

Enhancing Tumor-Specific Uptake of the Anticancer Drug Cisplatin with a Copper Chelator

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SUMMARY

Uptake of the anticancer drug cisplatin is mediated by the copper transporter CTR1 in cultured cells. Here we show in human ovarian tumors that low levels of *Ctr1* mRNA are associated with poor clinical response to platinum-based therapy. Using a mouse model of human cervical cancer, we demonstrate that combined treatment with a copper chelator and cisplatin increases cisplatin-DNA adduct levels in cancerous but not in normal tissues, impairs angiogenesis, and improves therapeutic efficacy. The copper chelator also enhances the killing of cultured human cervical and ovarian cancer cells with cisplatin. Our results identify the copper transporter as a therapeutic target, which can be manipulated with copper chelating drugs to selectively enhance the benefits of platinum-containing chemotherapeutic agents.

INTRODUCTION

Cisplatin is widely used for the treatment of solid tumors. It is a component of first-line treatment for testicular, ovarian, cervical, endometrial, bladder, head and neck, lung, and gastroesophageal cancers (Hussain et al., 2008; Omura, 2008; Gallagher et al., 2008; Yang et al., 2009; Lustberg and Edelman, 2007; Fischer and Arcaro, 2008; Gen and Ajani, 2007). It is also used as a second- or third-line treatment for prostate and pancreatic cancers, metastatic cancer of the breast, and for melanoma and gliomas (Carrick et al., 2004; Oh et al., 2007; Saif and Kim, 2007; Atallah and Flaherty, 2005; Glioma Meta-Analysis Trialists Group, 2002). The dosage and efficacy of cisplatin, however, are limited by its side effects, the most prominent being nephrotoxicity (Pabla and Dong, 2008).

Cisplatin exerts its cytotoxic effect by forming an intrastrand crosslink on DNA (Jamieson and Lippard, 1999). Studies of cisplatin-resistant cell lines have revealed drug uptake as a critical step that governs cisplatin sensitivity in vitro (Hall et al., 2008). A yeast genetic screen for cisplatin-resistant mutants identified the copper transporter *CTR1* as a major mediator of

cisplatin uptake in yeast and mouse cells (Ishida et al., 2002). Upon exposure to excess copper, Ctr1 protein undergoes endocytosis and degradation (Ooi et al., 1996; Petris et al., 2003). Pretreatment of cells with high copper results in decreased cisplatin uptake and increased resistance to this drug in a *CTR1*-dependent manner (Ishida et al., 2002), whereas chelating copper with bathocuprione disulphonate has opposite effects (S.I. and I. Herskowitz, unpublished data). These results demonstrated that cisplatin uptake can be modulated by copper levels in vitro through Ctr1p.

In this study, we examined the in vivo roles of *Ctr1* and copper in cisplatin uptake and response using a mouse model of human cervical cancer. Cervical cancer is the second most common cause of cancer-related death among women worldwide. Cisplatin has been one of the most active single agents and a component of combined-agent chemotherapy regimens for patients with advanced cervical cancer. The response rates for cisplatin alone range from 13%–19%, with an overall survival time of 6–9 months; the current combined-agent therapy is only modestly better, with response rates of 27%–36%, with overall survival of 8–10 months (Tao et al., 2008). Cervical cancer

Significance

Inefficient drug delivery and attendant toxicity represent impediments to successful chemotherapy. We present a strategy to mitigate these limitations for the anticancer drug cisplatin, whose uptake is mediated by the copper transporter CTR1. We show that therapeutic preconditioning with a copper chelator enhances cisplatin uptake and killing of cancer cells without affecting normal organs in a mouse model of cervical cancer. Concordantly, *Ctr1* levels in human ovarian tumors correlate with the efficacy of platinum-based chemotherapy. These results encourage clinical trials aimed to upregulate CTR1 in tumors with copper chelators so as to improve the benefits and potentially expand the scope of platinum-based chemotherapy.

is caused by integration and persistent expression of genes of human papilloma viruses (HPV), of which HPV type 16 is detected in a majority of cases of invasive carcinoma (Castellsague, 2008). In the mouse model of cervical carcinoma, K14-HPV16/E₂, the HPV16-encoded oncogenes are under the control of the keratin 14 promoter, resulting in targeted oncogene expression in keratin 14-positive squamous epithelial cells, a natural host-cell target for HPV infection in humans (Arbeit et al., 1996). When the estrogen levels of K14-HPV16 females are maintained at a level near their natural peak during estrus with time-released implants of 17 β -estradiol (estrogen, E₂), the female mice (referred to as HPV16/E₂ mice hereafter) develop progressive cervical intraepithelial neoplasia (CIN) lesions at the transformation zone, the site implicated in the genesis of human cervical cancer (Elson et al., 2000). By 6 months of age, these CIN lesions progress to invasive squamous cell carcinoma in 90% of the animals. Both male and female K14-HPV16 mice also develop dysplasia of the skin, and skin tumors arise later, with 50% penetrance by 12 months of age (Coussens et al., 1996).

Using this mouse model of human cervical carcinoma, HPV16/E₂, we ask whether a copper chelator selectively increases cisplatin uptake and killing of tumor cells without affecting normal organs, thereby increasing the antitumoral efficacy of cisplatin. We also analyze human ovarian tumors for expression of the copper transporter *Ctr1* to evaluate its association with clinical response to platinum drugs, and assess the capability of the copper chelator to enhance killing of human ovarian and cervical cancer cell lines with cisplatin.

RESULTS

Human *Ctr1* mRNA Levels in Tumors Are Associated with Clinical Response to Platinum-Based Chemotherapy in Ovarian Cancer

