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Serine phosphorylation of glutathione *S*-transferase P1 (GSTP1) by PKCα enhances GSTP1-dependent cisplatin metabolism and resistance in human glioma cells

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ABSTRACT

Recently, we reported that the human GSTP1 is phosphorylated and functionally activated by the PKC class of serine/threonine kinases. In this study, we investigated the contribution of this post-translational modification of GSTP1 to tumor cisplatin resistance. Using two malignant glioma cell lines, MGR1 and MGR3, the ability of PKCα-phosphorylated GSTP1 to catalyze the conjugation of cisplatin to glutathione was assessed and correlated with cisplatin sensitivity and cisplatin-induced DNA interstrand cross-links and apoptosis of the cells. The results showed PKCα activation and associated phosphorylation of GSTP1 to correlate significantly with increased glutathionylplatinum formation, decreased DNA interstrand cross-link formation and increased cisplatin resistance. Following PKC activation, the IC_{50} of cisplatin increased from 13.63 μM to 36.49 μM in MGR1 and from 20.75 μM to 38.45 μM in MGR3. In both cell lines, siRNA-mediated GSTP1 or PKCα transcriptional suppression similarly decreased cisplatin IC_{50} and was associated with decreased intracellular levels of glutathionylplatinum metabolite. Combined inhibition/transcriptional suppression of both PKCα and GSTP1 was synergistic in enhancing cisplatin sensitivity. Although, cisplatin-induced apoptosis was associated with the translocation of Bax to mitochondria, release of cytochrome *c* and caspase-3/7 activation, the levels of relocalized Bax and cytochrome *c* were significantly greater following GSTP1 knockdown. These results support a mechanism of cisplatin resistance mediated by the PKCα-dependent serine phosphorylation of GSTP1 and its associated increased cisplatin metabolism, and suggest the potential of simultaneous targeting of GSTP1 and PKCα to improve the efficacy of cisplatin therapy.

1. Introduction

Drug resistance is a frequent major obstacle to the success of chemotherapy with cisplatin, an anticancer agent used widely in the treatment of many solid cancers, including, pediatric brain tumors, and carcinomas of the breast, head and neck, lung, ovary and testes [1–5]. One of the most frequent correlates of cisplatin resistance, in the preclinical and clinical settings, is a significant elevation of glutathione *S*-transferase P1 (GSTP1) and its natural co-substrate, glutathione (GSH) [6]. In many of these tumors, both failure of patients to respond to therapy or relapse following initial response is frequently associated with increased tumor GSTP1 levels [7–12]. GSTP1 has also been found to be the most common and most predominant protein expressed in 58 of the panel of 60 tumor cell lines used by the NCI drug screening program [13].

A member of the multigene family of dimeric GSTs, GSTP1 is best known for its function in phase II metabolism in which it catalyzes the conjugation of exogenous and endogenous electrophilic compounds to GSH [14] and, more recently, for its ability to bind to and regulate several important cell signaling proteins, including, MAP kinases (JNK and ASK1/TRAF), transglutaminase 2 (TGM2), and the fanconi anemia group C DNA repair protein (FANCC) [15–19]. Many anticancer agents, including cisplatin, are GSTP1 substrates and the resulting metabolites are generally, non-toxic and, as in the case of glutathionylplatinum, are exported ATP-dependently from cells through an active GS-X efflux pump, primarily, the MRPs [20].

Recently, we reported that the GSTP1 protein undergoes phosphorylation at serines 42 and 184 by members of the protein kinase C (PKC) family of serine/threonine protein kinases [21]. PKCs play important roles in multiple cellular processes, including cell proliferation, differentiation, migration and apoptosis and, at least, 11 closely related PKC isozymes have been identified and

Abbreviations: GSTP1, glutathione *S*-transferase P1; GSH, reduced L-glutathione; PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; Bis-I, bisindolylmaleimide I.

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shown to differ in structure, biochemical properties, tissue distribution and substrate specificity [22]. The main PKC classes are the Ca^{2+}-dependent conventional cPKCs (α, β1, β2, γ), activated by both phosphatidylserine (PS) and diacylglycerol (DAG), the Ca^{2+}-independent novel nPKCs (δ, ε, η, θ, μ), also activated by both PS and DAG and the DAG and Ca^{2+}-independent atypical aPKCs (ζ, λ), regulated by PS [22].

The majority of human tissues and tumors examined to date express PKC, although, the levels of expression and the specific isoforms expressed vary significantly with the tissue and tumor type. Because of its high expression in malignant glioma cells and its high propensity to phosphorylate GSTP1 [21,23], we focused this study on PKCα. Suppression of PKCα by a dominant negative PKCα mutant or the small molecule inhibitor, UCN-01 (7-hydroxystaurosporine) has been shown to trigger apoptosis in glioma cells and to increase response to chemotherapy [24–26]. The mechanism(s) by which PKCα prevents apoptosis is, however, not well understood. One potential mechanism involves the anti-apoptotic and pro-survival protein, Bcl2 [27]. PKCα phosphorylates and stabilizes Bcl2 by rendering it resistant to proteasome-mediated degradation [27]. Other studies have shown that introduction of PKCα antisense oligonucleotides into cells is associated with suppression of the Bcl2 family of proteins and with increased cisplatin sensitivity [28]. The pro-apoptotic protein, Bax, a binding partner of Bcl2, is known to translocate to mitochondria upon exposure of cells to cisplatin and following its insertion into the mitochondrial membrane causes release of cytochrome *c* (cyt *c*) into the cytosol, resulting in activation of caspases and apoptosis [29]. Bcl2 prevents cyt *c* release by inhibiting the mitochondrial permeability transition and redistribution of Bax, and thus may result in cisplatin resistance [30]. The binding of Bcl2 with Bax thus results in suppression of Bcl2 and its anti-apoptotic function.

Against this background, we examined, in this study, the hypothesis that the enzymatically more active PKCα-phosphorylated GSTP1 will metabolize and inactivate cisplatin more efficiently than the unphosphorylated protein and, thereby, contribute to a higher level of tumor cisplatin resistance. Because PKCα may play a direct role in drug-induced apoptosis, we also investigated the effects of modulation of PKCα activity on cisplatin-mediated sub-cellular translocation of Bax and cyt *c*.

2. Materials and methods

2.1. Chemicals and other reagents

Recombinant GSTP1 protein, recombinant PKCα, aprotinin, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and bisindolylmaleimide-I (Bis-I) were purchased from Calbiochem, EMD Biosciences (San Diego, CA, USA). Reduced L-glutathione (GSH) and ethacrynic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cisplatin was obtained from Bedford Laboratories, (Bedford, OH, USA). The Cell Titer-Blue™ cell survival assay kit was from Promega (Madison, WI) and the SensoLyte™ fluorometric assay kit for assaying caspase-3/7 was from AnaSpec, San Diego, CA, USA. Qiagen RNeasy® RNA isolation kit was from Qiagen Inc. (Valencia, CA, USA). Lipofectamine™ and Superscript™ III First-Strand Synthesis System were from Invitrogen Corp. (Carlsbad, CA, USA). All antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA), unless otherwise stated. Tissue culture media, fetal bovine serum and other cell culture related reagents were from Gibco® (Invitrogen Corp. Carlsbad, CA).

2.2. Tumor cell lines

The MGR1 (anaplastic astrocytoma) and MGR3 (glioblastoma multiforme) cell lines were established in our laboratory from primary tumor specimens, as described previously [31]. Following establishment and characterization, the primary cultures were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells used in this study had undergone 25 to 30 *in vitro* passages and were all negative for mycoplasma.

2.3. Assay of cellular GSH content

Cellular GSH content was determined following its derivatization with *o*-phthalaldehyde [32], using a commercial kit (BioVision Research Products, Mountain View, CA, USA), with slight modifications of the manufacturer's recommended methodology. Briefly, cells treated as per experimental protocol were lysed in 50 mM Tris–HCl (pH 7.4), and the particle-free supernatants treated with 6N perchloric acid. After centrifugation, 60 μl of the supernatant were neutralized with 30 μl 3N KOH, re-centrifuged and to 10 μl of the final supernatant, was added 80 μl buffer and 10 μl *o*-phthalaldehyde. The samples were incubated at room temperature for 40 min, and the fluorescence of the product read at excitation and emission wavelengths of 360 nm and 460 nm, respectively (Synergy™ HT, Winooski, VT, USA). Cellular GSH concentration was determined using a previously established GSH standard curve and normalized to protein content.

2.4. Analysis of cellular GSTP1 and PKC activities

Glioma cultures treated as per experimental protocol were harvested by trypsinization, washed with cold PBS and sonicated in 50 mM Tris–HCl buffer (pH 7.4), containing 1 mM DTT and a protease inhibitor cocktail. The cell lysates were spun at 20,000 × *g* for 20 min, the protein concentration assayed by the Bradford method [33] and used to measure GSTP1 activity, as previously described [34,35]. Briefly, a 100 μl reaction mixture was set up containing 100 mM potassium phosphate buffer (pH 6.8), 10 μg cellular protein, 0.25 mM GSH and 0.1 mM ethacrynic acid. The rate of formation of the reaction product of ethacrynic acid with GSH was monitored spectrophotometrically at 270 nm (DU800 Spectrophotometer, Beckman Coulter, Brea, CA, USA).

