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Emetine as an Anti-Cancer Therapeutic in Bladder Cancer

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LOYOLA UNIVERSITY CHICAGO

EMETINE AS AN
ANTI-CANCER THERAPEUTIC
IN BLADDER CANCER

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN MOLECULAR BIOLOGY

BY
VALERIE J. DAVIDSON
CHICAGO, IL
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ABSTRACT

Bladder cancer is a serious health concern among the older population, as it is responsible for thousands of deaths annually in the United States. Patients that are diagnosed with muscle-invasive disease have a 5-year survival rate of only 20 percent. Additionally, muscle-invasive disease has a high metastatic potential; half of all patients develop metastatic disease within 3 years. Patients with muscle-invasive disease are presented with few treatment options aside from surgery. The current standard of care is a chemotherapeutic combination therapy of cisplatin and gemcitabine. This therapy is highly toxic, and due to the high instance of co-morbidities in these patients, approximately half are unfit for therapy. An alternative combination of carboplatin plus gemcitabine allows for the inclusion of more patients, but is an inferior therapy. Development of an alternative treatment option is necessary.

Previous studies in the Foreman lab have shown a synergistic decrease in bladder cancer cell proliferation when the natural alkaloid emetine is combined with cisplatin *in vitro*. Here, we expanded these studies to demonstrate that the addition of emetine to both the cisplatin-gemcitabine as well as carboplatin-gemcitabine standard of care regimens resulted in an additive decrease in bladder cancer cell proliferation. Moreover, the addition of low dose emetine allows for up

to a 10-fold decrease in effective dose of cisplatin or carboplatin. Treatment with this triple therapy appears to be inducing growth arrest in the cancer cells.

Hypoxia Inducible Factors (HIFs) are upregulated in response to low oxygen conditions, and regulate a wide range of genes responsible for giving tumor cells a selective advantage. HIF- α overexpression in bladder cancer corresponds to a poorer prognosis. This, as well as the concise regulation of HIFs, makes them an attractive target for anti-cancer therapy.

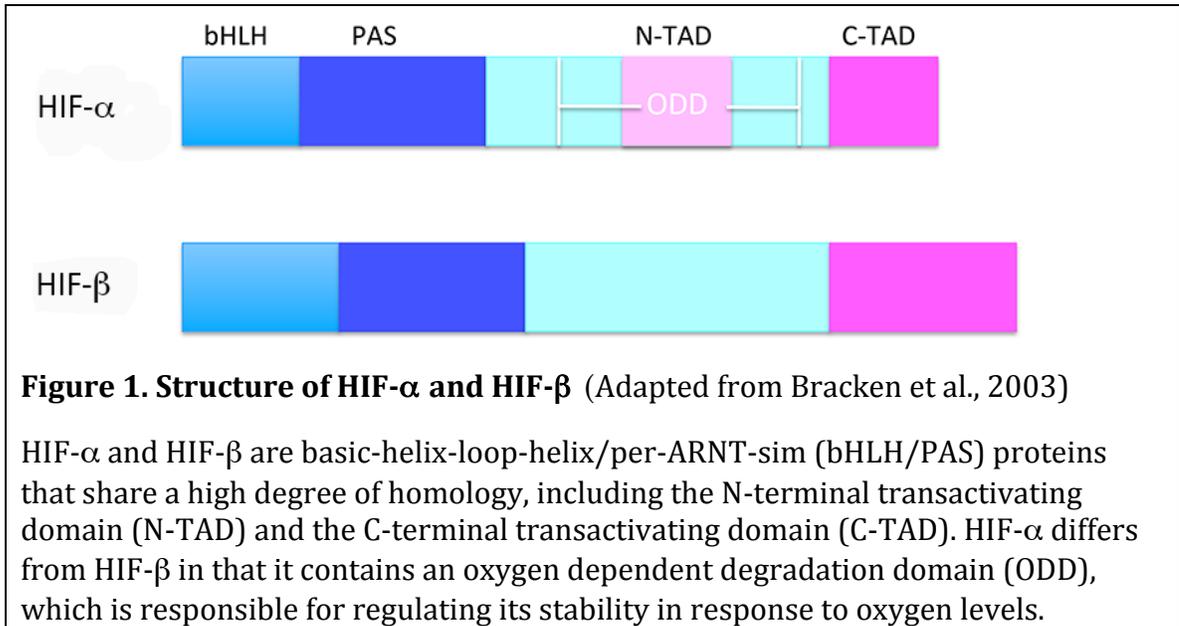
HIF-1 α and HIF-2 α are aberrantly upregulated under normoxia conditions in the invasive bladder cancer cell lines UMUC3, HT1376, and T24. Emetine is a known protein synthesis inhibitor at the micromolar level, but less is known about its actions at lower concentrations. We demonstrate here that low, nanomolar concentrations of emetine act to preferentially downregulate levels of HIF-1 α and HIF-2 α . Emetine appears to be acting via decreased HIF-1 α protein synthesis. We hypothesize that emetine is acting in a similar manner on HIF-2 α , but further work is necessary to confirm. This regulation of hypoxia signaling may act to decrease the cell's selective advantage and proliferative potential.

CHAPTER 1
INTRODUCTION
HYPOXIA

Oxygen homeostasis is a tightly regulated process in mammals. Hypoxia is a reduction in the normal level of tissue oxygen tension and occurs when oxygen demand exceeds supply. ¹ Hypoxia is an important factor in diseases such as vascular disease, pulmonary disease, and cancer. Tumors become hypoxic because they outgrow their blood supply, or their vasculature is aberrant or has poor blood flow. Cells will die if hypoxia is prolonged, but cancer cells have adapted ways to survive even in a hypoxic environment, leading to a more aggressive phenotype. ² Hypoxia Inducible Factors (HIFs) are transcription factors that upregulate many genes to promote angiogenesis and cell survival in response to low oxygen conditions. ^{3,4} HIFs regulate up to 2 percent of the genome, including genes involved in angiogenesis, erythropoiesis, cell proliferation, glucose metabolism, migration, invasion, and cell survival. ^{1,5} The expression of many response genes, including VEGF (vascular endothelial growth factor) occur in almost all cell types, while the expression of the majority of genes regulated by HIFs are cell-type specific. ^{6,7}

HIFs

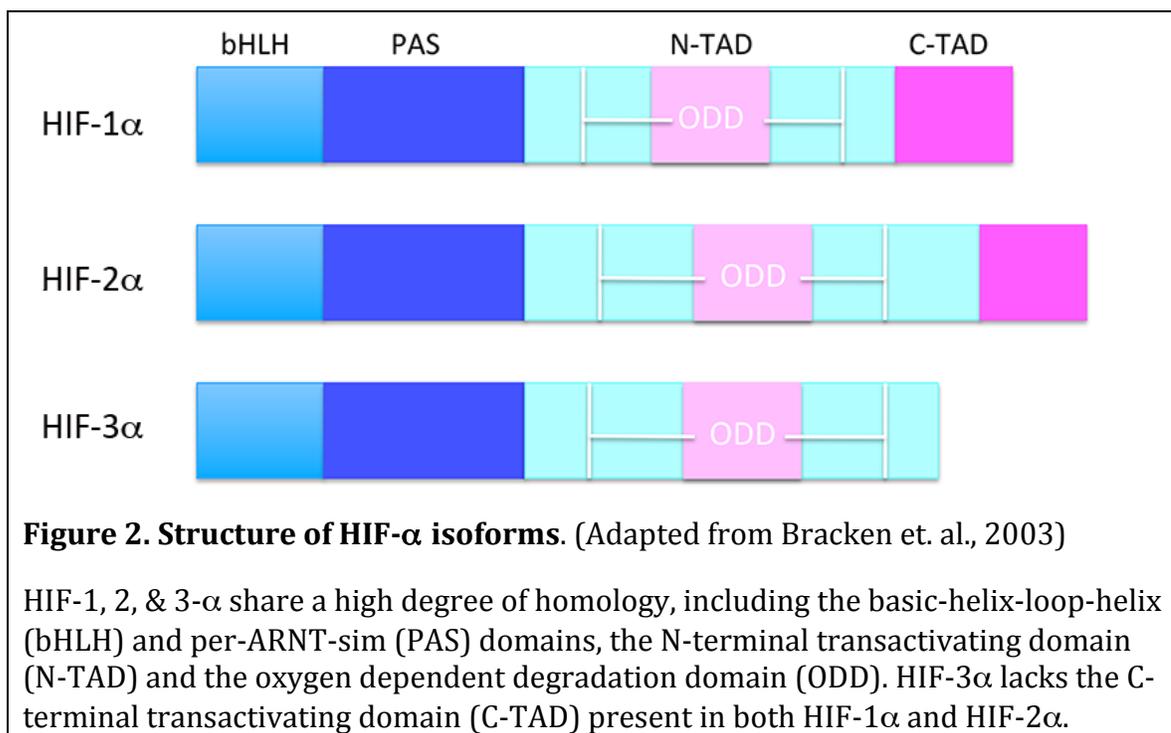
HIF is a heterodimeric transcription factor composed of an α subunit and a β subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT).⁸ Both subunits are of the basic-helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family. Oxygen levels affect the transcriptional activity of the alpha subunit, but have no effect on ARNT, which is constitutively expressed in the nucleus.⁹ The two subunits dimerize via the HLH & PAS domains, and together act as a transcription factor by binding to hypoxia response elements (HREs) at various gene loci on the DNA.¹⁰ HIF-1 α contains 2 transactivating domains, one involved in protein stability and the other interacts with co-activators and is required for full HIF activity.¹¹ The oxygen dependent degradation domain (ODD) encompasses a substantial portion of the protein and is the region that is post translationally modified for protein stability (Figure 1). HIF- α homologs are highly conserved across mammalian and non-mammalian species.¹



HIF- α Family

There are 3 members of the HIF- α family, HIF-1 α , HIF-2 α , and HIF-3 α .¹²

The three isoforms have overlapping, as well as unique functions.¹ HIF-1 α has been most extensively studied. HIF-2 α (also known as endothelial PAS domain protein, or EPAS) and HIF-1 α are both regulated in response to O₂ and share a high degree of structural similarity. HIF-1 α and HIF-2 α are observed to have nonredundant functions, which are cell or context-dependent.^{6,7,13} Both isoforms can be expressed in the same cell and may have different gene targets.¹⁴ HIF-3 α is structurally similar, but lacks the c-terminal transactivating domain, and does not regulate the same genes.^{1,5} In some instances, HIF-3 α may act to oppose HIF-1 and 2 α .⁵ (Figure 2).



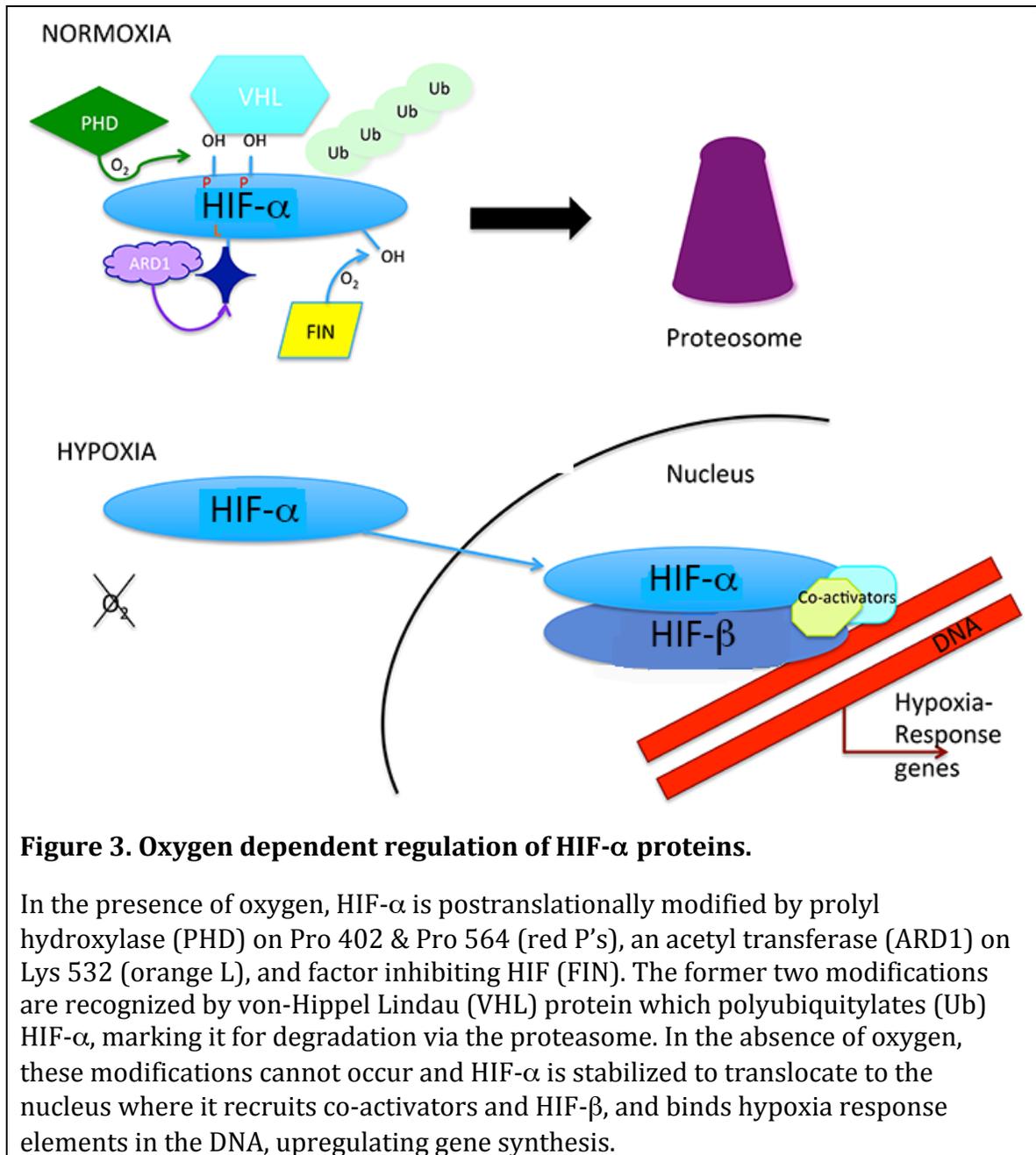
HIF- α Stability and Regulation

Hypoxia has no significant effect on levels of HIF-1 α mRNA, but drastically increases the abundance of HIF- α protein.¹⁵ Under normal oxygen conditions, PHD's use O₂ and 2-oxoglutarate in the cell to hydroxylate HIF at Proline 402 & Proline 564.^{5,16} (Figure 3) The E3 ubiquitin ligase, von Hippel-Lindau (VHL) recognizes this modification and polyubiquitylates HIF- α , targeting it for degradation via the proteasome.⁵ A second posttranslational modification that stabilizes HIFs interaction with VHL is the acetylation of Lys532 in the ODD by ARD1 acetyl transferase.¹⁷ FIN, or factor inhibiting HIF, further modifies HIF- α in the presence of O₂, 2-oxaloglutarate, and iron by hydroxylating an asparagine residue in the C-TAD, preventing the interaction of HIF with co-activators, reducing

its transcriptional activity.^{7,11} The turnover of HIF- α in normoxic conditions is very rapid, resulting in little detectable protein.^{18,19}

