GRP78 Up-regulation Leads to Hypersensitization to Cisplatin in A549 Lung Cancer Cells

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Abstract. *Background: GRP78 is one of the stress proteins linked to different functions in the cell. Previous reports have shown opposing functions of GRP78 in relation to drug resistance/sensitivity. In the current study, we examined the role of GRP78 in cisplatin-treated A549 cells. Materials and Methods: GRP78 was over-expressed in A549 cells with 2-deoxyglucose (2-dG) or tunicamycin (TM) treatments for 48 h and subsequently exposed to cisplatin for 2 h. Viability of these cells was determined at 0, 12, 24, 36 and 48 h afterwards. Results: We showed that A549 cells are hypersensitized to cisplatin following a transient GRP78 up-regulation. This hypersensitization is caused by the activation of JNK pathway and NF-κB, leading to early onset of apoptosis. Conclusion: Induction of GRP78 can be used as a potential tool to overcome drug resistance in lung cancer cells.*

Glucose regulated protein of 78-kDa (GRP78) is an endoplasmic reticulum (ER) protein of virtually all cells and has a high degree of amino acid sequence homology with the HSP70 and is, therefore, considered a member of the HSP70 family (1-3). GRP78 transiently associates with nascent proteins, facilitating their translocation into the ER and aiding in their folding and transport through the ER (4). This protein is crucial for survival and its loss leads to peri-implantation lethality in mice (5). Although GRP78 is constitutively expressed, its synthesis can be induced by a variety of stressful conditions or treatments that interrupt protein folding and assembly within the ER. Alteration of glucose metabolism by glucose starvation or treatment with 2-deoxyglucose (2-dG), hypoxia, depletion of calcium from intracellular calcium stores by thapsigargin or calcium ionophore treatment, inhibition of cellular glycosylation by agents such as tunicamycin (TM), and exposure to glucosamine could result in the induction of GRP78 (6-9). Other conditions which could trigger GRP78 synthesis are low extracellular pH (7, 9) and hypoxia (10-12).

Cisplatin is a platinum drug which forms interstrand-, intrastrand- and mono-adducts with neucleophilic N7 sites of purine bases in DNA (13). It is the most commonly used drug for the treatment of solid tumors *e.g.* testical, ovarian, head and neck, lung and the bladder (14). One main obstacle in the use of cisplatin, however, is the development of resistance in tumor cells (13, 15, 16) which has been attributed to various factors including reduction of DNA cross-linking due to drug accessibility to DNA and increased DNA repair (17). Mitochondrial pathways have been reported to be involved in the induction of apoptosis by cisplatin (18, 19) which could lead to release of cytochrome *c* and activation of caspase-12, which participates in ER stress-induced apoptosis (20-23). Another study shows that cisplatin could induce apoptosis in enucleated human melanoma and colon cancer cells by ER stress which is exhibited by alterations in calcium homeostasis required for calpain activation (24). On the other hand, chemoresistance in a wide spectrum of tumors in patients has been linked to the up-regulation of GRP78 (25). GRP78 is not exclusively localized to the ER, but also found in the cytoplasm and the intra-membrane which has been associated with chemoresistance (25).

In the present study, we examined if GRP78 over-expression could lead to resistance to the drug cisplatin in A549 human non-small cell lung cancer cells. GRP78 was

