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Mitigating cisplatin resistance in ovarian cancer.

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MITIGATING CISPLATIN RESISTANCE IN OVARIAN CANCER

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DEDICATION

This dissertation is dedicated to my parents

Kuyeb Edward Ndifor (of blessed memory)

and

Wuba Marguerite Sama

and

my dearest uncle

Dr. Nwana Sama

for giving me the opportunity to achieve my educational dreams

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ABSTRACT

MITIGATING CISPLATIN RESISTANCE IN OVARIAN CANCER

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Epithelial Ovarian cancer (EOC) is the leading cause of gynecological cancer death in the USA. Recurrence rates are high after front-line platinum chemotherapy and most patients eventually die from platinum-resistant disease.

P53 plays an important role in cellular response to platinum-DNA damage. It transcriptionally activates XPC, a platinum-DNA damage recognition protein in the global genome repair (GGR), sub-pathway of nucleotide excision repair (NER). The goal of this research is to investigate the effect of a novel combination of cisplatin, sodium arsenite ($NaAsO_2$) and hyperthermia (CPA 39 ^{o}C) on EOC cells with different p53 status. Human EOC cells were treated with cisplatin ± 20 μM $NaAsO_2$ for 1 h at 37 or 39 ^{o}C. $NaAsO_2$ ± hyperthermia selectively sensitized wild-type p53 EOC cells to cisplatin by suppressing XPC and enhancing cellular and DNA platinum accumulation. In contrast, only hyperthermia sensitized p53-mutated and p53-null EOC cells to cisplatin by enhancing cellular and DNA platinum accumulation. Cisplatin ± $NaAsO_2$ at 37 or 39 ^{o}C induced pseudo-G1 associated apoptosis in p53 expressing cells.

Co-treatment with HSP90 inhibitor 17-DMAG plus CPA 39 ^{o}C greatly sensitized EOC cells by enhancing cellular platinum accumulation. In order to

translate the *in vitro* findings in an *in vivo* model, metastatic ovarian cancer was established in nude mice by intraperitoneal injection of A2780/CP70 human EOC cells. Tumor bearing mice were perfused with 3 mg/kg body weight (BW) cisplatin ± 26 mg/kg BW $NaAsO_2$ for 1 h at 37 or 43 °C using a murine intraperitoneal chemotherapy system developed in our laboratory. Cisplatin induced NER proteins XPC and XPA and suppressed mismatch repair protein MSH2 that is associated with resistance. However, co-treatment with $NaAsO_2$ at 37 or 43 °C suppressed XPC, restored higher levels of MSH2 and enhanced tumor platinum uptake. Platinum and arsenic generally accumulated in systemic tissues during intraperitoneal lavage and decreased 24 h after perfusion.

In conclusion, CPA 39 °C alone or combined with 17-DMAG has the potential to sensitize EOC to cisplatin by attenuating NER, activating mismatch repair, enhancing tumor platinum accumulation and activating apoptotic cell death.

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CHAPTER 1

GENERAL INTRODUCTION

Every year, about 225,000 women are diagnosed with ovarian cancer (OC) worldwide and more than 50% of these patients die from the disease, making OC the 7th leading cause of cancer death in women globally (Jemal, et al., 2011). In the United States, an estimated 21,880 women were diagnosed with OC in 2010 and 13,850 died from the disease (Jemal, et al., 2010), making OC the leading cause of gynecological cancer death in the U.S. The high death rate is due to tumor spread beyond the ovary at the time of diagnosis and both innate and acquired resistance to chemotherapeutics (Cannistra, 2004).

The front-line treatment for advanced OC is cytoreductive surgery to excise the bulk of tumors followed by intravenous (IV) or combined IV and intraperitoneal (IP) cisplatin or carboplatin in combination with taxane therapy (McGuire, et al., 1996; Vasey, et al., 1999; Armstrong, et al., 2002; Markman, 1988). Although most women initially respond to treatment, the disease recurs in 60 – 70% of women with very poor prognosis (Ozols, 2005; Rubin, et al., 1999). Recurrent tumors that initially responded to platinum become resistant. In addition to this 'acquired' resistance, about 25% of OC are 'innately' resistant to platinum and respond poorly to initial chemotherapy.

Acquired or innate resistance to platinum compounds is a major limitation to clinical success. Ways of mitigating drug resistance include: increasing dose, changing route of drug administration or combining therapies. Increasing the dose of platinum agents will increase the risk for adverse side effects such as kidney failure (Zhang, et al., 2007). Changing route of drug delivery from intravenous to intraperitoneal makes sense because metastatic OC is usually confined to the peritoneal cavity. The rationale for intraperitoneal therapy in OC treatment is that tumors will receive sustained exposure to high concentrations of drugs while the peritoneal lining will protect against systemic distribution of drugs to limit toxicity (Echarri Gonzalez, et al., 2011). In response to three large randomized clinical trials showing benefit to incorporating intraperitoneal (IP) delivery in OC, the National Cancer Institute issued a clinical announcement recommending that patients with small volume disease at the end of frontline surgery be offered the chance of receiving IP chemotherapy (Trimble and Christian, 2008). Adding hyperthermia to chemotherapy agents delivered intraperitoneally (HIPEC) could improve outcome (Helm, 2009; Yang, et al., 2010; Dovern, et al., 2010).

Cisplatin is a potent chemotherapeutic against various forms of solid tumors including ovarian (Vasey, et al., 1999), testicular (Duale, et al., 2007) and bladder cancer (Ecke, et al., 2006). *In vitro* cell culture studies have shown that the cytotoxicity of cisplatin and its analogues such as carboplatin and oxaliplatin is directly related to total platinum bound to DNA (Knox, et al., 1986). Development of resistance to platinum drugs is a major challenge in

chemotherapy. Mechanisms of cisplatin resistance are multifactorial and include: decreased drug accumulation, enhanced detoxification by glutathionylation and efflux by multi-drug resistance proteins or binding to metallothionein, elevated DNA repair and DNA damage tolerance (Parker, et al., 1991; Stewart, 2007). The consequence of these mechanisms of resistance is decreased cell kill. In order to enhance OC cell kill, combined hyperthermia and platinum-based chemotherapeutics delivered intraperitoneally (HIPEC) has been used to treat women with advanced OC (Dovern, et al., 2010; Helm, et al., 2008). Hyperthermia enhances cisplatin cytotoxicity (Akaboshi, et al., 1994; Alberts, et al., 1980; Hahn, 1979; Herman, et al., 1988; Los, et al., 1994). Hyperthermia enhances the penetration of cisplatin into peritoneal tumor implant when delivered intraperitoneally (van, V, et al., 1998) and also enhances platinum-DNA adduct formation (Meyn, et al., 1980; van, V, et al., 1998). In addition, hyperthermia causes cellular stress and induces mitotic catastrophe (Hildebrandt, et al., 2002; Sekhar, et al., 2007). Even though HIPEC therapy increases survival, complete remission is not attained (Dovern, et al., 2010). Failure to achieve complete remission could be due to heat loss or uneven temperature distribution during HIPEC. Thus, there is need for pharmacological agents that will enhance the effect of hyperthermia on platinum-based chemotherapy. Arsenic has the potential to enhance hyperthermia cytotoxicity effect on cisplatin. Arsenic can potentially inhibit mechanisms of cisplatin resistance and also enhance mechanisms of hyperthermia induced cell death (Helm and States, 2009; Muenyi, et al., 2011).

Arsenic has a paradoxical effect because it is a chemotherapeutic as well as an environmental hazard (Desoize, 2004; Cui, et al., 2008). Arsenic (Fowler's solution ($KAsO_2$)) has been used as a chemotherapeutic to treat chronic myelogenous leukemia before the advent of radiation therapy (Waxman and Anderson, 2001). Arsenic trioxide (As_2O_3) has been used in Chinese traditional medicine for hundreds of years. The Food and Drug Administration approved the use of Trisenox® (As_2O_3) for the treatment of all-*trans* retinoic acid (ATRA) resistant acute promyelogenous leukemia in 2001 (Cohen, et al., 2001). There are several clinical trials underway using Trisenox® (As_2O_3) to treat hematological and solid tumors (Murgo, 2001; Murgo, et al., 2000).

Mechanisms of arsenic induced cytotoxicity include: inhibition of nucleotide excision repair (Hartwig, et al., 1997; Hartwig, et al., 2003; Nollen, et al., 2009; Muenyi et al., 2011), causation of oxidative stress (Shi, et al., 2004b; Shi, et al., 2004a), induction of mitotic catastrophe (Taylor, et al., 2008; McNeely, et al., 2008a) and induction of apoptotic cell death (HU, et al., 2005; Ramos, et al., 2005). Resistance to arsenic is associated with enhanced metallothionein binding, detoxification by glutathione conjugation and efflux by multi-drug resistance proteins (Leslie, et al., 2004), similar to cisplatin and its analogues (Cole, et al., 1994; Byun, et al., 2005; Surowiak, et al., 2005). Therefore, arsenic and platinum are expected to compete for the detoxification and efflux pathways. Hence, it is reasonable to predict that co-treatment with arsenic and cisplatin may increase the intracellular accumulation of platinum via competition for the detoxification and efflux systems. Increased cellular accumulation will likely

increase the amount of platinum binding to DNA and thus, increase cell death. Furthermore, arsenic mimics hyperthermia because it also causes mitotic catastrophe (Taylor, et al., 2006; McNeely, et al., 2008b; McNeely, et al., 2008a) and induces cellular stress similar to hyperthermia (Del Razo, et al., 2001).

The cytotoxicity of platinum-based chemotherapeutics is mediated through high levels of DNA damage leading to apoptosis, DNA repair, or cell cycle arrest. Cellular response to platinum-DNA damage is mediated through ATM/ATR and CHK1/CHK2 (Basu and Krishnamurthy, 2010) that phosphorylate and activate the tumor suppressor protein p53 (Canman, et al., 1998). P53 regulates cell death and cell survival following DNA damage. The role of p53 is to maintain the integrity of the genome. Thus, it is known as the "guardian of the genome" (Efeyan and Serrano, 2007). Activated p53 induces cell cycle checkpoint activation, apoptosis and DNA repair protein expression (Harris and Levine, 2005; Ford, 2005). Cisplatin is known to cause G2 arrest. P53 regulates G2/M transition by transcriptionally activating cyclin dependent kinase (CDK) inhibitor CDKN1A, GADD45 and 14-3-3σ (Abraham, 2001). These proteins inhibit CDK1/cyclin B and block entry into mitosis. Arsenic causes mitotic arrest and both arsenic and hyperthermia induce mitotic catastrophe (Taylor, et al., 2008; McNeely, et al., 2008a). Thus, it will be interesting to determine if arsenic and hyperthermia induce mitotic catastrophe in cisplatin treat cells.

Cisplatin forms bulky DNA adducts by binding to the N7 position of adjacent guanines to form predominantly intrastrand cross links and to a lesser extent interstrand cross links by linking guanines on opposite strands at CpG

sites. The bulky lesions resulting from intrastrand cross links are repaired primarily by the nucleotide excision repair (NER) pathway (Wood, 1996). Resistance to cisplatin is associated with enhanced NER (Martin, et al., 2008). There are five main steps involved in NER: damage recognition, assembly of repair complex, excision of damage, gap filling and ligation (Figure 1). There are two sub-pathways of NER: global genome repair (GGR) and transcription coupled repair (TCR). GGR removes DNA damage from the entire genome whereas TCR repairs DNA damage only on actively transcribing strands. These two pathways differ only in the lesion recognition step. In TCR, a stalled RNA polymerase II at the region of damage and Cockayne syndrome A and B (CSA and CSB) recognize damage on actively transcribing genes. In GGR, XPC and DDB2 are involved in DNA damage recognition (Fitch, et al., 2003). P53 regulates DNA repair by transcriptionally regulating XPC and DDB2 (Adimoolam and Ford, 2003; Ford and Hanawalt, 1995; Ford, 2005; Ford and Hanawalt, 1997). XPC is actively involved in cisplatin-DNA damage recognition (Neher, et al., 2010). Arsenic inhibits NER by suppressing XPC in fibroblast (Nollen, et al., 2009). This suggests that arsenic will sensitize OC cells to cisplatin by inhibiting XPC.

Following DNA damage recognition, downstream repair proteins XPA, RPA, ERCC1, TFIIH, DNA polymerase δ and DNA ligase are recruited to repair the damage in a common pathway (Figure 1). Over-expression of XPA and ERCC1 mRNA has been associated with cisplatin resistance in OC (Dabholkar,

et al., 1994). Thus, understanding if arsenic and hyperthermia modulate the expression of XPA and ERCC1 in order to sensitize cells to cisplatin is needed.

Figure 1

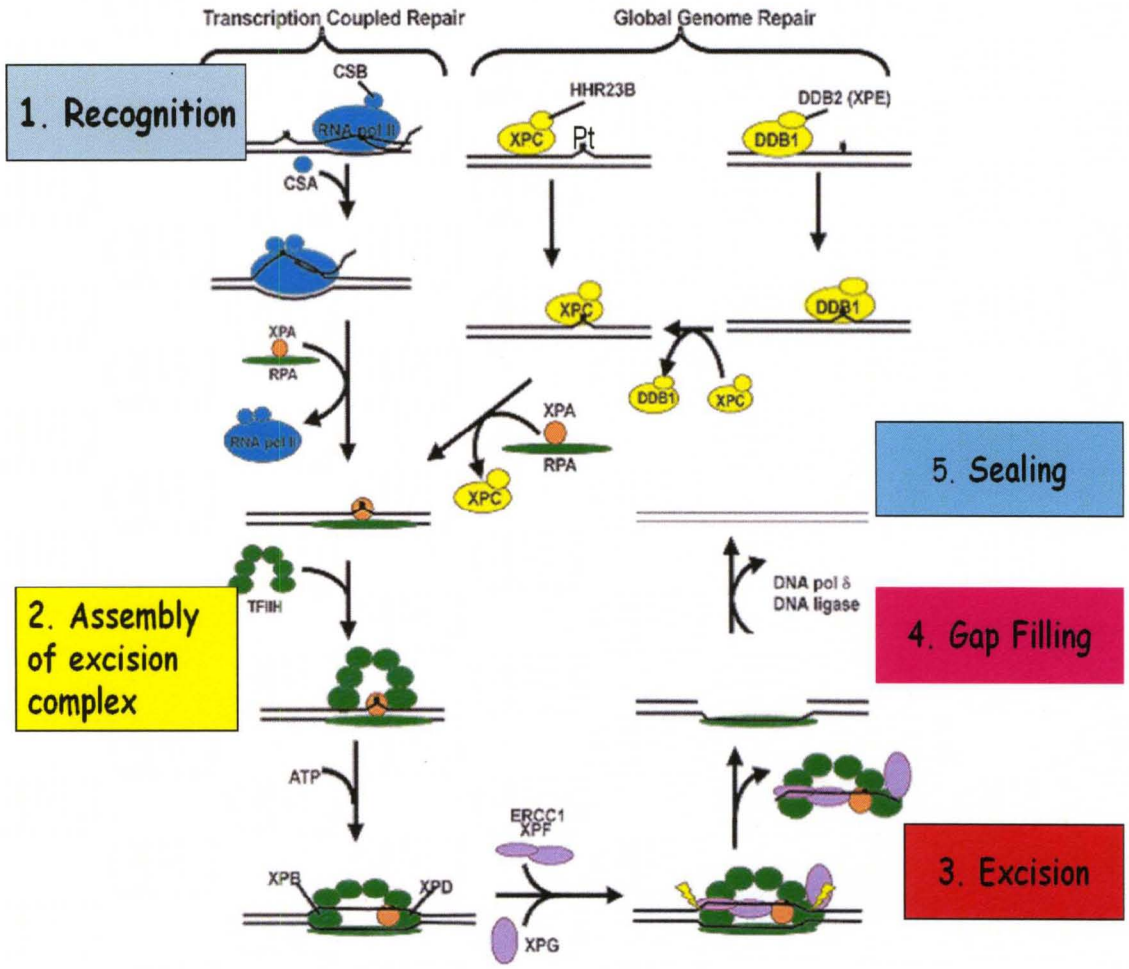

Figure 1. Nucleotide exicision repair pathway. The nucleotide excision repair pathway indicating the two sub-pathways: transcription coupled repair and global genome repair. This Figure was adapted from Paul Porter's dissertation.

In addition to NER, the mismatch repair (MMR) pathway has been implicated in cisplatin resistance (Fink, et al., 1997). In an effort to repair platinum-DNA damage by the MMR pathway, a futile MMR occurs leading to cell death (Martin, et al., 2008; Topping, et al., 2009). Ovarian cancer cells over-expressing MMR proteins such as MSH6 and MSH2 are sensitive to cisplatin (Ding, et al., 2009; Pani, et al., 2007; Topping, et al., 2009). Therefore, it is important to determine if arsenic and hyperthermia sensitize cells to cisplatin by activating the MMR pathway.

Although there has been tremendous effort in recent years to enhance the efficacy of platinum-based chemotherapy against OC by changing route of drug delivery and using combination therapy, over 50% of OC patients still die annually. The low response to chemotherapy and high death rate could be due to the heterogeneity of cancer cell population that result from constant mutation and alteration of important genes such as p53. This heterogeneous population of cancer cells cannot be treated successfully by a single agent or combination of drugs designed for a single target or cellular pathway. The need for combination therapy with each drug aimed at different targets or cellular pathways is paramount. In the following studies, I investigated the cytotoxicity effect of a new combination of sodium arsenite and hyperthermia on cisplatin cytotoxicity on a panel of OC cells expressing wild-type p53 or that are p53-null or p53-mutated. I used cells with different p53 status because about 50% of OC cells have non-functional p53 (Schuijer and Berns, 2003). In addition, p53 transcriptionally regulates genes involved in platinum-DNA damage repair (Ford, 2005).

Therefore, understanding how p53 status affects response to combined cisplatin, sodium arsenite and hyperthermia is important. I also examined how sodium arsenite and hyperthermia alter the mechanisms of cisplatin resistance. Particularly I examined the effects of sodium arsenite and hyperthermia on cellular and DNA accumulation of platinum, platinum-DNA repair pathways, induction of cellular stress, mitotic arrest/mitotic catastrophe induction and cell cycle regulation. I used both in *vitro* cell culture and human OC xenograft models for these studies. Cytotoxicity data indicate that combined cisplatin, sodium arsenite and hyperthermia sensitized wild-type p53 expressing human OC cells to cisplatin by suppressing p53 regulated protein XPC, enhancing cellular and tumor accumulation of platinum and enhancing platinum bound to DNA. I also observed that sodium arsenite ± hyperthermia suppressed XPC and enhanced platinum accumulation in metastatic tumors. Inhibition of the activity of stress response protein HSP90 greatly potentiated the cytotoxicity of combined cisplatin, sodium arsenite and hyperthermia in a p53-independent manner. Cisplatin, sodium arsenite and hyperthermia induced pseudo-G1 arrest associated apoptosis in p53 expressing cells.

In conclusion, combined cisplatin, sodium arsenite and hyperthermia (CPA 39 °C) or CPA 39 °C plus 17-DMAG has the potential to sensitize OC to cisplatin by suppressing NER, activating mismatch repair and enhancing cellular and DNA platinum accumulation, causing cellular stress and inducing apoptotic cell death. This combination chemotherapy will be effective because each of these drugs has a different mechanism of action.

CHAPTER 2

COMBINED SODIUM ARSENITE AND HYPERTHERMIA SELECTIVE SENSITIZATION OF WILD-TYPE P53 HUMAN OC CELLS TO CISPLATIN INVOLVES SUPPRESSION OF XPC AND ENHANCEMENT OF CELLULAR AND DNA PLATINUM ACCUMULATION

INTRODUCTION

Epithelial Ovarian cancer (OC) is the leading cause of gynecological cancer death among women in the United (Jemal, et al., 2010). Cisplatin and its analogues are front-line drugs to treatment OC (Armstrong, et al., 2002).

Cisplatin causes DNA damage to induce cell death (Cepeda, et al., 2007). However, cellular processes such as enhanced platinum-DNA damage tolerance, platinum-DNA repair, cisplatin metabolism and cellular export and reduced accumulation confer resistance to cisplatin (Stewart, 2007), and thus decrease the effectiveness of cisplatin.

Combined hyperthermia and cisplatin is used to treat OC (Helm, et al., 2008). Hyperthermia augments cisplatin-induced cytotoxicity and enhances the uptake of platinum and platination of DNA (Los, et al., 1994; van, V, et al., 1998). However, complete remission is not attained (Dovern, et al., 2010). The goal of this study is to determine if adding sodium arsenite (sodium

arsenite) to combined cisplatin and hyperthermia will further sensitize cisplatin-resistant OC cells to cisplatin.

In vitro studies demonstrate that arsenic induces apoptosis in solid cancer cells including gastric, colon, pancreatic, lung, prostate and OC (Cui, et al., 2008; Murgo, 2001). *In vivo*, arsenic inhibits the growth of orthotopic metastatic prostate cancer and peritoneal metastatic OC (Maeda, et al., 2001; Zhang and Wang, 2006). Results of combination chemotherapy studies demonstrate that arsenic sensitizes cancer cells to hyperthermia, radiation, cisplatin, adriamycin, doxorubicin, and etoposide (Chun, et al., 2002; Griffin, et al., 2003; Uslu, et al., 2000; Wang, et al., 2001). Arsenic has additive or synergistic effect with cisplatin following prolonged exposure (Uslu, et al., 2000; Wang, et al., 2001; Zhang, et al., 2009). Mechanisms of arsenic-induced cell death include formation of oxidative DNA damage (Nakagawa, et al., 2002), activation of the Fas pathway (Kong, et al., 2005), and inhibition of NER (Nollen, et al., 2009; Muenyi et al., 2011) and causation of mitotic catastrophe (McNeely, et al., 2008a).

In response to DNA damage, p53 is activated and stabilized by upstream DNA damage sensors. Activated p53 regulates cell cycle arrest, DNA repair and apoptosis. P53 is often mutated in human cancers (Olivier, et al., 2002), especially OC with about 50% of the tumors bearing p53 mutations (Schuijer and Berns, 2003). The role of p53 in OC response to platinum chemotherapy remains unclear. Most clinical studies suggest better response to platinum chemotherapy in patients with p53-mutated tumors than those with wild-type p53 tumors (Havrilesky, et al., 2003; Nakayama, et al., 2003; Okuda, et al., 2003). Most *in*

vitro studies also demonstrate that p53-mutated or p53–null cancer cells are more sensitive to cisplatin than those expressing wild-type p53 (Hagopian, et al., 1999; Havrilesky, et al., 1995; Yazlovitskaya, et al., 2001; Metzinger, et al., 2006). Therefore, presence of wild-type p53 generally confer resistance to cisplatin.

Enhanced DNA repair is an important mechanism of cisplatin resistance in OC cells (Parker, et al., 1991). XPC is required for platinum-DNA damage repair (Neher, et al., 2010). P53 is implicated in platinum-DNA repair because it transcriptionally regulates XPC (Ford, 2005). Therefore understanding the p53 status of tumors is important in developing personalized chemotherapy to effectively treat platinum-resistant cancer patients, and interfering with p53 function may suppress DNA repair and sensitize cancer cells to platinum therapy.

The present chapter addresses how the p53 status of OC cells affects response to new combination chemotherapy of cisplatin, sodium arsenite and hyperthermia. I show here for the first time that combined sodium arsenite and hyperthermia sensitize wild-type p53 expressing OC cells to cisplatin by suppressing XPC and enhancing cellular and DNA platinum accumulation. P53-mutated and-null cells were sensitized to cisplatin by hyperthermia only which involved enhancement of cellular and DNA platinum accumulation.

HYPOTHESIS

Sodium arsenite and hyperthermia sensitize OC cells to cisplatin by inhibiting DNA repair and enhancing accumulation of cisplatin.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, Tween 20, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), RNase A, cisplatin and sodium arsenite were purchased from Sigma-Aldrich (St. Louis, MO). DMSO was purchased from Fisher Scientific (Pittsburgh, PA). Stock solutions (cisplatin (1 mg/mL) in phosphate buffered saline (PBS) and sodium arsenite in water (10 mM)) were prepared freshly on the day of treatment and filter sterilized (0.22 μm) prior to use. It should be noted that Trisenox® (arsenic trioxide (As_2O_3) dissolved in 1 M NaOH) and sodium arsenite (sodium arsenite) both generate the same oxyanion [$As(OH)_3$] in solution (pharmacological form of arsenic). I used sodium arsenite for this research because it is readily soluble in water and is stable in water.

Cells and cell culture

Cisplatin-sensitive (A2780) and –resistant (A2780/CP70) human OC cells were the kind gift of Dr. Eddie Reed (The Mitchell Cancer Institute, University of South Alabama, Mobile, Alabama). SKOV-3 human OC cells were the kind gift of Dr. Donald Miller (Department of Medicine, University of Louisville). OVCA 420, 429, 432 and 433 cells were the kind gift of Dr. Zahid Siddik (Department of Gynecologic Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas). OVCAR-3 cells were purchased from American Type Culture Collection (Manassas, VA). A2780 and A2780/CP70 and OVCA 420, 429, 432 and 433 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 μg/mL penicillin/streptomycin, 2 mM L-glutamine and 0.2

units/mL insulin. SKOV-3 cells were maintained in McCoy's 5A media supplemented with 10% FBS and 100 µg/mL penicillin/streptomycin. OVCAR-3 cells were maintained in RPMI 1640 media supplemented with 20% fetal bovine serum, 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine and 0.01 mg/mL bovine insulin. Cells were cultured in an atmosphere of 95% humidity and 5% CO_2 at 37 °C. Cells were passaged twice weekly and replated at a density of 1 X 10^6 cells/150 mm dish.

Cell Viability assay

The growth inhibitory effects of cisplatin, sodium arsenite and hyperthermia were evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell viability assay (Mosmann, 1983). Briefly, 2500 cells/well were seeded overnight in 96-well plate. Cells were treated with drugs at 37 or 39 °C for 1 h. After treatment, cells were washed twice with PBS and refed with drug-free media and incubated at 37 °C for 5 days prior to assay. Control for no surviving cells ("blank") was cells treated with 0.1 mg/mL hygromycin B. The MTT assay evaluates the reduction of yellow tetrazolium salt to insoluble dark purple formazan crystals by mitochondrial succinate dehydrogenase in cells with functional mitochondria. The insoluble purple crystals that were formed were solubilized in DMSO and the absorbance measured at 570 nm. The absorbance values corresponded to the number of viable cells. Cell viability was calculated as follows and plotted against concentration of cisplatin.

$$\% \text{ cell viability} = \frac{100 \text{ x(Mean of triplicate treated samples - Blank)}}{\text{(Mean of triplicate untreated samples - Blank)}}$$

Data were expressed as means ± SEM of at least 3 independent experiments. Each experiment was done with triplicate wells for each treatment condition.

P53 and XPC siRNA transfections

One million cells were transfected with 400 nM of either XPC or p53 smart pool siRNAs (Dharmacon, # L-016040-00 and M-003329-01 respectively), non-targeting control pool (Dharmacon, # D-001206-13-05), or 1x universal buffer (Dharmacon, # B-001050-UB-015) using the Amaxa nucleofector kit V (Lonza, cat # VCA-1003, 2.5 mL). After transfection, 2500 cells/well were plated in 96-well plates for MTT assay and $1x10^5$ cells were plated in 6 cm dishes for western blot analyses. Cells were incubated at 37 °C for 23 h. Cells were then treated with cisplatin ± 20 μM sodium arsenite at 37 °C for 1 h. After treatment, cells were washed twice with PBS and refed with drug-free media and incubated at 37 °C for 5 days prior to MTT assay. Protein lysates were collected at 0 (immediately) and 24 h after treatment for western blot analyses.

Western blot analyses

Total cellular lysates were prepared from treated cells at 0 (immediately), 6, 12, 24, 36, 48 and 72 h after treatment. Cells were lysed with cell lysis solution (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% sodium dodecyl sulfate, 180 μg/ml phenylmethylsulphonylfluoride). After removal of debris by centrifugation at 13,000 xg for 45 min, total protein concentration in supernatant was determined by Bradford assay (Bio-Rad, Hercules, CA), using bovine serum albumin as standard. Proteins (30-40 μg/lane) were loaded and resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose

membranes. Membranes were probed with mouse monoclonal antibodies for p53 (Neomarkers, DO-1, dilution 1: 1000), DDB2 (Abcam, # ab51017, dilution 1:50), XPC (Abcam, # ab6264, dilution 1:1000), ß-actin (Sigma, # A 5441, dilution 1:10,000), or rabbit polyclonal antibodies for phospho-p53 Ser15 (Cell Signaling Technology, # 9284, dilution 1:500), XPC (Novus, # NB100-58801, dilution 1:10,000) and XPC (Santa Cruz, A-5, # SC-74411, 1:500). Secondary antibodies (rabbit anti-mouse IgG, # 81-6120 or goat anti-rabbit, # 81-6120, dilution 1:2500) conjugated to horseradish peroxidase (Zymed Laboratories, Inc. South San Francisco, CA) were bound to primary antibodies and protein bands detected using enhanced chemiluminescence (ECL) substrate (Pierce, Rockford, IL) followed by exposure to Kodak XAR x-ray film. ß-actin was used as the loading control.