For cellular PKC determination, tumor cells treated as required were lysed in 25 mM Tris–HCl buffer (pH 7.0), containing 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM EDTA, 1 mM EGTA and 5 mM 2-mercaptoethanol. PKC activity was determined using a non-radioactive enzyme-linked immunosorbent assay (MBL International Corporation, Woburn, MA, USA). To each well of a 96-microwell plate pre-coated with a PKC-specific peptide (RFARKGSLRQKNV) was added 25 μg protein, 50 μg/ml phosphatidylserine/Ca^{2+}, 0.1 mM ATP and 100 μl of a biotinylated antiphospho-tyrosine antibody (2B9). After 1-h at 25 °C, the plates were washed with PBS, treated with peroxidase-conjugated streptavidin for an additional 1 h, washed again and 100 μl of 30% H_2O_2 and *o*-phenylenediamine.2HCl added. After 5 min, the reaction was stopped with 100 μl of 20% H_3PO_4, and the absorbance at 492 nm measured (Synergy™ HT, BioTek plate reader, Winooski, VT, USA). Relative PKC activities in the extracts were computed from standard curves established with recombinant human PKCα and expressed as pmoles phosphate/mg protein.

2.5. Real-time quantitative RT-PCR of GSTP1 and PKCα gene transcripts

Total RNA was extracted from cells after experimental treatment and quantified by UV absorbance spectrometry. First strand cDNA was synthesized in a 20 μl reaction volume containing 20 ng of RNA, reverse transcription buffer, 6 mM $MgSO_4$, SuperScript™ III reverse transcriptase/Platinum® *Taq*

DNA polymerase, 0.4 mM deoxynucleotide triphosphate mixture (Invitrogen Corp, Carlsbad, CA, USA) and RNase-free water in a Mx3005P PCR instrument (Stratagene, La Jolla, CA, USA). Aliquots of cDNA and external standards were amplified in parallel reactions, using the FastStart SYBR Green master mix (Invitrogen Corp. Carlsbad, CA, USA). The mixture was heated at 95 °C for 5 min and 40 cycles of 95 °C (15 s) and 60 °C (30 s) were run using the following primers: 5′-TGCTGGACTTGCTGCTGATC-3′ and 5′-CACA-TATGCTGAGAGCAG-3′, (*GSTP1*), and 5′-AAGAAGGTGGTGAAG-CAGG-3′ and 5′-GTGTCGCTGTTGAAGTCAGA-3′ (*GAPDH*). The GAPDH coamplified product was used to normalize GSTP1 transcript levels and to adjust for differences in both the amount of total RNA and different RT reaction efficiencies. Controls lacking RNA template and reverse transcriptase were used to monitor for fluorescent contaminants. The ΔC_T values relative to GAPDH were determined and $\Delta\Delta C_T$ values of siRNA treated relative to untreated cells were computed as a measure of GSTP1 or PKCα gene expression.

2.6. Isolation of cytosolic and mitochondrial fractions of tumor cells

Cells treated as per experimental requirement were harvested, washed twice in cold PBS, and resuspended in MIB buffer (10 mM HEPES (pH 7.5), 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and a mixture of protease inhibitors). After a 20-min incubation on ice, the cells were homogenized (30 strokes) in a Dounce glass homogenizer (Sigma, St. Louis, MO, USA) and spun at 1000 × *g* to remove unbroken cells, nuclei, and heavy membranes. The supernatants were respun at 20,000 × *g* for 30 min to collect the mitochondria-rich (pellet) and the cytosolic (supernatant) fractions.

2.7. Western blotting

This was performed as we previously described [21]. Particle-free supernatants of whole extracts of tumor cells were resolved on 12% SDS-PAGE for GSTP1 or 8% SDS-PAGE for PKC isoforms. Cytosolic and mitochondrial proteins fractionated as described above were separated on 15% SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes, blocked with 5% BSA (Sigma, St. Louis, MO, USA) in Tris-buffered saline-Tween 20 (Sigma, St. Louis, MO, USA) and incubated with appropriately diluted antibodies for 1 h, washed and treated with horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were visualized by chemiluminescence (ECL, Thermo Scientific, Rockford, IL, USA) and scanned by densitometry. Band intensities were quantitated using the Image J digital image processing program (NIH, USA), after which the filters were stripped by boiling for 5 min, and reprobed with an antibody against beta-actin (cytosolic marker) or VDAC (mitochondrial marker). The level of expression of GSTP1 or PKCα was determined as the ratio of the band intensity of each protein relative to that of beta-actin band, while the ratio of Bax or cyt *c* band intensity relative to beta-actin or VDAC was used as a measure of each protein in the cytosol or mitochondria, respectively.

To examine PKC-dependent intracellular GSTP1 phosphorylation, we used a combination of immunoprecipitation and Western blotting. Cells treated as per experimental protocol were rinsed with cold PBS, harvested and lysed in 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, and a protease and phosphatase inhibitor cocktail (Calbiochem, Gibsstown, NJ). To the particle-free supernatant of the lysate (500 μg protein) was added 5 μl GSTP1 antibody (200 mg/ml) and the mixture incubated at 4 °C for 1 h on a rotator. A 50 μl slurry of protein A-Sepharose beads was then added, after incubation at 4 °C for another hour, the beads were washed four times with cold lysis buffer and subjected to Western blotting, with an anti-phospho-serine antibody specific to a phosphorylated epitope of PKC (motif recognized: Arg or Lys-X-Ser^{phos}-Hyd-Arg or Lys) and a GSTP1-specific antibody as probes.

2.8. siRNA-mediated GSTP1 and PKCα down-regulation

Exponentially growing glioma cells were transfected with 20 nM siRNA targeting GSTP1 (5′-ACCAGAUCUCCUUCGCUGACUA-CAA-3′) (Invitrogen Corp, Carlsbad, CA, USA) or PKCα (5′-CCAUCGGAUUGUUCUUUCUUCAUAA-3′) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine™, essentially as directed by the manufacturer, with some modifications to optimize the transfection conditions. Controls were transfected cells without any treatment or containing scrambled siRNA. After 6 h, the cultures were refed with fresh medium and incubated at 37 °C, 5% CO_2 for a further 24, 48 and 72 h, harvested and used to determine the level of GSTP1 and PKCα transcripts by RT-PCR, as described earlier. For the double knockdown of both PKCα and GSTP1, cells were first transfected with PKCα-specific siRNA, followed 6 h later with GSTP1-specific siRNA. After further 6-h incubation, the media was replaced and the cells incubated at 37 °C, 5% CO_2 for the time periods required by the experimental protocol. Under these optimized conditions, we generally achieve approximately, 80% transfection efficiency and kinetic studies have shown the level of gene suppression to be maintained for 96 h.

2.9. Assay of GSTP1-dependent cisplatin metabolism

This was determined by measuring the formation of the glutathionylplatinum metabolite in cell-free reaction mixtures or in cisplatin-treated cells. For the former, 1 μg of recombinant human GSTP1 protein was pre-incubated with 5 mM GSH for 30 min at 37 °C to mimic the physiologic GSH-bound state of GSTP1. The phosphorylation reactions were set up as we previously described [21]. Briefly, the GSH-GSTP1 mix was incubated at 30 °C for 1 h with a kinase reaction mixture containing 1 μg PKCα and 100 μM ATP (Calbiochem, EMD, Gibbstown, NJ). The phosphorylated or control unphosphorylated GSTP1 was then added to a mixture of 5 mM GSH and 1 mM cisplatin (1 ml total reaction volume) and incubated at 37 °C. At different time points 100 μl aliquots were drawn and used to determine the levels of glutathionylplatinum metabolite, as we previously described [20]. The peak absorbance at 260 nm used as a measure of the amount of glutathionylplatinum formed.

To determine the level of cisplatin metabolism in PKC-activated cells, exponentially growing cultures were treated with 50 nM TPA for 2 h to activate PKC, followed by 100 μM cisplatin. After 2 h, the cells were harvested, washed, homogenized and the protein concentration of the supernatants after centrifugation at 20,000 × *g* for 20 min, adjusted to 2 (g/μl. TCA was added to precipitate proteins, the samples recentrifuged and the particle-free supernatants used to determine the level of glutathionylplatinum, as described above.

The effect of siRNA-mediated GSTP1 or PKCα knockdown on cisplatin metabolism was similarly determined. MGR1 and MGR3, transfected as described earlier, for 48 h, were treated with 50 nM TPA for 2 h, followed by 100 μM cisplatin or with 50 nM TPA and 100 μM cisplatin simultaneously. 2 h post-treatment, the cells were harvested, homogenized and the level of glutathionylplatinum metabolite in cell-free supernatants determined, as described above.

2.10. Assay of residual non-metabolized cisplatin

Residual cisplatin in reaction mixtures was assayed spectrophotometrically as the formation of dichloro-(phenylenediami-

ne)platinum (DPDP), using a modification of a previously described method [36]. Briefly, 10 μl of GSTP1 phosphorylation and control reactions set up as described above, were added to 90 μl of 0.1 mM HCl and 100 μl of 1.4 mg/ml of *o*-phenylenediamine (OPDA) (Sigma, St. Louis, MO, USA) in dimethylformamide (DMF) (Sigma, St. Louis, MO), and heated for 30 min at 90 °C. The reactions were cooled to room temperature, adjusted to 1 ml with 70% DMF and the level of DPDP measured at 705 nm. The relative concentration of free cisplatin in the reaction was computed as $[A/A_{Cispt}].100$, where A and A_{Cispt} are the absorbances of the reactions with and without GSTP1-catalysis.

2.11. Effect of PKCα and GSTP1 gene knockdown on cisplatin-induced DNA interstrand cross-linking

MGR3 cells were treated with TPA or transfected with PKCα- and GSTP1-siRNAs, individually and combined, followed by 100 μM cisplatin. After 6 h, total genomic DNA from untreated control and cisplatin-treated cells was extracted and the level of DNA interstrand cross-linking (ICL) measured essentially, as we previously described [38] but using the thiazole orange DNA-binding agent [37] instead of ethidium bromide as the DNA binding agent. Briefly, 20 μg/ml genomic DNA in 20 mM potassium phosphate buffer (pH 11.75) were heated at 100 °C for 10 min in triplicate borosilicate tubes, cooled at 15 °C for 5 min and readjusted to 0.1 ml. After 10 min, 10 μl of 100 μM thiazole orange (AnaSpec, San Diego, CA, USA) was added to 90 μl aliquots of the solution, incubated at room temperature for 5 min and the fluorescence measured at an excitation wavelength of 508 nm and an emission wavelength of 528 nm (Synergy™ HT, Winooski, VT, USA). A DNA cross-link index (CLI) for each treatment was computed as we described previously [38]. Cisplatin CLI = ln [Xo/Xc]/-lnXo; where Xo and Xc are the respective fractional changes in DNA-thiazole orange fluorescence of DNA without and with cisplatin treatment.

