Emetine Dihydrochloride: A Novel Therapy for Bladder Cancer

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Purpose: Current cisplatin based therapies for stage IV bladder cancer show 4% to 20% 5-year survival, underscoring the need to develop novel therapies for these patients. In the 1970s the natural alkaloid emetine dihydrochloride demonstrated modest anticancer efficacy as a single agent in clinical trials but this was not pursued. Groups recently reported that emetine induced apoptosis in leukemia cell lines, which was enhanced by cisplatin. We determined the antiproliferative effects of emetine with and without cisplatin in bladder cancer cells.

Materials and Methods: Human bladder cancer cell lines and normal human urothelial cell cultures were treated with emetine and/or cisplatin. We measured cell proliferation and evaluated synergy using the Chou-Talalay method. The combination index was calculated. Cell cycle analysis was done and caspase activation was evaluated to assess growth arrest and apoptosis.

Results: Emetine and cisplatin individually inhibited bladder cancer cell proliferation. When combined, emetine and cisplatin acted synergistically to inhibit tumor cell proliferation with combination index values reflecting moderate to strong synergy. Normal urothelial cells were relatively resistant to this treatment. Emetine alone and combined with cisplatin appeared to primarily induce tumor cell growth arrest and not apoptosis.

Conclusions: To our knowledge this study demonstrates for the first time that emetine has in vitro antiproliferative activity against bladder cancer cell lines at nanomolar concentrations but little effect on normal urothelial cells. Moreover, emetine and cisplatin worked synergistically to inhibit tumor cell proliferation. Results suggest that combined emetine and cisplatin based chemotherapy may benefit patients with bladder cancer.

Key Words: urinary bladder, urothelium, carcinoma, emetine, cisplatin

BLADDER cancer is the fourth most common cancer in men and the ninth most common in women.1 It is estimated that in the United States in 2013 there were 72,570 new cases and 15,210 deaths from bladder cancer.1 The 5-year survival rate in patients with stage IV disease is 4% to 20% despite aggressive surgery and chemotherapy.2 There is an urgent need to develop new chemotherapy combinations.

Emetine dihydrochloride, a natural product alkaloid from the plant Psychotria ipecacuanha, is one of the active agents in ipecac syrup. Emetine or its synthetic derivative dehydroemetine is widely used as an emetic and to treat severe amebiasis.3 After sporadic reports of its efficacy in patients with cancer in the early 1900s emetine disappeared from use as an antineoplastic until it was used in phase I and II clinical trials.
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sponsored by the National Cancer Institute in the mid 1970s. Outcomes were mixed with some trials showing no benefit from emetine as a single agent and others indicating disease stabilization, symptom relief and occasionally tumor regression. Dose limiting effects were also documented, specifically muscle weakness and cardiotoxicity, similar to those in treated patients with amebiasis. Since then, emetine was reported to induce apoptosis in various human leukemic cell lines, including U937, CEM, HL-60 and Jurkat. Möller et al noted that emetine (0.5 to 10 μM) induced caspase dependent mitochondrial depolarization and DNA fragmentation in Jurkat cells and could potentiate cisplatin induced cell death.9,11

To date only 2 groups have evaluated emetine or dehydroemetine for bladder cancer. In 1969 Abd-Rabbo reported improvement in 9 patients with schistosomal bladder cancer who were treated with oral dehydroemetine. Patients experienced lessening of symptoms (local pain, dysuria, hematuria and incontinence) and tumor size gradually decreased. Although 7 of the 9 patients were lost to followup, at the time of publication 2 were reported to have been alive for 2 years. In 1971 Panettiere and Coltman reported a phase I study including a single patient with transitional cell bladder cancer. They reported that the patient withdrew from the study after marked improvement in symptoms following emetine treatment.

Surprisingly, to our knowledge these intriguing results have not been pursued. To fill this gap we investigated the effect of emetine treatment on bladder cancer cell lines and normal urothelial cell cultures. Because combination therapy is often superior to single agents, we also examined emetine combined with cisplatin, a standard of care chemotherapy agent used to treat stage IV bladder cancer.4–7 Outcomes have not been pursued. To fill this gap we investigated the effect of emetine treatment on bladder cancer cell lines and normal urothelial cell cultures. Because combination therapy is often superior to single agents, we also examined emetine combined with cisplatin, a standard of care chemotherapy agent used to treat stage IV bladder cancer.