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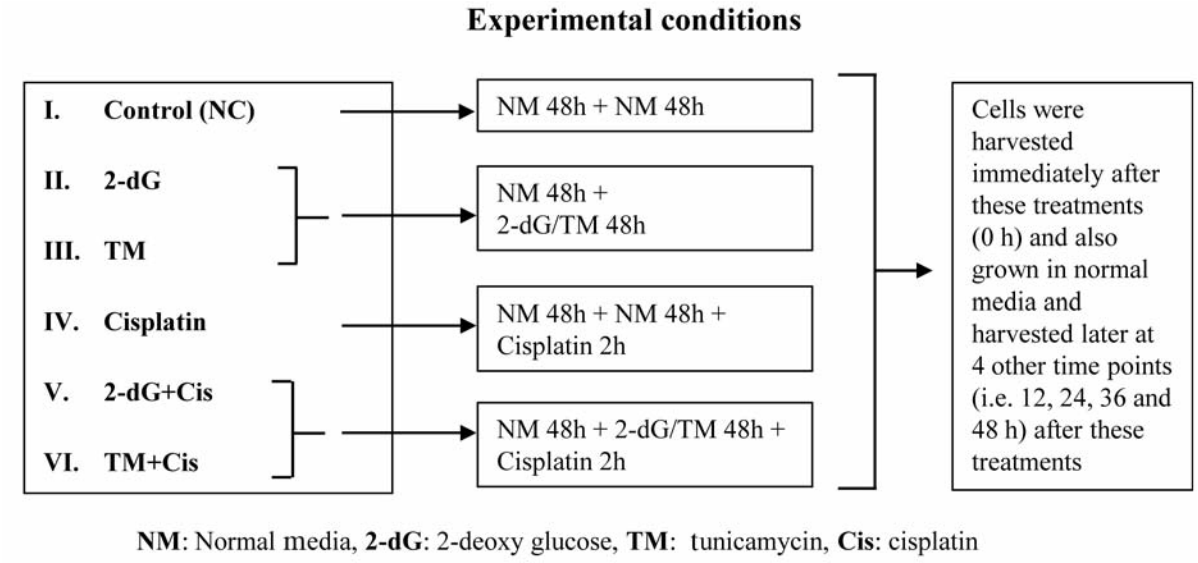

Figure 1. *A549 human lung cancer cells were grown for 48 h in normal media (NM) and then exposed to 10 mM 2-deoxy glucose (2-dG) and 250 nM tunicamycin (TM) separately for 48 h followed by 2 h cisplatin (Cis) treatment (V and VI). Cells were harvested immediately after cisplatin treatment (0 h), and also grown in normal media and harvested at 4 other time points after cisplatin treatment (i.e. 12, 24, 36 and 48 h). Three control groups were also used: untreated normal control (I), cisplatin treatment for 2 h (IV), and treatment with 2-dG/TM for 48 h (II and III). (NM- Normal Media; 2-dG - 2-deoxy glucose; TM- tunicamycin; Cis- cisplatin).*

overexpressed by exposing A549 cells to a) 2-dG, or b) TM. Subsequently, these cells were treated with cisplatin and it was expected that they become resistant to this drug upon GRP78 overexpression. To the contrary, A549 cells become hypersensitive to cisplatin treatment under GRP78-induced conditions. We further examined the possible involvement of c-Jun N-terminal kinase (JNK) and NF-κB pathways in the induction of apoptosis in this cell type under the described condition above.

Materials and Methods

Materials. 2-dG, TM, *cis*-Platinum (II) diammine dichloride (cisplatin) and propidium iodide were obtained from Sigma Chemicals Co. (St Louis, MO, USA). 2-dG was dissolved in distilled water to prepare stock solution of 2 M whereas TM was dissolved in DMSO to make 1 mM and 200 μM stock solutions, and aliquots were kept frozen at –20˚C. Cisplatin was prepared fresh in DMSO (10 mM). A549 human non-small cell lung cancer cells were obtained from ATCC.

Cell culture maintenance and clonogenic survival assay. A549 human non-small cell lung cancer cells were maintained in RPMI-1640 media (Cellgro, Manassas, VA, USA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Cellgro) and 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA). To determine the effect of GRP78 over-expression on the cytotoxicity of cisplatin by clonogenic survival assays, A549 cells were grown in 10 mM 2-dG or 250 nM tunicamycin separately for 48 h to induce up-regulation of GRP78. Subsequently, cells were washed twice with Hank's balanced salt solution (HBSS; Cellgro), detached with 0.05% trypsin (Cellgro), diluted in regular growth medium, and allowed to attach in 60-mm plates for 5 h. Following attachment, cells were subjected to cisplatin treatment (30, 60 and 90 μM) for 1 h at 37˚C in growth medium to determine clonogenic cytotoxicity. After 1 h of treatment, the drug-containing media were aspirated-off and cells were washed twice with HBSS and finally supplied with 8 ml of pre-warmed fresh medium. After 8 days of incubation at 37˚C, cells were fixed and stained with methylene blue-formaldehyde solution. Only those colonies containing more than 50 cells were visually counted. Surviving fraction for each concentration was calculated as the number of colonies for each concentration divided by the mean number of colonies for the control.