In the absence of O₂ (hypoxia) these processes are suppressed, leading to HIFs stabilization. The protein is no longer degraded, but recruits co-activators and dimerizes with HIF- β in the nucleus, where it activates expression of its response genes.^{5,7,13} The fact that the PHD function is dependent upon oxygen, implicates its role in the cell as the oxygen sensor.¹

Under physiological conditions, it is likely that FIN acts as the key regulator of HIF's transcriptional activity. For example, when levels of HIF are high in hypoxic or VHL-deficient cells, it outnumbers FIN, resulting in more HIF in the unhydroxylated, or transcriptionally active state.²⁰⁻²² Whereas in mildly hypoxic cells when the level of stabilized HIF is moderately elevated, FIN can play a more pronounced role in regulating HIFs transcriptional activity.²³



HIF- α Synthesis

HIF- α synthesis is regulated at the transcriptional level by the phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways.²⁴ These pathways are activated via tyrosine kinase receptors, non-tyrosine kinase receptors, and/or G-protein coupled receptors by a number of growth factors and cytokines (Figure 4). These include, fibroblast growth factor, angiotensin 2, hepatocyte growth factor, insulin, insulin-like growth factors 1 and 2, interleukin 1- β , platelet-derived growth factor, thrombin, transforming growth factor- β 1, tumor necrosis factor α , and epidermal growth factor.²⁴⁻³³ The growth factor mediated upregulation of HIF- α is limited to synthesis of the proteins, and has no effect on its stability.

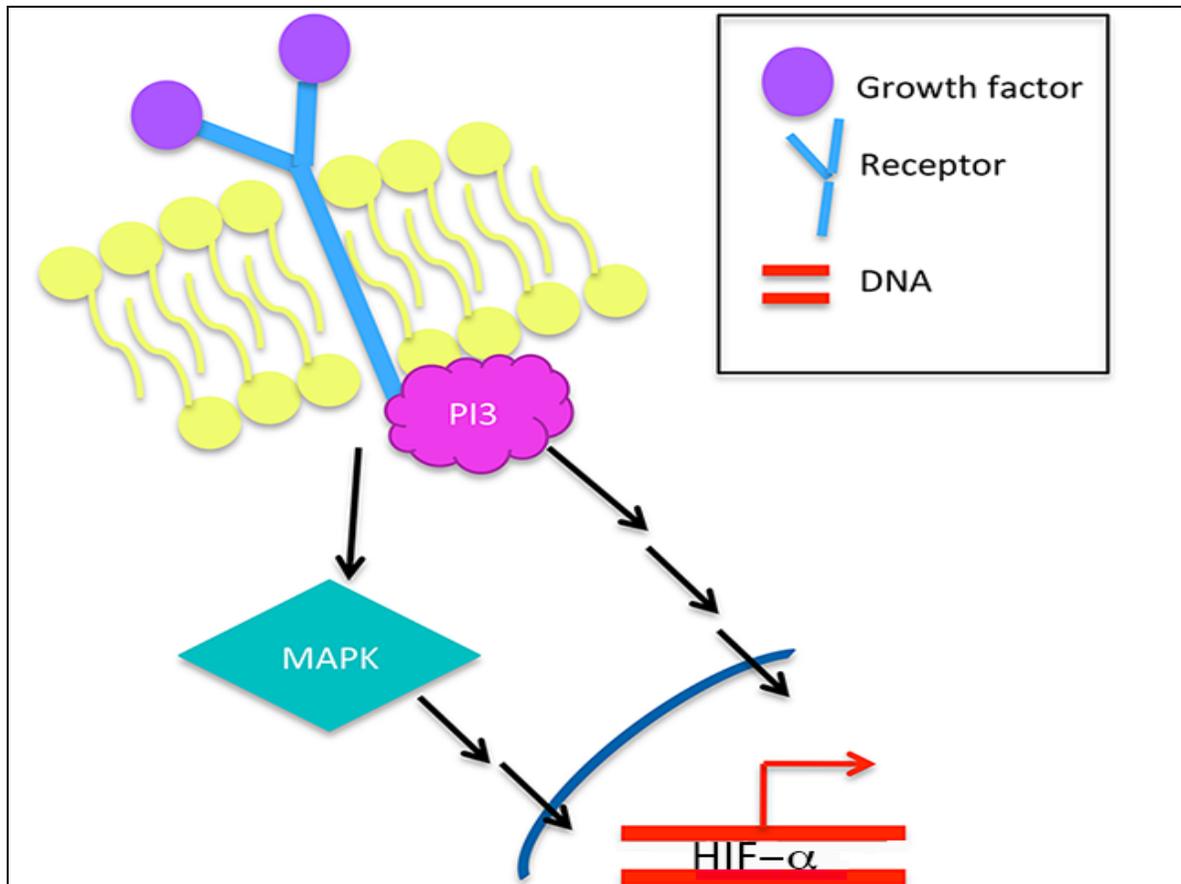


Figure 4: Growth factor initiated upregulation of HIF- α proteins.

Growth factors stimulate HIF- α transcription via activation of both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3) pathways.

HIF and Cancer

Hypoxia and HIFs are implicated in many diseases including cancer.¹ As HIFs upregulate genes involved in angiogenesis, glycolysis, and other pro-survival genes, their presence gives the tumor cells a selective advantage.¹ This, plus the tight regulation of HIF activity makes it an attractive therapeutic target.

The overexpression of HIF-1 α and/or HIF-2 α proteins have been implicated in various tumors, including invasive bladder cancer, brain tumors, breast cancer, cervical cancer, non-small-cell lung cancer, non-Hodgkin's lymphoma, oropharyngeal cancer, pancreatic cancer, colon, skin, gastric, prostate, and renal clear cell carcinomas.^{1,34} The importance of HIF in tumorigenesis is highlighted in clear cell renal carcinoma, where a majority of tumors overexpress HIF- α due to VHL mutations.^{35,36}

Enforced HIF- α overexpression in bladder cancer cells increases their tumorigenic potential-causing them to grow more rapidly and form larger tumors *in vivo*.³⁷⁻³⁹ In patient samples, HIF- α expression correlates with poor prognosis in bladder cancer.^{40,41}

Specifically, HIF-2 α protein expression in surgical specimens of bladder cancer is indicative of muscle invasive disease. HIF-2 α is not present in normal bladder epithelial or superficial disease. HIF-2 α is much more prevalent in the muscle invasive compartment of the tumor than in the superficial compartment. This suggests that HIF-2 α may be involved in the invasion of bladder cancer.⁴²

Moreover, studies indicate HIF-1 α is expressed in some bladder tumors adjacent to patent vasculature—an area where oxygen levels should be normal.³⁴ We have shown HIF-1 α and HIF-2 α are present in invasive bladder cancer cells grown under normoxic conditions (personal communication: K Foreman, PhD).

BLADDER CANCER

Bladder cancer is a carcinoma of the epithelial lining of the bladder. There is no known genetic abnormality associated with bladder cancer, but the best-known risk factor is cigarette smoking.⁴³ Bladder cancer is the fourth most common cancer in men and seventh most common in women in the United States. The American Cancer Society estimates that there will be 74,000 new cases diagnosed and 16,000 deaths due to bladder cancer in 2015.⁴⁴ This is a disease primarily of the elderly, as the median age at diagnosis is 65-70 years.⁴³

The tumor can be categorized into one of four stages: 0-IV. Stage 0 signifies a carcinoma in situ (CIS) or a papillary tumor that is growing in towards the hollow of the bladder, and has not infiltrated the connective tissue layer that surrounds the epithelial. Stage I signifies a tumor that has reached the connective tissue, but has not infiltrated the muscle of the bladder, nor has it spread beyond the bladder. Stage II signifies a tumor that has infiltrated, but has not passed all the way through the muscle layer of the bladder. Stage III signifies a tumor that has passed all the way through the muscle and into the fatty outermost layer of the bladder. A cancer at this stage may have infiltrated the vagina, prostate, or uterus, but has not reached the abdominal nor pelvic wall. Stage IV indicates a tumor that has broken through the bladder wall to the abdominal or pelvic wall, and may or may not have spread to lymph nodes or distant sites in the body.⁴³ Treatment strategies and outlook vary based on stage.

Any tumor that has not invaded the muscle is categorized as non-invasive, whereas a tumor that has infiltrated the muscle layer of the bladder is defined as muscle-invasive. Once the disease has infiltrated the muscle, the survival outlook is significantly diminished. The likelihood that a patient will live at least 5 years after diagnosis is defined as a 5-year survival rate. Based on information between 1988-2001, the 5-year survival rate for an individual diagnosed with non-muscle invasive disease is 88%. This number drops to 63% when the tumor has invaded the muscle, and to only 15% when the tumor has reached the abdominal or pelvic wall (stage 4, or advanced disease).⁴⁴ A contributing factor to the poor outlook is that 50% of patients with muscle invasive bladder cancer develop metastatic disease within 3 years.⁴⁵

The current standard of care for muscle invasive bladder cancer may begin with neoadjuvant platinum based chemotherapy regimen of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) or cisplatin plus gemcitabine in patients that are fit.⁴⁶⁻⁴⁸ This is followed with a radical cystectomy (removal of the bladder) accompanied with a hysterectomy in women and removal of the prostate in men. In less than 5% of cases, a partial cystectomy may be performed instead. Adjuvant treatment of platinum based chemotherapy regimen, may be administered in hopes of preventing disease recurrence. Patients at risk for recurrence may also be considered for radiation therapy.

Metastatic disease is responsible for approximately 12,500 deaths per year in the US, and remains an incurable disease.⁴⁹ In metastatic disease, cancer cells

accumulate in pelvic lymph nodes, and then travel to distant parts of the body via the lymph vessels. Bladder cancer primarily metastasizes to the lung and bone, as well as liver and brain. Prognosis of advanced disease is poor, with median survival of only 12 months.⁵⁰

To treat advanced disease, a chemotherapy regimen of cisplatin plus gemcitabine is used in patients that are fit. This treatment increases survival to 12.5-14.3 months.^{50,51} Cisplatin is a platinum-containing agent that works to crosslink DNA, resulting in programmed cell death.⁵² Cisplatin is neurotoxic, nephrotoxic, and ototoxic. Its neurotoxicity and nephrotoxicity are dose-limiting side effects. Many patients have concomitant kidney disease and are unable to tolerate even low doses of cisplatin.⁵³ Cisplatin's toxicity is thought to be attributed to the accumulation of reactive oxygen species, as well as protein dysregulation within various cellular compartments.⁵⁴

Due to other co-morbidities, such as poor renal function, lung or liver disease, half of patients with advanced disease are unfit for cisplatin therapy. In some of these patients, carboplatin is substituted for cisplatin, as it is less toxic. Carboplatin works in a similar manner to cisplatin, but has dose-limiting toxicities of its own, such as myelosuppression, primarily thrombocytopenia.⁵⁵ More importantly, carboplatin is known to be inferior in the treatment of metastatic disease.⁵⁵⁻⁶⁰ Gemcitabine is common to both therapies, and is a nucleoside analog that arrests tumor growth by inducing apoptosis.⁶¹ Gemcitabine acts

synergistically in combination with both carboplatin and cisplatin in the treatment of bladder cancer. ^{55,62,63}

EMETINE

Emetine is a natural alkaloid of the root of ipecac, or *ipecacuanha*, a plant indigenous to Brazil (Figure 5). Emetine's initial use dates back as early as the 17th century, and it continued to be used as an emetic and in the treatment of dysentery, caused by *Entamoeba histolytica* infection, until recent years.^{64,65} Emetine was available through the CDC for dysentery treatment until 10-15 years ago when better alternatives became available.⁶⁶ Emetine has been available over the counter as syrup of ipecac but was removed from the market a few years ago, when it was no longer recommended to induce vomiting in children who had swallowed toxic chemicals, and it was being abused by anorexia and bulimia patients.⁶⁷