2.12. Modulation of PKC and GSTP1 activity and effect on cisplatin sensitivity of glioma cells

This was examined using cell survival and induction of apoptosis as endpoints. MGR1 and MGR3 cells were plated at 1×10^3 to 1×10^4 per well in a 96-well microtiter plate and after 24 h, treated with Bis-I or TPA, or with PKCα- or GSTP1-targeted siRNA, as described above, and refed with fresh medium containing 0-50 μM cisplatin. Cell survival was measured after 48 h, using the Cell Titer-Blue assay, as per the manufacturer's instructions, with excitation and emission wavelengths of 560 nm and 590 nm, respectively. Surviving fractions, S.F., were computed using the equation: S.F. = F_t/F_c, where F_t and F_c are the fluorescence of treated and untreated cells, respectively, normalized against background fluorescence. The IC_{50} was computed by linear regression of the dose response curves using GraphPad® Prism software (San Diego, CA, USA).

The effect of the modulation on cisplatin-induced apoptosis was determined using caspase-3/7 activation as endpoint. Briefly, following treatment of tumor cells as described above, the caspase-3/7 substrate, $(Z\text{-}DEVD)_2$-Rh110 was added to 50 μM final concentration and after 3 h at room temperature in the dark, the fluorescence of the cleavage product was measured at excitation and emission wavelengths of 500 nm and 528 nm, respectively. The relative fluorescence, RF, after normalization against untreated controls, was used as a measure of apoptotic induction.

2.13. Quantitation of cell death by flow cytometry

Tumor cells were plated in 25-cm^2 flasks, treated as required, harvested, washed in PBS, and fixed in 70% ethanol at 4 °C for 1 h. The cells were rehydrated in PBS, treated with 100 μg/ml RNase A and stained with 50 μg/ml propidium iodide (Sigma, St. Louis, MO, USA) at room temperature for 30 min. Cells (20,000 per sample) were analyzed on a FACScan flow cytometer (Becton Dickinson, NJ). The percentage of cells in pre-G1 cell cycle phase was computed using WinMDI software and used as a measure of the apoptotic fraction.

2.14. Statistical analysis

All data points were determined in triplicates and the mean values computed and presented with one standard deviation. Differences between treatments were determined by one-way analysis of variance at a P-value of 0.05.

3. Results

3.1. Kinetics of modulating PKC and GSTP1 enzyme activity by TPA and Bis-I in glioma cells

The results of these studies, summarized in Fig. 1a–d and in Table 1. Table 1 shows that following treatment with 50 nM TPA, cellular PKC activity increased significantly from baseline levels of 106 pmol/mg protein (MGR1) and 192 pmol/mg protein (MGR3) to peaks of 424 pmol/mg protein and 931 pmol/mg protein, respectively at 2 h, followed by a steady decline to baseline levels after 24 h. TPA caused a dose-dependent increase in cellular PKC activity, reaching a maximum at 50 nM TPA (Fig. 1b). The kinetics of inhibition of cellular PKC following exposure to Bis-I was also time- and dose-dependent with a nadir of 50 pmol/mg protein (MGR1) and 55 pmol/mg protein (MGR3) at 6 h post-treatment. Under these conditions, PKC activity was completely inhibited by 250 nM Bis-I. In contrast to the activation, the suppression of PKC activity by Bis-I was sustained for up to 24 h.

The effects of the activation and suppression of PKC on GSTP1 activity are summarized in Table 1a. Baseline-specific GSTP1 activity in untreated control cells was 12.49 ± 0.14 nmol min^{-1} mg^{-1} in MGR1 and 19.17 ± 1.34 nmol min^{-1} mg^{-1} in MGR3. Following PKC activation, GSTP1 activity increased to 24.25 ± 1.61 nmol min^{-1} mg^{-1} in MGR1 and to 41.49 ± 2.57 nmol min^{-1} mg^{-1} in MGR3 cells. Conversely, a 6-h treatment with 250 nM Bis-I decreased GSTP1 activity to 3.45 ± 0.25 nmol min^{-1} mg^{-1} in MGR1 and 7.69 ± 0.24 nmol min^{-1} mg^{-1} in MGR3.

As summarized in Table 1, total intracellular GSH content in both MGR1 and MGR3 following treatment with TPA, Bis-I or GSTP1-/PKCα-siRNA did not change significantly ($P = 0.05$).

3.2. PKCα activation increases GSTP1-dependent cisplatin metabolism

As shown in Fig. 2a, in a cell-free reaction containing PKCα-phosphorylated GSTP1, the rate of glutathionylplatinum metabolite formation increased significantly relative to that in a reaction containing unphosphorylated GSTP1, with frequency turnover of 1.73 h^{-1} and 0.89 h^{-1}, for the former and latter, respectively (Fig. 2b). No metabolite was detected in reactions with cisplatin or GSH alone or with cisplatin and GSTP1 alone.

The spectral scan (Fig. 2c) of the reaction of cisplatin with OPDA to yield dichloro-phenylenediamine platinum, DPDP showed a peak at 705 nm that is proportional to the concentration of free cisplatin in the reaction mixture (Fig. 2c insert). After 1 h, the concentration of residual cisplatin in the reaction containing unphosphorylated GSTP1 was reduced to 50% of the initial concentration, compared to an 82% decrease in the reaction containing phosphorylated GSTP1 (Table 2a). There was no further change in residual cisplatin levels in reactions containing PKCα,

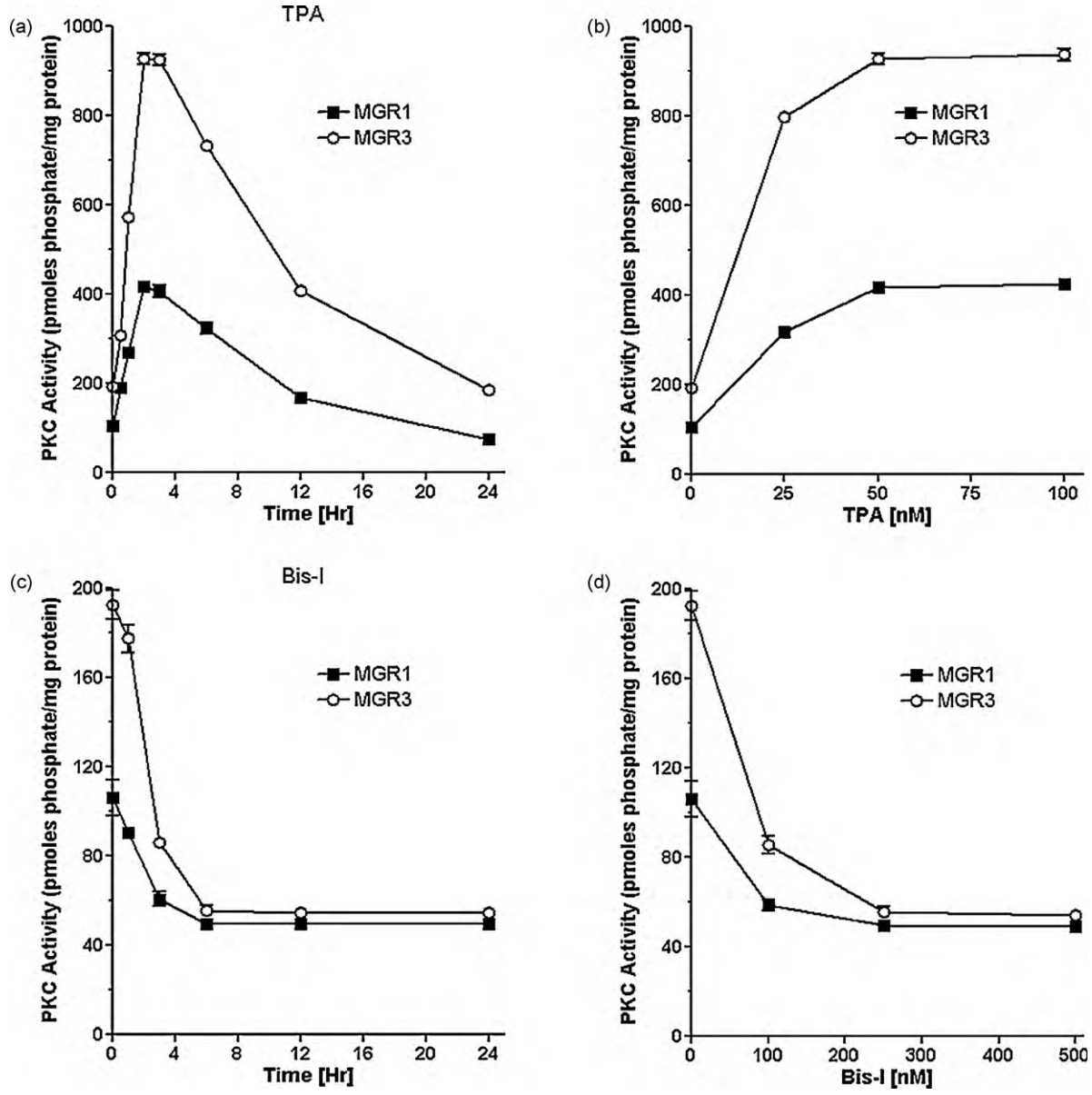

Fig. 1. Effect of the PKC activator, TPA, and the PKC inhibitor, Bis-I, on PKC activity in MGR1 and MGR3 cells. Values represent mean of three independent experiments ±SD (P-value < 0.05).

