In Vitro and In Vivo Experiments on Electrochemotherapy for Bladder Cancer

Juan Luis Vásquez, Per Ibsen, Henriette Lindberg and Julie Gehl*

From the Center for Experimental Drug and Gene Electrotransfer, Department of Oncology, Copenhagen University Hospital Herlev, Herlev and Department of Pathology, Copenhagen University Hospital Hvidovre (PI), Hvidovre, Denmark

Purpose: Electrochemotherapy is widely performed to treat solid tumors but experience with bladder cancer is limited. We investigated mitomycin C and cisplatin administered with electrochemotherapy for bladder cancer in vitro and in vivo.

Materials and Methods: The human bladder cancer cell line SW780 was used. Cells were treated with electroporation, drug alone or electroporation plus increasing concentrations of drug (mitomycin C 0.001 to 2,000 μM or cisplatin 1.56 to 300 μM). Electrochemotherapy parameters were 8 pulses of 1.2 kV/cm for 99 microseconds at 1 Hz. We investigated survival and apoptosis, the latter evaluated by caspase activity. NMRI-Fox1nu nude mice were inoculated subcutaneously and randomized to 1) electrochemotherapy plus NaCl, 2) NaCl alone, 3) electrochemotherapy plus drug or 4) drug alone (mitomycin C 5 mM or cisplatin 250 μM). Tumors were measured 3 times per week. A similar experiment was done to assess necrosis by histology at days 2 and 6.

Results: In vitro mitomycin C cytotoxicity and caspase activity was unaffected by electrochemotherapy (p = 0.9057 and 0.53, respectively). However, electrochemotherapy with cisplatin caused 6.6-fold increased cytotoxicity and higher caspase activity (p <0.0001 and <0.001, respectively). In vivo electrochemotherapy plus mitomycin C resulted in tumor volume reduction (p <0.0005). The survival rate in mice that received electrochemotherapy plus mitomycin C and mitomycin C alone was greater than in controls (p = 0.0004). The tumor response rate was 100% for electrochemotherapy plus mitomycin C, 53% for mitomycin C alone, 14% for electrochemotherapy plus NaCl and 0% for NaCl alone. In vivo electrochemotherapy plus cisplatin was associated with slower tumor growth over other combinations as well as significantly higher survival (p = 0.0005 and 0.0003, respectively). The tumor response rate was 47% for electrochemotherapy plus cisplatin, 0% for cisplatin alone, 0% for electrochemotherapy plus NaCl and 8% for NaCl alone.

Conclusions: In vivo electrochemotherapy with mitomycin C or cisplatin was more effective than chemotherapy alone in a bladder cancer tumor model, opening new perspectives in bladder cancer therapy.

Key Words: urinary bladder neoplasms, urothelium, electrochemotherapy, mitomycin, cisplatin

Electrochemotherapy is an emerging cancer treatment by which the effect of some chemotherapeutic agents is enhanced by increasing cellular uptake. This modality is based on EP, a physical method in which short
electric pulses are used to increase cell membrane permeability. This allows for the introduction of nonpermeable molecules (eg drugs or genes) into the cytoplasm.

Electrochemotherapy has efficacy in humans for cutaneous and subcutaneous tumors, and it is now being introduced to treat internal tumors. A single case report describes a CR in a patient with cutaneous metastasis of transitional cell carcinoma. However, to our knowledge electrochemotherapy for bladder cancer has not been investigated to date.

Most bladder tumors are nonmuscle invasive at diagnosis. Treatment for T1 disease is TURBT followed by intravesical instillation of MMC to target residual cancer cells in the bladder wall, which decreases the recurrence rate by 12%. T1 tumors are challenging since under staging and residual tumor are common after initial TURBT. In some of these patients radical cystectomy is considered, especially after bacillus Calmette-Guérin failure. This procedure carries a risk of morbidity and mortality, and affects quality of life. Not all patients are candidates for this procedure and some are reluctant to undergo major surgery. Minimally invasive but effective alternatives are desired for these patients and electrochemotherapy could be an option.

Bleomycin and CIS are suitable drugs to use clinically with electrochemotherapy. Furthermore, we previously reported an additive cytotoxic effect using the combination of EP and MMC in a bladder cancer cell line.

MMC is established as standard therapy for nonmuscle invasive tumors but it has no role in the treatment of more advanced disease stages. For invasive bladder cancer the most efficient drug is CIS in combination with other chemotherapeutic drugs. However, because anaphylaxis has been reported during intravesical therapy with CIS, its use has been limited.

MMC and CIS act by inducing a cascade of effects, including apoptosis of dividing cells. In this study we investigated these 2 drugs for use with EP in a bladder cancer cell line in vitro as well as in vivo to provide preclinical data on electrochemotherapy as bladder cancer therapy.

