ANTICANCER RESEARCH *24*: 2343-2348 (2004)

Antitumour Effectiveness of Hyperthermia is Potentiated by Local Application of Electric Pulses to LPB Tumours in Mice

KATARINA BARBARA KARNER, HOTIMIR LESNICAR, MAJA CEMAZAR and GREGOR SERSA

Institute of Oncology, Zaloska 2, SI-1000 Ljubljana, Slovenia

Abstract. *Background: The aim of our study was to determine whether local application of electric pulses to tumours, which induce transient reduction of tumour perfusion, could potentiate the antitumour effectiveness of hyperthermia. Materials and Methods: The antitumour effectiveness of local application of electric pulses (1300 V/cm, 100 μs, 1 Hz) and 910 MHz local hyperthermia at 43.5˚C, alone or in combination, was determined on LPB tumours in C57Bl/6 mice by measurement of tumour growth delay, changes in tumour perfusion using the Patent blue technique and extent of tumour necrosis. Results: When hyperthermia was performed immediately after application of electric pulses, at a time of maximally reduced tumour perfusion, greater than additive antitumour effectiveness was observed, resulting in 14.5±3.1 days growth delay of tumours that regrew and 43% complete responses. Single treatment, application of electric pulses or hyperthermia had minor or no effect on tumour growth. When hyperthermia was performed 24 hours after application of electric pulses, at a point when tumour perfusion was restored, the effect of both treatments was additive, resulting in 4.1±1.1 days growth delay and no cures. Conclusion: The probable mechanisms for the observed, more than additive, interaction when hyperthermia was performed immediately after application of electric pulses are the potentiation of thermic cytotoxicity, due to the reduced tumour perfusion induced by application of electric pulses and prolonged tumour perfusion reduction after combined treatment leading to additional cell kill, due to the protracted ischemia.*

The role of hyperthermia in the treatment of cancer has been emphasized in a number of reports in the last two decades. Hyperthermia has proved its effectiveness as an adjuvant to other cancer treatment modalities due to its direct cytotoxic, radio- and chemosensitizing effect (1-3). However, the exact mechanisms responsible for its antitumour action are still not fully understood and warrant further experimental investigation (2).

The extent of thermic cytotoxicity grows with the treatment temperature, increasing abruptly above 42.5˚C (4). At the cellular level, damage to proteins which may indirectly affect lipids or DNA/RNA processing is responsible for cell death after hyperthermia (2,5). At the same time hyperthermia has a profound effect on the integrity of tissue microcirculation. In general tumour blood flow during hyperthermia at temperatures of 42˚C and below increases, whereas at temperatures above 43˚C it decreases due to swelling of endothelial cells, shift of plasma fluid into the interstitium, microthrombosis because of activation of haemostasis and changes of viscosity of blood cell membranes (6-10).

In addition to that, it has been shown that treatment of tumours with vascular-targeted agents prior to hyperthermia predisposes tumours to heat damage by compromising the tumour blood flow. Reduction in tumour blood flow leading to hypoxia and induction of sub-optimal physiological conditions in the tumours, such as a nutrient-deprived environment (*e.g.* low glucose) and accumulation of metabolic by-products (*e.g.* lactic acid resulting in low pH) increases cell sensitivity to heat (2, 7, 11).

Exposure of cells *in vitro* and *in vivo* to electric pulses (electroporation) under controlled conditions increases plasma membrane permeability (12). This effect is transient and reversible and does not substantially affect cell viability. Electroporation is used to introduce various molecules into the cells, including chemotherapeutic agents (electrochemotherapy) (13). Electrochemotherapy with bleomycin and cisplatin has been extensively elaborated in preclinical studies and is also used in the treatment of cutaneous tumour nodules of various malignancies in cancer patients, resulting in good local tumour control (14, 15). At the level of the tumour microenvironment, electroporation

Correspondence to: Prof. Gregor Sersa, Institute of Oncology, Zaloska 2, SI-1000 Ljubljana, Slovenia. Tel/Fax: +386 1 5879 434, e-mail: gsersa@onko-i.si

Key Words: Electroporation, hyperthermia, tumour perfusion, animal tumour model.

induces an instant reduction of tumour perfusion, which is restored after several hours (16-18). The reduction of tumour perfusion results in tumour hypoxia (18), thus creating improved therapeutic conditions for the use of hyperthermia.

