Research article

Modulation of calcium-binding proteins expression and cisplatin chemosensitivity by calcium chelation in human breast cancer MCF-7 cells

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ABSTRACT

Cisplatin (CDDP) is currently one of the most effective FDA-approved treatments for breast cancer. Previous studies have shown that CDDP-induced cell death in human breast cancer (MCF-7) cells is associated with disruption of calcium homeostasis. However, whether the sensitivity of breast cancer cells to cisplatin is associated with dysregulation of the expression of calcium-binding proteins (CaBPs) remains unknown. In this study, we evaluated the effect of the intracellular calcium chelator (BAPTA-AM) on viability of MCF-7 cells in the presence of toxic and sub-toxic doses of cisplatin. Furthermore, this study assessed the expression of CaBPs, calmodulin, S100A8, and S100A14 in MCF-7 cells treated with cisplatin. Cell viability was determined using MTT-based *in vitro* toxicity assay. Intracellular calcium imaging was done using Fluo-4 AM, a cell-permeant fluorescent calcium indicator. Expression of CaBPs was tested using real-time quantitative PCR. Exposure of cells to increasing amounts of CDDP correlated with increasing fluorescence of the intracellular calcium indicator, Fluo-4 AM. Conversely, treating cells with cisplatin significantly decreased mRNA levels of calmodulin, S100A8, and S100A14. Treatment of the cells with calcium chelator, BAPTA-AM, significantly enhanced the cytotoxic effects of sub-toxic dose of cisplatin. Our results indicated a statistically significant negative correlation between calmodulin, S100A8, and S100A14 expression and sensitivity of breast cancer cells to a sub-toxic dose of cisplatin. We propose that modulating the activity of calcium-binding proteins, calmodulin, S100A8, and S100A14, could be used to increase cisplatin efficacy, lowering its treatment dosage while maintaining its chemotherapeutic value.

1. Introduction

Cisplatin continues to be among the best drugs used in clinical oncology. It has been used in the treatment of several tumors in both children [1,2], and adults [3]. However, cisplatin chemotherapeutic efficacy has been hampered by two serious limitations: acquisition of cisplatin resistance in cancer cells [4] and increase in cytotoxicity to vital organs, leading to adverse effects including nephrotoxicity [5,6] and ototoxicity [2,7]. Despite the extensive current knowledge on molecular mechanisms implicated in cisplatin cytotoxicity, there is still active research to lower its cytotoxicity while maintaining its cancer-specific therapeutic benefits.

Calcium (Ca^{2+}) is a key player in cellular signaling controlling cellular processes resulting in proliferation or cell death [8,9]. At rest, cells maintain low intracellular calcium $[\text{Ca}^{2+}]_i$ through removal of calcium from cytoplasm via Ca^{2+} extrusion at the cell membrane and Ca^{2+} intake into the endoplasmic reticulum (ER) [10]. In addition,

calcium-binding proteins (CaBPs), are utilized by cells to buffer $[\text{Ca}^{2+}]_i$ and to regulate Ca^{2+} -mediated cellular processes. Increased $[\text{Ca}^{2+}]_i$ is mediated through several pathways, including Ca^{2+} release from ER through inositol 1,4,5 trisphosphate receptor (IP_3R) [11], calcium influx through plasma membrane in response to different signals such as store depletion, phospholipase C (PLC)-associated receptors, and diacylglycerol signaling [12, 13, 14, 15].

The remodeling of calcium signaling in cancer cells is regulated by multiple pathways, including Ca^{2+} reuptake and release at the plasma membrane and from intracellular stores, store-operated calcium entry, and regulation effect of CaBPs [16,17]. The differential regulation of these components depends on the cancer type and tumor stage [18, 19, 20]. The association of $[\text{Ca}^{2+}]_i$ with cisplatin-resistance has been demonstrated by several studies. In cisplatin-resistant lung cancer cells, $[\text{Ca}^{2+}]_i$ level was reduced compared to that of the sensitive parental cell line [21]. Other studies showed that cisplatin elevates $[\text{Ca}^{2+}]_i$ leading to apoptosis in several tumor cell lines [22,23]. Schrödl *et al* [24]

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demonstrated that low level of cisplatin resistance in lung cancer cells was correlated with disruption in the expression of IP₃R. Xu et al [25] demonstrated that cisplatin-induced Ca²⁺ release from the ER led to mitochondrial calcium overload and promoted apoptosis in cisplatin-sensitive but not cisplatin-resistant ovarian cancer cells. The same group reported that cisplatin resistance in these cells is mediated by decrease oxidative stress associated with failure of calcium regulation [26]. On the other hand, cisplatin resistance in head and squamous carcinoma cells was associated with PLC-dependent Ca²⁺ mobilization [27].

Calcium-modulating drugs, including calcium chelators, have been shown to differentially affect cancer cells sensitivity to CDDP [28]. In breast cancer cells, Al-Taweel et al [29] demonstrated that cisplatin induced cell death is associated with disruption of calcium homeostasis and that [Ca²⁺]_i increase was reduced in resistant MCF-7 cells. Other studies have reported that several members of the so-called calcium-binding proteins (CaBPs) that participate in Ca²⁺ homeostasis and signaling, are highly expressed in several types of tumors, including breast cancer [30, 31, 32, 33]. However, the effect of the combination of calcium signaling modulators on the sensitivity of MCF-7 cells at sub-toxic and toxic doses of cisplatin, and potential changes in expression of CaBPs has not been explored. In this study, we evaluated the effect of intracellular calcium chelator, BAPTA-AM, on the viability of MCF-7 cells in the presence of toxic and sub-toxic doses of cisplatin. We further aimed to study the changes in the expression of specific calcium-binding proteins that have been associated with breast tumor progression in CDDP-treated cells with and without intracellular calcium chelator.

2. Materials and methods

2.1. Materials

Cisplatin (CDDP) was purchased from TCI (Tokyo, Japan). BAPTA-AM from Abcam (Cambridge, MA, USA), and Fluo-4 AM from Thermo Fisher Scientific (Waltham, MA, USA). Glass-bottomed culture dishes for imaging from MatTek (Ashland, MA). Primers were ordered from Gene Link (NY, USA).

2.2. Cell culture and treatments

Human breast cancer (MCF-7) cells were grown at 37 °C with 5% CO₂ in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM L-Glutamine. After split, cells were maintained overnight to reach 60–70% confluency before treatments. Cisplatin was added to cultures, where indicated, and the cells were grown for an additional 18 h. BAPTA-AM was dissolved in DMSO and added 30 min before cisplatin to final concentration of 10 μM.

