Emetine reduces the effective dose of cisplatin or carboplatin required to inhibit bladder cancer cell proliferation

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ABSTRACT

OBJECTIVE: We analyzed a novel therapeutic combination of emetine dihydrochloride added to standard of care chemotherapeutic agents (cisplatin, carboplatin, gemcitabine) in human muscle-invasive bladder cancer cell lines to determine if emetine enhanced their anti-tumor activity.

METHODS: Cells were treated with emetine, cisplatin (or carboplatin), and gemcitabine for 24–96 h *in vitro*. Cell proliferation, apoptosis, autophagy, cell cycle distribution and colony formation were analyzed.

RESULTS: Addition of low dose emetine enhanced the anti-proliferative activity of cisplatin-gemcitabine and carboplatin-gemcitabine against muscle-invasive bladder cancer, but not normal urothelial cells. Importantly, lower doses of cisplatin and carboplatin were required to achieve significant growth inhibition when emetine was included in the therapy. Treatment resulted in a combination of growth arrest, apoptosis and autophagy.

CONCLUSIONS: The inclusion of low dose emetine as part of multi-modal therapy for muscle-invasive bladder cancer could benefit patients by enhancing the anti-tumor activity of standard of care chemotherapy. It allowed for dose reduction of cisplatin and increased the efficacy of carboplatin. This may allow more patients currently unfit for cisplatin-based therapy to benefit from treatment.

Keywords: urothelial carcinoma, muscle-invasive bladder cancer, metastatic bladder cancer, cisplatin, carboplatin

INTRODUCTION

Bladder cancer is the second most common genitourinary malignancy in the USA with an estimated 79030 new cases and 16870 deaths in 2017 [1]. Bladder cancers are categorized as non-muscle invasive (NMIBC, 70%) or muscle-invasive (MIBC, 30%). Twenty percent of NMIBCs will progress to MIBC, 30%–50% of patients with locally invasive disease will develop metastases, and 15% patients will have metastatic disease at the time of diagnosis [2]. Despite aggressive therapy, advanced bladder cancer is a deadly disease with five-year overall survival rates of 20%–50% for deeply invasive and 5% for metastatic disease [2,3].

Treatment options for MIBC rely predominantly on neoadjuvant platinum-based chemotherapy followed by radical cystectomy. Methotrexate-vinblastine-doxorubicin-cisplatin (MVAC) and cisplatin-gemcitabine (CG) are common first line chemotherapeutic regimens for advanced bladder cancer, and modifications to these regimens (dose dense administration, addition of growth factor support) have lessened toxicity, but have not changed overall survival [2]. Approximately 50% of patients are unfit for cisplatin-containing chemotherapy due to poor performance status, impaired renal function and other co-morbidities. Carboplatin-gemcitabine (CaG) is often used in cases where cisplatin cannot be administered, but with reduced efficacy [4]. Last year, the FDA approved the first new systemic therapy for advanced or metastatic bladder cancer in over 20 years. Anti-PD-1 (programmed cell death

protein-1)/PD-L1 (PD- ligand 1) immunotherapy reduced tumor burden in approximately 15%–30% of patients and significantly increased overall survival [5-8].

Emetine dihydrochloride, a natural product alkaloid from the plant Psychotria ipecacuanaha is one of the active agents in ipecac syrup. It is well known as an emetic and has been widely used for the treatment of amoebic dysentery [9]. In the 1970s, the National Cancer Institute sponsored several phase I and II clinical trials evaluating the anti-tumor activity of emetine as a single agent in a variety of cancers; however, emetine was not further pursued due to modest efficacy and significant toxicity, particularly muscle weakness and cardiotoxicity [10-13]. More recently, emetine has been reported to induce apoptosis in leukemia cell lines and to sensitize ovarian carcinoma cells to cisplatin through downregulation of $Bcl-X_L$ [14-16].

Little is known about emetine and bladder cancer. Only a handful of patients were evaluated in early clinical trials, and the results suggested improvement of symptoms in most patients, but long-term follow-up data was not available [10,17]. We have previously demonstrated that emetine alone inhibits proliferation of bladder cancer cell lines in a dose dependent manner and acts synergistically with cisplatin to induce bladder cancer cell growth arrest [18]. Because patients are most often treated with multi-modal chemotherapy, the current study was designed to extend our original findings and explore emetine in combination with standard of care combination therapies CG and CaG as a novel therapeutic strategy for MIBC.

MATERIALS AND METHODS

Tissue culture

MIBC cell lines (UMUC3, T24, HT1376) were purchased from ATCC. The cells were originally isolated from urinary bladder tumor specimens and have been well characterized in the literature. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, except T24, which were cultured in McCoy's 5A media containing identical supplements. Normal urothelial cells were purchased from CellNTec and cultured following the manufacturer's recommendations.

MTT assays

Cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described [18]. Proliferation was calculated after subtraction of background and reported as a percentage relative to untreated control wells. At least three independent experiments, each run in triplicate or quadruplicate, were performed.

