*Radiol Oncol 2003; 37(1): 43-8.*

# Tumor blood flow modifying effects of electrochemotherapy: a potential vascular targeted mechanism

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***Background.*** *The aim of this study was to determine the tumor blood flow modifying, and potential vascular targeted effect of electrochemotherapy with bleomycin or cisplatin.*
***Materials and methods.*** *Electrochemotherapy was performed by application of short intense electric pulses to the tumors after systemic administration of bleomycin or cisplatin. Evaluated were antitumor effectiveness of electrochemotherapy by tumor measurement, tumor blood flow modifying effect by Patent blue staining technique, and sensitivity of endothelial and tumor cells to the drugs and electrochemotherapy by clonogenicity assay.*
***Results.*** *Electrochemotherapy was effective in treatment of SA-1 tumors in A/J mice resulting in substantial tumor growth delay and also tumor cures. Tumor blood flow reduction following electrochemotherapy correlated well with its antitumor effectiveness. Virtually complete shut down of the tumor blood flow was observed already at 24 h after electrochemotherapy with bleomycin whereas only 50% reduction was observed after electrochemotherapy with cisplatin. Sensitivity of human endothelial HMEC-1 cells to electrochemotherapy suggests a vascular targeted effect for electrochemotherapy in vivo with bleomycin as well as with cisplatin.*
***Conclusion.*** *These results show that, in addition to direct electroporation of tumor cells, other vascular targeted mechanisms are involved in electrochemotherapy with bleomycin or cisplatin, potentially mediated by tumor blood flow reduction, and enhanced tumor cell death as a result of endothelial damage by electrochemotherapy.*

*Key words: sarcoma experimental - drug therapy - blood supply; bleomycin; cisplatin; electroporation; drug delivery systems*

Received 1 October 2002
Accepted 15 November 2002

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## Introduction

Enhanced delivery of chemotherapeutic drugs into tumor cells by electroporation is termed electrochemotherapy.[1] A local increase in plasma membrane permeability, after exposure of tumor nodules to electric pulses (electroporation), results in increased

uptake of chemotherapeutic drugs into the tumor cells. Electrochemotherapy has been shown to be successful for drugs such as bleomycin and cisplatin, which normally exhibit impeded transport through the plasma membrane. The increased antitumor effectiveness of bleomycin and cisplatin combined with electroporation has already been demonstrated in experimental and clinical studies although the underlying mechanisms remain to be clarified. [1-4]

In addition to increased drug delivery into the cells, application of electric pulses to the tumors was found to exert tumor blood flow modifying effect.[5,6] Electric pulses, as used in preclinical and clinical studies were found to reduce tumor blood flow. Transient reduction in tumor blood flow down to 30% of control was found, but recovered to almost pre-treatment level within 24 hours.[5]

Application of electric pulses to solid tumors would not be expected to selectively electroporate tumor cells alone. All cells in all areas where electric field exceeds the critical threshold level would be electroporated.[1,7] Therefore endothelial cells are also potential targets for electroporation. Since the initial concentration of the drug is the highest in tumor blood vessels, during electroporation, electrochemotherapy is probably effective on endothelial cells in the tumor blood vessels. This may lead to severe damage to the vasculature of the tumors and consequently induce a secondary cascade of tumor cell death, *e.g.* by abrogating oxygen supply to the cells. This phenomenon, described as vascular targeted therapy, has been exploited in several studies.[8]

The aim of this study was to elucidate tumor blood flow modifying and vascular targeted effects of electrochemotherapy with bleomycin or cisplatin by measuring tumor perfusion, and cells survival of endothelial cells in relation to their antitumor effectiveness.

## Materials and methods

### *Animals, tumors and cell lines*

A/J mice of both sexes, purchased from the Institute Rudjer Bošković, Zagreb, Croatia, were used. Subcutaneous murine fibrosarcoma SA-1 tumors (The Jackson Laboratory, Bar Harbour, ME) were implanted, by injecting 0.1 ml NaCl (0.9%) containing 5 x $10^5$ viable tumor cells under the skin on the rear dorsum. Six to 8 days after implantation, when tumors reached approximately 40 $mm^3$ in volume (6 mm in diameter) mice were randomly divided into experimental groups, consisting of at least 6 mice. Treatment protocols were approved by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia No. 323-02-237/01.