**MATERIAL AND METHODS**

**In Vitro Experiments**

**Cell lines and drugs.** SW780 human bladder carcinoma cells were grown in Dulbecco's modified Eagle's medium (Gibco®), 10% fetal calf serum, penicillin and streptomycin at 37°C and 5% CO₂. MMC (Medac, Wedel, Germany) and CIS (Accord Healthcare UK, North Harrow, United Kingdom) were dissolved in phosphate buffered saline to a concentration of 0.01 to 2.000 and 1.56 to 300 μM, respectively.

**EP in vitro.** EP parameters were optimized to achieve high cell survival and efficient permeabilization. Eight pulses of 1.2 kV/cm with a pulse duration of 99 microseconds were delivered at a frequency of 1 Hz using a T820 Square Wave Electroporator (BTX, San Diego, California) and OptiMEM™ buffer.

Cells were treated with drug alone or EP plus drug. Cell survival was analyzed by MTS assay and apoptosis using the Caspase-Glo® 3/7 Assay System. Cells were harvested with trypsin and ethylenediaminetetraacetic acid, washed once and resuspended in OptiMEM. Viable cells were counted with a NucleoCounter® and killed on ice for at least 5 minutes. Cell suspension (270 μl of 5.5 × 10⁶ cells per ml) was placed in each of 2 (pulsed and control, respectively) 4 mm cuvettes (Molecular Bio Products, San Diego, California) with 30 μl OptiMEM as the control or increasing concentrations of drug. They were incubated for 20 minutes at 37°C, diluted in culture medium and transferred to 96-well plates at 10,000 cells per well.

Survival was assessed by MTS assay using the Multi-skran™ Ascent™ enzyme-linked immunosorbent assay (ELISA) after 24-hour incubation in 96-well plates. Caspase assay was also done as a surrogate marker for apoptosis. The Synergy HT Luminometer (BioTek®) was used to read luminescence. Results were normalized to the unpulsed control.

**In Vivo Experiments**

In vivo experiments were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and with the approval of the Danish Animal Experimentation Inspectorate.

SW780 human bladder cancer cells were tested for infection using the rapid MAP27 panel (Taconic, Skensved, Denmark). Cells were injected subcutaneously (5 × 10⁶ cells per 100 μl) in the left flank of 9 to 11-week-old NMRI- Fox1nu male mice, which we bred. The mice were housed in individual cages and had free access to food and water. Hypnorm® and Dormicum® were used as anesthesia, complemented with Rimadyll®. The mice were sacrificed by quick cervical dislocation.

We performed 2 separate experiments with MMC (5 mM) and CIS (250 μM). The concentrations were based on pilot experiments in which tumors were treated with different drug concentrations without EP. The drugs were dissolved in saline. Tumor volume of 117 mm³ the mice were randomized to 4 groups, which were treated with 1) injection of physiological NaCl and EP (8 pulses of 100 microseconds at 1.2 kV/cm and 1 Hz, the same as used in the clinic) using a 6 mm plate electrode and the square wave Cliniporator™ electroporator, 2) injection of NaCl alone, 3) drug and EP with the described parameters or 4) drug alone at the same concentrations described. The volumes injected were equal to tumor volume. Tumors were treated only once at day zero. Tumors were measured using a caliper 3 times weekly after treatment until death or 60 days after treatment. All mice that survived at least 7 days after treatment were included in...
analysis. Mice sacrificed due to infection or compromised health and mice that still survived by day 60 were censored from analysis. The last volume measurement was used to evaluate the response.

**Histology.** A separate experiment was performed to investigate histology in tumors at posttreatment days 2 and 6. At an average tumor volume of 100 mm³ the mice were randomized into 5 groups, including 1) physiological saline and EP using the same parameters described, 2) physiological saline without EP, 3) drug (MMC 5mM or CIS 250μM) and EP, 4) drug at the same concentrations described but without EP or 5) controls. In groups 1 to 4 tumors were removed 2 and 6 days after treatment, fixed immediately with 10% neutrally buffered formalin and paraffin embedded. Tissue sections (3 μm) were stained with hematoxylin and eosin. The area fraction of necrosis was estimated by stereological point counting by an observer blinded to treatment status.

**Statistics**
For statistical analysis of in vitro experiments and histology results we performed 2-way ANOVA with multiple comparisons and the Bonferroni correction using GraphPad® Prism® 6. The tumor response to treatment was analyzed using criteria similar to those of RECIST (Response Evaluation in Solid Tumors), including CR—tumor disappearance, PR—greater than 30% decrease in tumor diameter, disease progression—greater than 20% increase in tumor diameter and stable disease—smaller changes that did not meet the other criteria. For survival analysis Kaplan-Meier curves were used. The Mantel-Cox log rank test was used for statistical analysis. Event was defined as death from sacrifice when tumor volume was 905 mm³. Mice that survived after day 60 and those that were sacrificed due to compromised health were censored from analysis at that point. We evaluated differences in tumor volume as the consequence of the different treatments using linear mixed models with SPSS®, version 19. Coefficients of the slope, eg the change in tumor volume by measurement time, were compared as the treatment effect. Time of measurement was interpreted as every second day, although the interval from Friday to Monday was 3 days.

