Journal of Inorganic Biochemistry 191 (2019) 29–39

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

# Carbon monoxide sensitizes cisplatin-resistant ovarian cancer cell lines toward cisplatin via attenuation of levels of glutathione and nuclear metallothionein

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ARTICLE INFO

*Keywords:*
Carbon monoxide
Ovarian cancer
Cisplatin-resistance
Cystathionine β-synthase
Glutathione
Metallothionein

ABSTRACT

Cisplatin resistance remains a major impediment to effective treatment of ovarian cancer. Despite initial platinum responsiveness, thiol-containing peptides and proteins, glutathione (GSH) and metallothionein (MT), bind and inactivate cisplatin in cancer cells. Indeed, high levels of GSH and MT in ovarian cancers impart cisplatin resistance and are predictive of poor prognosis. Cystathionine β-synthase (CBS), an enzyme involved in sulfur metabolism, is overexpressed in ovarian cancer tissues and is itself associated with cisplatin resistance. Treatment with exogenous carbon monoxide (CO), a known inhibitor of CBS, may mitigate cisplatin resistance in ovarian cancer cells by attenuation of GSH and MT levels. Using a photo-activated CO-releasing molecule (photoCORM), $[Mn(CO)_3(phen)(PTA)]CF_3SO_3$ (phen = 1,10-phenanthroline, PTA = 1,3,5-triza-7-phosphaadamantane) we assessed the ability of CO to sensitize established cisplatin-resistant ovarian cancer cell lines to cisplatin. Cisplatin-resistant cells, treated with both cisplatin and CO, exhibited significantly lower cell viability and increased poly (ADP-ribose) polymerase (PARP) cleavage versus those treated with cisplatin alone. These cisplatin-resistant cell lines overexpressed CBS and had increased steady state levels of GSH and expression of nuclear MT. Both CO treatment and lentiviral-mediated silencing of CBS attenuated GSH and nuclear MT expression in cisplatin resistant cells. We have demonstrated that CO, delivered from a photoCORM, sensitizes established cisplatin-resistant cell lines to cisplatin. Furthermore, we have presented strong evidence that the effects of CO in circumventing chemotherapeutic drug resistance is at least in part mediated by the inactivation of endogenous CBS.

## 1. Introduction

Ovarian cancer is the fifth most prevalent and the most lethal gynecological cancer in the United States [1]. The overall 5-year survival rate for advanced ovarian cancer patients is only ~40% and has remained largely static over the past 20 years [2]. The current standard of care includes cytoreductive surgery and combination platinum/taxane chemotherapy [3]. However, ~90% of ovarian cancer deaths are caused by chemotherapeutic resistance and metastasis [4]. Clearly there is an unmet need for treatment modalities to mitigate chemotherapeutic resistance.

Cisplatin is one of the most widely used and effective anti-cancer drugs. In addition to ovarian cancer, it is the standard of care for other solid cancers of the head and neck, bowel and colon, cervix and lung. By localizing to the nucleus and binding to DNA, cisplatin gives rise to intrastrand DNA adducts and triggers G2 cell cycle arrest and subsequent apoptosis. The effectiveness of cisplatin, however, is limited by the high incidences of drug resistance [5,6]. In the cases of colorectal, lung and prostate cancers, intrinsic resistance is common [5]. In ovarian cancer, however, resistance is mainly acquired after initial treatment and response to cisplatin therapy [5]. Understanding the cellular changes that occur in the development of cisplatin resistance will help in developing more effective means of circumventing cisplatin resistance in ovarian cancer.

Exogenous carbon monoxide (CO) has recently been shown to decrease chemotherapeutic resistance and proliferation in various cancer cell types [7–9]. Our group has recently reported that CO increased the sensitivity of human breast cancer cells to doxorubicin mediated cell

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https://doi.org/10.1016/j.jinorgbio.2018.11.003
Received 8 June 2018; Received in revised form 31 October 2018; Accepted 4 November 2018
Available online 10 November 2018
0162-0134/ 

**Abbreviations**

| | |
|---|---|
| CBS | cystathionine β-synthase |
| CGL | cystathionine γ-lyase |
| CO | carbon monoxide |
| CTH | cystathionine |
| FBS | fetal bovine serum |
| GSH | glutathione |
| MT | metallothionein |
| NEM | *N*-ethylmalemide |
| OV | OVCAR-5 |
| OVcisR | cisplatin-resistant OVCAR-5 |
| PARP | poly (ADP-ribose) polymerase |
| PMSF | phenylmethane sulfonyl fluoride |
| SKV | SKOV-3 |
| SKVcisR | cisplatin-resistant SKOV-3 |

death by > 40% via the inhibition of endogenous cystathionine β-synthase (CBS) enzymatic activity [10]. CBS is overexpressed selectively in human breast cancer tissues and not in normal human breast tissues making it a potential therapeutic target [11]. Interestingly, CBS is overexpressed in only a few other neoplasms, one of which is ovarian cancer, where CBS has been implicated in resistance to cisplatin [12]. However, the mitigation of chemotherapeutic drug resistance, using a pharmacological inhibitor of CBS has not yet been demonstrated in ovarian cancer cells. This study for the first time assessed the pharmacological inhibition of CBS by a light-induced CO delivery modality to counter chemotherapeutic drug resistance in human ovarian cancer cells. The results underscore the important role of the transsulfuration pathway in the development of chemotherapeutic drug resistance in ovarian cancer.

The noxious nature of gaseous CO often poses challenging delivery issues in hospital settings. To avoid this problem, a designed metal carbonyl complex namely $[Mn(CO)_3(phen)(PTA)]CF_3SO_3$ (phen = 1,10-phenanthroline; PTA = 1,3,5-triza-7-phosphaadamantane; abbreviated "photoCORM" hereafter) has been employed as the exogenous CO source in this study (Scheme 1). This designed manganese carbonyl complex is water-soluble and rapidly releases CO only when exposed to low-power (10 mW/cm$^2$) broadband visible light. This photoactive CO-releasing molecule (photoCORM) has been a convenient source of CO in delivery under controlled conditions [10,13].

## 2. Materials and methods

### 2.1. Materials and reagents

$[Mn(CO)_3(phen)(PTA)]CF_3SO_3$ (photoCORM) was synthesized, analyzed to confirm purity, and applied as previously published [10,13]. Cisplatin (479306), protease inhibitor cocktail (P8340), puromycin (P8833), *N*-acetylcysteine (A7250), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and other chemicals were products of Sigma Aldrich (St. Louis, MO). GYY 4137 (13345) was purchased from Cayman Chemicals (Ann Arbor, MI). Primary antibodies against CBS (sc-133208), cystathionine γ-lyase (CGL, sc-365382), poly (ADP-ribose) polymerase (PARP-1, sc-56196), Lamin A (sc-71481), β-tubulin (sc-23949) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, sc-47724) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against metallothionein (ab12228) and rabbit polyclonal antibody against xCT (ab 37185) were purchased from Abcam (Cambridge, MA).

### 2.2. Cell culture

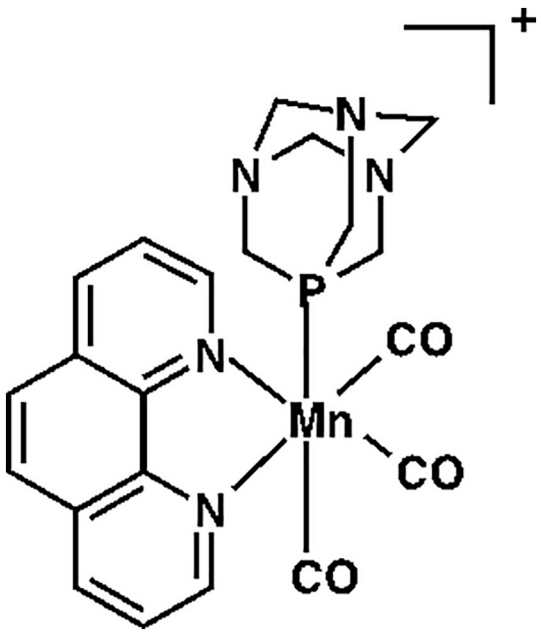
Ovarian cancer cell lines OVCAR-5 (OV), cisplatin-resistant OVCAR-5 (OVcisR), SKOV-3 (SKV), and cisplatin-resistant SKOV-3 (SKVcisR) were a gift from Dr. Sivakumar Ramadoss and Dr. Gautam Chaudhuri of the David Geffen School of Medicine at University of California at Los Angeles. Cells were grown in 1× RPMI media supplemented with 10% fetal bovine serum, 100 I.U./mL penicillin and 100 μg/mL streptomycin at 37 °C + 5% $CO_2$. Mycoplasma detection was performed regularly to confirm its absence with MycoAlert™ Mycoplasma Detection Kit (LT07-218) from Lonza (Basel, Switzerland). Cells were passaged no more than five times after being received. Unless otherwise indicated, cells were allowed to seed overnight prior to treatments, then assayed 24 h post-treatment.