Normal urothelial cells proliferate slower than bladder tumor cells and the use of MTT assays to measure their proliferation can be difficult. To confirm MTT based results, experiments with normal cells were also analyzed using trypan blue exclusion where 50–200 cells were examined per replicate. Two independent experiments were performed.

Synergistic activity of the drug combination was evaluated using constant drug ratios by the Chou-Talalay method and the combination index (CI) calculated as described [18]. Synergy was defined as CI ≤ 0.6 (synergy), 0.6–0.9 (moderate to slight synergy), 0.9–1.1 (additive) and > 1.1 (antagonism).

Cell cycle distribution and viability assays

Cell cycle analysis was performed by DNA-propidium iodide (PI) staining using standard methodologies [18]. Cell death was evaluated by flow cytometry using a PI viability assay. Cultured cells were lifted, washed once in phosphate buffered saline (PBS), and resuspended in 100 μl PBS. Immediately prior to analysis, PI (4 μg) was added.

All flow cytometry experiments were analyzed on a FACSCanto II flow cytometer with a minimum of 10000 cells analyzed per gated determination. At least three independent experiments were performed, and the data was analyzed using Flow Jo software (Tree Star Inc, Ashland, OR).

To evaluate autophagy, tumor cells grown on coverslips were treated under the indicated conditions and then labeled for 1 h with 25 μM monodansylcadaverine (MDC), an autofluorescent agent that accumulates in the acidic autophagic vacuoles that appear during autophagy. Coverslips were washed in PBS and immediately analyzed using a fluorescent microscope. Autophagy positive cells were determined using the ImageJ cell counter with a threshold setting of 65500-65535 to eliminate baseline fluorescence. A minimum of 900 cells in 5–9 random microscopic fields were examined for each treatment condition. Three independent experiments were performed.

Colony formation assays

DMEM-10% FBS media containing 0.5% Nobel agar (500 μl) was allowed to solidify in a 24-well plate. An equal volume of tumor cells (2500 cells) in complete media containing 0.3% Nobel agar and supplemented with the indicated dose of cisplatin, gemcitabine and/or emetine was layered on top. Plates were examined for colony formation for up to 28 d with fresh media without chemotherapeutic drugs added weekly. The results were quantitated by counting colonies and single cells in 10 random high powered fields. Two independent experiments were performed in duplicate.

Cell recovery assay

Cell monolayers (70% confluent) were treated with the indicated dose of cisplatin (or carboplatin), gemcitabine and/or emetine for 96 h. The drug-containing media was removed, the monolayers thoroughly washed, and fresh growth media added. The dishes were examined daily over 2–3 weeks for evidence of cell growth (colony formation) indicative of recovery and to estimate culture confluency. Fresh media was added every 4–5 d to ensure optimal growth conditions. Two-three independent experiments were performed.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (IC_{50} calculations, r^2 values) and Excel software (Student t-test). P values of < 0.05 were considered significant.

RESULTS

Emetine enhances the anti-proliferative effect of CG and CaG

We began by evaluating the anti-proliferative potential of each chemotherapeutic agent in MIBC cell lines (UMUC3, T24, HT1376) and normal urothelial cells using an MTT assay. As expected, treatment of the cells with any of the four agents (cisplatin, carboplatin, gemcitabine, or emetine) alone for 48 h inhibited cancer cell proliferation in

a dose dependent manner. Gemcitabine was the only exception where both HT1376 and normal urothelial cells were resistant. Inhibitory concentration 50 percent (IC_{50}) values were estimated from the log concentration effect curves and nonlinear regression analysis, and the results shown in **Table 1** for each cultured cell line.

Table 1. Estimated inhibitory concentration 50% (IC_{50}) for cultured normal urothelial cells and bladder cancer cell lines.

Cultured cells	Estimated IC_{50} values			
	Cisplatin	Carboplatin	Gemcitabine	Emetine
UMUC3	6.8 μM*	73 μM	30 nM	30 nM*
HT1376	4.2 μM*	38.9 μM	Resistant	24.6 nM*
T24	4.4 μM	69.2 μM	20 nM	66 nM
Bladder urothelium	24.6 μM*	281 μM	Resistant	3.6 μM*

Combined data from at least three independent experiments is presented. *Similar results were published [18] with the exception of normal bladder urothelium and emetine, which we previously reported as IC50 > 10 μM. The difference in the results may reflect the difference between individual urothelial cell donors for these primary cultures.

To investigate the combined effects of emetine, cisplatin, and gemcitabine, bladder cancer cells were treated with CG or CG plus emetine (CG&E) simultaneously for 48 h and cell proliferation evaluated with the MTT assay. The agents were combined at equipotent concentrations in a constant ratio at several combinations above and below the calculated IC_{50}. The concentration effect curves demonstrated enhanced inhibition of cell proliferation in UMUC3, HT1376, and T24 cells with the drug combinations compared with the single agents (data not shown). In each cell line, the addition of emetine to CG resulted in an additive effect on growth inhibition where the CI ranged from 0.9–1.1 across the drug concentrations tested (**Fig. 1A** and **1B**, for UMUC3, HT1376) and data not shown (T24). With respect to CaG, addition of emetine to the lower concentrations of CaG tested resulted in modest synergy (CI = 0.6–0.8) in UMUC3 (**Fig. 1C**) and HT1376 (**Fig. 1D**), but additive effects were found at higher drug concentrations (CI = 0.9–1.1) and in all conditions with T24 cells (data not shown).