Human dermal microvascular endothelial cells (HMEC-1) cells were generously provided by Dr. F.J. Candal (Center for Disease Control, Atlanta, USA). Cells were grown as monolayer in D-MEM supplemented with 10% fetal calf serum (FCS, Sigma, USA) in a humidified incubator at atmospheric oxygen supplemented with 5% $CO_2$ at 37°C. They were routinely subcultured twice per week.

### *Electrochemotherapy protocol*

Bleomycin (Bleomycin, Mack, Germany) was dissolved in phosphate buffered saline and the dose of 5mg/kg in 0.2 ml volume was injected intravenously. Bleomycin solution was prepared freshly for daily injections.

*cis*-Diamminedichloroplatinum (II) (Cisplatin) was obtained from Bristol Myers Squibb (Austria) as a crystalline powder and a stock solution prepared in sterile $H_2O$ at a concentration of 1 mg/ml. The final cisplatin solution was freshly prepared in 0.9% NaCl each day. Cisplatin at a dose of 4 mg/kg in 0.2 ml volume was injected intravenously.

Electric pulses were delivered by two flat, parallel stainless steel electrodes 8 mm apart (two stainless steel strips: length 35 mm, width 7 mm with rounded corners), which

were placed percutaneously at the opposite margins of the tumor. Good contact between the electrodes and the skin was assured by means of conductive gel (Parker Laboratories, New York, USA). Eight square-wave pulses of 1040 V amplitude (amplitude to distance ratio 1300 V/cm), with a pulse width of 100 μs and repetition frequency of 1 Hz were generated by electroporator Jouan GHT 1287 (Saint Herblaine, France). In the electrochemotherapy protocol, tumors were exposed to electric pulses 3 minutes after bleomycin or cisplatin injection. Treatments were performed without anesthesia and were well tolerated by the animals.

Tumor growth was followed by measuring three mutually orthogonal tumor diameters ($e_1$, $e_2$ and $e_3$) using a vernier caliper on each consecutive day following treatment. Tumor volumes were calculated by the formula $\Pi$ x $e_1$ x $e_2$ x $e_3$ /6. From the calculated volumes the arithmetic mean and SE were calculated for each experimental group. Tumor growth delay was calculated for each individual tumor by subtracting the doubling time of each tumor from the mean doubling time of the control group and then averaged for each experimental group.

*Assessment of tumor staining by Patent blue*

Patent blue (Byk Gulden, Switzerland) was used to estimate tumor perfusion. Patent blue (1.25%), diluted in 0.2 ml 0.9% NaCl, was injected into tail vein of animals after tumor treatment. The dye was distributed evenly through the blood at approximately 1 minute, thereafter animals were sacrificed and tumors were carefully dissected. Tumors were cut along their largest diameter and the stained versus non-stained tissue per cross-section was immediately estimated visually by two persons. The mean of both estimations was used as an indicator of tumor perfusion. The results of individual experiments were pooled and presented as arithmetic mean and SE for each experimental group.

*Cytotoxicity assay for SA-1 and HMEC-1 cells treated by electrochemotherapy*

The sensitivity of the SA-1 and HMEC-1 cells to combined treatment with bleomycin or cisplatin and electric pulses (electrochemotherapy) was determined by *in vitro* colony forming assay. The cells (2.2 x $10^7$ cells/ml) were mixed with bleomycin or cisplatin. One half of the mixture was exposed to 8 electric pulses (electric field intensity 1400 V/cm, pulse duration 100 μs, frequency 1 Hz) and the other half served as a control for bleomycin or cisplatin treatment alone. The bleomycin concentrations used ranged from 0.1 nM to 100 μM and the cisplatin concentrations from 16.7 to 670 μM. The cells were incubated with each drug for 5 min. The survival of cells treated with electrochemotherapy was normalized to electric pulses treatment alone. The $IC_{50}$ values (drug concentration that reduced cell survival to 50% of control) were determined for each treatment group.

*Statistical analysis*

The significance of differences between the mean values of the groups was evaluated by modified t-test (Newman Keuls test) after a one way analysis of variance was performed and fulfilled. Sigma Stat statistical package (SPSS, USA) was used for statistical analysis. P levels less than 0.05 were taken as statistically significant.