### 2.3. CO and cisplatin treatments of cells

Cells were seeded overnight, 100 × 10$^3$/well of 6-well tissue culture dishes or 1 × 10$^6$/well of 100 mm tissue culture dishes depending on the experiment being performed, and incubated at 37 °C + 5% $CO_2$ 24 h prior to cell culture treatments. Cells treated with CO were exposed to 30 μM photoCORM in the dark. To control for the effects of the non-CO backbone of the CO-releasing molecule, corresponding control cells were treated with 30 μM light-inactivated photoCORM (iCORM) in the dark. Upon addition of photoCORM or iCORM, cells were exposed to visible light for 30 min at room temperature, then returned back to 37 °C + 5% $CO_2$. In experiments involving cisplatin, 20 μM cisplatin or DMSO vehicle control was administered either by itself or together with 30 μM photoCORM or iCORM. In experiments utilizing NAC, 3 mM NAC or vehicle control were added to cells along with photoCORM and cisplatin. Subsequent assays were performed 24 h post-treatment.

### 2.4. Cell viability (Trypan blue exclusion and MTT) assay

When assessing cell viability via Trypan blue exclusion method, 100 × 10$^3$ cells/well of a 6-well tissue culture plate were allowed to seed overnight in a 37 °C incubator + 5% $CO_2$. The next day, cells were treated as indicated and allowed to incubate for 24 h. Following treatment, cells were harvested with 1 mL 0.05% Trypsin-EDTA, after which trypsin was neutralized with 1 mL cell culture media




**Scheme 1.** Structure of $[Mn(CO)_3(phen)(PTA)]CF_3SO_3$, the photoCORM used in this study.

supplemented with 10% FBS. This cell suspension was then visualized and quantified using a Vi-Cell XR cell viability analyzer from Beckman Coulter (Brea, CA). Cell viability was measured 24 h post-treatment and presented as the mean % of control or comparison group ± SEM of three independent experiments.

In cell viability experiments performed using the tetrazolium dye MTT, experiments were performed in 96-well tissue culture plates. Briefly, $4 \times 10^3$ cells were allowed to seed overnight in a 37 °C incubator + 5% $CO_2$. The next day, cells were treated as indicated, then assessed for viability 24 h post-treatment. Cell culture media was aspirated from cells, then replaced with 0.5 mg/mL MTT dissolved in fresh 1× DMEM and allowed to incubate for 2 h in a 37 °C incubator + 5% $CO_2$. Number of viable cells was assessed by quantifying the amount of MTT reduced to insoluble formazan. Insoluble formazan was solubilized in 10% SDS + 0.01 N HCl. The absorbance at 570 nm, with reference wavelength at 690 nm, was measured. Data are presented as average % change ± SEM from control(s) of n = 3 independent experiments.

### 2.5. Western blot analysis

Whole cell lysates were prepared after treatment in RIPA lysis buffer containing 150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS and 1× protease inhibitor cocktail. Soluble lysates were quantified using a Pierce™ BCA Protein Assay Kit (23225) from ThermoFisher Scientific (Waltham, MA), 20 µg cell lysates from samples were separated on 4–12% SDS-PAGE gel and transferred to poly(vinylidene difluoride) (PVDF) membrane. Membranes were blocked with 5% nonfat dried milk and incubated for 1 h room temperature or 4 °C overnight. Primary (1:1000 dilution) and horseradish peroxidase (HRP)-conjugated secondary (1:10,000 dilution) antibody incubations were performed at room temperature for 1 h. Immunofluorescent signals were detected with Pierce ECL Plus Western blotting substrate (32132) from ThermoFisher Scientific.

### 2.6. High performance liquid chromatography-mass spectrometry

Glutathione (GSH), γ-Glu-Cys, cysteine and cystathionine (CTH) were measured utilizing high pressure, liquid chromatography-mass spectrometry (HPLC-MS) as previously described [10]. ~$5 \times 10^6$ cells were lysed via three probe sonication in 200 µL of 10 mM *N*-ethylmaleimide (NEM) + 10 mM ammonium acetate, pH 7.4. To the lysate, 800 µL of methanol was added and the samples were vortexed. Samples were then centrifuged at 16,000 ×*g* for 5 min, then supernatants were collected into microcentrifuge tubes and dried via vacuum centrifugation. Samples were further dried with 100 µL methanol, then 100 µL benzene. The carboxy termini of metabolites were esterified with the treatment of 100 µL of 3 N methanolic HCl for 60 min, 60 °C. Samples were then dried via vacuum centrifugation, redissolved in 50 µL of water and transferred to liquid chromatography injector vials. Kinetex XB-C18, 100 × 2.1 mm, 1.7-µm particle size, 100 Å pore diameter, reverse phase column from Phenomenex (Torrance, CA) column was equilibrated with 85% 0.1 mM perfluorooctanoic acid in water (eulant A) and 15% 0.1 mM perfluorooctanoic acid in acetonitrile (eluant B). Samples were injected onto the equilibriated column and eluted at 100 µL/min with increasing concentration of eluant B (min/% B: 0/15, 5/15, 35/50, 33/ 15, 45/15). The eluants were directed toward an Agilent Jet Stream electrospray ionization (ESI) source connected to a triple quadrupole mass spectrometer (Agilent 6460) operating in positive ion tandem mass spectrometric multiple reaction-monitoring (MRM) mode. The intensities of the CTH parent to fragment transition (461 → 318, rt 27.88 min), cysteine-NEM conjugate (261 → 244, rt 24.06 min), GSH-NEM conjugate (461 → 318, rt 26.06 min) and γ-Glu-Cys-NEM conjugate (404 → 244, rt 26.59 min) were recorded using previously-determined settings. Standards were prepared containing known concentrations of CTH (0, 20, 40, 40, 80, 160 pmol), cysteine (0, 80, 160, 320, 640 pmol), GSH (0, 200, 400, 800, 1600 pmol) and γ-Glu-Cys (0, 100, 200, 400, 800 pmol). From these standards, calibration curves were constructed. The amount of the monitored metabolites in biological samples was calculated by interpolation from the curves. Signals for monitored metabolites were normalized to total µg protein and expressed as either average pmol/µg protein OR average percentage/fold-difference from control or comparison group ± SEM of n = 3 independent experiments.

### 2.7. D4-cystine uptake assay and $H_2S$ donation

1× DMEM with high glucose but no glutamine, methionine, and cystine (D0422) was procured from Sigma Aldrich. This media was then supplemented with 3.97 mM glutamine, 200 µM methionine, 10% FBS and 200 µM D4-CC (DLM-1000-PK), which was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). ~$1 \times 10^6$ cells were seeded in 100 mm tissue culture dishes and allowed to grow for 48 h at 37 °C + 5% $CO_2$. In experiments where $H_2S$-donor GYY 4137 was used, either 40 µM GYY 4137 or DMSO vehicle control was added for 48 h. Cells were harvested and processed in the identical manner described above (Section 2.6). Reported D4-CC uptake was measured by quantifying intracellular D2-cysteine levels normalized to total µg protein. Data are presented as average fold-difference from control or comparison group ± SEM of n = 3 independent experiments.

### 2.8. Nuclear fraction enrichment

$5 \times 10^6$ cells were scraped from 100 mm tissue culture dishes on ice and washed with cold, 1× PBS (phosphate buffer saline). Cells were suspended in cold lysis buffer containing the following: 10 mM HEPES pH 7.7, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% NP-40, 0.5 mM PMSF and protease inhibitor cocktail. Cells were incubated on ice for 15 min with intermittent mixing, then centrifuged at 12,000 ×*g* at 4 °C for 10 min. The supernatant was collected as the cytosolic enriched fraction. The nuclear pellet was washed three times with cold lysis buffer, then resuspended in cold nuclear extraction buffer containing 20 mM HEPES pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail. After incubation on ice for 30 min, the enriched nuclear fraction was collected by centrifugation at 12,000 ×*g* at 4 °C for 15 min.

### 2.9. Gene silencing experiments

Stable silencing of CBS expression in OVcisR and SKVcisR was achieved utilizing short hairpin RNA (shRNA) packaged in lentiviral particles purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with shRNA complimentary to CBS mRNA (sc-60335-V) and selected for in the presence of 1 µg/mL puromycin to generate CBS-silenced cell lines OVcisR(shCBS) and SKVcisR(shCBS). Cells were transfected with scrambled shRNA (sc-108080) and selected for in the presence of 1 µg/mL puromycin to generate transfection control cell lines OVcisR(scram) and SKVcisR(scram).

### 2.10. Statistical analysis

Data are presented as means ± SEM of n = 3 independent experiments. Statistical comparison between two groups was performed using a two-tailed, paired Student's *t*-test, while comparisons between more than two groups was performed using ANOVA with post hoc Tukey's test. p values < 0.05 were considered statistically significant (*p < 0.05). Regression analysis and IC50/ED50 estimations were determined using GraphPad Prism purchased from GraphPad Software (La Jolla, CA).