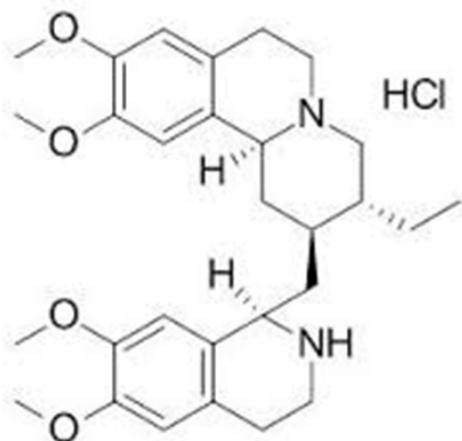


Figure 5: Structure of Emetine

Emetine as an Anti-Cancer Therapeutic

The first use of emetine as an anticancer agent in 1918 is thanks to the belief held by Lewisohn that cancer was of parasitic origin.⁶⁸ Upon the potent anti-parasitic effects observed of emetine, Lewisohn began the first study of emetine as an anticancer agent on human tumors. Apparent tumor regression was observed, but the studies were not pursued.⁶⁸ Van Hoosen also observed tumor regression with single agent emetine treatment and published her findings in 1919.⁶⁹

In the late 1960's investigators began to study emetine as an anti-tumor agent in more organized, clinical trials, and reported mixed results in a variety of malignancies.⁷⁰⁻⁷² An initial study done by Abd-Rabbo in the treatment of malignancy with emetine was in a patient that presented with a parasitic infestation. Due to the patient's symptoms attributed to chronic myeloid leukemia, the investigator felt the patient unfit for the current therapy and chose to substitute emetine as treatment for parasitic infestation. To his surprise, the treatment of 1 mg/kg/day dehydroemetine (synthetically derived emetine) alone brought about a robust hemopoietic remission, similar to that seen upon chemotherapy.⁷³

To date, there have been two studies examining emetine's anti-neoplastic effect on bladder cancer. After his initial study in leukemia, Abd-Rabbo conducted subsequent studies in the treatment of a variety of malignancies with dehydroemetine as a single-agent therapy. Carcinoma of the bladder was one of several solid malignancies in his study. Nine cases of bladder cancer were treated with 50 mg/day of dehydroemetine (DHE) orally for 21 to 30 days.⁷⁴ The

investigator notes the drug was surprisingly effective in the treatment of bladder carcinoma. The patients reported early improvement in symptoms of dysuria, hematuria, and incontinence, and a decrease in the size of tumor.⁷⁴ Two of the 9 patients were reported living after 2 years, the other 7 did not present for follow-up.⁷⁴ This same study reported mixed results in the treatment of other malignancies.⁷⁴

A few years later, in 1971, a phase I clinical trial of emetine as a single agent in the treatment of a variety of malignant tumors was conducted by Panettiere and Coltman. The study included one patient with bladder cancer, who demonstrated stabilization of the disease upon 17.4 mg/kg emetine. The investigators concluded that a minimum of 15 mg/kg emetine as a single-agent to be a reasonable treatment of various solid tumors.⁷²

Clinical studies with emetine were not pursued due to its marginal efficacy combined with severe side effects. Cardiotoxicity and muscle weakness caused the withdrawal of most patients enrolled in clinical studies examining the effectiveness of emetine as a single-agent therapy. These patients received substantial doses of emetine, up to 50 mg/kg/day for 30 days.^{72,74} Investigators suggest that lower doses are unlikely to have such severe side effects.⁷²

Minimal work has been done with emetine as part of a combination therapy. An investigation done by B.W. Street at Farnborough Hospital demonstrated a definite response in 6 distinct cases of lung carcinoma with combined therapy of emetine and cyclophosphamide. Street noted no toxic side effects from 1.5 mg/kg weekly IV emetine treatment, suggesting EKG monitoring as the only precaution in

this treatment.⁷⁵ However, further clinical work with emetine either as a single-agent or as part of a combination therapy has not been conducted.

More recently, Larsson examined emetine in combination with doxorubicin, etoposide, oxaliplatin, and docetaxel in neuroendocrine cell lines (atypical and typical bronchial carcinoid and pancreatic carcinoid).⁷⁶ His studies demonstrated a synergistic response in all three cell lines with emetine plus etoposide, oxaliplatin, and docetaxel, and an additive response of emetine plus doxorubicin.

Concentrations of emetine in these studies ranged from 0.0062-1.6 μ M.⁷⁶ His primary studies of synergism in cell culture suggest that combination therapy with emetine may be a reasonable candidate for clinical studies in patients with these, and possibly other, malignancies.⁷⁶

Emetine has also been shown to be anti-proliferative in combination with cisplatin in leukemia.⁷⁷ Studies in leukemia cells demonstrate that the combined treatment of emetine plus cisplatin has an additive effect and increases cisplatin-induced apoptosis.⁷⁷ The addition of emetine to many chemotherapy drugs has been demonstrated to be additive or synergistic in a number of malignant cell types, suggesting that its inclusion in a therapy regimen may be beneficial.

A recent study by our group reported emetine to act synergistically with cisplatin in the treatment of bladder cancer cells, but not in normal bladder epithelial.⁷⁸ The same study found that the combined treatment caused cells to undergo growth arrest, rather than die via apoptosis.⁷⁸ However, the addition of emetine to the full standard of care chemotherapeutic regimen for bladder cancer

has yet to be examined. The anti-proliferative effects of emetine alone and in combination against carcinomas, specifically of the bladder, suggest the need for further research.

Mechanism of Action

Emetine's primary mechanism of action is by inhibiting protein synthesis in mammalian and other cells. Grollman has extensively studied emetine's activity in HeLa cells. His studies demonstrate the rapid inhibition of protein biosynthesis at the micromolar level, with 50% inhibition at 0.04 μM and 99% inhibition at 1 μM .⁶⁴ He observed a corresponding effect on DNA synthesis, but inhibition was never complete, as it was with protein synthesis. He also observed a slight decrease in RNA synthesis.^{79,80}

Studies on emetine's effect on protein, DNA, and RNA synthesis have been conducted in other cell types and species. Emetine's effect on protein synthesis is consistent, but its inhibition on DNA and RNA synthesis is varied. Milimolar concentrations of emetine inhibit protein synthesis, but do not affect DNA synthesis in *Tetrahymena pyriformis*.⁸¹ Additionally, when rats were injected with a sublethal dose of emetine, their liver and kidney protein concentrations were reduced, but RNA levels were unaffected.⁸² At 10 mM, emetine inhibits protein synthesis by 75% in gametophytes of *Anemia Phylitidis*, a higher plant. At a ten-fold higher concentration, emetine inhibits protein synthesis by approximately 95% in rabbit reticulocytes and the yeast *S. cerevisiae*. Although emetine consistently inhibits protein synthesis in protozoa, plants, yeast, and mammalian cells and tissues, it is shown to be ineffective as a protein synthesis inhibitor in *E. coli* and other prokaryotes.⁸⁰

Treatment of HeLa cells with 1 mM emetine causes a decrease in the number of free ribosomes, and an increase in the number of polyribosomes, as well as nascent peptide.⁷⁹ Emetine's primary mechanism of action is by inhibiting protein biosynthesis. Emetine acts by interfering with the aminoacyl-(t)RNA transfer reaction, and is specific to this step in protein biosynthesis.⁸⁰ By preventing the transfer of amino acids on the polyribosome, emetine causes the nascent polypeptide to remain attached to the ribosome.⁸⁰ Additional studies done by Grollman suggest that emetine does not bind to the ribosome, either single or poly, but likely interferes with the enzymatic formation of the peptide bond between amino acids while docked in the ribosome.^{79,80} However, a study done by Jimenez et al. suggests that emetine binds directly with the 40S subunit of the ribosome to interfere with the aminoacyl-tRNA transfer reaction.⁸³ Although these two studies disagree about emetine's exact site of action, they confirm that its mode of action is by preventing movement of the mRNA along the ribosome.⁸³ Emetine's effect on protein synthesis is confined to the cytoplasm, as concentrations that completely block cytoplasmic protein synthesis do not affect mitochondrial protein synthesis.⁸⁴

The effects of emetine on macromolecule synthesis are structurally specific. Emetine's isomers, isoemetine, and O-methylpsychotrine, show less than 1% of the activity of emetine. The therapeutic and toxic effects of emetine may likely be accounted for by the metabolic disturbance caused by protein biosynthesis inhibition.⁶⁴

Emetine and HIF

Emetine has been shown to rapidly and preferentially downregulate HIF-2 α protein, but not mRNA, in a concentration dependent manner in clear cell renal carcinoma (CCRCC) cell lines at concentrations as low as 50 nM.¹³ In these cells, HIF-2 α protein appears to be degraded in the proteasome in response to emetine treatment.¹³ Moreover, 1 μ M emetine was shown to reduce the level of secreted VEGF and TGF- α (HIF-2 α transcription products) by 75%.¹³ This suggests that emetine's downregulation of HIF-2 α has a functional effect on the cell.

Emetine has also been found to downregulate levels of nuclear HIF-1 α but not HIF- β in a human breast cancer cell line at concentrations as low as 0.11 μ M. Additionally, 0.3 μ M emetine treatment decreased the level of VEGF secreted by these cells by 50%, again suggesting emetine may affect the function of the cell by downregulating HIF-regulated genes.⁸⁵ Emetine's effect on HIF proteins in other cancers has not yet been examined.

OBJECTIVES AND HYPOTHESIS

The goal of this project is to test emetine's efficacy as an anticancer therapeutic in combination with standard of care chemotherapeutic regimens in bladder cancer, and work to define the mechanism behind its action. We proposed the following hypothesis for this project.

Emetine, combined with standard of care chemotherapeutic regimens, may benefit bladder cancer patients by acting to enhance growth arrest and/or killing of bladder cancer cells. Emetine may act, at least in part, by altering hypoxia signaling.

SPECIFIC AIMS

AIM 1A: Determine if the addition of emetine to the standard of care chemotherapy regimen decreases proliferation in bladder cancer cells

Previous work in the Foreman lab has demonstrated that the addition of emetine to cisplatin causes a synergistic decrease in proliferation of bladder cancer epithelial cells.⁷⁸ It is important, however, to examine the addition of emetine to the standard of care therapy of cisplatin plus gemcitabine and carboplatin plus gemcitabine as they are a better representation of what patients receive in the clinic.

AIM 1B: Examine what is causing the decrease in cell proliferation

Using a variety of techniques, we will work to elucidate the mechanism behind the decrease in cell proliferation observed upon the addition of emetine to both cisplatin-gemcitabine and carboplatin-gemcitabine standard of care.

AIM 2: Examine the mechanism behind emetine's action

Previous work by Gupta and colleagues has demonstrated that emetine acts to downregulate HIF-2 α in CCRCC.¹³ We sought to examine if emetine acts in a similar manner in muscle invasive bladder cancer.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture

UMUC3 and HT1376 muscle invasive bladder cancer cell lines were cultured under standard conditions in Dulbecco's modified Eagle's media containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100µg/ml streptomycin. The T24 muscle invasive cell line was cultured under standard conditions in McCoys media containing 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100µg/ml streptomycin. Normal bladder epithelial cells (Cell-N-Tec) were cultured in standard conditions in CNT-PR media as recommended by the manufacturer. The cultures were confirmed mycoplasma negative through periodic testing using MycoAlert Mycoplasma Detection Kit (Lonza). Cells were maintained at 37°C in 5% CO₂ and sub-cultured when they reached 80-90% confluence (every 2-5 days).

Pharmaceutical Reagents

Drugs used in this project included emetine dihydrochloride hydrate (Sigma-E237) suspended in sterile Milli-Q water and stored at -4°C, Cisplatin (Selleck Chemical- S1166) suspended in sterile dimethyl sulfoxide (DMSO) and stored at -80°C, Carboplatin (Selleck Chemical- S1215) resuspended in sterile DMSO and

stored at -80°C, and Gemcitabine HCL (Selleck Chemical- S1149) resuspended in sterile Milli-Q water and stored at -80°C.

Proliferation Assay

Proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were plated at a density of 2,500-5,000 cells/well in a 96 well plate. Cells were allowed to adhere overnight and were then treated with individual drugs, drug combinations, or left untreated as a control. Forty-eight hours after treatment, MTT was added to each well. MTT (Sigma-M2128) was resuspended at 5 mg/ml in PBS, filtered to remove particulates and added for a final concentration of 0.5 mg/ml. The solution was gently mixed, and the cells incubated at 37°C for 2 hours. The media was removed, cells lysed in 100 µL DMSO, and the absorbance read at 560nm. Background absorbance at 670nm, as well as the absorbance from wells containing no cells, was subtracted from these values. Proliferation was reported as a percentage of no drug (untreated) control samples. Each condition was performed in quadruplicate.