2.3. In vitro toxicity assay

Cell proliferation was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (In vitro Toxicology Assay Kit, MTT based, Sigma M-5655, St. Louis, MO, USA) according to the manufacturer's protocol. Briefly, 10,000 cells were seeded per well on 96-well plate overnight to allow attachment. After treatment with drugs, the MTT drug was reconstituted with serum free media and 30 μl was added to each well. Following incubation at 37 °C for 4 h, 300 μl MTT solubilization solution was added to each well. The plate was incubated in the dark for ~20 min with occasional mixing to dissolve MTT formazan crystals. Absorbance was measured at 490 nm using Biotek ELx800 microplate reader (Ontario, Canada). Cell viability percentage was calculated based on the absorbance ratio between treated and the untreated control multiplied by 100.

2.4. Light microscopy and fluorescence microscopy

Cells were photographed with an inverted OPTIKA XDS-2 microscope (Optika, Italy) with 10X and 20X objective. Fluorescent imaging was done on cells plated on poly-D-lysine coated glass-bottomed plates (MatTek Corporation) to reach 50–60% confluency before treated, as indicated. Intracellular calcium imaging was done using Fluo-4 AM, a cell-permeant fluorescent calcium indicator (ThermoFischer Scientific, Waltham, MA, USA). Following treatment, cells were washed in Hanks' balanced salt solution (HBSS, pH 7.2) and loaded with 5 μM Fluo-4 AM for 30 min in the dark at 37 °C. Cells were washed in HBSS and then incubated in the same buffer for another 20 min before imaging by Optika XDS-2 inverted microscope with M-795 fluorescence system (Optika, Italy) using green/FITC filter.

To visualize nuclei, cells were fixed in 4 % paraformaldehyde (PFA) for 10 min, washed with PBS, and incubated with DAPI (Sigma Aldrich, Missouri, USA). Images were taken using 40X objective and were processed using Adobe Photoshop CS6.

2.5. Real time-PCR

Total RNA was extracted from MCF-7 cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). cDNA was produced from 1.8 μg of total RNA by FIREScript RT cDNA Synthesis KIT (Solis BioDyne, Tartu, Estonia) using random primers following manufacturer's protocol. Real-time quantitative PCR was performed with 5x HOT FIREPol® EvaGreen® qPCR Supermix kit (Solis BioDyne, Tartu, Estonia) using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The cycling protocol was as follows: Initial activation at 95 °C for 12 min, followed by 40 cycles at 95 °C for 15 s, annealing at 53 °C for 30 s, elongation at 72 °C for 30 s. GAPDH was used as the reference gene, and fold change in gene expression was calculated making use of the comparative CT (2^{-ΔΔCT}) method. The relative mRNA abundance was determined by the ratio of the sample to control. Primers for tested genes were as follows: Calmodulin: forward 5'-GGCATTCCGAGTCTTTGACAA-3' and reverse 5'-CCGTCTCCATCAATATCTGCT-3', S100A8: forward 5'-GGGATGACCTGAAGAAATTGCTA-3' and reverse 5'-TGTGATATCCAACCTCTTTGAACCA-3', S100A14: 5'-GTCGGTCAGCCAACGGCAGAG-3' and reverse 5'-CAGGCCACAGTTGCTCGG-3', P21: forward 5'-CATGTGGACCTGTCCTGTGTA-3' and reverse 5'-GAA-GATCAGCCGGCGTTTG-3', and GAPDH: forward 5'-AAGGCTGGGGCT-CATTTGCA-3' and reverse 5'-ATGACCTTGCCACAGCCTT-3'.

2.6. Statistical analysis

The results are reported as means ± SEM. The data were tested for statistical significance using a one-way analysis of variance followed by a Tukey's multiple comparison test using GraphPad Prism 5. For analysis between two groups, Student's *t*-test was used. Differences were considered significant when *P* < 0.05.

3. Results

3.1. Differential changes of intracellular Ca²⁺ [Ca²⁺]_i levels in response to sub-toxic and toxic levels of cisplatin in human breast cancer MCF-7 cells

To study whether cisplatin resistance in MCF-7 correlates with intracellular Ca²⁺ concentration, we evaluated disturbances of intracellular calcium with sub-toxic and toxic doses of cisplatin (CDDP). Cells were treated with increasing doses of cisplatin (5 μM, 10 μM, 20 μM, 40 μM, and 100 μM) for 18 h and viability was determined by light microscopy (Optika) (Figure 1A), and MTT assay (Figure 1B). Morphologically, cells showed increase in the percentage of detachment, rounding, and shrinkage starting at 40 μM, which was intensified at 100 μM (Figure 1A).