Emetine reduces the required dose of cisplatin or carboplatin

Based on our results, we wondered if addition of emetine might reduce the effective dose of cisplatin and/or carboplatin required to inhibit bladder cancer cell proliferation. Tumor cells were treated with increasing concentrations of cisplatin (**Fig. 2A** and **2B**) or carboplatin (**Fig. 2C** and **2D**) along with fixed doses (the IC_{50} concentration) of emetine and gemcitabine. With UMUC3 cells (**Fig. 2A** and **2C**), addition of 30 nM emetine resulted in at least a 10-fold decrease in cisplatin or carboplatin concentrations required to block proliferation. The results were more modest with HT1376 cells (**Fig. 2B** and **2D**, average 2-fold decrease, range 1.0 to 3.7-fold) in cisplatin and 4.4-fold decrease (range 2 to 6.7-fold) in carboplatin] and T24 cells [data not shown; average 2.6-fold decrease, range 1.5 to 4.5-fold in cisplatin). Addition of emetine did not significantly alter proliferation of T24 cells regardless of the concentration of carboplatin used (data not shown; average 1.2-fold decrease in carboplatin in the presence of emetine).

Proliferation of CG or CaG treated normal urothelial cells remains unchanged

We have previously demonstrated that normal urothelial cells are relatively resistant to emetine treatment alone or emetine combined with

cisplatin [18]. To evaluate the effect of emetine on normal urothelial cells treated with CG or CaG, we cultured the cells with increasing concentrations of the platinum agent in the presence of 30 nM emetine and 30 nM gemcitabine (identical conditions to UMUC3 experiments). The results demonstrated no significant difference in proliferation of normal cells treated with standard of care therapies CG or CaG with or without emetine (**Fig. 3A** and **3B**).

Cisplatin-gemcitabine-emetine causes a combination of apoptosis and autophagy

We previously published that emetine did not induce apoptosis in bladder cancer cell lines, but the combination of cisplatin and emetine modestly increased apoptosis compared to cells treated with cisplatin alone [18]. To further explore the fate of the cells following multi-drug therapy, we performed parallel PI viability and DNA-PI cell cycle distribution assays on the same samples. The results demonstrated CG- and CG&E-treated UMUC3 were on average 31% ± 6% and 38% ± 6% PI positive, respectively in the viability studies after 48 h of treatment (**Fig. 4A**, P = NS) while the paired DNA-PI cell cycle studies showed on average 15% ± 3% and 14% ± 3% of cells in the sub-G_0 region of the cell cycle, respectively (**Fig. 4B**, P = NS). After 96 h, the percentage of dead cells increased in both CG and CG&E-treated cultures. On average, cell viability studies demonstrated 70% ± 5% and 63% ± 4% PI-positive cells in CG and CG&E treated cultures respectively. The paired DNA-PI cell cycle studies demonstrated on average 50% ± 6% and 48% ± 8% sub-G_0 cells, respectively (**Fig. 4A** and **4B**, P = NS). Interestingly, at the later time point, CG treatment resulted in slightly higher levels of death than CG&E treatment, although the differences were not statically significant (**Fig. 4A** and **4B**, P = NS). Therefore, addition of emetine did not further augment CG-mediated killing of MIBC cell lines.

Studies from other laboratories have demonstrated increased autophagy in bladder cancer cells treated with CG [19]. To determine if autophagy was responsible, at least in part for our results, we stained UMUC3 cells with MDC, an autofluorescent pharmacologic agent that localizes in autophagocytic vesicles. Consistent with earlier reports, we found low levels of autophagy in untreated cells that increased following treatment with 3.4 μM cisplatin, 30 nM gemcitabine, or 30 nM emetine (**Fig. 5**). The combination of CG or CG&E did not further increase autophagy
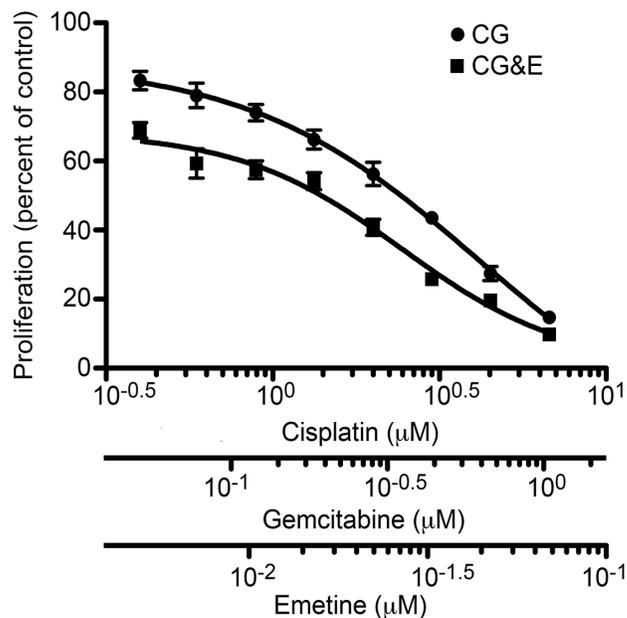
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compared to cisplatin or gemcitabine alone under our experimental conditions (**Fig. 5**). Thus, the addition of emetine to standard of care