The aim of our study was to determine whether local application of electric pulses to tumours, which induce reduction of tumour perfusion, could potentiate the antitumour effectiveness of hyperthermia. In order to find a time schedule suitable for further investigation, the effect of applied electric pulses on tumour perfusion was examined. Based on these results, a tumour response study was conducted wherein two different time intervals between the application of electric pulses and hyperthermia were examined. In addition, tumour perfusion and morphological changes in tumour sections after combined treatment were determined to disclose some of the potential mechanisms of the interaction between the two treatment modalities.

Materials and Methods

Animals and tumours. The animals used in the present experiments were C57Bl/6 mice of both sexes, purchased from the Institute of Pathology, University of Ljubljana, Slovenia. They were kept at constant room temperature (22˚C) with a natural day/night light cycle in a conventional animal colony with food and water *ad libitum*. The mice were 10-12 weeks old at the beginning of the experiments. The tumour used was fibrosarcoma LPB, which is a clonal derivative of TBL.Cl2, a methylcholanthrene-induced C57Bl/6 mouse sarcoma tumour. The cells were routinely maintained *in vitro* in Eagle minimal essential medium (EMEM) (Sigma, St. Louis, MO, USA) supplemented with 8% foetal calf serum (Sigma) and antibiotics. Subcutaneous tumours were initiated by injecting 0.1 ml NaCl (0.9%) containing 1.5 x 10^6 viable tumour cells under the skin in the upper part of the right leg of the mouse. When the tumours reached 40-50 mm^3 in volume, approximately 10 days after implantation, the mice were randomly divided into experimental groups, consisting of at least 6 mice. Treatment protocols were approved by the Department of Agriculture of the Republic of Slovenia No. 323-02-237/01. The ethical guidelines that were followed meet the standards required by the UKCCCR guidelines (19).

Treatment protocol. Electroporation of tumours was performed by the application of eight square-wave electric pulses to the tumours, delivered in two sets of four pulses in perpendicular directions, of 1040 V amplitude (amplitude / distance ratio 1300 V/cm), with pulse width of 100 μs and repetition frequency 1 Hz. The electric pulses were delivered by two flat, parallel stainless-steel electrodes 8 mm apart (two stainless-steel strips, width 7 mm, with rounded corners), placed closely apposed to the skin at opposite margins of the tumour. Good contact between the electrodes and the skin was achieved by means of conductive gel. The electric pulses were generated by an electroporator Jouan GHT 1287 (Jouan, St Herblain, France). Treatment with the electric pulses was performed without anaesthesia and was well tolerated by mice.

Hyperthermia was performed on anaesthetised mice. The mice were anaesthetised with 0.1 ml 0.2% xylazine (Rompun 2%, Bayer, Germany) and 0.1 ml 2% ketamine (Ketanest 50 mg/ml, Parke-Davis GMBH, Germany) injected intraperitoneally. While anaesthetised, the animals were kept on an automatically regulated heating pad to prevent hypothermia. The rectal temperature was kept as close as possible to 37˚C, with the contact temperature of the heating pad never exceeding 39˚C. The tumours were heated by local microwave hyperthermia. Briefly, the right hind leg with the tumour was immobilized without impairing the blood supply to the foot and the tumour was heated up to 43.5˚C for 30 minutes by the microwave probe (30 mm diameter) and the 910 MHz generator. The distance between the tumour and the probe was a few millimetres. The tumour temperature reached the set value of 43.5˚C after 4-5 minutes, the power input not exceeding 6-10 W. The tumour temperature was measured by ceramic thermocouple moving repeatedly along one track from one to the opposite margin of the tumour. The tumour temperature was kept at the set level 43.5±0.2˚C by adjusting the power input of the generator throughout the heating period.

In the combined treatment group, the mice were anaesthetised before application of electric pulses to the tumour. The heating of the tumours at 43.5˚C for 30 minutes started either immediately (1 minute) or 24 hours after the application of electric pulses. The tumour temperature was monitored throughout the treatment.