Glutathione-S-transferase (GST) activity assay

Cells ($2x10^6$ cells/6 cm dish) were treated with 40 µM cisplatin ± 20 µM sodium arsenite at 37 or 39 °C for 1 h. Immediately after treatment, cells were washed twice with phosphate buffered saline and collected in 500 µL phosphate buffered saline EDTA (PBSE) by scraping with a rubber policeman. Cells were sonicated (at 3 pulses for 2 seconds each) using a probe sonicator, centrifuged at 10,000 xg for 15 min at 4 °C. The supernatant was collected and stored at -80 °C until use (samples are stable for at least a month). On the day of the assay, 10 mM glutathione (GSH, Sigma Aldrich, St. Louis, MO) in PBSE and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB, Santa Cruz, # 237329) in ethanol were prepared fresh (these reagents are stable at room temperature during assay). For total

glutathione-S-transferase activity determination, 830 µL PBSE plus 100 µL GSH plus 60 µL protein sample (25 – 50 µg protein) were transferred into a quartz cuvette. The cuvette was covered with parafilm wrap and mixed six times. The parafilm wrap was then removed and the background absorbance read at 340 nm for 1 min using a Varian Cary UV Spectrophotometer (Agilent Technologies, Palo Alto, CA). Then 10 µL CDNB was added rapidly, reaction mix was mixed six times and the absorbance for glutathione conjugated CDNB (GS-CDNB) was read for 2 min.

$$\text{Total GST acitvity (nmol/min/mg protein)} = \frac{\text{(Abs/min (sample) - Abs/min (background))}}{A^m \text{ nmol/mL x mg protein added}}$$

The molar extinction coefficient for GS-CDNB (A^m) = 0.0096 umol-1cm-1.

Genomic DNA isolation

After treatment, cells were washed twice with PBS and lysed with DNA lysis buffer (0.5 M Tris-HCl (pH 8.0), 20 mM Na_2EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate (SDS), and 0.5 mg/mL proteinase K). Lysed cells were collected and incubated overnight at 37 °C. The lysates were mixed with 1/4 volume of saturated NaCl solution and centrifuged at room temperature for 30 minutes at 500 x g to pellet undigested proteins. The supernatants were collected, mixed with 2 volumes of 96% ethanol, and inverted several times and kept overnight at 4 °C. The precipitated DNA was recovered, rinsed in 70% ethanol, briefly air dried to remove excess ethanol, and resuspended in 1X TE buffer (10 mM Tris-HCl, pH 7.4 and 1.0 mM Na_2EDTA, pH 7.4). DNA was incubated at 37°C with 100 µg/ mL heat-treated RNase A for 3 h, mixed gently with 1/5 volume of 11 M ammonium acetate (pH 6.5) and 2 volumes of cold 96% ethanol. DNA was

allowed to precipitate overnight at -20 °C and then recovered, rinsed in 70% ethanol, briefly dried, and resuspended in 1X TE buffer. DNA was quantified by A_{260} and purity determined by A_{260}/A_{280} ratio. DNA was stored at -20 °C until use.

Platinum accumulation studies

Cells (1 X 10^6/10 cm dish) were treated with cisplatin ± 20 μM sodium arsenite at 37 or 39 °C for 1 h. The cell monolayers were washed twice with PBS, harvested and lysed with protein lysis solution. Samples were removed for protein determination using the bicinchoninic acid (BCA) method according to manufacturer's instruction (Pierce, Rockford, IL, micro-well plate protocol) (Smith, et al., 1985). Then 100 μL samples of lysates were transferred to 2 mL acid washed (washed with 0.1 M nitric acid) centrifuge tubes and lyphophilized using FTS systems Flexi-Dry MP (Stone Ridge, NY). Concentrated nitric acid (350 μL) was added to every sample and the samples were predigested overnight. After predigestion, 100 μL of each sample was transferred into 10 mL acid-washed microwavable digestion tubes (triplicate for each sample). The samples were microwave-digested at 150°C for 10 min using an automated focused beam microwave digestion system (Explorer™, CEM, Matthews, NC, USA). Then 1.9 mL of 18 Mohm H_2O containing 10 ppb internal standard (SPEX CertiPrep, Metuchen, NJ) was added into every sample to give final 5% nitric acid. Platinum was determined using Thermo X Series II Inductively Coupled Plasma Mass Spectrometry (ICP-MS) instrument (Thermo Fisher Scientific, Waltham, MA) located at the University of Louisville Center for Regulatory and Environmental Analytical Metabolomics (CREAM) facility. Concentrated nitric acid was

processed similarly and used as Blank. Platinum standard (SPEX CertiPrep, Metuchen, NJ) was used to generate a standard curve. Cellular platinum levels were expressed as ng platinum/mg protein. Results are the mean of 3 ICP-MS determinations for each data point from 3 independent experiments.

Statistical analysis

Results are expressed as the mean ± SD of three independent experiments. Statistical analyses were performed using Microsoft Excel built in formulas for one-way analysis of variance and student's t-test with significance level as $p < 0.05$, $n \leq 3$.

RESULTS

Sodium arsenite ± hyperthermia selectively sensitizes wild-type p53 expressing OC cells to cisplatin

P53 regulates DNA repair and is frequently mutated in OC cells. Thus, it is very important to determine if p53 status will affect response to DNA damaging chemotherapeutics such as cisplatin. I used wild-type p53 expressing (A2780 (cisplatin-sensitive) and A2780/CP70, OVCA 420, OVCA 429, OVCA 433 (cisplatin-resistant)), p53-null (SKOV-3) and p53 mutated (OVCAR-3 and OVCA 432) human OC cells to determine if the p53 status is an important determinant of OC cells response to a new combination of cisplatin, sodium arsenite and hyperthermia. Cells were treated with cisplatin ± 20 μM sodium arsenite at 37 or 39 °C for 1 h. The 20 μM sodium arsenite I used for all studies was determined to be non-toxic in A2780 and A2780/CP70 cells when treated for 1 h and MTT assay five days later (Figure 2).

Figure 2

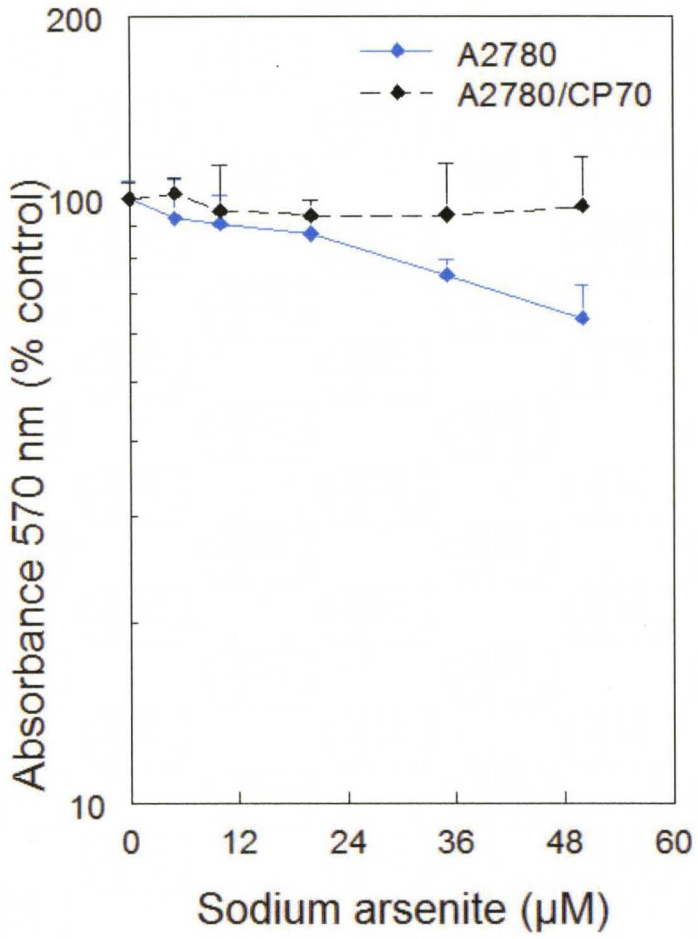

Figure 2. Cell viability in response to sodium arsenite. A2780 and A2780/CP70 cells were treated with the indicated concentrations of sodium arsenite and incubated at 37 °C for 1 h. Cells were then washed twice with PBS and refed with fresh media and incubated at 37 °C. Cell viability MTT assay was performed five days later. Data are expressed as percentage of untreated control and plotted as means ± SD of triplicate independent experiments each performed with triplicate wells.

Co-treatment of cells with cisplatin plus 20 μM sodium arsenite or hyperthermia moderately enhanced cisplatin cytotoxicity in cells expressing wild-type p53 (Figure 3A). However, combined sodium arsenite and hyperthermia greatly potentiated cisplatin cytotoxicity in wild-type p53 expressing cells. In contrast, only hyperthermia sensitized p53-null and mutated cells to cisplatin (Figure 3B), combining arsenite with hyperthermia did not further increase cisplatin sensitivity in cells lacking functional p53 (Figure 3B).

Figure 3

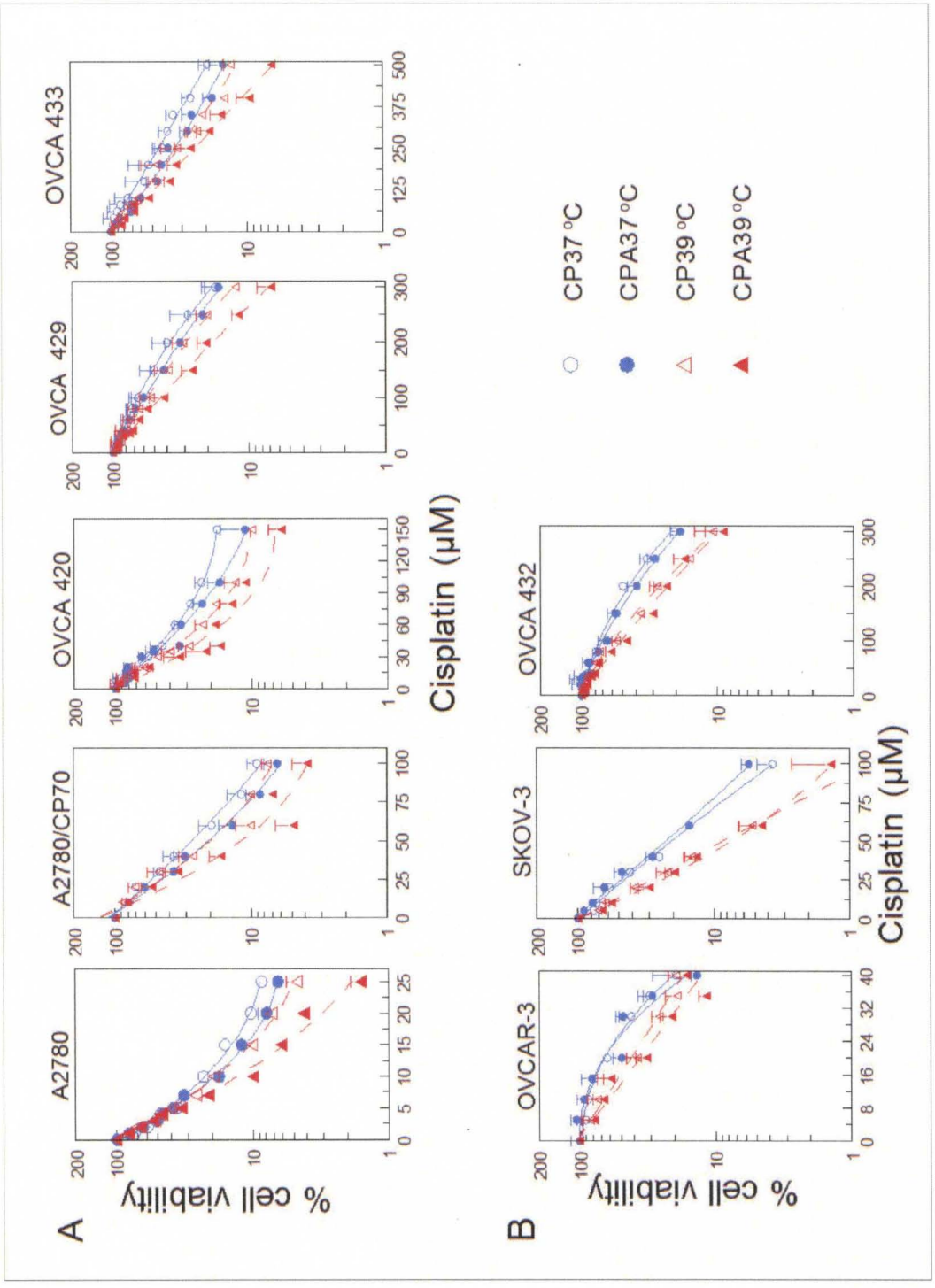

Figure 3. Cell viability as determined by MTT assay. **A.** Wild-type p53 expressing cells (A2780, A2780/CP70, OVCA 420, OVCA 429 and OVCA 433)

as indicated. **B.** P53-null (SKOV-3) and mutated (OVCAR-3 and OVCA-432) cells. Cells were co-treated with the indicated concentrations of cisplatin with (filled symbols) or without (open symbols) 20 μM sodium arsenite and incubated at 37 (circles) or 39 °C (triangles) for 1 h. Cells were then washed twice with PBS and refed with fresh media and incubated for 5 days at 37 °C. Cell viability MTT assay was performed on day 5. Data are expressed as percentage of untreated control and plotted as means ± SD of triplicate independent experiments each performed with triplicate wells. R-values for the best fitting polynomial curves were all >0.99.

Sodium arsenite requires functional p53 to sensitize cells to cisplatin

Data in Figure 3 indicate that sodium arsenite sensitizes only wild-type p53 expressing cells to cisplatin. To test whether sodium arsenite requires functional p53 to sensitize cells to cisplatin, I transfected A2780 and A2780/CP70 cells with p53 smart pool siRNA, non-targeting control (NSC) or universal buffer (mock). Western blot data confirmed suppression of p53 at 24 after transfection for A2780 cells (Figure 4A) and at 24 and 48 h for A2780/CP70 cells (Figure 4C). MTT cell viability data indicate that suppression of p53 abrogates sodium arsenite sensitization effect (Figures 4B and 4D). Moderate arsenite sensitization effect on cisplatin was observed in cells transfected with NSC and mock (Figures 4B and 4D). These data indicate that sodium arsenite requires functional p53 to sensitize cells to cisplatin.

Figure 4

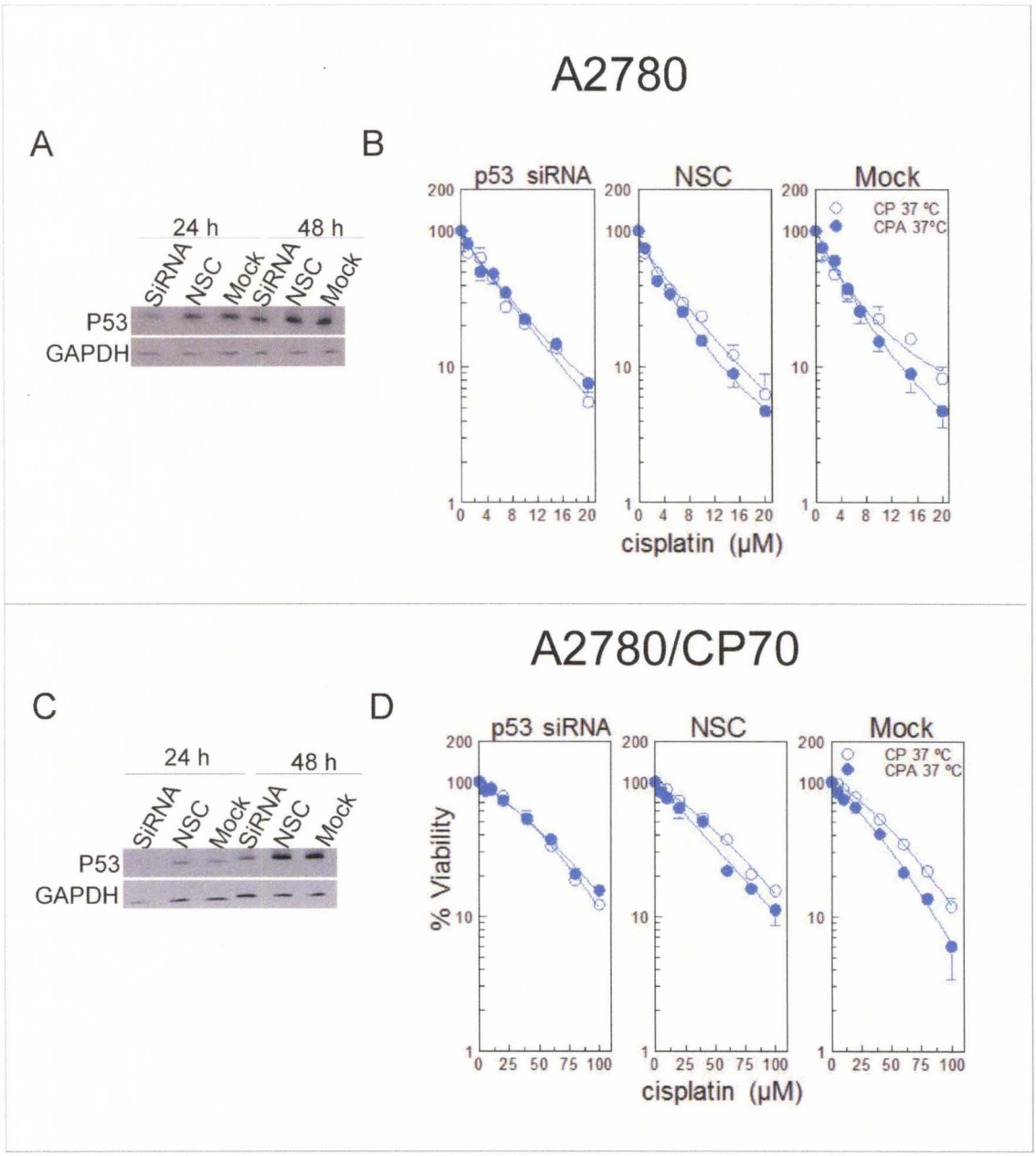

Figure 4. Cell viability and western blot analysis of cells transfected with p53 siRNA. A. Western blot analyses of A2780 cells transfected with p53 siRNA, NSC or mock. **B.** MTT cell viability assay of A2780 cells transfected with p53 siRNA, NSC or mock. **C.** Western blot analyses of A2780/CP70 cells transfected with p53 siRNA, NSC or mock. **D.** MTT cell viability assay of A2780/CP70 cells

transfected with p53 siRNA, NSC or mock. A2780 and A2780/CP70 cells were transfected with p53 smart pool siRNA, non-targeting control (NSC) or mock. At 23 h after transfection, cells were co-treated with the indicated concentrations of cisplatin with (filled symbols) or without (open symbols) 20 μM sodium arsenite for 1h at 37 °C. Protein lysates for western blot analyses were prepared at 24 and 48 h after transfection. Data in panels A and C are representative of 3 independent experiments. Data in panels B and D are means ± SD from 3 independent experiments.

Induction of XPC is p53-dependent

Cisplatin causes DNA damage that is repaired by the nucleotide excision repair (NER) system. P53 regulates the NER pathway by transcriptionally activating XPC and DDB2, DNA damage recognition proteins in the global genome repair – NER sub-pathway (Ford, 2005). Thus, the p53 dependence of arsenite sensitization may be related to the role of p53 in DNA repair. Post-translational modification of p53 is required for its stabilization and activity. P53 phosphorylated on ser392 (p53Ser392P) activates the DNA binding function of p53 by stabilizing tetramer formation and p53 phosphorylated on ser15 (p53Ser15P) stabilizes p53 (Boehme and Blattner, 2009). Therefore, I determine the expression of p53Ser15P and p53Ser392P and also DDB2 and XPC expression in A2780, A2780/CP70, OVCA 420 and SKOV-3 cells 24 h after cisplatin treatment (Figure 5). P53 and p53Ser15P induction occurred in a concentration-dependent manner in the wild-type p53 expressing A2780 and A2780/CP70 cells (Figure 5A and 5B). DDB2 induction in response to cisplatin treatment was robust in the wild-type p53 expressing cells but modest in p53-null SKOV-3 cells (Figure 5A and B). Basal XPC expression was observed in both wild-type p53 expressing and p53 null cells (Figures 5A, 5B and 5C) . However, induction of XPC occurred only in wild-type p53 expressing A2780, A2780/CP70 and OVCA 420 cells (Figure 5A, 5B and 5C). These results suggest that p53 and its phosphorylation on Ser15 (p53Ser15P) mediate XPC induction. Robust DDB2 induction in A2780 and A2780/CP70 cells is also regulated by p53 and p53Ser15P. P53Ser392P was stabilized in OVCA 420 cells (Figure 5C).

Figure 5

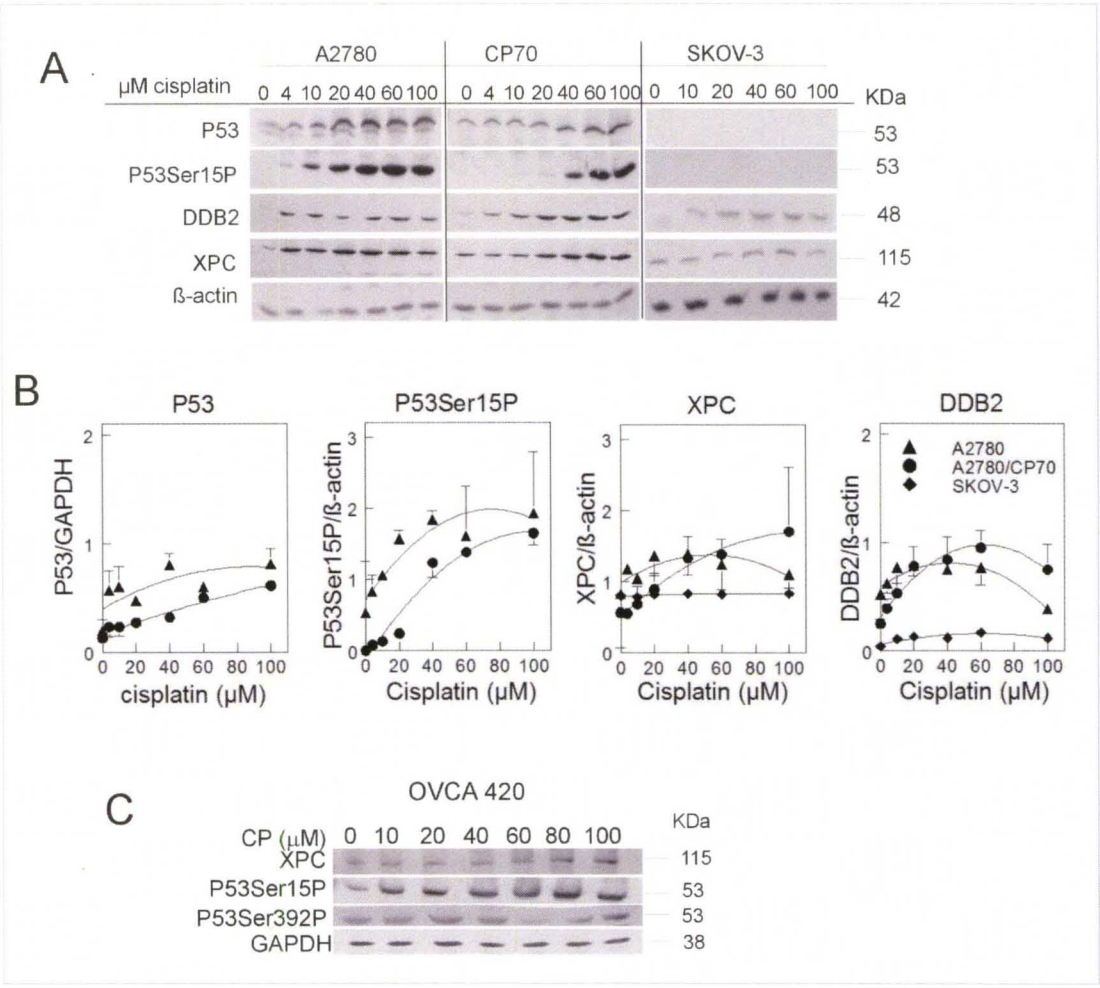

Figure 5. Expression of DDB2, p53, p53Ser15P, p53Ser392P and XPC in OC cells after cisplatin treatment. **A.** Western blot determination of DDB2, p53, p53Ser15P and XPC in A2780, A2780/CP70 and SKOV-3 cells. **B.** Densitometry analysis of DDB2, p53, p53Ser15P and XPC for A2780 and A2780/CP70 cells; DDB2 and XPC for SKOV-3 cells. **C.** Western blot determination of p53Ser15P, p53Ser392P and XPC in OVCA 420 cells. Cells were treated with the indicated concentrations of cisplatin (CP) at 37 °C for 1 h. After treatment, cells were washed twice with PBS and incubated in drug-free media for 24 h and protein lysates were then prepared. Blots were probed for DDB2, p53, p53Ser15P,

p53Ser392P and XPC as described in methods. ß-actin is the loading control. Blots are representative of 3 independent experiments.

Sodium arsenite ± hyperthermia sensitizes wild-type p53 expressing cells to cisplatin by suppressing XPC

Data in Figure 5 suggest that induction of XPC occurs only in wild-type p53 expressing cells. Therefore I predicted that sodium arsenite and/or hyperthermia sensitize wild-type p53 expressing cells to cisplatin by suppressing XPC. Cells were treated for 1 h with their respective IC50 cisplatin (A2780 = 4 μM and A2780/CP70 = 40 μM (Behrens, et al., 1987), SKOV-3 = 20 μM and OVCA 420 = 45 μM) ± 20 μM sodium arsenite at 37 or 39 °C. P53Ser15P and XPC were induced in a time-dependent manner in A2780, A2780/CP70 and OVCA 420 cells 24 h after cisplatin treatment (Figure 6A, B and C, CP 37 °C). P53Ser392P was induced in a time-dependent manner in OVCA 420 cells (Figure 6C, CP 37 °C). Co-treatment with sodium arsenite suppressed cisplatin-induced XPC in A2780, A2780/CP70 and OVCA 420 cells (Figure 6A, B and C, CPA 37 °C). Hyperthermia co-treatment did not alter cisplatin-induced XPC in A2780 and A2780/CP70 cells (Figure 6A and B, CP 39 °C), but it suppressed XPC in OVCA 420 cells (Figure 6C, CP 39 °C). Combined treatment with sodium arsenite and hyperthermia suppressed cisplatin-induced XPC in A2780, A2780/CP70 and OVCA 420 cells (Figure 6A, B and C, CPA 39 °C). Sodium arsenite and hyperthermia did not suppress p53Ser15P. XPC was not induced by cisplatin over time in p53-null SKOV-3 cells and co-treatment with sodium arsenite ± hyperthermia did not suppress XPC in these cells (Figure 6D).

Figure 6

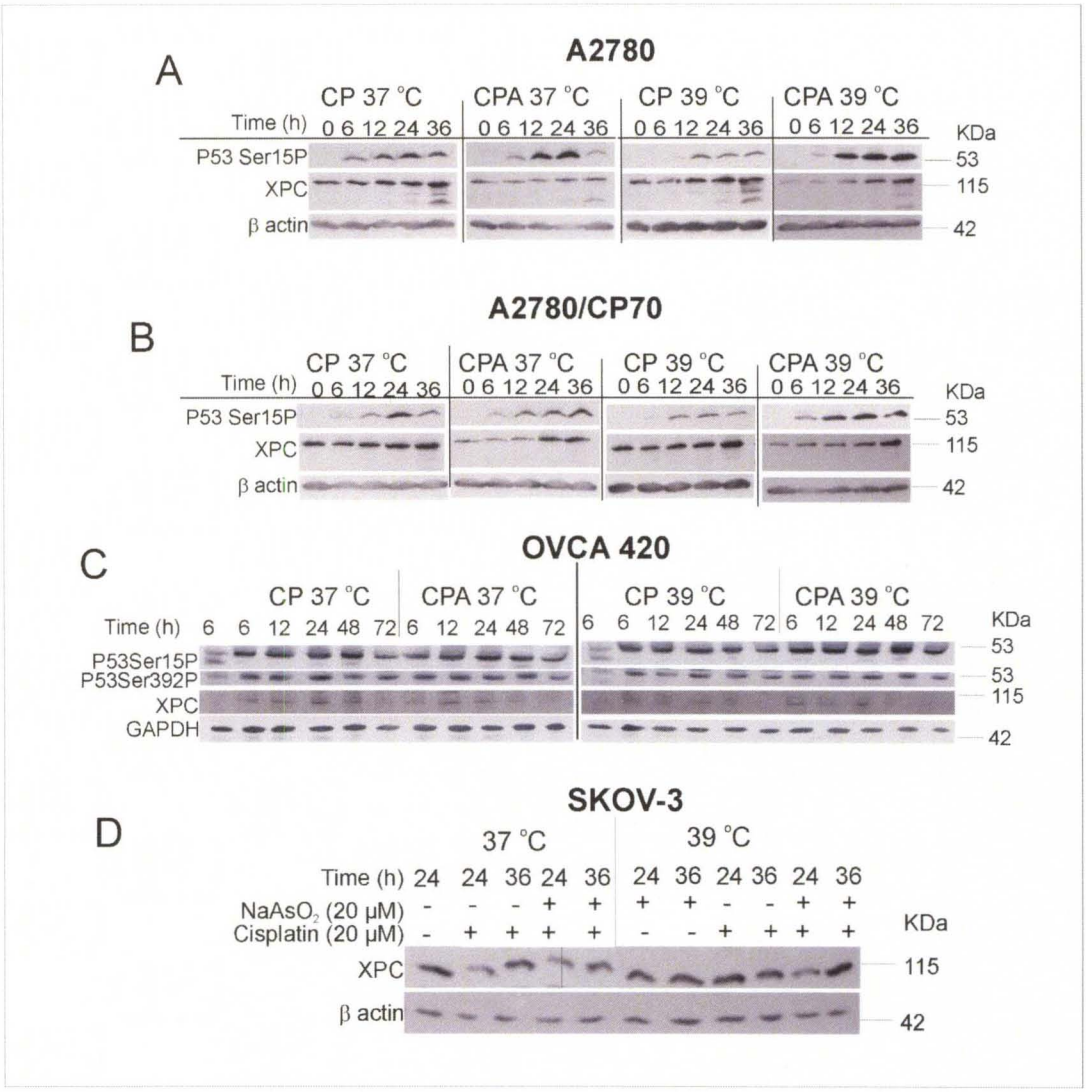

Figure 6. Effect of sodium arsenite and hyperthermia on XPC. **A, B, C** and **D** Western blot analyses of XPC, p53Ser15P and p53Ser392P. Cells were treated with their IC50 cisplatin (CP; A2780, 4 μM; A2780/CP70, 40 μM; SKOV-3, 20 μM and OVCA 420, 45 μM), or CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C for 1 h. After treatment, cells were washed with PBS and incubated in drug-free media. Protein lysates were prepared at the indicated time points for Western

Blot analysis. ß-actin and GAPDH are loading controls. Data are representative blots for 3 independent experiments.