## 3. Results

### 3.1. Cisplatin-resistant ovarian cancer cell lines were re-sensitized to cisplatin upon co-treatment with CO

Previous studies have demonstrated the ability of CO to sensitize cancer cells to chemotherapeutics [7,9,10], though cisplatin resistance and ovarian cancer had yet to be addressed. In this study, we wanted to assess the ability of CO, delivered by 30 μM photoCORM, to sensitize established cisplatin-resistant ovarian cancer cells. 30 μM of photoCORM was used in this study because CO concentrations higher than that delivered from > 30 μM photoCORM, were cytotoxic to the cells

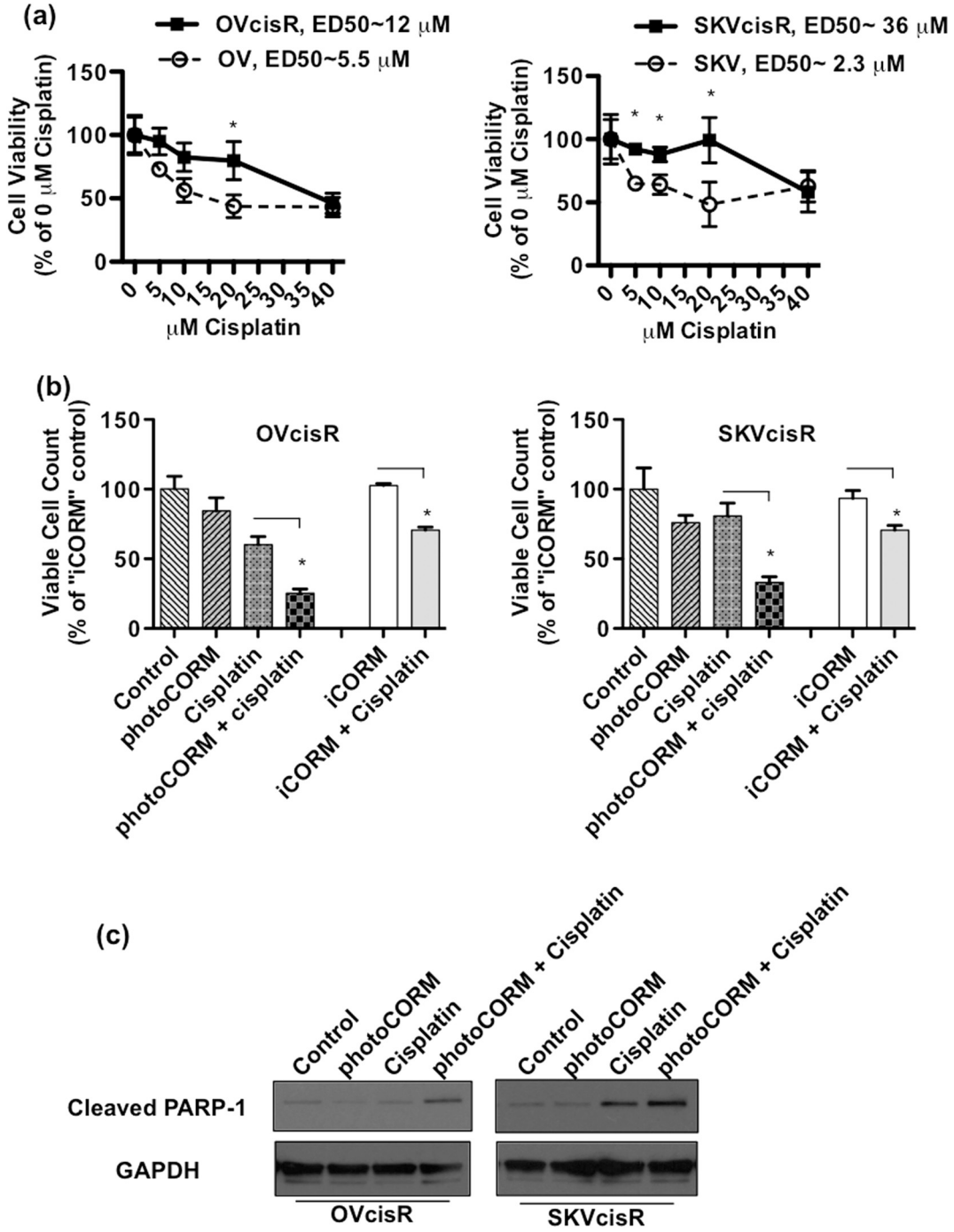



**Fig. 1.** Cisplatin-resistant ovarian cancer cell lines are sensitized to cisplatin upon co-treatment with carbon monoxide (CO), delivered from a photo-activated CO-releasing molecule (photoCORM). (a) Dose-dependency of cisplatin on cell viability in cisplatin-sensitive and resistant cell lines. Cells were treated with 0–40 μM cisplatin and/or DMSO vehicle control. Cell viability was then measured 24 h post-treatment via Trypan blue exclusion. Data are presented as average % of 0 μM cisplatin ± SEM of n = 3 independent experiments. (*$p < 0.05$). (b) Cell viability 24 h post-treatment, as measured by trypan blue exclusion, of cisplatin-resistant ovarian cancer cell lines, assessing the ability of CO to sensitize cisplatin-resistant ovarian cancer cell lines cells to cisplatin. iCORM, photo-inactivated photoCORM, was used as control for the non-CO scaffolding of the photoCORM. In lieu of cisplatin treatment, cells were treated with DMSO as vehicle control. Data are presented as average percentages of "Control" ± SEM of n = 3 independent experiments. (*$p < 0.05$). (c) Immunoblot of 20 μg whole cell lysate for cleaved PARP-1 in cisplatin-resistant ovarian cancer cell lines treated with CO and/or cisplatin. GAPDH was probed to assess equal protein loading. Blots presented are representative blots of n = 3 independent experiments.

used in this study (Supporting information, Fig. S1). Cisplatin-resistant versions of ovarian cancer cell lines OVCAR-5 and SKOV-3 (abbreviated OVcisR and SKVcisR respectively) were assessed for their resistance to therapeutically relevant concentrations of cisplatin compared to their respective parent cancer cell lines, OVCAR-5 and SKOV-3 (abbreviated OV and SKV respectively). Dose-response experiments revealed that OVcisR, and SKVcisR exhibited significant cisplatin resistance, with > 2-fold increased ED50 values for cisplatin compared with OV and SKV respectively (Fig. 1a). Because CO has been shown to sensitize certain cancer cells to chemotherapeutics, we wanted to assess whether CO, delivered from a photoCORM, could work similarly in an ovarian cancer model, attenuating drug resistance for platinum-based chemotherapies in ovarian cancer cell lines. To determine this, we treated cisplatin-resistant cell lines, OVcisR and SKVcisR, with 30 μM photoCORM and 20 μM cisplatin and compared these cell viability to cells treated with cisplatin alone. We observed that CO significantly enhanced the ability of cisplatin to reduce cell viability of cisplatin-resistant cell lines compared with cisplatin treatment alone (Fig. 1b). OVcisR and SKVcisR cells treated with cisplatin alone exhibited ~40% and ~29% decreases in cell viability, which was enhanced > 2-fold in both cell lines by CO, delivered from 30 μM photoCORM (Fig. 1b). Light-inactivated photoCORM (iCORM) neither significantly alter cell viability itself nor enhanced the cytotoxicity of cisplatin toward OVcisR and SKVcisR (Fig. 1b), demonstrating the negligible effect of the molecular scaffolding of the photoCORM toward increasing cisplatin sensitivity in OVcisR and SKVcisR. Lower doses of photoCORM also sensitized cisplatin-resistant cells to cisplatin in a concentration-dependent manner (Supporting information, Fig. S2). To determine whether the actions of CO and cisplatin together were apoptotic in nature, we assessed PARP-1 cleavage. Indeed, the sensitization of OVcisR and SKVcisR to cisplatin by co-treatment with 30 μM CO corresponded to increased PARP-1 cleavage in whole cell lysates, indicating an apoptotic process (Fig. 1c). Interestingly, we found 24 h treatment with that co-addition of 3 mM *N*-acetylcysteine (NAC), an antioxidant and efficient donor of cysteine, for 24 h was able to largely reverse CO's ability to re-sensitize of OVcisR and SKVcisR to cisplatin (Supporting information, Fig. S3). This finding indicated that NAC, and its effect on OVcisR and SKVcisR, disrupted the cellular processes by which CO sensitizes these cells to cisplatin. Since NAC is an efficient donor of cysteine, we measured steady state levels of intracellular cysteine by HPLC-MS. Indeed, 3 mM NAC treatment for 24 h increased intracellular levels of cysteine in ~4.4-fold in OVcisR and ~3.8-fold in SKVcisR compared with respective vehicle controls (Supporting information, Fig. S4). Together, these findings regarding NAC suggested that intracellular levels of cysteine, a sulfur-containing amino acid, might be mechanistically important for the cisplatin-resistance phenotype in OVcisR and SKVcisR.

