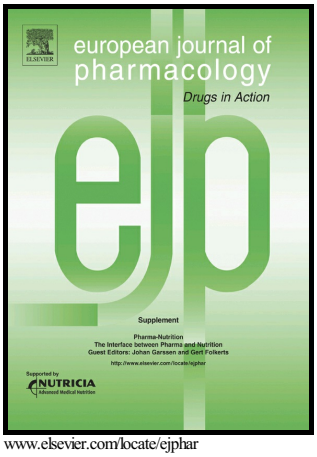

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Inhibition of Transglutaminase 2 activity increases cisplatin cytotoxicity in a model of human hepatocarcinoma chemotherapy

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Abstract

Transglutaminase 2 (TG2) is a ubiquitous multifunctional enzyme whose expression has been found to be altered in numerous studies of apoptosis and cell survival; its activity has been found to be increased in many types of cancer, where it is often over-expressed.

Cisplatin has long been used as an effective therapeutic drug to treat numerous cancers. Although its activity is based on cross-linking of DNA, cisplatin may also operate via other mechanisms that involve modification and alteration in the activity of protein and RNA modulators of the cell cycle and apoptotic processes; these mechanisms are less well characterised.

In this study, we investigated the effects of cisplatin-induced apoptosis on TG2 expression and activity in the human hepatocarcinoma (HepG2) cell line. Through a combination of Western blotting, enzymatic activity assays, flow cytometry and fluorescence microscopy we provide evidence that TG2 is inhibited during initiation of apoptosis by cisplatin, an observation that was reversed by increasing the expression of TG2, by treating cells with retinoic acid. We also report, for the first time, that cisplatin can directly inhibit transglutaminase activity *in vitro*.

Collectively, these studies increase our understanding of the mechanism(s) of action of cisplatin, as cisplatin–mediated reduction in TG2 activity appears to act as an early activator of apoptosis during chemotherapeutic treatment of hepatocarcinoma cells. This observation suggests an explanation as to how increased levels of TG2 activity in cancer cells could contribute to chemotherapeutic resistance to cisplatin, and so has implications for novel approaches to cisplatin therapy.

Keywords: Transglutaminase 2, apoptosis, protein expression, cisplatin, hepatocarcinoma, chemotherapy.

1. Introduction

Cisplatin (Fig. 1) is one of the most effective drugs in the treatment of several types of cancer. The way cisplatin works in the body is not fully understood, though it is well known that cisplatin cross-links DNA, and in this way inhibits DNA synthesis (Gonzalez et al., 2001; Qin and Ng, 2002; Zhang et al., 2010). Cisplatin thus targets rapidly-dividing cells, and so is preferentially toxic to cancerous cells. However, cisplatin is also toxic to healthy cells, and treatment therefore has side-effects that can cause damage to vital organs (Ishida et al., 2002; Florea and Busselberg, 2011); cisplatin cannot be used at high doses. This problem is further increased during repeated treatments, as cancer cells that are able to evade the cytotoxic effects of low dose treatment of cisplatin become resistant to the concentrations normally used for treatment (Zhou et al., 2010; Lopez-Ayllon et al., 2014; Gumulec et al., 2014). Eventually, the high concentrations of cisplatin that are required to mediate a continued anti-cancer effect become untenable to the patient (Galluzzi et al., 2014).

Transglutaminases are a family of calcium-dependent enzymes that were first discovered almost six decades ago (Clarke et al., 1957). One of the members of this family, TG2, has been found to be variously expressed in several cellular compartments, including the nucleus, cytoplasm, mitochondria, and extracellular matrix (ECM), depending on the cell's physiological and pathological conditions (Nurminskaya and Belkin, 2012; Eckert et al., 2014; Piacentini et al., 2014; Park et al., 2010). TG2 operates by post-translationally modifying exposed glutaminyl residues in protein substrates, and, in the later stages of cell death, cross-links proteins via exposed glutaminyl and lysyl residues during apoptotic body formation. Through these activities and associated specific protein-protein interactions, and its G-protein activity, TG2 contributes to multiple cellular processes (Nurminskaya and Belkin, 2012; Eckert et al., 2014). Indeed, its wide range of influence has led to TG2 being described as the molecular equivalent of a "Swiss army knife" (Gundemir et al., 2012). TG2 is a difficult protein to characterise in terms of its particular importance in cell biology, as it appears to be linked to so many processes. However, the fact that TG2 is now implicated in

several inflammatory diseases, including coeliac disease, diabetes, neurodegenerative diseases, and cancer (Siegel and Khosla, 2007; Odii and Coussons, 2014) is starting to suggest a common mechanistic theme in different cell types, and so beginning to highlight a hierarchy of activities of TG2 that are required for normal cellular health. A further, and possibly revealing, observation is that despite TG2 expression already being high in cancer cells, its levels are even further increased in drug resistant and metastatic cancer (Mehta et al., 2004; Herman et al., 2006; Kumar et al., 2010) - though the precise mechanism of its action remains currently poorly understood.

In order to investigate whether the mechanism of cisplatin cytotoxicity also operates on protein mediators of apoptosis and/or cell survival, we have developed a HepG2 cell line model of cisplatin therapy of human hepatocarcinoma. In this report we show that cisplatin treatment of hepatocarcinoma cells contributes to the induction of apoptosis by down-regulating both TG2 expression and its enzyme activity by approximately 30-50%. Further *in vitro* studies show that although most of the loss of TG2 activity is probably due to the loss of expression of TG2 protein in cells, a contributory inhibitory effect may also be contributed by direct inhibition of TG2 enzyme activity by interaction of cisplatin at the active site of TG2. On the basis of our observations, we speculate that the over-expression of active TG2 in cancer cells may serve to dilute the suppressing effects of cisplatin on TG2 expression and activity, and so contribute to the resistance of cells to cisplatin-induced apoptosis. Such an effect may contribute to one of the major problems in cancer treatment by increasing the drug-resistance of cancer cells in patients undergoing long-term repeated cisplatin-based chemotherapy. The inhibition of TG2 activity may therefore represent a novel combined approach to the clinical treatment of HCC with cisplatin.

2. Materials and methods

All the chemicals used in this study were from Sigma Aldrich, UK, unless otherwise stated.

2.1 Cell line and cell culture preparation

The HepG2 cell line used in our experiments was procured from the European Collection of Cell Cultures (ECCC) (Sigma Aldrich, UK) and was maintained in RPMI 1640 culture medium (Invitrogen, UK) fully supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, UK). Cell cultures were maintained at 37 ^{o}C in the presence of a humidified 5% CO_2 atmosphere and were kept free of mycoplasma contamination by adding the Plasmocin, a commercially available anti-mycoplasma reagent. Cells were observed on a regular basis and their morphological authentication was confirmed by comparison with online STR profile data (NCBI Biosample database).

2.2 Stock solution preparation

Stock solutions of 2 mM cisplatin were prepared in 18 mΩ ultrapure water under subdued light and stored in the dark at room temperature before use. Freshly-prepared cisplatin solution was used for every experiment, owing to its instability in aqueous solution. Special precautions were taken during preparation of retinoic acid solutions, as it is sensitive to UV light, air and oxidizing agents. Preparation of 10 mM (3 mg/ml) retinoic acid in absolute ethanol was performed under subdued light and in a glove bag under an atmosphere of inert gas and stored at -20 ^{o}C. Retinoic acid solutions were diluted with tissue culture medium prior to any treatments and used within two weeks. Freshly prepared stock solutions of 0.5 M cystamine were prepared in ultrapure water and stored at 4 ^{o}C for the duration of experiments prior to use.

2.3. Cytotoxicity assay using cell counting kit-8 assay

The cell counting kit-8 (CCK-8) assay is a colorimetric procedure based on the ability of viable cells to reduce a yellow-coloured formazan dye. The CCK-8 uses WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium

salt] in conjugation with an electron mediator, 1-methoxy-5-methylphenazinium methylsulphate, to assess cell viability.

HepG2 cells were detached by proteolysis using 1% trypsin/0.02% EDTA and were then re-suspended in fresh culture medium at a concentration of $1x10^5$ cells/ml. The cells were then seeded into 96-well cell culture plates at 10,000 cells per well in a 100 µl volume of fully-supplemented RPMI 1640 medium. Blank control wells were filled with 100 µl fresh culture medium with omission of the cells. The cells were then grown for 24 h at 37 ^oC in a humidified 5% CO_2 environment. Following incubation for 24 h, the culture medium was removed and 100 µl of fresh culture medium containing freshly-prepared cisplatin were added to give final concentrations over a range of 0-20 µM. The plates were incubated for 24 h and 48 h time intervals. Following exposure to the drug, 10 µl of the cell counting kit-8 reagent were added directly to the cell cultures and incubated for another 4 h at 37 ^oC in a humidified 5% CO_2 environment. The WST-8 formazan product was measured at 450 nm using a monochromator-based multi-mode microplate reader equipped with Megalan software (Sunrise, UK). The viable cell numbers were counted and the IC_{50} values, for 24 h and 48 h incubation, were calculated. All samples were prepared in triplicate and the data are derived from the average of five independent experiments.

2.4. Apoptosis analysis by propidium iodide staining with flow cytometry

Briefly, $1x10^6$ cells were grown in culture flasks for 24 h, and then treated with cisplatin for different time intervals, at 37 ^oC in a humidified 5% CO_2 atmosphere. After defined incubation times, floating cells from aspirated medium were collected by centrifugation at 300*g* for 5 min. Adherent cells were washed with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and harvested by trypsinization and the cell pellets were washed once with ice-cold PBS. Cell pellets were re-suspended in 0.5 ml ice-cold PBS and 70% ethanol was added to the cell suspension drop-wise, up to 5 ml, while

vortexing. Cells in 70% ethanol were incubated at 4 ^{o}C for 2 h, with gentle agitation to avoid clump formation after 1 h. Then, cells were spun at 600*g* for 10 min, and cell pellets were washed twice with ice-cold PBS. 400 µl of propidium iodide (PI) (50 µg/ml) solution were added to cells and the mixtures were incubated at room temperature for 30 min, before analysist by flow cytometry that was carried out on a FACScan equipped with Cell Quest Pro Software (Becton Dickinson, UK).