Table 1
Effect of modulating PKC on (a) specific GSTP1 activity and (b) GSH content in glioma cells. Cells were treated with 50 nM TPA for 2 h, 250 nM Bis-I for 6 h or transfected with PKCα-specific siRNA for 24 h. The values represent the means of three individual experiments ±SD.

(a)		
Treatment	Specific GSTP1 Activity (nmol/min/μg protein)	
	MGR1	MGR3
Untreated	12.49 ± 0.14	19.17 ± 1.34
TPA	24.25 ± 1.61	41.49 ± 2.57
Bisindolylmaleimide-I	3.45 ± 0.25	7.69 ± 0.24
PKCα-siRNA	1.75 ± 0.18	2.32 ± 0.36
(b)		
Treatment	GSH levels (nmol/mg protein)	
	MGR1	MGR3
Untreated	89.78 ± 0.19	163.54 ± 1.87
TPA	89.86 ± 0.38	159.95 ± 4.85
Bisindolylmaleimide-I	87.18 ± 2.95	159.14 ± 2.50
PKCα-siRNA	80.06 ± 1.35	157.23 ± 3.0
GSTP1-siRNA	85.67 ± 2.39	163.52 ± 0.45

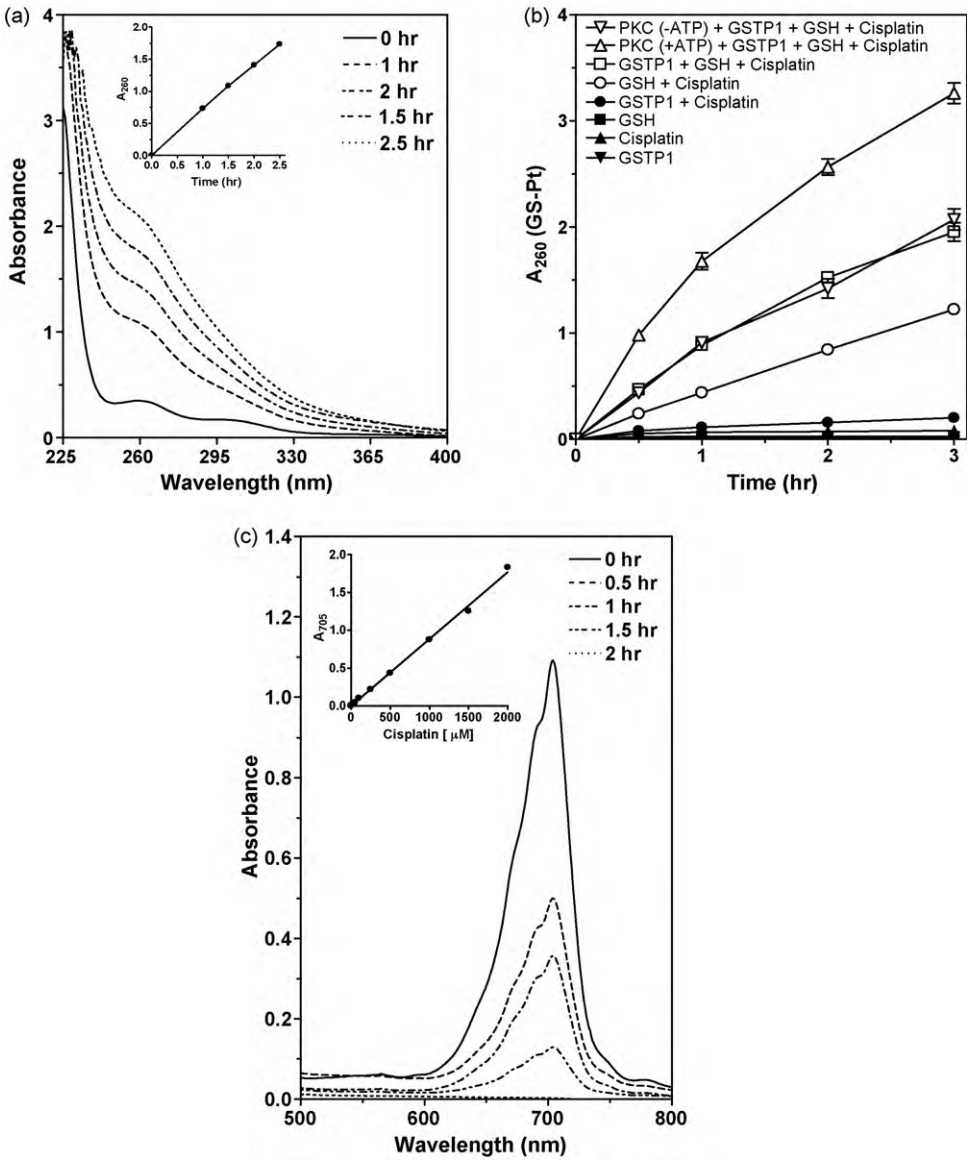

Fig. 2. Kinetics of glutathionylplatinum metabolite formation. (a) Wavelength scans over time of product of the PKCα-catalyzed GSTP1-dependent conjugation of GSH/cisplatin. (b) Kinetics of GSTP1-catalyzed cisplatin metabolism in a cell-free reaction. Spectral changes were monitored for reactions, with no GSTP1, with unphosphorylated GSTP1 and with PKCα-phosphorylated GSTP1. (c) Wavelength scans over time of residual-free cisplatin in a reaction mixture containing PKCα-phosphorylated GSTP1, GSH and cisplatin (inset shows the scan for cisplatin).

GSH and cisplatin, suggesting that cisplatin metabolism is GSTP1-dependent and any decrease in cisplatin concentration was due to the presence of GSTP1 in the reaction.

Table 2b shows that, following PKC activation, intracellular glutathionylplatinum metabolite increased by 2.8-fold (MGR1) and 2.4-fold (MGR3) relative to controls. Conversely, in cells treated with the GSTP1 inhibitor, ethacrynic acid, glutathionylplatinum metabolite levels decreased to 4% (MGR1) and 6% (MGR3) of untreated controls.

3.3. Cisplatin-induced DNA cross-linking is decreased in PKC-activated MGR3 cells

Table 3 shows that the amount of cisplatin-induced DNA interstrand cross-linking in the high GSTP1 expressing cells after a 6-h exposure to 100 μM of cisplatin was approximately 2-fold lower following PKC activation than in controls. Knockdown of GSTP1 in the cells prior to cisplatin treatment increased the cross-link index by 4.5-fold, while knockdown of PKCα resulted in a 2-fold increase in interstrand cross-links. Further, knockdown of both PKCα and GSTP1 protein increased the cross-link index by 6-fold.

3.4. Modulation of PKC sub-classes by TPA and Bis-I in glioma cells

To gain further insight into the PKC subclasses activated by TPA or inhibited by Bis-I in the tumor cells, we examined the Ca^{2+}-dependency of the activated PKC following the respective treatments. The results (Table 4) show that in both MGR1 and MGR3 cell lines, total PKC activity in extracts of TPA-treated cells decreased by more than 70% in the presence of the Ca^{2+}-chelating agent, EGTA. The Western blots in Fig. 3a show four PKC isoforms to be detectable in cells of both MGR1 and MGR3, namely, the Ca^{2+}-dependent and DAG-sensitive PKCα, and Ca^{2+}-independent PKCδ and ε and ζ. MGR3 cells also expressed PKCβI. Treatment with TPA or Bis-I had no effect on the protein levels of these isoforms. Although, under these conditions, GSTP1 protein levels remained largely unaltered by PKCα activation (2 h TPA) or inhibition (6 h

Table 2
(a) Analysis of residual cisplatin and glutathionylplatinum conjugate levels in cell-free PKC/GSTP1 phosphorylation reactions after 60 min incubation. The metabolite levels were normalized against a reaction containing cisplatin alone; (b) formation of glutathionylplatinum conjugate in MGR1 and MGR3 cells treated with 50 nM TPA (PKC activator), 50 μM of ethacrynic acid (EA; GSTP1 inhibitor) or a combination of TPA and EA, followed 1 h later with 100 μM cisplatin for 2 h. The values represent the means of three individual experiments ±1 SD.

(a)

Treatment	% Residual Cisplatin	% Glutathionylplatinum
Cisplatin/GSH	72.5 ± 0.7	37.3 ± 4.0
Cisplatin/ GSTP1/PKCα	95.0 ± 1.4	4.3 ± 1.2
Cisplatin/GSH/PKCα	75.7 ± 2.1	34.7 ± 0.6
Cisplatin/GSH/GSTP1	46.5 ± 0.7	81.7 ± 1.5
Cisplatin/GSH/GSTP1/PKCα	17.5 ± 2.1	161.0 ± 13.5

(b)

	% Increase in glutathionylplatinum conjugate				
	Cisplatin	TPA	TPA + Cisplatin	EA + Cisplatin	EA + TPA + Cisplatin
MGR1	31.7 ± 8.5	1.3 ± 1.2	90.0 ± 1.7	3.7 ± 0.6	10.3 ± 1.2
MGR3	44.3 ± 9.9	14.0 ± 0.2	104.3 ± 3.5	5.7 ± 0.6	16.7 ± 0.6

Normalized against controls.

Table 3
Effect of PKCα-dependent GSTP1 phosphorylation on DNA interstrand cross-link formation. MGR3 cells pre-treated with TPA for 2 h or transfected with PKCα- and GSTP1-siRNA alone and in combination for 48 h were treated with 100 μM cisplatin for 6 h and DNA inter cross-linking determined as described earlier.