## Results

*Antitumor effectiveness*

Electrochemotherapy with either bleomycin or cisplatin was effective in inducing cytotoxicity in subcutaneous SA-1 tumors (Table 1). Treatment of tumors with electric pulses alone had a minor effect on tumor growth, resulting in only 1.3 days tumor growth delay. Treatment of mice with bleomycin or cisplatin alone had also minor effects on tumor

**Table 1.** Antitumor effectiveness of electrochemotherapy on SA-1 tumors in mice (* P<0.05)

| Therapy | n | Tumor doubling time (Days, AM±SE) | Tumor growth delay (Days, AM±SE) | Cures |
|---|---|---|---|---|
| Control | 20 | 1.8 ± 0.05 | | 0 % |
| Electric pulses | 17 | 3.1 ± 0.2* | 1.3 ± 0.2 | 0 % |
| Bleomycin (5 mg/kg) | 20 | 1.9 ± 0.1 | 0.1 ± 0.1 | 0 % |
| Electrochemotherapy with bleomycin | 17 | 34.5 ± 2.9* | 32.7 ± 2.9 | 70 % |
| Cisplatin (4 mg/kg) | 10 | 3.7 ± 0.4* | 1.9 ± 0.4 | 0 % |
| Electrochemotherapy with cisplatin | 10 | 12.1 ± 1.6* | 10.3 ± 1.6 | 0 % |

growth; bleomycin having none, whereas cisplatin inducing 1.9 days tumor growth delay. When using bleomycin in electrochemotherapy, a highly significant growth delay of 32.7 days was achieved and 70% of the animals were cured (tumor free 100 days after the treatment). The animals tolerated the treatment well without scaring of the treatment area. Electrochemotherapy with cisplatin also resulted in good antitumor effect with reduction in tumor size at three days after the treatment, regrowth after 8 days, however no tumor cures were achieved. Tumor growth delay was 10.3 days, which was highly significant compared to the antitumor effectiveness of either single modality.

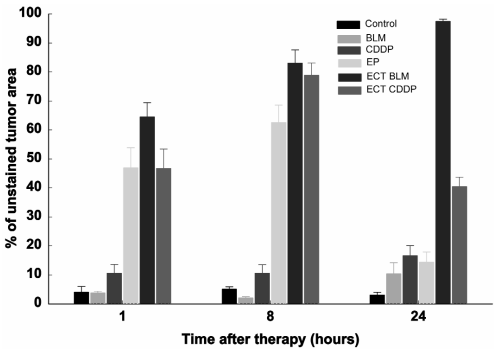


**Figure 1.** Changes in tumor blood flow at 1, 8 or 24 h after electrochemotherapy (ECT) with bleomycin (BLM) or cisplatin (CDDP), measured by Patent blue staining. Eight electric pulses (EP) were applied to the tumor (amplitude to distance ratio 1300 V/cm, pulse width 100 Ķs, repetition frequency 1 Hz) 3 minutes after intravenous injection of 5 mg/kg of bleomycin or 4 mg/kg of cisplatin. Mean values ± SE of the mean of at least 6 mice per point.

*Tumor blood flow changes*

Electrochemotherapy, either with bleomycin or cisplatin, induced substantial reduction of tumor blood flow. Untreated SA-1 tumors showed very low incidence of necrosis with approx. 90% of the tumor area stained with Patent blue. When electric pulses were applied to a tumor reduction in tumor staining was observed (Figure 1). By 1 hour after the application of electric pulses the percentage of unstained tumor section had increased to 45% and after 8 hours further increased to 65%, however tumor blood flow recovered almost completely within 24 hours after this treatment. Treatment with bleomycin alone did not induce changes in tumor blood flow. However, electrochemotherapy with bleomycin demonstrated substantial increase in unstained tumor area at 8 hours after treatment, and virtually complete shut down of tumor perfusion at 24 hours after therapy compared to electric pulses alone (Figure 1).

Treatment with cisplatin alone had minimal tumor blood flow modifying effect. However, electrochemotherapy with cisplatin demonstrated greatly increased unstained tumor area at 8 hours after treatment which remained significantly higher at 24 hours after the treatment compared to treatment with electric pulses alone (Figure 1).