Previous studies using mouse embryonic fibroblasts (MEFs) deleted for the mouse *Ctr1* gene demonstrated a correlation between *Ctr1* gene dosage and cisplatin sensitivity in vitro: cells that are heterozygous for a knockout allele of mouse *Ctr1* were more resistant than wild-type cells to cisplatin, and homozygous mutant cells were more resistant than heterozygous cells (Ishida et al., 2002). These findings raised the hypothesis that *Ctr1* mRNA levels correlate with and thus explain differential clinical responses to platinum drugs. To address this possibility, we analyzed tumor specimens and the clinical course of 15 ovarian cancer patients. The standard treatment for advanced ovarian cancer is primary cytoreductive surgery followed by chemotherapy that includes a platinum drug. Resistance to platinum-based chemotherapy remains a primary factor in disease recurrence. Figure 1A lists the baseline characteristics of the 15 patients, all with advanced-stage (stage III or IV), serous epithelial ovarian carcinoma. The tumors were of mixed grade (Figure 1A). Following optimal cytoreductive surgery resulting in residual tumors of 1 cm or less in diameter, the patients received regimens containing either cisplatin or carboplatin, and paclitaxel. Prior clinical studies have revealed equal efficacy for the two platinum compounds (Ozols et al., 2003; du Bois et al., 2003). The mechanisms of resistance are thought to be similar for the two drugs, which share the same putative trans-

porter CTR1 (Holzer et al., 2006). Of the 15 patients, 9 proved to be sensitive to platinum-based therapy (no evidence of recurrence within 6 months after completion of primary therapy), whereas the remaining 6 were refractory or resistant (progression or recurrence within 6 months of completion of therapy, as indicated by an increase in CA-125 levels, or by CT, ultrasonography, or second-look laparoscopy) (see Figure S1A available online). RNA was prepared from these patients' tumor samples, and *Ctr1* mRNA levels were measured by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). The mean relative *Ctr1* mRNA level was substantially higher in platinum-sensitive patients than in refractory/resistant patients (43% versus 18% of internal control gene expression, $p = 0.016$) (Figure 1B). Expression levels of the internal control gene human *GUS*, which encodes β -glucuronidase, were equivalent between the two patient groups (average \pm standard deviation [SD] Ct in sensitive group was 28.2 ± 0.8 versus 28.1 ± 1.3 in resistant/refractory group, $p = 0.69$) (Figure S1B). These findings suggest that *Ctr1* expression in ovarian tumors is associated with platinum drug response.

In order to validate these findings in an independent data set, we used clinical and array-based expression data from The Cancer Genome Atlas (TCGA) that are deposited at the Data Coordinating Center for public access (<http://cancergenome.nih.gov/>). We defined a subset of 91 patients with stage III or IV serous epithelial ovarian cancer who had undergone a cytoreductive surgery followed by adjuvant chemotherapy consisting of a platinum drug and a taxane (<http://tcga-data.nci.nih.gov/tcga/homepage.htm>) (Figure 1C). Results for *Ctr1* expression were extracted from expression data that were produced using Affymetrix HT Human Genome U133A Array Plate Set (HT_HG-U133A) (<http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm>). The patients were divided into two groups by the median *Ctr1* expression value, resulting in 45 "high *Ctr1*" (above median) and 46 "low *Ctr1*" (below median) patients (Figure S1C). High expression of *Ctr1* was associated with increased disease-free survival (Figure 1D; log-rank statistic 8.67, hazard ratio 0.53, $p = 0.003$), supporting our hypothesis that *Ctr1* levels are prognostic of treatment outcome. These findings suggest that mRNA levels of the human copper transporter *Ctr1* in tumors are clinically relevant to the efficacy of platinum therapy.

Ctr1 mRNA Levels Correlate with Cisplatin Adduct Levels in Mouse Tissues

To address the in vivo role of CTR1 in cisplatin uptake, we examined the levels of *Ctr1* mRNA and cisplatin-induced DNA adducts in various tissues of HPV16/E₂, the mouse model of human cervical cancer. Overall, the levels of cisplatin adducts correlated with *Ctr1* mRNA in most organs tested—skin, lung, liver, pancreas, and uterus (Figure 2). Kidney contained the highest amount of cisplatin adducts and *Ctr1* mRNA, which is interesting in light of the prevalence of nephrotoxicity among cisplatin-treated patients. The colon contained fewer adducts relative to *Ctr1* mRNA levels. In the duodenum and intestine, CTR1 protein is localized to the apical surface facing the intestinal lumen and in intracellular compartments (Nose et al., 2006; Kuo et al., 2006). It is possible that such localized CTR1 in the digestive tract is not involved in the uptake of cisplatin, which is administered intravenously to patients, and in this experiment was supplied

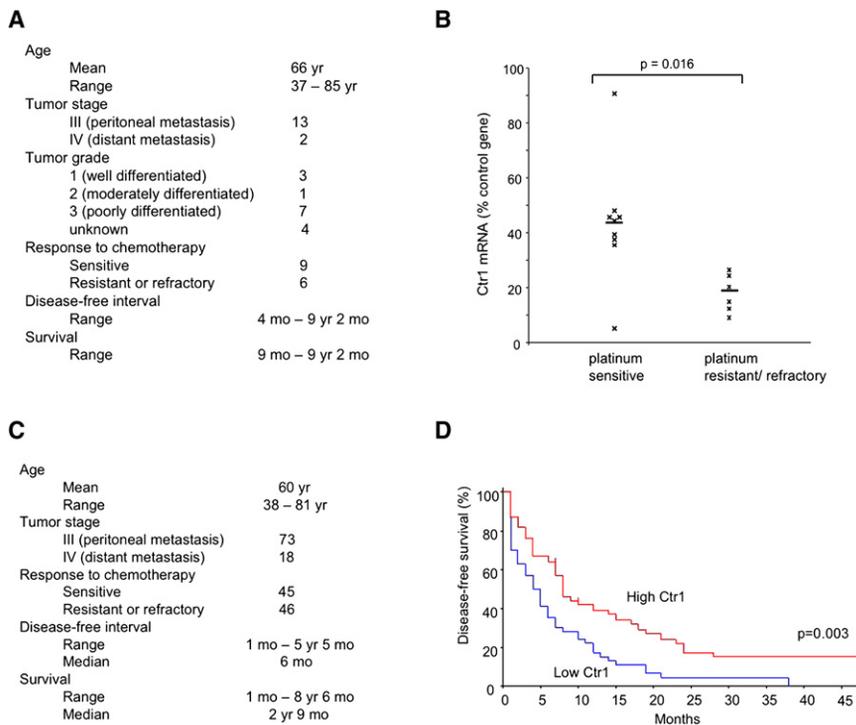


Figure 1. Low Levels of *Ctr1* mRNA in Ovarian Tumors Are Associated with Poor Clinical Outcome

(A) Baseline characteristics of the 15 patients with advanced-stage epithelial ovarian carcinoma, whose tumors were analyzed for relative expression of the *Ctr1* copper transporter. (B) Tumor *Ctr1* mRNA levels in platinum sensitive and refractory/resistant ovarian cancer patients. Bars represent mean values. *Ctr1* mRNA levels are expressed as a percentage relative to the internal control of human *GUS* mRNA. (C) Characteristics of 91 ovarian cancer patients from The Cancer Genome Atlas study. (D) Kaplan-Meier estimate of disease-free survival for 91 advanced-stage ovarian cancer patients according to *Ctr1* expression levels. Patients whose tumors expressed *Ctr1* mRNA at levels higher than the median value were classified as “High *Ctr1*” (red; 45 patients); the remaining patients were labeled “Low *Ctr1*” (blue; 46 patients). The vertical hash marks (at 7 and 10 months, High *Ctr1*) represent censored patients who were disease-free at the last follow-up time indicated. See also Figure S1.