Treatment conditions. A total of 6 experimental groups and five time points were considered (Figure 1). A549 cells were grown in media containing 2d-G (10 mM) or TM (250 nM) for 48 h followed by treatment with cisplatin (Cis) for 2 h (2-dG+Cis or TM+Cis conditions). Drug-containing media were aspirated-off; cells were washed with HBSS, supplied with pre-warmed fresh medium and allowed to grow. Cells were harvested at 0, 12, 24, 36 and 48 h after cisplatin treatment. Control groups *i.e.* those without any treatment (normal control or NC), cisplatin control (Cis-only) and 2 more groups treated with 2-dG- or TM-only were also run.

Cell lysate preparation, and western blotting. Cells were rinsed twice with HBSS after the treatment, trypsinized, suspended in medium and collected by centrifugation. The cell pellets were washed twice with cold HBSS and finally re-suspended at 4˚C in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA)

supplied with complete mini protease inhibitors (Roche Diagnostics, Basel, Switzerland) and PMSF (Sigma). Lysate was sonicated for 10 sec to shear the DNA. Aliquots were removed and protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Whole-cell protein samples were separated by SDS-PAGE using Bio-Rad Criterion pre-cast gels. 12.5% gels were used for detecting IκB, phospho-NF-κB, phospho-MKK4, phospho-c-Jun and phospho-JNK. For GRP78 and phospho-ATF-2, 10% gels were used albeit, for PARP 7.5% gels were used. Proteins were then transferred electrophoretically at 4˚C onto Immobilin-P PVDF membranes (Millipore, Bedford, MA, USA) for 30 min at 100V. Membranes were washed in the wash buffer (TBS/T; Tris buffered saline with 0.1% tween-20) for 5 min and later blocked for 1 h in a 5% solution of non-fat dry milk. Subsequently the blots were incubated overnight with primary antibodies in 5% BSA at 4˚C. After washing 3 times in wash buffer for 5 min each, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in 5% non-fat dry milk at room temperature for 1 h. All antibody dilutions and blocking buffer were prepared in the wash buffer. Antibodies for all the kinases were obtained from Cell Signaling Technology, anti-KDEL for GRP78 was obtained from StressGen Biotechnology (Victoria, British Columbia, Canada) and PARP antibody was purchased from Pharmingen. Appropriate secondary antibodies were purchased from respective vendors and dilutions were used as suggested. After washing three times in the wash buffer for 5 min each, the blots were placed in chemiluminescence detection reagent (Roche Diagnostics) for 1 min and exposed to Biomax Light-1 film (Kodak, Rochester, NY, USA) at room temperature. The level of histone H3 was obtained for each lane by re-probing the same membrane in anti-H3 antibody (Cell Signaling Technology) and subsequently in anti-rabbit secondary antibody. Protein band intensity were quantified by densitometric scanning using Un-Scan-It software (Un-Scan-It; UT, USA) and normalized to the intensity of the corresponding histone H3 bands that served as internal control.

Cell-cycle analysis by flow cytometry. A549 human lung cancer cells were harvested after treatment (as described earlier). Cells were trypsinized and the pellet was washed with pre-warmed HBSS. One million cells for each treatment group were fixed in 75% ethanol. After overnight fixation at 4˚C, cells were spun-down and incubated in pre-warmed HBSS for 1 min and centrifuged. Supernatant was removed and cells were re-suspended in propidium iodide stain containing triton-x 100 and incubated for 30 min at room temperature in dark. Cell-cycle analysis was performed and data were acquired by flow cytometry using Guava PCA (Guava's Personal Cell Analysis system; Guava Technologies Inc., Hayward, CA, USA). Data analysis was done using ModFit *LT* software.