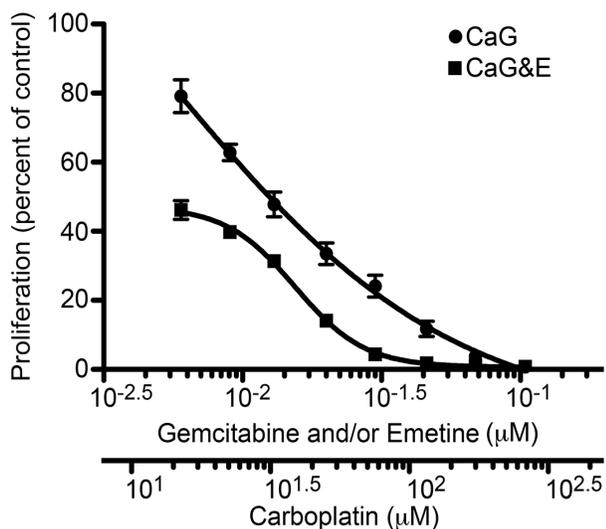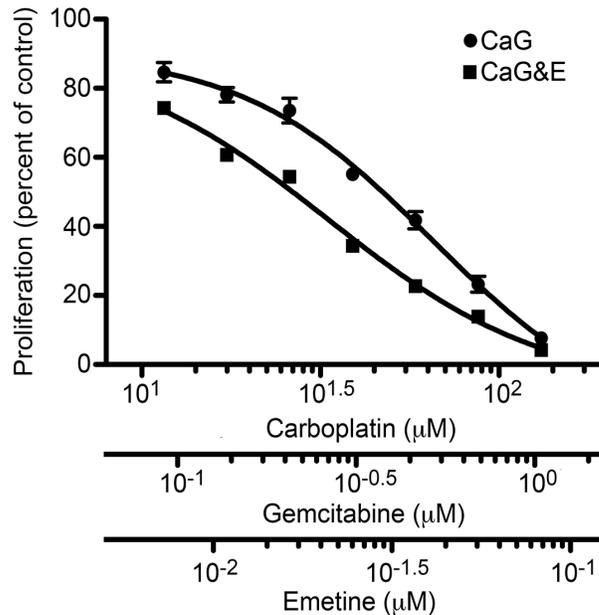therapy does not significantly enhance autophagy.
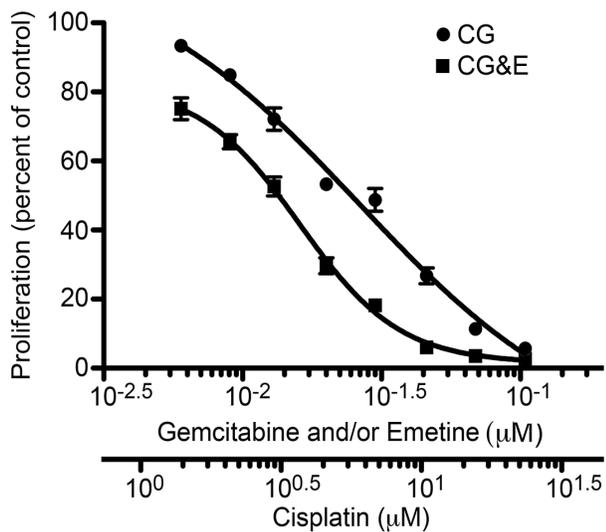
Figure 1. Concentration effect curves of bladder cancer cell lines UMUC3 and HT1376 treated with cisplatin or carboplatin combined with gemcitabine and emetine for 48 h (r^2 values ranged from 0.88–0.95). A and **B.** Combination therapy of cisplatin with gemcitabine and emetine. **C** and **D.** Combination therapy of carboplatin with gemcitabine and emetine. Data are shown as the mean ± SEM of 3–4 independent experiments performed in triplicate or quadruplicate.

Cisplatin-gemcitabine-emetine induces growth arrest

Taken together, our studies demonstrate that CG and CG&E result in similar levels of *in vitro* cell death, but CG&E consistently blocked cell proliferation more effectively than CG in MTT assays. To determine

if this effect on proliferation was transient or sustained, we utilized a colony formation assay where UMUC3 cells were embedded in soft agar containing the IC$_{50}$ or half the IC$_{50}$ of cisplatin, gemcitabine and/or emetine. After 28 d, no colony formation was found in any cisplatin-con-

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taining condition (cisplatin alone, CG or CG&E). This was unexpected as cells treated with suboptimal doses of cisplatin frequently develop resistance and continue to proliferate.