2.5. Apoptosis analysis by FITC-labelled Annexin-V with flow cytometry

HepG2 cells were harvested by trypsinization. The cell pellets were washed two times in ice-cold PBS. Cells were then diluted with ice-cold binding buffer (0.01 M Hepes/NaOH, 0.14 M NaCl, 2.5 mM $CaCl_{2,,}$ pH 7.4) to 1x10^{6} cells/ml density. To 100 µl cell suspension (10^{5} cells), 5 µl of Fluorescein isothiocyanate (FITC) labelled-Annexin V (5 µg/µl) and 10 µl of PI (20 µg/µl) (BD Biosciences, UK) were added, and mixtures were incubated for 15 min at room temperature in the dark. Then, 300 µl of binding buffer were added, and samples were analysed using a BD Accuri flow cytometer equipped with C6 software (BD Biosciences, UK).

2.6. Nuclear staining with DAPI

HepG2 cells were grown on coverslips in 6-well culture plates in a humidified 5% CO_2 environment. After 24 h, cells were treated with 8 µM cisplatin and incubated for 24 h. After incubation, cells were washed briefly with 1 x PBS. Then, samples of untreated and treated cells were fixed and permeabilized with 4% formaldehyde containing 0.2 M sucrose to open the cell membrane, for 20 min at 4 ^{o}C. Coverslips with cells were washed briefly with PBS, then mounted with mounting medium containing DAPI (4', 6-diamidino-2-phenylindole) (5 µg/ml). The slides were then observed by fluorescence microscopy.

2.7. Reverse transcription quantitative real time-PCR (qRT-PCR)

HepG2 cells were seeded at a density of ≥1x10^6 per 25 cm^2 culture flask and pre-incubated for 24 h. Following subsequent cisplatin treatment, total RNA was isolated from cells using the RNeasy kit (Quiagen, UK), according to the manufacturer's instructions. After DNase treatment, total RNA was collected in RNase-free H_2O and was quantified using a Nanodrop spectrophotometer (Thermo Scientific, UK).

The quantitative estimation of expression of TG2 and the "housekeeping" enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls were based on real-time monitoring of amplification and melting curves. The reverse transcription was performed at 50 ^oC for 10 min with 500 ng of total RNA and specific reverse primers (Eurofins, UK) using iScript reverse transcriptase from the one-step RT-PCR kit (Bio-Rad, UK), with some modifications in protocol as described by the manufacturer. Specific sequences of the primers for TG2 and other genes are summarized in Table 1. The reaction mixture contained: 12.5 µl 2x SYBR green PCR master mix; 1 µl (400 nM) forward primer; 1 µl (400 nM) reverse primer; 0.5 µl RT-enzyme; total RNA and RNase free-H_2O, in a total volume of 20 µl. Quantification based on real-time monitoring of amplification and melting curves was carried out using a Roche RT-PCR Thermal cycler equipped with light cycler 4.1 software (Roche-science, UK). The reaction mixtures were incubated in the following manner: cDNA synthesis during 1 cycle at 50 ^oC for 10 min; denaturation during 1 cycle at 95 ^oC for 5 min, 3 step amplification during 40 cycles, at 95 ^oC for 30 s, 60 ^oC for 30 s, and 72 ^oC for 90 s; final extension during 1 cycle at 72 ^oC for 10 min; melting curve generation during 1 cycle at 95 ^oC for 10 s, 44 ^oC for 1 min, and 72 ^oC for 1 s; and final step cooling during 1 cycle at 37 ^oC. All determinations were done in triplicate together with one control reaction, in which RT enzyme was omitted. The latter was used to test for potential DNA contamination, and to act as a negative control with respect to primer dimer-oligomerization. Absolute numbers of

mRNA molecules were normalised to GAPDH to correct for differences in RNA concentration. Sequences of the primers used for subsequent amplification reactions were designed from available data on specific genes obtained from NCBI website. Amplified products were monitored by running samples on a 1.2% agarose gel and confirmed against available data. The relative quantification was calculated using the $\Delta\Delta C_T$ ($\Delta\Delta C_q$) method.

Table 1

Primers sequences.

Transcript Name	Sequence
GAPDH	(F) – 5'-CACTAGGCGCTCACTGTTCTC-3'
	(R) – 3'-GACTCCACGACGTACTCAGC-5'
TG2	(F) – 5'-CTGGGCCACTTCATTTTGC-3'
	(R) – 3'-ACTCCTGCCGCTCCTCTTC-5'

F = Forward Primer, R = Reverse Primer.

2.8. Measurement of protein expression by Western blotting

Cell lysates were prepared using RIPA cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with freshly added 1% complete protease inhibitor cocktail (Aprotinin, Bestatin, Leupeptin, Pepstatin A), unless otherwise described. Cells were lysed in RIPA buffer and then centrifuged at 17,000*g* for 20 min at 4 ^{o}C to obtain supernatant enriched with proteins. The supernatant containing the proteins was collected and the total protein concentrations of samples were determined using the Bradford dye-binding assay (Bradford, 1976).

Samples were then mixed with Laemmli 2 x SDS sample buffer (1:1) and 20 µl were loaded and separated by one-dimensional SDS-PAGE using 10% Bis-Tris gels (Invitrogen, UK) under reducing conditions with 1 x MES SDS running buffer (Invitrogen, UK). The gels were electrophoretically transferred to nitrocellulose membranes (Abcam, Cambridge, UK) using

semi-dry transfer (Thermo Scientific, UK) for 1 h at 100 mA, 5 V. Transferred proteins were visualised by Ponceau S red staining before further processing. Blots were then blocked in a 5% non-fat dried milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h. The blots were then washed briefly with TBST and incubated with rabbit anti-TG2 polyclonal antibody (Sigma Aldrich, UK) at 1:3000 dilution in TBST for 3 h at room temperature or overnight at 4 ^{o}C with rotation agitation. The blots were subsequently washed three times for 5 min each with TBST, replacing the buffer each time. Then, HRP-conjugated anti-rabbit secondary antibody (Abcam, Cambridge, UK) was added at a 1:3000 dilution and the blots were incubated for 1 h at room temperature. The blots were then washed three times with TBST for 5 min each time. The blots were subsequently developed using an enhanced luminol-based chemiluminescence substrate for the detection of horseradish peroxidase (HRP) (ECL-HRP Western blotting substrate) (Pierce, Thermo Scientific, UK). The antibodies were stripped off of the blot in stripping solution (0.5 M Tris-buffer, pH 6.7, 2% SDS, 0.7% β-mercaptoethanol) for re-incubation with a loading control, an antibody against β actin (Abcam, Cambridge, UK). The stripped blots were re-incubated with anti-β actin antibody at 1:2000 dilution for 3 h at room temperature or overnight at 4 ^{o}C. Then, blots were washed as described above and incubated with secondary anti-rabbit antibody (1:3000) for 1 h, then washed and developed as previously described.

2.9. *In vitro* specific TG2 colorimetric micro-assay (TG2-CovTest)

Cell lysates were prepared from cisplatin-treated and non-treated HepG2 cells and were used to measure TG2 activity using a commercially-available specific TG2 colorimetric microassay kit (Covalab, France) according to the manufacturer's instructions. Briefly, the wells of a microtiter plate were pre-coated with amine substrate (spermine), and washed once with 1 x PBS for 15 min at 37 ^{o}C. The buffer was removed and 50 µl of assay mixture (containing freshly made DTT, biotin-pepT26/$CaCl_2$), were added. Then, 60 µl of lysate were

added to each well. Samples were mixed by pipetting and incubated at 37 ^{o}C for 30 min. Wells were washed once with PBS, then were washed once with 0.1 M NaOH, and then washed twice with PBS. Streptavidin-labelled peroxidase solution (SAv-HRP) was added (100 µl) and the samples were incubated for 15 min at 37 ^{o}C. Wells were washed thrice with PBS and 100 µl HRP-substrate (H_2O_2) were dispensed into each well. The mixtures were then incubated for 5 min at room temperature in the dark. The reaction was developed by adding tetramethyl benzidine stock solution (100 µl) and the colour that formed was measured on the basis of optical density at 450 nm using a microplate reader.

2.10. CBZ-Gln-Gly assay of transglutaminase activity

Guinea pig liver transglutaminase activity was measured by Benzyloxycarbonyl-Glutamine-Glycine (CBZ-Gln-Gly) assay using the method of Folk and Cole, (1966) with some modifications. The reaction was carried out in a final concentration of 200 mM Tris-HCL (pH 7.0), 10 mM CBZ-Gln-Gly, 100 mM hydroxylamine (NH_2OH), 6 mM $CaCl_2$, 1.6 mM dithiothreitol (DTT) and 0.02 mg/ml guinea pig liver transglutaminase in a total of 1 ml reaction mixture. Aliquots of freshly-prepared cisplatin (see stock solution preparation section) over a concentration range of 0-128 µM were added to reaction mixtures in final volumes of 1 ml and incubated for 1 h at 37 ^{o}C. The reactions were stopped by adding 0.3 ml of ferric chloride-trichloroacetic acid ($FeCl_3$/TCA/HCl) and the precipitated products were centrifuged at 700*g* for 3 min. The optical density of the supernatant was measured immediately at 520 nm against a blank in which TG2 was omitted. For determination of transglutaminase activity from cell lysates by CBZ-Gln-Gly assay, the protocol was the same as described above, except 100 µl of lysate were added to mixtures instead of commercially-available guinea pig liver transglutaminase.

2.11. Statistical analysis

All statistical analyses were performed using GraphPad prism 6 software (GraphPad, USA). A $P<0.05$ was considered to be statistically significant. The Student's t test and one way ANOVA with Dunnett's multiple comparisons test were used to calculate significant values.