### 3.2. Cisplatin resistance in ovarian cancer cells was associated with increased expression and activity of CBS

Cisplatin resistance is attributed to increased drug inactivation by sulfur-containing nucleophilic species GSH and nuclear metallothionein (MT) binding and inactivating the drug [14,15]. Increased generation of GSH and MT places a significant demand for cysteine, as both GSH and MT require the same sulfur-containing amino acid. One of the major sources of cysteine can be from the enzymatic actions of CBS and cystathionine γ-lyase (CGL), transsulfuration pathway enzymes that convert homocysteine to cysteine [16]. Immunoblot analysis of 20 μg whole cell lysates revealed dramatically increased expression of both CBS and CGL, transsulfuration pathway enzymes, in cisplatin-resistant versus the corresponding sensitive cells (Fig. 2a). Additionally, steady state levels of intracellular CTH, the enzymatic product of CBS, was present ~3.1-fold more in OVcisR and ~7.5-fold more in SKVcisR versus cisplatin-sensitive cell lines OV and SKV respectively (Fig. 2b).

### 3.3. Cisplatin-resistant ovarian cancer cell lines exhibited increased uptake of cystine and steady state levels of cysteine versus corresponding cisplatin-sensitive cell lines

In addition to overexpressing CBS, cisplatin-resistant ovarian cancer cell lines OVcisR and SKVcisR also exhibited overexpression of CGL (Fig. 2a), an enzyme downstream of CBS in the transsulfuration pathway. CGL breaks down CTH into the amino acid cysteine, which, like CTH, was found at higher steady state levels in cisplatin-resistant cell lines, ~2.7-fold more in OVcisR and ~1.4-fold compared to cisplatin-sensitive cells (Fig. 3a). CGL, however, is only one of several processes by which cysteine levels are regulated in the cell. In addition to the generation of cysteine through the transsulfuration pathway, uptake of cystine, an oxidized disulfide of two cysteine molecules, by the glutamate/cystine antiporter (xCT) is known to be an important source of intracellular cysteine for cancer cells, including ovarian cancer [18]. Furthermore, certain cisplatin-resistant ovarian cancer cells are reported to have increased cystine-uptake via xCT [18]. In consideration of this alternate source of cysteine, we measured relative uptake of extracellular deuterium-labeled cystine (D4-CC) by cisplatin-resistant ovarian cancer cells compared to their respective cisplatin-sensitive counterparts. The relative uptake of D4-CC was quantified by measuring intracellular levels of heavy labeled cystine and cysteine

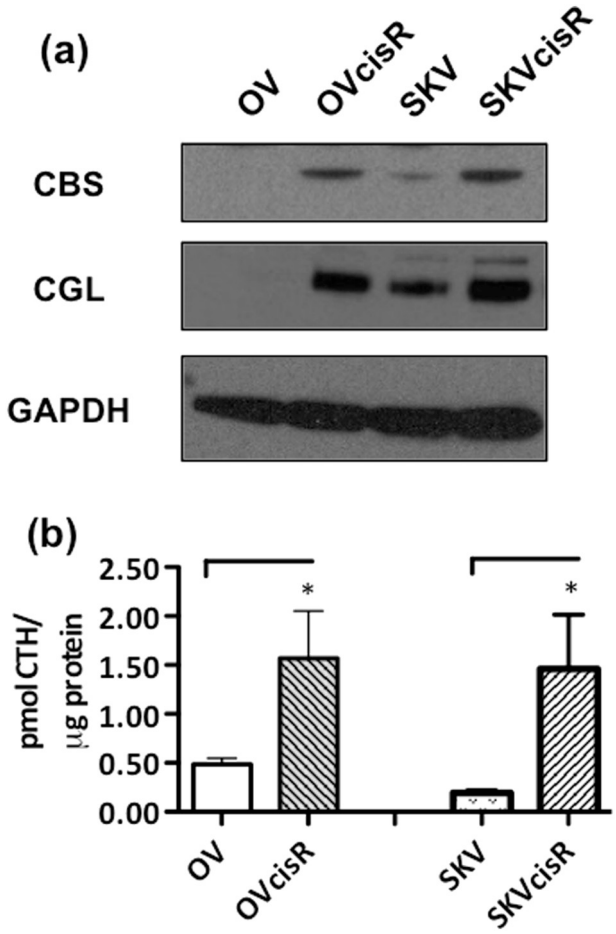



**Fig. 2.** Cystathionine β-synthase (CBS) expression and activity correlates with cisplatin-resistance in ovarian cancer cells. (a) Immunoblot for CBS and cystathionine γ-lyase (CGL) of 20 μg whole cell lysate of ovarian cancer cells, both cisplatin-sensitive and cisplatin-resistant cells. GAPDH expression was used as a loading control. Blots are representative of n = 3 independent experiments. (b) Steady state levels of cystathionine (CTH) in ovarian cancer cell lines, as determined by HPLC-MS. Data presented as average pmol CTH per μg protein ± SEM of n = 3 independent experiments. (*$p < 0.05$).

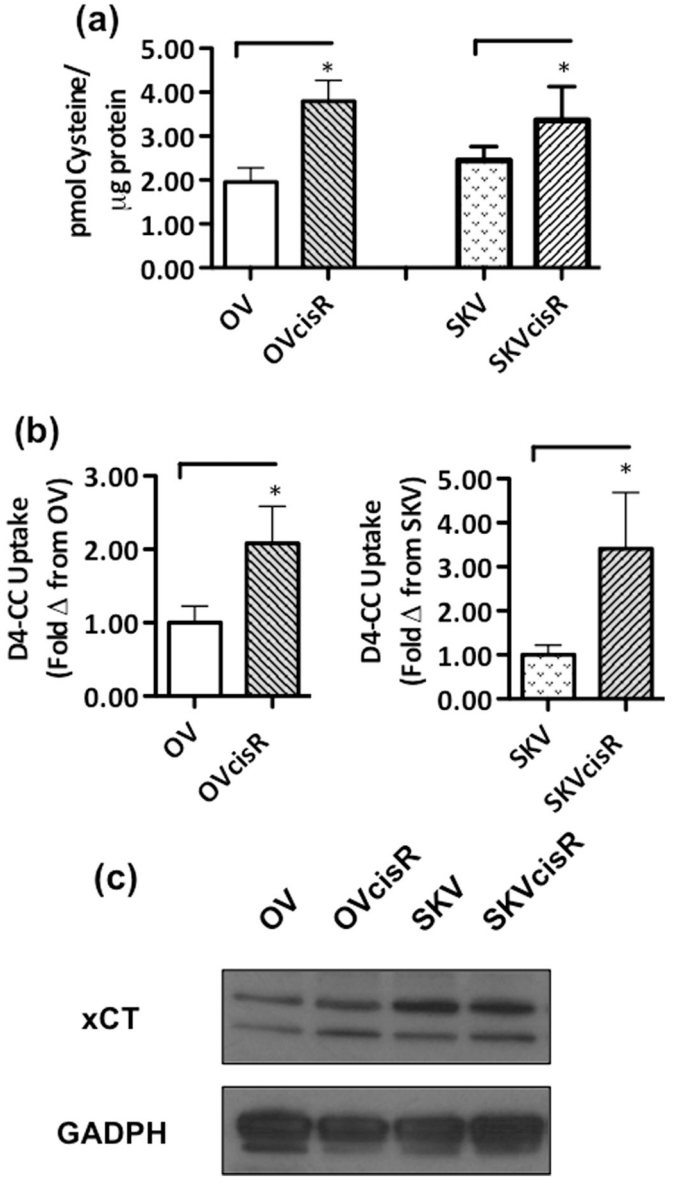



**Fig. 3.** Cisplatin-resistant ovarian cancer cell lines, OVcisR and SKVcisR, maintain higher levels of intracellular cysteine compared with cisplatin-sensitive cell lines. (a) Steady state levels of cysteine in OVcisR and SKVcisR, cisplatin-resistant ovarian cancer cell lines, compared with OV and SKV, cisplatin-sensitive ovarian cancer cell lines. Data are presented as average pmol cysteine/ µg protein ± SEM of n = 3 independent experiments. (*p < 0.05) (b) Relative uptake of D4-cystine (D4-CC) by OVcisR and SKVcisR compared to OV and SKV respectively. Data expressed as average fold difference from OV and SKV ± SEM of n = 3 independent experiments. (*p < 0.05). (e) Representative immunoblot of three independent experiments, showing the relative expression of xCT in 20 µg whole cell lysate of cisplatin-sensitive and cisplatin-resistant cell lines. GAPDH was used as a loading control.