Because cell proliferation may have been hindered by the stringent growth conditions inherent to colony formation assays, we performed a drug recovery experiment where 70% confluent UMUC3 cell monolayers were treated with chemotherapeutics for 96 h, the drugs removed, and the cultures examined for an additional 2–3 weeks. As shown in **Figure 6**, treatment with the IC_{50} concentrations of CG&E or CaG&E

resulted in a gradual decrease in culture confluency with no recovery in cell growth. Of the few lingering cells in these cultures, most were rounded up but remained attached to the plate while others were firmly attached but appeared granular and highly vacuolated. Control cultures and all cultures treated with half of the IC_{50} drug concentration showed a decrease in cell confluence over the first week, which plateaued before small clusters of growing cells re-emerged within 4–12 d (data not shown). Similar results were found with the T24 cell line (data not shown).
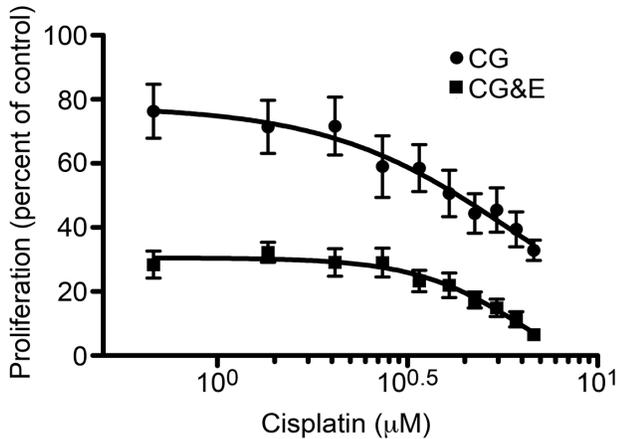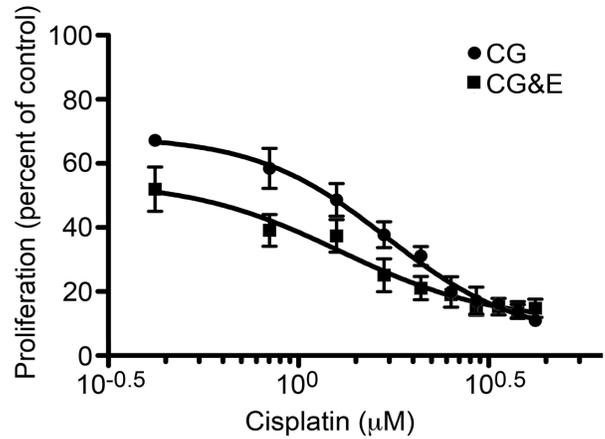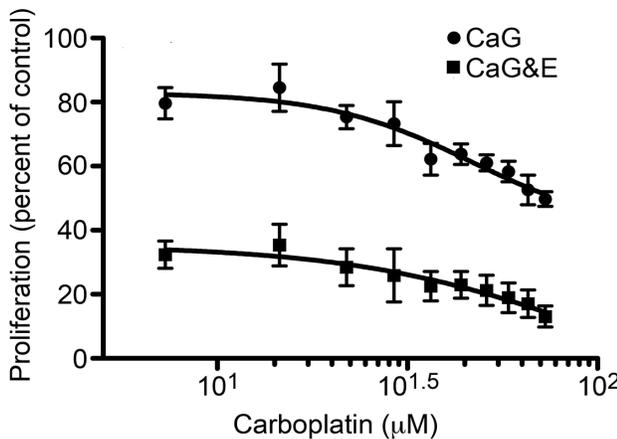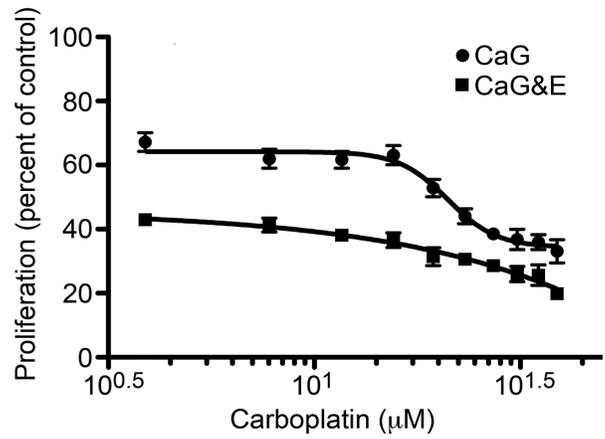

Figure 2. Concentration effect curves for the UMUC3 and HT1376 cell lines treated with increasing concentrations of cisplatin or carboplatin combined with emetine and gemcitabine. A and **B.** Increasing concentrations of cisplatin combined with 30 nM emetine and 30 nM gemcitabine (the IC_{50} concentrations). **C** and **D.** Increasing concentrations of carboplatin combined with 300 nM gemcitabine and 25 nM emetine (the IC_{50} concentration). HT1376 cells were resistant to gemcitabine; thus, we chose 300 nM gemcitabine based on previously published data in this cell line [33]. Data are shown as the mean ± SEM of three independent experiments performed in triplicate or quadruplicate.