Treatment	Interstrand cross-link index (ICL)
Cisplatin	0.50 ± 0.04
TPA + Cisplatin	0.29 ± 0.04
PKCα-siRNA + Cisplatin	1.10 ± 0.04
GSTP1-siRNA + Cisplatin	2.28 ± 0.09
PKCα- + GSTP1-siRNA + Cisplatin	2.95 ± 0.09

Bis-I) (Fig. 3b), Western blotting with a phospho-serine antibody showed that after 2 h exposure to TPA, there was a significant increase in the level of a 23-kD serine-phosphorylated protein, which, after stripping and reprobing with an anti-GSTP1 antibody was confirmed to be phospho-GSTP1. No detectable serine-phosphorylated GSTP1 was observed in control untreated MGR1 and MGR3 cells. No change in GSTP1 gene transcripts were observed after PKCα silencing (data not shown).

3.5. Modulation of PKCα activity and associated changes in serine-phosphorylated GSTP1 alter cellular sensitivity to cisplatin

As summarized in Fig. 4, the cisplatin IC_{50} for control MGR1 and MGR3 without PKC activation or inhibition of 13.63 ± 0.75 μM and 20.75 ± 0.36 μM, respectively, decreased to 6.32 ± 1.02 and 8.71 ± 0.11 μM following pre-treatment with Bis-I for 6 h. Conversely, a 2 h TPA pre-treatment increased cisplatin IC_{50} values to 36.49 ± 0.39 μM for MGR1 and 38.45 ± 0.62 μM for MGR3 (Fig. 4a and b). The decreased cisplatin sensitivity is consistent with the previously observed increases in PKCα activity, GSTP1 enzymatic activity and glutathionylplatinum metabolite level associated with TPA treatment. Treatment with Bis-I or TPA without cisplatin had no significant effect on cell growth in both glioma cell lines.

Table 4
Determination of total and classical (Ca^{2+}-dependent) PKC activities in glioma cells. Cells were treated with 50 nM TPA for 2 h and PKC activity assayed in the presence of 2 mM $CaCl_2$, and in the presence of 5 mM of the Ca^{2+}-chelating agent, EGTA. The values represent the means of three independent experiments ±1 SD.

	Total and classical (Ca^{2+}-dependent) PKC Activity (pmol phosphate/mg protein)			
	MGR1		MGR3	
Treatment	Untreated	50 nM TPA	Untreated	50 nM TPA
2 mM $CaCl_2$	100.89 ± 10.67	434.66 ± 11.61	186.48 ± 8.35	936.47 ± 23.10
5 mM EGTA	34.27 ± 4.18	145.98 ± 11.05	57.50 ± 3.95	249.55 ± 14.86

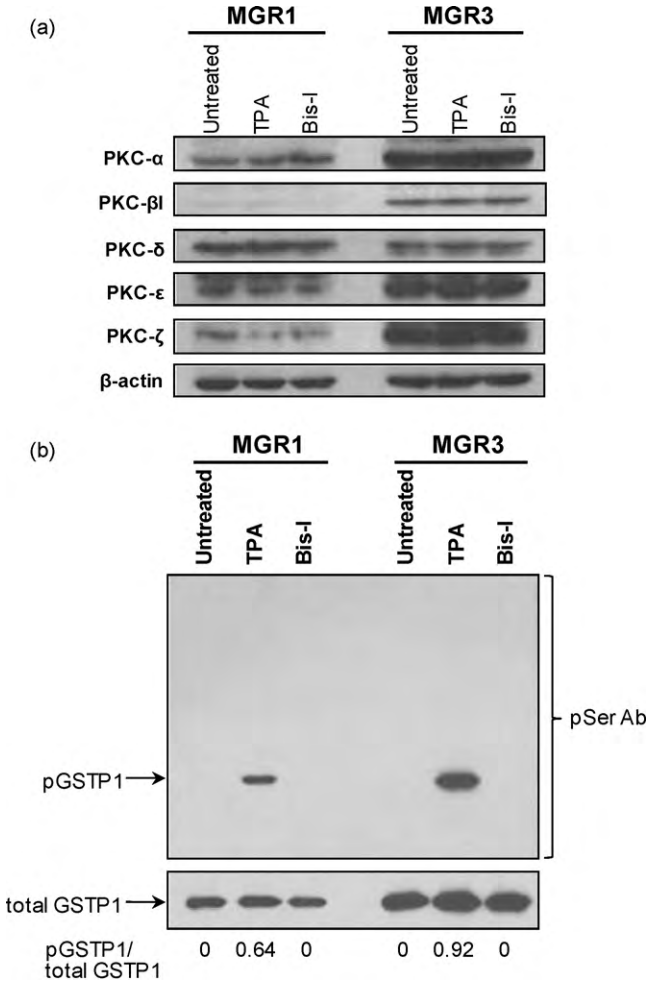

Fig. 3. (a) Western blotting of PKC isoforms expressed in MGR1 and MGR3 cells. (b) Combined immunoprecipitation/Western blotting for serine phosphorylated GSTP1 in gliuoma cells following PKC down-regulation. After stripping and reprobing with anti-GSTP1 antibody, the 23-kDa protein immunoreactive with the phosphoreine antibody was confirmed to be GSTP1. PKC activation with 50 nM TPA (2 h) and inhibition with 250 nM Bis-I (6 h) had no significant effect on GSTP1 protein levels in cells of both cell lines.

Fig. 4c and d show that PKC activation with 50 nM TPA decreased cisplatin-induced apoptosis, viz, caspase-3/7 activation by 57% and 45% in MGR1 and MGR3, respectively. Conversely, inhibition of PKC activity with Bis-I for 6 h increased caspase-3/7 activation by 86% in MGR1 and by 112% in MGR3 cells, 48 h following treatment with 50 μM cisplatin.

3.6. Effect of siRNA-mediated down-regulation of GSTP1 and PKCα on cisplatin metabolism and sensitivity in glioma cells

Western blotting (Fig. 5a, upper panel) showed a significant decrease in expression of both GSTP1 and PKCα protein levels in MGR1 and MGR3 cells after siRNA treatment with the decrease in GSTP1 levels more pronounced in MGR1 than in MGR3, consistent with the higher levels of GSTP1 in the latter compared to the former, with no effect on the expression of β-actin. Fig. 5b shows that 48 h after siRNA transfection, GSTP1 and PKCα gene transcripts in both MGR1 and MGR3, were reduced by more than

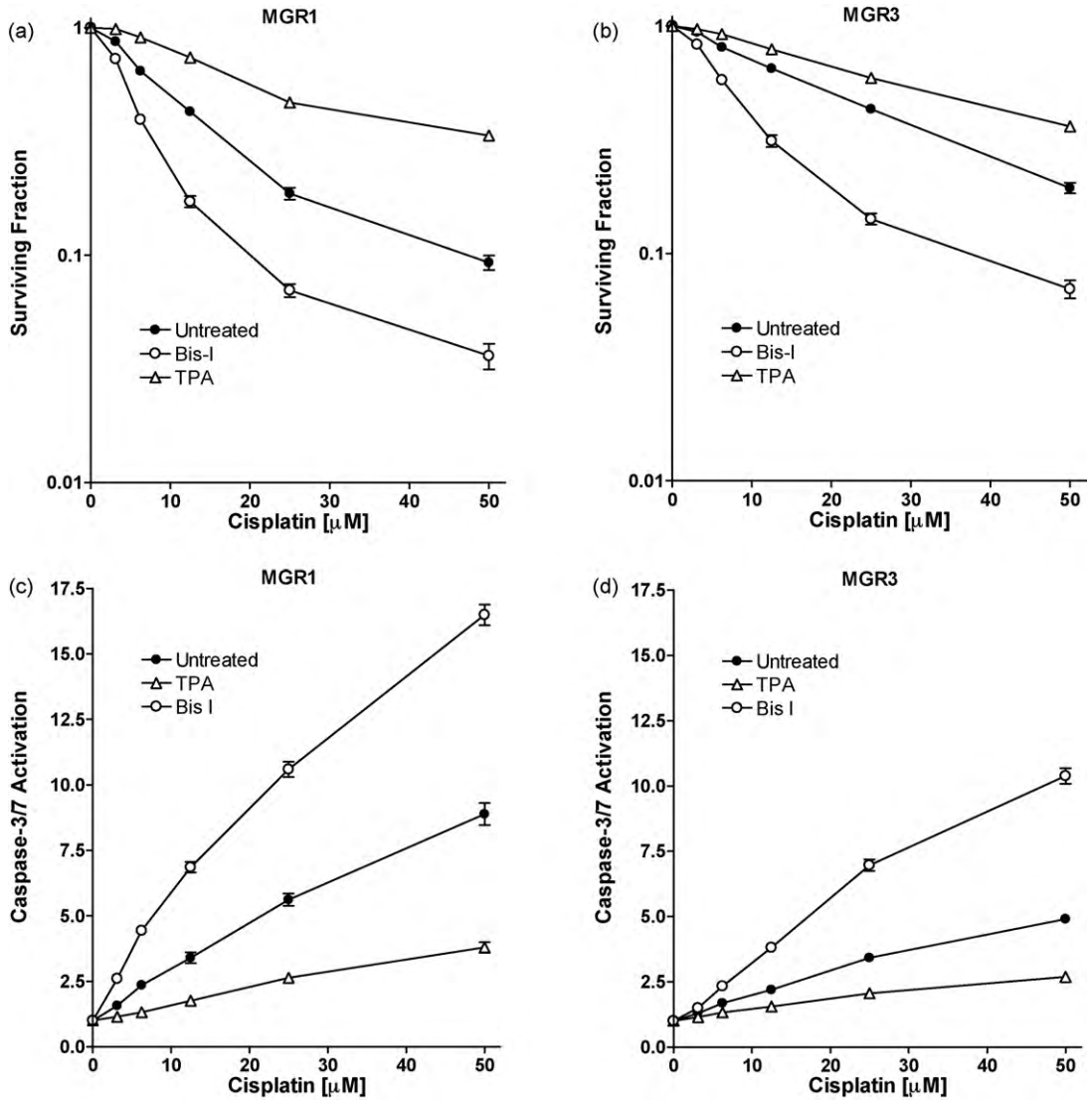

Fig. 4. Effect of modulating PKC activity on cisplatin sensitivity and on cisplatin-induced caspase 3/7 activation in MGR1 and MGR3 cells. Solid circles (●) represent untreated control cells; open circles (○) represent cells treated with Bis-I and open triangles (△) represent cells treated with TPA. Each data point is the mean of three independent experiments ±1 SD.