Assesment of tumour perfusion by Patent blue. Patent blue (Patent blau, Byk Gulden, Kreuzlingen, Switzerland) was used to estimate tumour perfusion (16, 17). The dye solution (1.25%) was diluted 2 times in physiological saline and 0.2 ml injected at different time points after treatment into the tail vein of animals from the control, electric pulses alone, hyperthermia alone and the combination of electric pulses and hyperthermia groups. After the dye had evenly distributed through the tissue for approximately 1 minute, the animals were killed by cervical dislocation and the tumours carefully dissected. The tumours were cut along their largest diameter and the percentage of stained *versus* unstained (perfused *versus* unperfused) cross-section was immediately estimated visually by two investigators. The mean value of both estimations was used as an indicator of tumour perfusion. The results were presented as arithmetic mean (AM) and standard error of the mean (SEM) for each experimental group. The Patent blue staining technique is a reliable method to determine tissue perfusion since the good correlation of this technique and the ^{86}RbCl extraction method that measures plasma flow through the tumour has been established (17).

Assessment of tumour response and statistical analysis. The tumour growth was followed every 2 days by measuring three mutually orthogonal diameters (d_1,d_2,d_3) with Vernier calliper. The tumour volume was calculated by the formula $d_1 \times d_2 \times d_3 \times \pi/6$. On the basis of these measurements, the AM and SEM were calculated for each experimental group. The tumour doubling-time (DT) was determined as the time in days for tumours to double their volume from the beginning of the treatment. The DT was determined for each individual tumour and the tumour growth delay (GD) from the mean DT of experimental groups was calculated by: $GD=DT_x-DT_c$, where DT is the mean doubling-time, subscript x–experimental group, subscript c-control group. The results were graphically presented as tumour growth curves. The response to treatment was scored as complete when the tumours became

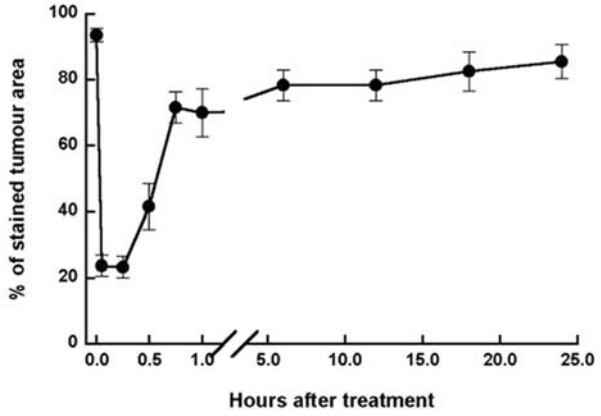

Figure 1. *Percentage of stained (perfused) tumour area of LPB tumours measured by the Patent blue staining technique. Perfusion was estimated in tumours treated by application of electric pulses (8 pulses, 1300 V/cm, 100 µs, 1 Hz) before and at different time points after the treatment. Values are AM ± SEM from 6 mice.*

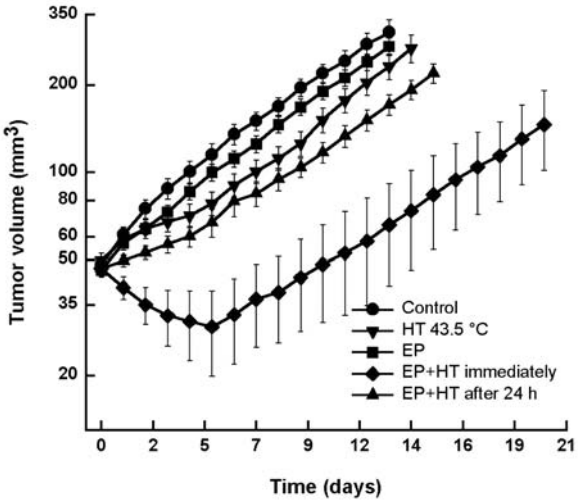

Figure 2. *Tumour growth curves of LPB fibrosarcoma. C57Bl mice were treated with electric pulses (EP; 8 pulses, 1300 V/cm, 100 µs, 1 Hz), 910 MHz microwave hyperthermia at 43.5°C for 30 minutes (HT), or with combination of both methods. In groups where the combination of methods was applied, hyperthermia followed electric pulses immediately (EP + HT immediately) or was postponed for 24 hours (EP + HT after 24 h). Values are AM ± SEM from 9-15 mice. Tumours with complete response (CR) were not included in the growth curves.*