Results

2-dG or TM exposure leads to GRP78 over-expression in A549 cells. We observed that 2-dG and tunicamycin caused a strong induction of GRP78 in A549 human lung cancer cells (Figure 2a and b). At 0-h time point, the level of GRP78 in cells exposed to 2-dG or TM was at least 7-fold higher in comparison to unexposed cells and it was reduced to 2-fold at 48 h, albeit, in condition where A549 cells were exposed to 2-dG followed by 2 h of cisplatin treatment, the level of GRP78 was significantly higher (at least 10-fold) and was consistent until 48 h. In the case of TM exposure followed by 2 h of cisplatin treatment, the level of GRP78 slightly decreased at later time points. Cisplatin-alone did not cause a significant increase in GRP78 expression (Figure 2a and b).

Cisplatin treatment causes mild level of cell death in A549 cells. Cleavage of the DNA repair enzyme poly-ADP-ribose polymerase-1 (PARP-1) was used as a read-out of apoptotic cells. PARP-1 is 116-kDa DNA repair protein and is cleaved into 85 kDa and 25 kDa fragment upon apoptosis (Figure 2a and b). We found that A549 cells did not respond to 2-dG or TM at any time point in terms of PARP cleavage. In case of Cis treatment, a cleavage of the DNA repair enzyme PARP at 0 h was not observed, whereas a very light cleavage band was observed at later time points indicating the induction of apoptosis.

Cisplatin treatment of GRP78 overexpressing cells causes hypersensitization. In another condition where cells were pre-exposed to 2-dG or TM for 48 h to up-regulate GRP78 followed by 2 h cisplatin treatment, PARP cleavage bands were very prominent (Figure 2a and b). Apoptosis starts in these 2-dG/TM pre-exposed cells immediately after drug treatment and reaches to its maximum at later time points, indicating an increased occurrence of apoptosis or hypersensitization of A549 cells by cisplatin under the GRP78 up-regulated condition. Diminishing top bands and appearance of the bottom bands of PARP at later time points support the idea of hypersensitization (Figure 2a and b).

A549 cells become hypersensitive to cisplatin in GRP78 up-regulated condition as evidenced by clonogenic survival assay. We examined the consequences of cisplatin treatment in GRP78 over-expressed condition in A549 cells by the clonogenic survival assay (Figure 3). Consistently, it was seen that A549 cells were hypersensitized to Cis when treated in GRP78 up-regulated condition and very few colonies were found at the 60-μM concentration (Table I) whereas, at 90 μM concentration, number of colonies was further diminished. When treated with cisplatin alone, the effect on colony formation was comparatively mild (survival fraction 0.0686) (Table I). In 2-dG+Cis or TM+Cis conditions, where GRP78 was up-regulated, survival fractions were 0.0020 and 0.0034 respectively at 90 μM concentration of Cis (Table I), which was highly significant, as determined by two-tailed T-test and ANOVA.

Hypersensitization is mediated by c-Jun and NF-κB. To elucidate the possibility of JNK and NF-κB signaling pathways involvement in cisplatin-induced apoptosis in GRP78 over-expressing cells, we performed western blot analysis for phosphorylated forms of MKK4, JNK, ATF2, c-