3. Results

3.1. Determination of IC_{50} for cisplatin

When HepG2 cells were treated with 0-20 μM cisplatin for 24 h and 48 h, it was confirmed from the cell survival curve that cisplatin had the expected concentration- and time-dependent cytotoxic effects on HepG2 cells. The IC_{50} values for 24 h and 48 h were 8 μM and 4 μM, respectively (Fig. 2A). The IC_{50} values were confirmed by flow cytometric analysis of cell death (Figs. 2Bi and ii). The IC_{50} values obtained were different from the IC_{50} previously reported from various studies (Table 2). However, the cells' response to this drug may be dependent on the cells' condition/passage number and/or methods used for the cytotoxicity assay.

Table 2

IC_{50} values in HepG2 cell line obtained from different studies after 24 h incubation

IC_{50}	**Method**	**References**
4 μM	CCK-8	Odii and Coussons, 2012.
8.3 μM	MMT	Luo, Jin and Huang, 2012.
33.3 μM	MMT	Uray, Davies and Fesus, 2001.
40 μM	MMT	Zhang, Niu and Zhou, 2010
106.3 μM	MMT	Qin and Ng, 2002.

3.2. Apoptosis determination after cisplatin treatment

Following treatment of HepG2 cells with 8 μM of cisplatin, induction of apoptosis was measured by harvesting cells and estimating DNA-based propidium iodide levels after 12 h and 24 h. Although cisplatin is known to induce apoptosis as early as at 12 h post-treatment in the HepG2 cell line at high dose (50 μM) (Qin and Ng, 2002), our results indicated that there were no signs of apoptosis at this point with 8 μM cisplatin (Figs. 3A and B). However, following incubation for 24 h, cisplatin-treated cells generated an extra apoptotic peak before G0/G1 phase (indicated by the arrow in Fig. 3D) that was not apparent in untreated control cells (Fig. 3C). As the incubation time was increased, more cells became apoptotic. The morphological nuclear changes and the formation of apoptotic cell bodies were confirmed by DAPI staining observed by fluorescent microscopy. While untreated cells had a regular nuclear shape and showed no DNA condensation and/or fragmentation (Fig. 3G), cisplatin-treated cells revealed larger and irregularly-shaped nuclei, and showed signs of DNA condensation and fragmentation (Fig. 3H). Further evidence that apoptosis had been induced by cisplatin treatment was provided by FITC-labelled Annexin-V flow cytometric analysis (Figs. 3E and F) that indicated the presence of phosphatidylserine on the surface of cisplatin-treated cells following incubation for 24 h.

3.3. TG2 transcriptional expression after treatment with cisplatin

Whereas Uray *et al.,* (2001) have shown that TG2 mRNA was overexpressed, initially as early as six hours following treatment of HepG2 cells with cisplatin, examination of TG2 mRNA from cells that had been treated with 4 and 8 μM of cisplatin for 24 h showed that TG2 gene expression was decreased in cisplatin-treated cells compared to control groups (Fig. 4A). This result was independently confirmed by isolating PCR products by agarose gel electrophoresis (Fig. 4B).

3.4. TG2 protein expression after cisplatin treatment

After incubation of cells for 12 h, there was little change in TG2 protein expression with increasing cisplatin concentration, over the 0-16 μM range (Fig. 5A). However, after incubation for 24 h, TG2 protein expression was reduced in cisplatin-treated cells compared to untreated cells (Fig. 5B), and apoptosis was induced (41% apoptosis) (Figs. 3D and F).

3.5. Effects of cisplatin treatment on TG2 transamidation activity

The pattern of TG2 activity broadly reflected that of TG2 protein expression. After incubation for 12 h, TG2 transamination activity was little changed in cisplatin-treated cells compared to untreated cells (Fig. 6A). After 24 h, as cisplatin concentration was increased, TG2 transamidation activity decreased in a concentration-dependent manner, by approximately 30-50% compared to untreated cells (Fig. 6B), although the P value was not statistically significant when compared between groups (P=0.5). The enzyme activity appeared to fall proportionately less than the levels of TG2 protein expression, thus suggesting some compensation for loss of TG2 expression (possibly through an increase in specific activity or expression of other members of the family following cisplatin treatment).

3.6. Inhibition of TG2 by cystamine induced apoptosis from 12 h post-treatment

Cisplatin is known to induce apoptosis as early as at 12 h post-treatment in the HepG2 cell line at a higher dose (50 μM) (Qin and Ng, 2002), but induction of cell death at higher concentration creates toxicity to other organs. Induction of apoptosis at significantly lower concentrations would be likely to increase patient responses to cisplatin treatment. Therefore, to determine whether TG2 transamidating activity has a role in the induction of apoptosis at relatively low concentration, HepG2 cells were pre-incubated with, or without a non-cytotoxic dose of 1 mM cystamine for 48 h. Cells were subsequently left without further

treatment (control), or treated with 8 μM cisplatin and incubated for another 6 to 12 h. Results indicated that induction of apoptosis in cystamine treated cells was induced as early as 12 h post cisplatin-treatment (Fig. 7F), whereas apoptosis was not induced earlier in cisplatin-treated cells that had not been exposed to cystamine (Fig. 3B).

3.7. Effect of pre-treatment of cells with retinoic acid

Low concentrations of retinoic acid (1 μM, 5 μM) had little, or no effect on TG2 expression, but like other studies (Ou et al., 2000; Kweon et al., 2004), 10 μM treatments induced an increase (of approximately 50%) in TG2 protein expression and this was reflected by a comparable increase in enzyme activity; we therefore used this concentration of retinoic acid in subsequent experiments. As previously observed, such treatments increased the general viability of cells after 72 h in control experiments (Kweon et al., 2004) (Fig. 8) and also increased the resistance of cells to the cytotoxic effects of cisplatin, compared to controls (Fig. 9) – an effect that was reversed by treatment of cells with the TG2 inhibitor, cystamine (Fig. 10).

3.8. Direct effect of cisplatin on commercially-available exogenous guinea pig liver TG2

The inclusion of cisplatin into CBZ-Gln-Gly assay mixtures containing purified guinea pig liver transglutaminase caused a direct concentration-dependent inhibition of CBZ-Gln-Gly transamination over the whole 0-128 μM range assayed. The results obtained were consistent with, though less pronounced than, the decrease in transamination activity measured following treatment of HepG2 cells with cisplatin, requiring 128 μM of cisplatin to reduce the activity by 50% *in vitro* (Fig. 11). Interestingly, the incubation of cisplatin together with TG2 over 6 h resulted in a loss in apparent cisplatin activity; this may reflect deactivation of cisplatin by aqueous hydrolysis or TG2 (Fig. 12). This observation may also explain why 106 μM cisplatin was required to induce its cytotoxic effect after 24 h, as observed in other studies (Table 2).

4. Discussion

Over the last decade, it has emerged that increased TG2 expression has been linked with cell survival in several cancer cell lines (Cao et al., 2008; Cho et al., 2012; Kweon et al., 2004). Some reports have also shown that TG2 overexpression and activation have been linked to the initiation of apoptosis, after treatment of cancer cells with chemotherapeutic drugs (Robitaille et al., 2008; Fok and Mehta, 2007; Hsieh et al., 2013). Other studies show that down-regulation of TG2 appears to sensitize cancer cells to apoptosis (Han and Park, 1999; Budillon et al., 2013). This is interesting, as TG2 expression has been not only associated with apoptosis-related pathways (Yoo et al., 2012; Han and Park, 1999; Fok and Mehta, 2007) but also with anti-apoptosis pathways (Cho et al., 2010) - though the precise mechanism of its action remains currently poorly understood. Indeed, whether TG2 facilitates or ameliorates apoptosis appears to be dependent on the types of stressors and cells involved (Ientile et al., 2007; Chhabra et al., 2009; Gundemir et al., 2013). Interestingly, Fesus and Zondey (2005) have suggested that loss of TG2 activity in mice could cause compensatory induction of other transglutaminases (TG1, 3, 5 and 7).

Hepatocellular carcinoma (HCC) is the most common liver malignancy and its incidence is increasing on a worldwide basis, possibly due to the associated rise in hepatitis C and B (Attwa and El-Etreby, 2015). Conventional cytotoxic chemotherapy is not currently effective in prolonging survival in patients with late diagnosis of HCC. However, cisplatin treatment has shown some encouraging results, although the response rate is still inadequate (Toru, 2009). Further studies of the mechanism of action of cisplatin are needed in order to optimise dosing schedules in clinical studies.

Although cisplatin is more toxic to cancer cells than normal cells, it could be considered to be a relatively non-specific drug, as it can bind to and modify any susceptible molecules that contain nucleophilic sites (Gonzalez et al., 2001; Florea and Busselberg,

2011). For example, it is not necessarily the case that simple arrest of DNA synthesis is sufficient to induce apoptosis, as there are many other biologically-susceptible molecules that are involved in cisplatin-induced apoptosis (Galluzzi et al., 2014; Dasari and Tchounwou, 2014). Such molecules may include RNA, and it has been suggested that cisplatin can also initiate apoptosis by damaging cytoplasmic proteins (Gonzalez et al., 2001; Florea and Busselberg, 2011).

In this study during the time course of treatment of HepG2 cells with cisplatin, the cytotoxic effects of cisplatin were accompanied by both a loss of enzyme activity and TG2 expression (Figs. 4-7). However, the change in the loss of activity of TG2 was proportionately less than that of the protein expression, suggesting that extra levels of control (possibly compensatory) of TG2 activity may be responsible for the observed cellular response to toxicity. However, taken together the general patterns of TG2 expression reported here suggest that TG2 down-regulation by cisplatin may be a necessary step in the initiation of apoptosis in the HepG2 cell line.