unpalpable. Mice that had complete response 100 days after the treatment were considered cured. Statistical significance was evaluated by modified *t*-test (Bonferroni *t*-test) after one-way ANOVA was performed and fulfilled. Sigmastat and Sigmaplot software (SPSS inc. Chicago, IL, USA) were used for statistical calculation and graphic presentation of the data. Spector's formula was used to assess the combined effects (additivity, synergism and antagonism) of two treatment modalities (20).

Histology of tumours. Morphological changes were assessed in the same tumours as those that were used for the Patent blue staining. The specimens were fixed in 10% buffered-formalin. One tissue block cut through the largest diameter of the tumour was embedded in paraffin and stained with haematoxilyn-eosin by standard method. Slides of 6 tumours per group were examined in a blind fashion. Tumour necrosis was determined in a whole mount tumour section through the largest tumour diameter by two investigators and expressed as percentage of necrosis in relation to whole tumour cross-section. The mean of both estimations was used as an indicator of tumour necrosis. The results were presented as AM and SEM for each experimental group.

Results

Tumour perfusion changes after application of electric pulses alone. In order to determine the time point with maximal reduction of tumour perfusion after application of the electric pulses to the tumours, the time course of tumour perfusion changes was determined. Several time points, immediately after and up to 24 hours after the application, were evaluated. Untreated LPB tumours were well perfused as demonstrated by 94% of the tumour section stained by Patent blue. Application of 8 electric pulses to the tumours reduced the tumour perfusion at 5 minutes post-treatment to 24% and it remained at this level for 15 minutes. Reperfusion began after this time and was evident by 30 minutes. After 45 minutes it returned to 70% and after 24 hours to 85% (Figure 1).

Antitumour effectiveness of hyperthermia combined with application of electric pulses. According to data obtained on tumour perfusion changes after application of electric pulses alone in this set of experiments, hyperthermia was combined with application of electric pulses at two time points. Hyperthermia was performed immediately after application of electric pulses in order to determine the antitumour effectiveness of hyperthermia at maximally reduced tumour perfusion and at 24 hours when tumour perfusion returned almost to the pre-treatment level.

More than additive antitumour interaction was obtained when hyperthermia was performed immediately after application of the electric pulses to the tumours. This treatment combination resulted in a significantly prolonged tumour growth delay and 43% of complete responses up to 100 days after the treatment. In contrast, when hyperthermia was performed 24 hours after the application of electric pulses, no complete responses were obtained and only 4.1±1.1 days tumour growth delay. The effect of combined treatment with a 24-hour interval resulted in an additive interaction between the application of electric pulses and hyperthermia. Single treatment application of electric pulses or hyperthermia had a minor or no effect on tumour growth (Figure 2, Table I).

Table I. *Antitumour effectiveness of hyperthermia (HT) combined with the application of electric pulses (EP) to the tumours. Tumours were treated with electric pulses (8 pulses, 1300 V/cm, 100 μs, 1 Hz), hyperthermia at 43.5˚C for 30 minutes or with a combination of both methods. In the combined treatment one group was heated immediately after the application of electric pulses to the tumours (EP + HT immediately) and the other group in the 24-hour interval between the treatments (EP + HT 24-h interval).*

Group	n[a]	DT[b] (days) AM ± SEM	*P*-value	GD[c] (days) AM ± SEM	CR[d]
Control	15	4.1±0.3			0
EP	15	4.9±1.6	0.64	0.7+1.6	0
HT	17	7.6±1.2[e]	<0.05	3.4+1.2	1/17
EP + HT immediately	14	18.6±3.1[e]	<0.05	14.5+3.1	6/14
EP + HT 24-h interval	9	8.2±1.1	<0.05	4.1+1.1	0