The half maximal inhibitory concentration (IC₅₀) was calculated using PRISM software. Drug synergy was assessed via the Chou-Talalay Median Effects method with the combination index (CI) calculated via CalcuSyn software (Biosoft).^{86,87} A CI value of 0.2-0.8 indicates synergy, a CI value from 0.9-1.1 indicates an additive effect and a CI value >1.1 indicates an antagonistic response.^{86,87}

Trypan Blue Exclusion

Normal bladder epithelial cells were plated at a density of 3×10^4 – 8×10^4 cells/well in a 24-well plate. Cells were treated with drug alone or in combination when cells were at least 50% confluent (1-3 days after plating). Forty-eight hours after treatment, cells were lifted with accutase (Cell-N-Tec) and resuspended in a 1:1 ratio of media:trypan blue. Cells were counted using a hemocytometer. Several of the higher concentrations inhibited proliferation to the extent that there were fewer than 100 cells recovered, limiting the total cell count for that condition. A minimum of 150 cells were counted per condition, whenever possible.

Nuclear Protein Extraction

Cultured cells at 80-90% confluency were placed in an ice bath and rinsed with cold PBS. Cells were scraped into 1 mL cold PBS and transferred to a microfuge tube. The cells were pelleted with a low speed centrifugation at 4°C. The pellets were weighed, and 5 μ L/mg KCl buffer (10 mM Hepes- pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA) with HALT protease and phosphatase inhibitors was added to dissociate and swell cells. Samples were incubated on ice for 10 minutes. 10% NP-40 solution was added at a volume of approximately 1/18 of the KCL buffer, the cells vortexed, and then incubated on ice for 1 minute to lyse cells. Samples were centrifuged on top speed for 30 seconds to pellet nuclei. NaCl buffer (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA) at approximately 1/8 the volume of the first salt buffer was added to nuclear pellet. Samples placed in shaker at 1450 rpm at 4°C

for 40 minutes. Samples were centrifuged at top speed for 10 minutes to pellet debris. Nuclear extracts were stored at -80°C in 20-30 μ L aliquots.

Whole Cell Protein Extraction

Cultured cells at 80-90% confluency were placed in an ice bath and rinsed with cold PBS. Cells were scraped into 1 mL cold PBS and transferred to a microfuge tube. Plates were rinsed with cold PBS to collect any remaining cells, which was added to the microfuge tubes. Samples were centrifuged at low speed for 5 minutes, and supernatant removed. 100 μ L of ice cold RIPA buffer (PBS with 1% NP-40 and 0.1% SDS) with 5 mg sodium deoxycholate per mL RIPA plus HALT protease and phosphatase inhibitors was added to pellet. Sample was sonicated on ~10% duty cycle and output of 3.5 for 4-5 pulses and repeated. Samples were shaken at 1450 rpm at 4°C for 1 hour, and centrifuged for 20 minutes at top speed at 4°C. Supernatant was frozen at -80°C in 20-40 μ L aliquots.

Immunoblotting

Protein concentration of nuclear or whole cell extracts was determined using Pierce micro plate BCA-Protein Assay Kit according to manufacturer's instructions. 50-100 μ g protein samples were added to an appropriate amount of SDS-PAGE loading buffer containing beta-mercaptoethanol and heat denatured for 10 minutes prior to loading onto an SDS-PAGE denaturing gel. Samples were run at 100 volts then transferred to Immobilon PVDF membrane. Even protein loading was verified by Ponceau red stain of membrane after transfer. Primary antibodies used included

HIF-1 α (1:500, BD Bioscience-610959), HIF-2 α (1 μ g/ml, R&D Systems-AF2886), HIF- β /ARNT (1:1000, Cell Signaling-D28F3), Lamin A/C (1:1000, Cell Signaling-2032S). Primary antibodies were detected using species-specific, horseradish peroxidase labeled secondary antibodies (1:1000, BD Bioscience). Membranes were imaged using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific-34078) on a Fujifilm imager.

Detection of Autophagy: monodansylcadaverine staining of autophagosomes

Cells were plated on sterile cover slips at a density of 1.1×10^5 cells per well of a 6-well plate. Cells were allowed to adhere to the cover slips overnight, then treated with single-agent or combination of emetine, cisplatin, and/or gemcitabine. Forty-eight hours after drug treatment, 50 μ M monodansylcadaverine (MDC, resuspended in sterile DMSO) (Sigma-D4008) was added and cells incubated at 37°C for one hour. Cells were washed 3X with 1% FA (Difco) buffer and mounted on slides using Vectasheild mounting medium (Vector Laboratories) and immediately imaged with a fluorescent microscope under excitation wavelength of 335nm. Ten random high powered images were captured per slide using Olympus cellSens Dimensions 1.6 software.

MDC is an auto fluorescent compound that accumulates in autophagosomes that accrue during autophagy.⁸⁸ To quantitate the percentage of autophagy positive cells, ImageJ software was used. The cell counter add-in was utilized to determine the total number of cells in each image and then fluorescent cells above a pre-set

threshold were highlighted and counted as positive. The intensity threshold was set so that 3% or less of untreated cells were positive. A minimum of 500 cells per condition were counted.

Immunoprecipitation

Cultured cells at 80-90% confluency were rinsed twice in ice-cold PBS. 900 μ L of 1X Cell Lysis Buffer (Cell Signaling-9803) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Roche; diluted in DMSO) and HALT protease and phosphatase inhibitors was added drop wise to one T150 plate, and incubated on ice for 5 minutes. Cells were scraped into a microfuge tube and flash frozen in ethanol and dry ice, or overnight at -80°C . Lysates were thawed, and sonicated 4-5X on a duty cycle of $\sim 10\%$ and an output setting of 4. Samples were spun at $14,000 \times g$ to pellet cellular debris. The protein concentration was determined using Pierce micro plate BCA-Protein Assay Kit according to manufacturer's instructions. Samples were diluted with 1X lysis buffer accordingly to equalize the protein concentration. Lysates were pre-cleared by adding 5 μ g of irrelevant antibody of appropriate species and isotype control to 1 mL of lysate, and rotated at 4°C for 60 minutes [abcam-ab18469 (mouse IgG2b) & ab27472 (rabbit IgG), and Santa Cruz Biotechnology- sc2028(goat IgG)]. 75 μ L of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology-sc2003) pre-washed in 1X lysis buffer plus HALT protease and phosphatase inhibitors were added to each tube and rotated at 4°C for 30-60 minutes. Beads were pelleted by centrifuge at low speed for 2-3 minutes.

Supernatant was removed and transferred to new, pre-chilled microfuge tube. 5 μ g of antibody, either HIF-1 α (Santa Cruz- sc10790, or abcam- ab1) or HIF-2 α (R&D Systems-AF2886) was added to each tube and rotated overnight at 4°C. 50 μ L of conditioned beads were added and rotated at 4°C for 1-2 hours. Beads were pelleted at low speed for 2-3 minutes at 4°C, and washed in 1X lysis buffer plus HALT protease and phosphatase inhibitors 4 times, and resuspended in 30 μ L 50 μ M Tris pH 8.

Click-iT Metabolic Labeling of Proteins

Cells were incubated in DMEM containing 2%FBS with or without 100 μ M CoCl₂ for 6 hours. The media was replaced with methionine-free media containing 2% FBS, and increasing concentrations of emetine were added for 1 hour. Media was removed, and plates rinsed with warm PBS. AHA, or L-azidohomoalaine (Invitrogen-C10102), was added to media at a final concentration of 50 μ M for three hours. For immunoprecipitation experiments, cells were lysed and either HIF-1 α or HIF-2 α total protein was immunoprecipitated as stated above. Newly synthesized proteins were selected using Click-iT Protein Reaction Buffer Kit (Invitrogen-C10276). 50 μ L alkyne solution was added to beads, and vortexed for 5 seconds. 5 μ L of CuSO₄ solution was added to each tube. 5 μ L of reconstituted component D was added and tube was vortexed for 5 seconds. Tubes were held on ice for at least 2, but no more than 3 minutes. 10 μ L of reaction buffer additive 2 was added to each tube, and vortexed for 5 seconds. Tubes were rotated at 4°C for 1 hour. Beads were

washed 1X in TBS. Proteins were eluted by heating in 40 μ L of 2X Laemmli Sample Buffer (Bio Rad- 161-0737EDU) plus beta-mercaptoethanol for 5 minutes at 95°C. Beads were precipitated by centrifuge at high speed for 1 minute. The entire supernatant was loaded onto 8% SDS-Page denaturing gel for western blot analysis. Primary antibodies used were HIF-1 α , or HIF-2 α . Streptavidin-HRP (1:5,000-1:7,000, Invitrogen-43-4323) was used to detect biotin.

To evaluate total protein synthesis, proteins were precipitated following the click reaction, as stated above, by adding 600 μ L methanol, 150 μ L chloroform, and 400 μ L of milli-Q water to microfuge tubes immediately after the 1 hour rotation. Tubes were briefly vortexed, and centrifuged at high speed for 5 minutes. Upon removal of the upper aqueous phase, another 450 μ L of methanol was added, samples were vortexed and centrifuged at high speed to pellet protein. Samples were washed once with methanol and allowed to air dry in microfuge tube covered with a Kim wipe 15 minutes to overnight. Protein was resuspended in 1X loading buffer plus beta-mercaptoethanol, vortexed for 10 minutes and boiled at 70°C for 10 minutes. Samples were run on an 8% SDS-Page denaturing gel for western blot and Streptavidin-HRP was used to detect biotin.

Propidium Iodide Staining

Cells were plated at a density of 5×10^5 in p60 dishes and allowed to adhere overnight. Cells were treated with drug alone or in combination, media removed, and cells were retreated where applicable. When treatment schedule was complete,

media from plates were collected in a 15 ml conical tube. Attached cells were rinsed once with PBS, lifted with trypsin and added to tube. Cells were pelleted by low speed centrifugation at 4°C. Supernatant was removed, and cells resuspended in FACS buffer (1% FA buffer, 1% FBS, 0.1% sodium azide). At least 1×10^6 cells were transferred to a FACS tube and washed 1X with FACS buffer. Supernatant was decanted, and 100 μ L cold FBS was added to each tube. 600 μ L ice cold 100% ethanol was added to each tube while gently vortexing. Cells were incubated on ice for 30 minutes. 2 mL of cold FACS buffer was added, and cells were pelleted by centrifuge at low speed at 4°C. Liquid was decanted, and 10 μ L of 10 μ g/ml RNase, DNase Free (Thermo Scientific-ENO531) was added to cells plus 490 μ L PBS. Solution was mixed and incubated at 37°C for 15 minutes. 10 μ L of 5 mg/ml propidium iodide in PBS (Sigma P-4170) plus 490 μ L PBS was added to suspension. Samples were mixed gently and incubated for at least 1 hour at 4°C before analysis.

For cell cycle analysis via propidium iodide (PI) incorporation, samples were run on CantoII flow cytometer. Results were analyzed via FlowJo software. Gates were drawn manually for each experiment based on the untreated sample, and applied to the remaining samples for analysis.

Detection of Apoptosis: Annexin V/PI staining.

Cells were plated at a density of 5×10^5 in p60 dishes, and allowed to adhere overnight. Cells were treated with drug alone or in combination, media removed, and cells were retreated where applicable. When treatment schedule was complete,

media from plates were collected in 15 ml conical tubes. Plates were rinsed with PBS, which was added to conical. Cells were trypsinized for 5 minutes and added to conical. Samples were centrifuged for 5 minutes at low speed at 4°C. Cells were resuspended in 5 mL of cold PBS. The Beckman coulter Annexin V-FITC kit (PN-IM3546) was used to detect Annexin V positive cells following the manufacturer's instructions. Briefly, 1×10^5 – 3×10^5 cells were transferred to a FACS tube on ice and resuspended in 100 μ L cold 1X binding buffer from Annexin V-FITC kit. 1 μ L Annexin V-FITC was added followed by 5 μ L of PI. Samples were incubated on ice for 15 minutes in the dark. 400 μ L 1X binding buffer was added and samples were analyzed on Cantoll flow cytometer within 30 minutes. Data was analyzed using FlowJo software. Quadrants were drawn so that populations of cells were uninterrupted, and fit squarely into one quadrant. ⁸⁹

CHAPTER 3

RESULTS

AIM IA: DETERMINE IF THE ADDITION OF EMETINE TO THE STANDARD-OF-CARE CHEMOTHERAPY REGIMEN DECREASES PROLIFERATION IN BLADDER CANCER CELLS

To answer this question, we first determined the value of each drug that inhibited cell growth in vitro by 50%, or the IC₅₀ value, via an MTT proliferation assay. We used these values as reference points on which to base drug concentrations used in all subsequent experiments, including synergy proliferation assays. With the aid of PRISM software, we determined the following IC₅₀ values for cisplatin, carboplatin, gemcitabine, and emetine:

Table 1: Experimentally Determined IC₅₀ values.