This observation is in stark contrast to the delay and/or prevention of cisplatin-induced apoptosis that was observed following induction of increased levels of TG2 expression by retinoic acid pre-treatment of cells (Figs. 8-10). As this retinoic acid effect could be partially reversed by cystamine - induced inhibition of cellular TG2 enzyme activity, this study supports the notion that TG2 may be involved in the early decision-making phase of apoptosis in cisplatin-treated HepG2 cells (Fig. 7). Currently, there are no other published reports that show a direct chemical inhibitory effect of cisplatin on TG2 activity and this effect in combination with the effects on down regulation of TG2 by cisplatin contribute to the overall cytotoxic effects of cisplatin *in situ*.

In conclusion, the induction of apoptosis in hepatocarcinoma cancer cells by cisplatin therefore appears not only to be dependent on drug-dependent DNA modification, but may also be due to functional damage caused by this drug to one of the known protein modulators of apoptosis, i.e., TG2 (Fig. 13). Collectively, these results may explain why

increased TG2 expression in cancer cells contributes to the development of their resistance to chemotherapeutic drugs such as cisplatin; higher doses may be expected to be required to reduce TG2 expression to levels consistent with non-resistance. Our observations also support the contention that there may be potential for improving cisplatin-based cancer treatment by enhancing the effectiveness of patient-tolerable cisplatin doses, especially if cisplatin chemotherapy is combined with relatively non-toxic doses of TG2 inhibitors, such as cystamine, which has been shown to be cytoprotective towards healthy cells in a number of studies. Such treatment strategies for patients might also include avoidance of rich nutritional sources of retinoids such as carrots, which have been counter-indicated in several epidemiological nutritional studies where recipients of these supplements became more prone to development of cancer(s) than those consuming placebos (Omenn et al., 1996; Cancer study group report, 1994).

Competing interests

No competing interest.

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Figure legends:

Fig. 1. Chemical Structure of Cisplatin.

Fig. 2. Cytotoxicity of cisplatin to HepG2 cells. HepG2 cells were incubated with increasing concentrations of cisplatin over 24 h and 48 h. (A) Cytotoxicity measured by CCK-8 assay, (B) Cytotoxicity and IC_{50} values confirmed by flow cytometry using FITC-Annexin-V labellelling. Figure B.i indicates 24 h incubation with 8 μM cisplatin and Figure B.ii indicates 48 h incubation with 4 μM cisplatin. The IC_{50} value from the 24 h incubation was statistically different from the IC_{50} value from the 48 h incubation. The ***P=0.0007 by two-tailed Student's paired t-test. Results are means ±S.D. of triplicates from five independent experiments.

Fig. 3. Apoptosis induction following cisplatin treatment**.** Apoptosis in HepG2 cells was measured by flow cytometry using propidium iodide-staining. Cells were treated or not treated with 8 μM cisplatin for 12 h (A - control; B - cisplatin treated), or 24 h (C - control; D - cisplatin treated). FITC-labelled Annexin-V flow cytometric analysis of apoptosis was conducted after incubation for 24 h (E - control; F - cisplatin treated). In Figs E and F, Q1 indicates apoptotic cells; Q2 live cells; Q3; late apoptotic cells and Q4 early apoptotic cells). DNA condensation and fragmentation analysis by fluorescent microscopy with DAPI staining is shown after treatment for 24 h with or without 8 μM cisplatin (G - control; H - cisplatin treated), where the red arrows indicate cells undergoing apoptosis (x 200 magnification); Scale bar = 50 μm. Results are representatives of triplicate experiments.

Fig. 4. Cisplatin effects on TG2 at the transcriptional level. (A) TG2 mRNA expression measured by quantitative RT-PCR. HepG2 cells were incubated with cisplatin for 24 h. Results are means ±S.D. of duplicates of three independent experiments. The *P=0.01 by one-way ANOVA with Dunett's multiple comparison test relative to the control. (B) PCR products analysed by 1.2% agarose gel electrophoresis (lanes: 1 - control; 2 - 4 μM cisplatin;

3 - 8 μM cisplatin). PCR products were confirmed against available online data from which primers were designed (NCBI Nucleotide database).

Fig. 5. Effects of cisplatin on TG2 protein expression. HepG2 cells were treated with cisplatin (lane 1 - untreated; lane 2 - 4 μM; lane 3 - 8 μM; lane 4 - 12 μM; lane 5 -16 μM) and expression was measured by Western blot analysis following treatment for (A) 12 h, and (B) 24 h. Results are typical representatives of triplicate experiments.

Fig. 6. Effect of cisplatin on transglutaminase 2 activity. Transglutaminase 2 activity was measured with the specific TG2-CovTest kit. Cells were treated with 0-16 μM cisplatin for A) 12 h and B) 24 h. Samples were derived from 60 μl of cell lysate and the results are the mean ±S.D. of duplicate samples from three independent experiments.

Fig. 7. Apoptosis induction after inhibition of TG2 by cystamine. HepG2 cells were pre-incubated with or without a non-lethal dose of 1 mM cystamine for 48 h. Then, cystamine-treated cells were left untreated or were treated with 8 μM cisplatin for 6 h and 12 h, and apoptotic cells were analysed by flow cytometry using propidium iodide staining.

Fig. 8. Effect of retinoic acid on TG2 expression in HepG2 cells. HepG2 cells ($2x10^5$ cells/25cm^2 flask) were treated with 10 μM retinoic acid (RA) continuously for 72 h. After incubation, (A) changes in cell morphology were observed by inverted phase light microscope, (B) toxicity of 0-20 μM retinoic acid to cells was measured by flow cytometry, (C) TG2 protein expression was measured by Western blotting and densitometry analysis (D) Transglutaminase activity was measured by CBZ-Gln-Gly assay. Plates A, C and D show effects of 10 μM retinoic acid; TG2 was maximally expressed after 72 h of incubation.

Fig. 9. Effect of retinoic acid pre-treatment of HepG2 cells on cisplatin toxicity. HepG2 cells ($2x10^5$ cells) were treated or not treated with 10 μM retinoic acid (RA) and incubated for 72 h. After incubation, cells were either not treated or were treated with 8 μM cisplatin for a further

48 h. Then samples were analysed by FITC-labelled Annexin-V by flow cytometry. The results are means ±S.D. of triplicate samples from two independent experiments.

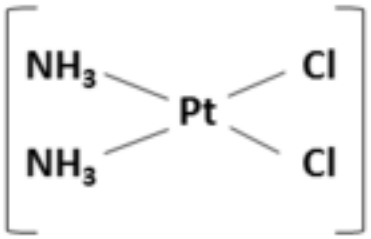
Fig. 10. Effect of pre-treatment of HepG2 cells with retinoic acid on cystamine potentiation of cisplatin toxicity. HepG2 cells ($2x10^5$ cells) were pre-treated with 10 μM retinoic acid for 72 h. Then, cells were treated or were not treated with 1 mM cystamine (cys) for another 48 h. After incubation with cystamine, cells were treated or not treated with 8 μM cisplatin for a further 48 h, and cell death was analysed by FITC-labelled Annexin-V by flow cytometry. Results are means ±S.D. of triplicate samples from two independent experiments.

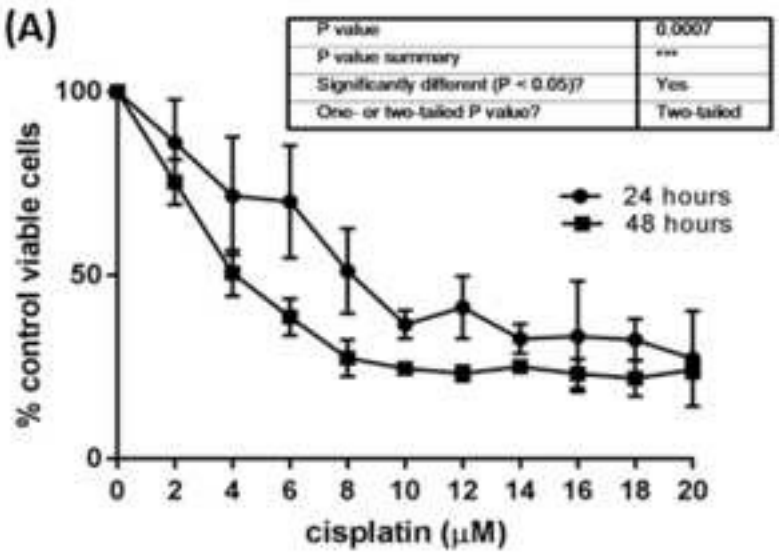
Fig. 11. Short-term effect of cisplatin on guinea pig liver transglutaminase activity *in vitro.* Transglutaminase activity was measured by CBZ-Gln-Gly assay. Cisplatin was used over the range of 0-128 μM on a 0.02 mg/ml guinea pig liver transglutaminase solution for 1 h. Results are means ±S.D. of duplicates of three independent experiments. All cisplatin-treated groups were compared with control groups using one way ANOVA with Dunnett's multiple comparison test. ****P=0.0001.

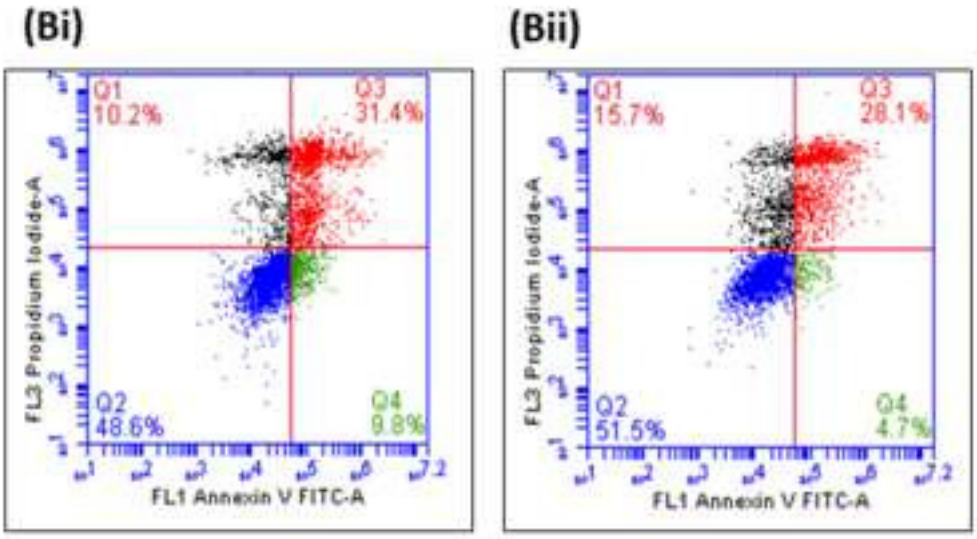
Fig. 12. Time-dependent effect of cisplatin on Tgase activity. Transglutaminase activity was measured by CBZ-Gln-Gly assay. Cisplatin was used over the range of 0-128 μM on a 0.01 mg/ml guinea pig liver transglutaminase solution for 0-24 h. Results are means ±S.D. of duplicates of three independent experiments.