(D4-CC and D2-cystine respectively). We observed cisplatin-resistant cells, OVcisR and SKVcisR, contained ~2.1 and ~3.4 times more intracellular D2-cysteine (the reduced form of D4-CC) than OV and SKV, their respective cisplatin-sensitive cells, indicating a greater capacity to uptake cystine (Fig. 3b). Intracellular D4-CC was not detected at levels above background (data not shown). This is in accordance with previous observations because intracellular cystine is quickly reduced to two cysteine residues, due to the reducing environment of the cytosol [18]. Despite the increased uptake of cystine in cisplatin-resistant cells, xCT protein expression was not remarkably different between the

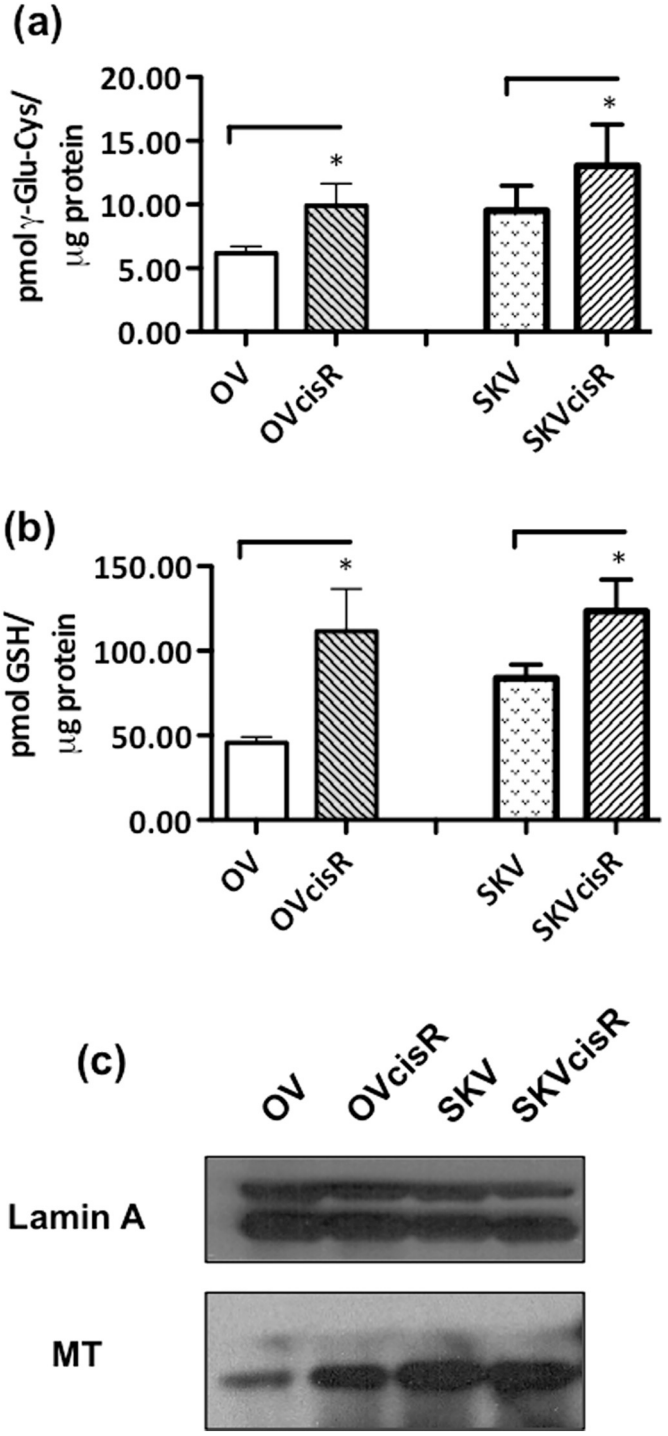



**Fig. 4.** Glutathione (GSH) and metallothionein (MT) are up regulated in cisplatin-resistant ovarian cancer cell lines. (a–b) Steady state levels of γ-Glu-Cys and GSH as measured by HPLC-MS in cisplatin-resistant cell lines, OVcisR and SKVcisR, and cisplatin-sensitive cell lines, OV and SKV. Data are presented as average pmol metabolite per total µg protein ± SEM of n = 3 independent experiments. (*p < 0.05). (c) Immunoblot of MT in 15 µg nuclear-enriched fractions of ovarian cancer cells. Lamin A was probed for determining equal loading of nuclear fractions. Blot is representative of n = 3 independent experiments.

cisplatin-resistant and cisplatin-sensitive cells (Fig. 3c). This suggested that xCT activity per se, and not its expression, was important for the differential uptake of D4-CC.

### 3.4. GSH and MT were found at increased levels in cisplatin-resistant ovarian cancer cell lines compared with cisplatin-sensitive ovarian cancer cell lines

Next, we wanted to determine whether increased cysteine levels observed in cisplatin-resistant cell lines was correlated with increased biosynthesis of GSH and MT, cysteine-containing species that bind and inactivate cisplatin [14]. γ-Glu-Cys, the product of the rate-limiting step in the biosynthesis of GSH, was present at higher steady state levels, ~1.6-fold higher in OVcisR and ~1.4-fold higher in SKVcisR, versus OV and SKV respectively (Fig. 4a). Further connecting the dependence of γ-Glu-Cys with intracellular cysteine, 3 mM NAC significantly increased steady state levels of γ-Glu-Cys: > 2-fold in both OVcisR and SKVcisR (Supporting information, Fig. S5). Such increased levels of γ-Glu-Cys in OVcisR and SKVcisR were supported by concomitant increases in the steady-state levels of GSH. Intracellular levels of GSH were ~2.4-fold higher in OVcisR and ~1.4-fold higher in SKVcisR when compared with OV and SKV, their respective, cisplatin-sensitive, parent cell lines (Fig. 4b). NAC-treated OVcisR and SKVcisR cells both exhibited ~3.4-fold and ~2.4-fold higher steady state levels of GSH compared with their respective, vehicle treated controls (Supporting information, Fig. S6), demonstrating intracellular cysteine levels can influence GSH levels in cisplatin-resistant ovarian cancer cells.

In addition to GSH, MT, a cysteine-rich protein, is known to bind and inactivate cisplatin specifically when localized in the nucleus [14,15]. Qualitative measurement of nuclear MT indicated that OVcisR and SKVcisR cells exhibited considerably increased expression of nuclear MT when compared with OV and SKV cells (Fig. 4c). Addition of 3 mM NAC, a donor of cysteine, also resulted in modest increased nuclear MT expression as determined by Western analysis (Supporting information, Fig. S7). Together, these findings demonstrate the importance of elevated, intracellular cysteine levels toward maintaining higher levels of GSH and nuclear MT, thiols known to bind and inactivate cisplatin.

### 3.5. Stable silencing of CBS expression in cisplatin-resistant ovarian cancer cell lines sensitized those cells to cisplatin

We next wanted to assess if and, if so, how CBS expression/activity was imparting cisplatin resistance. Toward this end, we prepared stable, lentiviral-mediated CBS-silenced cisplatin-resistant ovarian cancer cell lines [OVcisR(shCBS) and SKVcisR(shCBS)]. Western analysis on whole cell lysates of OVcisR(shCBS) and SKVcisR(shCBS) revealed decreased CBS protein expression in those lysates compared to control shRNA-transfected cells OVcisR(scram) and SKVcisR(scram) respectively (Fig. 5a). Reduced expression of CBS also resulted in reduced bioactivity, as measured by its enzymatic product CTH. Steady state levels of CTH in OVcisR(shCBS) were ~51% lower compared to transfection control OVcisR(scram) (Fig. 5b, left). Similarly, SKVcisR (shCBS) exhibited ~31% lower levels of intracellular CTH when compared to SKVcisR(scram) (Fig. 5b, right). To determine whether CBS over-expression in cisplatin-resistant cell lines was at least in part mediating cisplatin resistance, we measured the effects of cisplatin on cell viability in CBS-silenced cells versus scrambled controls. OVcisR (shCBS) and SKVcisR(shCBS), over a range of concentrations of cisplatin, exhibited significantly reduced cell viability compared to OVcisR(scram) and SKVcisR(shCBS) respectively (Fig. 5c). Calculated ED50 values for cisplatin in OVcisR(shCBS) an SKVcisR(shCBS) were ~2.5 μM and ~3.6 μM respectively. These values were lower than the ED50 values for OVcisR(scram) and SKVcisR(scram), ~11 μM and ~30 μM respectively, reflecting increased sensitivity to cisplatin due to silencing CBS (Fig. 5c).