	Cisplatin	Carboplatin	Gemcitabine	Emetine
UMUC3	6.8 μ M	73 μ M	31 nM	30 nM
HT1376	4.2 μ M	38.9 μ M	>1000 μ M	24.6 nM
T24	4.4 μ M	69.2 μ M	20 nM	66 nM
Normal Bladder Epithelial	24.6 μ M	281 μ M	>100 μ M	3.22 μ M

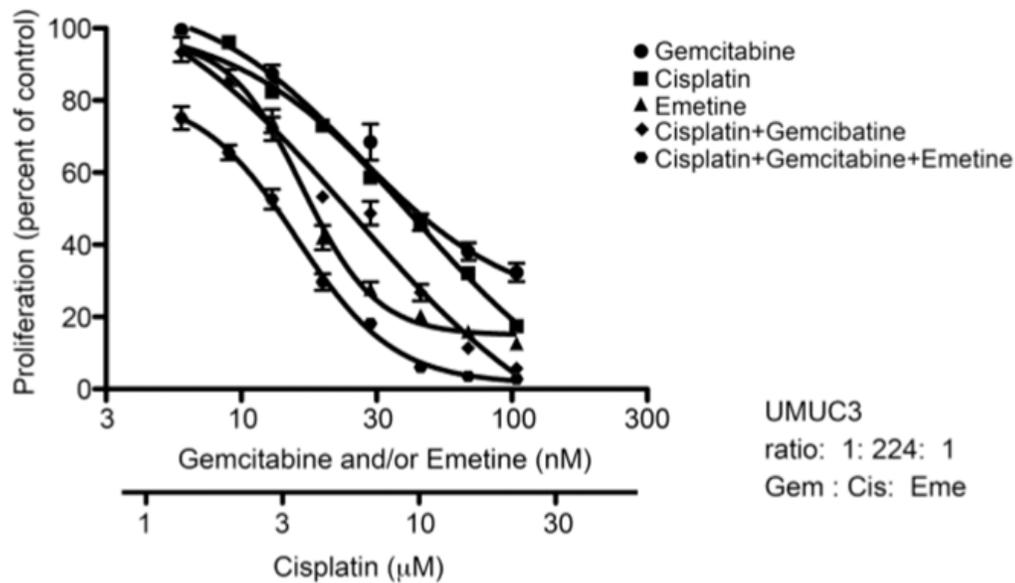
The Addition of Emetine to Cisplatin-Gemcitabine Standard of Care Results in an Additive Decrease in Cell Proliferation

Previous work in the Foreman lab has demonstrated that emetine acts synergistically with cisplatin to decrease cell proliferation in invasive bladder cancer cell lines.⁷⁸ Here, we examine the addition of emetine to cisplatin plus gemcitabine, or carboplatin plus gemcitabine, as it is a better representation of what patients receive in the clinic. As in our previous work, we used constant drug ratios to assess synergy.^{78,86,87} That is, each drug in a condition was applied at its IC₅₀ value, or a fraction of the IC₅₀ value. For this set of experiments, eight concentrations were tested. Proliferation was assessed using an MTT assay, and the combination index (CI, a measure of the combined effect) was calculated using CalcuSyn software. Previous work in our lab demonstrated that a 48-hour drug exposure followed by a 2-hour MTT incubation was optimal for these assays with the invasive bladder cancer lines.⁷⁸ These conditions were maintained for all MTT assays described below.

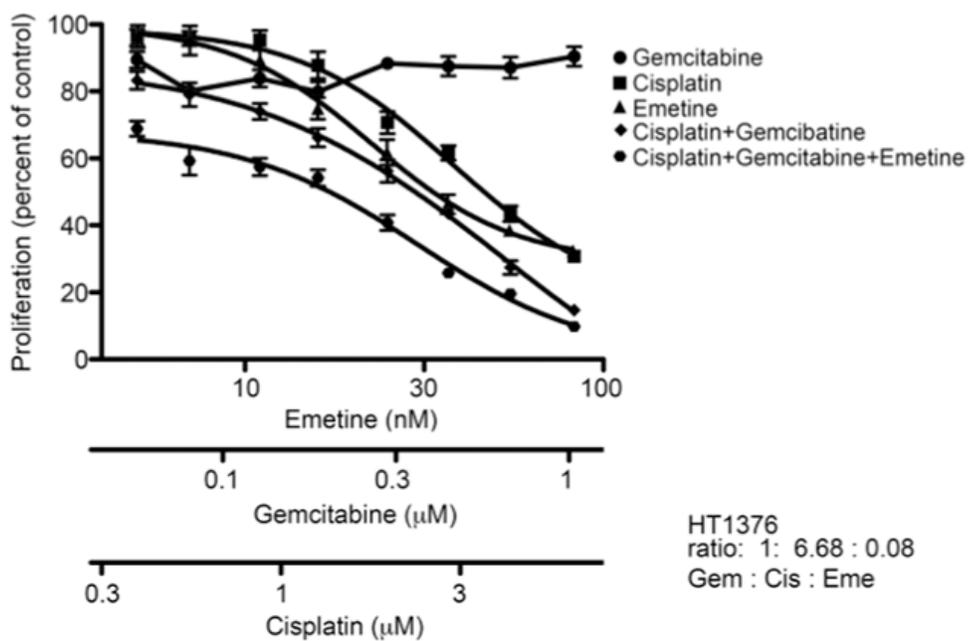
In the UMUC3 cell line, the addition of emetine to the cisplatin-gemcitabine therapy caused an additive decrease in cell proliferation, as demonstrated by a combination index between 0.9-1.1 at all tested drug ratios. The most effective drug ratio resulted in a decrease in proliferation of 28% +/- 2.6% between cells treated with emetine plus cisplatin-gemcitabine and those treated with cisplatin-gemcitabine alone (Figure 6A). Similar results were found with the HT1376 and T24 cell lines where the addition of emetine further decreased cell proliferation by 18%

+/- 4.7% (HT1376; Figure 6B), and 20% +/- 5.3% (T24; Figure 6C) in the most effective drug ratio.

A



B



C

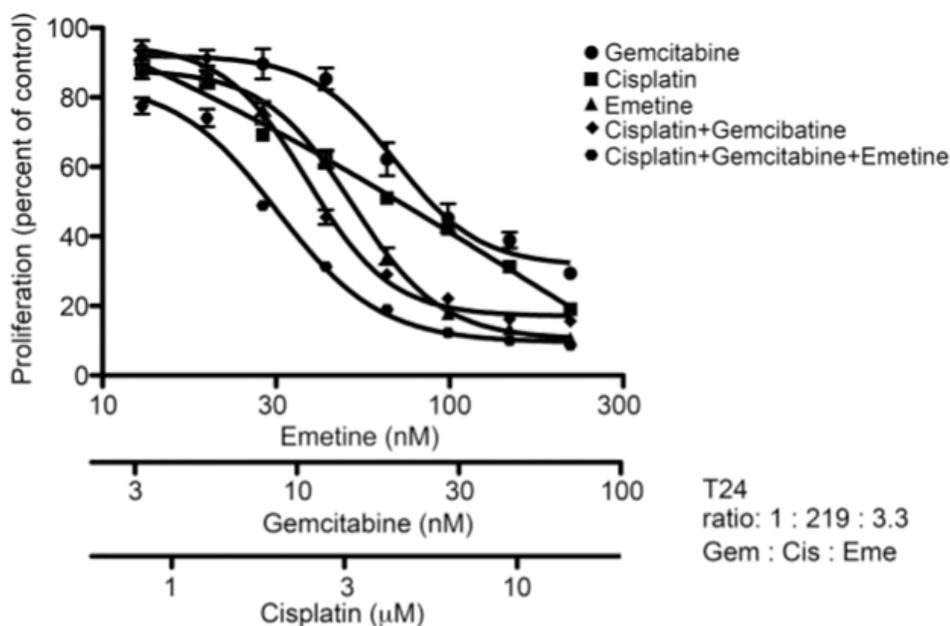


Figure 6: Addition of Emetine to Cisplatin-Gemcitabine Standard of Care Therapy

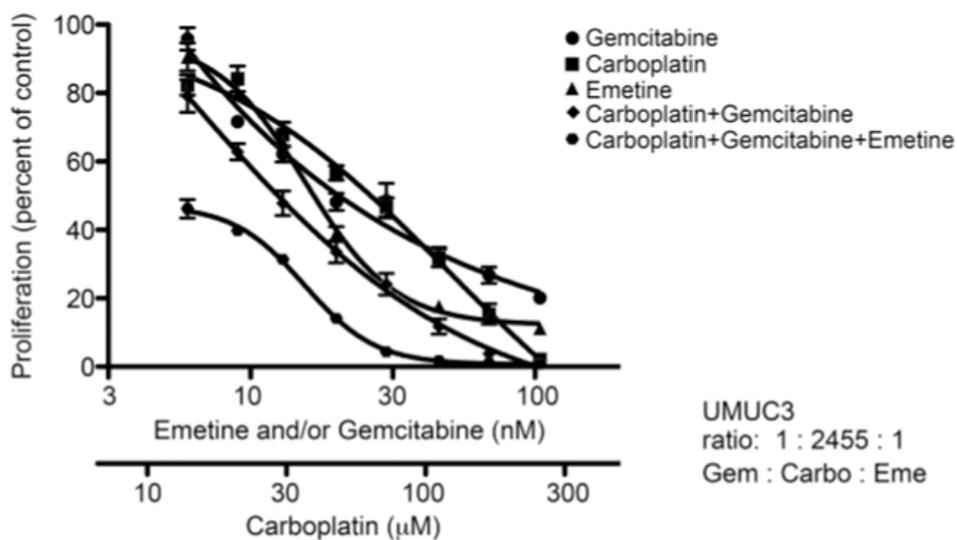
Cells were treated with drug alone or in combination at a constant ratio as indicated. Proliferation was analyzed via MTT assay. Data represent combined results from three independent experiments performed in quadruplicate. A) UMUC3 cells, B) HT1376 cells, C) T24 cells

Addition of Emetine to Carboplatin-Gemcitabine Standard of Care Results in an Additive Decrease in Cell Proliferation

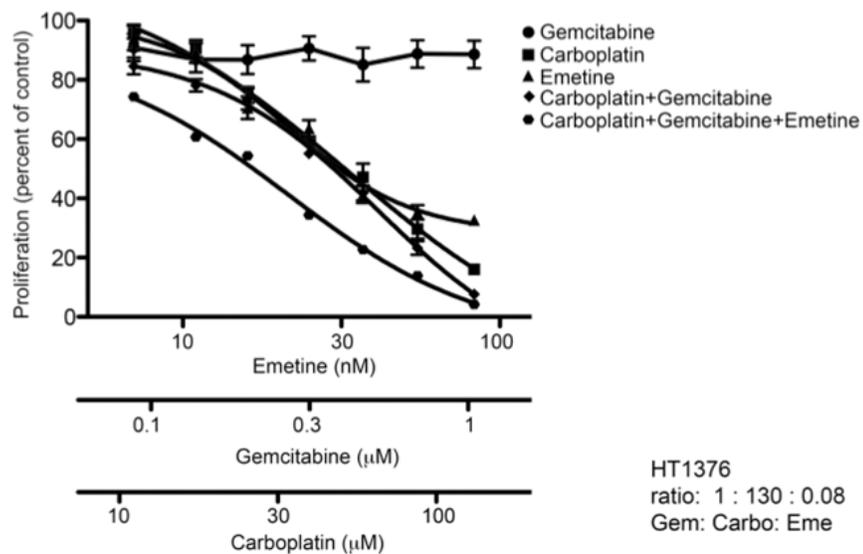
Next, we sought to examine the effect of adding emetine to carboplatin-gemcitabine therapy, a common chemotherapy regimen given to patients that are clinically unfit to receive cisplatin. As with the previous set of experiments, we used a constant ratio of drugs based on their individual IC_{50} values, again testing eight

concentrations. In UMUC3 and HT1376 cell lines, the two lowest concentrations resulted in modest synergy ($CI=0.6-0.8$), but additive effects were found at higher drug concentrations ($CI=0.9-1.1$), and in all conditions in T24 cells. For UMUC3, optimal conditions demonstrated a decrease in proliferation of $36\% \pm 2.7\%$ with the addition of emetine (Figure 7A), while the decrease in HT1376 was $25\% \pm 4.3\%$ (Figure 7B) and T24 cells showed a decrease of $24\% \pm 5.2\%$ (Figure 7C).

A



B



C

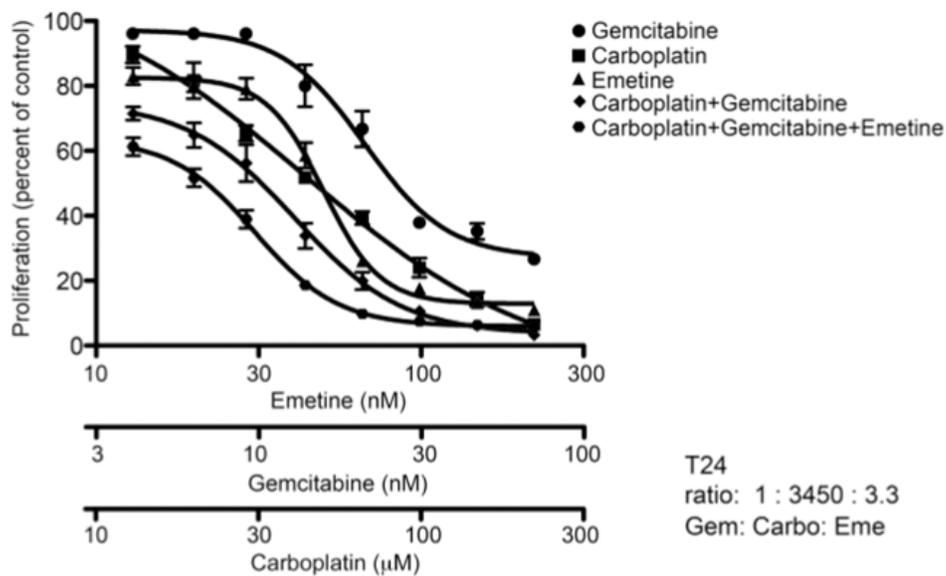


Figure 7: Addition of Emetine to Carboplatin-Gemcitabine Therapy

Cells were treated with drug alone or in combination at a standard ratio 24 hours after plating. Proliferation assessed via MTT assay 48 hours after treatment. Data represent combined results from three independent experiments performed in quadruplicate. A) UMUC3 cells, B) HT1376 cells, C) T24 cells.