intraperitoneally. Notably, the cancerous HPV16/E₂ cervix contained more adducts relative to *Ctr1* mRNA levels. Expression in the cervix of the internal control gene mouse *L19*, to which *Ctr1* mRNA was normalized, was equivalent to that of other

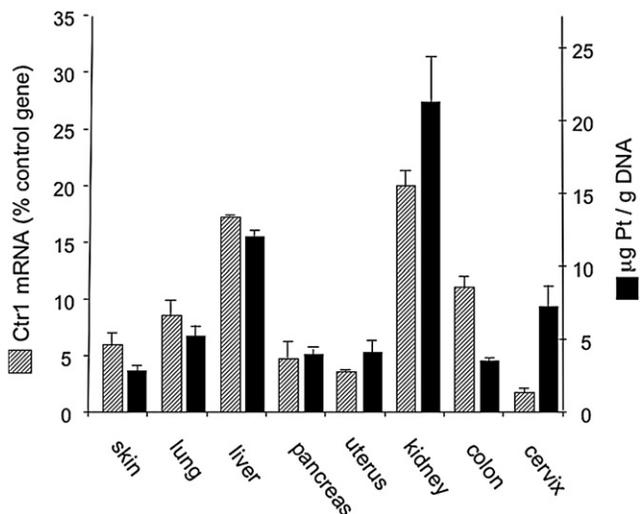


Figure 2. Levels of *Ctr1* mRNA and Cisplatin Adducts in Various Organs of the HPV16/E₂ Female Mice

Organs were harvested from three 6-month-old HPV16/E₂ females and *Ctr1* mRNA levels were determined by quantitative RT-PCR (hatched bars). Mouse *L19* mRNA levels were used as internal controls. For measurement of cisplatin adducts, genomic DNA was purified from each organ (n = 8) 2 hr after 6 mg/kg cisplatin was injected. Platinum was measured by ICP-MS and normalized to the amount of DNA (solid bars). Error bars represent the standard error of the mean. See also Figure S2.

tissues (average ± SD tissue *L19* Ct 23.5 ± 1.1 versus 23.3 in the cervix) (Figure S2A), indicating that the observed low levels of *Ctr1* mRNA in the cervix is not a result of higher internal control gene expression in the tumors. Despite the low *Ctr1* mRNA levels, CTR1 protein was substantially expressed in the cancerous cervix (Figure 3D), suggesting increased protein synthesis and/or reduced protein turnover in this neoplastic tissue. We also measured mRNA levels of the copper exporters, *ATP7A* and *ATP7B*, which are involved in exporting cisplatin in vitro (Komatsu et al., 2000; Samimi et al., 2004). Consistent with prior reports (Bull et al., 1993; Tanzi et al., 1993), *ATP7B* was primarily expressed in the liver (Figure S2B). We were able to detect *ATP7A* mRNA in all the tissues tested, but did not observe an inverse correlation between *ATP7A* mRNA levels and tissue platinum levels, further supporting our proposition that the copper transporter *Ctr1* is the major determinant for cisplatin accumulation in vivo as well.

The Copper Chelator Tetrathiomolybdate Increases Cisplatin Adduct Levels in Tumors but Not Normal Organs

Having observed an in vivo correlation between levels of the copper transporter *Ctr1* and cisplatin adducts in the majority of tissues examined, we next asked whether reducing systemic copper could result in increased cisplatin adducts in tumors. We treated HPV16/E₂ mice with the copper chelator tetrathiomolybdate (TM), which is currently granted orphan designation in the United States and Europe for the treatment of Wilson’s disease, an autosomal recessive disorder characterized by excess copper deposition in various organs. TM reduces bio-available copper levels, primarily by forming a tripartite complex with ingested copper and protein, preventing the absorption of