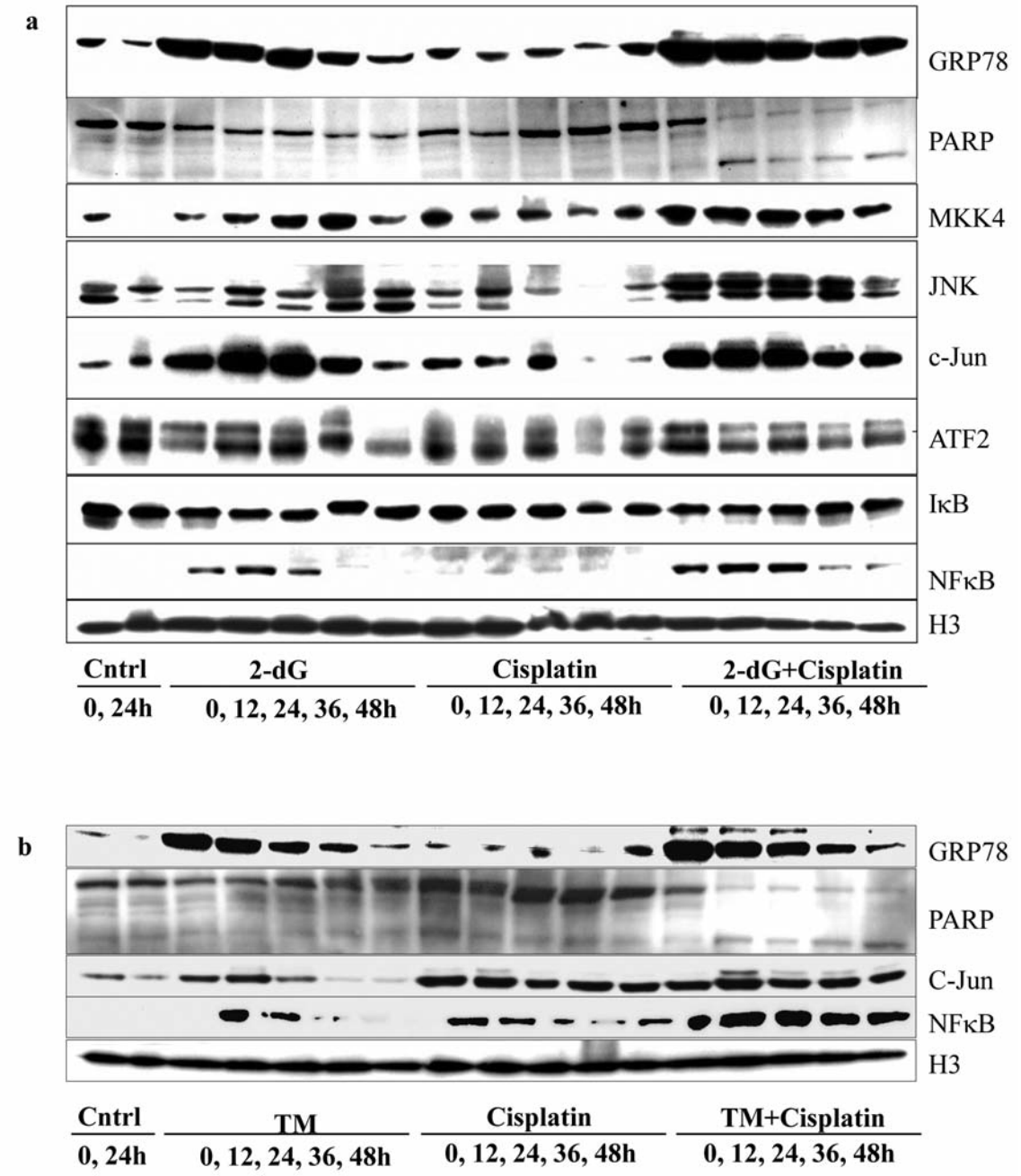

Figure 2. *A549 lung cancer cells were exposed to +/– 2-dG (a) or +/– TM (b) for 48 h followed by +/– cisplatin treatment for 2 h and levels of GRP78, PARP, c-Jun, NFκB and H3 were quantified by western blot at different time points after the treatment.*

Jun, IκB and NF-κB (Figure 2a and b). MKK4 phosphorylates JNK which in turn activates its downstream pro-apoptotic targets, transcription factors, c-Jun and ATF2 by phosphorylating them (26, 27, 28). NF-κB is also a transcription factor which enters the nucleus and binds to DNA after dissociation from its inhibitor IκB by IKK (29). We found that although c-Jun is phosphorylated in all conditions we studied, 2-dG/TM exposure followed by cisplatin treatment for 2 h caused hyperactivation of c-Jun in every time point (Figure 2a and b). This hyper-activation of c-Jun was well-associated with hypersensitization of A549 cells when GRP78 is up-regulated and this suggests the