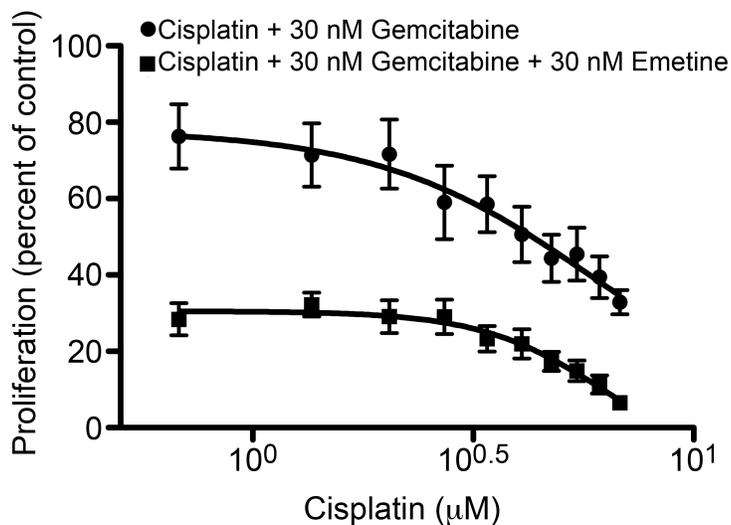
Low-dose Emetine Decreases the Effective Cisplatin and Carboplatin Dose Required to Inhibit Bladder Cancer Cell Proliferation

Because emetine enhanced inhibition of bladder cancer cell proliferation by standard therapeutic regimens, we wondered if emetine could decrease the dose of cisplatin or carboplatin required to effectively inhibit proliferation. To answer this question, we used an MTT proliferation assay, but instead of using constant drug ratios, we treated with a fixed, low-dose of emetine and gemcitabine, while decreasing the dose of cisplatin or carboplatin. We began with all the drugs at their calculated IC_{50} , and then sequentially decreased the dose of cisplatin or carboplatin by 10% until reaching zero.

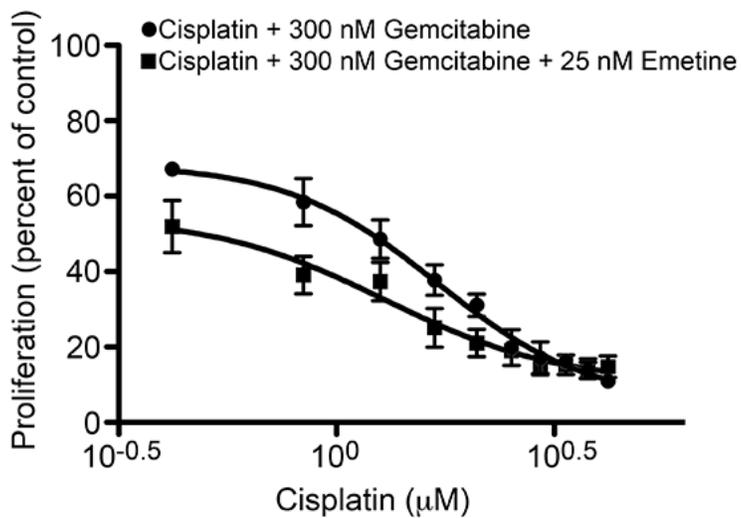
Strikingly, in the UMUC3 cell line, when only 0.68 μ M cisplatin was added to emetine (30 nM) and gemcitabine (30 nM), proliferation was blocked to the levels seen upon treatment with 6.8 μ M cisplatin and 30nM gemcitabine (figure 8A). These results suggest that inclusion of nanomolar concentrations of emetine may allow a 10-fold reduction in the cisplatin dose while maintaining comparable anti-proliferative effects, *in vitro*. The results were more modest with HT1376 [figure 8B,

average 2-fold decrease in cisplatin (range 1.0-3.7-fold)] and T24 cells [figure 8C, average 2.6-fold decrease in cisplatin (range 1.5-4.5-fold)].

A



B



C

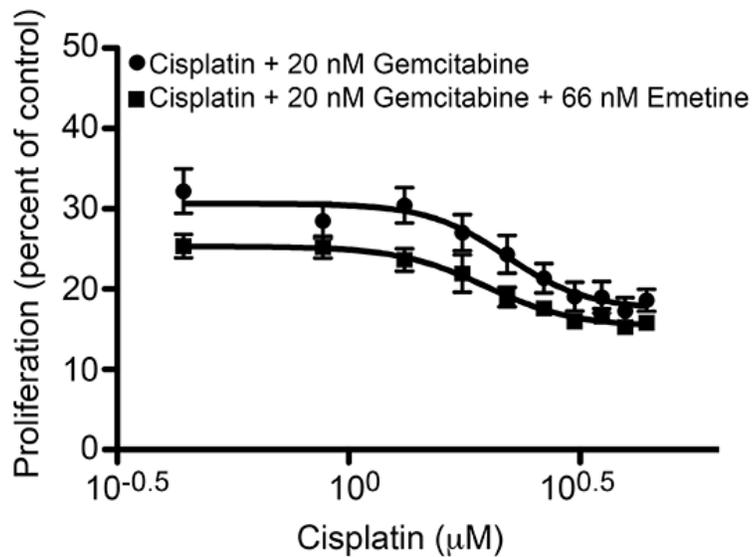


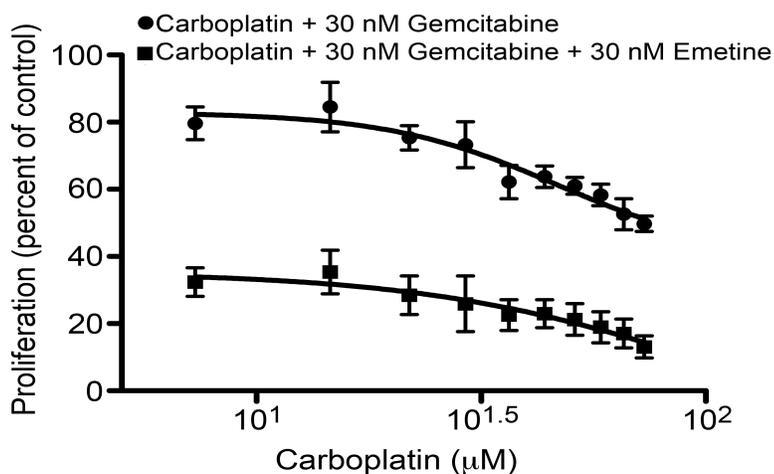
Figure 8: Low Dose Emetine Reduces Effective Dose of Cisplatin

Cells were treated with constant dose of gemcitabine with or without a constant dose of emetine in the presence of increasing concentrations of cisplatin. Data represent combined results from three independent experiments performed in quadruplicate. A) UMUC3 cells, B) HT1376 cells, C) T24 cells

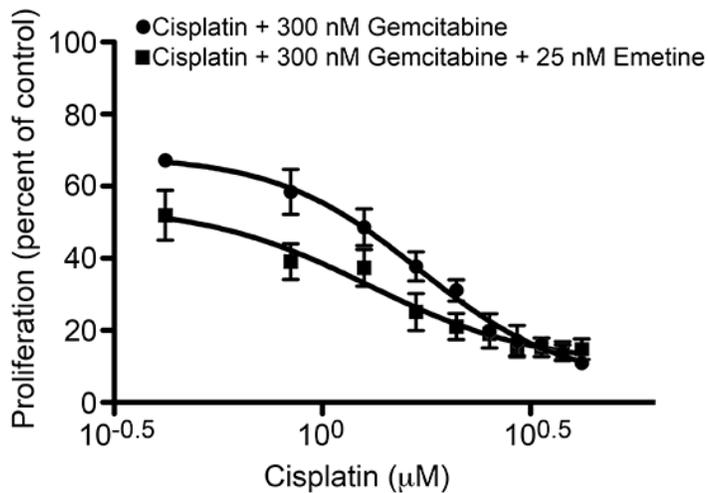
With respect to carboplatin, we also found that low dose emetine could reduce the dose of carboplatin required for anti-proliferative effects. Again, we found 30 nM emetine resulted in a 10-fold decrease in the dose of carboplatin needed to block UMUC3 cell proliferation seen with carboplatin and gemcitabine at IC₅₀ concentrations (Figure 9A). Similar, albeit more modest, results were observed in HT1376 [figure 9B; 4.4-fold decrease in carboplatin (range: 2-6.7-fold)]. With

respect to T24 cells, there was no significant difference in proliferation in the presence of emetine regardless of the carboplatin dose (Figure 9C; 1.2-fold decrease in carboplatin).

A



B



C

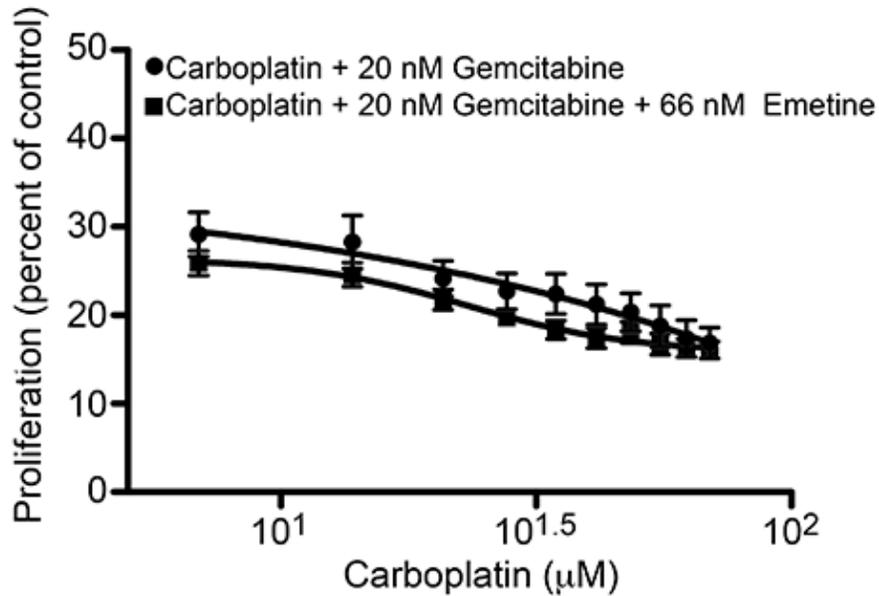


Figure 9: Low Dose Emetine Reduces Effective Dose of Carboplatin

Cells were treated with constant dose of gemcitabine with or without a constant dose of emetine in the presence of increasing concentrations of carboplatin. Data represent combined results from three independent experiments performed in quadruplicate. A) UMUC3 cells, B) HT1376 cells, C) T24 cells

Cisplatin-Gemcitabine-Emetine has a Greater Effect on Proliferation of Invasive Bladder Cancer Cells than on Normal Bladder Epithelial

When normal bladder epithelial cells were used in similar experiments, emetine had little or no additional affect on cell proliferation. The results were virtually identical in cells treated with increasing doses of cisplatin and a constant

dose of gemcitabine whether or not emetine was added to the cultures (Figure 10A).

The results were similar with carboplatin (Figure 10B). When proliferation was

compared with invasive bladder cancer cell lines, proliferation of the normal

bladder epithelial cells was at least 20% greater in all conditions (data not shown).

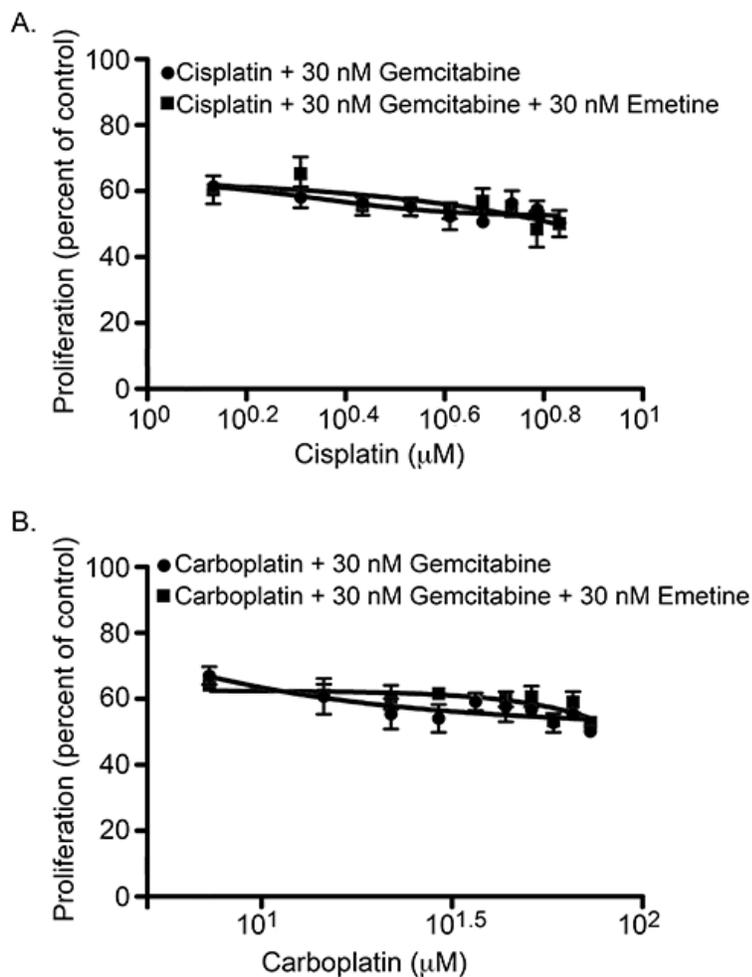


Figure 10: Addition of Emetine does not Enhance Anti-Proliferative Effect of Normal Bladder Epithelial Cells Treated with Cisplatin-Gemcitabine or Carboplatin-Gemcitabine.