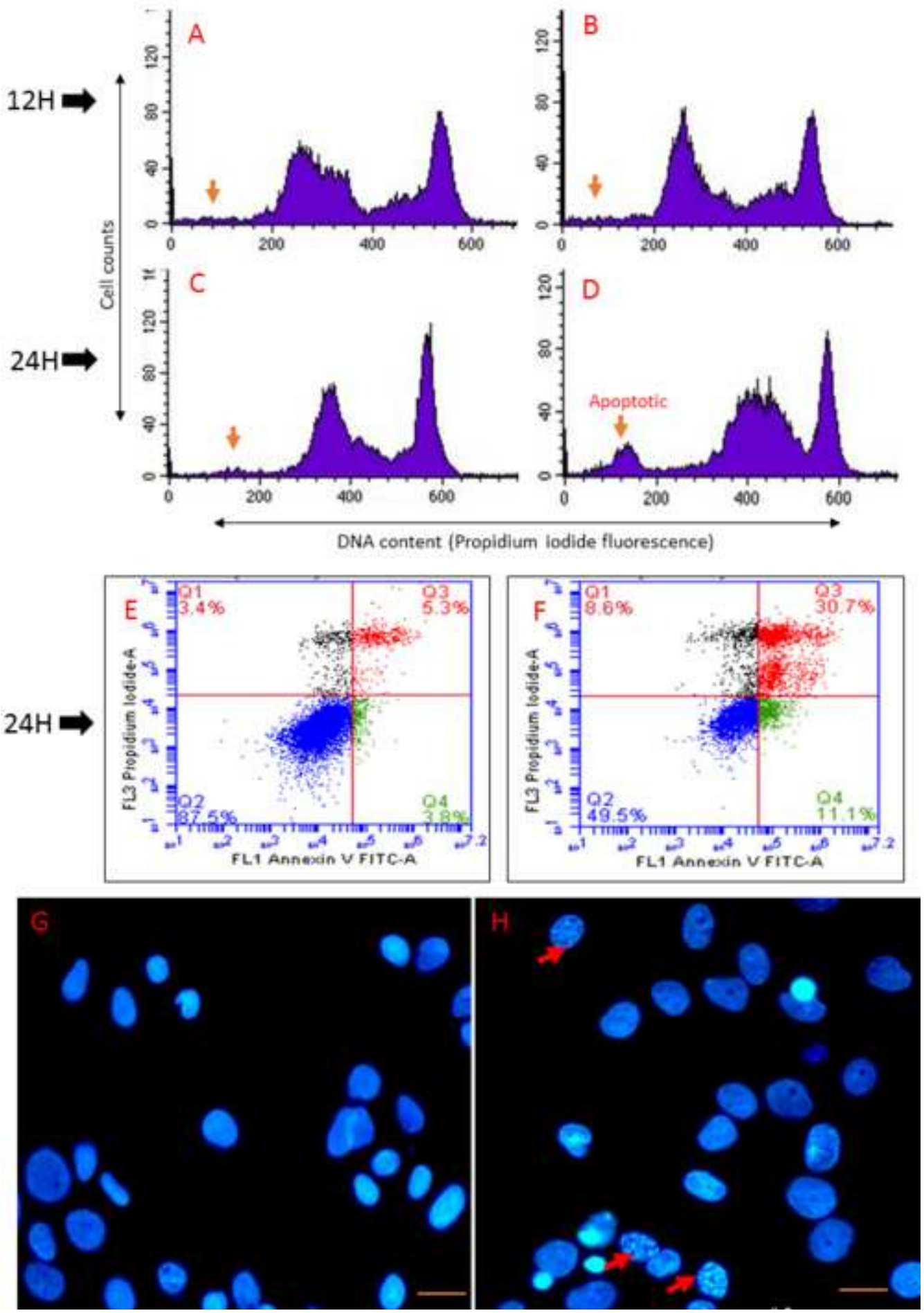
Fig. 13. Proposed mechanism of cisplatin-induced apoptosis by TG2. In HepG2 cells, cisplatin inhibited the expression and activity of TG2. Once TG2 is inhibited, it may increase the expression of caspases and trigger apoptosis (Tatsukawa et al., 2011). On the other hand, overexpression of TG2 by retinoic acid may inhibit caspases and thus protect cells from cisplatin toxicity.