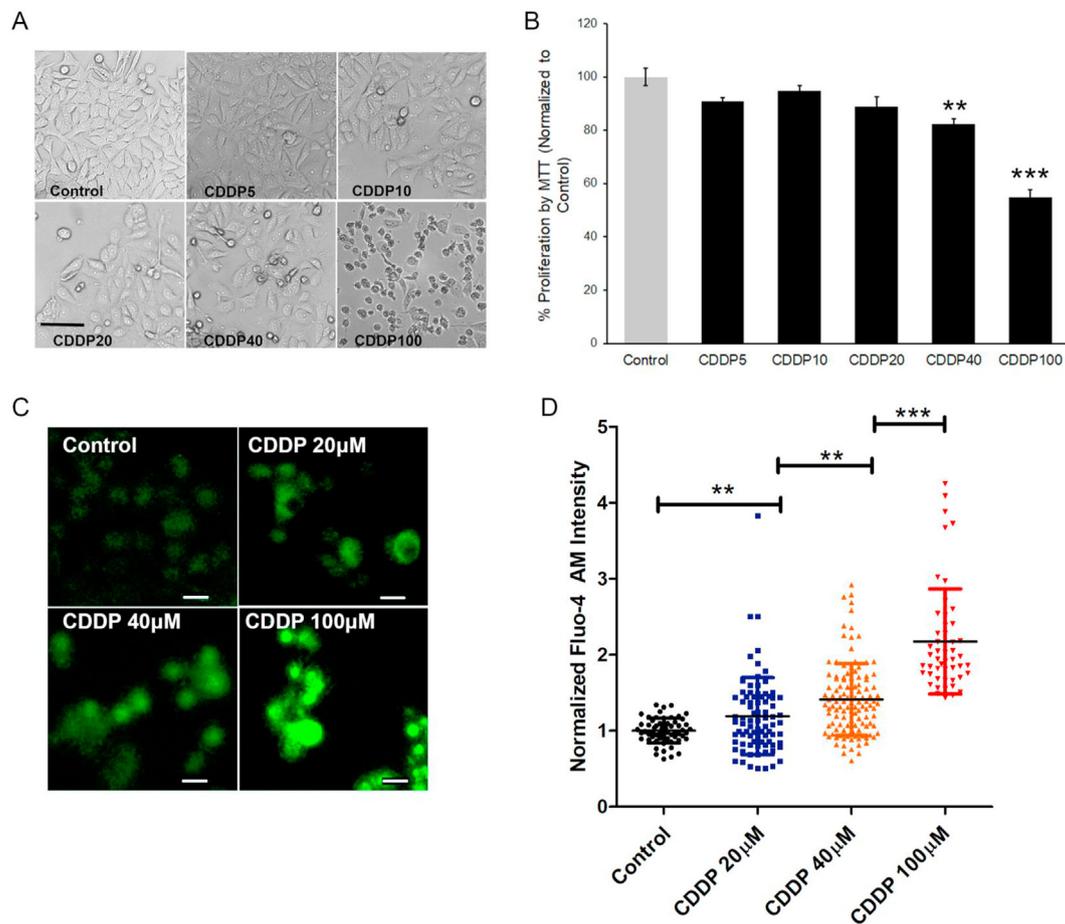


Figure 1. Differential changes of $[Ca^{2+}]_i$ levels in response to sub-toxic and toxic levels of cisplatin in human breast cancer MCF-7 cells. A. Light microscopy of MCF-7 cells untreated or treated with increasing doses of cisplatin, CDDP (5 μ M, 10 μ M, 20 μ M, 40 μ M, 100 μ M) for 18 h. Cells were photographed with an inverted microscope (Optika) (scale, 100 μ m). B. MTT analysis of MCF-7 cells treated as in A. After treatment, cell viability was determined using MTT-based in vitro toxicity assay. Data are represented as a percentage of MTT activity where untreated cells were taken to be 100%. Experiments were repeated twice, each sample in triplicates. Bar graphs represent mean \pm SEM. C. Cells were treated with cisplatin (20, 40, or 100 μ M) for 18 h, and incubated with the fluorescent calcium indicator, Fluo-4 AM. Calcium concentrations in the cytosol were observed by Optika XDS-2 inverted fluorescence microscope using FITC filter. D. Vertical scatter plots showing Fluo-4 AM fluorescence intensity in individual cells treated with CDDP 20 μ M (blue dots), CDDP 40 μ M (orange dots), and CDDP 100 μ M (red dots), or control cell (black dots). Black line represent mean and whiskers are error bars. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Cells treated with cisplatin (5 μ M and 10 μ M) had no obvious morphologic changes showing signs of cell death, while at cisplatin (20 μ M), cells showed minimal signs of apoptosis (Figure 1A). MTT assay revealed a significant reduction in cell viability following cisplatin 40 μ M and 100 μ M, consistent with morphological changes. Cisplatin at 20 μ M showed a slight decrease in cell viability but it did not reach statistical significance. To evaluate $[Ca^{2+}]_i$ levels, we monitored intracellular Ca^{2+} using a fluorescent Ca^{2+} indicator (Fluo-4 AM) in cells treated with sub-toxic (20 μ M), 40 μ M, and 100 μ M cisplatin doses (Figure 1C). Fluorescence microscopy was used to observe alterations in free $[Ca^{2+}]_i$ levels. As shown in Figure 1C, cisplatin treatment resulted in a dose-dependent increase in Fluo-4 AM fluorescence intensity, suggesting an increase in $[Ca^{2+}]_i$. Cell-by-cell quantification of Fluo-4 signal showed that fluorescent intensity was significantly increased with increasing doses of cisplatin (Figure 1D). These results suggested a higher increase in $[Ca]_i$ in MCF-7 cells in response to CDDP at toxic concentrations compared to sub-toxic dose.

3.2. Differential and dose-dependent regulation of gene expression of calmodulin, S100A8, and S100A14 in MCF-7 cells treated with cisplatin

We examined the expression of calcium-binding proteins, calmodulin, S100A8, and S100A14, in breast cancer cells in response to cisplatin (Figure 2). These CaBPs, in particular, has been associated with cell survival and apoptotic pathways in several breast cancer models [34, 35,

36]. Cells treated with a sub-toxic dose (20 μ M) of cisplatin showed significant decrease by 46% of control levels in calmodulin mRNA levels but no change in S100A14 mRNA levels. Conversely, S100A8 mRNA levels were significantly increased to 273% of control levels at this dose. At 40 μ M dose, cisplatin resulted in significant decrease by approximately 60% in calmodulin and 29% of control levels in S100A14 mRNA levels with a further decrease at 100 μ M dose. To test whether the decrease in the expression of three tested CaBPs mRNAs at toxic dose of CDDP resulted from a global decrease in gene expression following cisplatin, we studied in parallel the expression of p21, cyclin-dependent kinase inhibitor [37], known to be induced by cisplatin and contributes to the maintenance of cell cycle arrest [38, 39, 40, 41]. Cisplatin treatment induced a significant increase by 193%, 570%, and 450% of control levels in p21 mRNA at cisplatin 20 μ M, 40 μ M, and 100 μ M, respectively (Figure 2, top left panel), suggesting that the decrease in CaBPs mRNAs was not due to a global reduction in gene expression in cisplatin treated cells.