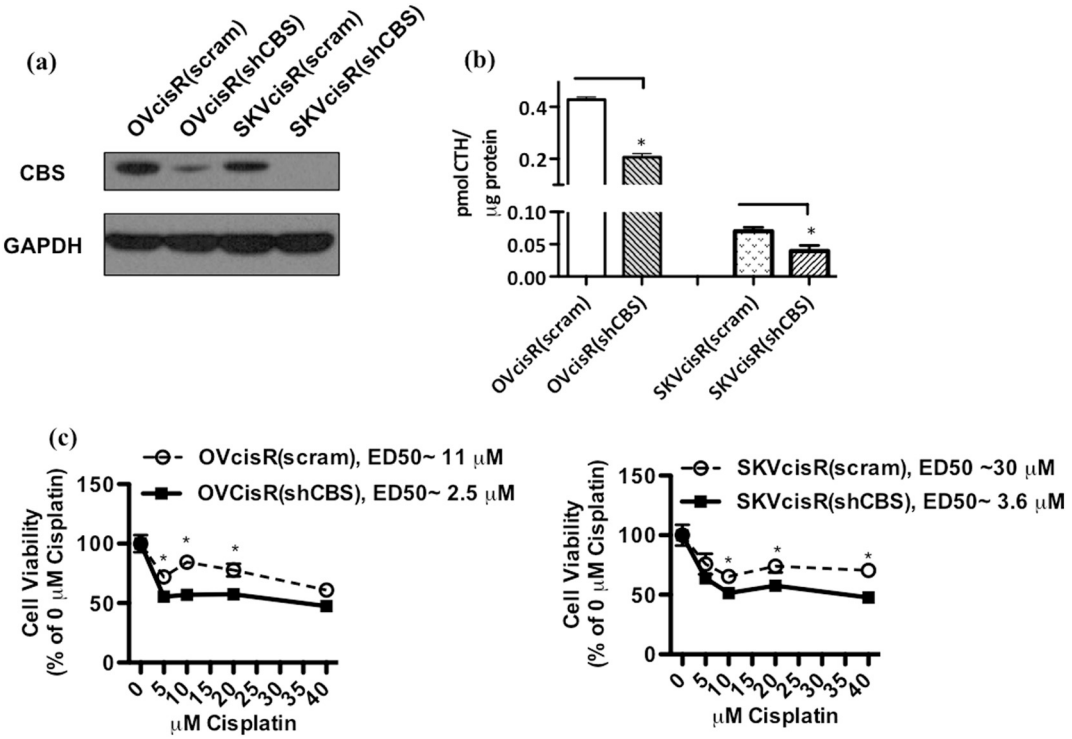



**Fig. 5.** Silencing cystathionine β-synthase (CBS) protein expression attenuates cisplatin resistance in cisplatin-resistant ovarian cancer cell lines OVcisR and SKVcisR. (a) Immunoblot for CBS of 20 μg whole cell lysate to measure efficacy of lentiviral-mediated gene silencing of CBS. Blots are representative of n = 3 independent experiments. (b) Intracellular cystathionine (CTH), as measured by HPLC-MS, to determine relative CBS enzymatic activity in CBS-silenced cell lines versus scrambled controls. Data presented as average pmol CTH per total μg protein ± SEM of n = 3 independent experiments. (c) Graph depicting the dose-dependency of cisplatin on cell viability 24 h post-treatment in cisplatin-resistant cell lines silenced for CBS (shCBS) versus those treated with scrambled controls (scram). ED50 values calculated from modeling of data shown. Data presented as average percentage of 0 μM cisplatin treatment ± SEM of n = 3 independent experiments. (*$p < 0.05$).

### 3.6. CBS-silencing in cisplatin-resistant ovarian cancer cell lines decreased steady state levels of intracellular cysteine

Intracellular cysteine levels were ~60% lower in OVcisR(shCBS) and ~77% in SKVcisR(shCBS) compared to their respective scrambled lentiviral controls OVcisR(scram) and SKVcisR(scram) (Fig. 6a). CBS-silenced cell lines exhibited ≥50% reduced D4-CC uptake compared with their respective controls (Fig. 6b) strongly implicating a role for CBS in regulating cystine uptake. The uptake of cystine, as addressed in Results Section 3.3, may be dependent on the activity and/or expression of xCT. Expression of xCT was however not noticeably different compared with respective scrambled controls (Fig. 6c). To partially elucidate the connection between CBS and cystine uptake, we turned our attention to $H_2S$. $H_2S$ is a possible enzymatic product of CBS, whereby CBS catalyzes the condensation of homocysteine with cysteine, rather than serine [18]. It has been shown that $H_2S$ can allosterically upregulate xCT activity and cystine uptake in neurons [19], though it had not yet been demonstrated in ovarian cancer cells. We therefore wanted to determine if $H_2S$ could at least partially restore the attenuated uptake of D4-CC. CBS-silenced cells treated with 40 μM GYY 4137, a slow releaser of $H_2S$, exhibited significant yet highly variable increases in D4-CC uptake versus those cells treated with vehicle control: > 600% in OVcisR(shCBS) and > 33% in SKVcisR(shCBS) (Fig. 6b).

### 3.7. Cystathionine β-synthase (CBS) in cisplatin-resistant ovarian cancer cell lines regulated GSH biosynthesis and expression of nuclear MT

In our next step we sought to determine whether the observed reduction in steady state levels of cysteine, caused by CBS-silencing (Fig. 6a), affected the biosynthesis of GSH. Steady state levels of γ-Glu-Cys, the metabolic precursor to GSH, were significantly lower, ~30% less in OVcisR(shCBS) and ~60% less in SKVcisR(shCBS) versus OVcisR (scram) and SKVcisR(scram) respectively (Fig. 7a). Additionally, we observed steady state levels of GSH in CBS-silenced cell lines, 63 pmol/μg in OVcisR(shCBS) and 58 pmol/μg SKVcisR(shCBS), were > 50% lower than that observed in scrambled controls, 130 pmol/μg in OVcisR (scram) and 150 pmol/μg in SKVcisR(scram) (Fig. 7b). CBS-silenced cell lines also expressed relatively less nuclear MT compared with scrambled controls (Fig. 7c).

### 3.8. CO inhibited CBS, thereby decreasing nuclear metallothionein and GSH

Following confirmation of a substantial connection between CBS and cisplatin-resistance in OVcisR and SKVcisR, we investigated whether CO-induced sensitization of cisplatin-resistant cells to cisplatin (Fig. 1b–c) was in part mediated by inhibition of CBS and the subsequent suppression of nuclear MT and GSH. CO, delivered by 30 μM photoCORM, significantly lowered CBS bioactivity, as measured by decreased steady state levels of intracellular CTH. CTH decreased ~2.7-fold in OVcisR and ~4.5-fold in SKVcisR (Fig. 8a). In a similar manner to lentiviral-mediated silencing of CBS (Fig. 6a), CO treatment reduced steady state levels of cysteine > 55% in the both cisplatin-resistant cell lines, OVcisR and SKVcisR (Fig. 8b). Concomitantly, CO reduced D4-CC uptake, ~73% in OVcisR and ~56% in SKVcisR (Fig. 8c). Steady state levels of γ-Glu-Cys, the product of the rate-limiting step of GSH synthesis, was also significantly lower in cisplatin-resistant cells treated with CO, ~63% lower in OVcisR and ~66% lower in SKVcisR, versus respective controls (Fig. 8d). Expression of nuclear MT, the thiol-containing peptide that is known to bind and inactivate cisplatin, was also lower in OVcisR and SKVcisR cells following treatment with CO (Fig. 8e). Treatment of cells with CO significantly lowered steady state levels of GSH in cisplatin-resistant cells, by ~40% in OVcisR and 65% SKVcisR (Fig. 8f).

## 4. Discussion

The results reported above require critical evaluation. In previous studies, investigators have shown that silencing of CBS (by lentiviral transduction) enhances drug sensitivity in cisplatin-resistant ovarian cancer cells [12]. Also, CO delivered by tricarbonyldichlororuthenium (II) dimer, has been shown to promote global methylation in U937