*Cytotoxicity of electrochemotherapy to tumor and endothelial cells*

The sensitivity of SA-1tumor cells and human endothelial cells HMEC-1 to bleomycin and

cisplatin as well as to electrochemotherapy was evaluated by *in vitro* colony forming assay (Table 2). Endothelial cells were more sensitive to bleomycin than tumor cells. The potentiation of bleomycin cytotoxicity by electroporation was ~5000-fold for endothelial cells and ~2400-fold for tumor cells. Electrochemotherapy with cisplatin was less effective on endothelial as on tumor cells, but potentiation of cisplatin cytotoxicity by electroporation was bigger for endothelial cells (~10-fold), as for tumor cells (~8-fold).

## Discussion

This study shows tumor blood flow modifying and vascular targeted effect of electrochemotherapy with bleomycin as well as with cisplatin. The sensitivity of endothelial cells to electrochemotherapy with either, bleomycin or cisplatin correlates well with the enhanced reduction of tumor blood flow induced by electrochemotherapy in vivo and its antitumor effectiveness.

As many preclinical and clinical studies have shown, electrochemotherapy either with bleomycin or cisplatin leads to high percentage of tumor cures, on many tumor types tested so far.[1,3,4,9,10] Electroporation was shown to significantly increase drug accumulation in the tumor cells.[1,11] In electrochemotherapy treated tumors more than twice the amount of platinum was determined in whole tumors, as well as bound to DNA compared with cisplatin treatment alone.[11] In view of our previous study observing that electrochemotherapy with cisplatin induced more than 20-fold increase in cell kill compared with cisplatin treatment alone, we proposed that, in addition to direct electroporation of tumor cells, other mechanisms may be involved in antitumor effectiveness of electrochemotherapy.[11]

**Table 2.** Cytotoxicity of electrochemotherapy to human endothelial HMEC-1 and mouse tumor SA-1 cells *in vitro*

| Cell line / Group | HMEC-1 ($IC_{50}$; μM) | SA-1 ($IC_{50}$; μM) |
|---|---|---|
| Bleomycin | 20.0 | 60.0 |
| Electrochemotherapy with bleomycin | 0.004 | 0.025 |
| Cisplatin | 380.0 | 166.0 |
| Electrochemotherapy with cisplatin | 40.0 | 20.0 |

The direct blood flow modifying effect of electric pulses applied to the tumors has now been established. Application of electric pulses reduces blood flow selectively at the site of its application, *i.e.* within the tumor site, without modifying flow in normal tissues.[5] Recently, a new method by staining of tumors with Patent blue was evaluated, giving data on tumor blood flow, in support of that found using the $^{86}$RbCl extraction technique.[12] Since the two methods correlated well, Patent blue staining technique was preferred in this study, because of its simplicity. The present study confirms the results of our previous study that application of electric pulses to the tumors induces transient reduction in tumor blood flow.

Tumor blood flow modifying effect of electrochemotherapy was greater than after application of electric pulses alone. This effect was especially dramatic in electrochemotherapy with bleomycin, but in lesser extent after electrochemotherapy with cisplatin. Tumor blood flow after electrochemotherapy with bleomycin was completely shut down already at 24 hours after therapy, indicating that tumor vasculature was irreversibly damaged.[12] Since HMEC-1 endothelial cells were more sensitive to electrochemotherapy with bleomycin *in vitro* than SA-1 tumor cells, this vascular shut down may be ascribed in large part to the death of endothelial cells. In contrast, endothelial cells were less sensitive to electrochemotherapy with cisplatin *in vitro*, which was also reflected in less severe tumor blood flow changes induced by this therapy with flow partly restored after 24 hours.[13]

In summary, several mechanisms are involved in antitumor effectiveness of electrochemotherapy. Electroporation of the tumors increases delivery of cytotoxic drugs into the tumor cells, potentiating their cytotoxicity. Additionally, the current study demonstrates that nonselective electroporation of solid tumors enables cytotoxic action of electrochemotherapy to endothelial cells and enhanced tumor cytotoxicity by a vascular targeted mechanism.

## Acknowledgment

This work was supported by the Ministry of Education, Science and Sport of the Republic of Slovenia.

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