DISCUSSION

Neoadjuvant platinum-based chemotherapy followed by radical cystectomy is the gold standard of care for MIBC. CG is a common first-line chemotherapeutic regimen with similar efficacy to MVAC, but with less toxicity. CaG, although considered inferior, is often used as an alternative for patients unfit for cisplatin. Yet, even cisplatin-based

chemotherapy is effective for only 30%–40% of patients, and the majority will recur and progress [20]. Moreover, no standard second-line therapy exists for those who fail treatment. There is clearly an urgent need to develop novel strategies to treat MIBC.

Cisplatin is one of the most effective chemotherapeutic agents for solid tumors; however, its toxicity, particularly nephrotoxicity, is a major impediment to clinical management of patients. Cisplatin is primarily

excreted through the kidneys, and during excretion, it actively accumulates in renal tubule epithelial cells through a transport-mediated process. Once in the cells, cisplatin damages the nuclear and mitochondrial DNA, activates mitogen activated protein kinase (MAPK) signaling, and induces oxidative stress, inflammation, fibrogenesis, and apoptosis [21]. Unfortunately, efforts to reduce nephrotoxicity often result in lower anti-tumor activity, and significant effort has been made to identify targets to protect the kidneys without compromising cisplatin efficacy. To date, these interventions have demonstrated partial protection against nephrotoxicity, but the effect on cisplatin efficacy remains unclear [21].
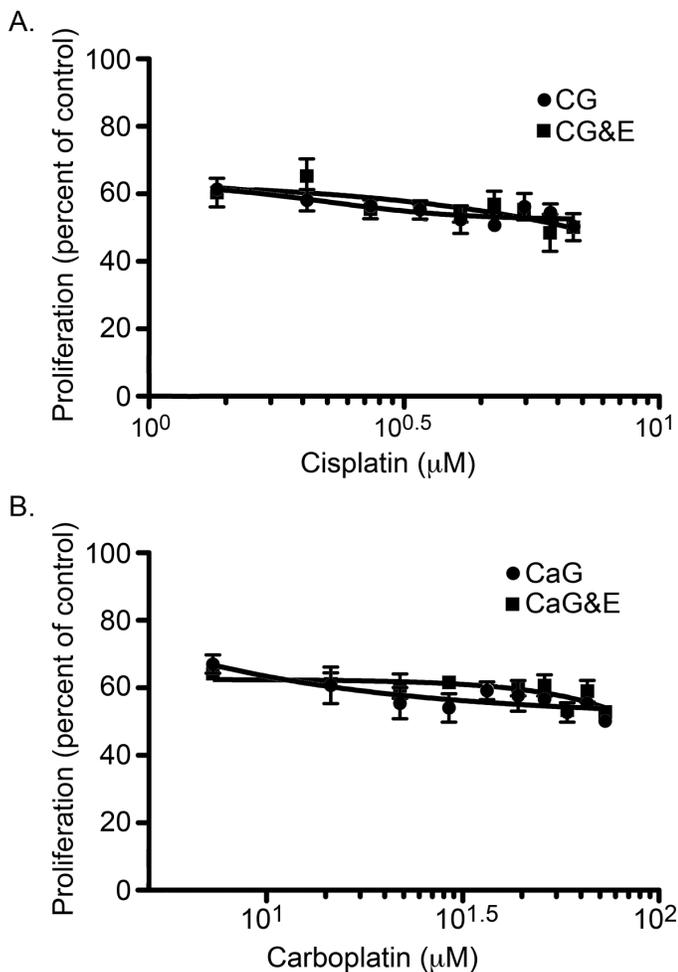
A.

B.

Figure 3. Concentration effect curves for normal bladder urothelial cells cultured with increasing concentrations of cisplatin (A) or carboplatin (B) combined with emetine (30 nM) and gemcitabine (30 nM). Data are shown as the mean ± SEM of three independent experiments performed in triplicate.

Here, we report for the first time that addition of nanomolar concentrations of emetine dihydrochloride to standard of care chemotherapy, CG or CaG, reduces the dose of platinum drug required to induce growth arrest and killing of MIBC cell lines *in vitro*. Moreover, low dose emetine improved the anti-cancer efficacy of carboplatin in combination with gemcitabine. The concentrations of single agent cisplatin and carboplatin used in our studies are in the middle to lower end of the clinically relevant range based on published pharmacokinetic data [22,23]. Al-

though it is impossible to directly compare treatment of tissue culture monolayers with the complexity of treating patients, we can extrapolate that an average bladder cancer patient may have a tumor concentration of ~19 μM cisplatin compared with the IC$_{50}$ concentration of ~7 μM in UMUC3 cells used in our experiments [22]. Similarly, the peak plasma concentration of carboplatin in an average patient is calculated at ~105 μM compared with the IC$_{50}$ of ~73 μM for UMUC3 cells [23]. Our concentrations of gemcitabine are about 1000-fold less than used in the clinic. This is due to the rapid metabolism of gemcitabine *in vivo* with the primary product being 2',2'-difluorodeoxyuridine, a much less cytotoxic compound [24]. Thus, only a fraction of the dose is required for *in vitro* studies. Early clinical studies using emetine reported that treatment of patients with 1 mg/kg emetine daily for 10 d did not result in adverse side effects [11]. This equates to ~3 μM emetine, which is far higher than the 30 nM concentration used in the majority of our studies. Because cisplatin-induced nephrotoxicity is dose-dependent, the addition of low dose emetine to cisplatin-containing chemotherapy may allow lower drug doses to be used and reduce the toxic side effects. Moreover, patients currently deemed unfit for cisplatin may be able to receive this preferred therapy if lower doses can be effectively used. Importantly, our results indicate that addition of emetine to standard of care should have no greater or lesser effect on normal urothelial cells than standard of care alone.