90% of control levels and remained at this low level for up to 72 h. Transfection of cells of either cell line with scrambled siRNAs did not affect the GSTP1 and PKC gene transcripts and protein levels.

Relative to un-transfected controls, GSTP1-siRNA transfection decreased cisplatin IC_{50} values from 12.99 ± 0.50 μM to 4.36 ± 0.10 μM in MGR1 and from 24.41 ± 0.54 μM to 6.17 ± 0.15 μM in MGR3 (Fig. 6a). Treatment of cells with TPA following siRNA-mediated GSTP1 down-regulation did not significantly alter sensitivity to cisplatin (Fig. 6a) suggesting that the protective role of PKCα against cisplatin cytotoxicity in glioma cells is GSTP1-dependent. In PKCα-siRNA treated cells, cisplatin IC_{50} decreased to 7.06 ± 0.09 μM (MGR1) and 8.9 ± 0.19 μM (MGR3). The combined down-regulation of PKCα and GSTP1 showed a more than additive sensitization to cisplatin in both glioma cell lines, with a decrease in cisplatin IC_{50} values to 0.78 ± 0.03 μM in MGR1 and 0.89 ± 0.04 μM in MGR3.

The increased sensitivity of the cells to cisplatin following siRNA treatment was also associated with an increased level of cisplatin-induced apoptosis (activation of caspase-3/7). Fig. 6b shows that, 48 h following transfection with GSTP1-siRNA, the baseline apoptotic fractions of 8.3 (MGR1) and 4.7 (MGR3) increased by 2.4-fold in MGR1 and 4-fold in MGR3 transfected cells after treatment with 50 μM cisplatin. Similarly, PKCα knockdown increased cisplatin-induced caspase-3/7 activity by 1.5-fold in MGR1 and 2.2-fold in MGR3 after 48 h. Dual down-regulation of PKCα and GSTP1 in MGR1 and MGR3, followed by treatment with 50 μM cisplatin further increased caspase-3/7 activation by 3.2-fold and 5.9-fold, respectively over the baseline levels.

The histograms in Fig. 6c show that treatment with GSTP1-specific siRNA decreased intracellular levels of glutathionylplatinum metabolite by 93% in MGR1 and 92% in MGR3, compared to controls. Conversely, glutathionylplatinum levels increased by 33% in MGR1 and 28% in MGR3, following TPA treatment. PKC activation in cells in which GSTP1 was suppressed did not have any significant effect on glutathionylplatinum levels. Consistent with this, in cells with unperturbed GSTP1, the suppression of PKCα alone reduced the formation of the glutathionylplatinum metabolite by 61% in MGR1 and 54% in MGR3 cells.

3.7. Translocation of Bax to mitochondria in MGR3 following treatment with cisplatin is altered with PKC activated and serine-phosphorylated GSTP1

To investigate the mechanism of cisplatin-induced apoptosis following PKC modulation and associated alterations in GSTP1

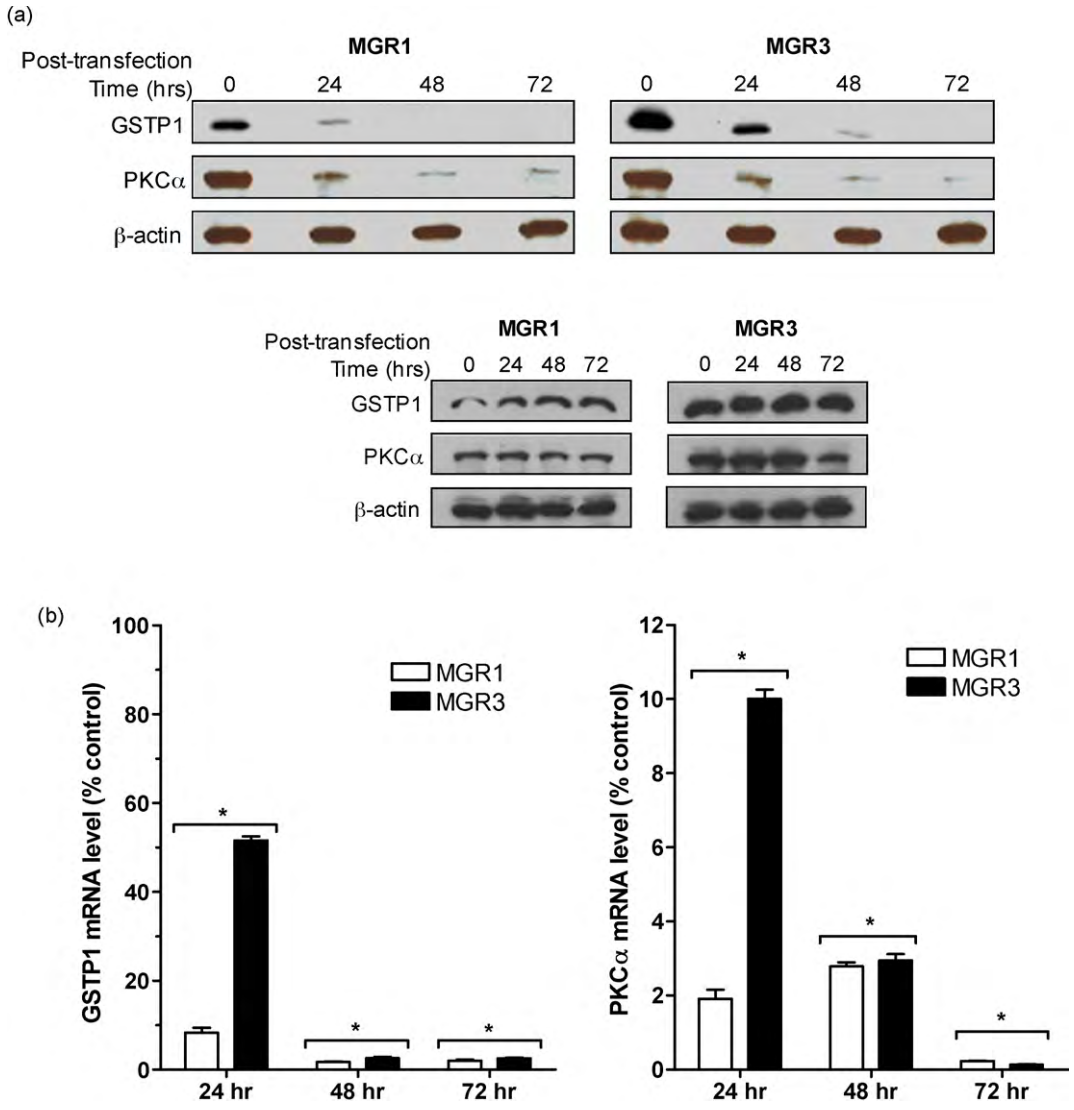

Fig. 5. (a) Effect of GSTP1-specific siRNA on GSTP1 protein levels and of PKCα-specific siRNA on PKC protein levels in gliomas, measured at 24, 48 and 72 h following treatment (upper panel). The lanes are (1) un-transfected; (2) 24 h post-siRNA transfection; (3) 48 h post-transfection and (4) 72 h post-transfection with 20 nM siRNA. Lower panel represents control with scrambled siRNA treatment.(b) Effect of 20 nM GSTP1-siRNA on GSTP1 mRNA levels (left panel) and 20 nM PKCα-siRNA on PKCα mRNA levels (right panel) in MGR1 and MGR3 cells. RNA levels were normalized to the level of GAPDH mRNA (*P-value < 0.05; ** not detected).

phosphorylation, we examined changes in Bax subcellular translocation in MGR3 cells. As shown in Fig. 7a, cytosolic Bax decreased by 50% and mitochondrial Bax increased significantly in response to cisplatin. Activation of PKC by TPA, prior to cisplatin treatment, resulted in a 25% decrease in mitochondrial Bax, while inhibition of PKCα induced a relocalization of, approximately, 90% of cytosolic Bax to mitochondria. Knockdown of GSTP1 followed by cisplatin treatment of the cells resulted in complete translocation of Bax from the cytosol to mitochondria. Treatment with TPA alone or siRNA-mediated suppression of PKCα or GSTP1 did not alter Bax levels or subcellular localization. These results indicate that the redistribution of Bax between the cytosol and mitochondria induced by cisplatin is partially inhibited by PKCα and GSTP1.

3.8. Cisplatin-induced release of cytochrome c from mitochondria into the cytosol is altered by PKC activation and associated GSTP1 phosphorylation

The efflux of cyt *c* from mitochondria to the cytosol in MGR3 cells after different treatments was examined by Western blotting (Fig. 7a). Cisplatin-induced release of cyt *c* from mitochondria to cytosol decreased by approximately, 50% in cells following PKC activation, relative to controls without PKC activation. Conversely, siRNA-mediated knockdown of PKCα and GSTP1 followed by cisplatin resulted in an almost complete release of mitochondrial cyt *c* to the cytosol. These results demonstrate that cisplatin-induced apoptosis and Bax translocation in glioma cells is associated with efflux of cyt *c* from mitochondria into the cytosol and supports the hypothesis that PKCα can prevent this translocation by increasing GSTP1-mediated metabolism of cisplatin.