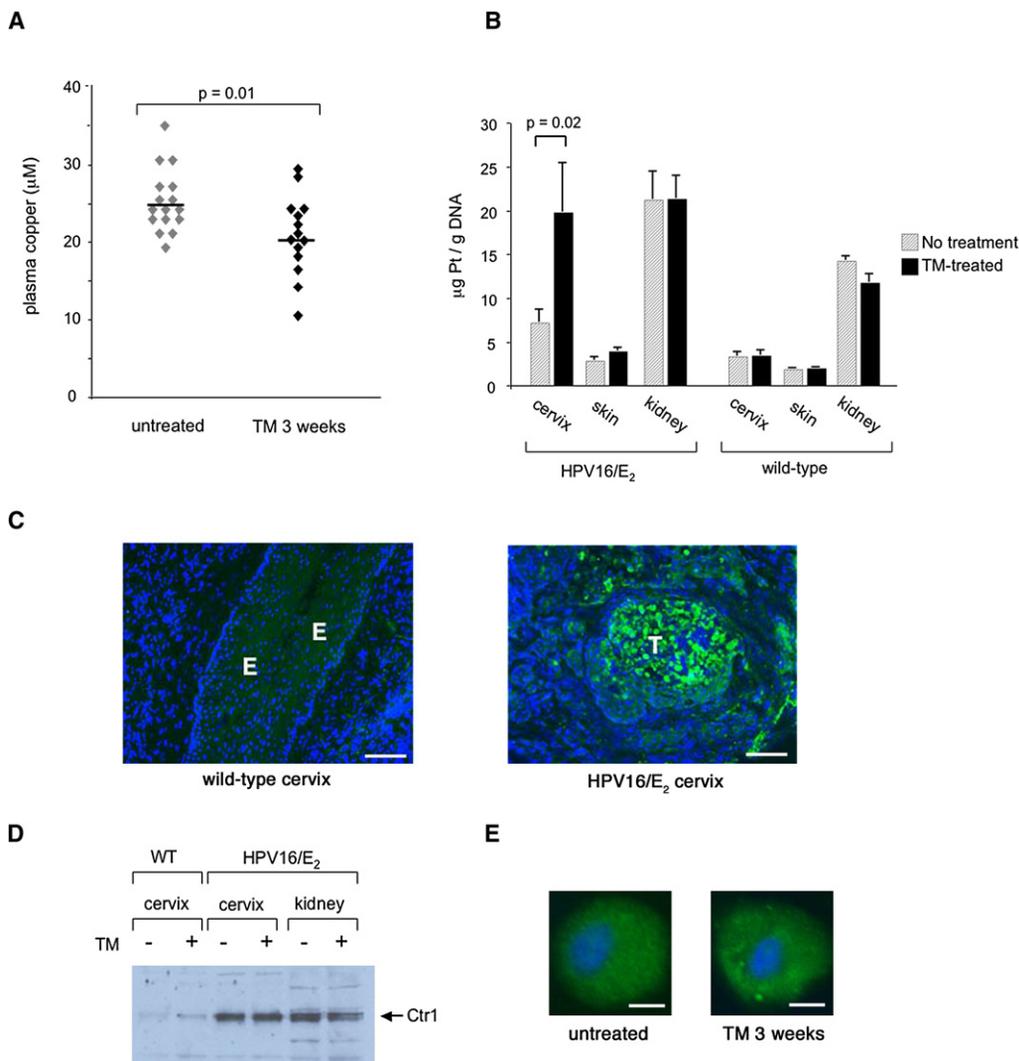


Figure 3. The Copper Chelator Tetrathiomolybdate Enhances Cisplatin Uptake into the Cervix of HPV16/E₂ Mice

(A) Plasma copper levels in six-month-old HPV16/E₂ females after a 3 week daily treatment with 1 mg tetrathiomolybdate (TM). Bars represent mean values. (B) Cisplatin adduct levels in the cervix, skin, and kidney of six-month-old HPV16/E₂ females (n = 12) and wild-type females (n = 5) that received either the three-week TM treatment (solid bars) or no treatment (hatched bars). 6 mg/kg cisplatin was injected two hours before organs were harvested. Adduct levels were determined as in Figure 2. Error bars represent the standard error of the mean. (C) CTR1 immunohistochemistry reveals positive cells (green) in tumors (T) of the 6-month-old HPV16/E₂ cervix (right panel) but not in the epithelium (E) of the wild-type cervix (left panel). DAPI staining is shown in blue. The panels shown are representative images of 12 fields in three tissue sections per mouse collected from three mice. Scale bars represent 100 µm. (D) Tissue CTR1 protein levels are elevated in the neoplastic cervix, but unchanged in the context of TM treatment. Wild-type and HPV16/E₂ mice were either treated with 1 mg TM daily for 3 weeks prior to being sacrificed at 6 months of age, or did not receive such treatment. A total of 5 µg membrane extracts from tissues was pooled from three mice and applied on each well. (E) CTR1 protein (green) is predominantly localized in intracellular compartments in cervical carcinoma cells, and is unchanged by TM treatment. HPV16/E₂ mice were either treated with 1 mg TM daily for 3 weeks prior to being sacrificed at 6 months of age, or did not receive such treatment. Tissue sections were immunostained with anti-CTR1 (green). Nuclei are stained with DAPI (blue). The images were collected using a 20x objective lens on a fluorescent microscope, and are representative of > 500 cells in 12 sections from six tumors. Scale bars represent 5 µm.

copper in the intestine (Brewer and Merajver, 2002). We determined the maximally tolerated daily dose of TM for the HPV16/E₂ female mice to be 1 mg (data not shown). When the mice are subjected to this daily dose of TM, plasma copper levels start decreasing after 3 days, and reach a plateau after 2 weeks. We did not observe any side effects during 3 weeks of daily TM treatment, at which point the plasma copper levels had decreased by 23% (Figure 3A). This TM dosage resulted in a 2.7-fold increase

in cisplatin adduct levels in the cancerous cervix of the HPV16/E₂ mice (7.2 µg Pt/ g DNA in control versus 19.8 µg Pt/ g DNA in TM-treated mice, p = 0.02), but not in the wild-type cervix (Figure 3B). Such a difference in the number of adducts is considered significant because it can result in a 1000-fold differential in cell viability in vitro (Hall et al., 2008). Notably, this TM regimen did not increase adducts in the kidney (Figure 3B), which is the major organ affected by cisplatin toxicity, nor in