Normal bladder epithelial cells were treated with constant dose of gemcitabine with or without emetine in the presence of increasing doses of A) cisplatin or B) carboplatin. Data represent combined results from three independent experiments performed in quadruplicate.

AIM 1B: EXAMINE WHAT IS CAUSING THE DECREASE IN CELL PROLIFERATION

The Addition of Emetine to Cisplatin-Gemcitabine Standard of Care Results in Little to No Increase the Number of Cells Undergoing Apoptosis *in vitro*

Previous studies in the Foreman lab demonstrated that the addition of emetine to cisplatin caused a modest increase in the number of cells undergoing apoptosis.⁷⁸ We anticipated that the inclusion of gemcitabine, an apoptosis-inducing drug, would further increase apoptosis in the cultured cells. To test our hypothesis, we followed the same treatment schedule as in the proliferation assays. Apoptosis was assessed after 48 hours using the ANNEXIN V-FITC apoptosis detection kit and flow cytometry. No significant change in apoptosis (3.5% +/-1.3% decrease on average; p=ns.) was observed when emetine was added to cisplatin-gemcitabine therapy in the UMUC3 cell line.

When cells were treated twice, a slight decrease of 1.5% was observed upon the same treatment schedule. We expanded the treatment conditions to include T24 cells treated twice with the drug combination. When cells were treated twice and collected 48 hours after the second treatment, no significant increase in apoptosis (3.5% +/- 3.5% increase on average; p=ns.) was observed when emetine was added to cisplatin-gemcitabine therapy (Figure 11).

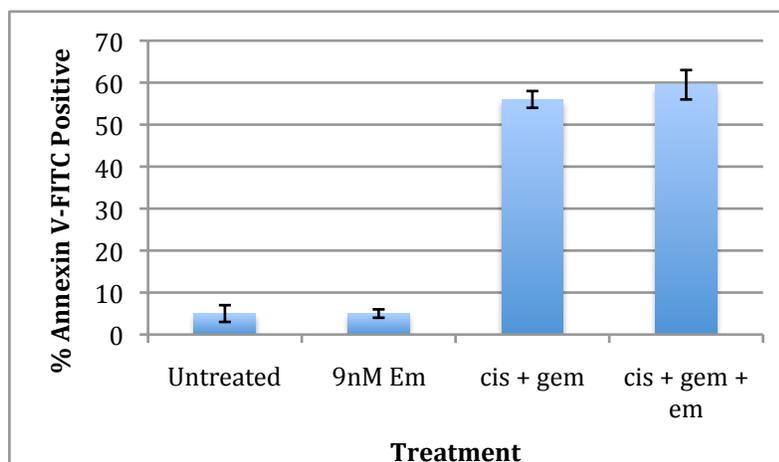


Figure 11: The Addition of Emetine to Cisplatin-Gemcitabine Has Little to No Effect on Apoptosis

T24 cells treated twice with drug alone or in combination (2 μ M cisplatin, 9 nM gemcitabine, 30 nM emetine), and collected for apoptosis analysis via ANNEXIN-V-FITC 48 hours after second treatment. Data is combined from 2 experiments

To extend these studies, we also examined the cell cycle profile of invasive bladder cancer cells treated with cisplatin-gemcitabine with or without emetine. Treatment with cisplatin-gemcitabine resulted in an accumulation of the cells in the S-phase and G2/M phase of the cell cycle with a modest increase in the number of cells in the sub-G₀ region. Addition of emetine did not increase the sub-G₀ population, but did partially reverse the shift of cells into the S and G2/M phases (Figure 12). This finding is consistent with our published report.⁷⁸

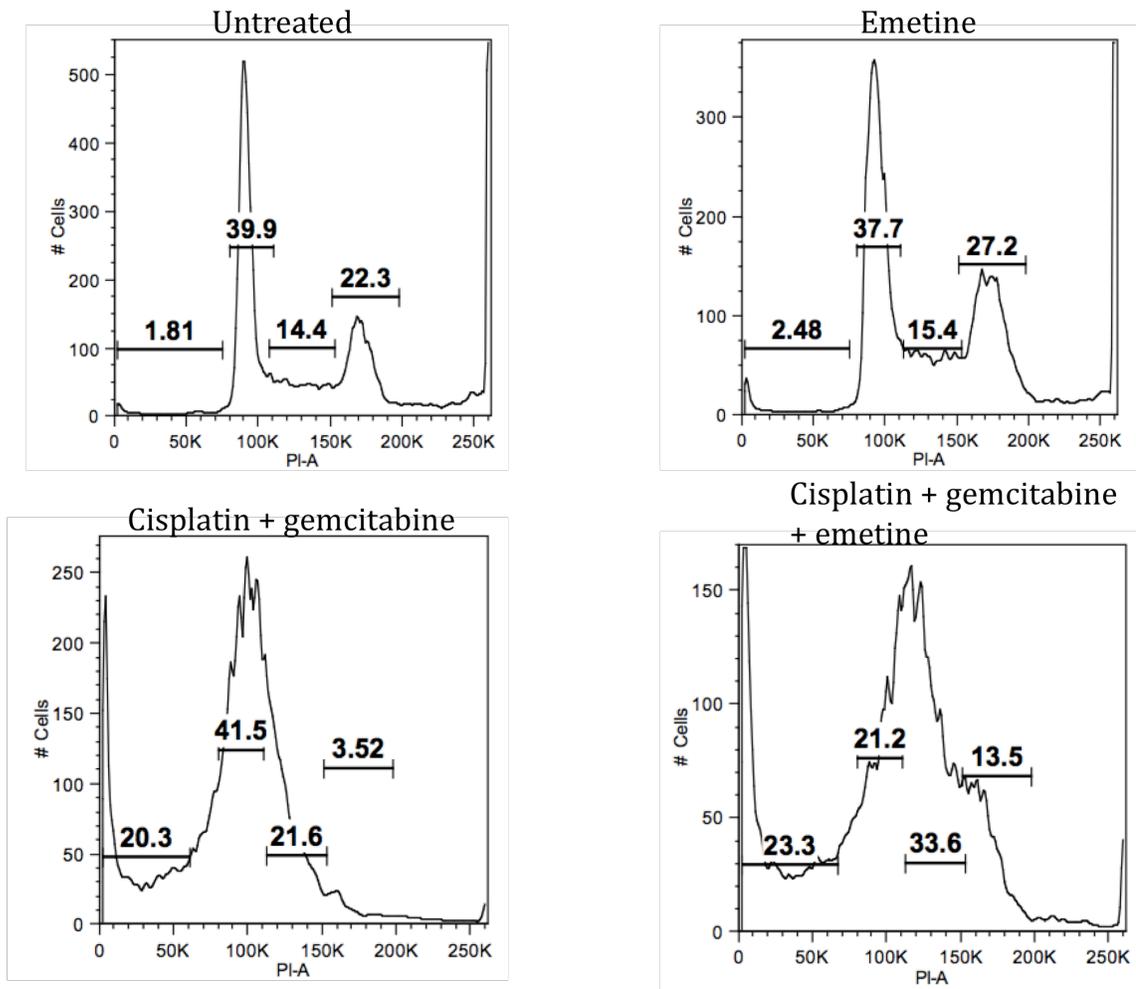


Figure 12: Effect of the Addition of Emetine to Cisplatin-Gemcitabine on the Cell Cycle

T24 cells treated with drug alone or in combination (2 μ M cisplatin, 9 nM gemcitabine, and 30 nM emetine) 24 hours after plating, and again 72 hours later. Collected for DNA-PI analysis 24 hours after 2nd treatment.

Given the significant decrease in tumor cell proliferation, but lack of increase in apoptosis following emetine-cisplatin-gemcitabine treatment, the results suggest

that the cells are either undergoing growth arrest or are dying via a mechanism other than apoptosis.

The Addition of Emetine to Cisplatin-Gemcitabine Results in a Modest Increase in Autophagy

Studies from other laboratories have demonstrated increased autophagy in bladder cancer cells treated with cisplatin and gemcitabine.⁹⁰ To determine if autophagy was responsible, at least in part, for our results, we examined the bladder cancer tumor cells for staining with MDC, an autofluorescent pharmacologic agent that localizes in autophagocytic vesicles. The results demonstrated that a substantial portion (approximately 20%) of bladder cancer cells undergo autophagy in response to cisplatin-gemcitabine treatment. However, there was only a modest increase in MDC-positive cells when treated for 48 hours with cisplatin-gemcitabine plus emetine compared to cisplatin-gemcitabine alone (Figure 13). While this increase was statistically significant ($p < 0.01$), the difference was not significant when comparing cisplatin-gemcitabine plus emetine and cisplatin alone ($p = 0.1$). Taken together, the results suggest that addition of emetine causes, at best, a modest increase in autophagy in invasive bladder cancer cells.

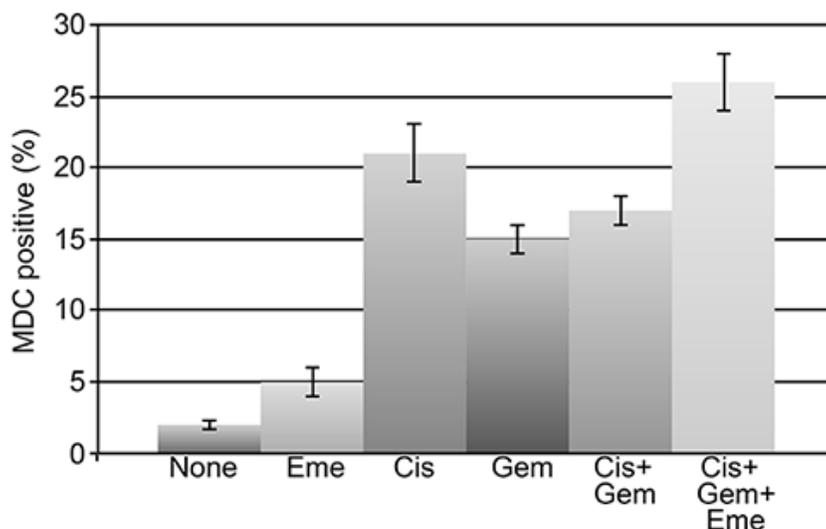


Figure 13: Emetine Modestly Increases Autophagy in Cisplatin-Gemcitabine Treated Bladder Cancer Cells

UMUC3 cells were treated for 48 hours with chemotherapeutic agents, labeled for 1 hour with MDC, and then analyzed by fluorescent microscopy immediately after mounting. Positive cells were counted in ImageJ as described in the materials and methods. Results represent combined data from two independent experiments. Eme = emetine, Cis = cisplatin, & Gem = gemcitabine.

Cisplatin-Gemcitabine-Emetine Treatment May Permanently Growth Arrest Bladder Cancer Cells

UMUC3 cells were treated with 30 nM emetine, 30 nM gemcitabine and half and full IC_{50} concentrations of cisplatin (3.4 μ M or 6.8 μ M) or half and full IC_{50} concentrations of carboplatin (36.5 μ M or 73 μ M) for 24 or 48 hours. Cells were unable to recover when observed for 14 days post drug removal. Similar results were observed in T24 cells.

AIM 2: EXAMINE THE MECHANISM BEHIND EMETINE'S ACTION

Effect of Emetine on Global Protein Synthesis

Emetine is a known inhibitor of protein synthesis at high micromolar / low millimolar concentrations.⁷⁹ Because low nanomolar concentrations of emetine effectively inhibited tumor cell proliferation in our experiments, we wondered if emetine was acting via inhibition of protein synthesis or through some other unidentified mechanism. To answer this question, we used the Click-iT metabolic labeling system. The Click-iT procedure incorporates an alkyne-labeled methionine into newly synthesized proteins. The cells are lysed, and a biotin-labeled alkane is then covalently linked to the alkyne (Click-reaction), so that all newly synthesized proteins are biotin labeled.

We treated UMUC3 cells with or without 30 nM emetine during alkyne labeling, performed the Click reaction, and then detected newly synthesized proteins using a western blot for biotin. The results showed similar levels of protein synthesis in both the treated and untreated cells suggesting that low dose of emetine has no effect on global protein synthesis (Figure 14).