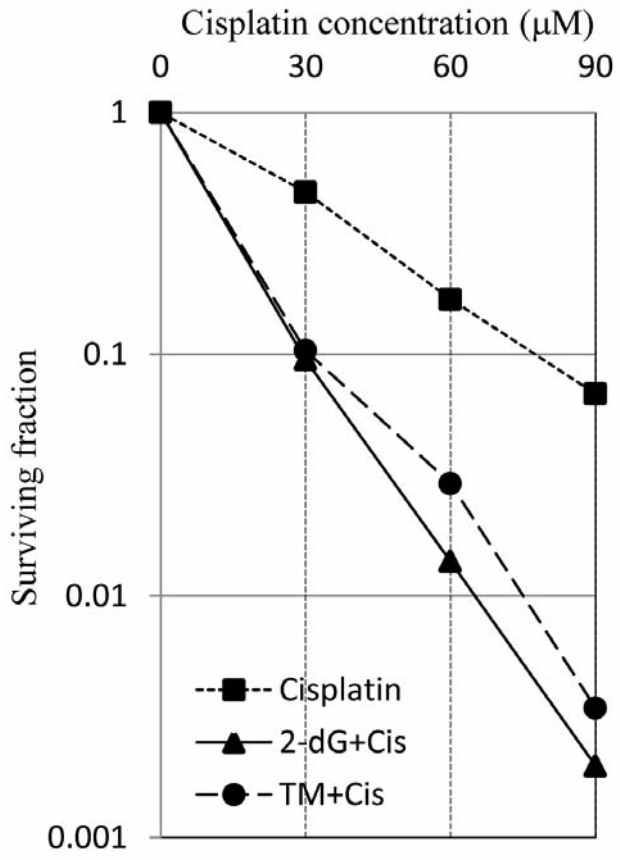

Figure 3. *Clonogenic survival assay in A549 human lung cancer cells after exposure to +/– 2-dG/TM for 48 h followed by 1 h cisplatin treatment. Cells were grown for 8 days to develop colonies.*

Table I. *Surviving fraction of A549 cells in GRP78-induced conditions after cisplatin treatment (average of three replicates).*

Cisplatin (μM)	Cisplatin	2-dG+Cis	TM+Cis
0	1	1	1
30	0.4688	0.0950	0.1038
60	0.1691	0.0139*	0.0292*
90	0.0686	0.0020**	0.0034**

*$p<0.05$ compared to 60 μM cisplatin treated condition, **$p<0.01$ compared to 90uM cisplatin treated condition.

involvement of JNK pathway in this condition. NF-κB was found to be phosphorylated in almost all GRP78 up-regulating conditions, but it was hyperactivated in those cells which were hypersensitized by cisplatin after up-regulation of GRP78 (Figure 2a and b). These results, again, suggest a strong involvement of NF-κB in the apoptosis induced by cisplatin after up-regulation of GRP78.