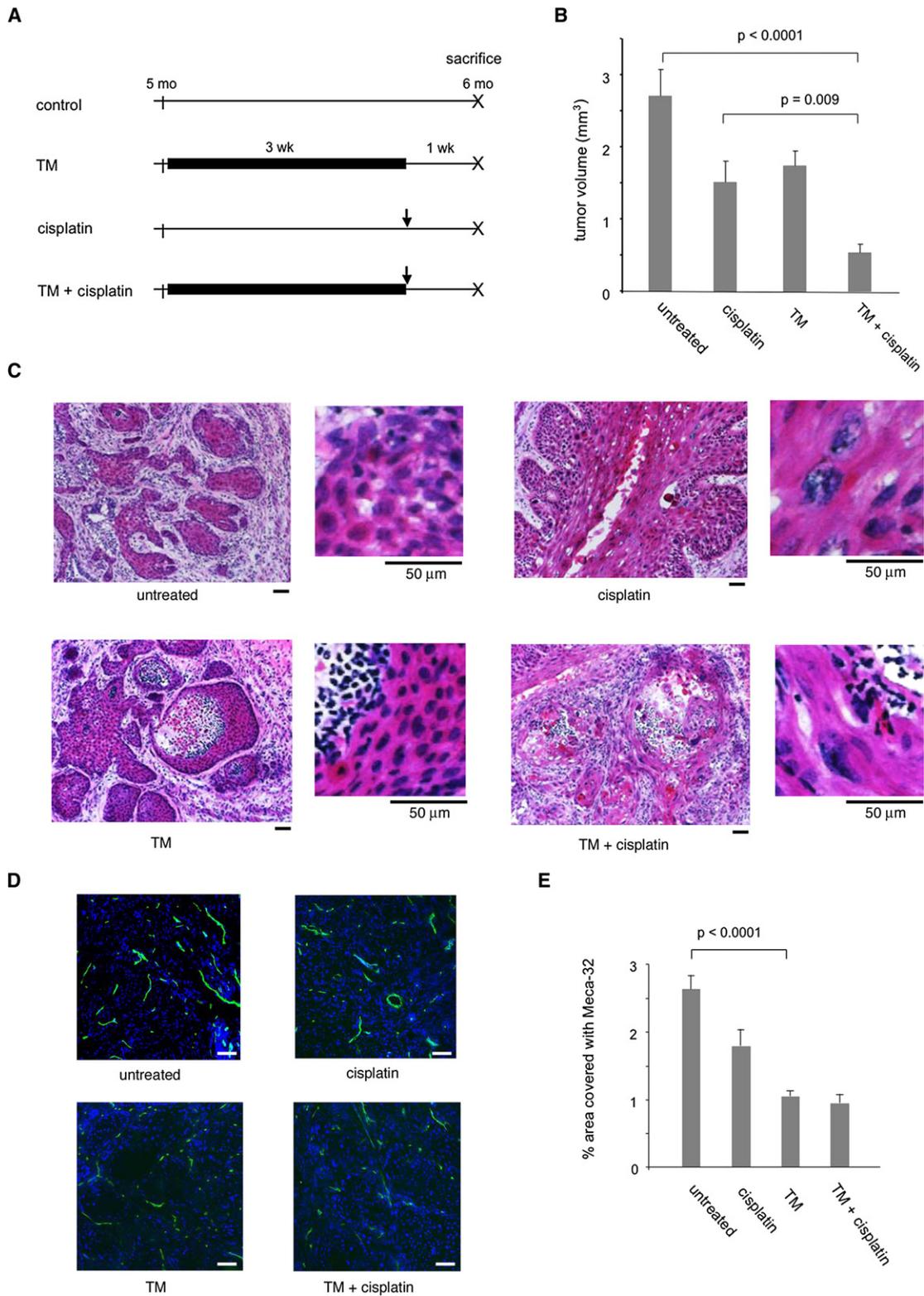


Figure 4. Preclinical Trials Assessing Therapeutic Effects of the Copper Chelator Tetrathiomolybdate in Combination with Cisplatin on De Novo Cervical Carcinomas

(A) Schematic diagrams of each therapeutic arm. Cohorts of 14 female HPV16/E₂ mice were subjected either to a 3 week oral administration of 1 mg tetrathiomolybdate (TM) daily starting at 5 months of age, or to a single intraperitoneal injection of 6 mg/kg cisplatin at 5.75 months of age, or to both. All the mice were sacrificed at 6 months of age.

other noncancerous organs of the mice. Interestingly, in the absence of TM treatment, the kidneys of HPV16/E₂ mice accumulated 50% more platinum adducts than those of wild-type mice (Figure 3B). This increase in the adduct level in the kidneys of HPV16/E₂ mice may be due to renal failure from urinary tract obstruction, which is not uncommon both in cervical cancer patients and in this mouse model. TM treatment also produced a marginal 1.4-fold increase in the adduct level in the dysplastic skin of the HPV16/E₂ mice (2.8 $\mu\text{g Pt/g DNA}$ in control versus 3.9 $\mu\text{g Pt/g DNA}$ in TM-treated mice, $p = 0.03$), but not in the skin of wild-type mice, suggesting that neoplastic tissues may become more sensitive to changes in copper levels. Notably, we found that CTR1 protein is highly expressed in tumors of the HPV16/E₂ cervix but not in the epithelium of the wild-type cervix, the origin of cervical carcinoma (Figure 3C). We were, however, unable to detect any change in *Ctr1* mRNA (data not shown) or protein levels (Figure 3D) in response to TM treatment in the neoplastic HPV16/E₂ cervix or in normal tissues. In tumor cells of the HPV16/E₂ cervix, CTR1 protein was predominantly localized in intracellular compartments; CTR1 remained intracellular in TM-treated HPV16/E₂ cervix (Figure 3E). It is possible that the modest change in systemic copper (23% reduction) by TM (Figure 3A) is not sufficient to cause measurable changes in the levels of CTR1 protein, which is already high in these tumors, or in its localization. However, TM treatment resulted in increased cisplatin uptake in the HPV16/E₂ cervix (Figure 3B), demonstrating that even a small reduction in systemic copper levels can increase the transport activity of CTR1 in tumors. These data indicate that expression of the copper transporter CTR1 is upregulated in neoplastic tissues compared to cognate normal tissues, and that its transport activity could be further elevated selectively in tumors by reducing systemic copper.

The Copper Chelator Tetrathiomolybdate Enhances Cisplatin Efficacy

For assessment of possible therapeutic benefits of using TM to enhance the uptake and consequent cytotoxicity of cisplatin in tumors, cohorts of 14 female HPV16/E₂ mice were subjected either to a 3 week regimen of oral 1 mg TM daily starting at 22 weeks of age, or to a single intraperitoneal injection of 6 mg/kg cisplatin at 25 weeks of age, or to both (Figure 4A). At 22 weeks, all HPV16/E₂ females have high-grade dysplasias (CIN 2/3), and about half have invasive cervical carcinomas. Animals from all treatment arms were sacrificed at 26 weeks of age,

when 90% have cervical carcinomas. Tumor volumes were calculated by measuring tumor areas on serial sections. Cisplatin treatment reduced tumor volumes to 55% of untreated cervix (2.7 mm³ in control versus 1.5 mm³ with cisplatin, $p = 0.01$) (Figure 4B). In the cisplatin-treated cervix, cells had more abundant cytoplasm that appeared keratinized (Figure 4C). Nuclei were enlarged and bizarre in shape, characteristic features of human cervical cancer treated with radiation (Robboy et al., 2002), which causes DNA damage. TM alone also modestly decreased tumor volumes in the cervix, to 65% of controls (2.7 mm³ in control versus 1.7 mm³ with TM, $p = 0.04$) (Figure 4B). In the TM-treated cervix, the center of a tumor mass was necrotic and filled with inflammatory cell infiltrates (Figure 4C). The percentage of area covered with meca-32-positive endothelial cells in the TM-treated tumors was 2.6-fold less compared with those from untreated mice (Figures 4D and 4E), consistent with prior observations that TM is antiangiogenic (Brewer and Merajver, 2002). When cisplatin was combined with the TM treatment, tumor volume decreased to 19% of untreated cervix (2.7 mm³ in control versus 0.5 mm³ with TM and cisplatin, $p < 0.0001$) (Figure 4B). Microscopically, the cervical carcinoma from the combination arm showed histologic features of the effects seen with both cisplatin and TM monotherapies: enlarged nuclei, necrotic centers, inflammatory cell infiltrates, and reduced vascularity (Figures 4C and 4D). These results demonstrate that TM enhances efficacy of cisplatin synergistically.