Our findings were confirmed in three established MIBC cell lines. Recently, several studies have reported the molecular subdivision of MIBC into two large groups, basal and luminal, with further subdivisions possible along the spectrum between these groups based on gene expression patterns [25]. In general, luminal cancers have a better prognosis and are more responsive to chemotherapy. They are enriched for mutations in fibroblast growth factor receptor 3 (FGFR3) and express genes associated with urothelial differentiation (FOXA1+, GATA3+, PPARγ+). Basal MIBC has a worse prognosis, and the tumors express genes associated with a squamous phenotype (CK5/6+, CK14+). By examining three different MIBC cell lines, our data are representative of a wider range of tumors than studies based on a single cell line. HT1376 represent the most basal-like tumor in our study. They express the lowest levels of FOXA1 and the highest levels of CK14 with moderate expression of GATA3, CK5, and PPARγ [26]. UMUC3 cells express mid-range levels of CK5 and CK14, but lower levels of GATA3 and little to no PPARγ while T24 cells have moderate expression levels of all these markers. Warrick and colleagues referred to this group as non-basal/non-luminal with T24 having more similarities with luminal tumors than UMUC3 [26]. As studies continue to decipher the genetic alterations associated with different subtypes of bladder cancer, a consensus on the biomarkers and terminology will become more apparent and uniform for the field [27].

We previously reported that emetine acted synergistically with cisplatin to promote growth arrest and killing of MIBC; however, in the current report, we demonstrate emetine acts additively with CG to block tumor cell proliferation, but not cell death [18]. This is not surprising as gemcitabine enhances the anti-tumor effects of cisplatin compared to either drug alone, and the addition of emetine, although able to further enhance anti-proliferative activity, did not meet the definition of synergy. The mechanisms involved in multi-drug interactions are complex and difficult to decipher [28]. Both gemcitabine and cisplatin are primarily recognized as inhibitors of DNA replication. Emetine is well-recognized as a potent inhibitor of protein synthesis. It irreversibly

blocks translation elongation by binding to the 40S ribosomal subunit and prevents its movement alone the mRNA [29,30]. Emetine also inhibits DNA replication, although this seems to be secondary to its effects of protein synthesis [31,32]. Emetine appears to act early in the S-phase of DNA synthesis by interfering with the processing of the Okazaki fragments that are required for synthesis of the lagging DNA strand [32]. The mechanisms by which emetine is enhancing the anti-proliferative effects of CG and CaG are currently unknown. Studies are ongoing in the laboratory to determine if emetine is acting as a global protein synthesis inhibitor under our experimental conditions or if its effects are focused on a more specific target. These mechanistic studies combined with *in vivo* experiments using an orthotopic bladder cancer model will provide the essential preclinical data to move us forward toward translation into the clinic.
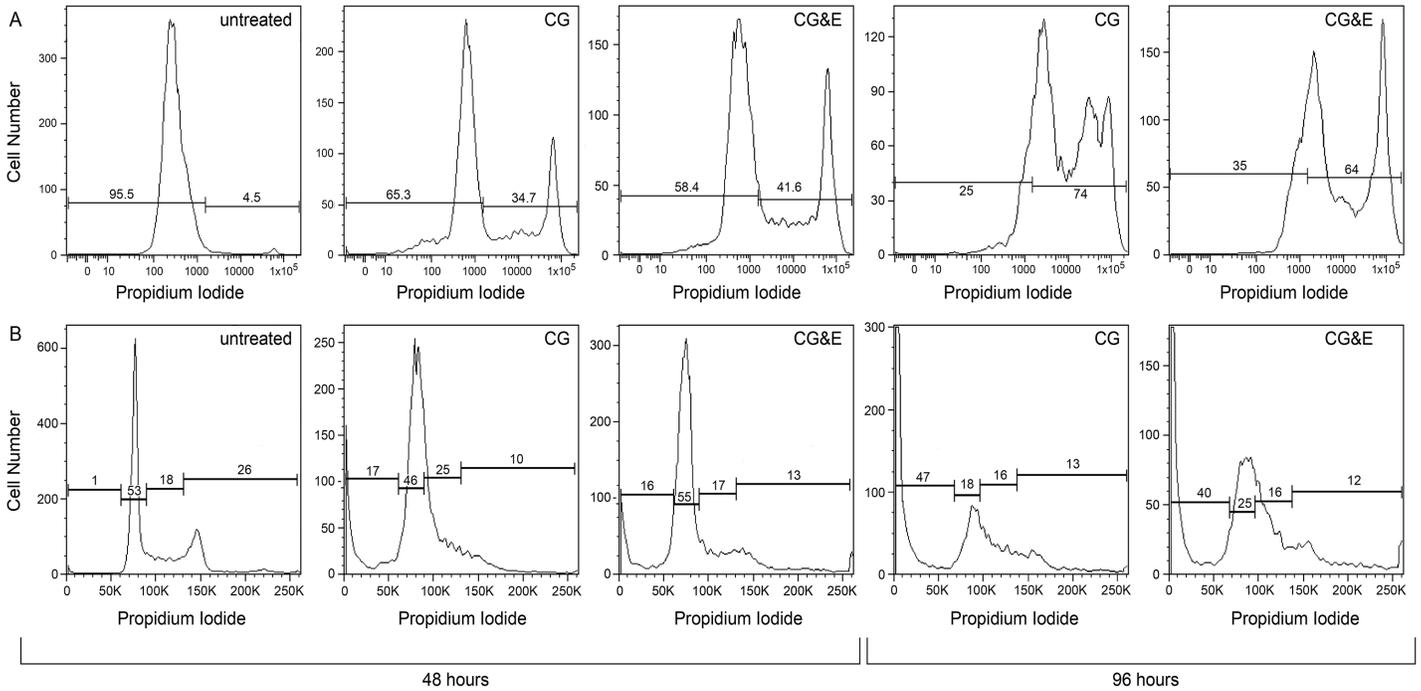