3.3. Intracellular calcium chelator, BAPTA-AM, sensitizes MCF-7 to sub-toxic dosage of cisplatin

We investigated the effect of BAPTA-AM, an intracellular Ca^{2+} chelator, on cisplatin-induced cell death by light microscopy, MTT assay, and nuclear morphology using DAPI staining (Figure 3). Morphologic

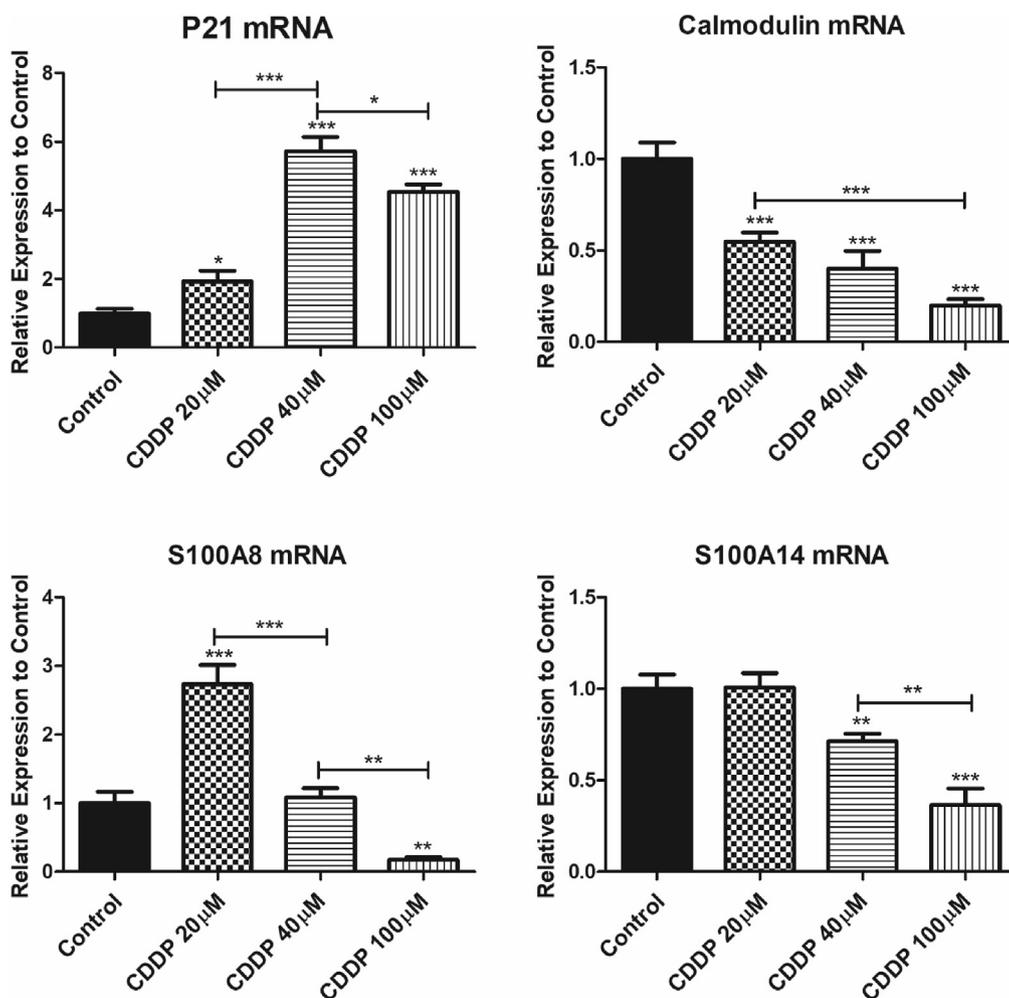


Figure 2. Differential and dose-dependent effect of cisplatin on mRNA expression levels of p21, calmodulin, S100A8, and S100A14. After cells were exposed to sub-toxic (20 μM), 40 μM, and 100 μM of cisplatin for 18 h, mRNA expression levels were quantified by qPCR. All mRNA expression levels were normalized against GAPDH. Statistically significant differences were determined by one-way ANOVA. Statistical comparison between two groups was performed using *t*-test. Each value is the mean ± SEM from three independent experiments, each with three replicates. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

assessment of cells pre-treated with BAPTA-AM showed an obvious increase of cells showing typical apoptotic features such as rounding, shrinkage, and detachment at 20 μM and 40 μM (Figure 3A). Quantifying cell viability by MTT assay showed approximately 30% and 15% decrease in viability of MCF-7 pre-treated with BAPTA-AM at 20 μM and 40 μM, respectively, compared to cisplatin alone (Figure 3B). However, no significant changes were observed in cells treated with toxic-dose (100 μM) in the presence of BAPTA-AM. Cells treated with BAPTA-AM or cisplatin (20 μM), showed no significant change in cell viability percentage (Figure 3B). To further confirm the decrease in viability at 20 μM and 40 μM doses, in the presence of BAPTA-AM, nuclear changes were examined by DAPI staining. As shown in Figure 3C (arrows), morphological characteristics of apoptosis, including nuclear fragmentation, nuclear condensation, and nuclear shrinkage, were apparent in cells treated with cisplatin (40 μM). However, few changes were observed in the cells treated with a sub-toxic dose of cisplatin (20 μM), BAPTA-AM, or untreated group where cells had normal nuclear morphology (Figure 3C, arrowheads). Cells treated with cisplatin (20 μM) in the presence of BAPTA-AM showed increased signs of nuclear changes typical of apoptotic cells, suggesting that BAPTA-AM enhances apoptosis and increases the sensitivity of MCF-7 to a sub-toxic dose of cisplatin.

3.4. Quantifying relative changes in intracellular calcium in the presence of calcium chelator, BAPTA-AM

We monitored intracellular calcium alterations in the presence of BAPTA-AM at single-cell level using Fluo-4 AM imaging (Figure 4A). In cells treated with 20 μM cisplatin we detected a significant increase in

Fluo-4 AM fluorescence compared to control cells (Figure 4A and B), consistent with our previous results demonstrating increase in intracellular calcium in a dose-dependent manner (Figure 1C and Figure 1D). Cells treated with CDDP 20 μM in the presence of BAPTA-AM showed a decrease in mean Fluo-4 signal compared to CDDP 20 μM alone. Interestingly, cells treated with cisplatin (20 μM) in the presence of BAPTA-AM exhibited distinct cell heterogeneity. We chose the cutoff value of 2 based on the maximum value obtained in the control sample. Interestingly, 8.3% of cells (11 out of 131) treated with CDDP 20 μM in the presence of BAPTA-AM exhibited a two-fold or higher increase in Fluo-4 AM signal, compared to 1.6% (2 out of 118 cells) in CDDP 20 μM as shown in Figure 4B. This may be due to saturation of the BAPTA-AM buffering capacity as previously reported [42,43]. Cells treated with CDDP 40 μM showed a significant increase in mean intensity compared to CDDP 20 μM. Similarly, in the presence of BAPTA-AM, cells treated with CDDP 40 μM showed a decrease in mean Fluo-4 signal, as compared to CDDP 40 μM alone. However, 5.2% (7 out of 134 cells) showed two-fold or higher increase in Fluo-4 AM signal in CDDP 40 μM in the presence of BAPTA, compared to 11.5% (15 out of 130 cells) in CDDP 40 μM alone. These results suggest that moderate elevation of [Ca²⁺]_i contribute to survival at sub-toxic dose of cisplatin.