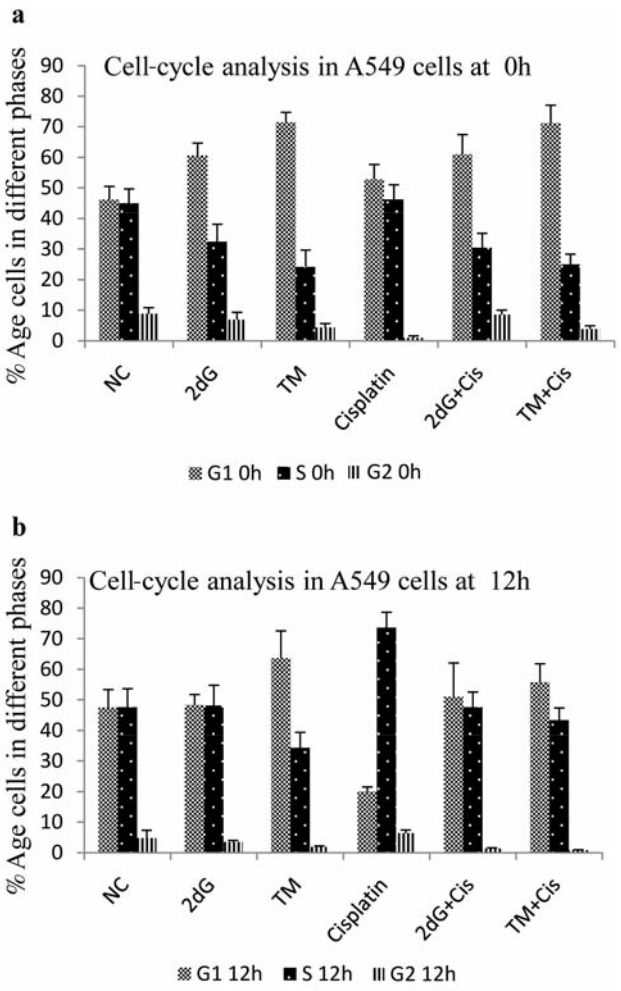

Figure 4. *Cell-cycle analysis by flow cytometry in A549 human lung cancer cells exposed to 2-dG/TM for 48 h followed by 2 h cisplatin treatment. Analysis was done at 0h and 12h after the treatment.*

Cell-cycle analysis by flow cytometry. Cell-cycle analysis was performed in A549 cells at 0 and 12 h time points to look at the possible cell-cycle arrest by any of the GRP78 up-regulating agents and to understand the mechanism of hypersensitivity (Figure 4a and b) (Tables II and III). At 0-h time point, 2-dG and TM arrested the cells in G_1 phase of the cell cycle with 60.66% and 71.48% cells respectively, whereas the number of cisplatin treated cells in S phase were 46.29%, which was similar to the untreated control. In case of combination of 2-dG/TM with cisplatin, most of the cells were arrested in G_1 with 61.01% in 2-dG+Cis and 71.17% in TM+Cis, which was comparable to 2-dG/TM-alone. In these two conditions at 0 h where GRP78 is being first induced followed by cisplatin treatment, A549 cells are undergoing apoptosis even when the majority of cells are

Table II. *Cell-cycle analysis by flow cytometry in A549 human lung cancer cells exposed to 2-dG/TM for 48 h followed by 2-h cisplatin treatment. Analysis was performed at 0 h after the treatment.*

Treatment conditions	G_1 (%)	S (%)	G_2 (%)
Normal control	46.14	45.02	8.84
2-dG 10 mM (48 h)	60.66	32.47	6.87
TM 250 nM (48 h)	71.48	24.17	4.35
Cisplatin 60 μM (2 h)	52.8	46.29	0.91
2-dG+Cis (48+2 h)	61.01	30.45	8.54
TM+Cis (48+2 h)	71.17	25.03	3.8

Table III. *Cell-cycle analysis by flow cytometry in A549 human lung cancer cells exposed to 2-dG/TM for 48 h followed by 2-h cisplatin treatment. Analysis was performed at 12h after the treatment.*

Treatment conditions	G_1 (%)	S (%)	G_2 (%)
Normal control	47.53	47.625	4.845
2-dG 10 mM (48 +12 h)	48.285	48.145	3.575
TM 250 nM (48 +12 h)	63.66	34.365	1.975
Cisplatin 60 μM (2+12 h)	19.98	73.645	6.375
2-dG+Cis 12 h	51.015	47.56	1.43
TM+Cis (48+2+12 h)	55.79	43.365	0.85

arrested in G_1. The number of cells in untreated controls was 46.14%, 45.02% and 8.84% in G_1, S and G_2 respectively. At 12-h time point, number of cells in 2-dG- and TM-treated groups were found decreasing in G_1 phase and shifting towards S phase in comparison to 0 h. In cisplatin-treated group, 73.65% cells were arrested in S phase at 12 h. In combination conditions at 12 h, more cells were being released from G_1 and shifting to S phase, thus being available for cisplatin action causing further hypersensitization.