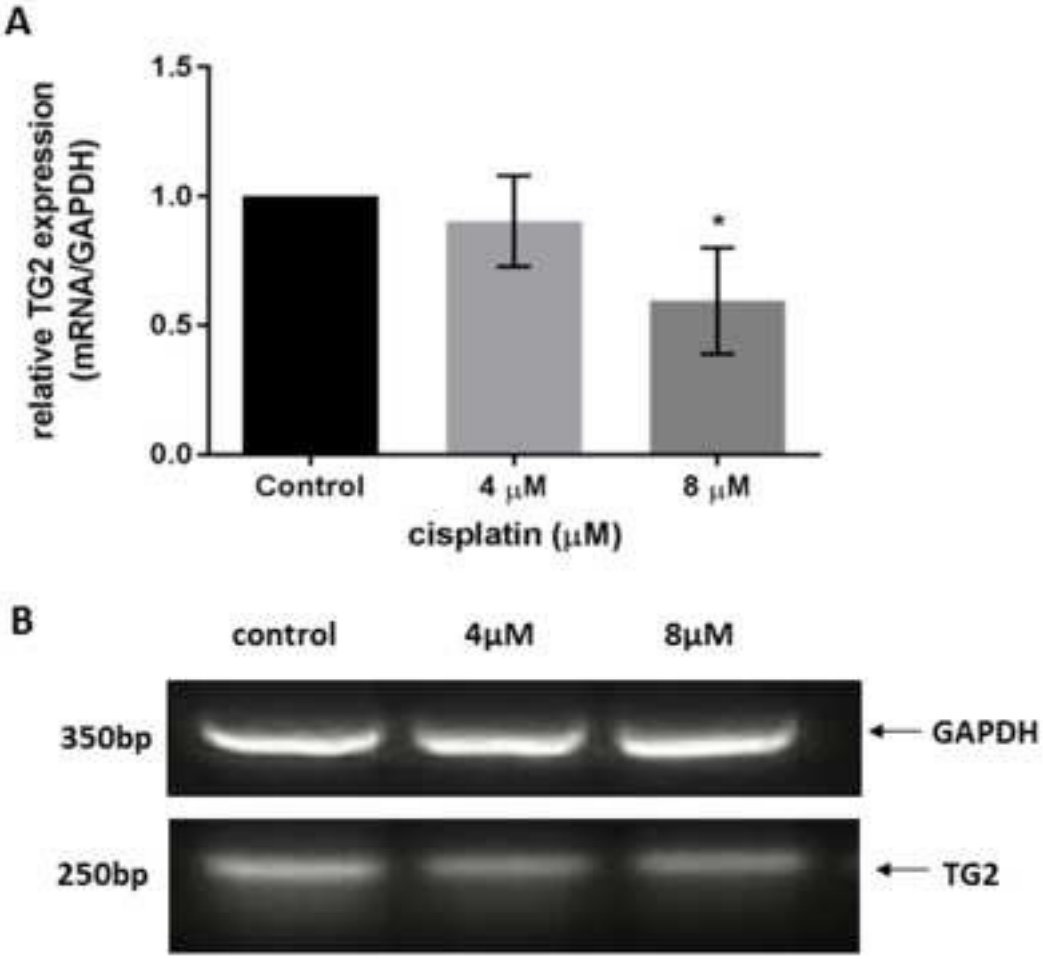
Figure

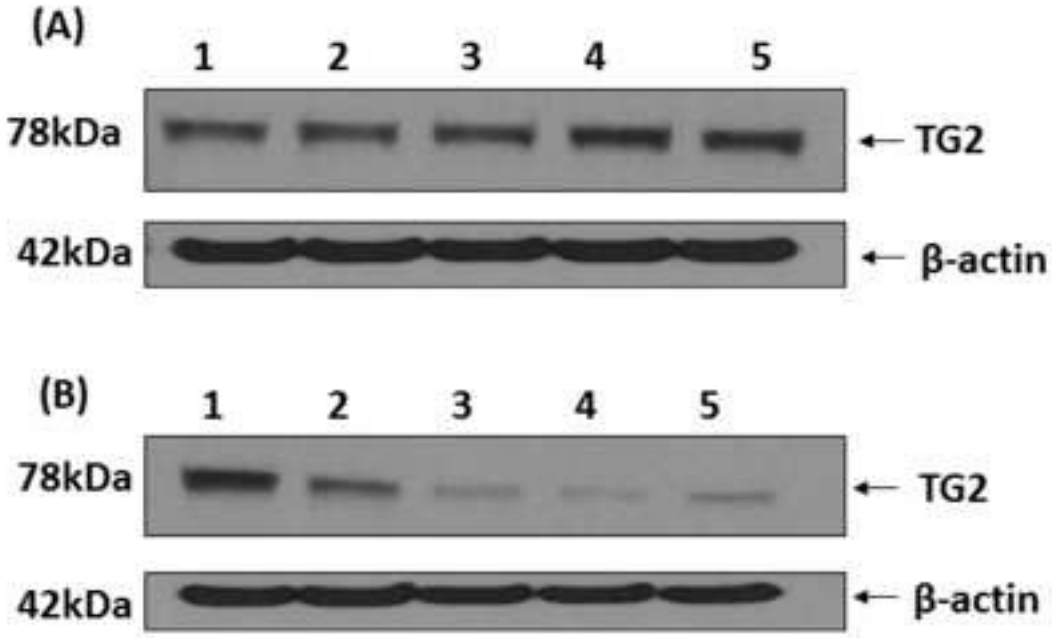


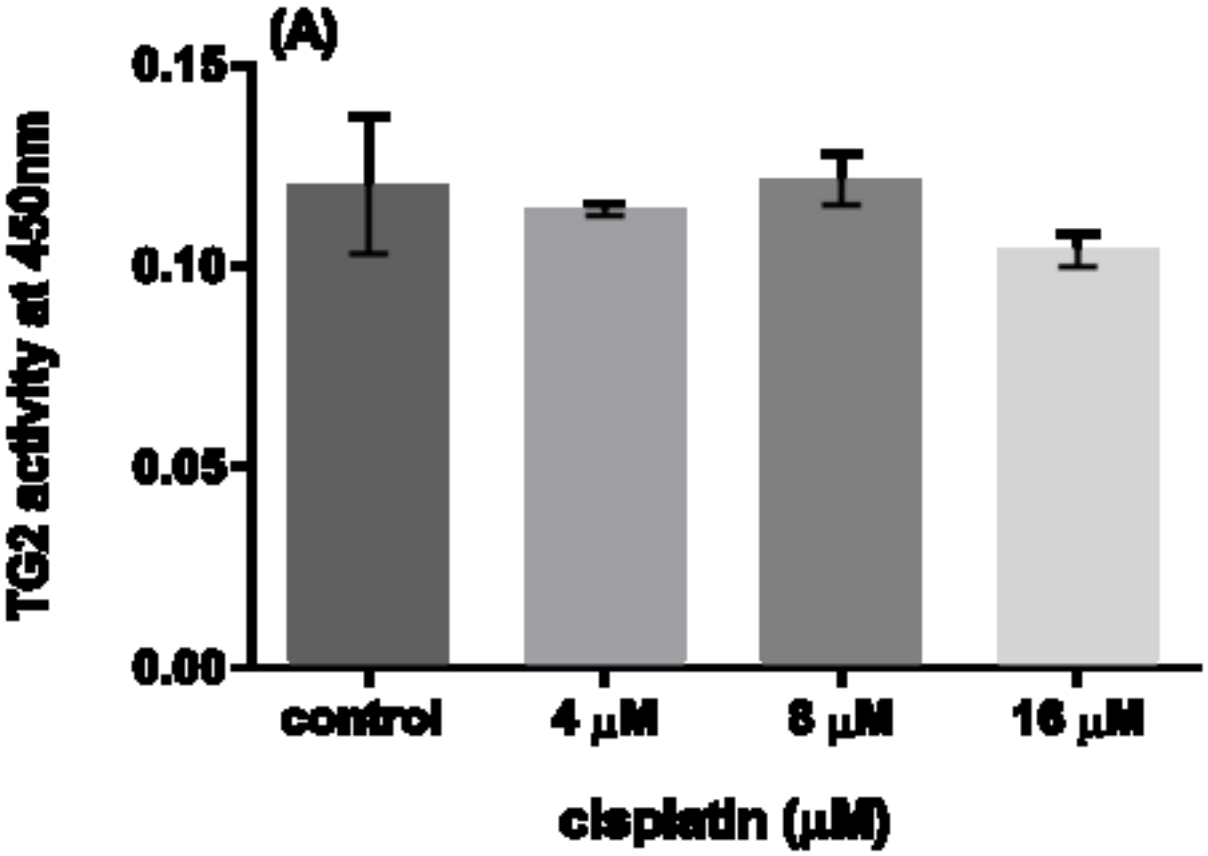


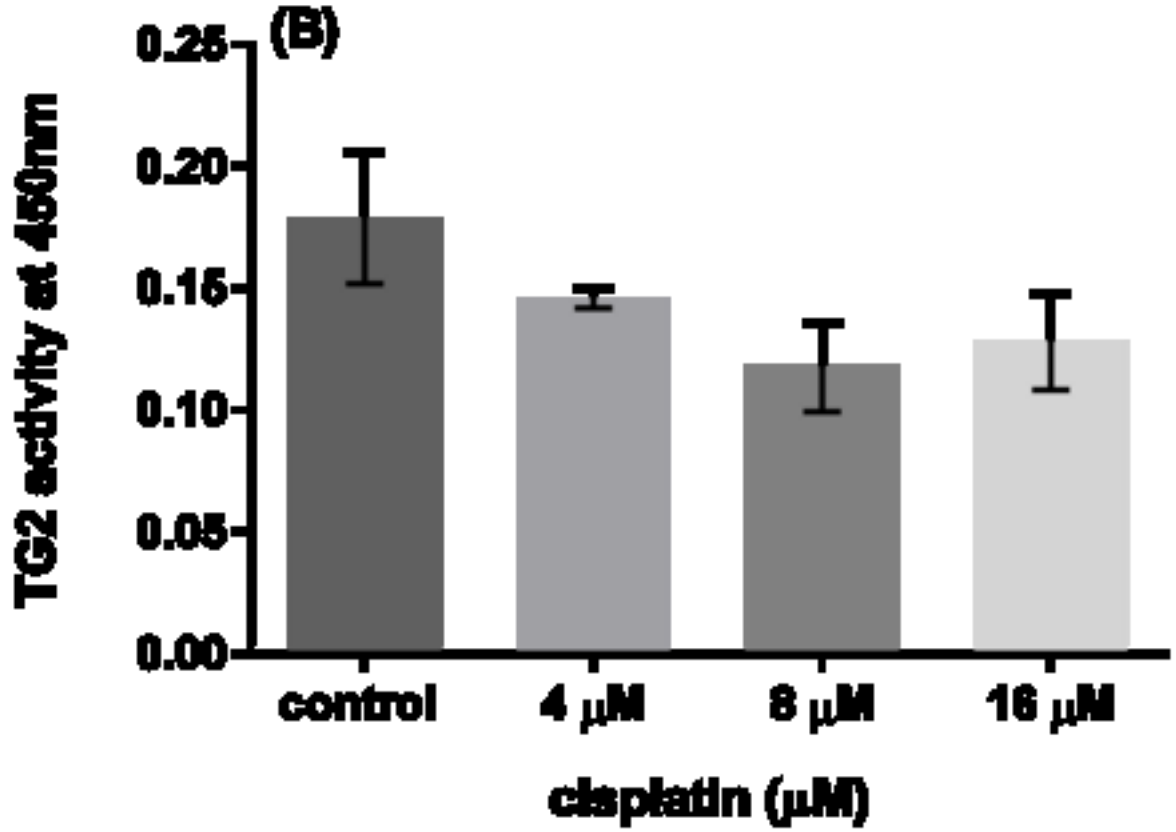