3.9. Modulation of GSTP1 and PKC alter cisplatin-induced apoptosis in tumor cells.

The results of these studies are summarized in Fig. 7b and Table 5. As shown, exposure of tumor cells to 10 μM cisplatin for 48 h induced an 18% higher level of apoptosis compared to controls. Pre-treatment of the cells with TPA decreased their cisplatin sensitivity, as shown by a decrease in the apoptotic fraction to 8%. In contrast, transfection with GSTP1-siRNA enhanced the cisplatin-induced apoptotic fraction to 53% and, similarly, siRNA-mediated suppression of PKCα, increased cisplatin-induced apoptosis to 27%

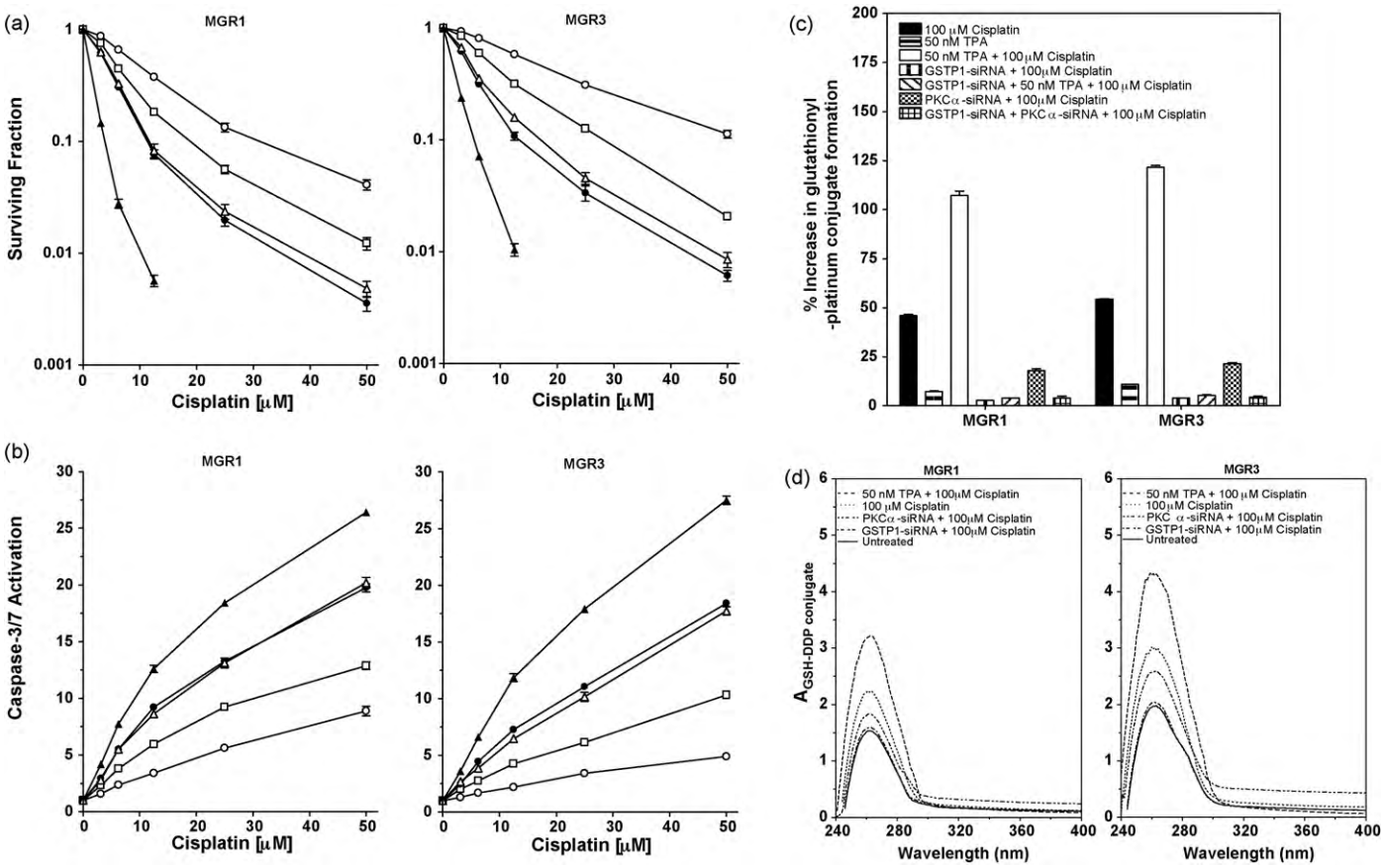

Fig. 6. Effect of GSTP1-siRNA on the cisplatin sensitivity of MGR1 and MGR3 cells. (a) Cell survival and (b) caspase 3/7 activation (apoptosis) were assessed 48 h post-cisplatin treatment. Open circle (○): untreated controls; open square (□): cells transfected with PKCα-siRNA; closed circle (●): cells transfected with GSTP1-siRNA; open triangle (△): GSTP1-siRNA transfected cells treated with TPA and solid triangle (▲): cells with knockdown of both PKCα and GSTP1. (c) Effect of siRNA-mediated GSTP1 knockdown on the formation of glutathionylplatinum metabolite in gliomas. (d) Wavelength scans of glutathionylplatinum metabolite in glioma cell extracts. Cells were treated with cisplatin, TPA or combination of TPA and cisplatin following siRNA transfection.

of controls. Although modulation of PKCα levels alone had no effect on the level of apoptosis, siRNA-mediated GSTP1 knockdown resulted in a 26% increase in apoptotic cells (the underlying mechanism is under investigation in our laboratory). The combined siRNA-mediated suppression of PKCα and GSTP1 increased cisplatin-mediated apoptotis fraction to 65%. Transfections with non-specific siRNAs had no effect on the level of apoptosis with or without cisplatin post-treatment.

4. Discussion

Alterations, primarily increases, in the levels of gene transcripts, protein and activity of specific PKC isoforms have been reported in a variety of human cancers, including gliomas [39,40]. In high grade gliomas, the inhibition or down-regulation of PKC has also been shown to reduce tumor cell growth and invasiveness, while overexpression has been associated with increased proliferation

Table 5
Effect of modulating PKCα and GSTP1 on cisplatin-induced apoptosis in MGR3 cells. Tumor cells pre-treated with 50 nM TPA (2 h) or 20 nM of PKCα or GSTP1-specific siRNA were exposed to 10 μM cisplatin (48 h) and the apoptotic cell population analyzed by flow cytometry after staining with PI. Twenty thousand cells were evaluated in each sample and the values represent the means of three independent experiments ±SD.

Treatment	% Cell cycle phase distribution			
	sub-G1	G0/G1	S	G2/M
Untreated	2.55 ± 0.04	69.69 ± 0.99	12.75 ± 0.70	15.02 ± 0.33
TPA	2.90 ± 0.24	67.52 ± 0.5	11.49 ± 0.22	18.08 ± 0.96
PKCα-siRNA	5.44 ± 0.72	72.40 ± 1.44	10.75 ± 0.01	11.41 ± 0.73
GSTP1-siRNA	25.51 ± 1.67	54.45 ± 1.73	9.09 ± 0.01	10.95 ± 0.07
PKCα- + GSTP1-siRNAs	28.22 ± 3.01	52.23 ± 2.84	8.73 ± 0.32	10.82 ± 0.15
Cisplatin	17.94 ± 1.33	62.03 ± 1.39	9.49 ± 0.03	10.54 ± 0.03
TPA + Cisplatin	8.33 ± 0.65	67.77 ± 2.15	9.84 ± 0.01	14.06 ± 1.49
PKCα-siRNA + Cisplatin	27.11 ± 0.28	55.08 ± 0.93	8.25 ± 0.70	9.55 ± 0.05
GSTP1-siRNA + Cisplatin	52.74 ± 1.21	33.96 ± 1.17	5.65 ± 0.01	7.65 ± 0.02
PKCα- + GSTP1-siRNAs + Cisplatin	64.92 ± 1.89	22.61 ± 1.83	5.44 ± 0.03	7.04 ± 0.04
Scrambled siRNA (PKCα)	4.16 ± 0.20	73.34 ± 0.31	10.24 ± 0.69	12.27 ± 0.79
Scrambled siRNA (GSTP1)	4.40 ± 0.07	73.51 ± 0.20	10.57 ± 0.06	11.52 ± 0.07
Scrambled siRNA (PKCα) + Cisplatin	18.60 ± 1.89	61.01 ± 1.77	9.58 ± 0.06	10.82 ± 0.06
Scrambled siRNA (GSTP1) + Cisplatin	18.72 ± 1.05	59.59 ± 1.15	10.05 ± 0.05	11.64 ± 0.05