XPC siRNA sensitizes OC cells to cisplatin

Data in Figure 6 suggest that sodium arsenite suppressed XPC in wild-type p53 expressing cells. In order to better understand the significance of XPC suppression, I transfected A2780, A2780/CP70 and SKOV-3 cells with XPC smart pool siRNA, NSC or mock. Suppression of XPC was confirmed by western blot (Figure 7A). Suppression of XPC moderately sensitized A2780 and A2780/CP70 cells to cisplatin but had no effect on SKOV-3 cells (Figure 7B). These data suggest that suppression of XPC by sodium arsenite ± hyperthermia is the mechanism of sensitizing wild-type p53 expressing OC cells to cisplatin.

Figure 7

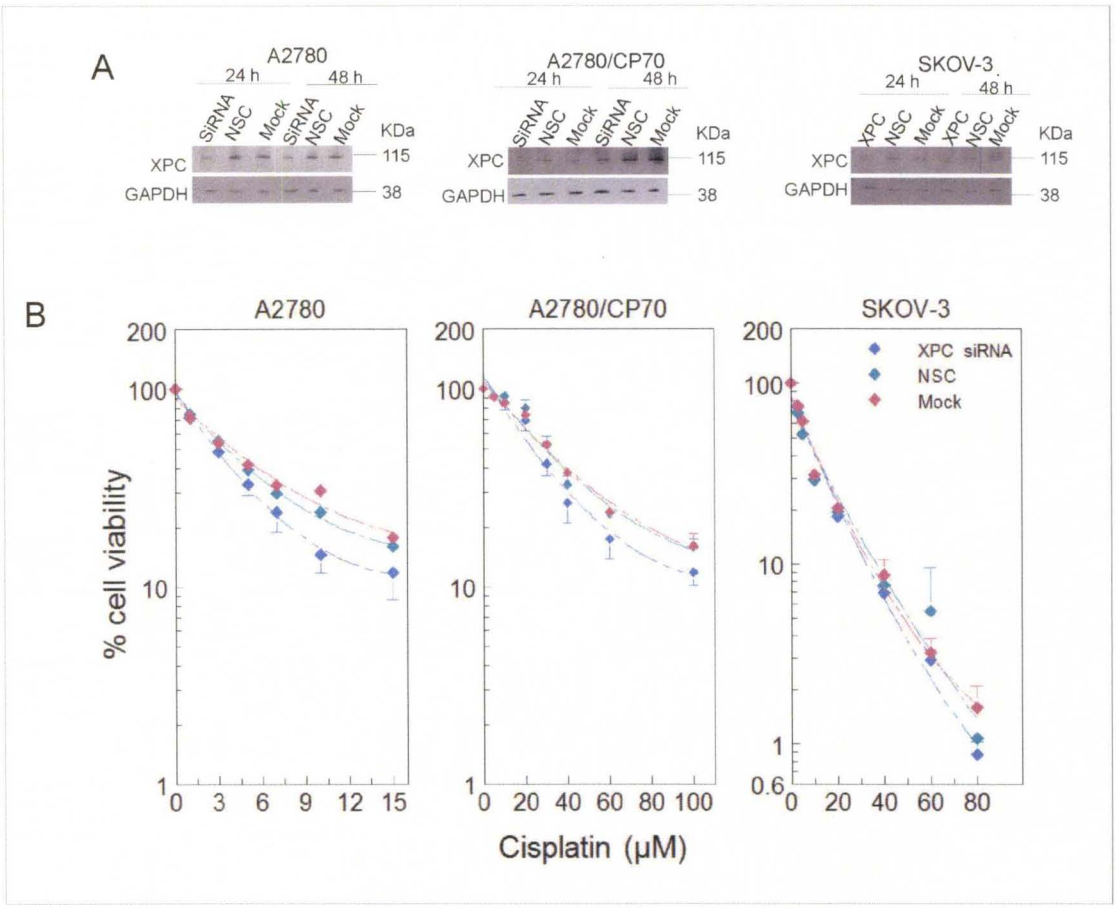

Figure 7. Effect of XPC siRNA on cisplatin cytotoxicity. **A.** Western blot analysis of XPC after siRNA transfection. **B.** MTT cell viability assay of A2780, A2780/CP70 and SKOV-3 cells after siRNA transfection. Cells were transfected with XPC smart pool siRNA, NSC or mock. At 23 h after transfection, cells were treated with the indicated concentrations of cisplatin (D) or respective IC50 cisplatin (CP; A2780, 4 μM; A2780/CP70, 40 μM; SKOV-3, 20 μM). After treatment, cells were washed twice with PBS and incubated in drug-free media. MTT assay was performed 5 days after treatment. Protein lysates for western blot analyses were prepared at 24 and 48 h after transfection. Blots are

representative for 3 independent experiments. Data are means ± SD from 3 independent experiments.

Effect of cisplatin on ERCC1, XPA and MSH2

Western blot analysis of downstream NER proteins ERCC1 and XPA and the mismatch repair protein MSH2 revealed that cisplatin did not induce these proteins in A2780, A2780/CP70 and OVCA 420 cells (Figure 8A, B and C). However, cisplatin-resistant A2780/CP70 cells expressed higher levels of XPA and low levels of MSH2 compared to cisplatin sensitive A2780 cells (Figure 8A). ERCC1 levels were similar in A2780 and A2780/CP70 cells (Figure 8A). ERCC1 and MSH2 were induced in SKOV-3 cells (Figure 8C).

Figure 8

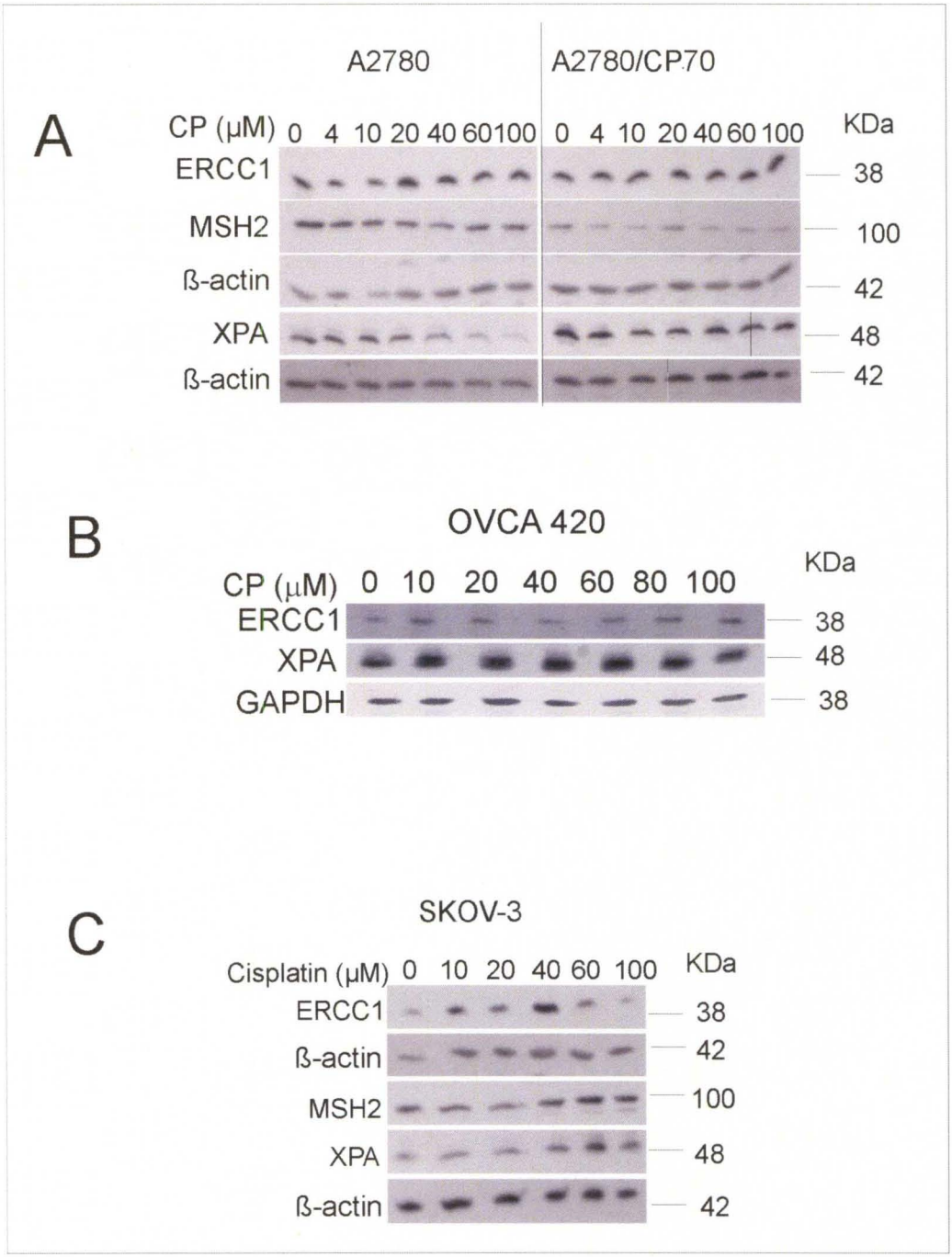

Figure 8. Expression of ERCC1, MSH2 and XPA in OC cells after cisplatin treatment. Cells were treated with the indicated concentrations of cisplatin (CP) at 37 °C for 1 h. After treatment, cells were washed twice with PBS and

incubated in drug-free media for 24 h and protein lysates were then prepared. Blots were probed for ERCC1, MSH2 and XPA as described in methods. Blots are representative of 2 independent experiments. ß-actin and GAPDH are loading controls.

Sodium arsenite and/or hyperthermia alter ERCC1, XPA and MSH2 expression

Data in Figure 8A indicate that cisplatin resistance in A2780/CP70 cells is associated with high levels of XPA and low levels of MSH2. Also, high levels of XPA were observed in OVCA 420 cells (Figure 8B). ERCC1 was induced in SKOV-3 cells in response to cisplatin treatment (Figure 8C). Therefore, I performed western blot analysis to determine if sodium arsenite and/or hyperthermia altered the expression of ERCC1, XPA and MSH2. The data suggest that sodium arsenite and/or hyperthermia did not suppress ERCC1 or XPA in A2780, A2780/CP70 and SKOV-3 cells (Figure 9A, B and C). MSH2 levels were suppressed by cisplatin ± sodium arsenite at 37 or 39 °C in A2780/CP70 cells (Figure 9B), suggesting reduced mismatch repair. Reduced mismatch repair is associated with increased cisplatin resistance (Fink, et al., 1997). Co-treatment with sodium arsenite induced higher levels of ERCC1 in OVCA 420 cells (Figure 9D, CPA37). ERCC1 and XPA levels decreased over time in OVCA 420 cells (Figure 9D). XPA was completely suppressed by sodium arsenite plus hyperthermia co-treatment with cisplatin in OVCA 420 cells (Figure 9D, CPA39).

Figure 9

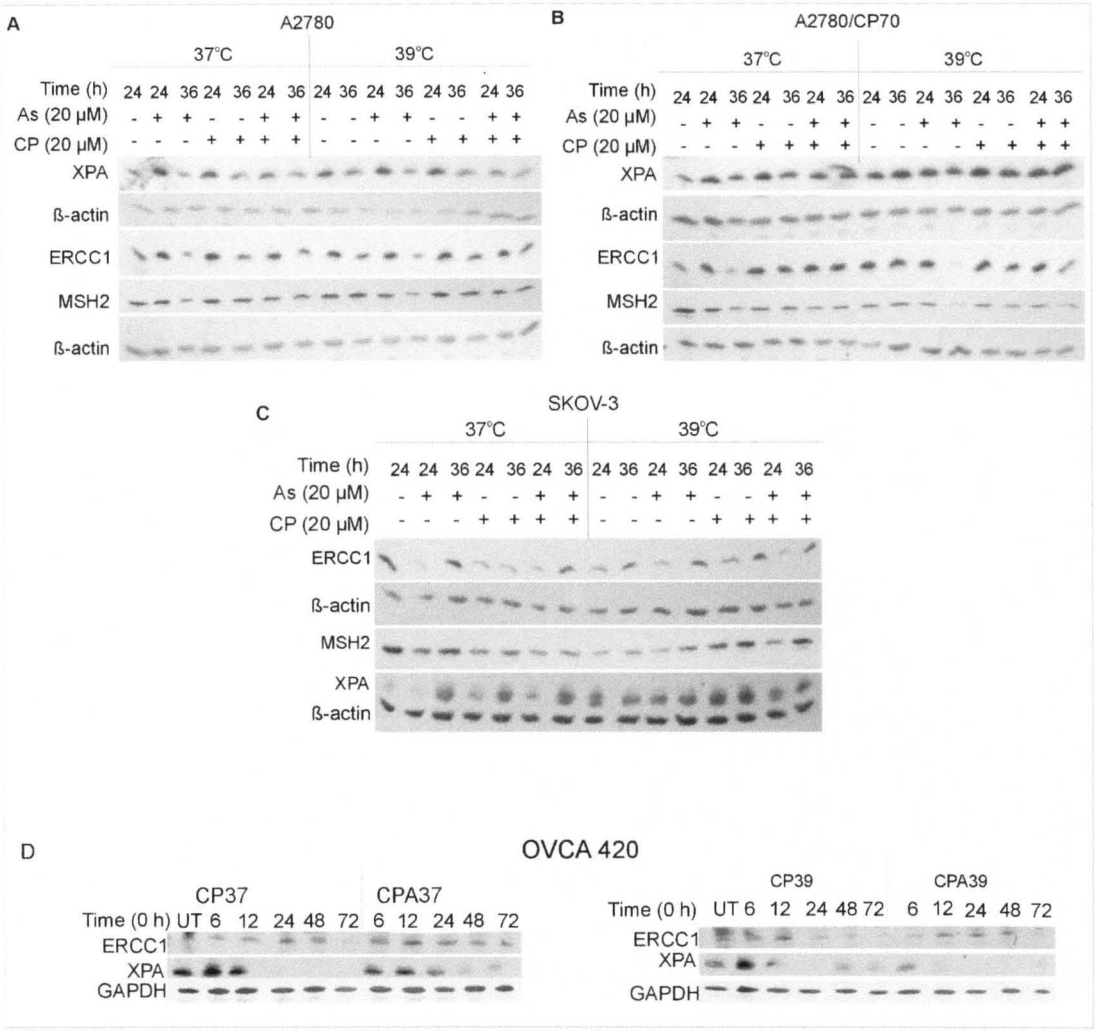

Figure 9. Effect of cisplatin, sodium arsenite and hyperthermia on ERCC1, XPA and MSH2. Cells were treated with their IC50 cisplatin (CP) (A2780, 4 µM; A2780/CP70, 40 µM; SKOV-3, 20 µM; OVCA 420, 45 µM), or CP plus 20 µM sodium arsenite (CPA) at 37 or 39 °C for 1 h. After treatment, cells were washed with PBS and incubated in drug-free media. Cell lysates were prepared at the indicated time points for Western Blot analyses. UT is untreated control. ß-actin and GAPDH are loading controls. Data are representative blots from 2 independent experiments.

Hyperthermia ± sodium arsenite enhance cellular accumulation of cisplatin

Decreased cellular accumulation is an important mechanism of cisplatin resistance (Parker, et al., 1991; Cepeda, et al., 2007). I used inductively coupled plasma mass spectrometry (ICP-MS) to determine if differential cellular platinum (platinum) accumulation contributes to resistance and also to determine if sodium arsenite and hyperthermia alter platinum accumulation (Figure 10). The concentration response of platinum accumulation (Figure 10A) in the p53 (+) cisplatin-sensitive cell line (A2780) had a linear correlation of $Y = 0.87X + 0.95$, $R = 0.99$. In the resistant cell line (A2780/CP70) the equation was $Y = 0.38X + 0.55$, $R = 0.99$. P53-null SKOV-3 cells had a linear correlation of $Y = 0.75X + 0.11$, $R = 0.99$. A2780 and SKOV-3 cells accumulated similar levels of platinum and ~2-fold more platinum than A2780/CP70 cells. Hyperthermia alone or combined with sodium arsenite enhanced platinum accumulation in A2780 cells (Figure 10B, left panel). Co-treatment with sodium arsenite and hyperthermia significantly enhanced cellular platinum accumulation in A2780/CP70 cells (Figure 10B center panel). In contrast, only hyperthermia enhanced cellular accumulation of platinum in SKOV-3 cells (Figure 10B, right panel).

Figure 10

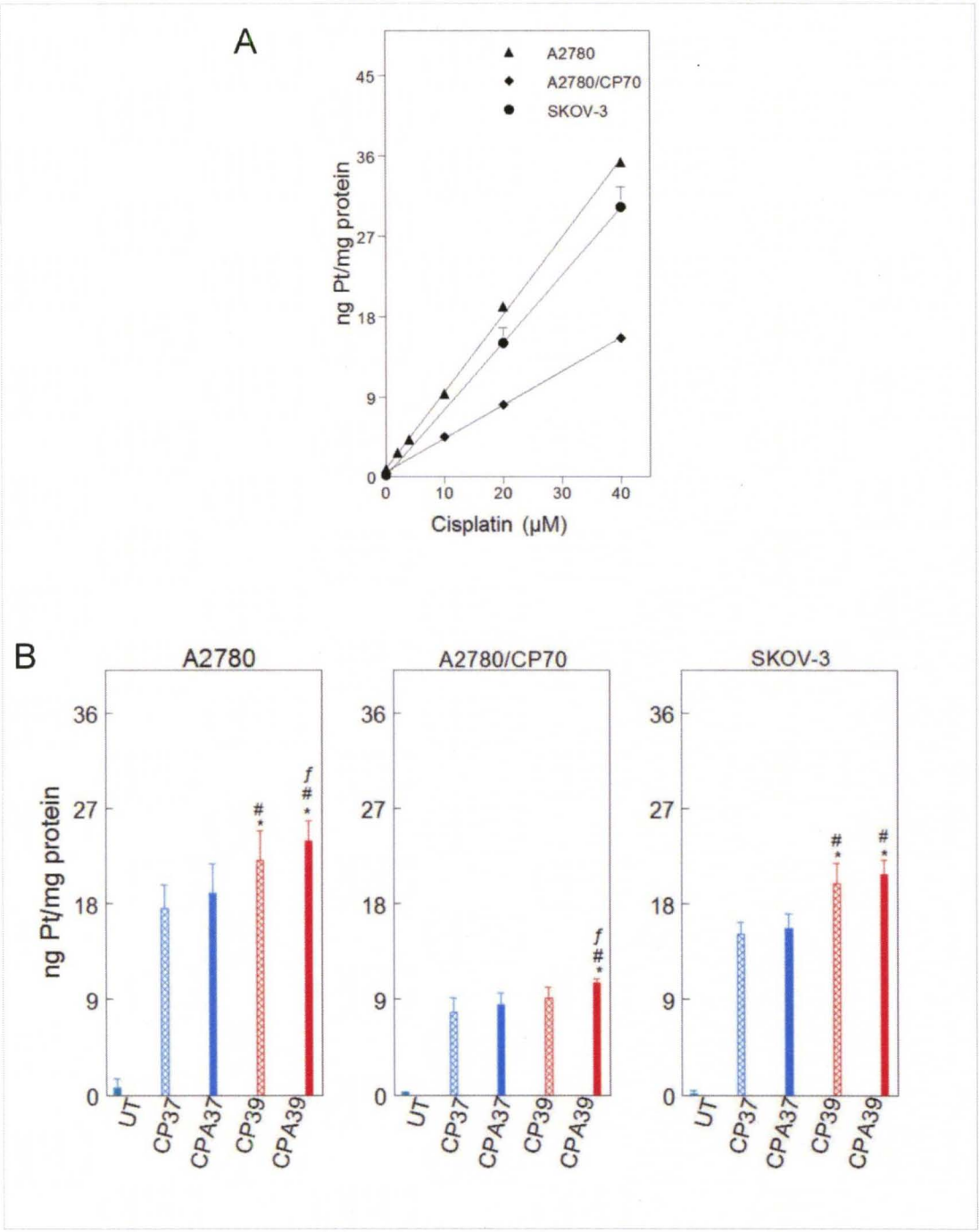

Figure 10. ICP- MS analysis of cellular platinum accumulation and effect of sodium arsenite and hyperthermia. A. Concentration-dependent accumulation of platinum in cells. **B.** Effect of sodium arsenite and hyperthermia on cellular

platinum accumulation. A2780, A2780/CP70 and SKOV-3 cells were treated with the indicated cisplatin concentrations for 1 h at 37 °C (**A**) or with 40 μM cisplatin with (CPA 37 or CPA 39) or without 20 μM sodium arsenite (CP 37 or CP 39) at 37 or 39 °C for 1 h (**B**). Cells were harvested immediately after treatment for total cellular platinum determination. Data are means ± SD from 3 independent experiments. $P<0.05$. * compared to CP37, # compared to CPA37 and *f* compared to CP39.

Enhanced cellular accumulation of platinum by hyperthermia and sodium arsenite is not associated with decreased glutathione-S-transferase acitivity

Increased glutathionylation and export of platinum-GSH and arsenic-GSH conjugates by the multidrug resistance proteins is a mechanism of resistance to cisplatin and arsenic respectively (Leslie, et al., 2004; Stewart, 2007). Glutathione (GSH) conjugation is mediated by glutathione-S-transferase (GST). I hypothesized that co-treatment with sodium arsenite, hyperthermia and cisplatin might decrease GST activity and enable more platinum to accumulate in the cells. I determined total GST activity as described in Materials and Methods section. GST activity for all treatment conditions was similar to that of untreated control (UT) in A2780 and A2780/CP70 cells (Figure 11, left and center panels). GST activity significantly increased in SKOV-3 cells co-treated with cisplatin, sodium arsenite and hyperthermia (Figure 11, right panel, CPA 39 °C). These data indicate that decreased GST activity is not a mechanism of enhancement of cellular platinum accumulation.

Figure 11

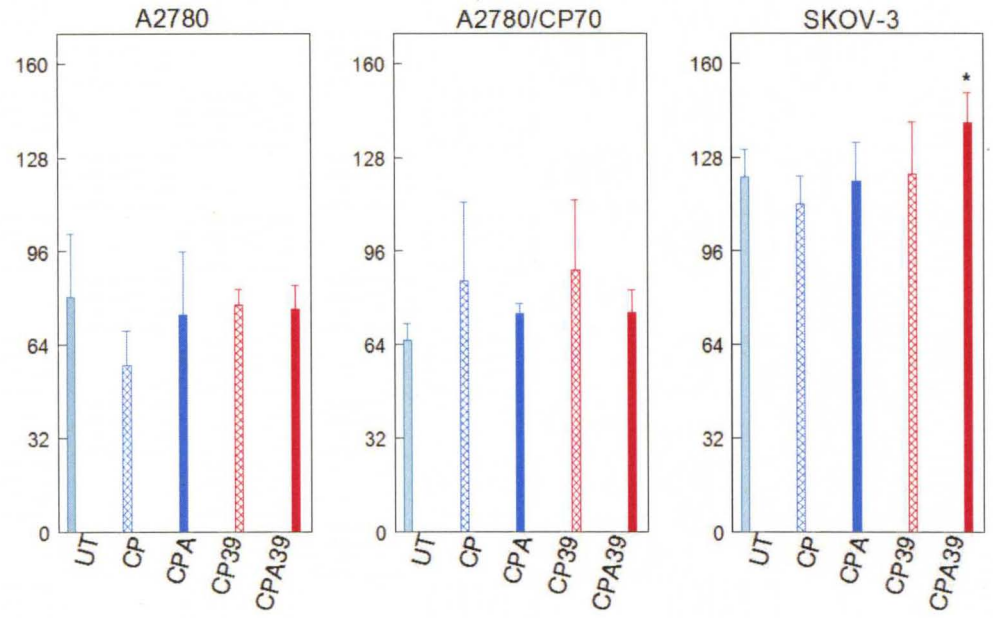

Figure 11. Total GST activity determination. A2780, A2780/CP70 and SKOV-3 cells were treated with 40 μM cisplatin (CP) or CP plus 20 μM sodium arsenite (CPA) at 37 °C (CP or CPA) or 39 °C (CP39 or CPA39) for 1 h. Cells were harvested immediately after treatment for total GST activity determination. Data are means ± SD from triplicate biological experiments. $P<0.05$. * compared to untreated cells (UT).

Hyperthermia ± sodium arsenite enhances accumulation of platinum on DNA

The cytotoxicity of cisplatin is known to depend on its direct interaction with DNA to form bulky platinum-DNA adducts. I tested the hypothesis that sodium arsenite and hyperthermia sensitize cells to cisplatin by increasing platinum (platinum) binding to DNA. Using ICP-MS, I determined platinum bound to DNA immediately after exposure (0 h) to measure initial platinum binding to DNA and DNA bound platinum remaining 24 h after treatment to determine repair of platinum-DNA adducts. I observed that hyperthermia alone or combined with sodium arsenite significantly increased platinum binding to DNA in A2780 and A2780/CP70 cells (Figure 12, left and center panels). Retention of platinum on DNA in A2780 cells was favored by sodium arsenite, hyperthermia or the combination. In A2780/CP70 cells retention was favored by hyperthermia only. In SKOV-3 cells, only hyperthermia favored initial binding (0 h) and retention (24 h) of platinum on DNA (Figure 12, right panel). These data suggest that in the wild-type p53 expressing cells, platinum binding to DNA was favored by both sodium arsenite and hyperthermia, whereas, in p53-null cells only hyperthermia favored platinum binding to DNA.

Figure 12

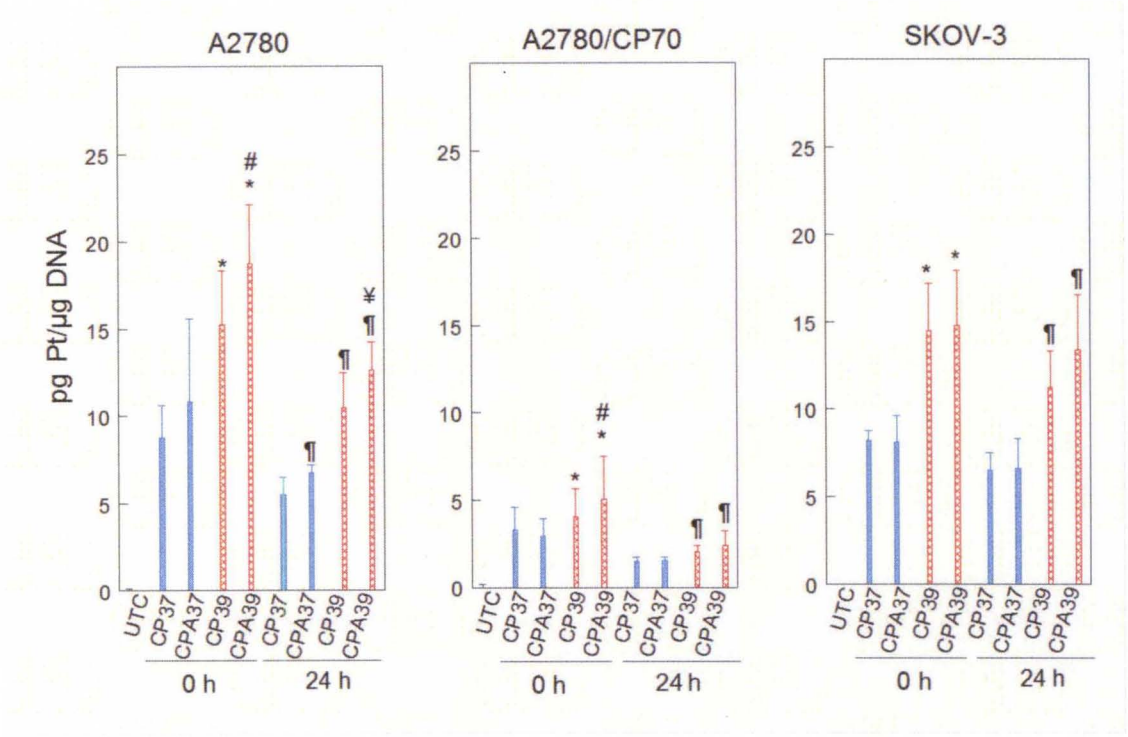

Figure 12. Determination of sodium arsenite and hyperthermia effect on DNA-bound platinum. A2780, A2780/CP70 and SKOV-3 cells were treated with 40 µM cisplatin for 1 h at 37 or 39 °C. Total genomic DNA was isolated at 0 (immediately) and 24 h after treatment for platinum on DNA determination. Data are means ± SD from 3 independent experiments. $P<0.05$, ∗ compared to CP37 0 h, # compared to CP39 0 h, ¶ compared to CP37 24 h and ¥ compared to CP39 24 h.