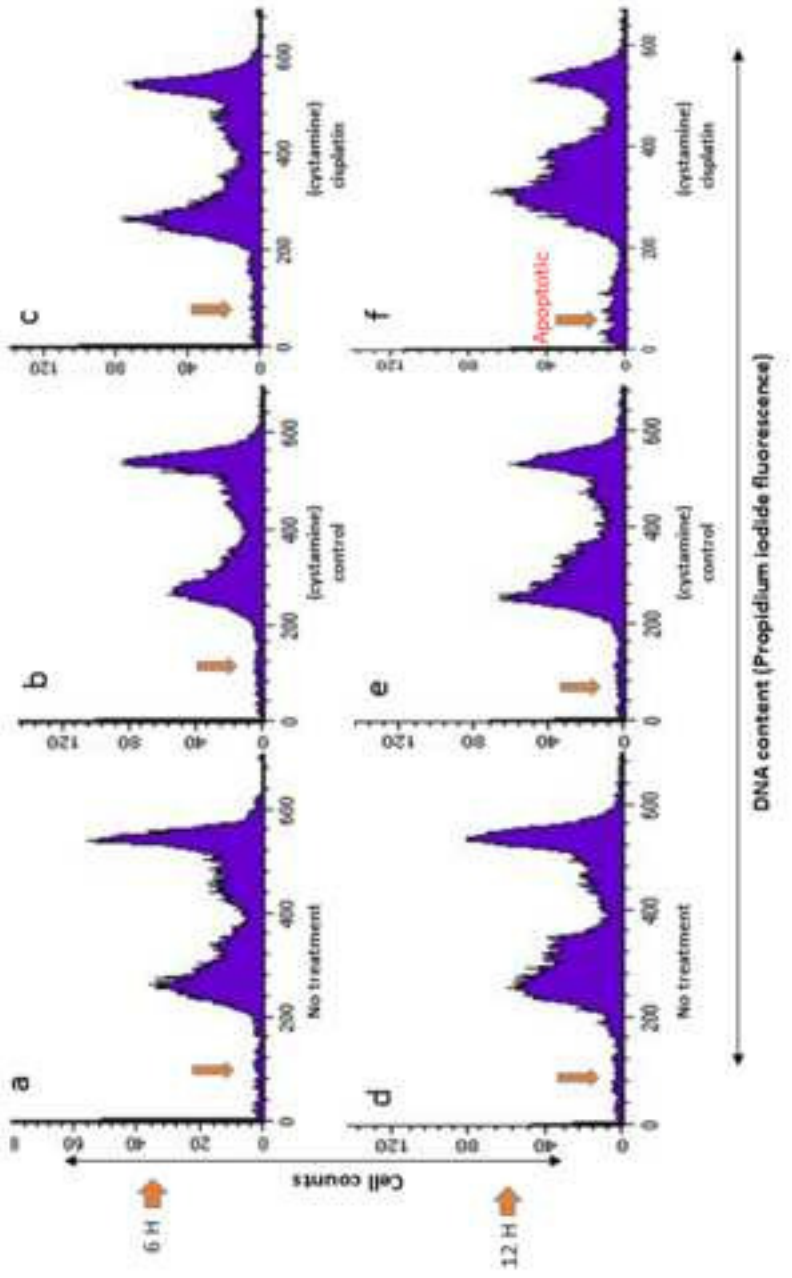












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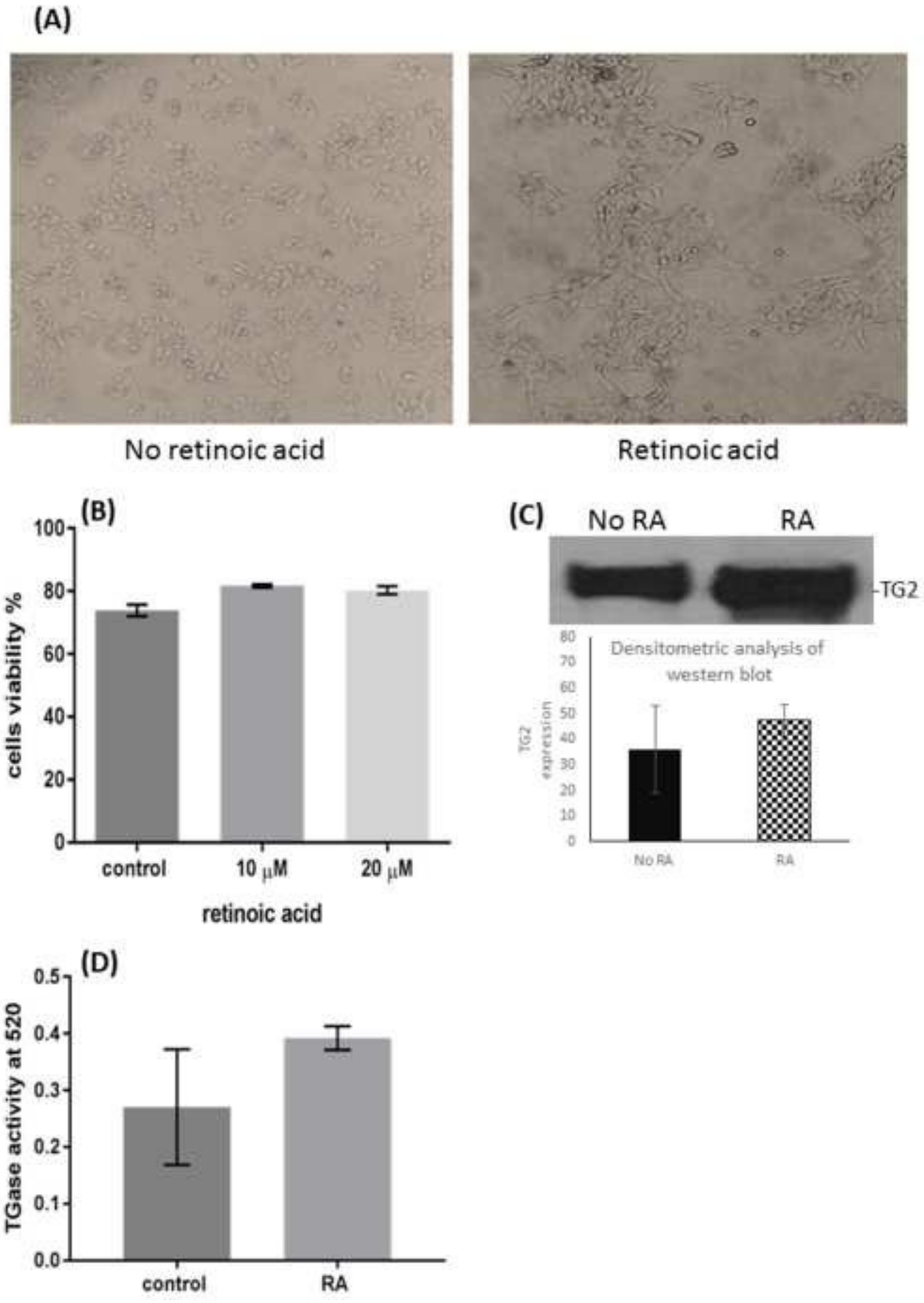