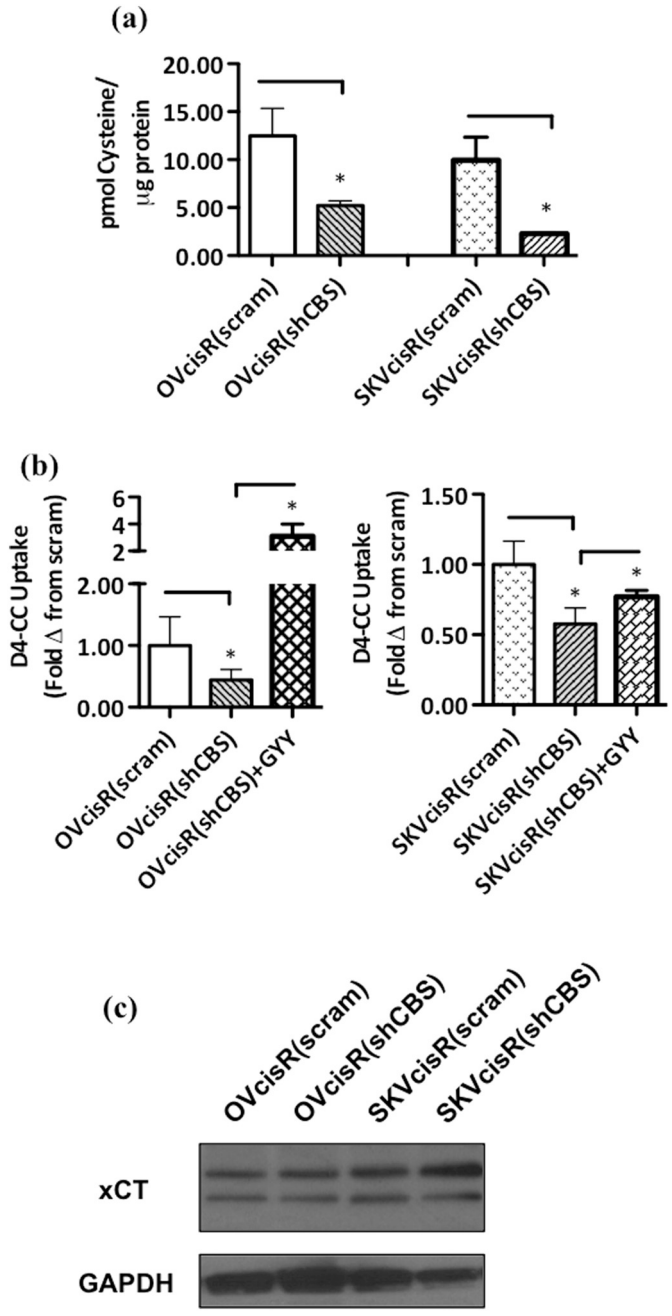



**Fig. 6.** Cystathionine β-synthase (CBS) expression in cisplatin-resistant ovarian cancer cells correlates to the bioavailability of cysteine. (a) Steady state levels of intracellular cysteine, as measured by HPLC-MS, normalized to μg protein. Data presented as averages ± SEM of n = 3 independent experiments. (b) Relative D4-cystine (D4-CC) uptake from extracellular media in CBS-silenced cells. Cells treated with either 40 μM GYY or DMSO vehicle control, then incubated for 48 h. Data are presented as average fold difference from scrambled controls ± SEM of n = 3 independent experiments. (*$p < 0.05$). (c) Expression of xCT in 20 μg whole cell lysates of CBS-silenced ovarian cancer cells versus respective scrambled controls. GAPDH was used as a loading control. Blot representative of n = 3 independent experiments.

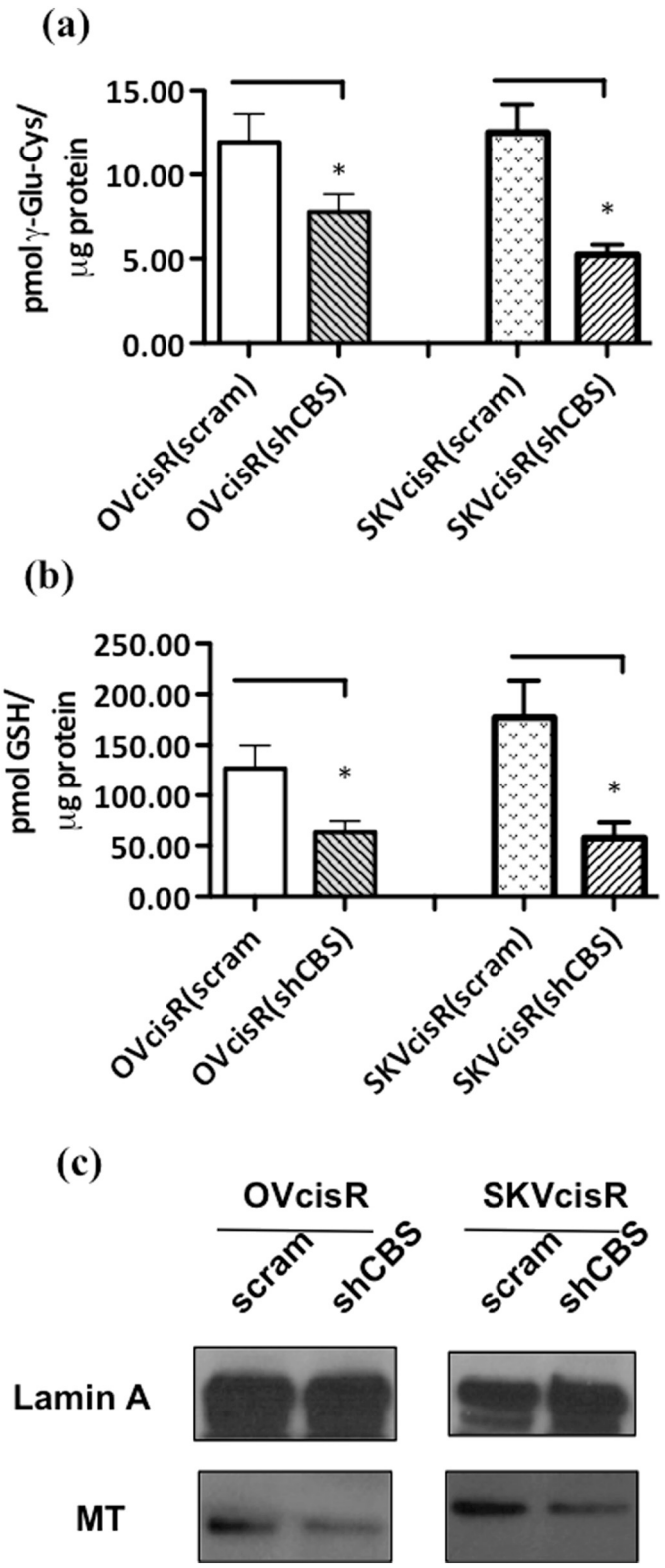



**Fig. 7.** Cisplatin resistance and cystathionine β-synthase (CBS) expression correlate to glutathione (GSH) biosynthesis and expression of nuclear metallothionein (MT) in cisplatin-resistant ovarian cancer cells. (a-b) Steady state levels of γ-Glu-Cys and GSH, normalized to total μg protein, in CBS-silenced cell lines (shCBS) compared with respective scrambled controls (scram), measured by HPLC-MS. Data presented as average pmol of metabolite per total μg protein ± SEM of n = 3 independent experiments. (c) Immunoblot of MT in 15 μg nuclear extracts of CBS-silenced cells (shCBS) versus transduction controls (scram). Lamin A is used as a nuclear fraction loading control. Blots representative of n = 3 independent experiments.

human monoblastic leukemia cell, presumably by CO-mediated inhibition of CBS [20]. However, direct demonstration of a viable pharmacological inhibition of CBS to alleviate drug resistance in ovarian cancer has not been achieved. The present study for the first time provides evidence that CO, delivered from a photoCORM, inhibits CBS and such delivery could be a viable option to circumvent chemo-resistance in ovarian cancer. Specific small molecule inhibitors of CBS, such as benserazide, have shown promise in eradicating cancer cells [21]. However, if further developed, these could only be used as a systemic therapy that will not spare normal cells. Such systemic therapy will result in serious adverse side effects, as CBS in the liver is the primary source of GSH, the major antioxidant in physiology [22]. On the other hand, light-activated CO delivery via appropriate catheters, prototypes for which have already been developed in our laboratory [23], could be used to inhibit CBS locally in accessible cancer tissues. This would bypass systemic delivery and increase the exposure of affected tissues to effective concentrations of the inhibitor and at the same time minimize adverse side effects. In fact the feasibility of CO-releasing molecules for cancer treatment has recently been reviewed [24].

Platinum-containing anti-cancer drugs react readily with the sulfur-containing amino acids in proteins namely methionine and cysteine. Unlike methionine, whose reaction with platinum is readily reversible by replacement with thiols or nucleotide bases, the cysteine-platinum complex is more stable [25]. Therefore, cysteine-rich proteins such as GSH and MT, both of which are present at high concentrations in the cancer cell, readily bind and inactivate platinum drugs. Indeed, formation of a cisplatin-$(GS)_2$ complex has been characterized (by NMR spectroscopy and HPLC-atomic absorption spectroscopy) in vitro and in vivo (in murine L1210 cells) in addition to efflux of the complex across the cell membrane [26]. Along the same line, formation of a ternary complex between MT and Platinum-DNA adduct followed by release of platinum from the DNA (and formation of $[(NH_3)_2Pt(S_2\text{-MT})]$ species) has been suggested to modulate DNA-repair and gene transcription leading to drug resistance [27]. Immunohistochemical MT expression in various human tumours has been associated either with processes related to carcinogenesis or with resistance against radiation and chemotherapy [28]. In ovarian tumours an increasing percentage of MT expression has been observed during the progression of malignancy [29]. Results shown in Fig. 8 now clearly indicate that CO-mediated CBS inhibition leads to the reduction of both GSH and MT (implicated in cisplatin inactivation) in ovarian cancer cells. Our observations are supported by earlier findings that CBS positively regulates GSH levels in breast cancer cells [11]. *The fact that exogenous CO could interfere with MT expression as well in refractory ovarian cancer cells, is in itself a novel finding in this study*. The data that CBS silencing could lower levels of nuclear MT underlines the important role of CBS in regulating two major thiol moieties (GSH and MT) implicated in chemotherapeutic drug resistance [28–31]. Nuclear MT expression is induced by cisplatin and seems to protect DNA in cells from toxic effects of the drug. The proportion of the individual contributions of GSH and MT in inactivating cisplatin is however not explored in this work.