Data in Figure 12 were used to determine platinum-DNA repair as follows:

$$\text{Percentage platinum-DNA repair} = \frac{100 \times (\text{pg Pt/}\mu\text{g DNA at 0 h}) - (\text{pg Pt/}\mu\text{g DNA at 24 h})}{(\text{pg Pt/}\mu\text{g DNA at 0 h})}$$

A2780/CP70 cells repaired platinum-DNA damage better than A2780 and SKOV-3 cells (Figure 13). However, sodium arsenite and hyperthermia did not alter overall platinum-DNA repair at 24 h after treatment.

FIGURE 13

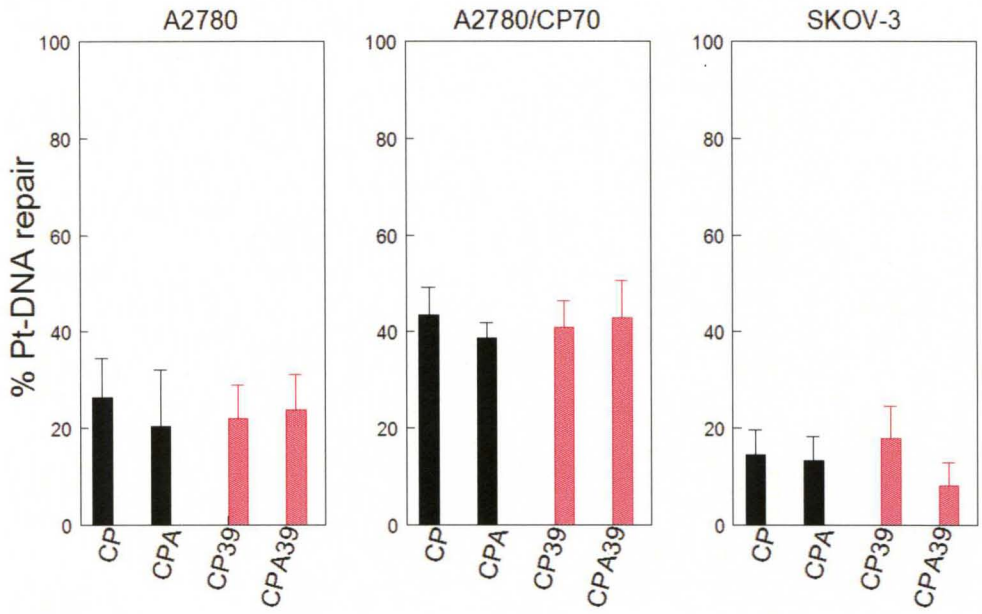

Figure 13. **Determination of platinum-DNA repair.** A2780, A2780/CP70 and SKOV-3 cells were treated with 40 μM cisplatin for 1 h at 37 or 39 °C. Total genomic DNA was isolated at 0 (immediately) and 24 h after treatment for platinum on DNA determination. Percentage of platinum-DNA repair was calculated using platinum bound to DNA data in Figure 12. Data are means ± SD from 3 independent experiments.

DISCUSSION

The present study was aimed at determining the efficacy and mechanisms of action of a novel combination of cisplatin, sodium arsenite and hyperthermia in human OC cells with different p53 status. A panel of wild-type p53 expressing (A2780 (cisplatin-sensitive) and A2780/CP70, OVCA 420, OVCA 429, OVCA 433 (cisplatin-resistant)) and p53-null (SKOV-3) and p53-mutated (OVCA 432 and OVCAR-3) human OC cells were used for this study. I showed for the first time that combining sodium arsenite and hyperthermia sensitizes wild-type p53 expressing cells to cisplatin (Figure 3A). The combination of all three drugs is more effective than the individual combination. P53-null and p53-mutated cells were sensitized only by hyperthermia to cisplatin (Figure 3B). Suppression of p53 abrogated sodium arsenite sensitization to cisplatin effect (Figure 4), indicating that the arsenite effect was p53-dependent.

The mechanism of cisplatin cytotoxicity involves DNA damage formation. However, resistance to cisplatin is a major problem. Mechanisms of cisplatin resistance are multifactorial and include reduced cellular drug accumulation, enhanced drug metabolism by glutathionylation, export by multidrug resistance proteins, enhanced DNA damage tolerance and enhanced DNA repair (Stewart, et al., 2007). Enhanced cisplatin-DNA repair by the nucleotide excision repair pathway (NER) confers resistance to cisplatin (Martin, et al., 2008). P53 regulates NER by transcriptionally regulating XPC and DDB2, critical DNA damage recognition proteins in global genome repair, a sub-pathway of NER (GGR-NER) (Ford, 2005). XPC is actively involved in cisplatin-induced DNA

damage recognition (Neher, et al., 2010). Basal expression of XPC was observed in both wild-type p53 and p53-null cells, consistent with a report that, in addition to p53, other proteins are involved in regulating basal XPC levels (Lin, et al., 2009). However, cisplatin-dependent induction of XPC occurred only in wild-type p53-expressing cells but not in p53-null cells (Figure 4), indicating that cisplatin-induction of XPC is regulated by p53 in these cells. Co-treatment with sodium arsenite alone or combined with hyperthermia attenuated cisplatin-induced XPC in the wild-type p53 cells (Figure 6A, B and C, CPA 37 °C and CPA 39 °C). Suppression of XPC by sodium arsenite ± hyperthermia is a mechanism of sensitizing wild-type p53 expressing cells to cisplatin because XPC siRNA moderately sensitizes A2780 and A2780/CP70 cells to cisplatin (Figure 7). Given that XPC is critical to platinum-DNA damage recognition in GGR-NER, suppression of XPC could diminish the assembly of the downstream NER repair complex (Nollen, et al., 2009) and subsequently decrease DNA repair. Arsenic has been shown to inhibit p53 DNA binding activity by suppressing p53S392P via inhibition of CK II (Tang, et al., 2006). My goal was to investigate whether p53S392P is required for the transcriptional activation of XPC by p53 and if arsenic suppression of XPC is via inhibition of p53S392P. However, arsenite did not inhibit p53Ser392P in OVCA 420 cells, suggesting that arsenite is not acting by inhibiting CK2.

Decreased cellular accumulation is an important mechanism of cisplatin-resistance (Stewart, et al., 2007). It has been shown previously using atomic absorption spectroscopy that A2780 cells accumulate ~2-fold more cellular

platinum than A2780/CP70 cells (Parker, et al., 1991). Here, I used a more sensitive analytical technique (ICP-MS) and showed that A2780 cells accumulate ~2.3 fold more platinum than A2780/CP70 cells (Figure 10A), consistent with the earlier platinum measurements. Cisplatin-resistance in SKOV-3 cells has been linked partially to decreased cellular platinum accumulation (Mistry, et al., 1992). However, a comparative cellular cisplatin accumulation study by Johnson et al indicated that A2780 and SKOV-3 cells accumulated similar levels of platinum during 4 h incubation with equimolar concentrations of cisplatin (Johnson, et al., 1997). In the current study, I showed that A2780 and SKOV-3 cells accumulated similar level of cellular platinum when treated with 40 μM cisplatin; similar to previous findings by Johnson et al. Co-treatment of A2780 cells with hyperthermia ± sodium arsenite significantly enhanced cellular accumulation of platinum. Combined sodium arsenite and hyperthermia significantly enhanced platinum accumulation in A2780/CP70 cells (Figure 10B). Consistent with my findings that only hyperthermia sensitized SKOV-3 cells to cisplatin, I also observed that only hyperthermia increased cellular platinum accumulation in these cells (Figures 10B). Therefore, increasing cellular platinum accumulation is a mechanism by which sodium arsenite and hyperthermia are sensitizing wild-type p53 expressing cells to cisplatin. Enhanced platinum accumulation is not associated with decreased GST activity (Figure 11).

Reduced platinum bound to DNA contributes to cisplatin resistance (Parker, et al., 1991). When A2780 and A2780/CP70 cells were treated with 40 μM cisplatin, wild-type p53 A2780 (cisplatin-sensitive) and p53-null SKOV-3

(cisplatin-resistant) cells accumulated similar amounts of platinum on DNA (Figure 12A), contrary to previous findings by Johnson et al. (Johnson, et al., 1997). The observed difference in amount of platinum bound to DNA between my study and that of Johnson et al could be due to differences in drug exposure and platinum determination times. Johnson et al treated cells for 4 h and assayed for platinum at 0 (immediately after treatment) and 8 h after treatment, whereas, I did a 1 h exposure and determined platinum levels at 0 and 24 h after treatment. My data suggest that cisplatin-resistance in SKOV-3 cells is not due to decreased platinum bound to DNA. However, cisplatin-resistance in wild-type p53 A2780/CP70 cells is due to decreased platinum bound to DNA, consistent with previous findings (Parker, et al., 1991). Hyperthermia alone or combined with sodium arsenite significantly increased initial (0 h) platinum bound to DNA in both A2780 and A2780/CP70 cells (Figure 12B). Meanwhile, platinum retention (24 h) on DNA was favored by sodium arsenite, hyperthermia or the combined treatment in A2780 cells. Retention was favored by hyperthermia in A2780/CP70 cells (Figure 12B). Therefore, increased accumulation of platinum bound to DNA is a mechanism by which sodium arsenite and hyperthermia are sensitizing wild-type p53 cells to cisplatin. In p53-null SKOV-3 cells, only hyperthermia increased platinum bound to DNA accumulation (Figure 12B). Sodium arsenite and hyperthermia did not alter platinum-DNA damage repair (Figure 13).

In summary, I have shown for the first time that combining sodium arsenite and hyperthermia with cisplatin sensitizes wild-type p53 expressing human OC cells to cisplatin by attenuating XPC induction in response to cisplatin and

enhancing cellular and DNA bound platinum accumulation. The combination of cisplatin, sodium arsenite and hyperthermia works better than the individual combinations because each drug has a different mechanism of action. Further studies using *in vivo* cancer models are necessary to determine whether this combination therapy may improve clinical outcomes.

CHAPTER 3

SODIUM ARSENITE AND HYPERTHERMIA MODULATE CISPLATIN-DNA DAMAGE RESPONSES AND ENHANCE PLATINUM ACCUMULATION IN MURINE METASTATIC OC XENOGRAFT AFTER HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY (HIPEC)

(This chapter has been published as Muenyi et al., JOR, 2011.)

INTRODUCTION

Epithelial OC (OC) is the leading cause of gynecological cancer death in the U.S. (Jemal, et al., 2010). Most women are diagnosed only after peritoneal dissemination has occurred. The standard treatment for patients with OC is cytoreductive surgery (CRS) followed by intravenous platinum-taxane chemotherapy (Armstrong, et al., 2006). Even though initially effective, relapse from residual disease and/or drug resistant cancer reduces the 5-year survival rate to about 20% (Ozols, 2005). Despite research efforts to improve on platinum-based chemotherapy, or to develop new drugs against OC, most patients still die from metastatic disease. Since metastatic OC is usually confined in the peritoneal cavity, it makes theoretical sense to deliver chemotherapy intraperitoneally rather than intravenously since higher levels of drug can be delivered to the disease site by that route (Markman, 2001; van, V, et al., 1998). In response to three large randomized clinical trials showing benefit to incorporating intraperitoneal (IP) delivery in OC, the National Cancer Institute

issued a clinical announcement recommending that patients with small volume disease at the end of frontline surgery be offered the opportunity to receive IP chemotherapy (Trimble and Christian, 2008). Adding hyperthermia to chemotherapy agents delivered intraperitoneally (HIPEC) could improve outcome (Helm, 2009; Yang, et al., 2010; Dovern, et al., 2010).

Cisplatin is a DNA damaging chemotherapeutic used to treat solid tumors including OC. However, resistance to cisplatin limits clinical success (Stewart, et al., 2007). Since platinum-containing chemotherapy drugs remain the major weapon against OC, improving their efficacy could have a great impact on mortality. The combination of hyperthermia with cisplatin administered intraperitoneally (HIPEC) has been reported for the treatment of OC (Helm, et al., 2008). However, complete remission is not achieved with HIPEC therapy (Dovern, et al., 2010). Adding arsenic could potentially sensitize cancer cells to cisplatin and hyperthermia (Helm and States, 2009; Wang, et al., 2001; Griffin, et al., 2003). *In vivo* studies also show that arsenic inhibits the growth of orthotopic metastatic prostate cancer and peritoneal metastatic OC (Maeda, et al., 2001; Zhang and Wang, 2006). The mechanism of arsenic-induced cell death *in vitro* is suggested to include formation of oxidative DNA damage (Nakagawa, et al., 2002), activation of the Fas pathway (Kong, et al., 2005), inhibition of DNA repair (Hartwig, et al., 1997; Nollen, et al., 2009), and causation of mitotic arrest and induction of apoptosis in the mitotic cells (McNeely, et al., 2008a; Taylor, et al., 2008).

Enhanced repair of platinum-DNA damage by the nucleotide excision repair (NER) pathway (Earley and Turchi, 2010), decreased mismatch repair (MMR) (Fink, et al., 1997) and reduced accumulation contribute to cisplatin resistance (Parker, et al., 1991). Adding arsenic to cisplatin and hyperthermia could potentially decrease cisplatin resistance by decreasing NER, enhancing MMR and increasing accumulation. Arsenic and cisplatin are detoxified by glutathionylation and exported by multidrug resistant family transport pumps (Leslie, et al., 2004; Stewart, 2007), suggesting a potential for competition for the detoxification pathway if arsenic and cisplatin are used in combination. This competition might enhance cisplatin accumulation in tumors.

The goal of this study is to determine how sodium arsenite and hyperthermia modulate mechanisms of cisplatin resistance *in vivo*. We developed murine models of HIPEC treatment and metastatic human OC to investigate if sodium arsenite and hyperthermia alter the expression of DNA repair proteins, tumor platinum levels and systemic distribution of platinum. I show that sodium arsenite and hyperthermia either as single agents or in combination reverse key DNA repair proteins (XPA, XPC and MSH2) responsible for cisplatin resistance and also enhanced tumor platinum uptake suggesting decreased platinum detoxification. Sodium arsenite and hyperthermia did not alter systemic platinum distribution.

HYPOTHESIS

Sodium arsenite and hyperthermia modulate cisplatin-induced DNA damage responses and enhance platinum accumulation in metastatic tumors.

MATERIAL AND METHODS

Animals

Female NCr athymic nude mice (7 – 9 weeks old), were purchased from Taconic (Cambridge City, IN). Animals were kept in a temperature-controlled room on a 12 h light-dark schedule. The animals were maintained in cages with paper filter covers under controlled atmospheric conditions. Cages, covers, bedding, food, and water were changed and sterilized weekly. Animals were fed autoclaved animal chow diet and water. All procedures were performed under sterile conditions. This experiment was approved by the Institutional Animal Care and Use Committee of the University of Louisville in an AALAC approved facility in accordance with all regulatory guidelines.

Establishment of intraperitoneal metastatic ovarian tumors in mice

A2780/CP70 cell suspension ($1x10^6$ cells in 500 µL of serum-free RPMI 1640 media) was injected into the peritoneum of anesthetized mice using an 18-gauge needle. The needle was flushed with 500 µL physiological saline. The abdomen of injected animals was massaged to ensure even distribution of cells. By 3 – 4 weeks after injection, the mice had developed multiple small disseminated intraperitoneal tumors (1 – 7 mm) (Figure 14). Tumors were monitored by microCT scanning in the Brown Cancer Center Small Animal Imaging Facility.

Figure 14

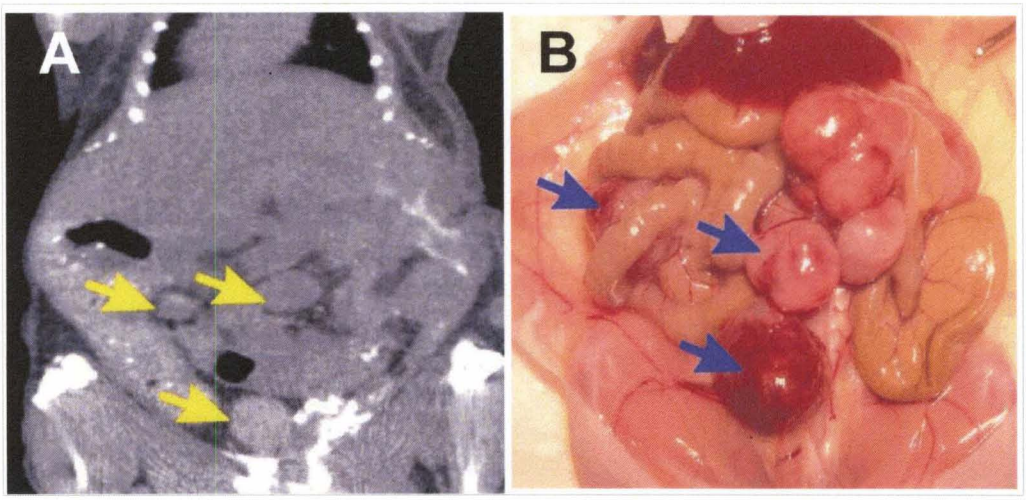

Figure 14. Mouse with multiple small intraperitoneal tumors. **A.** MicroCT scan of tumors in live mouse. **B.** Direct visualization of tumors at necropsy of mouse. Three tumors are denoted by arrow in panels **A** and **B**.

Intraperitoneal chemotherapy

Tumor-bearing mice were anesthetized with 3% isoflurane in an inhalation chamber and maintained on 1% isoflurane during surgery. Incisions (~0.5 cm) were made on both sides of the lower abdominal wall allowing entry into the peritoneal cavity (Figure 15). Inflow and outflow tubes were inserted into the peritoneal cavity and secured with skin sutures. The tubes were connected to a bag containing 100 mL normal saline with added cisplatin (3 mg/kg body weight (BW)) ± sodium arsenite (26 mg/kg BW) and cefazolin (0.01 mg/mL). (The dose of cisplatin used for this study was determined from human dose of cisplatin (100 mg/m^2) administered intravenously to a 70 kg (body surface area = 1.87 m^2) (Reagan-Shaw, et al., 2008) cancer patient and sodium arsenite dose was calculated from a single daily dose of Trisenox (0.15 mg/kg/day) administered intravenously to a 70 kg acute promyelocytic leukemia patient. The underlying assumption in the calculations is that the drugs are mixed in 2 L saline solution for HIPEC therapy in humans). The saline bag was submerged in a water bath to maintain the perfusate temperature at either 37 or 43 °C. Perfusion was performed at a rate of 3 mL/min for 60 min using a Masterflex pump (Cole-Palmer Instrument Co, Cat # 07524-50). The inflow and outflow temperatures were monitored by thermocouple probes with temperature maintained within 1°C. The core temperature of the animals was monitored using an anal temperature probe and maintained using a heating pad and heat lamp. After 60 min perfusion, most of the perfusate in the peritoneum was sucked out using sterile cotton balls with a light abdominal massage. Wounds were sutured closed and animals were

injected intraperitoneally with 1 mL physiological saline containing 0.01 mg ketoprofen for pain. Mice were kept in warm cages (single mouse/cage) and monitored for recovery and discomfort. Immediately (0 h) and 24 h after perfusion, mice were euthanized and tumors, kidneys, liver, spleen, heart and brain were dissected and snap frozen in liquid nitrogen and stored at -80 ^{o}C until use.

Figure 15

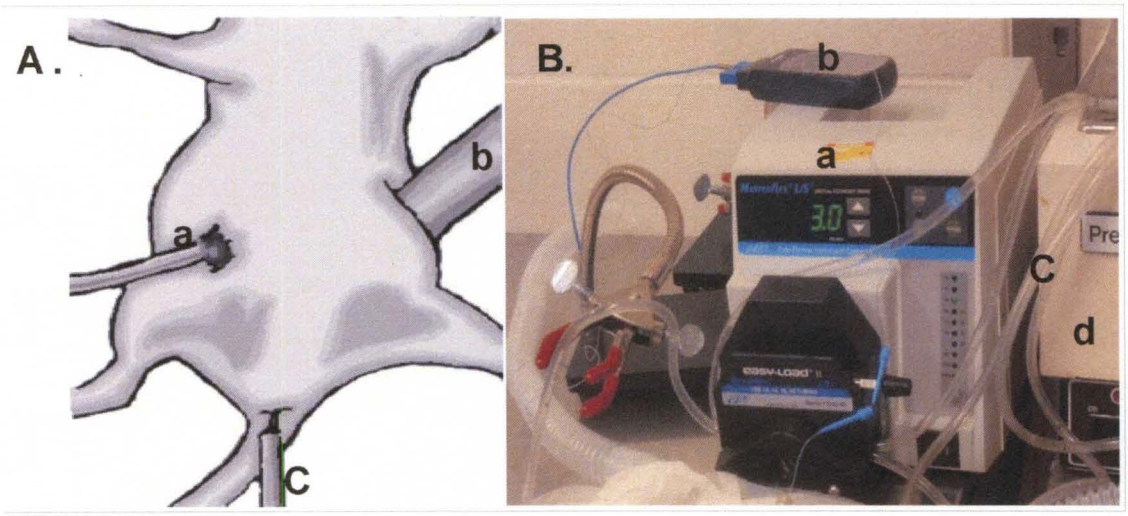

Figure 15. Murine hyperthermic intraperitoneal chemotherapy model. A. Drawing of tumor bearing mouse undergoing HIPEC. Depicted are inlet (a) and outlet (b) ports and anal temperature probe (c) to monitor internal temperature of mouse during perfusion. **B.** Photograph showing perfusion pump (a), temperature monitor (b), flow tubes (c) and heating bath (d). Mice were perfused for 1 h at the rate of 3 mL/min with cisplatin (3 mg/kg) ± sodium arsenite (26 mg/kg) at 37 or 43 °C.

Western blot analysis

Tumors of ~ 3-5 mm in diameter were homogenized in protein lysis solution (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% sodium dodecyl sulfate, 180 µg/ml phenylmethylsulphonylfluoride) using a tissue grinder. After removal of debris by centrifugation (45 min, 14,000 x g), total protein concentration in supernatant was determined by bicinchoninic acid (BCA) method according to manufacturer's instructions (Pierce, Rockford, IL, micro-well plate protocol) (Smith, et al., 1985). Fifteen µg protein samples were resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membranes. Membranes were probed with antibodies to XPA (Neomarkers, MS-650-P1, dilution 1:1000), XPC (Novus, # ab6264, dilution 1:10,000), GAPDH (Sigma, # A 5441, dilution 1:10,000), p53 (DO-1, Cell Signaling Technology, # 9284, dilution 1:1000), MSH2 (Santa Cruz, # SC-494, dilution 1:1000), and ERCC1 (Santa Cruz, # SC-10785, dilution 1:1000). Secondary antibodies (rabbit anti-mouse IgG, # 81-6120 or goat anti-rabbit, # 81-6120, dilutions 1:2500) conjugated to horseradish peroxidase (Zymed Laboratories, Inc. South San Francisco, CA) were bound to primary antibodies and protein bands detected using enhanced chemiluminescence (ECL) substrate (Pierce, Rockford, IL). GAPDH was used as the loading control. Films were scanned with a Molecular Dynamics Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed with ImageQuaNT software (Molecular Dynamics) to determine band density.

ICP-MS analysis

Samples of tumor homogenates were lyphophilized using Heto vacuum centrifuge (ATR, Laurel, MD) and 350 µL concentrated nitric acid was added to each sample. Wet weight of brain, heart, spleen, liver and kidney was recorded and concentrated nitric acid (350 - 500 µL) was added to samples. Samples were predigested overnight, and then 100 µL of each dissolved sample was transferred into 10 mL acid washed microwavable digestion tubes (triplicate for each sample). The samples were microwave-digested at 150°C for 10 min using an automated focused beam microwave digestion system (Explorer™, CEM, Matthews, NC, USA). After digestion, 1.9 mL of 18 Mohm H_2O containing 10 ppb internal standard (SPEX CertiPrep, Metuchen, NJ) was added into every sample to give final 5% nitric acid and ICP-MS analyses was performed using Thermo X Series II ICP-MS (Thermo Fisher Scientific, Waltham, MA) at the University of Louisville Center for Regulatory and Environmental Analytical Metabolomics facility. Concentrated nitric acid was processed similarly as blank. Platinum standard (SPEX CertiPrep, Metuchen, NJ) was used to generate a standard curve. Platinum and arsenic levels in tumors and tissues were expressed as ng metal/mg protein and ng metal/mg wet weight respectively. Results are presented as the means of three ICP-MS determinations for each data point ± SD from 3 individual mice.

Immunocytochemistry

Cells ($1x\ 10^5$) were plated on poly-D-lysine coated coverslips (BD Biosciences) in a 24-well plate and allowed to acclimate for 24 h. Cells were then

treated with 40 μM cisplatin for 1 h. After treatment, cells were washed twice with PBS and incubated in drug-free media for 24 h. Cells were fixed in ice-cold acetone for 10 min at room temperature and washed twice with ice cold PBS and samples incubated for 10 min with PBS containing 0.25% Triton X-100 (PBST). Cells were then washed with PBS three times for 5 min and incubated in 3% hydrogen peroxide for 30 min to quench endogenous peroxidase. Cells were washed three times with PBS and incubated in 1% BSA in PBST for 30 min to block unspecific binding of the antibodies. Cells were incubated overnight at 4°C in primary antibodies (1:200 dilution in PBST containing 1% BSA). The primary antibodies used were XPA (Neomarkers, MS-650-P1), XPC (H-300, SantaCruz Biotechnology, # sc-30156), p53 (DO-1, Cell Signaling Technology, # 9284), MSH2 (Santa Cruz, # SC-494) and ERCC1 (Santa Cruz, # SC-10785). After incubation, the primary antibody solution was decanted and cells were washed three times with PBS for 5 min each wash. Cells were incubated with secondary antibodies (rabbit anti-mouse IgG, # 81-6120 or goat anti-rabbit, # 81-6120, dilution 1:200 in PBST containing 1% BSA) conjugated to horseradish peroxidase (Zymed Laboratories, Inc. South San Francisco, CA) for 1 h at room temperature. Secondary antibody solution was decanted and cells were washed three times with PBS for 5 min. Cells were stained with 3,3'-diaminobenzidine (DAB) substrate solution by incubating cells in 200 μL premixed DAB solution (mix 30 μL (one drop) of the DAB liquid chromogen solution to 2 mL of the DAB liquid buffer solution (Sigma, # D 3939)) for 10 min. DAB solution was removed and cells rinsed briefly with PBS. Cells were counterstained with 20% Wright

Giemsa solution for 1 min. Coverslips were mounted on microscope slides using a drop of permount mounting medium. Slides were viewed under a Nikon Eclipse E600 Microscope (Fryer Company Inc, Scientific Instruments, Cincinnati OH 45240) and pictures taken using MetaMorph software (Universal Imaging Corporation). DAB-positive cells were counted per 1000 cells using MetaMorph software.

Statistical analysis

Statistical analyses were performed using wilcoxon rank sum test with significance set as $p < 0.05$, $n \geq 3$.

RESULTS

Murine intraperitoneal chemotherapy model

Multiple disseminated tumors were established in the peritoneal cavity of nude mice as described in Materials and Methods. Mice were scanned using microCT scan to determine the location and estimate the size of tumors (Figure 14A). This was confirmed upon necropsy (Figure 14B).

Tumor bearing mice were treated by peritoneal lavage for 1 h with cisplatin ± sodium arsenite at 37 °C (normothermia) or 43 °C (hyperthermia) (Figure 15A and B) as described in Materials and Methods.

During treatment, the required inflow temperature was reached within 2-5 min after the start of perfusion. Inflow, outflow and rectal temperatures were recorded every 15 min and remained stable within 1 °C throughout the 60 min perfusion (Table 1).

Table 1: Inflow, outflow and body temperatures of mouse during intraperitoneal perfusion.

Inflow temperature	Outflow temperature	Body temperature
37.4 ±1.1 °C	36.4 ± 0.8 °C	35.5 ± 1.0 °C
43.0 ± 0.7 °C	39.7 ± 0.6 °C	36.3 ± 2.1 °C

Mice were perfused for 1 h with cisplatin (CP/37; CP/43) or cisplatin + sodium arsenite (CPA/37; CPA/43) at 37 or 43 °C respectively. Inflow, outflow and body temperatures were recorded every 15 min. Data are presented as means ± SD of readings taken from five mice.

Platinum and arsenic accumulation and retention in metastatic tumors

I determined platinum and arsenic accumulation in tumors immediately (0 h) and 24 h after perfusion using ICP-MS. platinum and arsenic accumulated in tumors during treatment (0 h) and generally decreased after treatment (24 h), compared with the untreated control (Figure 16). Co-treatment with sodium arsenite and cisplatin at 37 °C (CPA/37) or 43 °C (CPA/43) caused significantly more platinum to accumulate in tumors. By 24 h after perfusion, tumor platinum levels for CPA/37 and CPA/43 treatment conditions decreased to levels similar to CP/37. Hyperthermia did not increase tumor platinum levels nor alter platinum retention in tumors 24 h after treatment. More arsenic initially accumulated in tumors when co-treated with cisplatin and sodium arsenite at 37 °C (CPA/37) than with hyperthermia treatment (CPA/43). Arsenic decreased to similar levels at 24 h.