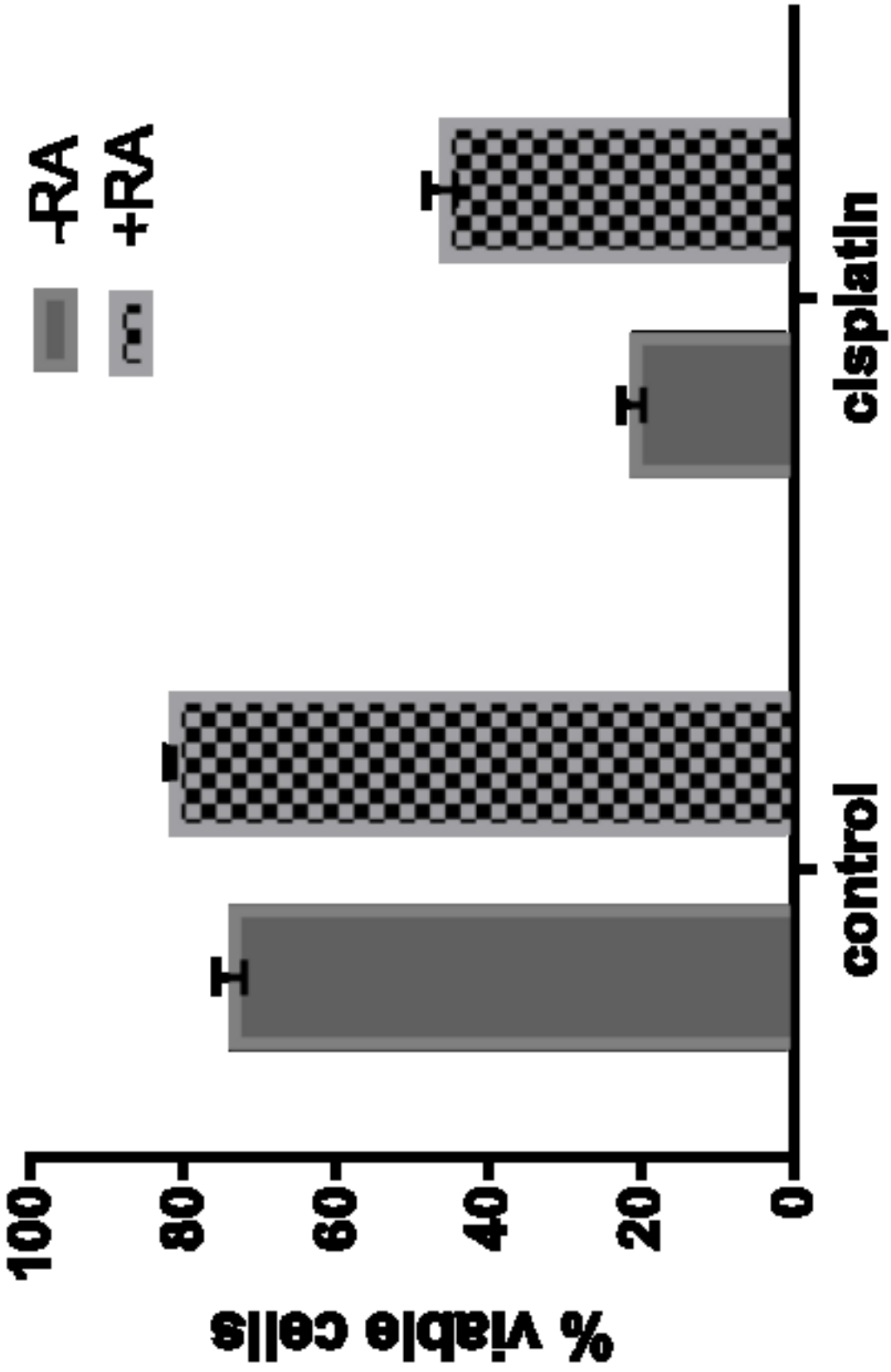

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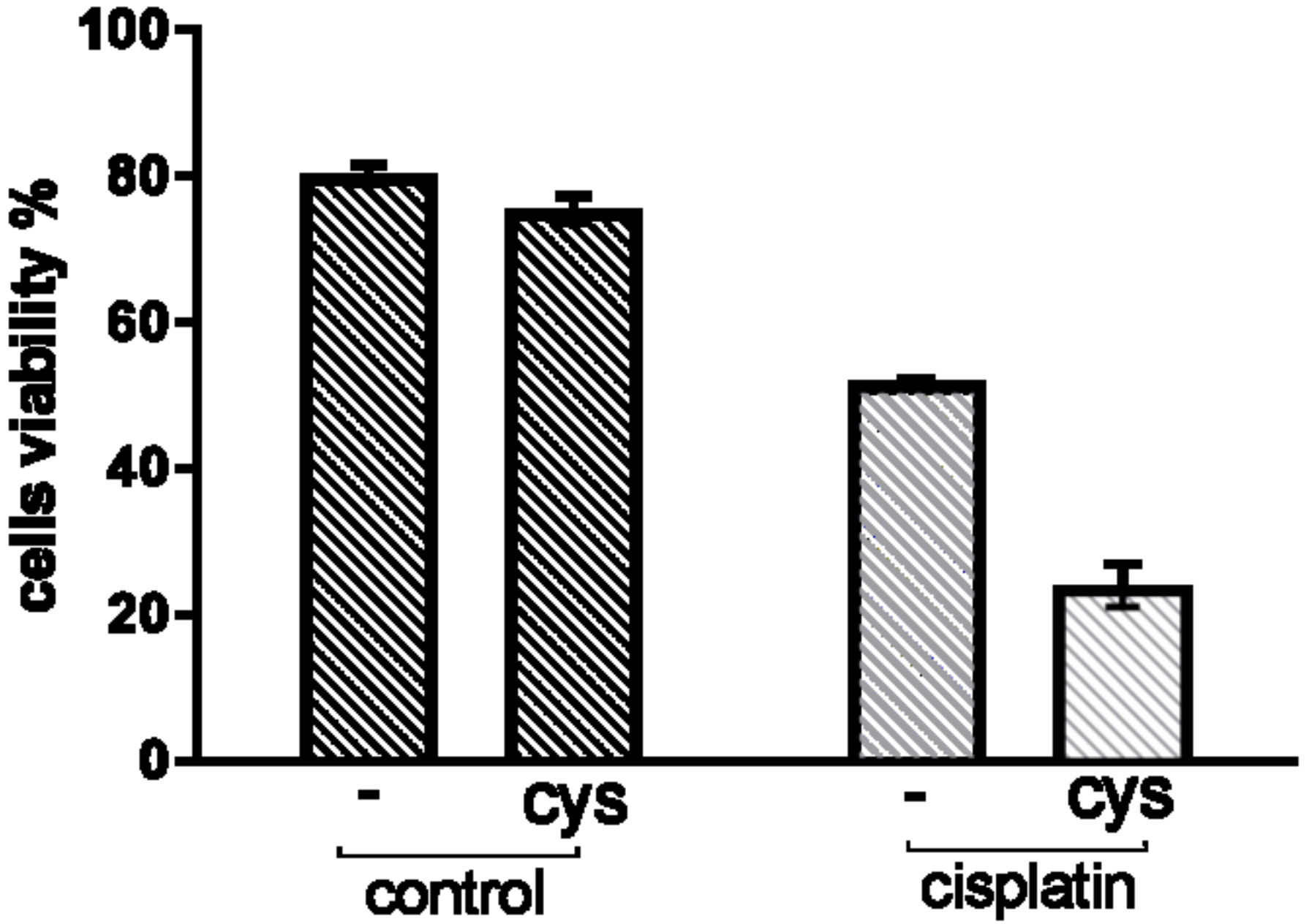

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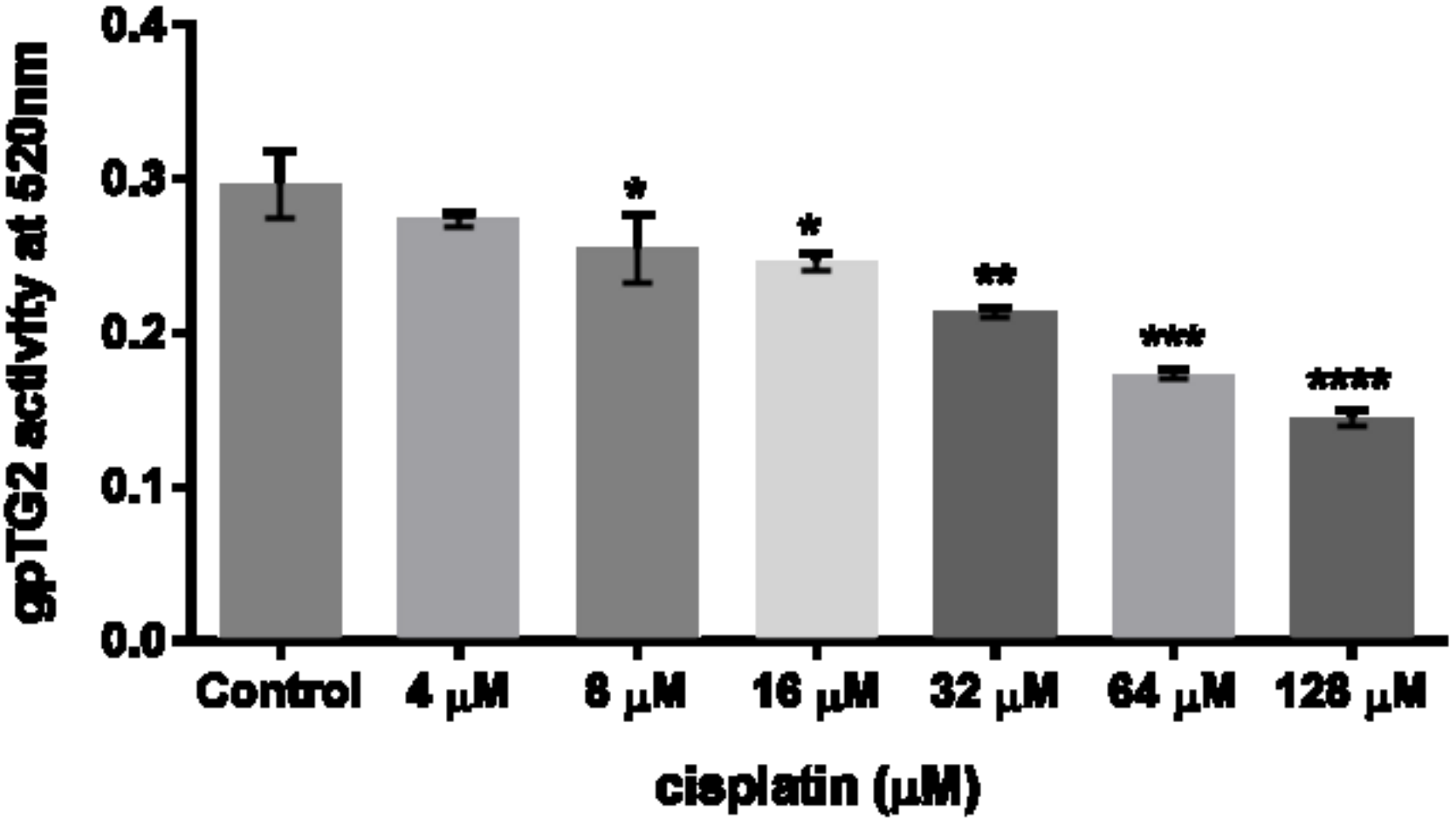

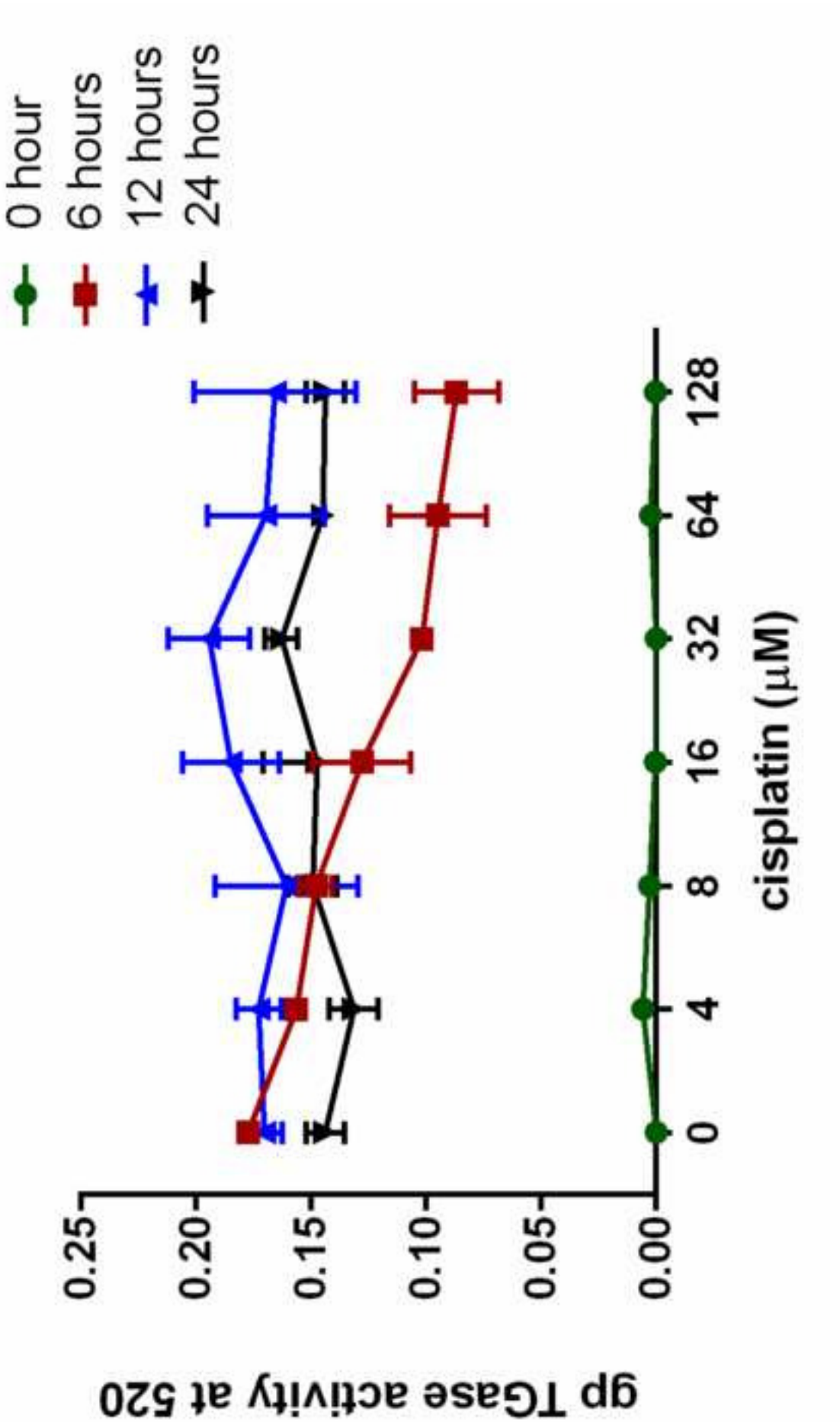
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cisplatin (μM)

Figure