3.5. BAPTA-AM induced sensitization to sub-toxic-dose cisplatin is associated with decrease in p21, calmodulin, S100A8, and S100A14 mRNA expression

We next evaluated the changes, in the presence of BAPTA-AM, on the expression of p21, calmodulin, S100A8, and S100A14. As expected, sub-

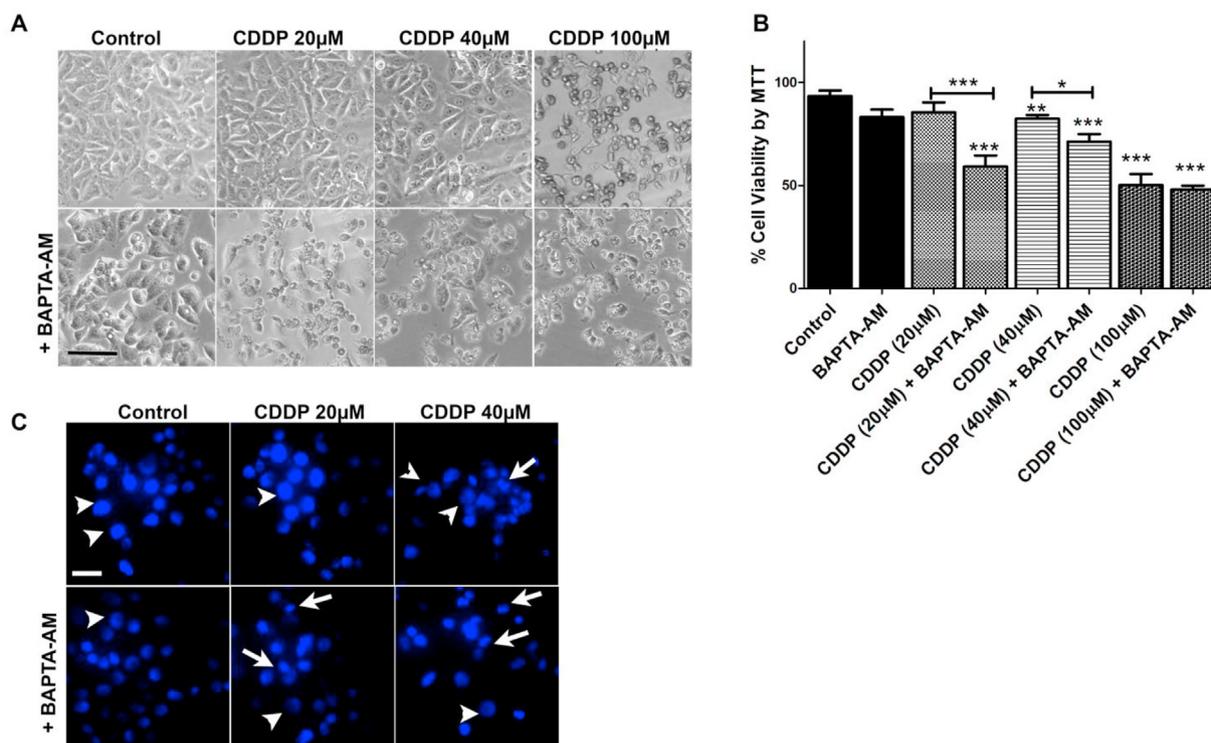


Figure 3. Intracellular calcium chelator, BAPTA-AM, sensitizes breast cancer MCF-7 cells to sub-toxic dose of cisplatin. **A.** Light microscopy images of MCF-7 cells from untreated (control), and those treated with BAPTA-AM (10 µM) for 30 min before exposure to 20 µM, 40 µM, or 100 µM cisplatin for 18 h. Scale bar: 100 µM. **B.** Cell viability determined using MTT assay. Bars denote the percentage of cell viability mean ± SEM from two independent experiments performed in triplicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **C.** Representative fluorescent images of MCF-7 cells after DAPI staining. Cells were grown on glass-bottom plates before treated as in **A**. Cells were then fixed with 4% paraformaldehyde, and stained with DAPI. Apoptosis was studied with a fluorescence microscope with DAPI filter and 40X oil objective. Scale bar: 20 µM. White arrows indicate apoptotic nuclei, and white arrowheads indicate normal nuclei.

toxic CDDP induced a significant increase in p21 mRNA, and this was augmented at higher doses, 40 µM, and 100 µM (Figure 5A). In the presence of BAPTA-AM, p21 mRNA levels were decreased by 44% when compared with cisplatin (20 µM) alone. Similarly, the induction of p21 at cisplatin (40 µM) and (100 µM) was significantly decreased in the presence of BAPTA-AM, suggesting that p21 expression is inversely associated with cell death in these conditions. Cells treated with BAPTA-AM showed no effect on p21 mRNA, but significant increase in calmodulin, S100A8, and S100A14 mRNA, compared to control cells, suggesting possible compensation for greatly decreased cytosolic calcium. Calmodulin mRNA levels decreased by 50% after cisplatin (20 µM) (Figure 5B) where cells showed minimal cell death. In the presence of BAPTA-AM, calmodulin mRNA showed further decrease up to 62% after cisplatin (20 µM), and decrease to 86% after cisplatin (40 µM) and (100 µM) (Figure 5B). Cells treated with sub-toxic (20 µM) cisplatin showed an increase in S100A8 and this was reduced to 32% in the presence of BAPTA-AM (Figure 5C). Similarly, S100A14 mRNA was significantly decreased with BAPTA-AM as compared to cisplatin alone (Figure 5D). S100A14 mRNA levels were similar in control and CDDP (20 µM) treated samples. Cells treated with CDDP (40 µM) in the presence of BAPTA-AM showed significant decrease in S100A8 mRNA but no change in S100A14 mRNA. No changes in S100A8 and S100A14 mRNA levels in the presence of BAPTA-AM at CDDP (100 µM) compared to CDDP (100 µM) alone.

Overall our results demonstrate that increased sensitivity to sub-toxic cisplatin in the presence of BAPTA-AM is correlated with decrease mRNA levels for p21 and calcium-binding proteins calmodulin, S100A8, and S100A14. At higher CDDP doses (40 µM and 100 µM), BAPTA-AM has differential effects on the expression of calmodulin, S100A8, and S100A14.