[a] n, number of animals in the group; [b] DT, doubling-time of the tumours that were not cured; [c] GD, growth delay; [d] CR, complete responses

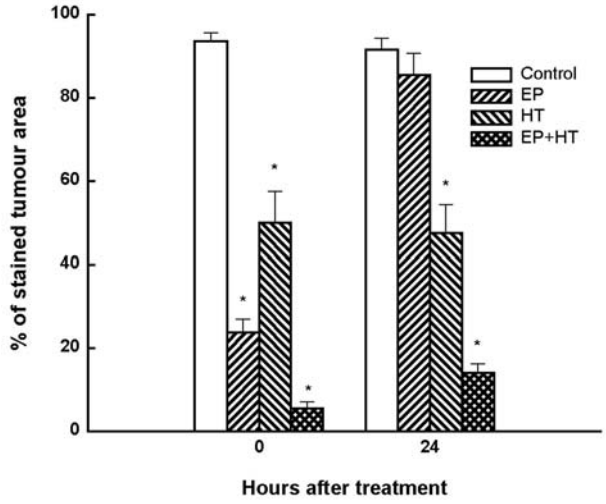

Figure 3. *Percentage of stained (perfused) tumour area of LPB tumours measured by the Patent blue staining technique. Tumour perfusion was assessed immediately or 24 hours after the application of electric pulses (EP), hyperthermia (HT) or combination of electric pulses and immediate hyperthermia (EP + HT). Values are AM ± SEM from 6 mice.*

Side-effects associated with application of the electric pulses were instantaneous contractions of the muscles located beneath the site of treatment, which disappeared immediately thereafter. Hyperthermia induced, to some degree, normal tissue damage; 17% of mice developed foot oedema 24 hours after treatment, which resulted 2 days later in skin ulceration without macroscopically visible damage to the underlying muscle. The normal tissue reaction to the combined treatment (hyperthermia immediately after application of electric pulses) was the same as to hyperthermia alone, however the percentage of the affected animals was slightly higher (21%).

Tumour perfusion changes after combined treatment, when hyperthermia was performed immediately after application of electric pulses. In order to assess how the combined treatment, when hyperthermia was performed immediately after application of the electric pulses, affects tumour perfusion, Patent blue staining of the tumours was performed at two time points, immediately and 24 hours after the treatment. Tumour perfusion was abrogated immediately after the combined treatment and restored to only 14% at 24 hours post-treatment. Hyperthermia alone reduced tumour perfusion to a lesser extent (50%), but remained at the same level also at 24 hours post-treatment. As already described in the previous section, the effect of the electric pulses was a profound reduction of tumour perfusion, which was restored to 85% at 24 hours post-treatment (Figure 3).

Morphological changes after hyperthermia performed immediately after application of electric pulses. Morphological changes in the tumours, when hyperthermia was performed immediately after application of the electric pulses, were assessed 24 hours after the treatment. Tumour necrosis after the combined treatment was observed in 90.0±2.6% tumour cross-section and was largely central. This correlated well with the observed tumour perfusion changes and antitumour effectiveness. Hyperthermia alone resulted in substantial tumour necrosis (71.6±3.0%), which was also observed in the central region, despite the moderate antitumour effectiveness of this treatment. The application of electric pulses alone resulted in less tumour necrosis (26.0±6.7%), which was also reflected in negligible antitumour effectiveness. Control untreated tumours had 2.5±1.1% necrosis, which was distributed in multiple small foci.

Discussion

The results of our study show that the application of electric pulses to tumours potentiates the antitumour effectiveness of hyperthermia. The effect was more than additive when hyperthermia followed the application of electric pulses immediately, resulting in significantly prolonged tumour growth delay and 43% tumour cures. The probable mechanisms responsible for the observed interaction are potentiation of thermic cytotoxicity due to the reduced tumour perfusion induced by the application of electric

pulses, and prolonged tumour perfusion reduction after the combined treatment leading to additional cell kill due to protracted ischemia. This observation is supported by the fact that the antitumour effectiveness of the combined treatment was only additive when the interval between the treatments was 24 hours. At that time point, tumour perfusion was already restored to the pre-treatment level after application of the electric pulses to the tumours.