**RESULTS**

**In Vitro Experiments**
Mean ± SD cell viability after applying only electric pulses was 95% ± 4.76%. Figure 1 shows dose-response curves as raw data and as data normalized to unpulsed controls. MMC experiments showed that EP did not increase cytotoxicity and the IC₅₀ was similar in the 2 arms at 350 μM for EP plus MMC and 400 μM for MMC alone (p = 0.9057, fig. 1, A and B).

Cell survival was significantly decreased by EP plus CIS compared to CIS alone. For EP plus CIS the IC₅₀ was 90 μM. For CIS alone the IC₅₀ was not

---

**Figure 1.** Dose-response curves show mean ± SE effects in 6 SW780 cell preparations. A and C, raw data on optical density (OD) measured by MTS assay at 550 nm wavelength, representing cell viability. B and D, percent of surviving cells with optical density values normalized to each control. No drug, pulsed and unpulsed control.
attained but when the curve was extrapolated, the estimated IC_{50} was approximately 590 μM, resulting in a 6.6-fold increase in IC_{50} for CIS plus EP (p < 0.0001, fig. 1, C and D).

Figure 2 shows that caspase activity in the MMC and CIS experiments had a bell-shaped pattern. In the MMC experiment caspase activity was highest at the 500 μM concentration (fig. 2, A). When the MMC concentration was greater than 500 μM, caspase activity decreased. No statistically significant difference was noted for EP plus MMC vs MMC alone. In CIS experiments caspase activity was highest at the 1,000 μM concentration when the drug was combined with EP. Caspase activity was 6 times greater for EP plus CIS than for CIS alone (p < 0.0001, fig. 2, B).

In Vivo Experiments
Figure 3 shows mean tumor volumes, survival rates and the response to treatment of mice exposed to MMC or CIS.

**Mitomycin C.** For EP plus MMC the tumor volume reduction was 3 mm^3 per day (p < 0.0005), for MMC alone tumor volume increased 5 mm^3 per day (p < 0.0005), for EP plus NaCl tumor volume increased 24 mm^3 per day (p < 0.0004) and for NaCl alone tumor volume increased 140 mm^3 every 2 days (p = 0.023) and 44 mm^3 per day for NaCl alone (p < 0.0005, fig. 3, A). Median survival was 60 days or greater for EP plus MMC and MMC alone, 32 days for EP plus NaCl and 25 days for NaCl alone (p = 0.0004, fig. 3, B). The best treatment response was observed for EP plus MMC (fig. 3, C).

**Cisplatin.** Linear mixed model analysis revealed that tumor volume increased 5 mm^3 per day for EP plus CIS (p < 0.0005), 24 mm^3 per day for CIS alone (p = 0.049), 140 mm^3 every 2 days for EP plus NaCl (p = 0.023) and 44 mm^3 per day for NaCl alone (p = 0.0005, fig. 3, D). Median survival was 60 days or greater for EP plus CIS, 35 days for CIS alone, 11 days for EP plus NaCl and 14 days for NaCl alone (p = 0.0003, fig. 3, E). The best treatment response was observed in the EP plus CIS group (fig. 3, F).

**Histology.** Figure 4 shows examples of histological findings. At baseline mean ± SE necrosis in untreated tumors was 5.93% ± 1.4% and 7.25% ± 2.2% in MMC and CIS experiments, respectively. In each experiment the degree of necrosis at day 2 was significantly increased compared to baseline only for EP plus MMC (24%, p = 0.04) and EP plus CIS (19%, p = 0.03). At day 6 no statistically significant change was observed (fig. 5).

**DISCUSSION**
Electrochemotherapy with CIS or MMC in a bladder cancer model was more effective than chemotherapy alone. CIS cytotoxicity increased by a factor of 6.6 using EP in accordance with previous studies in other cell lines. In the bladder cancer cell line T24 EP increased MMC cytotoxicity by 30% at the approximate concentration present in the bladder during intravesical instillation with 40 mg MMC. In that series EP alone caused cell mortality with phosphate buffered saline used as the buffer to simulate urine. We used a different buffer to imitate conditions in a tumor and found no difference.

MMC alone penetrates the cell membrane by passive diffusion so that in vitro cell permeabilization would not enhance the MMC effect. In contrast, only about 50% of CIS penetrates by passive diffusion. Therefore, membrane permeabilization improves the cytotoxicity of CIS in vitro, as our results show.