4. Discussion

Several studies demonstrated the interaction between cisplatin activated molecular pathways and calcium signaling in multiple types of cancer, including breast cancer cells [44]. In cisplatin-sensitive and resistant MCF-7 cells, real-time calcium imaging demonstrated reduced increase in $[Ca^{2+}]_i$ in cisplatin-resistant MCF-7 cells compared to sensitive cells, and that was associated with decreased cytotoxicity in CDDP-resistant MCF-7 at lower CDDP doses [29]. However, the effect of combination of calcium signaling modulators on the sensitivity of MCF-7 cells to sub-toxic and toxic doses of cisplatin, and potential changes in expression of calcium-binding proteins (CaBPs) have not been explored. Initially, we investigated the expression of CaBPs, calmodulin, S100A8, and S100A14 in MCF-7 cells exposed to sub-toxic (20 µM) and toxic doses (40 µM, 100 µM) of cisplatin. Several CaBPs were reported to be highly expressed in several types of tumors, including S100A proteins in gastric cancer [45], S100A8 and S100A9 in skin [46], and colorectal cancer [47], and calmodulin, S100A8 and S100A14 in breast cancer [30, 31, 32, 33]. Calmodulin has been reported to be overexpressed in breast tumors [48,49]. Calmodulin antagonists have been shown to overcome resistance to TRAIL-based therapy in triple-negative breast cancer [50]. Several S100 proteins are differentially expressed in breast cancer tissue compared with normal tissue [51], suggesting that these proteins may be important in tumor development and progression [52,53]. High expression of S100A8 and S100A14 was associated with poor survival and higher incidence in breast cancer patients [32,54]. In addition, increased S100A8 expression enhanced the resistance of breast cancer cells to chemotherapy by activating pro-survival pathways [55]. Here in our study, we aimed to compare the expression of calmodulin, S100A8, and

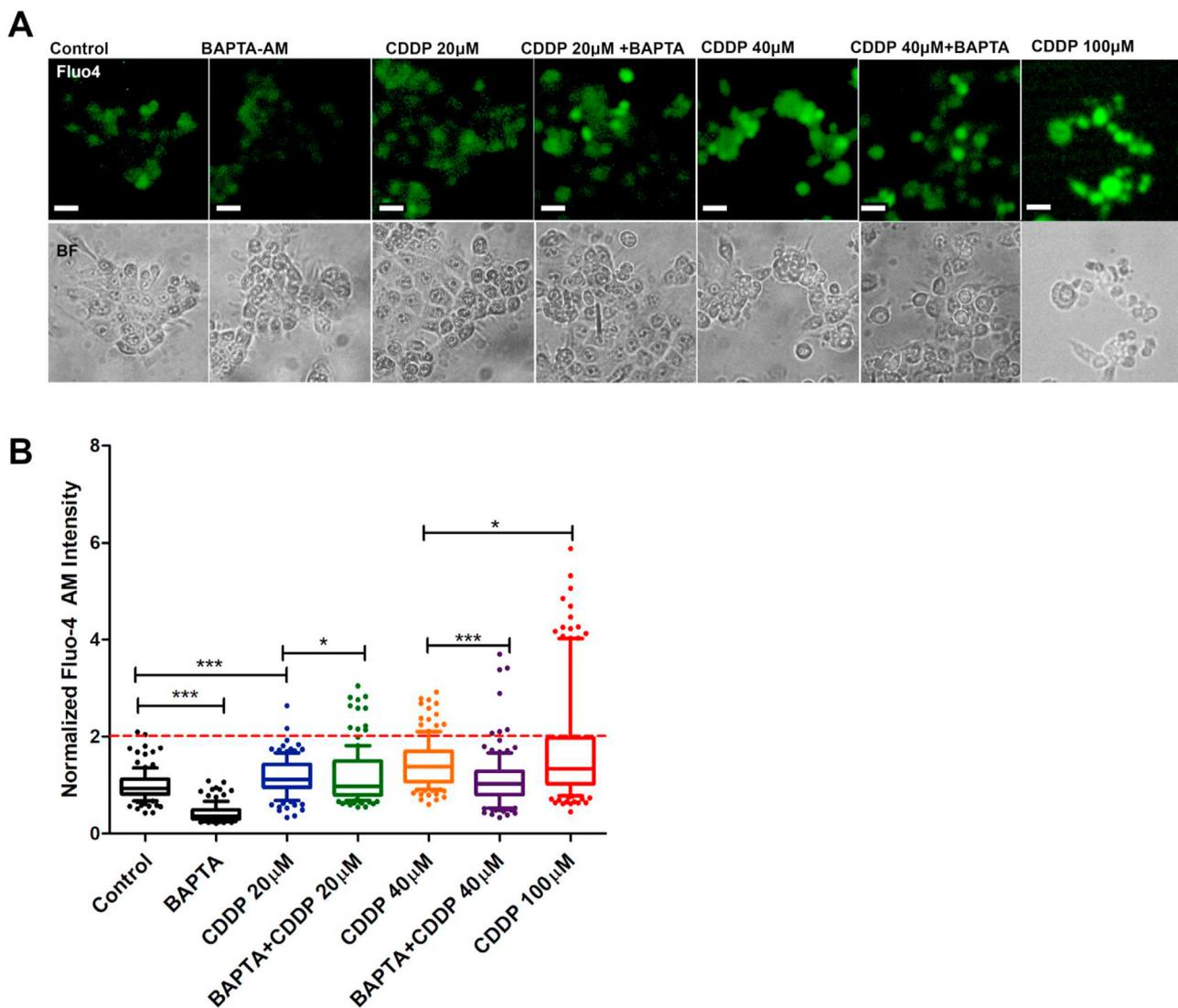


Figure 4. A. Effects of BAPTA-AM on cisplatin-induced intracellular Ca^{2+} changes. Cells grown on glass-bottom dishes were either untreated (control) or treated with BAPTA-AM (10 μM) for 30 min before exposure to 20 μM or 40 μM cisplatin. Some cells were exposed to 100 μM CDDP. After treatment, cells were washed HBSS (pH 7.2) and loaded with 5 μM Fluo-4 AM for 30 min in the dark at 37 $^{\circ}\text{C}$ as described in under “Materials and Methods”. The intracellular fluorescence of Fluo-4 was imaged using fluorescence microscopy with a green emission filter. BF, bright field. Scale bar: 20 μm . B. Fluo-4 fluorescence from individual cells was quantified and shown as a Box plot with horizontal lines depicting medians and 10–90 percentile whiskers. Dashed red line represent cutoff of maximum value obtained in control sample. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 89$ –127 cells).