The Copper Chelator Tetrathiomolybdate Increases Cisplatin Sensitivity of Cancer Cells

Seeking to separate the antiangiogenic effect of TM on tumor vasculature from its effect on tumor cell uptake of cisplatin, we asked whether TM treatment increases cisplatin sensitivity and uptake in cultures of SiHa cells, which are derived from an HPV16-positive human cervical carcinoma (Friedl et al., 1970). Plasma TM levels in the HPV16/E₂ females range from 150 μM to 4 μM , over the period from 3 to 24 hr after oral administration of 1 mg TM, respectively (data not shown). We determined that 24 hr incubation with up to 10 μM TM does not inhibit proliferation of SiHa cells (Figure 5A). When SiHa cells were incubated with 10 μM TM for 24 hr and then subjected to a 2 hr cisplatin treatment, we observed a 2-fold increase in cisplatin sensitivity and a 45% increase in cisplatin accumulation inside TM-treated cells compared to cells that were not preincubated with TM (Figures 5B and 5C). We also assessed effectiveness of the

(B) Tumor volumes in the cervix of 6-month-old HPV16/E₂ mice from each treatment arm. Approximately 30 sections per mouse from cohorts of 14 female HPV16/E₂ mice were analyzed as described in [Experimental Procedures](#). Error bars represent the standard error of the mean.

(C) Representative H&E-stained cervixes from each treatment arm, with the treatment indicated below each panel. Cells are shown at a higher magnification on the right. The magnification is scaled by a bar below each panel indicating 50 μm . In the nontreated cancerous cervix (top left), invasive squamous cell carcinomas contain large keratinized cells with hyperchromatic nuclei. In the cisplatin-treated cervix (top right), the cancer cells have more abundant cytoplasm that appears keratinized, with enlarged, bizarre-shaped nuclei. In the TM-treated cervix (bottom left), the center of a tumor mass is necrotic and filled with inflammatory cell infiltrates. The cervix treated with both TM and cisplatin (bottom right) shows histological features of the effects seen with both cisplatin and TM monotherapies: enlarged nuclei, necrotic centers, and inflammatory cell infiltrates. The panels shown are representative of four fields in 30 tissue sections collected from cohorts of 14 HPV16/E₂ mice.

(D and E) Vascular density of tumors after treatment. 15 tumor sections from five mice per treatment arm were stained with a pan-endothelial cell antibody Meca-32, followed by FITC-conjugated secondary antibody to visualize tumor endothelial cells. Images were collected from five to seven fields per section, and vascular density was determined using the Metamorph Angiogenesis Tube Formation program. Data are expressed as the percentage of area covered with Meca-32-positive tube structures (E). Error bars represent the standard error of the mean. Representative images of Meca-32 (green) staining in the cervix of control and TM-treated HPV16/E₂ mice are shown in (D). Nuclei are stained with DAPI (blue). Scale bars represent 50 μm .

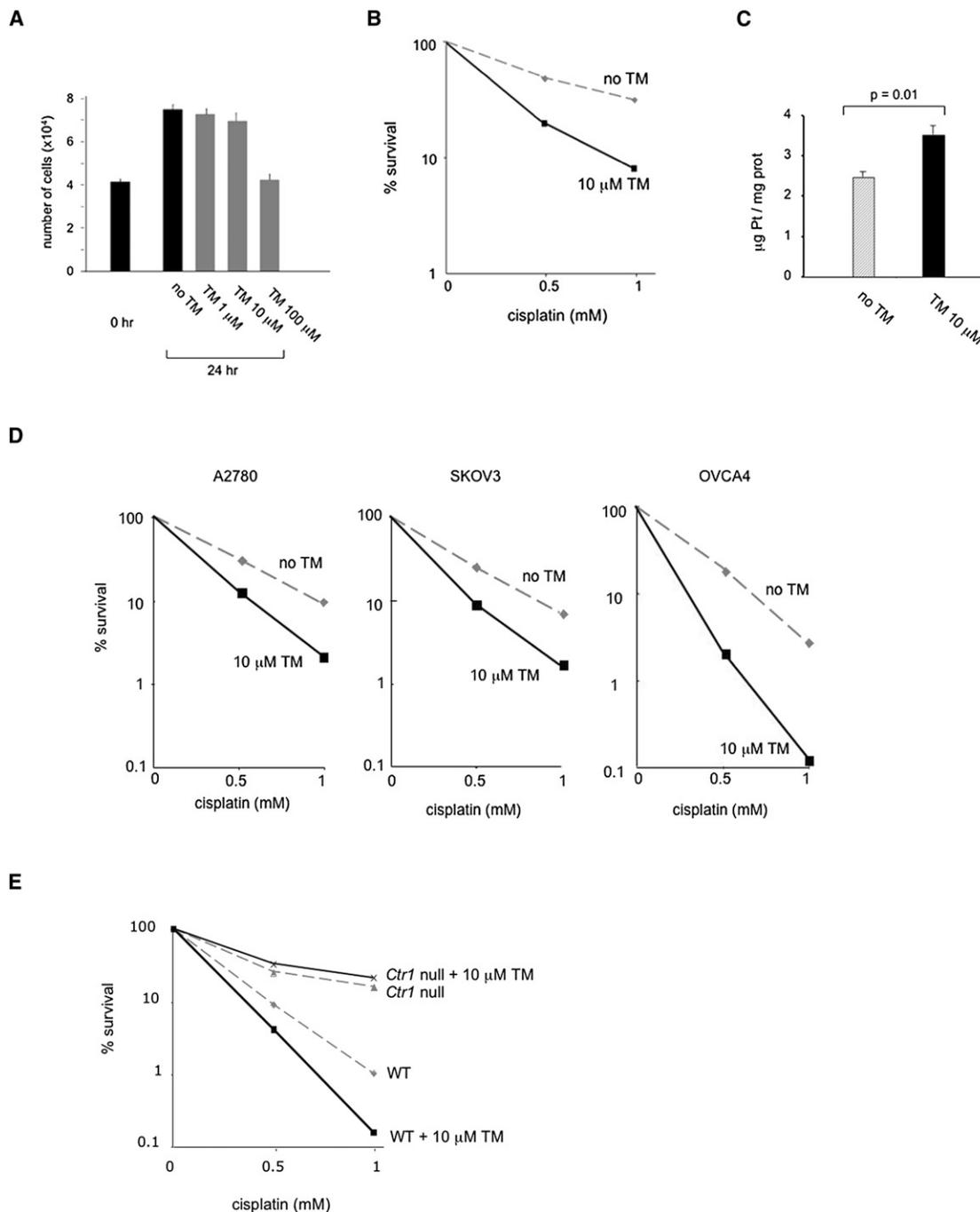


Figure 5. Effects of the Copper Chelator Tetrathiomolybdate (TM) on Cisplatin Sensitivity in Cultured Mammalian Cells

(A) Effect of TM on cell proliferation in human cervical carcinoma cells. SiHa cells were plated in triplicates and treated with 0, 1, 10, or 100 μ M TM for 24 hr. Numbers of cells before and after the treatment are shown. Error bars represent the standard error of the mean.