Electroporation of tissues *in vivo* has several biomedical applications. Due to its principle mechanism of action *i.e.* increasing membrane permeability, it is used for delivery of molecules into the cells of different types of tissues (13-15). Besides increased membrane permeability, application of electric pulses to the tissues *in vivo* affects blood perfusion. After application of electric pulses a profound reduction of blood perfusion is observed, with quick restoration in normal tissues, while in tumours restoration of blood perfusion takes a longer time. It is presumed that the application of electric pulses causes reflexive constriction of arterioles in the electroporated area. In addition, electroporation affects permeability and the viability of cells in the vessel's wall, leading to increased inerstitital fluid pressure, disruption in water balance and, consequently, also slower blood flow (16-18, 21-23). In addition, in our previous study it was demonstrated that reduced tumour perfusion correlated with reduced tumour oxygenation (18). Tumour hypoxia induced by application of electric pulses increased the antitumour effectiveness of the bioreductive drug tirapazamine (24).

Numerous studies have demonstrated an increased response of tumours to heat after a decrease in tumour perfusion by various methods, such as treatment of tumours with combretastatin A-4 disodium phosphate, hydralazine, 5,6-dimethylxanthenone-4-acetic acid and flavone acetic acid (11, 25-28). The results of our study, combining the application of electric pulses to tumours with hyperthermia, are in the line with the concept that reduced tumour perfusion leads to increased tumour response to heat. We have shown that the local application of electric pulses to tumours significantly decreased tumour perfusion and increased the necrotic fraction. However, the perfusion changes were somewhat transient, having recovered by 24 hours. Reduced tumour perfusion induced by the application of electric pulses led to tumour hypoxia. Factors, which accompany hypoxia, *i.e.* lowered pH and nutritional deprivation, can increase thermosensitivity and thus enhance the tumour response, which we observed when hyperthermia was performed immediately after application of electric pulses. Tumour growth was delayed for 14.5 days and 43% tumours were cured. Therefore, to some extent the more than additive interaction of electric pulses and hyperthermia could be due to the tumour hypoxia induced by application of the electric pulses to the tumours. However, to fully prove this assumption an *in vivo-in vitro* clonogenic assay should be performed immediately after the combined treatment to determine whether the application of electric pulses has a direct effect on the heat-induced damage to the tumours.

Hyperthermia alone in our LPB tumour model reduced tumour perfusion to 50% immediately after the treatment and it remained at this level up to 24 hours post-treatment. As speculated in other studies using different tumour vascular-targeted agents in combination with hyperthermia, we can also presume that application of the electric pulses did not induce sufficient perfusion changes to explain the more than additive interaction between the treatments when hyperthermia was performed immediately after the application of electric pulses. Therefore, the more than additive interaction may be in part due to the perfusion reduction from both, hyperthermia and application of electric pulses, which resulted in the prolonged low perfusion level obtained after the combined treatment (14%). In the study of Honess *et al.,* the *in vivo-in vitro* clonogenic assay, performed immediately after the combined treatment of hyperthermia and hydralazine, showed an additive effect. Therefore, it was suggested that the reason for the more than additive tumour growth delay of the combined treatment was due to the destructive effect of the heat on non-perfused tumour blood vessels, causing prolonged reduction in tumour perfusion, which may in turn lead to extended growth delay or tumour cures (25). Indeed, our results demonstrated that, besides prolonged tumour growth delay, 43% of tumours were cured by combining hyperthermia immediately after the application of electric pulses to the tumours. Furthermore, our results on applying hyperthermia 24 hours after the electric pulses support the concept of the vascular damaging effect of the combined treatment.

In conclusion, the local application of electric pulses to the tumours potentiated the antitumour effectiveness of hyperthermia, when hyperthermia immediately followed the application of electric pulses.

Acknowledgements

This work was supported by the Ministry of Education, Science and Sport of the Republic of Slovenia. The authors would like to thank Simona Kranjc, Matej Bracko and Smiljan Ogorelec for their assistance.