MATERIALS AND METHODS

Tissue Culture
UMUC3 and HT1376 muscle invasive bladder cancer cell lines (ATCC®) were cultured in Dulbecco modified Eagle medium supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Urothelial cells isolated from de-identified normal human bladder specimens were maintained in Eagle minimal essential medium with Earle salts supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

MTT Assay
Cell proliferation was evaluated by MTT assay. Cells were plated in triplicate wells at 5 × 10^3 per well in growth medium in 96-well plates and incubated overnight. Cells were treated with the stated concentrations of emetine dihydrochloride (Sigma-Aldrich®) and/or cisplatin (Selleck Chemicals, Houston, Texas) agents for 24 to 96 hours. MTT (0.5 mg/ml) (Sigma-Aldrich) was subsequently added to each well and the plates were incubated for 2 hours. The formazan precipitate was solubilized in 100 μl dimethyl sulfoxide and absorbance was measured at 560 nm. Proliferation under the various treatment conditions was calculated after subtracting background (absorbance at 670 nm and average absorbance from no cell control wells). It is reported as a percent relative to untreated control wells. Statistical analysis was performed with Prism® (IC50 calculations and r² values) and Excel® (the Student t-test). Initial studies demonstrated that a 48-hour time point was optimal for our experimental conditions.

Activity of the drug combination was evaluated using constant drug ratios using the Chou-Talalay method. The CI was calculated using CalcuSyn (Biosoft®). Synergy was defined as a CI of 0.2 to 0.4—strong synergy, 0.4 to 0.6—synergy, 0.6 to 0.8—moderate synergy, 0.8 to 0.9—slight synergy, 0.9 to 1.1—additive and greater than 1.1—antagonism as previously described.

Cell Cycle Analysis and Apoptosis Assays
DNA PI staining was performed using standard methods. Briefly, 1 × 10^6 cells were permeabilized with 100% ethanol in the presence of 15% FBS. The cells were washed and treated for 15 minutes at 37C with 10 μg/ml ribonuclease. PI (5 μg/ml) was added and cells were incubated for 1 hour at 4C before analysis on a FACSCanto™ II flow cytometer with a minimum of 10,000 cells analyzed per gated determination. Results were confirmed by annexin V-PI staining using the ApoScreen™ Annexin V-FITC Apoptosis Kit according to manufacturer instructions. Data were analyzed using FlowJo (Tree Star, Ashland, Oregon).

Activated caspase 3 and 8 were detected by flow cytometry and previously described intracellular staining techniques with cells permeabilized with 0.3% saponin during washes and antibody incubations. Cleaved caspase 3 and 8 were detected using rabbit monoclonal antibodies 5A1E and 18C8 (Cell Signaling Technology®), respectively. Primary antibodies were detected by Alexa Fluor® 488 labeled species specific secondary antibodies. Results were analyzed with FlowJo.

RESULTS

Emetine

Inhibited bladder cancer cell line proliferation. The antiproliferative potential of emetine in bladder cancer cell lines and normal bladder urothelial cell cultures was evaluated by MTT assay. Cell treatment with emetine for 48 hours decreased cancer cell proliferation in a dose dependent manner (fig. 1). IC50 values were estimated from the log concentration effect curves using Prism and nonlinear regression analysis. Average IC50 values were 63 and 26 mM in the UMUC3 and HT1376 bladder cancer cell lines, respectively. Urothelial cell cultures were more...
resistant with an estimated IC\textsubscript{50} of greater than 10 \textmu M.

Similar studies were performed to estimate the cisplatin IC\textsubscript{50} under our experimental conditions. As expected, cisplatin treatment decreased cell proliferation in a dose dependent manner and normal urothelial cells were less sensitive to the drug (data not shown). At 48 hours the average IC\textsubscript{50} values were 7.6 \textmu M for UMUC3, 1.9 \textmu M for HT1376 and 36 \textmu M for urothelial cells.

Acted synergistically with cisplatin to inhibit bladder cancer cell line proliferation. To investigate the combined effects of emetine and cisplatin we simultaneously treated bladder cancer cell lines with both drugs for 48 hours and evaluated cell proliferation by the MTT assay. Emetine and cisplatin were combined at equipotent concentrations in a constant ratio and we tested several combinations above and below the calculated IC\textsubscript{50}. Concentration effect curves demonstrated enhanced inhibition of cell proliferation in UMUC3 and HT1376 cells treated with the drug combination compared to that of emetine or cisplatin alone (fig. 2). The combination resulted in a synergistic effect on the growth inhibition of UMUC3 with a CI of 0.3 to 0.4 (strong synergy) at the higher drug concentrations tested. The effect was additive at the lower drug concentrations tested under our experimental conditions (CI 0.9 to 1.0). Synergistic effects were also seen for the drug combination in HT1376 cells with a calculated CI of 0.6 (synergy) for the low drug concentrations, which moved toward moderate synergy or additive effects (CI 0.7 to 1.0) at higher concentrations.