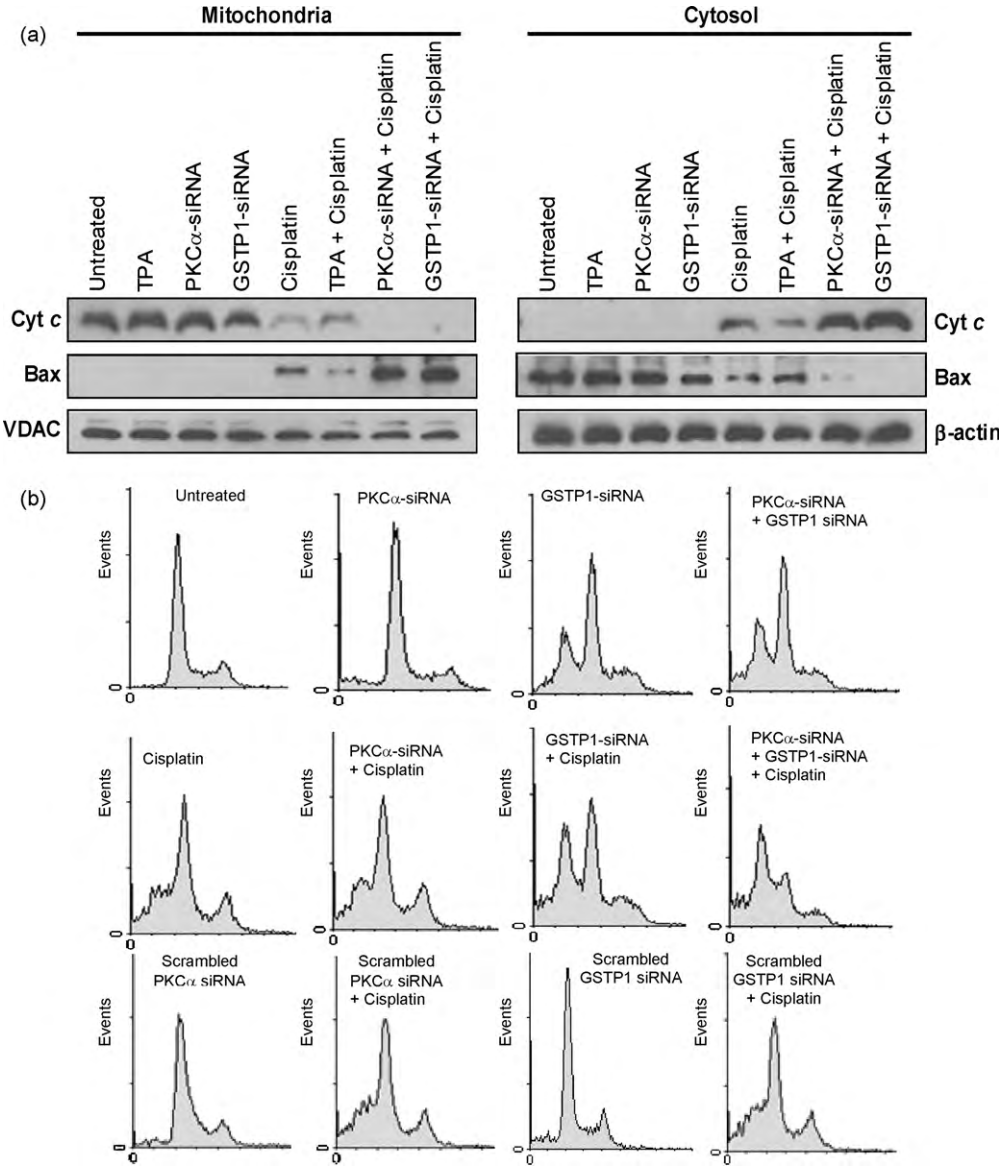

Fig. 7. (a) Relocalization of Bax and cyt *c* by cisplatin. To detect Bax and cyt *c* translocation, MGR3 cells, pre-treated with TPA or transfected with PKCα or GSTP1-siRNA, were treated with 25 μM cisplatin, homogenized and fractionated into cytosolic and mitochondrial fractions and subjected to Western blotting. VDAC and beta-actin were used as specific mitochondrial and cytosolic markers, respectively. (b) Effect of PKCα and GSTP1 down-regulation on cell cycle distribution of MGR3 cells, treated with and without cisplatin. Cells transfected with PKCα- or GSTP1-siRNA for 48 h were treated with 10 μM cisplatin for an additional 48 h and subjected to flow cytometry, as described earlier.

and decreased apoptotic response [41]. Despite the associations between high PKC expression and tumor drug resistance [24,41–43], the mechanisms underlying this relationship remain poorly understood. In the case of cisplatin, these are likely to be complex, as several mechanisms that can potentially regulate cellular sensitivity to cisplatin, including, cisplatin uptake and metabolism, and DNA repair can all be regulated by PKCs, notably, PKCα [6,44]. Elevated expression of GSTP1 has now been established as a major factor in tumor resistance to therapy with cisplatin and other alkylating and free radical generating agents [16,45–49].

Recently, we reported that several PKC isoforms phosphorylate the human GSTP1 protein and increase its enzymatic activity in glioma cells [21]. This, together with the large body of evidence showing that overexpression of the GSTP1 gene is associated with drug resistance in several cancers [8,9, 12, 48], raises the important question of whether PKC-dependent phosphorylation of GSTP1 could contribute to GSTP1-mediated tumor drug resistance. Our findings show that the effect of PKC activation or inhibition on cisplatin sensitivity or resistance in the two malignant glioma cell lines was GSTP1-dependent. PKC activation-induced cisplatin resistance was associated with increased intracellular levels of both phosphorylated GSTP1 and of the glutathionylplatinum metabolite. Conversely, pharmacologic inhibition and siRNA-mediated suppression of PKCα and GSTP1 increased cellular cisplatin sensitivity in the two glioma cell lines. Interestingly, in the absence of GSTP1, PKC activation had no significant change on cisplatin sensitivity in either cell line. These results are consistent with the pattern of cisplatin-induced DNA interstrand cross-link formation in MGR3 cells. Cells transfected with GSTP1-siRNA formed significantly higher levels of DNA-cisplatin interstrand cross-links than in un-transfected cells. While in PKC activated cells the level of DNA interstrand cross-links formation was reduced by about 2-fold, consistent with the more than 2-fold increase in GSTP1 enzymatic activity observed after TPA treatment.

In the two malignant glioma cell lines, MGR1 and MGR3, we identified four PKC isoforms (α, δ, ε and ζ) to be highly expressed,

with a fifth, PKCβI expressed in MGR3 but not in MGR1. Although, a 2-h TPA treatment did not affect PKC protein levels, it increased activity of the Ca^{2+}-dependent classical PKCs by more than 4-fold. These findings provide evidence for a mechanism of GSTP1-mediated cisplatin resistance in which the resistance is driven by PKC-dependent serine phosphorylation of GSTP1 resulting in increased metabolism of cisplatin by the phosphorylated GSTP1. Such a mechanism of cisplatin resistance will be most dominant in tumors with elevated GSTP1 and PKCα levels.

Our observations that endogenous Bax relocalizes to mitochondria with concomitant release of cyt *c* during cisplatin-induced apoptosis is consistent with previous reports [29,30]. Interestingly, however, we showed that activation of PKC partially prevented this cisplatin-mediated sub-cellular redistribution of Bax and cyt *c*, while, PKCα knockdown enhanced both Bax and cyt *c* mitochondria-cytosol translocation upon cisplatin treatment. Similar results were observed when cells were transfected with GSTP1-siRNA before cisplatin exposure. Interestingly, modulation of PKC or GSTP1 inactivation alone did not induce Bax or cyt *c* sub-cellular relocalization. These results, taken together with those on cisplatin metabolism and cisplatin induction of DNA interstrand cross-link formation suggest that Bax and cyt *c* translocation depends on the levels of non-metabolized cisplatin. Since GSTP1 is a cytosolic protein and Bax is predominately located in the cytosolic compartment, expression of GSTP1 and PKCα might partially inhibit the activation of Bax by reducing the amount of cisplatin.

The changes in cisplatin-induced activation of caspase-3/7 in the glioma cells, following PKCα activation or inhibition, indicate a central role for altered programmed cell death in the mechanism of PKCα/GSTP1-mediated cisplatin resistance in these cells. This is consistent with observations in other tumor types [50,51]. The absence of any significant alteration in caspase-3/7 activation following the short-term (2-h) TPA treatment in cells in which GSTP1 expression is suppressed suggests that the effects on cisplatin-induced apoptosis are related to the changes in GSTP1 activity. In this context, it is noteworthy that PKCα has been shown to phosphorylate the anti-apoptotic Bcl2 at serine 70, thereby suppressing the induction of apoptosis by anticancer agents [27]. Furthermore, inhibition of PKCα expression by a nuclease resistant ribozyme was shown to lead to the induction of apoptosis in the human glioblastoma cell lines, U87MG and T98G, through suppression of $Bclx_L$ gene expression [28]. Bcl2 protects cells against cisplatin apoptosis through blockage of the activation of Bax and Bak [52]. The exact mechanism by which this blockade is mediated is not fully understood. Bcl2 has been shown to sequester Bax, Bak and BH3-only proteins, such as, Bik, Bid and Bad, thereby preventing Bax- and Bak-mediated apoptosis [53]. It would be interesting to investigate how PKCα-mediated phosphorylation of Bcl2 affects the interaction of Bcl2 with Bax, Bak and BH3-only proteins. We hypothesize that PKCα confers chemotherapeutic resistance firstly by phosphorylating and enhancing GSTP1-dependent drug metabolism and secondly, by phosphorylating Bcl2 and altering its engagement of the mitochondrial apoptotic machinery.

It has been reported that intracellular GSH is depleted before the onset of apoptosis induced by various agents and that, TPA-induced differentiation of leukemic cells (U937) was associated with a decrease in intracellular GSH content rendering the cells sensitive to apoptotic-inducing agents [54]. Results in this study, however, showed that, under our experimental conditions, treatment with TPA, Bis-I or siRNA did not have any significant effect on intracellular GSH levels in both glioma cell lines, an indication that the altered cisplatin resistance and associated cisplatin metabolism following PKC modulation was more related to GSTP1 activity and less to changes in intracellular GSH levels.

In summary, this study provides strong evidence linking phosphorylation of GSTP1 by PKCα and tumor drug resistance. We demonstrate that glioma cisplatin resistance results, at least, in part, from an enhancement of the ability of the phosphorylated GSTP1 to metabolize and detoxify cisplatin. Thus, the enhancement of GSTP1 metabolic inactivation of cisplatin could function together with the direct effects of PKCα on the apoptotic pathway to mediate cisplatin resistance. Although, both PKCα and GSTP1 have been targeted individually to improve the efficacy of cancer chemotherapy in pre-clinical and clinical studies, the dual targeting of both proteins has not yet been explored. In tumors with high levels of PKCα and GSTP1, the results we present in this study provide a rationale for such a strategy, which will likely improve the efficacy of their therapy with chemotherapeutic agents metabolized by GSTP1.

Acknowledgements

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