The caspase activity seen with MMC was unaffected by the combination with EP, as demonstrated by the same bell-shaped pattern (fig. 2, A). With CIS...
caspase activity was also bell-shaped but EP improved the CIS effect, causing higher caspase activity at lower CIS concentrations (p < 0.001, fig. 2, B).

EP potentiated the effect of CIS and MMC in this animal model, reducing the tumor burden and improving the treatment response and disease specific survival. Previous studies showed the efficacy of electrochemotherapy using bleomycin and CIS in different cell lines. In an animal study using a pancreatic cancer cell line electrochemotherapy with MMC had higher efficacy than MMC alone for tumor growth.

Electrochemotherapy affects blood vessels and inhibits blood flow, causing drug entrapment. This counteracts dilution and allows the drug to act for a longer time. Furthermore, electrochemotherapy with bleomycin or CIS induces a vascular disrupting effect by which the tumor vasculature is damaged, leading to increased cell death. To our knowledge this effect has not been studied for MMC but since tumor endothelial cells are dividing cells, one might suppose that they would be susceptible to electrochemotherapy with MMC. These mechanisms generate severe tumor hypoxia, which can be
advantageous for bioreductive drugs such as MMC. These facts may explain the discrepancy between MMC cytotoxicity in vitro and in vivo.

Bleomycin is the ultimate drug used with electrochemotherapy due to at least 300-fold increase in cytotoxicity and it was also effective for bladder cancer. Nevertheless, any increase in the CIS or MMC cytotoxicity is appreciated because bladder cancer is already sensitive to these drugs. Our study shows that electrochemotherapy was effective using intratumor MMC or CIS. MMC seems to be relatively superior to CIS. The concentration of CIS used in this study was 25 times lower than the concentration recommended for electrochemotherapy using intratumor CIS in patients. The distribution volume is far less in mice, likely leading to a lower level of systemic washout. It is likely that if a higher CIS concentration had been used, the response rate would have been higher. Our data did not enable us to directly compare electrochemotherapy with MMC vs CIS because these experiments were performed separately.

This study shows that electrochemotherapy with MMC or CIS is more effective than chemotherapy alone in our tumor model. This opens new possibilities to develop electrochemotherapy for bladder tumors. We chose intratumor injection because it is recommended in standard operating procedures for CIS use. To our knowledge MMC has not been used previously in clinical trials and data are limited on intravenous CIS. Furthermore, an advantage of electrochemotherapy is that it is a local treatment and intratumor administration might limit systemic side effects. However, intravenous bleomycin was effective in clinical studies with limited side effects. In addition, new studies indicate that EP in the presence of supraphysiological doses of calcium could be an efficient treatment. This would also be potentially interesting to test.

Electrochemotherapy is effective treatment of cutaneous and subcutaneous metastases independent of primary tumor histology. Novel electrodes were designed to use electrochemotherapy for brain tumors, colorectal cancer, bone and liver metastasis, soft tissue sarcoma, etc. The bladder is relatively easy to access. Novel electrodes adaptable to cystoscopes are in demand and their development is planned. Electrochemotherapy might be applied immediately after TURBT at the site and borders of the resected tumor after intratumor injections of MMC or CIS. Intravenous bleomycin could also be considered. A patient subgroup might benefit from this strategy, including those with recurrent high grade Ta tumors, recurrent carcinoma in situ or T1 tumors for which radical cystectomy is indicated but the patient is unfit for or reluctant to undergo major surgery. Another possibility is palliative treatment of bleeding tumors, for which the antihemorrhagic effect of electrochemotherapy might be advantageous. Electrochemotherapy opens new and exciting perspectives in therapy for bladder cancer, for which less morbid yet effective alternative strategies are needed.

**CONCLUSIONS**

Electrochemotherapy is an effective method that potentiates certain anticancer drugs by applying electric pulses to permeabilize the tumor cell membrane, enabling enhanced drug uptake. Our study shows that electrochemotherapy using MMC or CIS was more effective than chemotherapy alone for experimental bladder cancer tumors. The development of novel electrodes that could be used on
bladder tumors is being explored, opening promising perspectives in bladder cancer treatment.

ACKNOWLEDGMENTS

Marianne Fregil, Laboratory of Oncology, Copenhagen University Hospital Herlev, assisted with in vitro experiments. Anne Boye and Lone Christiansen assisted with animal experiments. Professor Bente Pakkenberg, Research Laboratory for Stereology and Neuroscience, assisted with stereological point counting analysis. Dr. Lars Dyrskjot Andersen, Department of Molecular Medicine, Aarhus University Hospital, provided the SW780 cell line.

REFERENCES

2. Mir LM, Gehl J, Sersa G et al: Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator by means of invasive or non-invasive electrodes. EJC Suppl 2006; 4: 14.