Figure 16

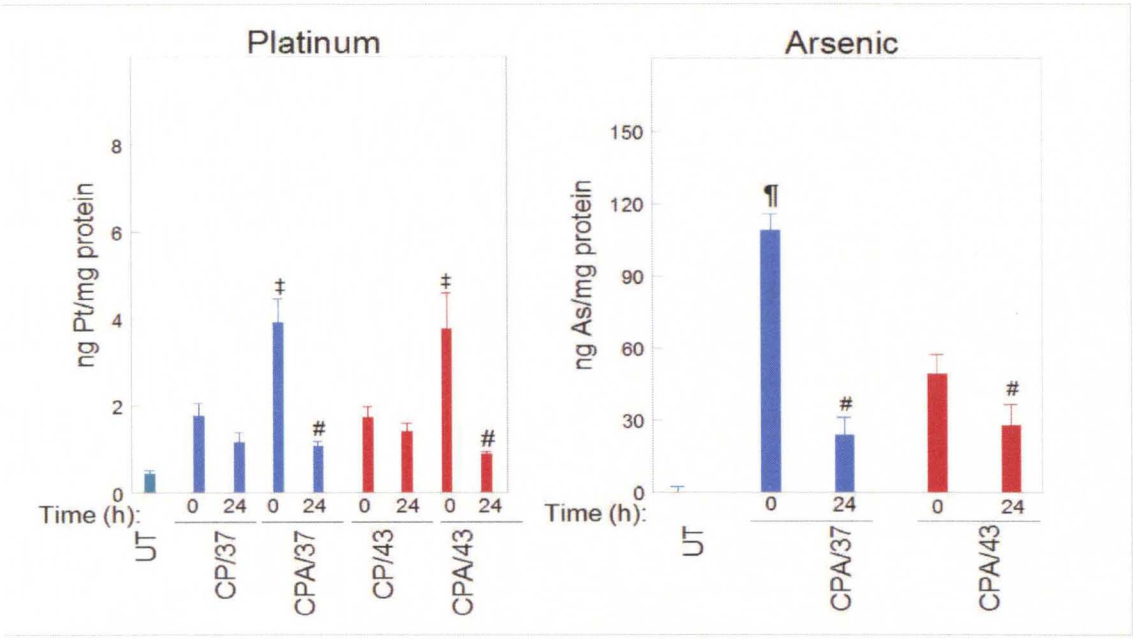

Figure 16. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) determination of platinum and arsenic in tumors. Mice were perfused for 1 h with cisplatin (CP/37; CP/43) or cisplatin plus sodium arsenite at 37 or 43 °C respectively (CPA/37; CPA/43). Tumors from untreated (UT) and treated mice were harvested at 0 and 24 h after treatment. Tumors were homogenized and samples of the homogenate were analyzed for protein concentration by BCA or digested in nitric acid for ICP-MS analysis for platinum and arsenic. Data are presented as means ± SEM of ≥3 tumors each from different mice. Statistical analysis was performed using wilcoxon rank sum test. $P < 0.05$, $N \geq 3$: # = lower than 0 h partner, ‡ = higher than CP/37 at 0 h and CP/43 at 0 h, ¶ = higher than CPA/43 °C at 0 h.

Effect of cisplatin, arsenic and hyperthermia on DNA repair protein expression

Cisplatin causes bulky DNA damage that is repaired mostly by the nucleotide excision repair system (NER). Cellular response to cisplatin-DNA damage involves the induction of DNA repair proteins to initiate DNA repair (Cepeda, et al., 2007). I determined if sodium arsenite and hyperthermia modulated the expression of XPC, a platinum-DNA damage recognition protein in global genome repair (GGR) (Neher, et al., 2010) sub pathway of NER, and of ERCC1 and XPA, downstream NER proteins that have been implicated in cisplatin resistance (Dabholkar, et al., 1994). I also determined the expression of p53, which is involved in the activation of the GGR pathway by transcriptionally activating XPC (Ford, 2005). In addition to NER, decreased mismatch repair (MMR) has been implicated in cisplatin resistance (Fink, et al., 1997; Jensen, et al., 2008). Thus, we also investigated the expression of MSH2, an important MMR DNA damage recognition protein. Western blot analysis of p53, XPC, XPA, ERCC1and MSH2 revealed mouse-to-mouse and tumor-to-tumor variabilities (Figure 17A). Some tumors failed to express the protein of interest while others either expressed high, moderate or very low levels of the proteins. I determined band intensities for the expressed proteins by scanning the films using a Molecular Dynamics Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzing bands of interest using ImageQuaNT software (Molecular Dynamics). Each protein value was normalized to its respective GAPDH (loading control) value. Data were further normalized to untreated control (Figure 17B).

Tumors that failed to express the protein of interest were not considered in the densitometry analyses. P53 (Figure 17B, panel a) and XPC (Figure 17B, panel b) were significantly induced during treatment (0 h) by cisplatin at 37 °C (CP/37) or 43 °C (CP/43) and cisplatin plus arsenite at 43 °C (CPA/43). P53 significantly decreased at 24 h after treatment with CPA/43 (Figure 17B, panel a). XPC decreased at 24 h after perfusion with both CP/43 and CPA/43 treatments (Figure 17B, panel b). P53 (Figure 17B, panel a) and XPC (Figure 17B, panel b) did not significantly increase during (0 h) and after (24 h) peritoneal lavage with sodium arsenite and cisplatin co-treatment at 37 °C (CPA/37). XPA (Figure 17B, panel c) was significantly induced during (0 h) and 24 h after perfusion with CP/37, CPA/37 and CPA/43 but not with CP/43. ERCC1 remained generally low for all treatment conditions except with CPA/37 (Figure 17B, panel d). The suppression of MSH2 by CP/37 and CP/43 treatments was not seen in tumors co-treated with arsenite (CPA/37, CPA/43) (Figure 17B, panel e).

Figure 17

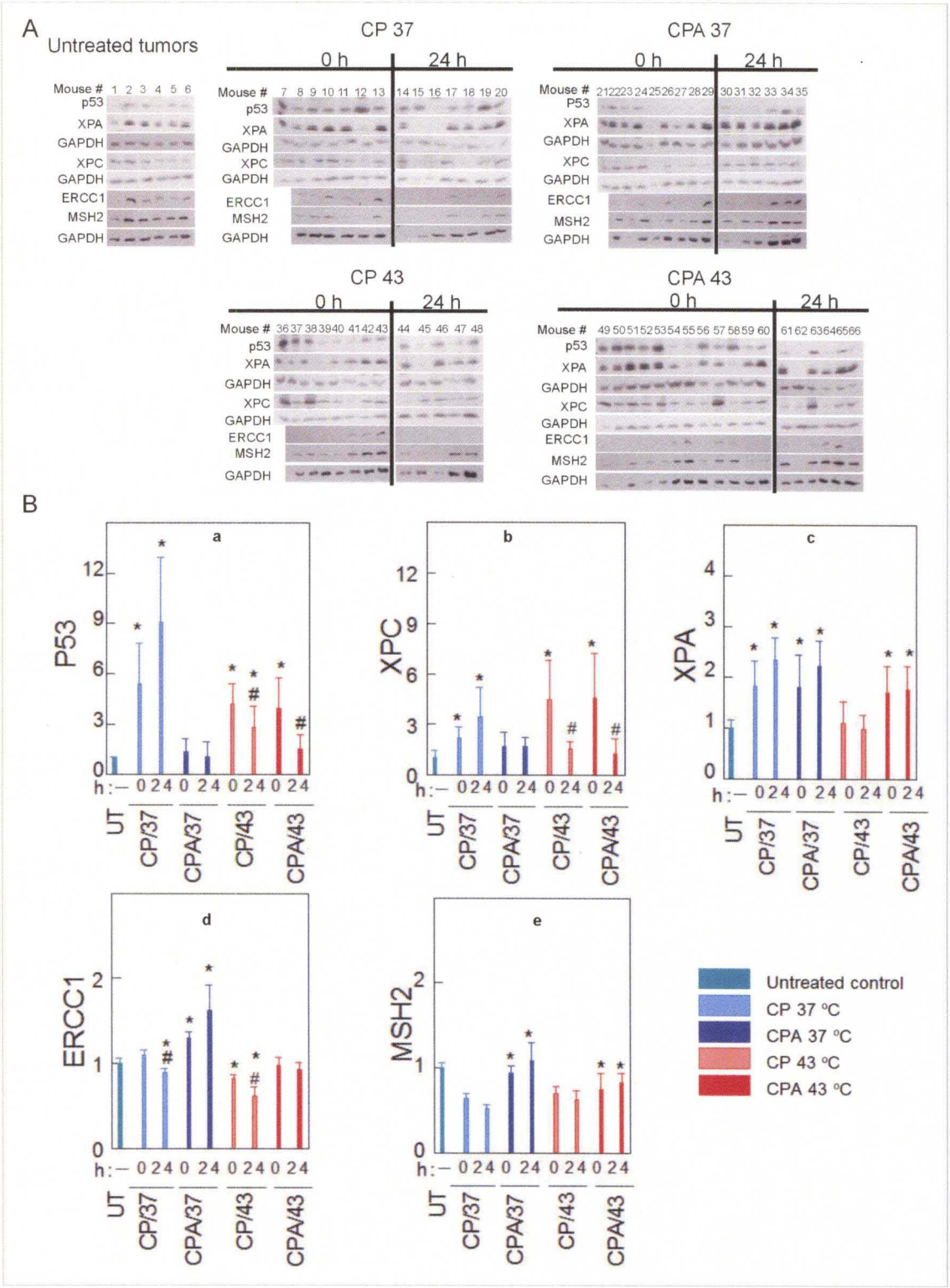
A
Untreated tumors
CP 37
CPA 37
CP 43
CPA 43
0 h
24 h
Mouse #
p53
XPA
GAPDH
XPC
ERCC1
MSH2
B
a
b
c
d
e
P53
XPC
XPA
ERCC1
MSH2
h: — 0 24 0 24 0 24 0 24
UT
CP/37
CPA/37
CP/43
CPA/43
Untreated control
CP 37 °C
CPA 37 °C
CP 43 °C
CPA 43 °C

Figure 17. DNA repair protein expression in tumors. A. Western blot determination of p53, XPC, XPA, ERCC1 and MSH2 in tumors. GAPDH is loading control. **B**. Densitometry analyses of (**a**) p53, (**b**) XPC, (**c**) XPA, (**d**) ERCC1 and (**e**) MSH2 normalized to GAPDH loading control and untreated tumors. Mice were perfused for 1 h with cisplatin (CP/37; CP/43) or cisplatin plus sodium arsenite (CPA/37; CPA/43) at 37 or 43 °C respectively. Tumors from untreated (UT) mice and treated mice were harvested at 0 and 24 h after treatment. Protein extracts were prepared from the tumors and 20 μg loaded per lane for SDS-PAGE. Data are presented as means ± SD of ≥5 tumors each from different mice. Statistical analysis was performed using wilcoxon rank sum test. $P < 0.05$, $N \geq 5$. # = compared to 0 h partner, *= compared to UT.

Expression of P53, XPA and MSH2 in OC cells

Western blot determination of P53, XPC, XPA, ERCC1 and MSH2 in metastatic tumors revealed that some tumors failed to express p53 (6%), XPC (3%), XPA (8%), ERCC1 (40%) and MSH2 (9%) (Figure 17A). Failure to express these proteins could be an inherent feature of the cells that were used to establish the tumors or due to mutations and alteration of genes during tumor development that could result in lack of protein expression. I therefore performed immunocytochemistry studies using A2780/CP70 cells to determine expression of P53, XPA and MSH2 in these cells (Figure 18A). Immunocytochemistry data revealed that 25% of cells do not express p53 as evident by lack of 3,3'-diaminobenzidine (DAB) brown staining and ~3% and 60% of cells did not stain positive for XPA and MSH2 respectively (Figure 18B). Full-length western blots for XPC and ERCC1 had several non-specific bands in addition to the band of interest (data not shown) making it impossible to perform Immunocytochemistry with specificity for these proteins.

Figure 18

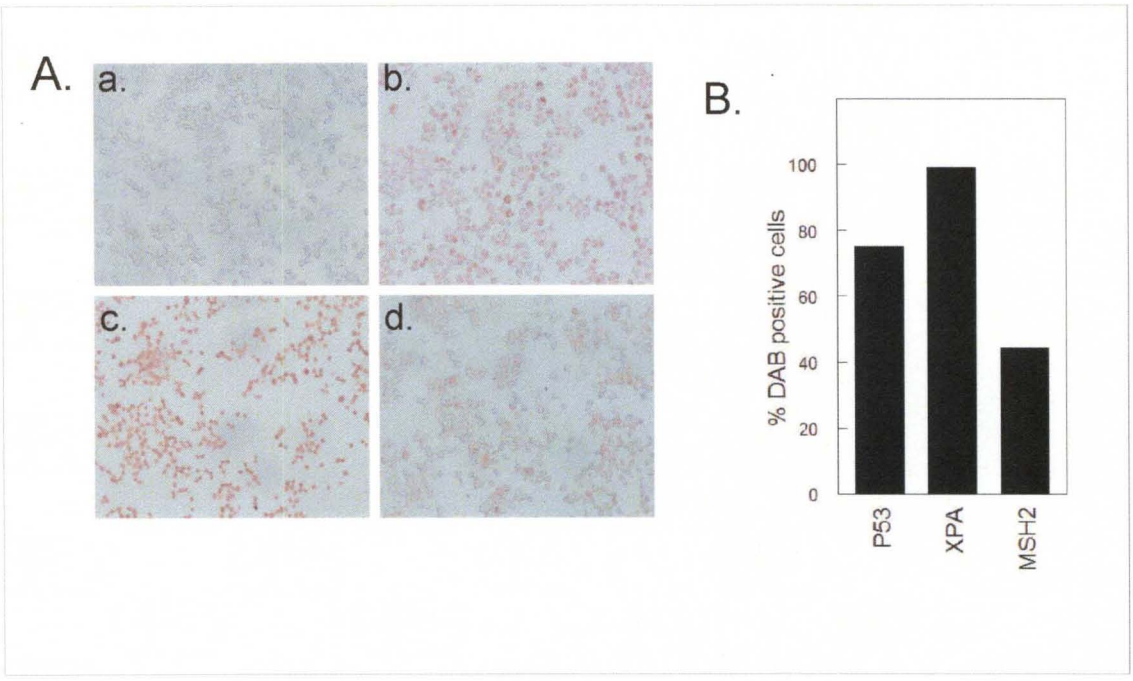

Figure 18. Immunocytochemical determination of p53, XPA and MSH2 expression in OC cells. A. A2780/CP70 cells were treated for 1 h with 40 μM cisplatin. Cells were washed and incubated in drug-free media for 24 h and immunohistochemistry was performed. Representative pictures at 20x magnification of cells for secondary antibody only control (a), p53 (b), XPA (c) and MSH2 (d). **B.** Plot of percentage of 3,3'-diaminobenzidine (DAB)-positive cells. Data are single biological experiment performed in duplicate. Four different fields were counted per coverslip.

Platinum and arsenic biodistribution in somatic tissues

The clinical use of anticancer chemotherapeutic agents is limited by adverse toxicities. For cisplatin, these include toxicity to the kidney, peripheral nerves, liver, heart, bone marrow and brain (Hartmann and Lipp, 2003; Gesson-Paute, et al., 2008). Clinical use of arsenic is known to cause liver, kidney and neurological damage, cardiovascular and gastro-intestinal toxicity, anemia and leucopenia (Senkus and Jassem, 2010; Emadi and Gore, 2010; Au and Kwong, 2008). Therefore, I determined cisplatin and arsenic accumulation in mouse tissues including kidney, liver, heart, spleen and brain (Figures 19A and B). Samples were prepared as described in Methods. During perfusion, platinum accumulated in all tissues examined regardless of the treatment condition, in the order: kidney > liver = spleen > heart > brain. At 24 h after perfusion, significant decrease of platinum was observed in the kidney for all treatment conditions. The combination treatment (CPA/43) favored the removal of platinum from the liver, spleen and heart at 24 h after perfusion. Arsenic also significantly accumulated in all the tissues examined, in the order: liver > kidney = spleen > heart > brain and it significantly decreased in all tissues by 24 h after perfusion.

Figure 19

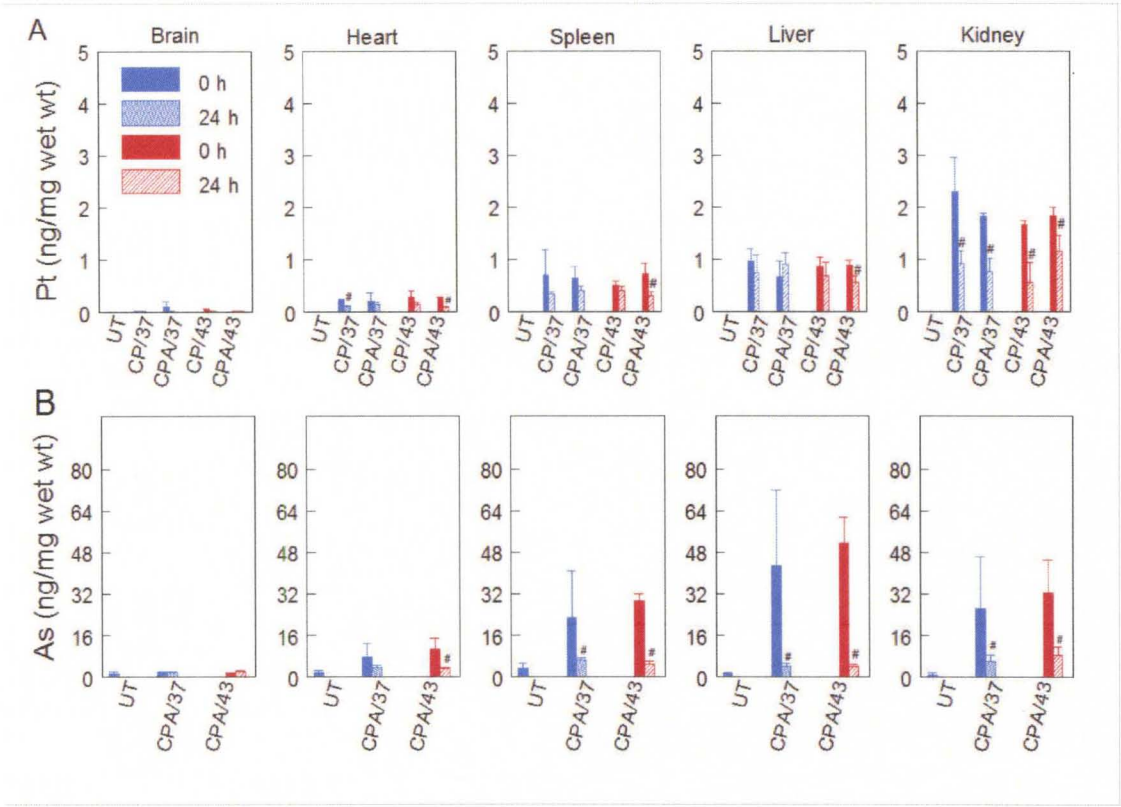

Figure 19. Platinum and arsenic accumulation in somatic tissues. Mice were perfused for 1 h with cisplatin (CP/37; CP/43) or cisplatin plus sodium arsenite (CPA/37; CPA/43) at 37 or 43 ºC respectively. Tissues from untreated (UT) and treated mice were harvested at 0 and 24 h after treatment. Tissue samples were weighed and digested in nitric acid for ICP-MS analysis for platinum (**A**) and arsenic (**B**). Data are presented as means ± SD of triplicate samples each from different mice. Statistical analysis was performed using wilcoxon rank sum test. $P < 0.05$, N = 3. # = compared to 0 h partner.

DISCUSSION

Although the platinum analogues (cisplatin and carboplatin) are at the forefront of combination chemotherapy for OC, acquired or inherent resistance limits clinical success. In the current study, I used metastatic OC xenograft in nude mice to investigate how sodium arsenite and hyperthermia modulate response to cisplatin *in vivo*. I focused on three key mechanisms of cisplatin resistance: enhanced NER, diminished MMR and decreased platinum accumulation. My data suggest that cisplatin induces resistant phenotype in metastatic tumors by inducing XPC and XPA and suppressing MSH2. Sodium arsenite alone or combined with hyperthermia inhibits mechanisms of cisplatin resistance by suppressing XPC induction, maintaining higher levels of MSH2 and increasing tumor uptake of cisplatin.

Decreased platinum accumulation is an important mechanism of cisplatin resistance. Hyperthermia has been reported to increase both cellular and DNA bound platinum levels *in vitro*. However, *in vivo* data remain controversial. Los et al used rats bearing metastatic colon cancer to show that hyperthermia suppressed tumor growth by increasing platinum accumulation in tumors (Los, et al., 1994). Zeamari et al used a similar colon cancer xenograft model in rats and reported that hyperthermia did not increase tumor platinum levels (Zeamari, et al., 2003). Similar to Zeamari, I observed that hyperthermia does not increase platinum accumulation in tumors. The observed discrepancies with Los et al could be due to differences on how HIPEC was performed. Los et al injected hyperthermic cisplatin intraperitoneally; whereas I and Zeamari et al performed

peritoneal lavage similar to what is done clinically. Unlike hyperthermia, I observed that sodium arsenite at 37 or 43 °C increased initial tumor platinum levels. Since arsenic and cisplatin are detoxified by glutathionylation and export by the multidrug resistant family proteins, potential competition for the detoxification/export pathways might have resulted in more platinum accumulating in the tumors when cisplatin is co-administered with sodium arsenite.

Cisplatin is a DNA damaging agent and p53 is implicated in platinum-DNA damage response (Ford, 2005). P53 is frequently mutated in OC (Berchuck, et al., 1994). The p53 phenotype of A2780/CP70 cells remains controversial. Some studies have demonstrated that A2780/CP70 cells have non-functional p53 (Jones, et al., 1998; Lu, et al., 2001), while other studies have shown that these cells have wild type p53 (Brown, et al., 1993; Yazlovitskaya, et al., 2001). My data indicate that A2780/CP70 cell population is heterogeneous: ~75% of cells express wild type p53 and ~25% are p53 null (Figure 18). In addition, 6% of the tumors derived from A2780/CP70 are p53 null (Figure 17A). My *in vitro* data also demonstrate the induction of p53 target genes CDKN1A, XPC and DDB2 in A2780/CP70 cells (Figures 5 and 25), which strongly suggests that a large fraction of these cells have wild type p53. The observed heterogeneity might have resulted from mutations and alterations that occur during serial propagation of cells in culture leading to cell line drift (Hughes, et al., 2007). The observed heterogeneity may impact response to chemotherapy and result in treatment failures because p53 wild type and null cells will respond differently to

chemotherapy especially DNA damaging agents such as cisplatin. This heterogeneity explains why targeting master regulators such as p53 or AKT in cancer cells has not been successful (Zeimet and Marth, 2003; Engelman, 2009). Therefore, combination chemotherapy such as cisplatin, sodium arsenite and hyperthermia with different mechanisms of action might be more beneficial than using a single drug to target a single protein or pathway.

Cisplatin predominantly forms intrastrand DNA crosslinks that are repaired by the nucleotide excision repair (NER) system. There are two sub-pathways of NER; transcription coupled repair (TCR) which removes damage from actively transcribing DNA and global genome repair (GGR) which removes lesions from the entire genome (Martin, et al., 2008). These two pathways differ only in the proteins that are involved in damage recognition. In TCR, CSA and CSB along with RNA pol II recognize damage, whereas in GGR, XPC and DDB2 are important for lesion recognition. XPC is actively involved in the recognition and initiation of cisplatin-DNA damage repair in GGR (Earley and Turchi, 2010; Neher, et al., 2010). Arsenic has been shown to inhibit NER by inhibiting XPC expression (Nollen, et al., 2009). In the current study, I observed that P53 and XPC were induced by cisplatin. However, sodium arsenite alone or in combination with hyperthermia prevented the induction of p53 and XPC by cisplatin (Figure 17B, panels a and b). Since p53 is known to transcriptionally induce XPC (Ford, 2005), my data suggest that sodium arsenite ± hyperthermia might be inhibiting p53, which in turn might be suppressing XPC induction. Suppression of XPC will potentially sensitize tumors to cisplatin because my *in*

vitro data indicate that inhibition of XPC using siRNA sensitizes OC cells to cisplatin (Figure 7). Therefore, the suppression of XPC could potentially sensitize tumors to cisplatin in a similar fashion. Following DNA damage recognition, downstream DNA repair proteins (XPA, RPA, TFIIH complex, ERCC1/XPF and XPG) are recruited to the DNA damage recognition complexes in both TCR and GGR to remove the damage in a common pathway. Over-expression of XPA and ERCC1 mRNA has been associated with cisplatin resistance in OC (Dabholkar, et al., 1994). In the current study, cisplatin induced XPA (Figure 17B, panel c) that was suppressed by hyperthermia co-treatment (Figure 17 panel c). Suppression of XPA might decrease repair of cisplatin-DNA damage. ERCC1 was modestly induced (<1.5 fold) by sodium arsenite co-treatment with cisplatin at 37 °C (CPA37) (Figure 17B, panel d).

In addition to the NER pathway, the mismatch repair (MMR) system has been implicated in cisplatin resistance (Fink, et al., 1997). In an effort to repair platinum-DNA damage by the MMR system, a futile MMR occurs leading to cell death (Martin, et al., 2008; Topping, et al., 2009). Ovarian cancer cells over-expressing MMR proteins are sensitive to cisplatin (Ding, et al., 2009; Pani, et al., 2007; Topping, et al., 2009). I report for the first time that tumors treated with cisplatin at 37 °C (CP37) significantly suppressed MSH2 consistent with resistance. The observed suppression of MSH2 by cisplatin was reversed in tumors co-treated with sodium arsenite at 37 or 43 °C (CPA/37 and CPA/43 respectively). Thus, sodium arsenite at 37 or 43 °C has the potential to sensitize tumors to cisplatin by maintaining functional MMR.

Cisplatin causes serious and dose-limiting side effects including kidney damage, peripheral sensory neuropathy, cardiovascular toxicity, myelosuppression and anemia which occur as a result of diffusion of chemotherapy from the peritoneal to systemic compartment. In addition, arsenic also causes adverse side effects including cardiovascular toxicity, kidney damage, myelosuppression and anemia, liver damage and peripheral sensory neuropathy. Understanding the biodistribution of these drugs during peritoneal perfusion of chemotherapy is important in order to predict the occurrence of these adverse side effects and determine the risk:benefit balance in performing intraperitoneal perfusion with cisplatin and arsenic. For this reason, I determined platinum and arsenic accumulation in the brain, heart, liver, kidney and spleen during (0 h) and 24 h after perfusion. I observed that platinum and arsenic accumulated to similar extent in these tissues regardless of the treatment condition. The greatest accumulation of platinum was observed in the kidney, the site of platinum elimination. Likewise, greatest level of arsenic was observed in the liver, the organ for arsenic metabolism and detoxification. Even though I did not observe any toxicity with the short-term survival study, accumulation of arsenic and platinum in assayed organs suggests that potential adverse side effects such as encephalopathy, cardiotoxicity, liver damage, renal damage and myelosuppression/anemia respectively may occur during long-term survival studies.

In summary, treatment of tumors with cisplatin plus sodium arsenite or combined cisplatin, sodium arsenite and hyperthermia is most likely to enhance

cisplatin efficacy because sodium arsenite ± hyperthermia impairs NER by inhibiting induction of p53 and XPC and activates MMR by maintaining high levels of MSH2 and enhances platinum accumulation in tumors. Sodium arsenite and hyperthermia might not produce added systemic toxicity to cisplatin chemotherapy; on the contrary, the combined treatment might help in the clearance of platinum from tissues. Long-term survival studies are required to determine the efficacy of this new combination chemotherapy. The murine HIPEC model may serve as a useful tool to study in vivo mechanisms of platinum resistance and explore ways to sensitize tumors to platinum chemotherapy.

CHAPTER 4

CISPLATIN, SODIUM ARSENITE AND HYPERTHERMIA INDUCE PSEUDO-G1 ASSOCIATED APOPTOTIC CELL DEATH IN OC CELLS

INTRODUCTION

Cisplatin and its analogues are widely used to treat ovarian, testicular, head and neck, bladder, gastric and lung cancer (Vasey, et al., 1999; Winter and Albers, 2011; Pan, et al., 2009; Mitsui, et al., 2011; Norman, et al., 2010; Blackhall and Faivre-Finn, 2011). The cytotoxicity of cisplatin is mediated through cisplatin-DNA damage formation. Cisplatin primarily forms 1,2-intrastrand cross links between adjacent purines and to a lesser extent 1,3-intrastrand cross links, monoadducts and interstrand crosslinks (Basu and Krishnamurthy, 2010). In addition to DNA damage, cisplatin can also form covalent bonds with biologically important macromolecules such as protein and RNA.

Inherent and/or acquired resistance to cisplatin limits clinical success (Armstrong, 2002a). One approach to mitigate cisplatin resistance is the use of combination chemotherapy. My dissertation research project focuses on investigating the cytotoxic effects and mechanisms of action of a new combination of cisplatin, sodium arsenite and hyperthermia. I previously showed that combined sodium arsenite and hyperthermia sensitize wild-type p53 expressing OC cells to cisplatin (Figure 3, Chapter 2). Mechanistically, sodium arsenite and hyperthermia attenuated DNA repair protein XPC *in vitro* and *in vivo*

(Figure 6 and Figure 17) and enhanced cellular and DNA accumulation of cisplatin (Figures 10B and 16).

In response to platinum-DNA damage, p53 is stabilized and activated by upstream kinases. Activated p53 activates cell cycle checkpoints, DNA repair and apoptosis (Abraham, 2001; Horvath, et al., 2007). P53 regulates cell cycle checkpoints by inducing transcription of cyclin dependent kinase inhibitor CDKN1A, GADD45 and 14-3-3 σ (Basu and Krishnamurthy, 2010; Taylor and Stark, 2001). CDKN1A blocks cell cycle progression by binding and inactivating cyclin/CDK complexes which are required for pRb phosphorylation in order to release E2F for cell cycle progression through G1. Cisplatin is known to cause G2 arrest (Horvath, et al., 2007). CDKN1A blocks G2 to M transition by binding and inactivating CDK1/cyclin B complex (Taylor and Stark, 2001).