In contrast, normal urothelial cells were minimally affected by combination therapy (fig. 2). Although emetine and cisplatin acted synergistically to inhibit urothelial cell proliferation (CI 0.5 to 0.7), at the highest cisplatin and emetine concentrations tested the overall effect on proliferation was modest. Figure 3 shows the effect of select concentrations of cisplatin combined with emetine on the proliferation of bladder cancer cell lines and urothelial cells. Results showed significantly more inhibition of tumor cell growth than of urothelial cells at each drug concentration tested (data not shown and fig. 3).

**Figure 1.** Concentration effect curves of bladder cancer cell lines and normal bladder urothelial cultured cells treated with emetine for 48 hours (each r\textsuperscript{2} >0.94). Data are shown as average \pm SEM of 3 or 4 independent experiments done in triplicate or quadruplicate.

**Figure 2.**

**Figure 3.**

**Figure 4.**

**Figure 5.**

Emetine and Cisplatin Treatment Sequence Altered Synergistic Activity

Our findings suggest that simultaneous treatment of bladder cancer cell lines with emetine and cisplatin resulted in synergistic inhibition of cell growth. To determine whether the order of drug addition enhanced or decreased growth inhibition we pretreated UMUC3 bladder cancer cells with emetine or cisplatin for 24 hours before adding the other agent. Medium was not removed after pretreatment and a small volume of the second agent was added so that the final concentration of the first drug was not significantly altered. Thus, emetine and cisplatin were present during the last 24 hours of treatment. Simultaneous addition of the drugs resulted in optimal synergistic activity compared to pretreatment with either drug (fig. 4). Adding emetine before cisplatin was also effective but synergy was demonstrated for only a portion of the drug doses tested and the calculated CI was higher (fig. 4). Interestingly, cisplatin followed by emetine did not result in synergistic interaction (fig. 4).

Emetine Induced Bladder Cancer Cell Line Growth Arrest

To better understand the mechanism by which emetine and cisplatin inhibited bladder cancer cell proliferation we examined DNA PI staining of UMUC3 cells treated with each drug alone or in combination and determined the cell cycle profile by flow cytometry (fig. 5). Using drug concentrations that synergistically inhibited UMUC3 bladder cancer cell proliferation we found that emetine alone caused an increase in the number of cells in the G\textsubscript{1}/G\textsubscript{0} phase of the cell cycle compared to untreated control cells. As expected, cisplatin induced modest apoptosis after 48 hours, as shown by the increased percent of cells in the subG\textsubscript{0} region. However, most cells shifted to the S and G\textsubscript{2}/M regions of the cell cycle profile. Interestingly, the emetine and cisplatin combination resulted in a cell cycle profile that more closely resembled that of untreated or emetine treated cells. There was a
slight increase in the number of apoptotic cells but most cells remained in the G0/G1 region and did not show the characteristic S-G2/M growth arrest induced by cisplatin alone (fig. 5).

Because DNA PI staining detects DNA cleavage, which occurs in the later stages of apoptosis, we confirmed the results using 2 assays that detect earlier apoptotic events, including detection of cleaved (activated) caspases and annexin V-PI staining. Flow cytometry revealed no change in caspase 3 or 8 activation in cells treated with emetine alone and emetine did not enhance caspase activation in combination treated cells (fig. 6). Results were similar in annexin V-PI studies (fig. 7). Taken together the results suggest that emetine and cisplatin act synergistically to promote growth arrest in bladder cancer cells but they do not induce significant levels of apoptosis.

**DISCUSSION**

To our knowledge this study reveals for the first time that emetine dihydrochloride causes dose dependent growth inhibition of bladder cancer cell lines in vitro. Emetine works synergistically with cisplatin to inhibit bladder cancer cell proliferation better than either agent alone and the drug combination had little effect on normal bladder urothelial cells compared with tumor cells. Our study also suggests that emetine treatment results
predominantly in a growth arrest when combined with cisplatin.