S100A14 at sub-toxic and toxic doses of cisplatin. Based on cell viability, we considered 20 μM of cisplatin as a sub-toxic dose as cells treated with this dose showed slight decrease in cell viability but it did not reach statistical significance. At sub-toxic CDDP (20 μM), we found differential effects of cisplatin on calmodulin, S100A8, and S100A14 mRNA levels, while these genes showed consistent decrease at CDDP (40 μM) and (100 μM) (Figure 2). The decrease in mRNA levels is unlikely due to a global decrease in expression following cisplatin as the expression of p21, cell cycle inhibitor, was increased in these conditions. The correlation between decrease of calmodulin, S100A8, and S100A14 mRNA levels (Figure 5) and decreased viability (Figure 3), suggests that these CaBPs may be an integral part of the survival mechanism against sub-toxic cisplatin in MCF-7 cells.

Monitoring $[\text{Ca}^{2+}]_i$ demonstrated a significant increase in Fluo-4 AM intensity in cells treated with sub-toxic CDDP, with a greater increase in intensity at higher doses (Figures 1C and 1D). Similar results, while using different doses and time-course of cisplatin, were reported by Al-Taweel et al. [29]. We were interested to see the changes in the gene expression profile of several CaBPs associated with breast cancer cells under

sub-toxic dose of cisplatin that has been enhanced by calcium-dependent modulation. We tested cisplatin toxicity in the presence of different calcium signaling modulators (preliminary data) and selected BAPTA-AM to correlate changes for CaBPs with sub-toxic (20 μM) cisplatin.

The combined effect of cisplatin and calcium signaling modulators on the viability of cancer cells has been tested in numerous studies. Shen et al [56] reported that combining a toxic-dose of cisplatin with BAPTA-AM markedly decreased the cell growth inhibition and rate of apoptosis in Hela cells. This study also demonstrated that intracellular Ca^{2+} participated in ER stress-associated apoptosis induced by cisplatin. On the other hand, Ma et al. [26] demonstrated that BAPTA-AM prevented the increase in cytosolic Ca^{2+} and inhibited cisplatin-induced ROS generation in ovarian cancer cells. This study suggested that the maintenance of intracellular Ca^{2+} homeostasis protects cells from cisplatin-induced apoptosis. Here we show that BAPTA-AM enhanced the sensitivity of MCF-7 cells to sub-toxic dose of cisplatin suggesting the importance of maintaining of low levels of $[\text{Ca}^{2+}]_i$ in the survival of these cells under sub-toxic dose of the drug. Cell-by-cell analysis of Fluo-4 AM fluorescence