Sodium arsenite causes mitotic arrest and mitotic arrest associated apoptosis (mitotic catastrophe) (McNeely, et al., 2008b; McNeely, et al., 2008a). P53 has been implicated in sodium arsenite induced mitotic arrest. Presence of functional p53 promotes mitotic exit (McNeely, et al., 2006), whereas, cells with non-functional p53 are more susceptible to sodium arsenite-induced mitotic arrest (Taylor, et al., 2006). In addition, a functional spindle checkpoint is required for arsenite-induced mitotic arrest and apoptosis (McNeely, et al., 2008b; Wu, et al., 2008). Similar to sodium arsenite, hyperthermia has been reported to induce mitotic catastrophe in cancer cells (Grzanka, et al., 2008; Michalakis, et al., 2005; Nakahata, et al., 2002). Therefore, I hypothesized that combined sodium arsenite and hyperthermia will enhance mitotic catastrophe in cisplatin treated OC cells.

The purpose of the studies in this chapter is to determine if a new combination of sodium arsenite and hyperthermia alters cisplatin-induced G2 cell cycle arrest and causes mitotic arrest and mitotic arrest associated apoptosis (mitotic catastrophe) in wild-type p53 expressing OC cells treated with cisplatin. I show that cisplatin, sodium arsenite and hyperthermia fails to activate spindle assembly checkpoint protein BUBR1 and causes cells to exit mitosis without dividing. Cells accumulate in pseudo-G1 with 2C DNA content and subsequently underwent apoptotic cell death.

HYPOTHESIS

Sodium arsenite and hyperthermia sensitize OC cells to cisplatin by causing mitotic arrest associated apoptotic cell death.

MATERIALS AND METHODS

Western blot analyses

Cells were treated and protein lysates prepared and resolved by SDS-polyacrylamide gel electrophoresis as previously described in Materials and Methods section in Chapter 2. Membranes were probed with mouse monoclonal antibodies for ß-actin (Sigma, # A 5441, 1:10,000 dilution), GAPDH (Ambion, # AM4300, 1:10,000 dilution), cyclin A (Cell Signaling, # 4656, 1:1000 dilution), cyclin B (BD Biosciences PharMingen, # 55477, 1:1000 dilution), histone H3 Ser10 phosphorylation (Cell Signaling, # 9706S, 1:1000 dilution) and cyclin E (BD Biosciences PharMingen # 51-1459GR, 1:1000 dilution) or rabbit polyclonal antibodies for CDKN1A (H-164, Santa Cruz, # SC 756, 1:1000 dilution), CDK1 (Upstate Cell Signaling Solutions, # 06-141, 1:1000 dilution) and pRbSer807/811 phosphorylation (cell signaling, # 9308, 1:1000 dilution). Secondary antibodies (rabbit anti-mouse IgG, # 81-6120 or goat anti-rabbit, # 81-6120) conjugated to horseradish peroxidase (Zymed Laboratories, Inc. South San Francisco, CA) were bound to primary antibodies and protein bands detected using enhanced chemiluminescence (ECL) substrate (Pierce, Rockford, IL). Bands for BUBR1, cyclin E, CDK1 Y15P, histone H3 Ser10P and GAPDH were detected using enhanced chemiluminescence (ECL) plus western blotting detection system (GE Healthcare, RPN2132) and bands were developed using Molecular Dynamics Storm 860 (GE Healthcare BioSciences) in blue fluorescence mode. ß-actin and GAPDH served as the loading control.

Flow cytometry analyses

Cells (1 X 10^6) were cultured in 10 cm dishes overnight and treated with IC50 cisplatin (A2780 = 4 μM and A2780/CP70 = 40 μM) ± 20 μM sodium arsenite at 37 or 39 °C for 1 h. Cells were washed twice with PBS and refed with fresh media and incubated at 37 °C. Whole cells were trypsinized and collected at 0 and 36 h, washed twice with PBS and fixed in 70% ethanol overnight at 4 °C. Cells in 0.5 mL PBS were incubated at 37 °C with RNase A (100 U/mL) for 30 min. After adding propidium iodide (5 μg/mL), cells were incubated at room temperature for 30 min protected from light. Propidium iodide fluorescence (DNA content) was determined by flow cytometry using FACScalibur (BD Biosciences, San Jose, CA). A minimum of 20,000 cells/sample were analyzed. Data were collected and analyzed using FLOWJO software (FLOWJO, Ashland, OR).

Mitotic Index determination

Cells (5 X 10^5/ 6 cm dish) were treated with IC50 cisplatin (A2780 = 4 μM and A2780/CP70 = 40 μM) ± 20 μM sodium arsenite at 37 or 39 °C for 1 h. The cell monolayers were washed twice with PBS and incubated in drug-free media for 36 h. Cells were washed twice with cold PBS, trypsinized using 1x trypsin and collected by centrifugation at 500 xg for 5 min. Cells were resuspended in 150 μL serum free media and 2.5 mL of 0.4% KCl was added. The cell suspension was incubated for 10 min at 37 °C. Methanol:acetic acid (3:1 v/v) fixation solution was added to 2% v/v and cells collected by centrifugation at 500 xg for 5 min. Cells were resuspended in 2.5 mL fixation solution and fixed at room temperature for 20 min. Samples were centrifuged at 500 xg for 5 min and

pellets resuspended in 0.5 mL fixation solution and chilled on ice for a minimum of 20 min. Aliquots of the suspensions were dropped onto slides (2 slides per sample), air dried for about 1 min and stained with Wright Giemsa solution (States, et al., 2002). Slides were examined under a microscope and a minimum of 200 nuclei were counted on each slide for determination of mitotic index and mitotic catastrophe index. Chromosomal spreads with sharp features were scored as mitotic nuclei (Taylor, et al., 2006)

Annexin V-FITC apoptosis assay

Cells (5 X 10^5/ 6 cm dish) were treated with IC50 cisplatin (A2780 = 4 μM and A2780/CP70 = 40 μM) ± 20 μM sodium arsenite at 37 or 39 °C for 1 h. The cell monolayers were washed twice with PBS and incubated in drug-free media for 36 h. Cells were trypsinized using 1x trypsin and collected by centrifugation at 500 xg for 5 min. Cells were resuspended in 500 μL of 1X binding buffer and 5 μL of annexin V-FITC and 5 μL of propidium iodide (50 μg/mL). Samples were incubated at room temperature in the dark for 5 min. Annexin V-FITC binding was analyzed by flow cytometry (excitation = 488 nm; emission = 530 nm) using FACScalibur (BD Biosciences, San Jose, CA). Minimum of 20,000 cells/sample were analyzed. Annexin V assay was performed following manufacturers' instructions (Cat #: K101-25, BioVision Research Products, Mountain View, CA).

Statistical analysis

Results were expressed as the mean ± SD of three independent experiments. Statistical analyses were performed using Microsoft Excel built in

formulas for one-way analysis of variance and student's t-test with significance level as $p < 0.05$, $n = 3$.

RESULTS

Flow cytometry determination of cell cycle arrest

Cisplatin is a DNA damaging agent. Cellular response to DNA damage involves cell cycle arrest to allow time to repair damaged DNA (Basu and Krishnamurthy, 2010). Cisplatin is known to cause G2 arrest (Cepeda, et al., 2007). The goal of this experiment was to determine if sodium arsenite and hyperthermia alter the accumulation of cells in the G2/M compartment of the cell cycle following cisplatin treatment. Data suggest that both A2780 and A2780/CP70 cells accumulated in the G2/M compartment 36 h after cisplatin IC50 exposure (Figure 20). A2780/CP70 cells accumulated in the G2/M compartment to a greater extent than A2780 cells. Accumulation was not altered by sodium arsenite and/or hyperthermia co-treatment with cisplatin.

Figure 20

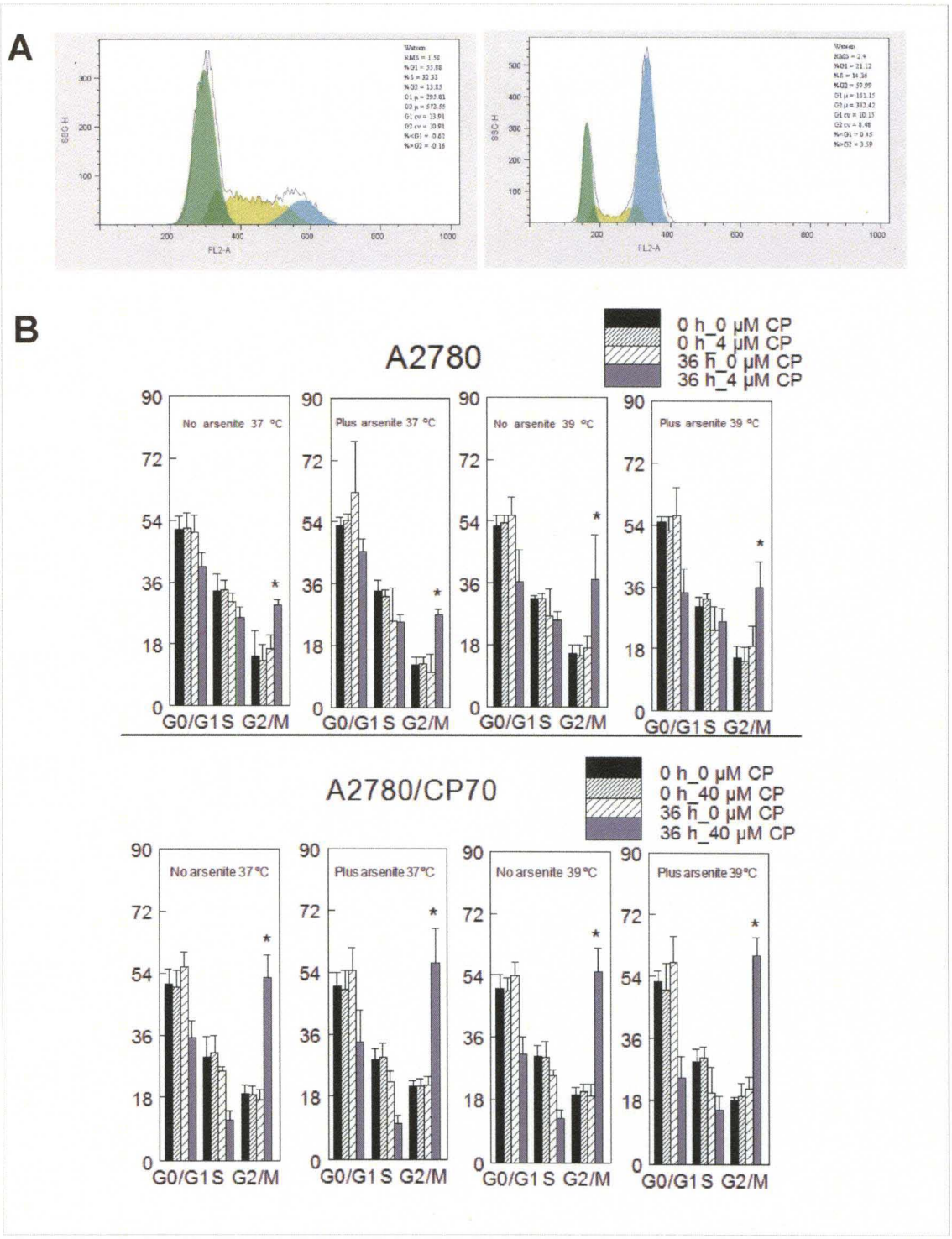

Figure 20. Cell cycle analyses by flow cytometry. A. Representative histogram of untreated cells (left panel) showing no cell cycle arrest and cisplatin

treated cells (right panel) accumulating in the G2/M compartment. **B.** Plot of percentage of cells in each phase of the cell cycle. A2780 and A2780/CP70 cells were treated with their IC50 cisplatin (CP) (A2780, 4 μM; CP70, 40 μM), CP plus 20 μM sodium arsenite at 37 or 39 °C for 1 h. Cells were harvested at 0 and 36 h after treatment. DNA content was analyzed by flow cytometry after propidium iodide staining. Data are the means ± SD of samples from 2 independent experiments. Each experiment was performed in duplicate dishes. Statistically analysis was performed using one way ANOVA and student t test. $P<0.05$, * = compared to G2/M partners.

Sodium arsenite and hyperthermia effect on G2 and M cell cycle regulatory proteins

Flow cytometry determination of DNA content using propidium iodide does not distinguish between G2 and M cells because these cells both have 2C DNA content. In order to determine if cells are in the G2 or M phase of the cell cycle at 36 h after treatment, the expression of cyclin A and cyclin B and cyclin dependent kinase CDK1 were determined. Furthermore, I determined if sodium arsenite and hyperthermia alter the expression of cyclin A, cyclin B and CDK1 in response to cisplatin treatment. G2 to M progression requires degradation of cyclin A and accumulation of cyclin B (Malumbres and Barbacid, 2009). Data in Figure 21 indicate that cisplatin treatment at 37 ^{o}C stabilized CDK1, cyclin A and cyclin B (Figure 21, panel a), suggesting G2 arrest. Adding hyperthermia to cisplatin decreased the levels of both cyclin A and cyclin B in A2780 cells suggesting G1 arrest, whereas; in A2780/CP70 cells cyclin A and cyclin B were stabilized suggesting G2 arrest (Figure 21, panel b). Co-treatment with cisplatin and sodium arsenite decreased both cyclin A and cyclin B in A2780 cells suggesting G1 arrest; while in A2780/CP70 cells, cyclin A was undetected, while cyclin B was stabilized, suggesting mitotic arrest (Figure 21, panel c). Combined cisplatin, sodium arsenite and hyperthermia stabilized cyclin B and CDK1 but attenuated the expression of cyclin A in both cell lines at 36 h after treatment (Figure 21, panel d), suggesting mitotic arrest. These data suggest that combined sodium arsenite and hyperthermia induced mitotic arrest in cisplatin treated cells.

Figure 21

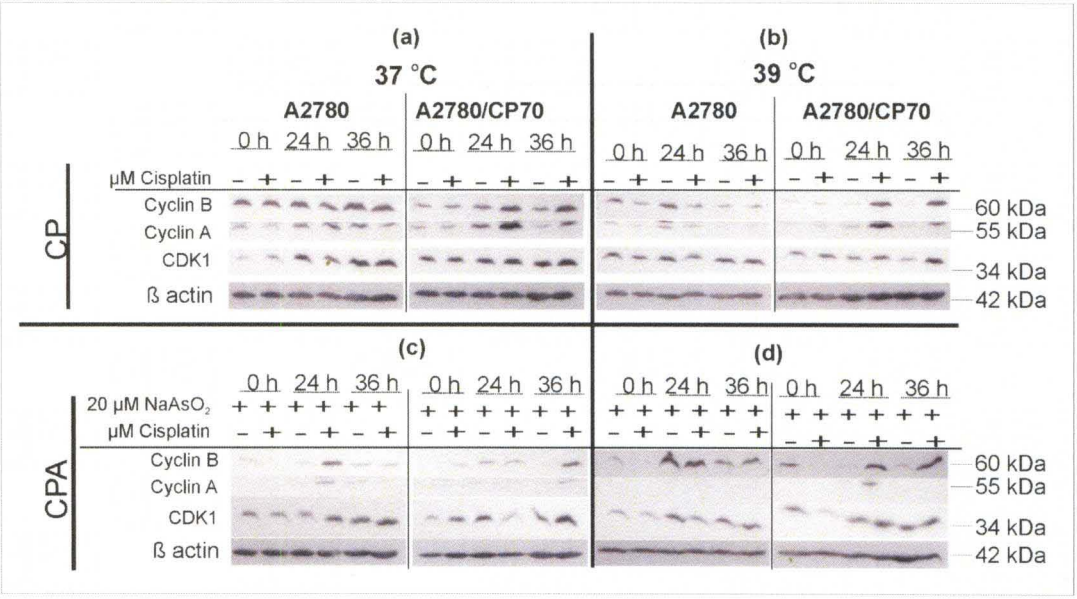

Figure 21. Western blot analyses of G2/M cell cycle regulatory proteins. Cells were treated with their respective IC50 cisplatin (CP) (A2780, 4 µM; CP70, 40 µM), or CP plus 20 µM sodium arsenite (CPA) at 37 or 39 °C (hyperthermia) for 1 h, then washed with PBS and refed with fresh media and incubated at 37 °C. Cells lysates were prepared at 0, 24 and 36 h. ß-actin is the loading control. Blots shown are representative of 2 independent experiments.

Sodium arsenite and hyperthermia do not enhance mitotic index in cisplatin treated cells and also failed to induce histone H3 Ser10 phosphorylation

Data in Figure 21 suggest that combining sodium arsenite and hyperthermia with cisplatin is causing treated cells to arrest in mitosis. In order to confirm if indeed these cells are in mitosis, I determined mitotic index as described in Materials and Methods section. Adding sodium arsenite and/or hyperthermia to cisplatin did not increase the mitotic index in both A2780 and A2780/CP70 cells (Figure 22). The observed low mitotic index suggested that sodium arsenite and hyperthermia do not induce mitotic arrest in cisplatin treated cells.

Figure 22

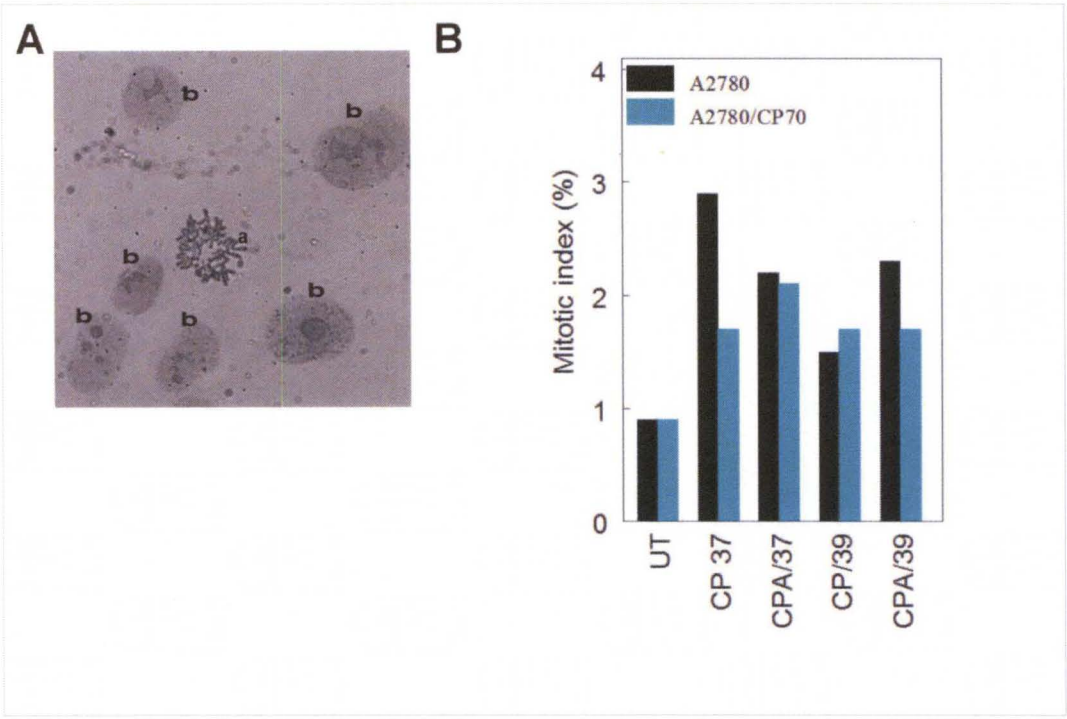

Figure 22. Mitotic index determination. A. Representative picture of (a) mitotic spread and (b) interphase nuclei. **B.** Plot of means of percentage of mitotic index for duplicate slides. Cells were treated with their respective IC50 cisplatin (CP) (A2780, 4 μM; CP70, 40 μM), or CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C (hyperthermia) for 1 h. Treated cells were washed with PBS and refed fresh media and incubated at 37 °C for 36 h. Mitotic index was determined at 36 h after treatment. Data are single biological experiments performed in duplicate dishes.

Mitotic index data in Figure 22 indicate that less than 3% of cells were undergoing mitotic arrest. In order to confirm this finding, I performed western blot analysis of histone H3 phosphorylated on Serine 10 (H3Ser10P), a mitotic marker, (Figure 23). H3Ser10P was undetected in both A2780 and A2780/CP70 cells treated with cisplatin ± sodium arsenite at 37 or 39 °C. These data confirmed that a large fraction of cells were not in mitosis.

Figure 23

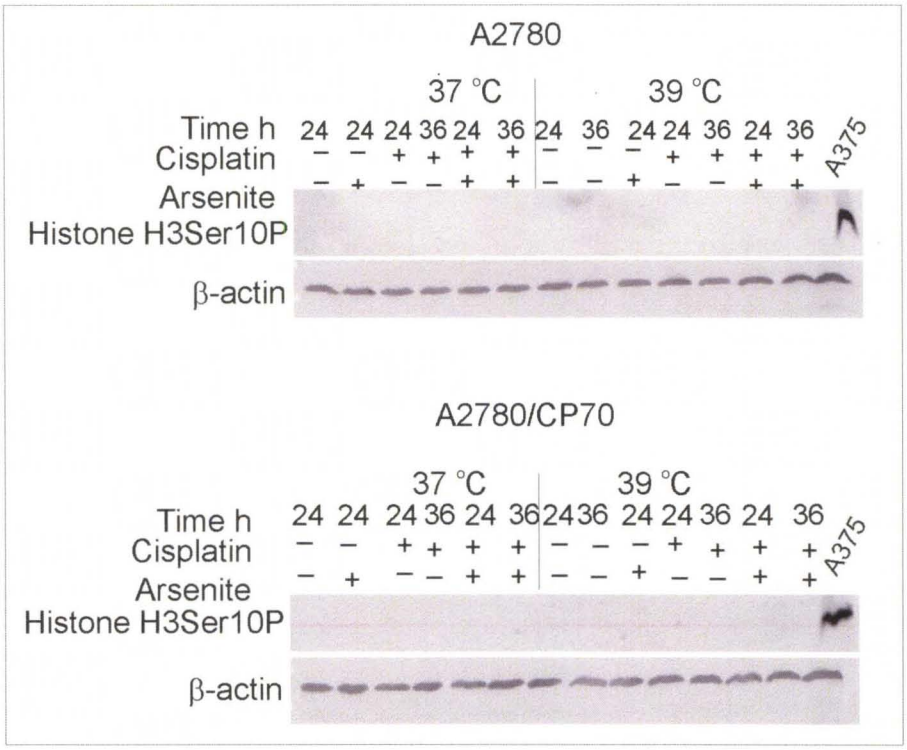

Figure 23. . Western blot analysis of protein marker of mitotic arrest.

Western blot analysis of H3Ser10P. Cells were treated with their respective IC50 cisplatin (CP) (A2780, 4 μM; A2780/CP70, 40 μM), or CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C (hyperthermia) for 1 h. Treated cells were washed with PBS and refed fresh media and incubated at 37 °C. Protein lysates were prepared at 36 h for western blot analysis. Data are representative from duplicate biological experiments. A375 cells were treated with 5 μM sodium arsenite for 24 h served as positive control for mitotic cells. ß-actin served as loading control.

Cisplatin, sodium arsenite and hyperthermia prevent BUBR1 phosphorylation

A functional spindle assemble checkpoint is required for mitotic arrest. The mitotic spindle checkpoint complex consists of MAD3/BUBR1, BUB3 and MAD2 (Tan, et al., 2005). The mitotic spindle checkpoint induces mitotic arrest by inhibiting anaphase onset by associating with CDC20, a subunit of the anaphase promoting complex (APC). Therefore, I determined if A2780 and A2780/CP70 cells have functional spindle checkpoint by determining the phosphorylation of BUBR1 following 100 nM taxol treatment for 16 h. I observed that taxol treatment induced BUBR1 phosphorylation in A2780 and A2780/CP70 cells (Figure 24), indicating that these cells have functional spindle assembly check point. However, cisplatin or its co-treatment with sodium arsenite and hyperthermia failed to induce BUBR1 phosphorylation (Figure 24); suggesting that mitotic spindle checkpoint is disrupted.

Figure 24

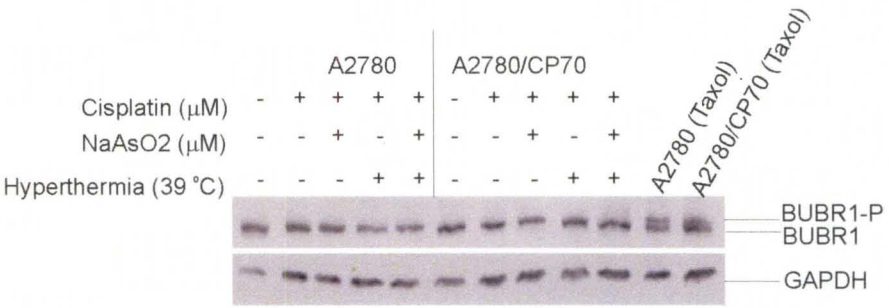

Figure 24. Western blot analysis of mitotic spindle assembly checkpoint protein. Western blot analysis of BUBR1 and phosphorylated BUBR1 in control lysates treated with 100 nM taxol 16 h. Cells were treated with their respective IC50 cisplatin (CP) (A2780, 4 μM; A2780/CP70, 40 μM), or CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C (hyperthermia) for 1 h. Treated cells were washed with PBS and refed fresh media and incubated at 37 °C. Protein lysates were prepared at 36 h after treatment for western blot analysis. Data are representative of triplicate biological experiments. GAPDH served as loading control.

Cisplatin, sodium arsenite and hyperthermia induced pseudo-G1 arrest in OC cells

Data in Figure 24 suggest that cisplatin ± sodium arsenite at 37 or 39 °C are disrupting the mitotic spindle assembly checkpoint in A2780 and A2780/CP70 cells. Absence of mitotic spindle checkpoint activation allows cells with damaged DNA to exit mitosis and progress through the cell cycle to G1 without dividing and end up with double DNA content (2C) (Lanni and Jacks, 1998). Therefore I determined if the cells were undergoing pseudo-G1 arrest by determining the expression of CDKN1A, pRbSer807/811P and cyclin E using western blot analysis. Data in Figure 25 suggest that cisplatin stabilized CDKN1A over time and decreased pRbSer807/811P at 36 h after treatment (Figure 25A panel a). Adding sodium arsenite ± hyperthermia (Figure 25A, panels c, b and d respectively) caused stronger CDKN1A induction and decreased levels of pRbSer807/811P compared with cisplatin alone at 37 °C (Figure 25, panels b, c and d). These data confirmed that G1 arrest is taking place at 36 h after treatment. I also observed that cyclin E was stabilized in both A2780 and A2780/CP70 cells when compared to mitotic positive A2780 and A2780/CP70 cells that did not express cyclin E (Figure 25B). These data suggest that cisplatin alone or combined with sodium arsenite and hyperthermia is inducing pseudo-G1 arrest in A2780 and A2780/CP70 cells.

Figure 25

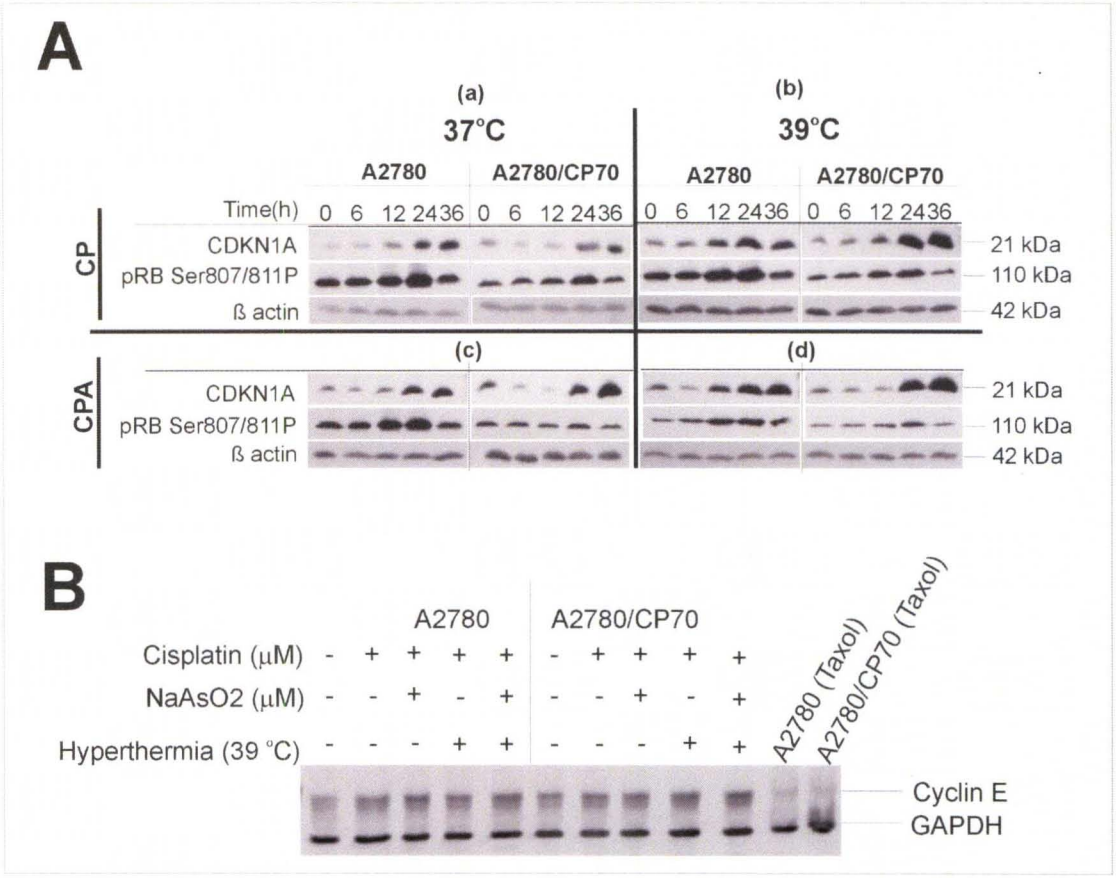

Figure 25. Western blot analysis of protein markers of G1 arrest. A. Western blot analysis of CDKN1A and pRbSer807/811P. Panel (a) is CP 37 ºC, (b) is CP 39 ºC, (c) is CPA 37 ºC and (d) is CPA39 ºC. **B.** Western blot analysis of cyclin E. Cells were treated with their respective IC50 cisplatin (CP; A2780, 4 μM; CP70, 40 μM), or CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C (hyperthermia) for 1 h. Cells were washed with PBS and refed with fresh media and incubated at 37 °C. Cell lysates were prepared at 0, 6, 12, 24, and 36 h. ß-actin and GAPDH are loading controls. A2780 and A2780/CP70 cells were treated with 100 nM Taxol for 16 h and served as negative control for cyclin E.