Increases in GSH and MT require adequate maintenance of intracellular cysteine levels. The intimate connection between GSH/MT and intracellular cysteine is highlighted by the ability of extracellular treatment of cisplatin-resistant ovarian cancer cells with NAC. Treatment of OVcisR and SKVcisR with 3 mM NAC treatments increased both GSH and nuclear MT (Fig. S6 and S7, Supporting information). These results strongly suggest that regulation of sulfur metabolism in cisplatin-resistant ovarian cancer cells by CBS consists of two processes: flux through the transsulfuration pathway and uptake of extracellular cystine. Previous studies have shown that some cancer cell types maintain intracellular cysteine levels by importing cystine via the glutamate/cystine antiporter, xCT [32,33]. Elevated expression and activity of xCT in response to oxidative stress has been reported in breast cancer cells, alluding to a role for xCT toward protecting cancer cells

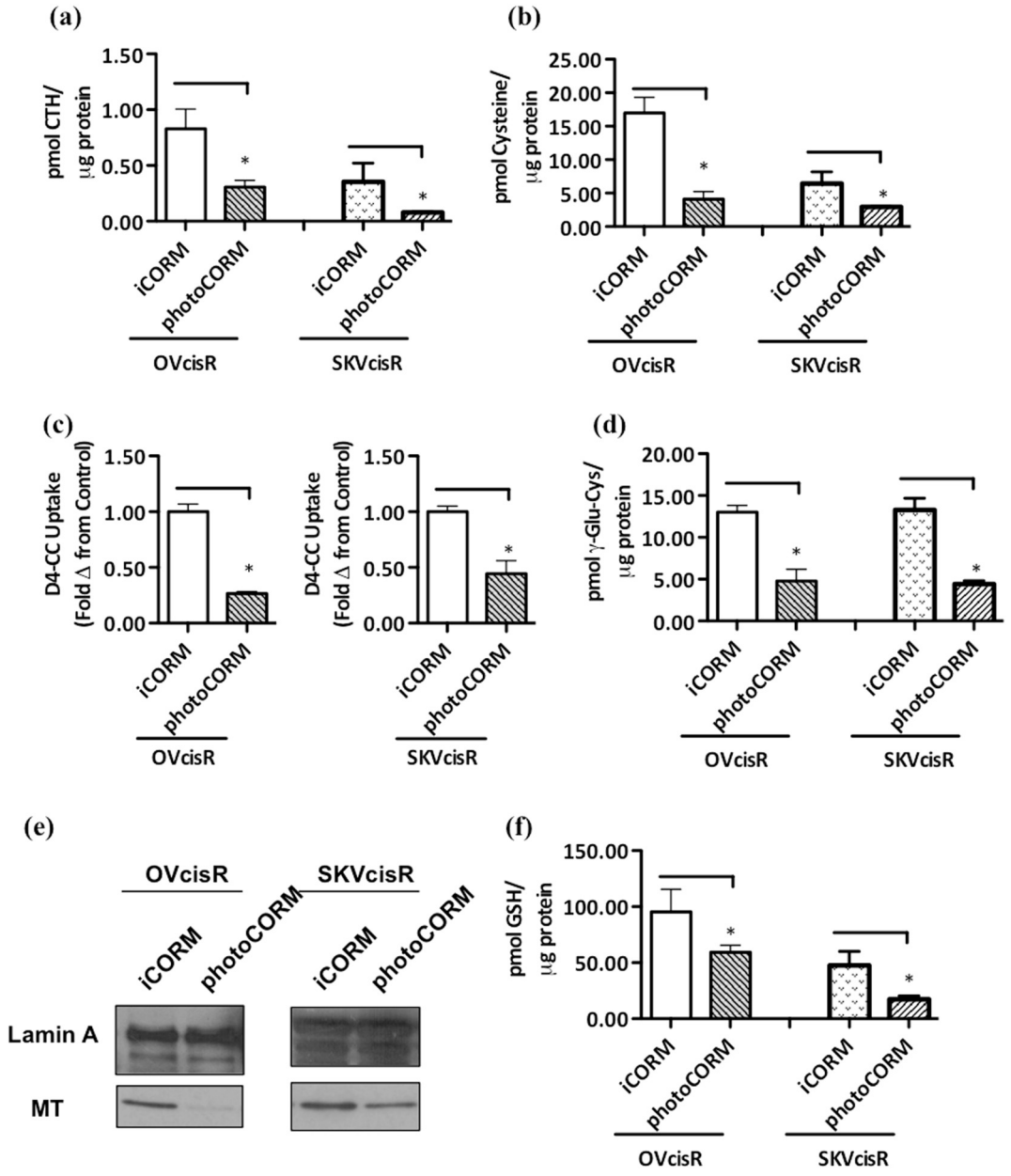



**Fig. 8.** Carbon monoxide (CO) inhibits CBS activity in cisplatin-resistant ovarian cancer cell lines OVcisR and SKVcisR. (a) CBS activity as measured by intracellular cystathionine (CTH). Levels of (b) cysteine, (d) γ-Glu-Cys and (f) GSH as measured by HPLC-MS in cisplatin-resistant ovarian cancer cell lines (normalized to total µg protein). Cells treated with either 30 µM photoCORM or 30 µM of inactivated photoCORM (iCORM) for 24 h. Data presented as averages ± SEM of n = 3 independent experiments. For GSH, data presented as average pmol GSH/µg protein ± SEM of n = 3 independent experiments (*$p < 0.05$). (c) Effect of 30 µM photoCORM treatment on D4-cystine (D4-CC) uptake in OVcisR and SKVcisR. Cells grown for 48 h in media supplemented with D4-CC. Data presented as average fold difference from "iCORM" control ± SEM of n = 3 independent experiments. (e) Immunoblot of MT expression in 15 µg nuclear enriched fractions of cisplatin-resistant cell lines. Cell treated with 30 µM photoCORM or respective iCORM controls for 24 h prior to nuclear fraction isolation.

against oxidative stress induced loss of cell viability (a mechanism often exploited by chemotherapeutic drugs) [34]. Overexpression of xCT has been shown to increase intracellular GSH and increase resistance to cisplatin [17]. In addition, loss of xCT from cancer cells resulted in suppressed tumor growth of gastric cancer in pre-clinical models [35]. In cisplatin-resistant ovarian cancer cells, we observed increased xCT activity, but not increased expression per se. We have provided strong evidence for the regulation of xCT activity by CBS via $H_2S$, as lentiviral-mediated silencing of CBS in OVcisR(shCBS) and SKVcisR(shCBS) reduced the activity of xCT, which was partially reversed by the addition of exogenous $H_2S$, known to allosterically increase xCT activity (Fig. 6b) [19]. To the best of our knowledge, this is the first report that demonstrated that CBS can upregulate GSH synthesis independent of the transsulfuration pathway in cancer cells by inducing the upregulation of cystine uptake in cancer cells. This study further emphasized that CO mediated inhibition of CBS could exploit this mechanism to compromise the anti-oxidant potential of cancer cells resulting in their increased sensitivity to cisplatin.

The importance of overexpression of CBS in imparting cisplatin resistance was observed in SKV cells, which despite being sensitive to cisplatin, express detectable levels of CBS and CGL. This is in contrast to OV cells, where the expression of CBS and CGL is nearly undetectable. Interestingly, regression analysis of the effects of cisplatin on ovarian cancer cell lines in this study revealed that the ED50 of cisplatin for SKV cells is higher than that of OV cells (5.5 µM versus 2.3 µM, respectively, data not shown). The lower sensitivity of SKV cells to cisplatin versus

OV (Fig. 1a) could possibly be attributed to the higher protein expression of CBS and CGL in the former (Fig. 2a).

Finally, it is important to note that overexpression of CBS is observed in select malignancies, namely breast and ovarian cancers while the corresponding normal tissues in all such cases exhibit very low levels of expression or none at all [36]. For this reason, CBS could be an important target in case of these two types of cancer where modulation of CBS activity by exogenous CO could thwart resistance to conventional chemotherapy. The effect of CO on non-transformed ovarian tissues in the context of resistance to cisplatin is intriguing, as the effect (s) of CO in non-cancerous cells are likely independent of CBS. Elucidation of other binding partners of CO in non-transformed ovarian cell lines could be an interesting follow-up study.

## 5. Conclusions

Previous work from this laboratory demonstrated that light-triggered CO delivery by the photoCORM can attenuate antioxidant capacity in human breast cancer cells through inhibition of CBS and sensitizes such cells to doxorubicin and paclitaxel [10]. Sensitization of human ovarian cancer cells to cisplatin therapy through administration of CO now demonstrates the general concept of CBS inhibition as a treatment modality to overcome chemoresistance encountered in ovarian cancer therapy. Strategies that overcome cisplatin resistance may dramatically reduce the mortality of ovarian cancer [4]. Here we have presented that CO, delivered from a photoCORM, sensitizes established cisplatin-resistant cell lines to cisplatin. Furthermore, we have provided strong evidence that the effects of CO in circumventing chemotherapeutic drug resistance is at least in part mediated by the inactivation of endogenous CBS (as evidenced by the reduction in CTH, the direct metabolic product of CBS).

## Notes

The authors declare no competing financial interests.

## Acknowledgments

Financial support from the NSF grant DMR-1409335 (to PM) and Departmental funding from the Department of Department of Obstetrics and Gynecology, David Geffen School of Medicine at UCLA is gratefully acknowledged. CJ acknowledges support from the NIH U01 grant HD087221. Statistical analysis by Ariel Wen, M.B.A. of the UCLA Anderson School of Management is gratefully acknowledged.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2018.11.003.

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