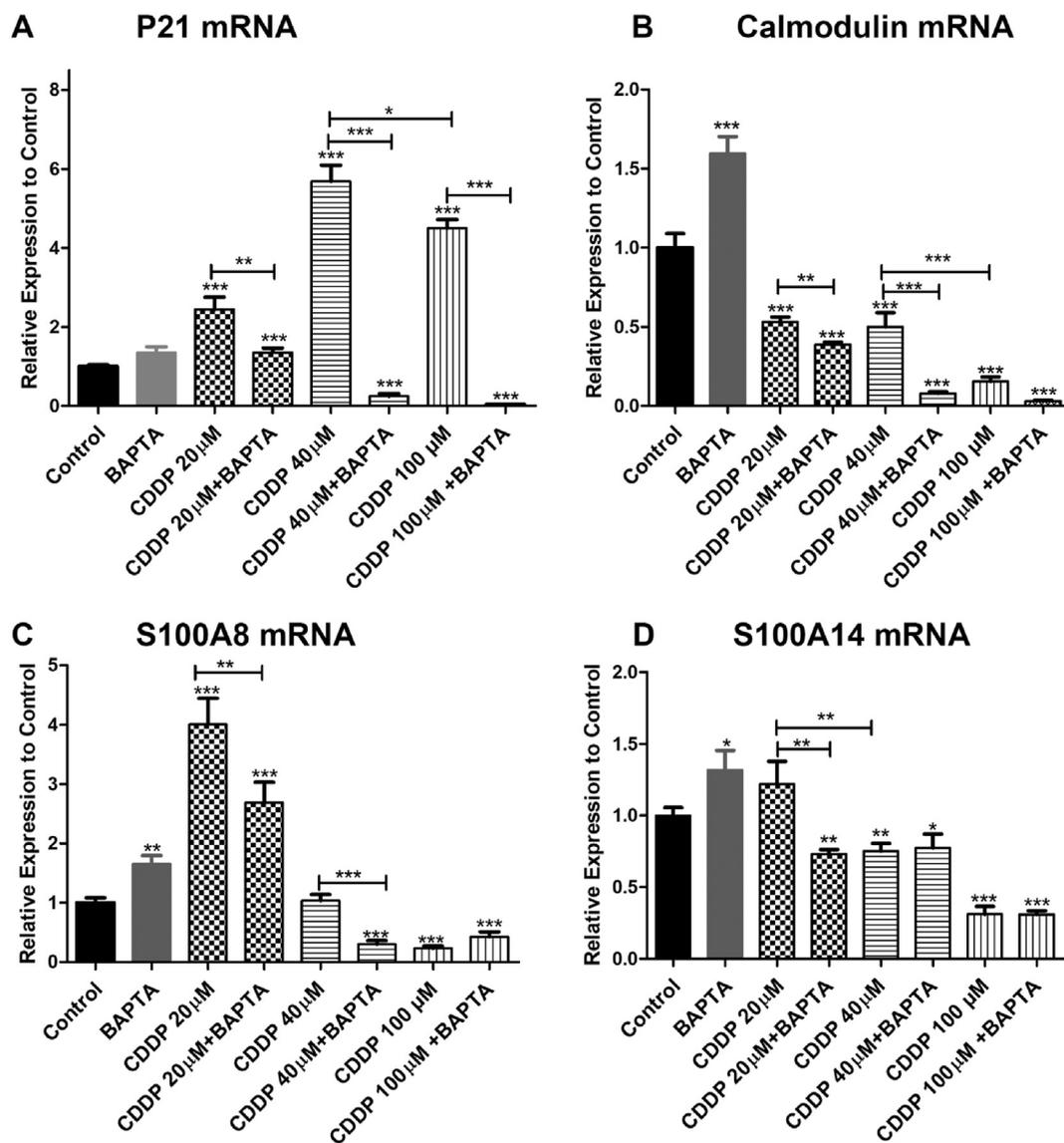


Figure 5. BAPTA-AM induced sensitization by sub-toxic-dose of cisplatin is associated with a decrease in mRNA expression of p21, calmodulin, S100A8, and S100A14. A. Cells were exposed to sub-toxic (20 μM), 40 μM, and 100 μM of cisplatin for 18 h, in the presence or absence of BAPTA-AM. After treatment, total RNA was extracted from cells followed by cDNA synthesis and real-time qPCR. All mRNA expression levels were normalized against GAPDH. The data show the normalized expression of (A) p21, (B) calmodulin, (C) S100A8, and (D) S100A14, relative to the expression of the control. Each value is the mean ± SEM. Experiments were conducted with triplicate samples and repeated at least twice. Statistically significant differences were determined by one-way ANOVA. Statistical comparison between two groups was performed using *t*-test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

showed significant decrease in mean intensity, in the presence of BAPTA-AM, at sub-toxic (20 μM) and toxic dose (40 μM) of cisplatin. This might suggest that BAPTA-AM is enhancing cisplatin cytotoxicity by disrupting $[Ca^{2+}]_i$ homeostasis necessary for survival. The profound effect of BAPTA-AM with sub-toxic cisplatin compared to a higher dose might be explained by studies showing dose-dependent rise in $[Ca^{2+}]_i$ associated with apoptotic death by CDDP [57]. This is partially supported by analyzing cells showing high Fluo-4 fluorescence (higher than the cutoff value used) in 11.5% of cells treated with CDDP 40 μM compared to 1.6% with sub-toxic CDDP (Figure 4). This might explain the weak effect of BAPTA-AM with CDDP 40 μM where increase in $[Ca^{2+}]_i$ could be a stressor signal compared to survival signal with sub-toxic cisplatin. Although this remains a speculation at this point, previous studies have shown activation of survival pathways promoting autophagy at low dose cisplatin [58].

On the other hand, S100 proteins have been shown to be closely associated with calcium-mediated signalling, similar to IP3R1 which

plays a critical role in the calcium-associated apoptotic pathway [8,17,18]

Calcium-binding proteins, including calmodulin and S100 proteins, have been shown to be an integral component of cellular events controlled by Ca^{2+} [59]. Calmodulin has been shown to play crucial roles in cell cycle progression through G1 and mitosis [60,61]. Several chemical inhibitors of calmodulin have been used to inhibit several types of tumors [62]. In human ovarian cells, combination of calmodulin antagonist and cisplatin potentiated the effect of cisplatin in these cells [63]. Using a comparative analysis of expression, we show a significant decrease in calmodulin mRNA levels by ~46%, 60%, and 80% in cells treated with sub-toxic CDDP (20 μM), 40 μM, and 100 μM, respectively. The enhanced sensitivity of MCF-7 cells treated with sub-toxic CDDP combined with BAPTA-AM is associated with a further decrease by 30% in calmodulin expression, suggesting a negative correlation between calmodulin expression and cisplatin sensitivity in MCF-7 cells. To test whether cisplatin treatment is causing a global reduction of gene expression, we studied the expression of p21 which is known to be

induced by cisplatin. As expected, p21 levels in cells treated with sub-toxic CDDP was elevated although to a lesser degree compared to CDDP higher doses. In the presence of BAPTA-AM, the observed increase in p21 at CDDP 20 μM was significantly blunted and tended to reverse as the concentration of cisplatin was increased (Figure 5), suggesting that increase in p21 is modulated by intracellular $[\text{Ca}^{2+}]_i$. These results support studies demonstrating enhanced apoptosis rate to cisplatin in p21 knockdown ovarian and testicular cancer cells [64,65], and kidney cells [40]. Similarly, we found negative correlation between S100A14 expression and cisplatin sensitivity in cells treated with sub-toxic CDDP in the presence of BAPTA-AM, while no significant change in CDDP alone. Xu et al [25] reported that siRNA-mediated silencing of S100A14 in MCF-7 cells caused inhibition of HER-mediated proliferation. Our results suggest a positive role for calmodulin, S100A8, and S100A14 in the survival of MCF-7 cells at sub-toxic levels of cisplatin. S100A8 mRNA levels increased after cisplatin treatment at sub-toxic levels (Figures 2C and 5C), consisting with the previous reports associating increased S100A8 expression with the poor prognosis in breast cancer [66]. Cells treated with cisplatin in the presence of BAPTA-AM had significantly blunted the induction of S100A8 mRNA levels as compared to cisplatin alone. In the presence of BAPTA-AM, CDDP 40 μM caused significant decrease by 70% in S100A8 mRNA levels, while no significant change in S100A8 expression was observed at CDDP 100 μM , suggesting a differential dose-dependent regulation of S100A8 by cisplatin, in the presence of BAPTA-AM. Our findings are consistent with studies showing that knockdown of S100A8 decreased proliferation in thyroid carcinoma cells [67] and enhanced arsenic trioxide-induced apoptosis in leukemia cells [68].

5. Conclusions

In conclusion, our data demonstrated decrease in mRNA levels in calcium-binding proteins, calmodulin, S100A8, S100A14 at toxic doses of cisplatin (40 μM and 100 μM). At sub-toxic CDDP (20 μM) levels, we found differential gene expression changes in calmodulin and S100A8 while no significant change in S100A14 mRNA levels at this dose. We also demonstrated that the sensitivity of sub-toxic cisplatin is enhanced by intracellular calcium chelator, BAPTA-AM. Furthermore, BAPTA-AM prevented the increase in intracellular calcium induced by cisplatin. Enhancing sub-toxic cisplatin sensitivity by BAPTA-AM is accompanied by decrease in calmodulin, S100A8, and S100A14, compared to sub-toxic dose alone. We propose that modulating the activity of CaBPs plays a role in sensitivity of MCF-7 cells to sub-toxic cisplatin doses. Future studies investigating the biological function of calmodulin, S100A8, and S100A14 in breast cancer should verify the possibility that these proteins are targets to enhance CDDP-based chemotherapy.

Declarations

Author contribution statement

Rawad Hodeify: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Shoib Sarwar Siddiqui: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rachel Matar, Maxime Merheb, Cijo George Vazhappilly: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Hussain Al Zouabi, John Marton: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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