To date few groups have evaluated emetine combined with other anticancer agents. In 1972 Street treated 6 patients with lung cancer with emetine and cyclophosphamide, and reported definite responses in each patient.16 Subsequent groups focused on cultured cell lines and noted an additive effect with cisplatin in leukemia cells, additive effects with doxorubicin and synergy with etoposide, docetaxel and oxaliplatin in neuroendocrine cell lines.11,17 Möller et al found that emetine induced significant apoptosis in Jurkat leukemia cells whether used alone or combined with cisplatin.9,11 On MTT assay they observed that 500 nM emetine almost completely blocked the proliferation of Jurkat cells after 48 hours and induced death in 46% of cells, which was largely reversed by Bcl-2 over expression. This was in contrast to our results, which showed that less than 5% emetine induced death at the same time point. The difference in our results may be due in part to our use of 50 nM emetine. However, even when using 500 nM emetine, we noted significantly less apoptosis in bladder cancer cells, that is 15% vs 46% in the Jurkat cell study by Möller et al.11 Although it is unclear why bladder cancer cell lines preferentially show growth arrest in response to emetine, it is important to note that our results may have clinical relevance. In an early clinical trial of emetine Siddiqui et al reported a lack of disease progression in 51 patients with various advanced tumors.7 They concluded that emetine was not beneficial for treating patients with cancer and commented that arresting tumor growth or spread was not thought to be an adequate criterion for the antineoplastic effect of emetine.

Other clinical studies also demonstrated the clinical efficacy of emetine in patients with various late stage cancers.4,12 Although it decreased symptoms and tumor regression was seen in some patients, emetine was associated with significant cardiac and musculoskeletal toxicities that led investigators to conclude that the benefit of emetine did not outweigh the risk. Mastrangelo et al...
reported that patients treated with 1 mg/kg emetine daily via subcutaneous injection for 10 days did not experience notable toxicity, that is there was no muscle weakness, myalgia or pain at the injection site. To compare the doses used in our tissue culture studies with the pharmaceutical doses used by Mastrangelo et al we calculated that a 70 kg patient who received 1 mg/kg emetine would have received about 3,000 nM emetine. While we cannot directly compare treatment of a tissue culture monolayer to the complexity associated with treating humans, our results suggest that doses of emetine below those previously used in clinical trials and reported to cause little to no toxicity may be beneficial when combined with cisplatin.

To our knowledge the mechanism by which emetine acts synergistically with cisplatin has not been identified. Emetine is a potent inhibitor of protein synthesis. It irreversibly blocks translation elongation by binding to the 40S ribosomal subunit and preventing its movement along with mRNA. In early studies 1 μM emetine rapidly blocked greater than 95% of protein synthesis in HeLa cells and later studies revealed that it also reduced DNA synthesis but had little effect on RNA synthesis in these cells.

However, groups have reported that emetine alters numerous cellular processes through mechanisms other than protein synthesis inhibition. Emetine regulates the expression of several apoptosis associated genes and significantly inhibits HIF-1 and 2 expression. Since cancer cells must adapt to hypoxic environments to survive, decreased HIF expression and the resulting decrease in HIF responsive genes could be detrimental to cancer cells. Emetine also inhibited NF-κB signaling via the inhibition of IκB phosphorylation.

Although each of these mechanisms potentially has a role in promoting synergy between cisplatin and emetine, a particularly interesting possibility involves the recent identification of emetine as an inhibitor of the ABC transporter ABCB1. ABC transporters are a family of transmembrane proteins that function as pumps to move specific substrates across cellular membranes. ABCB1, also known as MDR1 P-glycoprotein, is an important mediator of cancer drug resistance. Emetine inhibits ABCB1 activity but not that of the related transporter ABCC1 (MRP1). Studies showed that ABCB1 is over expressed in more than 50% of clinical bladder cancer specimens at the protein level. ABCB1 expression is increased in higher grade tumors and its expression correlates with shorter progression-free survival. It is possible that emetine acts synergistically with cisplatin by inhibiting ABCB1 activity and decreasing efflux of the chemotherapy agent from tumor cells. Therefore, emetine may be particularly effective as part of combination therapy for tumors that over express ABCB1 transporters. Further investigation is required to determine whether emetine indeed acts via this type of mechanism in bladder cancer cells.

CONCLUSIONS

Our study suggests that treatment of bladder cancer with combination chemotherapy that includes
Emetine dihydrochloride could be beneficial in patients with stage IV bladder cancer. The combination of emetine and cisplatin may be appropriate for clinical studies in these patients.

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References

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