Discussion

GRP78 is known to be a cytoprotective protein overexpressed by factors causing ER stress, such as unfolded protein response (UPR), intracellular Ca^{++} efflux, glucose deprivation and pathological conditions (30). Up-regulation of GRP78 is expected to promote cell survival, thereby helping the normal cells in tissue preservation and organ protection. On the other hand, in tumor cells, it is believed to cause immune resistance, tumor progression, malignancy and drug resistance. Contrary to the previous reports, our results suggest that up-regulation of GRP78 can be used as a tool to make the cancer cells hypersensitive to DNA platinating agent cisplatin. GRP78 was up-regulated using two different agents *i.e.* 2-dG and TM. 2-dG is a glucose antagonist and interferes with glycolysis at the very first step of conversion of glucose into glucose-6-phosphate making the cells hypoglycemic as well as interfering with glycosylation of proteins, thereby up-regulating GRP78, albeit tunicamycin is an antibiotic and a known glycosylation inhibitor which generates UPR. Therefore, as expected, GRP78 is up-regulated after treatment of A549 cells with 2-dG/TM and decrease in GRP78 level at later time points could be attributed to the lack of 2-dG/TM in the media and reduced ER stress. Surprisingly, exposure of A549 cells to these two agents followed by 2 h of cisplatin treatment causes a drastically high increase in GRP78 level which is persistent until later time points. Cisplatin has earlier been reported to cause ER stress itself and persistent increase in GRP78 level after combination treatment can be attributed to increased ER stress by cisplatin and the GRP78 up-regulating agents. Increased PARP cleavage in all combination treatments (2-dG+Cis or TM+Cis) indicates hypersensitization of these cells in comparison to cells treated only with cisplatin (Figure 2 and 3) (Table I).

Furthermore, 2-dG and TM caused arrest in the G_1 phase of the cell cycle (0 h) (Figure 4a) (Tables II and III) possibly due to lack of ATP, inhibition of glycosylation and/or interference with calcium signaling and therefore, in combination conditions, there were not many cells available in the S phase of the cell cycle to be arrested by cisplatin. At 12-h time point (Figure 4b) (Tables II and III) cells were being slowly released to the S phase where they could be arrested by cisplatin. Hypersensitization (Figure 2) (PARP cleavage) at 12 h could be attributed to more cells in S phase becoming vulnerable to cisplatin and continuing ER stress at the same time (31-34). Thus, after induction of GRP78 causing ER stress, treatment of A549 lung cancer cells with a DNA damaging agent, Cis, leads to a synergistic effect and causes hypersensitization of these cells. Additionally c-Jun, a downstream component of SAPK/JNK pathway (26, 35), was phosphorylated upon 2-dG/TM exposure and cisplatin exposure indicative of ER stress and DNA damage induced activation respectively. In 2-dG+Cis condition, MKK4 hyperactivation is followed by JNK hyperphosphorylation, leading to increased activation of c-Jun (Figure 2a). In TM+Cis condition also c-Jun was found to be hyperactivated at every time point examined. This hyperactivation of c-Jun is strongly associated with hypersensitization of A549 cells in these two conditions as shown by PARP cleavage and the clonogenic survival assay. ER stress-induced activation of NF-κB has been previously reported, which we observed at 0 and 12 h after 2-dG/TM treatment (36) however, in 2-dG+Cis and TM+Cis conditions, NF-κB was highly activated. Higher activation of NF-κB in hypersensitized cells suggests its role in apoptosis, synergistically caused by ER stress and

mitochondrial damage. It appears that up-regulation of GRP78 causes NF-κB activation in ER stress-induced apoptosis, which is further mediated by c-Jun hyperactivation.

We conclude that GRP78 up-regulation in the A549 human non-small cell lung cancer cells followed by the treatment with a DNA platinating agent, cisplatin, leads to an early and increased apoptosis. This hypersensitization occurs due to a possible activation of pro-apoptotic pathway inside the cells where DNA damage induced apoptosis is mediated by JNK pathway involving the release of cytochrome *c* from mitochondria and activation of caspase-9 and caspase-3, which leads the cell towards death (37-39). In summary, GRP78 over-expression hypersensitizes A549 cells to cisplatin by DNA damage- and ER stress-induced apoptosis. This observation requires further examination with other tumor cell lines. Nevertheless, our study is a step closer to overcoming the ever-existing problem in the development of cisplatin resistance to solid tumors and this approach will be confirmed *in vivo* in the near future.

Acknowledgements

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