Figure 4. Propidium iodide viability (A) and DNA-PI cell cycle profiles (B) of UMUC3 cells at 48 and 96 h after treatment with the IC$_{50}$ concentration of the indicated drugs. Representative data is shown. No significant differences were noted between cisplatin-gemcitabine and cisplatin-gemcitabine-emetine profiles when combined data from three independent experiments was analyzed.
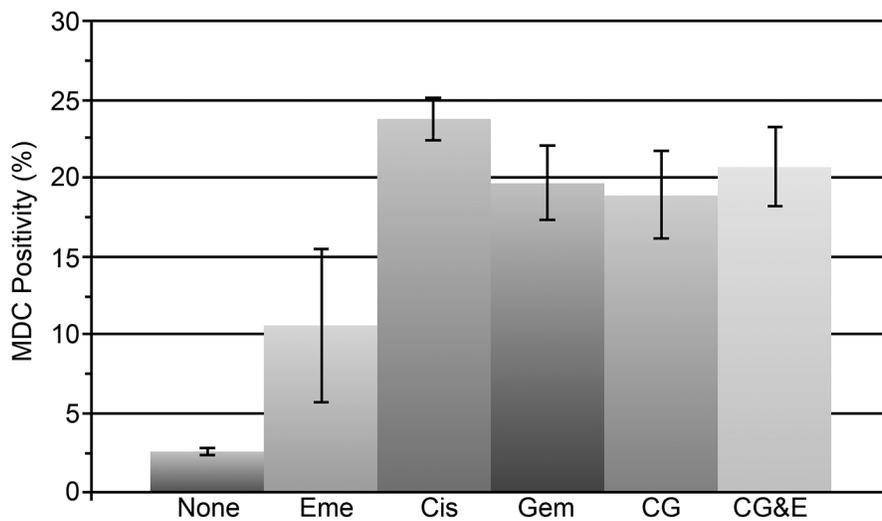

Figure 5. Autophagy in UMUC3 cells as determined by percent MDC positivity. Cells were treated with the IC$_{50}$ concentrations of indicated agents for 48 h. Data are shown as the mean ± SEM of three independent experiments. Eme: emetine alone; Cis: cisplatin alone; Gem: gemcitabine alone.
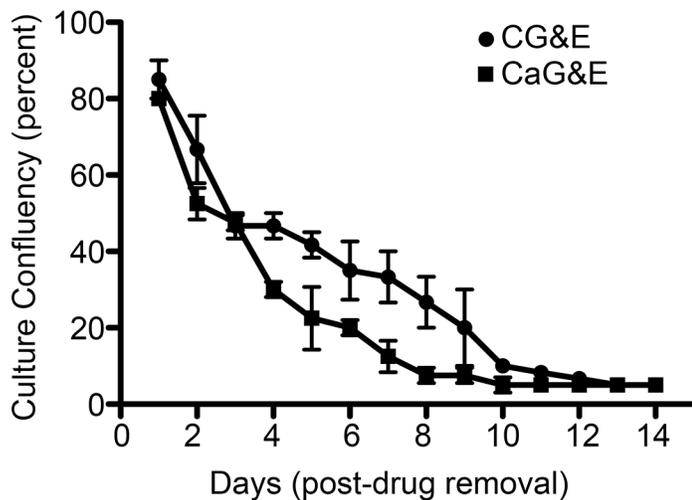
Figure 6. UMUC3 cell culture confluency following treatment with CG&E or CaG&E at the IC$_{50}$ concentration of each drug. Data are shown as the mean ± SEM of two independent experiments.

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