Cisplatin, sodium arsenite and hyperthermia induce apoptotic cell death in pseudo-G1 arresting cells

Data in Figure 25 suggest that cisplatin, sodium arsenite and hyperthermia are causing pseudo-G1 arrest in OC cells. I performed FITC Annexin V propidium iodide assay to determine if pseudo-G1 cells were undergoing apoptosis. Data suggest that cisplatin alone or combined with sodium arsenite and/or hyperthermia significantly induced apoptotic cell death (~15%) at 36 h after treatment (Figure 26).

Figure 26

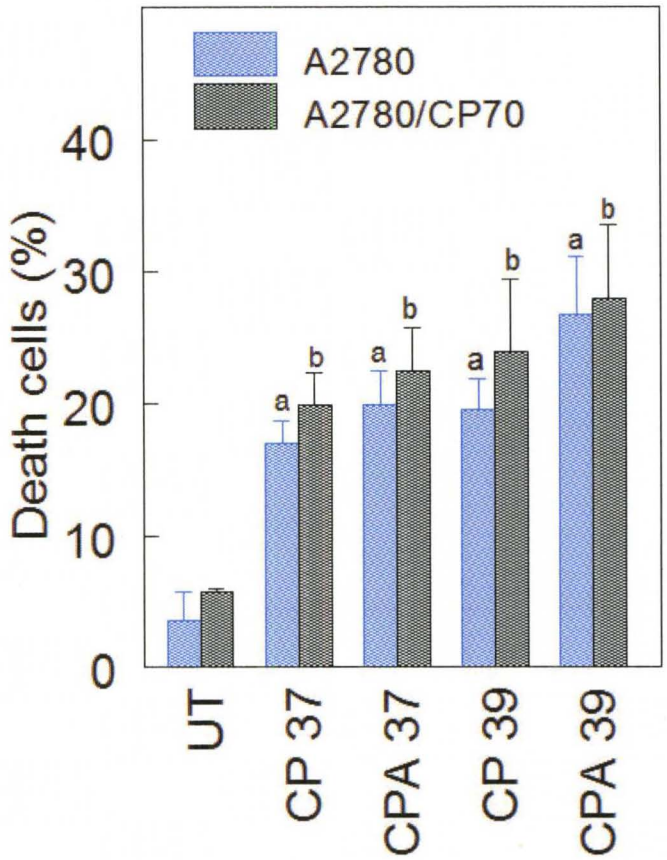

Figure 26. Apoptotic cell death determination using FITC Annexin V propidium iodide assay. Cells were treated with their respective IC50 cisplatin (CP) (A2780, 4 μM; CP70, 40 μM), or CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C (hyperthermia) for 1 h. Treated cells were washed with PBS and refed fresh media and incubated at 37 °C for 36 h. Cell death was determined at 36 h after treatment. Data are means ± SD of triplicate biological experiments. Statistically analysis was performed using one way ANOVA and Turkey test. $P<0.05$, a = compared to untreated (UT) A2780 cells, b = compared to untreated A2780/CP70 cells.

DISCUSSION

This study was aimed at determining if a new combination of sodium arsenite and hyperthermia altered cisplatin-induced G2 cell cycle arrest and induced mitotic arrest and mitotic arrest associated-apoptosis (mitotic catastrophe) in OC cells treated with cisplatin. I used a well characterized and widely used human OC cell model for cisplatin-sensitive (A2780) and its cisplatin-resistant subline (A2780/CP70) for this study. I showed that sodium arsenite and hyperthermia does not alter cisplatin-induced accumulation of cells in the G2/M compartment when analyzed by flow cytometry for DNA content. However, cisplatin alone or combined with sodium arsenite and/or hyperthermia failed to activate the mitotic spindle checkpoint which allowed cells to exit mitosis and enter pseudo G1 with 2C DNA content. These cells then underwent apoptotic cell death.

Flow cytometry determination of DNA content using propidium iodide does not distinguish between G2 and M cells because they both have 2C DNA content. Therefore I used western blot analysis to determine the expression of G2/M regulatory proteins cyclin A and cyclin B. Cyclin A is usually degraded before cells enter mitosis, whereas cyclin B is stabilized during mitosis (Malumbres and Barbacid, 2009). Stabilization of cyclin A and cyclin B by cisplatin (CP 37 ^{o}C) in both A2780 and A2780/CP70 cells suggest G2 arrest (Figure 21, panel a), consistent with previous findings (Cepeda, et al., 2007). Decreased expression of cyclin A and cyclin B by hyperthermia co-treatment with cisplatin (CP 39 ^{o}C) in A2780 cells is consistent with G1 arrest (Figure 21, panel

b). Decreased expression of cyclin A and cyclin B by sodium arsenite co-treatment with cisplatin (CPA 37 °C) in A2780 cells is consistent with G1 arrest (Figure 21, panel c). Sodium arsenite co-treatment with cisplatin (CPA 37 °C) decreased expression of cyclin A and stabilized cyclin B in A2780/CP70 cells (Figure 21, panel c), consistent with mitotic arrest. Sodium arsenite plus hyperthermia co-treatment with cisplatin (CPA 39 °C) attenuated expression of cyclin A and it stabilized cyclin B in both cell lines (Figure 21, panel d), consistent with mitotic arrest. However, mitotic arrest in A2780/CP70 cells treated with CPA 37 °C or in A2780 and A2780/CP70 cells treated with CPA 39 °C was not supported by mitotic index or histone H3Ser10P data (Figures 22 and 23 respectively). Sodium arsenite and hyperthermia did not increase mitotic index in cisplatin treated cells and undetected levels of histone H3 phosphorylation (H3Ser10P) clearly indicated that arsenite co-treated cells are not arresting in mitosis.

Mitosis is the phase of the cell cycle where cells divide to produce two genetically identical cells from one cell. In order to ensure proper division, the mitotic spindle checkpoint must ensure that all chromosomes are attached to the kinetochores by microtubules and that proper tension is exerted on the kinetochores before mitotic exit (Zhou, et al., 2002; Tan, et al., 2005). Cells with defective spindle checkpoint will proceed through the cell cycle with aberrant chromosomes. The mitotic spindle checkpoint complex consists of MAD3/BUBR1, BUB3 and MAD2 (Tan, et al., 2005). The mitotic spindle checkpoint inhibits anaphase onset by associating with CDC20, a subunit of the

anaphase promoting complex (APC). APC is an E3-ubiqitin ligase that mediates degradation of securin and cyclin B. Degradation of securin and cyclin B is required for anaphase onset and subsequent mitotic exit. Treatment of A2780 and A2780/CP70 cells with a mitotic arrest inducing drug taxol caused phosphorylation of BUBR1 in these cells (Figure 24). Phosphorylation of BUBR1 suggests that these cells have functional spindle assembly checkpoint. However, cisplatin ± sodium arsenite treatment at 37 or 39 °C failed to activate BUBR1phosphorylation in OC cells (Figure 24). Disruption of mitotic spindle checkpoint may allow cells to exit mitosis without undergoing cytokinesis.

In response to DNA damage, p53 is stabilized and activated and it transcriptionally activates CDKN1A expression. Induced CDKN1A binds to and inhibits CDK2/cyclin E complex, preventing the phosphorylation of pRb, thus blocking cells in G1. I observed strong induction of CDKN1A and suppression of pRb Ser807/811P (Figure 25) at 36 h after treatment suggested that G1 arrest is taking place. Accumulation of cells in G1 was supported by the stabilization of cyclin E (Figure 25B). The data clearly suggest that the cells accumulating in the G2/M compartment underwent endoreduplication and exited mitosis without cytokinesis and subsequently accumulated in G1 with 2C DNA content. The phenomenon of cells with disrupted spindle checkpoint undergoing endoreduplication and exiting mitosis without cytokinesis was previously observed by Lanni and Jacks (Lanni and Jacks, 1998). Lanni and Jacks showed that when mouse embryo fibroblasts were treated with spindle inhibitor nocodazole, these cells accumulated transiently in mitosis and progressed into

G1 with 4N DNA content. These cells were classified as pseudo-G1 cells because they showed upregulated cyclin E and hypophorylated pRb, but had 4N DNA content similar to G2/M cells. Cells with disrupted spindle checkpoint are expected to be resistant to sodium arsenite induced mitotic arrest and apoptosis (McNeely, et al., 2008b; Wu, et al., 2008). However, these pseudo-G1 cells underwent apoptotic cell death in response to cisplatin treatment and co-treatment with sodium arsenite and/or hyperthermia resulted in greater percentage of dead cells (Figure 26).

In summary, I have shown for the first time that cisplatin ± sodium arsenite at 37 or 39 ^{o}C disrupts the mitotic spindle checkpoint and causes cells to exit mitosis without dividing and subsequently accumulating in G1. These pseudo-G1 cells with 2C DNA content stabilized cyclin E and induced CDKN1A and decreased pRb Ser807/811P. In addition these pseudo-G1 cells underwent apoptotic cell death. These data suggest potential new mechanisms by which cisplatin or its combination with sodium arsenite and hyperthermia induce cell death in wild-type p53 expressing OC cells.

CHAPTER 5

HSP90 INHIBITOR 17-DMAG ROBUSTLY ENHANCES THE CYTOTOXICITY OF COMBINED CISPLATIN, SODIUM ARSENITE AND HYPERTHERMIA AGAINST OC

INTRODUCTION

Ovarian cancer is the most common cause of gynecologic cancer death in women in the United States (Jemal, et al., 2010). Cisplatin or carboplatin with taxane is used in the frontline treatment of OC following cytoreductive surgery (McGuire, et al., 1996). Inherent and/or acquired resistance decreases effectiveness of platinum-based chemotherapy, and only about 20% of women survive for 5 years once diagnosed with OC (Armstrong, 2002; Rubin, et al., 1999). Thus, finding a new effective treatment regimen to mitigate cisplatin resistance is highly needed.

Cisplatin exerts its cytotoxic effect by causing DNA damage. In addition to its effect on DNA, cisplatin binds to non-DNA targets such as glutathione, metallothionein, protein and RNA (Cepeda, et al., 2007). Cisplatin also binds to the C-terminus of HSP90 and interferes with ATP binding (Donnelly and Blagg, 2008; Landriscina, et al., 2010). Binding of cisplatin to non-DNA targets may give rise to cytotoxic oxidative and electrophile stress (Jacobs and Marnett, 2010). Presence of cellular stress induces drug metabolizing enzymes and antioxidant proteins such as hemoxygenase (HO-1), quinine oxidoreductase (NQO1) and

nicotinamide adenine dinucleotide phosphate (NAD(P)H) via NRF2 binding to antioxidant and electrophile response elements (Nerland, 2007). In addition, heat shock factor 1 (HSF1) protects against oxidative and electrophile-induced toxicity by inducing pro-survival heat shock genes such as HSP70, HSP110 and HSP40 that function to fold denatured proteins (Landriscina, et al., 2010). These defensive mechanisms additionally contribute to cisplatin resistance. Combination chemotherapy has been implemented to enhance cisplatin cytotoxicity. I previously showed that combined sodium arsenite and hyperthermia sensitized wild-type p53 expressing OC cells to cisplatin (Figure 3, Chapter 2) by suppressing XPC (Figure 6, Chapter 2) and enhancing cellular (Figure 10B, Chapter 2) and DNA (Figure 12, Chapter 2) accumulation of platinum. Cisplatin and sodium arsenite can generate oxidative and electrophile stress and hyperthermia can generate oxidative and heat stress (Jacobs and Marnett, 2010). Cellular response to stress involves induction of stress response proteins to protect the cells from dying.

The goal of this study is to investigate if inhibiting the activity of the molecular chaperone, HSP90 will sensitize both p53 (+) and p53 (-) OC cells to cisplatin. Cisplatin, sodium arsenite and hyperthermia induced HSP60 and HSP70 and stabilized HSP90 in A2780 and A2780/CP70 cells. PARP-1 cleavage was observed in these cells, suggesting that apoptosis is occurring. Inhibition of HSP90 activity using 17-(Dimethylaminoethyamino)-17-demethoxygeldanamycin (17-DMAG) remarkably potentiated the cytotoxicity of combined cisplatin, sodium arsenite and hyperthermia (CPA 39 °C) against OC cells in a p53-independent

manner. Enhanced cellular accumulation of cisplatin was observed with 17-DMAG co-treatment with CPA 39 ºC. These data suggest that 17-DMAG has the potential to reverse cisplatin resistance when co-treated with CPA 39 ºC.

HYPOTHESIS

Cisplatin, sodium arsenite and hyperthermia induce cellular stress. Inhibition of HSP90, a key modulator of stress response will potentiate the cytotoxicity of combined cisplatin, sodium arsenite and hyperthermia against OC.

MATERIALS AND METHODS

Chemicals

Stock and working solutions of 17-DMAG (17-(Dimethylaminoethyamino)-17-demethoxygeldanamycin) (InvivoGen, San Diego, California, # NSC 707545) were prepared in sterile water and stored at -20 °C protected from light.

Cell Viability assay

The growth inhibitory effects of cisplatin, sodium arsenite, hyperthermia and 17-DMAG were evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell viability assay (Mosmann, 1983) as described in the Materials and Methods section of Chapter 2 with some modifications. Briefly, 2500 cells/well were seeded overnight in 96-well plates. Next day, cells were treated with cisplatin ± 20 μM sodium arsenite and/or 100 nM of 17-DMAG at 37 or 39 °C for 1 h. After treatment, cells were washed twice with PBS and refed with drug-free medium and incubated at 37 °C for 5 days prior to assay. Blank was cells treated with 0.1 mg/mL hygromycin B. MTT assay was measured at 570 nm. The absorbance values corresponded to the number of viable cells. Cell viability was calculated as follows and plotted against concentration of cisplatin.

$$\% \text{ cell viability} = \frac{100 \text{ x (mean of triplicate treated samples - Blank)}}{\text{(Mean of triplicate untreated samples - Blank)}}$$

Data were expressed as means ± SEM of at least four independent experiments. Each experiment was done with triplicate wells for each treatment condition.

Western blot analyses

Total cellular lysates were prepared at 2 and 24 h after treatment. Cell lysates were prepared and proteins were separated on 10 % SDS-polyacrylamide gel electrophoresis as previously described in Materials and Methods section of Chapter 2. Membranes were probed with mouse monoclonal antibodies for HSP90 (F-8, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1: 1000), ß-actin (Sigma, # A 5441, dilution 1:10,000), or rabbit polyclonal antibodies for PARP-1, HSP70 (Cell Signaling Technology, dilution 1:1000) and HSP60 (Chemicon International, Millipore, Temecula, CA, 1:1000). Secondary antibodies (rabbit anti-mouse IgG, # 81-6120 or goat anti-rabbit, # 81-6120, dilution 1:2500) conjugated to horseradish peroxidase (Zymed Laboratories, Inc. South San Francisco, CA) were bound to primary antibodies and protein bands detected using enhanced chemiluminescence (ECL) substrate (Pierce, Rockford, IL) followed by exposure to Kodak XAR x-ray film. ß-actin was used as the loading control.

Platinum accumulation studies

Cells (5 X 10^5/6 cm dish) were treated with 40 µM cisplatin ± 20 µM sodium arsenite with or without 100 nM of 17-DMAG at 37 or 39 °C for 1 h. Samples were prepared and analyzed using ICP-MS as previously described in Materials and Methods section in Chapter 2.

Statistical analysis

Results were expressed as the mean ± SD of three independent experiments. Statistical analyses were performed using Microsoft Excel built in

formulas for one-way analysis of variance and student's t-test with significance level as $p < 0.05$, $n = 3$.

RESULTS

Cisplatin, sodium arsenite and hyperthermia induced stress response proteins in OC cells

Treatment of cells with cisplatin or sodium arsenite can generate reactive oxygen species and electrophiles that may cause oxidative DNA, lipid, RNA and protein damage (Sahin, et al., 2010; Davison, et al., 2002). In addition, hyperthermia may generate heat stress and denature important cellular proteins (Hildebrandt, et al., 2002). Thus, combined cisplatin, sodium arsenite and hyperthermia may enhance cytotoxic cellular stress. Following cellular stress, heat shock proteins are induced to fold denatured proteins and protect against stress-induced cell death. This experiment was aimed at investigating if cisplatin, sodium arsenite and hyperthermia induce stress response proteins in OC cells in response to cellular stress. Cells were treated as described in Materials and Methods Section. HSP90 was unchanged for all treatment conditions. HSP60 and HSP70 were induced at 2 and 24 h after treatment (Figure 27). Robust induction of HSP70 occurred at 24 h after treatment for all treatment conditions and in both cell lines. These data suggest that cisplatin, sodium arsenite and hyperthermia both individually and in combination induce cellular stress.

Figure 27

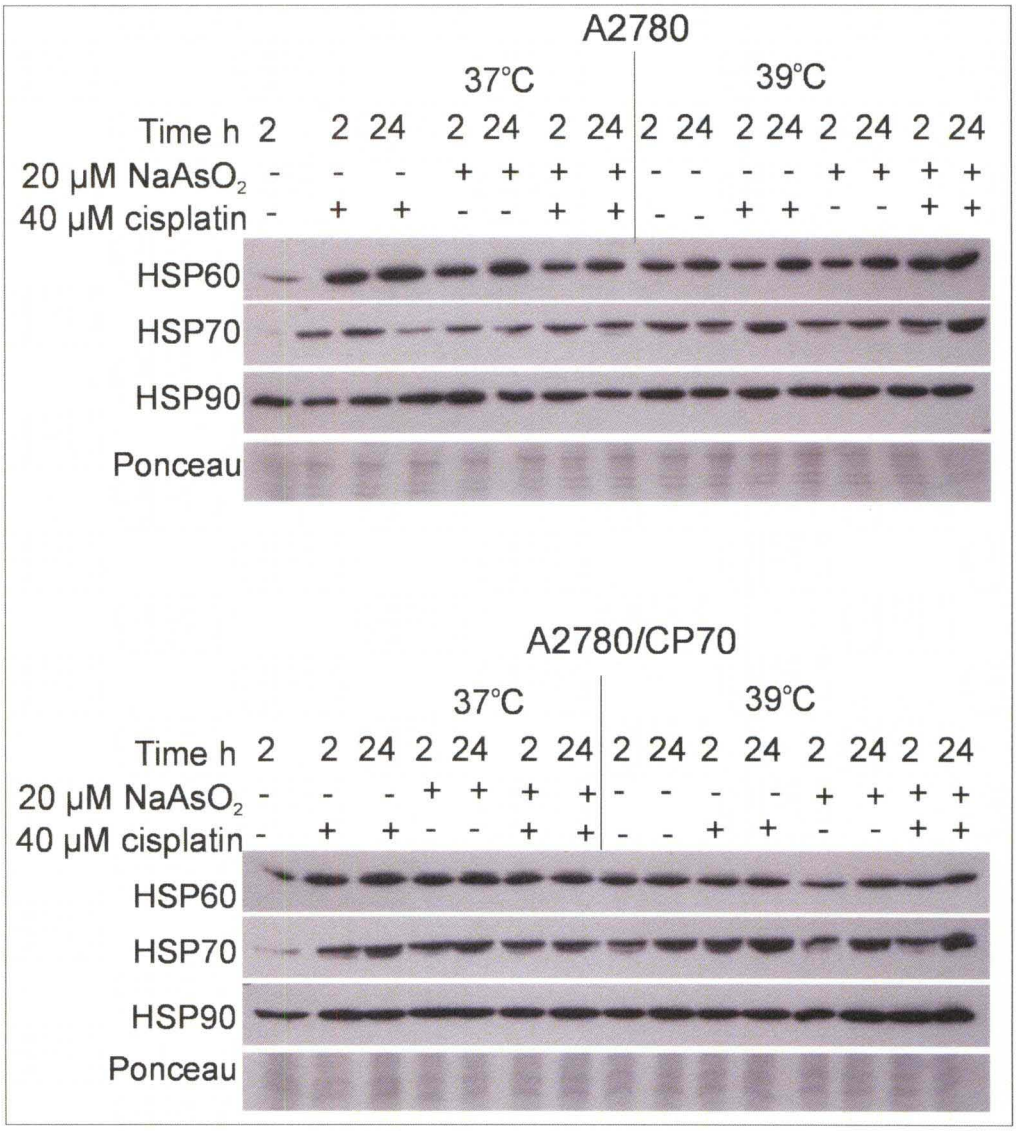

Figure 27. Western blot analysis of stress response proteins. A2780 and A2780/CP70 cells were treated with their IC50 cisplatin (CP) (A2780, 4 μM; CP70, 40 μM), CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C for 1 h. After treatment, cells were washed with 1X PBS, incubated in fresh media at 37 °C. Protein lysates were collected at 2 and 24 h after treatment for western blot analysis. Blots are representative of two independent experiments. Ponceau stained picture serves as loading control.

Cisplatin, sodium arsenite and hyperthermia induce apoptotic cell death in OC cells

Data in Figure 27 indicate that cisplatin, sodium arsenite and hyperthermia each induced stress response proteins HSP60 and HSP70 and did not change HSP90 levels. I then determined if induction of stress response proteins is associated with apoptotic cell death. Western blot analysis technique was used to determine PARP-1 cleavage (Figure 28). Data indicate that PARP-1 is cleaved 24 h after treatment, suggesting that apoptotic cell death is occurring in these cells at 24 h.

Figure 28

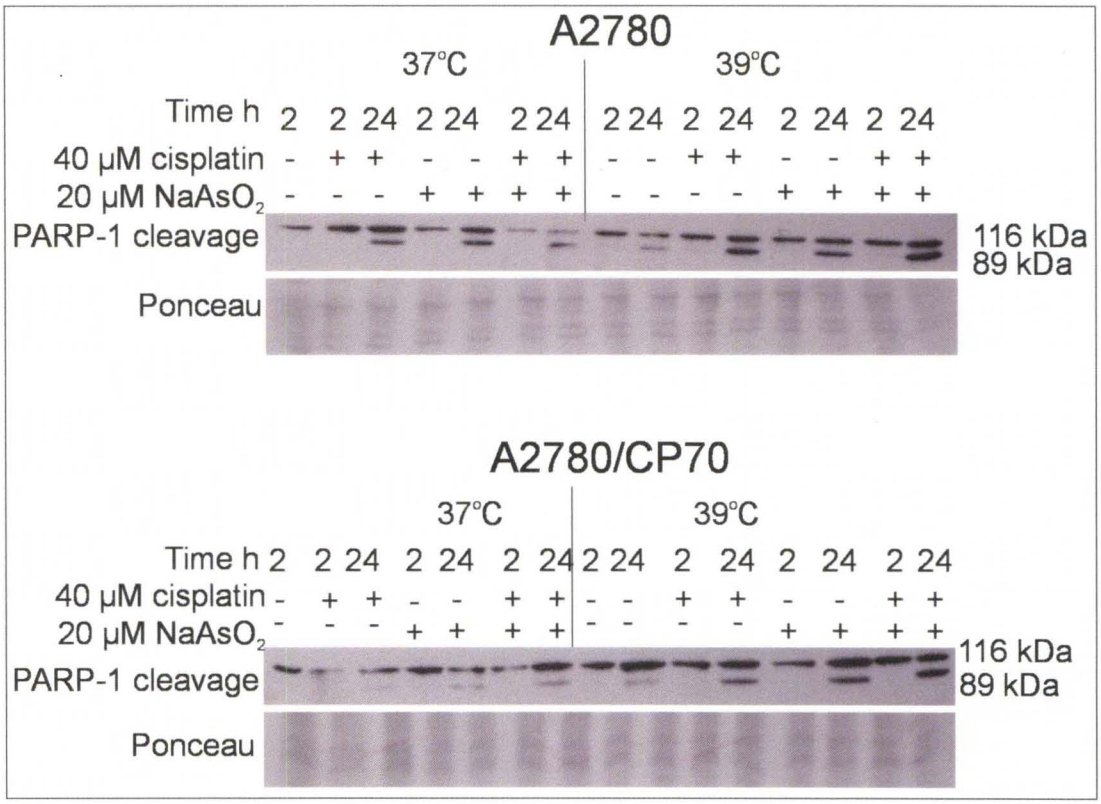

Figure 28. Western blot analysis of apoptotic proteins. A2780 and A2780/CP70 cells were treated with their IC50 cisplatin (CP) (A2780, 4 μM; CP70, 40 μM), CP plus 20 μM sodium arsenite (CPA) at 37 or 39 °C for 1 h. After treatment, cells were washed with 1X PBS, incubated in fresh media at 37 °C. Protein lysates were collected at 2 and 24 h for western blot analysis. Blots are representative of two independent experiments. Ponceau stained picture serves as loading control.

HSP90 inhibitor 17-DMAG potentiates cisplatin, sodium arsenite and hyperthermia cytotoxicity against OC

Data in Figure 27 suggest that cisplatin, sodium arsenite and hyperthermia are inducing cellular stress as evident by the induction of HSP60 and HSP70, while HSP90 remained stable. Inhibiting the activity of heat shock proteins may enhance the cytotoxicity of anticancer agents. HSP90 is a druggable target because it is ubiquitously expressed in most cancer cells and it is integrally involved in cell signaling, survival and proliferation (Taipale, et al., 2010). Furthermore, several HSP90 inhibitors have been extensively investigated as anticancer agents and most are undergoing clinical trial (Messaoudi, et al., 2011). I determined if inhibition of HSP90 activity using 17-DMAG would potentiate the cytotoxicity of cisplatin, sodium arsenite and hyperthermia against OC. I used a non-toxic dose of 17-DMAG (100 nM) (Figure 29) to treat the cells.

Figure 29

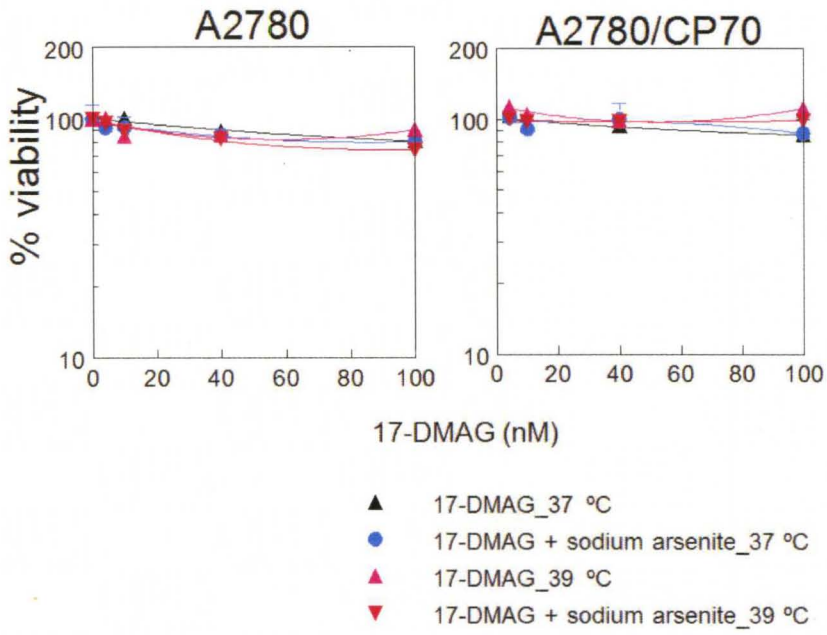

Figure 29. Cell viability effect of 17-DMAG on sodium arsenite ± hyperthermia. Cells were treated with the indicated concentrations of 17-DMAG ± 20 μM sodium arsenite at 37 or 39 °C for 1 h. Cells were then washed twice with PBS and refed with fresh media and incubated for 5 days at 37 °C. Cell viability MTT assay was performed 5 days after treatment. Data are expressed as percentage of untreated control and plotted as means ± SD of at least four independent experiments each performed with triplicate wells.

Cell viability data suggest that 17-DMAG moderately enhanced the sensitivity of wild-type p53 expressing A2780 and A2780/CP70 cells to cisplatin (CP 37 °C) or cisplatin plus arsenite (CPA 37 °C) or cisplatin plus hyperthermia (CP 39 °C) (Figure 30A). Combined 17-DMAG and CPA 39 °C greatly decreased cell viability (Figure 30B). P53-null SKOV-3 cells were not sensitized by 17-DMAG when co-treated with CP 37 °C or CPA 37 °C (Figure 30A, right panel). However, 17-DMAG enhanced the cytotoxicity of CP 39 °C and CPA 39 °C in SKOV-3 cells (Figure 30B, right panel). The enhancement of CPA 39 °C was greater than that of CP 39 °C in SKOV-3 cells.