(B and C) Effect of TM on cisplatin sensitivity and accumulation in human cervical carcinoma cells. SiHa cells were plated in triplicates and either pretreated with 10 μ M TM or mock treated for 24 hr before a 2 hr incubation with cisplatin. For cell survival (B), data are expressed as percentage of viable cells compared with control cultures not exposed to cisplatin. For determining cisplatin accumulation (C), cells were lysed and, after a centrifugation, the supernatant was used to determine the platinum content and protein concentration. Cellular platinum readings were normalized to protein concentration. Mean values of the triplicates are shown. Error bars represent the standard error of the mean.

(D) TM increases cisplatin sensitivity in human ovarian cancer cells. Cells were plated in triplicates and either pretreated with 10 μ M TM or mock treated for 6 hr (A2780), 12 hr (SKOV3), or 24 hr (OVCA4) before cisplatin treatment. Data are expressed as percentage of viable cells compared with control cultures not exposed to cisplatin.

(E) TM increases cisplatin sensitivity in a *Ctr1*-dependent manner. Isogenic mouse embryonic fibroblasts that are either wild-type or homozygous for a knockout allele of *Ctr1* were either pretreated with 10 μ M TM or mock treated for 24 hr before a 2 hr incubation with cisplatin. The percentage of viable cells compared with control cultures not exposed to cisplatin is shown.

combination therapy for ovarian cancer, using three human ovarian cancer cell lines derived from patients' tumors, A2780, SKOV3, and OVCA4 (Eva et al., 1982; Fogh and Trempe, 1975). These cells had different growth properties: the doubling time was 16 hr for A2780, 24 hr for SKOV3, and 36 hr for OVCA4 (data not shown). We determined that incubation of these cells with 10 μ M TM did not have an antiproliferative effect for up to 6 hr in A2780, 12 hr in SKOV3, and 24 hr for OVCA4 (data not shown). When these cells were subsequently treated with cisplatin, they exhibited 1.8- to 2.2-fold increased sensitivity compared with cells that were not preincubated with TM (Figure 5D), demonstrating that TM can increase cisplatin sensitivity of ovarian cancer cells as well. To establish that the effects of TM on enhancing cisplatin-mediated killing were dependent on *Ctr1*, we compared isogenic MEFs that were either wild-type or deleted for the *Ctr1* gene (Lee et al., 2002). The ability of TM to increase cisplatin killing proved to be dependent on *Ctr1*: in wild-type MEFs, the percentage of cells that survived a 2 hr treatment with 1 mM cisplatin was reduced to 0.16% in cultures pretreated with TM, as compared with 1% in control cultures (Figure 5E). In contrast, the percentage of cells surviving cisplatin treatment was not affected by TM treatment in *Ctr1* null MEFs (Figure 5D). These results further support the role of the copper chelator TM and the copper transporter CTR1 in enhancing cisplatin uptake and killing of cancer cells.

DISCUSSION

Our studies in a mouse model of HPV16-induced cervical carcinoma demonstrate that the copper chelator tetrathiomolybdate has a synergistic antitumor effect when combined with cisplatin treatment, by increasing uptake of cisplatin into tumors but not normal tissues while concomitantly inhibiting tumor angiogenesis. We show that cisplatin adduct levels in various mouse organs correlate with mRNA levels of *Ctr1*, supporting its in vivo involvement in cisplatin uptake. Pretreatment of cultured human cervical and ovarian cancer cells with TM resulted in increased cisplatin sensitivity, encouraging applicability to human cancers. TM-enhanced cisplatin killing was dependent on *Ctr1* because mouse cells deleted for the *Ctr1* gene were not sensitized to cisplatin by TM, in comparison to isogenic cells with wild-type *Ctr1*. Notably, low levels of *Ctr1* mRNA in human ovarian tumors are associated with decreased disease-free survival after platinum-based therapy in patients, suggesting that *Ctr1* may serve as a clinical predictor of response to platinum agents.

Previous genetic experiments with yeast and mouse cells demonstrated that the copper transporter *Ctr1* is a major determinant of cisplatin uptake and sensitivity, which could be modulated by copper levels. These in vitro results suggested a strategy to increase cisplatin uptake, but a challenge was to find a clinically feasible method to do so selectively in tumors, without affecting normal organs to avoid untoward toxicities of cisplatin. Surprisingly, we were able to achieve this goal by reducing systemic copper levels with a copper chelator in a mouse model of cervical cancer. We do not fully understand the mechanism by which reduced systemic copper leads to increased cisplatin uptake only in tumors. Copper is essential for a variety of key cellular processes such as respiration, free

radical detoxification, and iron uptake (Kim et al., 2008). Cancer cells may have a greater demand for copper than normal cells for proliferation and survival. Indeed, the copper transporter CTR1 was more highly expressed in cervical carcinoma of the HPV16/E₂ mice than in the wild-type cervix. Concordant with our observation is a recent demonstration using microbeam synchrotron X-ray fluorescence, showing that copper is concentrated in tumor regions of tissue specimens obtained from invasive ductal carcinoma of the breast (Farquharson et al., 2008).

Although pretreatment of mice with the copper chelator TM resulted in increased cisplatin sensitivity and adducts in tumors, we were unable to detect any increase in the levels of *Ctr1* mRNA or protein. In vitro, elevated extracellular copper causes endocytosis and degradation of human CTR1 protein (Petris et al., 2003). We did not, however, observe any change in CTR1 protein localization in the TM-treated cervix of HPV16/E₂ mice. It is possible that copper starvation enhances cisplatin transport activity by changing the conformation of CTR1, allowing more cisplatin to be transported into cells. Exogenous copper has been shown to induce structural rearrangements in yeast Ctr1p (Sinani et al., 2007). Further biochemical studies will be required to elucidate the mechanism by which copper chelation causes increased cisplatin uptake.

Drug-induced toxicity is a common cause for discontinuation or dose reduction of chemotherapeutic drugs during cancer treatment. Cancers that are deemed "resistant" to a chemotherapeutic drug fail to respond to the dosage tolerated by patients without untoward side effects. These "resistant" cancers might in principle still respond to a higher dosage if toxicities were not manifest. By reducing bioavailable copper with the copper chelator TM, we were able to increase cisplatin activity in highly metabolic tumors while comparatively sparing normal organs. TM was developed for the treatment of patients with Wilson's disease, an autosomal recessive disorder of copper transport that results in excessive accumulation of copper and toxicity. Phase II and III trials with Wilson's disease patients have demonstrated that TM is a fast-acting and well-tolerated drug (Brewer, 2009). The major side effect is anemia and leukopenia due to copper depletion, which can be reversed with a drug holiday or dose reduction.