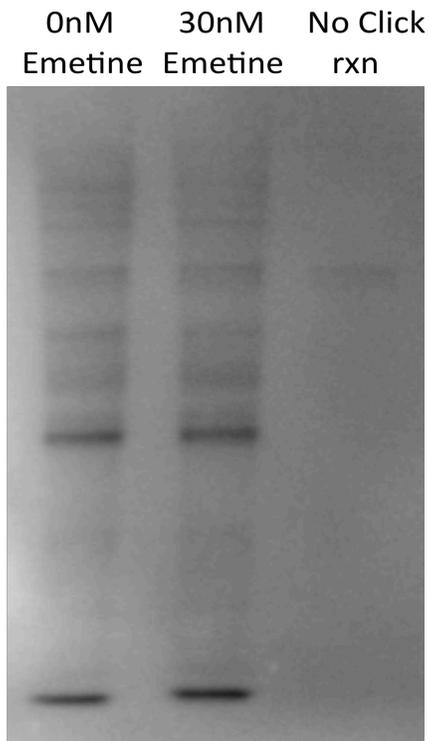


Figure 14: Effect of Emetine on Global Protein Synthesis

Western blot for biotin. UMUC3 cells were treated as indicated for four hours during the metabolic labeling. Lane 1: untreated, Lane 2: 30 nM Emetine, and Lane 3: untreated control sample not subjected to Click reaction with biotin-labeled alkane

Emetine Reduces Levels of Nuclear HIF-1 α and HIF-2 α but not HIF- β

Previous studies by Gupta and colleagues demonstrated that 100 nM emetine significantly decreased HIF-2 α , but not HIF- β expression in clear cell renal cell carcinoma cell lines.¹³ To determine if emetine altered HIF protein expression in invasive bladder cancer cells, we treated UMUC3 cells with increasing concentrations of emetine for 4 and 24 hours. Nuclear proteins were analyzed by western blot for HIF-1 α , HIF-2 α , and HIF-1 β . The results showed emetine rapidly

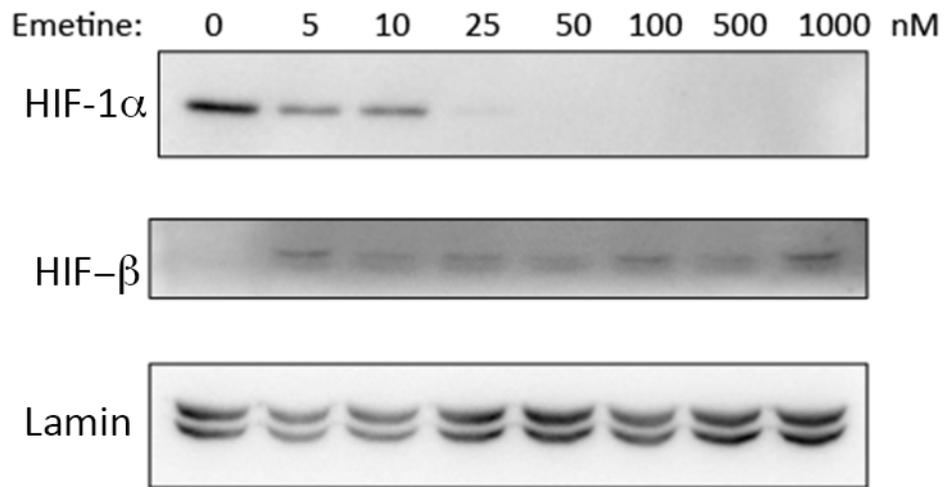
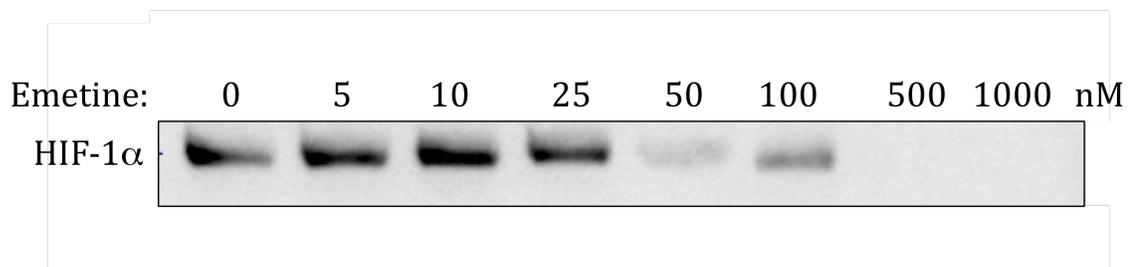
B**C**

Figure 15: Emetine Reduces Nuclear HIF-1 α and HIF-2 α but not HIF- β Expression

A) UMUC3, B) HT1376, and C) T24 cells treated with increasing concentrations of Emetine for 4 (A & B) and 24 (C) hours. Even quantities of protein were loaded onto SDS-Page gel. Western blot for HIF-1 α , HIF-2 α , and HIF- β proteins. Even protein loading was verified via Ponceau red stain and/or Lamin protein detection.

Low Dose Emetine Inhibits Synthesis of HIF-1 α

Taken together, our results suggest that low dose (30 nM) emetine does not inhibit global protein synthesis in invasive bladder cancer cells, but instead appears

to preferentially decrease expression of HIF-1 α . Experiments demonstrated that emetine (10 nM-1 μ M) did not alter HIF-1 α or HIF-2 α mRNA expression as determined by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR, personal communication: K Foreman, PhD). Moreover, Western blot analysis revealed HIF-1 α expression did not change following emetine treatment in presence or absence of MG132, a proteasome inhibitor (personal communication: K Foreman, PhD). This finding suggested that emetine-induced inhibition of HIF-1 α is not mediated through enhanced HIF-1 α degradation.

To determine if emetine preferentially affects HIF-1 α protein synthesis, we labeled newly synthesized proteins with the Click-iT system and then immunoprecipitated HIF-1 α (Figure 16A). The Click reaction was performed on the product to label newly synthesized proteins with biotin. Western blot analysis to detect biotin revealed that 25nM emetine was sufficient to decrease synthesis of HIF-1 α protein (Figure 16B).

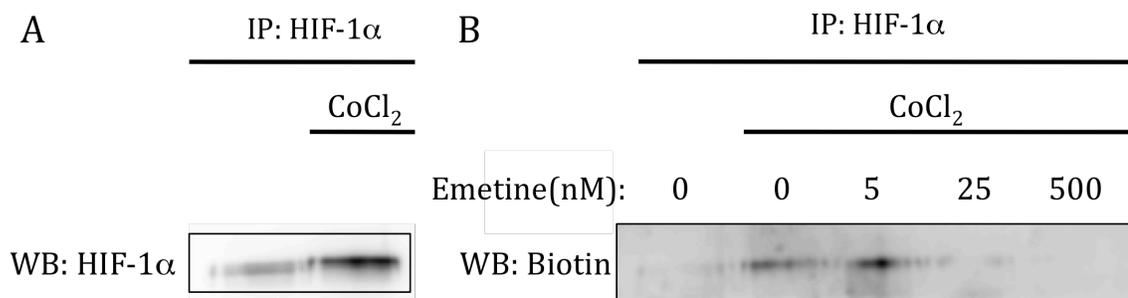


Figure 16: Emetine Inhibits Synthesis of HIF-1 α in a Dose Dependent Manner

UMUC3 cells were treated with increasing concentrations of emetine for 4 hours. Total HIF-1 α (A) was distinguished from newly synthesized HIF-1 α (B) using the Click-iT Biotin Protein Analysis Detection Kit as described previously. Due to the scarcity of HIF-1 α protein in whole cell lysates, cells were pre-treated with media containing 2% FBS and CoCl₂ 6 hours prior to emetine treatment in order to boost levels of HIF-1 α protein. Representative data from 2 independent experiments.

CHAPTER 4

DISCUSSION

The standard of care for patients with muscle invasive bladder cancer consists of removal of the bladder and a chemotherapy regimen of cisplatin plus gemcitabine.^{46,48,91} Unfortunately, bladder cancer is characterized by chemoresistance, and is only modestly responsive to this therapy.⁹² Moreover, cisplatin is neurotoxic and nephrotoxic, limiting its use in patients.⁵³ As the median age of bladder cancer diagnosis is 65, approximately 50% of patients have co-morbidities preventing them from receiving cisplatin.⁴³ These patients may be eligible to receive carboplatin and gemcitabine, an inferior chemotherapeutic regimen with less toxicity. With no comparable alternative, it is imperative to develop novel, effective therapies for these patients.

In the current project, we set out to determine if the addition of emetine to the cisplatin-gemcitabine or carboplatin-gemcitabine therapy enhanced inhibition of bladder cancer cell proliferation and/or cell death. Using MTT proliferation assays, we found that addition of emetine to cisplatin-gemcitabine treatment substantially decreased cell proliferation across a wide range of concentrations. Importantly, we demonstrated that addition of low dose, nanomolar concentrations of emetine allowed the effective dose of cisplatin or carboplatin to be reduced up to 10-fold. Given the significant toxicity of platinum-based therapies, the ability to reduce the

effective dose of these agents could allow patients previously deemed unfit for cisplatin or carboplatin to receive treatment.

Previous work in our lab demonstrated that emetine acts synergistically with cisplatin to decrease bladder tumor cell proliferation.⁷⁸ We initially hypothesized that a similar synergistic response would occur upon the addition of emetine to the full standard of care regimen of cisplatin plus gemcitabine. Instead, the response was primarily additive with a few conditions showing slight synergy. Because gemcitabine is an anti-proliferative drug, it is likely that the inclusion of gemcitabine reduced proliferation sufficiently that the effect of adding emetine was not as dramatic as in our initial work.

Our previous studies also showed that emetine caused only a modest increase in apoptosis when added to cisplatin chemotherapy.⁷⁸ Similarly, the present studies show little to no increase in apoptosis with the emetine, cisplatin and gemcitabine combination. Because our cells look like they are injured and/or are dying under the microscope, (i.e. enlarged, granular and flattened), we explored whether the cells were dying from a mechanism other than apoptosis.

Autophagy can be a pro-survival response in a cell by degrading old or damaged components, or by re-allocating its resources to a particular task. However, in certain cellular contexts autophagy can be a pro-death response, particularly in cells that are apoptosis-defective.^{93,94} Other studies have demonstrated autophagy as a response in bladder cancer cells treated with cisplatin and or gemcitabine.⁹⁰ We demonstrate that autophagy is, at best, modestly

increased upon the addition of emetine to cisplatin plus gemcitabine. If autophagic death were responsible for the observed decrease in cell proliferation, we would have expected to see a greater increase in the percentage of cells positive for autophagy. When cells were treated with the triple combination for 24 or 48 hours, they failed to proliferate upon drug removal. Therefore, autophagy is not acting as a pro-survival mechanism in the cells under our experimental conditions.

The triple drug therapy of emetine, cisplatin and gemcitabine, does not point to one particular mechanism as the cause behind the decrease in proliferation observed in muscle invasive bladder cancer cells. It is likely that up to 50% of our cells are dying from apoptosis, 25% are undergoing autophagy, and the remainder are either permanently growth arrested or senescent.

Muscle invasive bladder cancer is associated with an increase in HIF- α proteins expression.⁴² HIF- α proteins are transcription factors that induce transcription of genes involved in angiogenesis, glucose metabolism, proliferation, and migration, giving the cell a selective advantage.^{1,5} In the three human muscle invasive bladder cancer cell lines used throughout this project, HIF- α proteins are aberrantly expressed under normoxic conditions (personal communication, K Foreman, PhD). As emetine has been previously shown to decrease HIF-2 α expression in clear cell renal cell carcinoma cell lines¹³, we examined if emetine altered hypoxia signaling in order to decrease muscle invasive bladder cancer cell proliferation.

Treatment of cells in culture with nanomolar levels of emetine preferentially reduced levels of HIF-1 α and HIF-2 α , but not HIF- β proteins. This effectively reduced the transcription of their response genes, likely decreasing the cell's selective advantage, and leading to a decrease in proliferation. Emetine's precise regulation of the HIF- α subunit suggests a potential therapeutic role in cancers where HIF- α proteins are upregulated, such as muscle invasive bladder cancer. Since HIF- α proteins are not upregulated to the same extent in non-muscle invasive bladder cancer cells, it is unlikely that emetine would be as effective in these tumors.

In vivo work is necessary to supplement these results, and is currently being pursued by our lab. Preliminary studies show that emetine-cisplatin-gemcitabine treatment significantly inhibits tumor growth compared to cisplatin-gemcitabine treatment in a subcutaneous tumor model of invasive bladder cancer (personal communication, K Foreman, PhD). Orthotopic models of bladder cancer need to be studied next before possible clinical trials can be initiated. Results from this work will be telling in our hopes of clinically testing the effectiveness of the triple therapy in patients suffering from invasive bladder cancer.

Additional clinical studies are necessary to assess emetine's toxicity as part of the proposed therapy. Emetine has been examined as part of a combination therapy with cyclophosphamide in 1 trial of 6 lung cancer patients, where the investigator noted a definite response in all 6 patients.⁷⁵ This study, as well as those examining emetine as a single-agent, cite cardiotoxicity and muscle weakness as

dose-limiting considerations in the administration of emetine.^{70,72,75} Mastrangelo reports the absence of these toxicities when administering emetine at 1mg/kg daily for 10 days.⁷¹ In order to relate doses used in our tissue culture studies to those used in clinical trials, our lab has previously calculated that a 70 kg individual would have received 3,000 nM emetine in a 1 mg dose.⁷⁸ While it is difficult to directly compare treating a tissue culture monolayer with treating a tumor in a patient, our results suggest a smaller effective dose than what has previously been demonstrated to be free of toxic side effects.

In conclusion, our results suggest that the inclusion of a low dose of emetine to the cisplatin-gemcitabine or carboplatin-gemcitabine standard of care allow for up to a 10-fold reduction in effect dose of cisplatin or carboplatin. This is important, as the toxicity of these drugs limit the number of patients able to undergo chemotherapy. Moreover, we predict that the effective dose of emetine will not be accompanied with toxic side effects of its own. Additionally, our results suggest that emetine acts to regulate hypoxia signaling in invasive bladder cancer cells by decreasing protein synthesis of the HIF- α subunit. This may effectively reduce the tumor cell's selective advantage and proliferative potential.

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