Figure 30

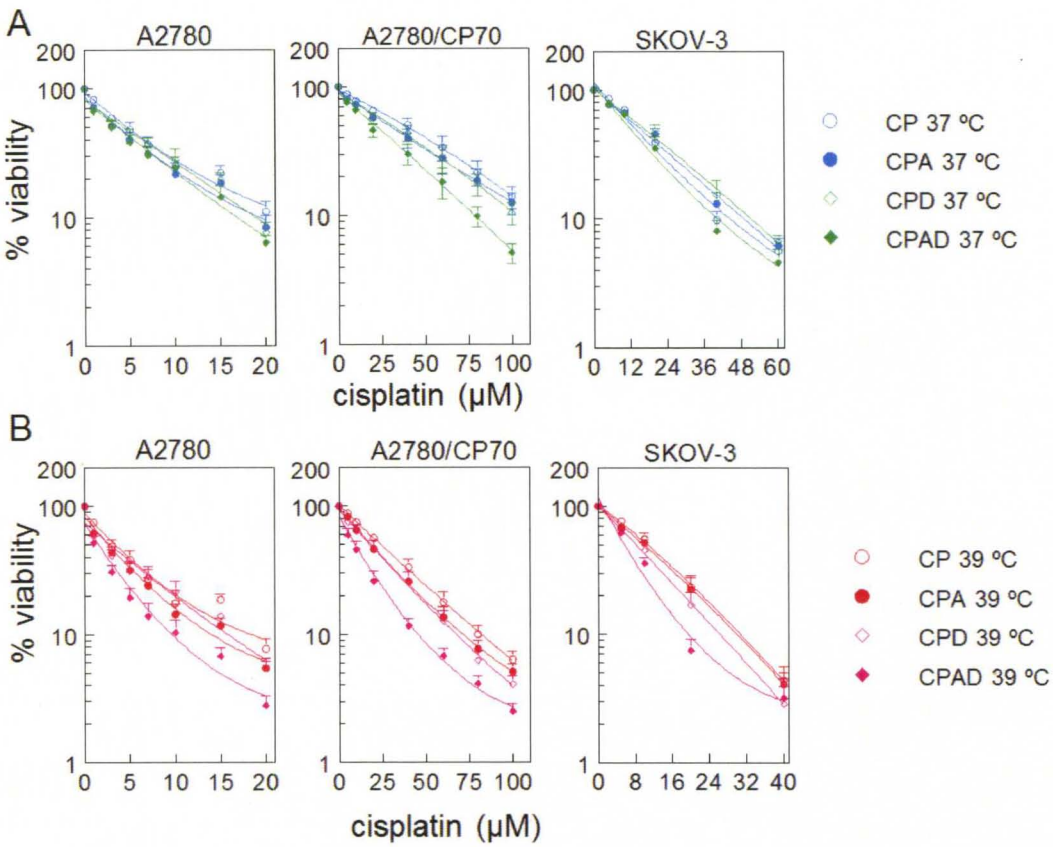

Figure 30. Cell viability as determined by MTT assay. **A.** Cells treated at 37 °C. **B.** Cells treated at 39 °C. Cells were treated with the indicated concentrations of cisplatin with (CPA) or without (CP) ± 20 μM sodium arsenite in the presence (diamond symbol) or absence (circle symbol) of 100 nM of 17-DMAG at 37 or 39 °C for 1 h. Cells were then washed twice with PBS and refed with fresh media and incubated for 5 days at 37 °C. Cell viability MTT assay was performed 5 days after treatment. Data are expressed as percentage of untreated control and plotted as means ± SEM of at least four independent experiments each performed with triplicate wells. R-values for the best fitting polynomial curves were all >0.99.

HSP90 inhibitor 17-DMAG enhanced cellular accumulation of cisplatin

Data in Figure 30B suggest that 17-DMAG has the potential to reverse cisplatin resistance when used in combination with cisplatin, sodium arsenite and hyperthermia (CPA 39 °C). In order to understand the mechanism involved in 17-DMAG sensitization, I determined if 17-DMAG enhanced cellular accumulation of cisplatin because decreased cellular accumulation of cisplatin is an important mechanism of resistance (Parker, et al., 1991). The results suggest that 17-DMAG moderately increased platinum accumulation in A2780 and A2780/CP70 cells when co-treated with CPA 39 °C (CPAD 39) (Figure 31, left and center panels). Cisplatin accumulation in SKOV-3 cells was significantly higher (Figure 31, right panel).

Figure 31

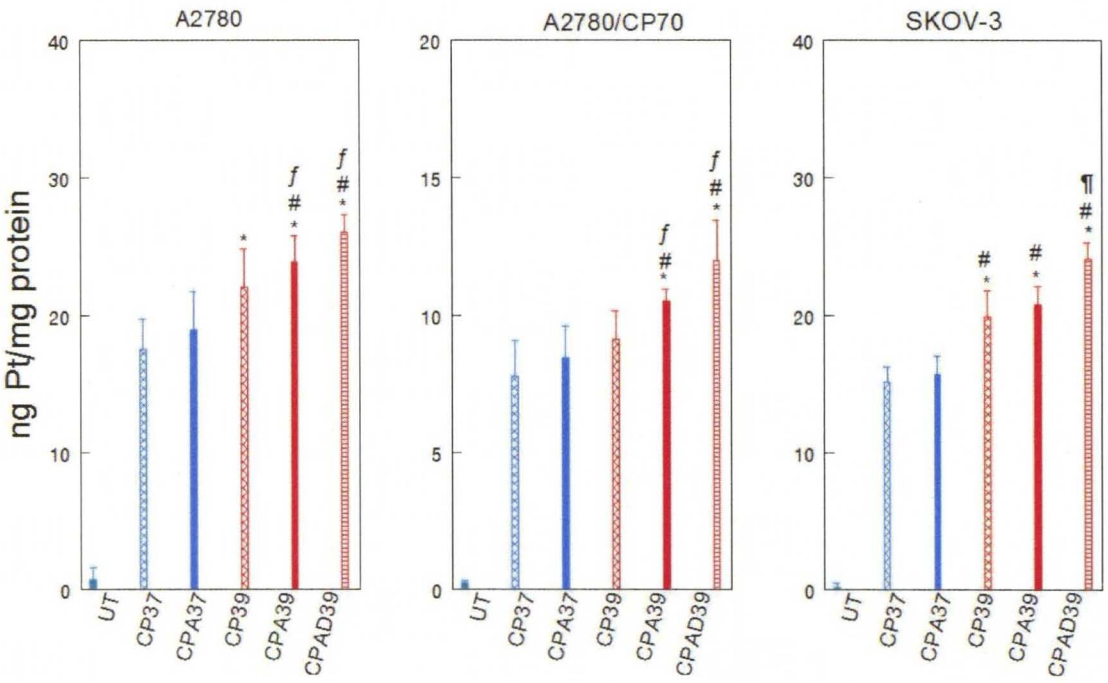

Figure 31. ICP- MS analysis of cellular platinum accumulation. A2780, A2780/CP70 and SKOV-3 cells were treated with 40 μM cisplatin (CP37 and CP39)) ± 20 μM sodium arsenite (CPA37 and CPA39) and/or 100 nM of 17-DMAG (CPAD39) at 37 or 39 °C for 1h. Cells were harvested immediately after treatment for total cellular platinum determination. Data are means ± SD from 3 independent experiments. N = 3, $P<0.05$. ∗ compared with CP37, # compared with CPA37, *f* compared with CP39 and ¶ compared with CPA39.

DISCUSSION

Cisplatin remains the drug of choice to treat OC. However, resistance limits clinical success. Combination chemotherapy is at the forefront to improve the efficacy of cisplatin and its analogues against OC. I showed in chapter 2 that combined sodium arsenite and hyperthermia (CPA 39 °C) sensitized wild-type p53 expressing cells to cisplatin by mitigating mechanisms of cisplatin resistance such as inhibiting DNA repair protein XPC and enhancing cellular platinum accumulation and platinum bound to DNA. A potential mechanism of cytotoxicity for combined cisplatin, sodium arsenite and hyperthermia (CPA 39 °C) is induction of oxidative, electrophile and heat stress (Davison, et al., 2002; Pandita, et al., 2009; Hildebrandt, et al., 2002; Jacobs and Marnett, 2010). Cellular stress may denature key proteins required for signal transduction, cell survival and proliferation by cancer cells. Heat shock proteins are intimately involved in refolding these denatured proteins to the correct conformation, thereby promoting survival (Landriscina, et al., 2010). Therefore, inhibiting heat shock protein function could sensitize cells to anticancer agents. The goal of this study was to investigate if inhibition of HSP90 activity would further sensitize OC cells to the combination of cisplatin, sodium arsenite and hyperthermia (CPA 39 °C) in a p53-independent manner. HSP90 inhibitor 17-DMAG at a non-toxic concentration potentiated CPA 39 °C cytotoxicity against OC cells. Presence of cellular stress was evident by the induction of HSP60 and HSP70 in response to cisplatin ± sodium arsenite treatment at 37 or 39 °C. In addition, PARP-1

cleavage occurred at 24 h after treatment, suggesting that apoptotic cell death is occurring following cellular stress induction.

HSP90 is a highly conserved and ubiquitously expressed in most tissues and cancer cells. It is a molecular chaperone and forms complexes with HSP70, HSP40, CDC37/p50, p23, AHA1 and accessory molecules such as HSP organizing protein (HOP), HSP-interacting protein (HIP) and immunophilin (Taipale, et al., 2010). HSP90 chaperone complex acts on its client proteins to stabilize them and prevent aggregation, facilitate cell membrane crossing, stabilize conformations and target client proteins for degradation (Taipale, et al., 2010). Because HSP90 regulates diverse cellular processes that promote cell survival and proliferation, it is a good molecular target to sensitize cancer cells to anticancer drugs. Cisplatin binds to HSP90 at its C-terminus and inhibits its ATP binding ability (Donnelly and Blagg, 2008; Landriscina, et al., 2010). In addition to the C-terminus domain, the N-terminal domain has an ATP binding site that is highly conserved. HSP90 inhibitors such as geldanamycin and 17-DMAG compete with ATP for this binding site. ATP binding and hydrolysis is required for HSP90 function. Several inhibitors of the ATP binding site of HSP90 are undergoing clinical trials as anticancer agents (Messaoudi, et al., 2011). In the current study, I have showed that HSP60 and HSP70 were induced and HSP90 remained unchanged in response to CPA 39 °C. Inhibition of HSP90 activity using 17-DMAG potentiated CPA 39 °C cytotoxicity in A2780, A2780/CP70 and SKOV-3 cells. The robust cytotoxicity observed with 17-DMAG co-treatment with CPA 39 °C could be due partially to enhanced cellular accumulation of cisplatin.

In summary, I have showed that co-treatment with HSP90 inhibitor 17-DMAG and CPA 39 °C (CPAD 39 °C) may reverse cisplatin resistance in OC cells independent of p53 status. Co-treatment enhanced cellular accumulation of cisplatin. Further studies are needed to better understand the precise mechanisms involved in 17-DMAG enhancement of the CPA 39 °C cytotoxicity.

CHAPTER 6

OVERALL DISCUSSION AND CONCLUSIONS

The goal of this research project was to mitigate cisplatin resistance in OC. I investigated the effect of a new combination of cisplatin, sodium arsenite and hyperthermia against OC cells. Sodium arsenite alone or combined with hyperthermia selectively sensitized wild-type p53 expressing human OC cells (A2780, A2780/CP70, OVCA 420, OVCA 429 and OVCA 433) to cisplatin (Figure 3A). In contrast, only hyperthermia sensitized p53-null (SKOV-3) and p53-mutated (OVCAR-3 and OVCA 432) cells to cisplatin (Figure 3B). Sodium arsenite selective sensitization of p53 expressing cells to cisplatin could be due to its effect on the nucleotide excision repair (NER) pathway that repairs platinum-DNA damage and confers resistance to cisplatin. Arsenic has been shown to inhibit induction of the global genome repair – NER protein XPC (Nollen, et al., 2009). XPC is intimately involved in the repair of platinum-DNA damage (Neher, et al., 2010). P53 is implicated in platinum-DNA repair because it transcriptionally regulates XPC (Ford, 2005), which is required for efficient global genome repair. I have shown that cisplatin induces XPC only in wild-type p53 expressing cells and in tumors derived from p53 expressing A2780/CP70 cells. XPC siRNA transfection enhanced cisplatin cytotoxicity, indicating that induction of XPC confers resistance to cisplatin. Furthermore, sodium arsenite ± hyperthermia inhibited XPC in p53 expressing OC

cells and in tumors derived from p53 expressing A2780/CP70 cells. These data indicated that sodium arsenite selectively sensitized p53 expressing cells to cisplatin by suppressing XPC. Inhibition of XPC will decrease the recruitment of downstream NER proteins and suppress DNA repair (Nollen, et al., 2009).

In addition to enhanced DNA repair, decreased cellular platinum and platinum bound to DNA contributes to cisplatin resistance (Parker, et al., 1991). Hyperthermia ± sodium arsenite enhanced cellular and platinum bound to DNA in p53 expressing A2780 and A2780/CP70 cells. Whereas, only hyperthermia enhanced cellular and platinum bound to DNA in p53 null SKOV-3 cells. Platinum accumulation in xenograft tumors was enhanced by sodium arsenite co-treatment with cisplatin at 37 or 39 °C. GST activity was not altered by cisplatin alone or by its combination with sodium arsenite and/or hyperthermia, suggesting that the GSH/GST system does not contribute to cisplatin resistance in these cells.

In response to cisplatin treatment, p53 induces G2 arrest by transcriptionally activating CDKN1A, GADD45 and 14-3-3σ which inhibit cyclin B/CDK1 to prevent cell cycle progression through G2 phase (Taylor and Stark, 2001). Cell cycle analysis data suggested that A2780 and A2780/CP70 cells treated with cisplatin ± sodium arsenite at 37 or 39 °C accumulated in the G2/M compartment 36 h after treatment. Western blot analyses of cyclin A and cyclin B suggested that sodium arsenite ± hyperthermia is caused cisplatin treated cells to arrest in mitosis. However, lack of histone H3Ser10P and relative low mitotic index clearly indicated that cells are not in mitosis. Furthermore, I observed that

cisplatin alone or combined with sodium arsenite and hyperthermia did not induce spindle checkpoint protein BUBR1 phosphorylation. Since the spindle assembly complex is dependent on BUBR1 phosphorylation for it to function, lack of BUBR1 phosphorylation weakens the mitotic spindle checkpoint and cause cells to exit mitosis without dividing (McNeely, et al., 2008b). Similar to a report by Lanni and Jacks (Lanni and Jacks, 1998), these postmitotic cells had biochemical properties similar to G1 cells: induction of CDKN1A, stabilization of cyclin E and hypophosphorylation of pRb. Thus, cells with 2C DNA content that accumulated in the G2/M compartment were postmitotic or pseudo-G1 cells that failed to divide in mitosis. These cells underwent apoptosis. These data suggest a new mechanism by which cisplatin alone or combined with sodium arsenite and/or hyperthermia induces cell death.

Cisplatin, sodium arsenite and hyperthermia induce stress-mediated cell death by generating reactive oxygen and electrophile species (Del Razo, et al., 2001; Pandita, et al., 2009). These reactive species can cause oxidative damage to DNA, protein, RNA and lipids. Stress response proteins promote survival by refolding unfolded proteins and degrading damaged proteins. These stress response proteins or heat shock proteins therefore confer resistance to anticancer drugs (Landriscina, et al., 2010). In response to cisplatin, sodium arsenite and hyperthermia treatment, I observed induction of HSP60 and HSP70 in A2780 and A2780/CP70 cells as early as 2 h after treatment and robustly at 24 h. In addition, HSP90 was stabilized in these cells. These data suggested that cisplatin, sodium arsenite and hyperthermia generated cellular stress that led to

the induction of stress response proteins. I also observed PARP-1 cleavage at 24 h after treatment, suggesting that apoptotic cell death occurred in response to cellular stress. HSP90 is ubiquitously expressed in cancer cells and it regulates signaling pathways, cell survival and proliferation (Taipale, et al., 2010), making it a good molecular target in cancer cells. HSP90 inhibitor 17-DMAG greatly sensitized A2780, A2780/CP70 and SKOV-3 cells to combined cisplatin, sodium arsenite and hyperthermia (CPA 39 °C). This sensitization was associated with enhanced cellular platinum accumulation.

Cancer cell populations are heterogeneous and not homogenous because cancer cells frequently undergo mutations and alterations in key genes such as p53 (Zeimet and Marth, 2003). Immunocytochemistry data suggest that A2780/CP70 cell population is heterogeneous in p53 expression. About 75% of the cells expressed functional p53 while 25% did not express the protein. Also, about 6% of tumors derived from A2780/CP70 cells were p53 null. Presence of heterogeneity makes it ineffective to treat cancer with a single drug or multiple drugs aimed at single key regulatory protein such as p53 or a signaling pathway. Therefore, My findings that combined cisplatin, sodium arsenite and hyperthermia (CPA 39 °C) suppressed DNA repair protein, enhanced cellular platinum and platinum bound to DNA accumulation, induced cytotoxic cellular stress, disrupted mitotic spindle checkpoint and caused cells to accumulate in pseudo-G1 and subsequently undergo apoptosis, indicate that this new combination chemotherapy has multiple mechanisms of sensitizing cancer cells to cisplatin and inducing cell death. Thus, this new combination therapy may be

beneficial clinically in treating patients with cisplatin resistant disease regardless of the p53 status. Adding 17-DMAG to counteract the heat shock protein response will potentially reverse cisplatin resistance. Therefore CPA 39 °C or CPA 39 °C plus 17-DMAG delivered intraperitoneally may be useful in increasing overall survival of OC patients. In the future, *in vivo* survival studies using the metastatic OC model and murine HIPEC system is required in order to determine if this combination chemotherapy is effective *in vivo* to suppress tumor burden. Long-term survival data generated from the *in vivo* studies will be useful in determining the potential clinical application of the new combination of cisplatin, sodium arsenite and hyperthermia (CPA 39 ^{o}C) and also CPA 39 ^{o}C plus 17-DMAG.

CHAPTER 7

FUTURE STUDIES

In the future, long-term survival studies are needed to determine response of peritoneal tumors to the new combination of cisplatin, sodium arsenite and hyperthermia (CPA 39 °C) and also CPA 39 °C plus 17-DMAG. These studies will require a non-invasive method of visualizing the tumors. Therefore, one means of visualizing is to establish OC cells stably expressing ß actin-pTurboFP635 (katushka) fusion protein. These cells can be injected into the peritoneum of nude mice to establish metastatic tumors stably expressing red fluorescent katushka protein. Tumors with red fluorescent katushka protein should be easily visualized by non-invasive fluorescence imaging. Tumor bearing mice will be treated with cisplatin, sodium arsenite and/or 17-DMAG at 37 or 39 °C for 1 h using the HIPEC model. Mice will be maintained for up to 60 days and response to chemotherapy will be determined by fluorescence imaging of tumors. The fluorescence intensity will be used to calculate the tumor burden. Data generated from this survival study will provide a better understanding of the *in vivo* response of peritoneal tumors to this new combination chemotherapy and its potential clinical use.

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APPENDIX

Abbreviations

APC	Anaphase promoting complex
APL	Acute promyelocytic leukemia
BSA	Bovine serum albumin
BUBR1	Budding uninhibited by benzimidazoles related 1
CDK	Cyclin dependent kinase
CP	Cisplatin
CP37	Cisplatin at 37 °C
CP39	Cisplatin at 39 °C
CPA	Cisplatin plus sodium arsenite
CPA37	Cisplatin plus sodium arsenite at 37 °C
CPA39	Cisplatin plus sodium arsenite at 39 °C
DDB2	DNA damage binding protein 2
DMSO	Dimethyl sulfoxide
17-DMAG	17-(Dimethylaminoethyamino)-17-demethoxygeldanamycin
ERCC1	Excision repair cross-complementing 1
FBS	Fetal bovine serum
GADD45	Growth arrest and DNA damage-inducible protein 45
GGR	Global genome repair

GSH	Glutathione
GST	Glutathione S-transferase
HIPEC	Hyperthermic intraperitoneal chemotherapy
ICP-MS	Inductively coupled plasma mass spectrometry
MSH2	MutS homolog 2 protein
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
$NaAsO_2$	Sodium arsenite
NER	Nucleotide excision repair
NSC	Non-specific control
OC	Ovarian cancer
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
p53Ser15P	p53 phosphorylated on Ser15
pRBS807/811P	Retinoblastoma protein phosphorylated on Ser807/811
Pt	Patinum
TCR	Transcription Coupled repair
XPA	Xeroderma pigmentosum group A protein
XPC	Xeroderma pigmentosum group C protein

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EDUCATION

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08/2006 – 08/2008	University of Louisville, Louisville, KY
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08/2003 – 08/2005	East Tennessee State University, Johnson City, TN
	M.S. in Chemistry

09/1999 – 12/2002 University of Buea, Buea, Cameroon

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PROFESSIONAL EXPERIENCE

2005 - 2006 Quality Assurance Analyst, GlaxoSmithKline Pharmaceutical Company, Bristol, TN.

2002-2003 High School Chemistry Teacher, Comprehensive High School Bambui, Bamenda, Cameroon.

HONORS & AWARDS

03/2011 Society of Toxicology - Metals Specialty Section 3rd place outstanding research award and travel award to attend the 50th annual SOT meeting.

03/2010 Society of Toxicology - Metals Specialty Section 3rd place outstanding research award and travel award to attend the 49th annual SOT meeting.

01/2010 Recipient of the University of Louisville CGeMM travel award to attend the 49th annual SOT meeting.

03/2009 Honorary Mention, National Society of Toxicology, Poster presentation to visiting undergraduate students.

01/2009 Society of Toxicology travel award to attend annual SOT meeting.

11/2008 Battelle Minority/Women Travel award to attend annual SOT meeting.

11/2008 University of Louisville Graduate Student Council travel award to attend annual SOT meeting.

04/2008 University of Louisville School of Medicine Research Committee travel award to attend annual SOT meeting.

04/2008 University of Louisville Graduate student Council travel award to attend annual SOT meeting.

11/2007 Honorable Mention, Ohio Valley Society of Toxicology Student poster.

08/06-06/08 University of Louisville Integrated Programs in Biomedical Sciences Predoctoral Fellowship Award.

04/2006 Best Masters Thesis Award, Faculty of Art and Science, East Tennessee State University.

10/2003 Margaret Sells Memorial Scholarship Award in recognition of outstanding achievement in the field of chemistry in the college of Arts and Sciences, East Tennessee State University.

11/2002 University of Buea, Buea, Cameroon, 7th annual convocation award:

- The Prime Minister's prize for the best female student in the University
- Senate prize for the best student in Chemistry
- Professor Chumbow's prize in memory of Prof. Johnson Foyere Ayafor for the best student in Chemistry

- FOPROW's prize for the best female undergraduate student
- LESAN's prize for the best female student in the University
- Brasserie's prize for the best female student
- Guinness prize for the best student in Chemistry

PROFESSIONAL SOCIETIES

American Association for Cancer Research

National Society of Toxicology

Society of Toxicology - Metals Specialty section

Ohio Valley Chapter of the Society of Toxicology

PUBLICATIONS

Articles published in peered reviewed journals

1. **Muenyi CS**, States VA, Masters JH, Fan TW, Helm CW and States JC (2011) Sodium arsenite and hyperthermia modulate cisplatin-DNA damage responses and enhance platinum accumulation in murine metastatic OC xenograft after hyperthermic intraperitoneal chemotherapy (HIPEC). *J Ovarian Res* **4**:9.

Articles in preparation

1. **Clarisse S. Muenyi**, Teresa W. Fan, C. William Helm, and J. Christopher States. Combined sodium arsenite and hyperthermia selective sensitization of

wild-type p53 human OC cells to cisplatin involves suppression of XPC and enhancement of cellular and DNA platinum accumulation.

2. **Clarisse S. Muenyi**, Teresa W. Fan, C. William Helm, and J. Christopher States. Cisplatin, sodium arsenite and hyperthermia induce postmitotic cell death in OC cells.
3. **Clarisse S. Muenyi**, Teresa W. Fan, C. William Helm, and J. Christopher States. Hsp90 inhibitor 17-DMAG robustly enhances the cytotoxicity of combined cisplatin, sodium arsenite and hyperthermia against OC

Published abstracts

1. **Clarisse S. Muenyi**, Vanessa A. States, Joshua H. Masters, Teresa Fan, C. William Helm, J. Christopher States. "Sodium arsenite and hyperthermia alter expression of XPA, XPC and MSH2 in response to cisplatin-induced DNA damage and increase accumulation of platinum in OC". 50th annual Society of Toxicology meeting, Washington, D.C. (March 2011)
2. **Clarisse S. Muenyi**, Vanessa A. States, Joshua H. Masters, Teresa Fan, C. William Helm, J. Christopher States. "Murine HIPEC model for study of in vivo effects of chemotherapy against metastatic human OC" 49th annual Society of Toxicology meeting, Salt Lake City, UT (March 2010)
3. **Clarisse Muenyi**, Abhaya A. Pandit, Teresa Fan, C. William Helm, J. Christopher States. "Augmentation of cisplatin cytotoxicity associated with altered DNA damage response and cellular Platinum accumulation" 48th annual Society of Toxicology meeting, Baltimore (March 2009)

4. V. A. States, J. H. Masters, **C. S. Muenyi**, J. States and C. Helm. "Mouse Model for treating metastatic human OC with hyperthermic intraperitoneal chemotherapy" 48th annual Society of Toxicology meeting, Baltimore (March 2009)
5. **Clarisse Muenyi**, Abhaya A. Pandit, J.C. States. "Arsenite modifies p53 responsive gene products after cisplatin DNA damage in OC cells" Society of Toxicology. Seattle, Washington (March 2008)

Abstracts for Regional and National meetings

1. **Clarisse S. Muenyi**, Teresa Fan, C. William Helm, J. Christopher States. "Arsenic and hyperthermia sensitization of p53+/+ OC cells to cisplatin is associated with decreased XPC protein and increased cellular platinum accumulation" 12th annual MidWest DNA Repair Symposium, Louisville, KY (May, 2010)
2. **Clarisse S. Muenyi**, Vanessa A. States, Joshua H. Masters, Teresa Fan, C. William Helm, J. Christopher States. "Murine HIPEC model for study of in vivo effects of chemotherapy against metastatic human OC" University of Louisville Graduate Research Symposium (March 2010)
3. **Clarisse S. Muenyi**, Vanessa A. States, Joshua H. Masters, Teresa Fan, C. William Helm, J. Christopher States. "Murine HIPEC model for study of in vivo effects of chemotherapy against metastatic human OC" Ohio Valley Society of Toxicology annual meeting, Cincinnati, OH (November 2009)

4. **Clarisse Muenyi**, Abhaya A. Pandit, Teresa Fan, C. William Helm, J. Christopher States. "Co-treatment with sodium arsenite and hyperthermia sensitizes cisplatin-resistant OC cells to cisplatin by suppressing XPC induction and increasing drug uptake" Research Louisville (October 2009)
5. **Clarisse Muenyi**, Abhaya A. Pandit, Teresa Fan, C. William Helm, J. Christopher States. "Augmentation of cisplatin cytotoxicity associated with altered DNA damage response and cellular Platinum accumulation" 11th annual DNA Repair Symposium, Michigan (May 2009)
6. **Clarisse Muenyi**, Abhaya A. Pandit, Teresa Fan, C. William Helm, J. Christopher States. "Augmentation of cisplatin cytotoxicity associated with altered DNA damage response and cellular Platinum accumulation" University of Louisville Graduate Research Symposium (March 2009)
7. **Clarisse Muenyi**, Abhaya A. Pandit, Teresa Fan, C. William Helm, J. Christopher States. "Augmentation of cisplatin cytotoxicity associated with altered DNA damage response and cellular Platinum accumulation" Ohio Valley Chapter Society of Toxicology (November 2008)
8. **Clarisse Muenyi**, Abhaya A. Pandit, Teresa Fan, C. William Helm, J. Christopher States. "Augmentation of cisplatin cytotoxicity associated with altered DNA damage response and cellular Platinum accumulation" Research Louisville (October 2008)
9. **Clarisse Muenyi**, Abhaya A. Pandit, J. Christopher States. "Arsenite and hyperthermia modify the levels of XPC, DDB2 and p21$^{CIP1/WAF1}$ after

cisplatin DNA damage in OC cells" 10th Annual Midwest DNA Repair Symposium (May 2008)

10. **Clarisse Muenyi**, Abhaya A. Pandit, J.C. States. "Arsenite modifies p53 responsive gene products after cisplatin DNA damage in OC cells" Ohio Valley Chapter Society of Toxicology (November 2007)
11. **Clarisse Muenyi** and J.C. States. "Resistance of Ovarian Cancer Cells to Cisplatin is Not Due to Differential Induction of XPC and DDB2" Research Louisville (October 2007)
12. **Clarisse Muenyi**, Heather L. Miller and J.C. States. "Combating cisplatin-resistance in OC: concurrent treatment with arsenic and/or hyperthermia"9th Annual Midwest DNA Repair Symposium (April 2007)

PLATFORM PRESENTATIONS

1. **Clarisse S. Muenyi**, Teresa Fan, C. William Helm, J. Christopher States. "Arsenic and hyperthermia sensitization of p53+/+ OC cells to cisplatin is associated with decreased XPC protein and increased cellular platinum accumulation" 12th annual MidWest DNA Repair Symposium, Louisville, KY (May, 2010)
2. **Clarisse S. Muenyi**, Vanessa A. States, Joshua H. Masters, Teresa Fan, C. William Helm, J. Christopher States. "Murine HIPEC model for study of in vivo effects of chemotherapy against metastatic human OC" Ohio Valley Society of Toxicology annual meeting, Cincinnati, OH (November 2009)