Additionally, the independent anti-angiogenic effects of the copper chelator TM present it as a drug that targets both tumor parenchyma and stroma, by enhancing cisplatin efficacy against the cancer cells while inhibiting angiogenesis in the tumor microenvironment. Combination regimens involving copper chelating and platinum-containing drugs may improve the treatment of cervical, ovarian, and other cancers for which cisplatin is currently in use, and for cancers that are treated with carboplatin, whose uptake is also mediated by CTR1 (Holzer et al., 2006). Such a therapeutic strategy may even prove effective in treating cancers that are inherently resistant to cisplatin or have developed resistance. With the development of a second-generation TM analog that depletes copper more quickly and is more stable (Lowndes et al., 2008), it may be possible to manipulate the activity of CTR1 even more effectively. The mechanistic principles and results elaborated in this report should motivate discussion of analogous clinical trials in patients with cervical, ovarian, and other platinum-responsive cancers.

EXPERIMENTAL PROCEDURES

Tumor Samples

We obtained ovarian tumor RNA samples from the UCSF Ovarian Cancer Tissue/Pathology Bank, which has approval from the UCSF Institutional Review Board to collect ovarian cancer specimens and clinical outcomes data for research. Written informed consent was obtained from all patients contributing specimens to the bank. Fifteen samples were available from women with stage III/IV serous epithelial ovarian carcinoma who underwent optimal cytoreduction (resulting in residual tumor of 1 cm or less in diameter), received platinum-based therapy, and had clinical outcome data available. Tumor samples were snap frozen in liquid nitrogen at the time of surgery and maintained in a monitored -80°C freezer. Adjacent sections were used for pathology and to confirm that they contained more than 70% tumor cells. RNA was isolated from snap-frozen tumors using Trizol and its integrity was determined by Agilent 2100 Bioanalyzer. Responses to initial chemotherapy were recorded as either refractory/resistant (if there was progression or recurrence of the disease within 6 months after completion of chemotherapy, as indicated by an increase in CA-125 levels, a positive finding on CT or ultrasonography, or second-look laparoscopy) or sensitive (if there was no such evidence of recurrence within 6 months after completion of chemotherapy).

Determination of mRNA Levels with Real-Time PCR

A total of 5 ng cDNA was used for quantitative RT-PCR to measure mRNA levels of copper transporters in human ovarian tumors and in mouse tissues. The following TaqMan gene expression assay kits (Applied Biosystems) were used: Hs00977268_g1 (human *CTR1*), Mm00612987_m1 (mouse *CTR1*), Mm00437663_m1 (mouse *ATP7A*), and Mm00599675_m1 (mouse *ATP7B*). The human *GUS* gene, which encodes β -glucuronidase, and the mouse *L19* gene were used as internal amplification controls for human and mouse, respectively.

Mouse Studies

All animal studies were performed in compliance with the UCSF Guide for the Care and Use of Laboratory Animals and under approved protocols by the UCSF Institutional Animal Care and Use Committee.

Platinum Measurement

After cisplatin treatment, organs were harvested and ~ 30 mg tissues were digested in 500 μl lysis buffer (50 mM Tris [pH 8.0], 20 mM NaCl, 1 mM EDT [pH 8.0], 1% SDS) with 2 mg/ml proteinase K at 55°C for 16 hr. Digests were treated with 0.2 mg/ml RNase at 37°C for 1 hr, followed by phenol/chloroform extraction three times. DNA was precipitated with sodium acetate and isopropanol, resuspended in 500 μl H_2O , and DNA concentration was measured with a spectrometer. A total of 2 ppb iridium was added to each sample as an internal control for inductively coupled plasma mass spectrometry (ICP-MS) analysis. The platinum reading was normalized to DNA concentration. For measuring cellular platinum, cells were trypsinized, washed with PBS, and lysed with 1% Triton X-100/0.1% SDS on ice for 30 min. Lysates were spun at top speed in a microcentrifuge at 4°C , and the supernatant was used to determine protein concentration (Bio-Rad). An equal volume of nitric oxide was added to the supernatant and digested at 75°C for 2 hr for ICP-MS analysis. The platinum reading was normalized to protein concentration.

Plasma Copper Measurements

Plasma was separated from blood using PST tubes with lithium heparin (BD microtainer) and digested at 75°C for 2 hr with an equal volume of nitric oxide. A total of 100 ppb cobalt was added to each sample as an internal control for ICP-MS analysis.

Immunohistochemistry

Optimal cutting temperature (OCT)-embedded frozen tissues were sectioned at 10 μm thickness. Sections were air-dried, acetone treated, and blocked with 5% BSA and 2.5% goat serum. Primary antibodies were applied at a dilution of 1:100 for anti-CTR1 and 1:200 for meca-32. Secondary antibodies were used at 1:500.

Western Blotting

Membrane protein extracts were prepared as described (Nose et al., 2006). Protein concentrations were measured with the BioRad Protein DC Assay Kit. 5 μg of membrane extracts from tissues were pooled from three mice and applied on each well. Anti-CTR1 antibody was used at 1:1000. Peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson Immuno-Research Laboratories) was used as secondary antibodies at 1:10,000. For detection, Amersham ECL Western Blotting Reagents were used.

Measurement of Tumor Volume

OCT-embedded cervixes were serially sectioned at 10 μm thickness and stained with hematoxylin and eosin (H&E) every 100 μm . Areas of squamous cell carcinoma on H&E-stained sections were measured using an Axio Imager program. Tumor volume (V) was calculated using the following formula $V = 2/3 \times A \times Z$, where A is the maximal tumor area measured on a section and Z is the depth of tumor determined by the number of sections containing tumors.

Tissue Culture

SiHa, A2780, SKOV3, and OVCA4 cells were cultured in RPMI medium containing 10% fetal bovine serum and penicillin/streptomycin. *CTR1* +/- (wild-type) and -/- mouse embryonic fibroblasts were cultured in DMEM supplemented with 20% fetal bovine serum, 2 mM glutamine, 1x nonessential amino acids, 55 μM 2-mercaptoethanol, 50 mg/L uridine, 110 mg/L pyruvate, and penicillin/streptomycin. For determining survival, cells were stained with trypan blue to detect dead cells; unstained cells were counted using a hemocytometer.

Statistics

Wilcoxon analyses were performed to determine two-tailed p values. For Kaplan-Meier survival estimates, p values were determined from a log-rank statistic with one degree of freedom.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.ccr.2010.04.011.

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