References

1 Falk MH and Issels RD: Hyperthermia in oncology. Int J Hyperther *17*: 1-18, 2001.

2 Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R and Riess H: The cellular and molecular basis of hyperthermia. Crit Rev Oncol Hemat *43*: 33-56, 2002.

3 Takemoto M, Kuroda M, Urano M, Nishimura Y, Kawasaki S, Kato H, Okumura Y, Akaki S, Kanazawa S, Asaumi J, Joja I and Hiraki Y: The effects of various chemotherapeutic agents given with mild hyperthermia on different types of tumours. Int J Hyperther *19*: 193-203, 2003.

4 Dewey WC: Arrhenius relationships from the molecule and cell to the clinic. Int J Hyperther *10*: 457-483, 1994.

5 Roti Roti JL, Kampinga HH, Malyapa RS, Wright WD and vanderWaal RP: Nuclear matrix as a target for hyperthermic killing of cancer cell. Cell Stress Chaperon *3*: 245-255, 1998.

6 Stewart F and Begg A: Blood flow changes in transplanted mouse tumours and skin after mild hyperthermia. Br J Radiol *56*: 477-482, 1983.

7 Song CW: Effect of local hyperthermia on blood flow and microenvironment: a review. Cancer Res *44*: 4721-4730, 1984.

8 Song CW, Shakil A, Osborn JL and Iwata K: Tumour oxygenation is increased by hyperthermia at mild temperatures. Int J Hyperther *12*: 367-373, 1996.

9 Brown SL, Hunt JW and Hill RP: Differential thermal sensitivity of tumour and normal tissue microvascular response during hyperthermia. Int J Hyperther *8*: 501-514, 1992.

10 Horsman MR and Overgaard J: Can mild hyperthermia improve tumour oxygenation? Int J Hyperther *13*: 141-147, 1997.

11 Horsman MR and Murata R: Combination of vascular targeting agents with thermal or radiation therapy. Int J Radiat Oncol Biol Phys *54*: 1518-1523, 2002.

12 Orlowski S and Mir LM: Cell electropermeabilisation: a new tool for biochemical and pharmacological studies. Biochim Biophys Acta *1154*: 51-63, 1993.

13 Mir LM: Therapeutic perspectives of *in vivo* cell electropermeabilization. Bioelectrochemistry *53*: 1-10, 2000.

14 Jaroszeski MJ, Heller R and Gilbert R: Electrochemotherapy, Electrogenetherapy and Transdermal Drug Delivery. Electrically Mediated Delivery of Molecules to Cells. New Jersey, Humana Press. 2000.

15 Sersa G, Cemazar M and Rudolf Z: Electrochemotherapy: advantages and drawbacks in treatment of cancer patients. Cancer Therapy *1*: 133- 142, 2003.

16 Sersa G, Cemazar M, Parkins CS and Chaplin DJ: Tumour blood flow changes induced by application of electric pulses. Eur J Cancer *35*: 672-677, 1999.

17 Sersa G, Cemazar M, Miklavcic D and Chaplin DJ: Tumor blood flow modifying effect of electrochemotherapy with bleomycin. Anticancer Res *19*: 4017-4022, 1999.

18 Sersa G, Krzic M, Sentjurc M, Ivanusa T, Beravs K, Kotnik V, Coer A, Swartz HM and Cemazar M: Reduced blood flow and oxygenation in SA-1 tumours after electrochemotherapy with cisplatin. Br J Cancer *87*: 1047-1054, 2002.

19 Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin DJ, Double J, Embleton J, Newell D, Rymond R, Stables J, Stephens T and Wallace J: United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia. Br J Cancer *77*: 1-10, 1998.

20 Spector SA, Tyndall M and Kelley E: Effects of acyclovir combined with other antiviral agents on human cytomegalovirus. Am J Med *73*: 36-39, 1982.

21 Gehl J and Geertsen PF: Efficient palliation of haemorrhaging malignant melanoma skin metastases by electrochemotherapy. Melanoma Res *10*: 585-589, 2000.

22 Cemazar M, Parkins CS, Holder AL, Chaplin DJ, Tozer GM and Sersa G: Electroporation of human microvascular endothelial cells: evidence for anti-vascular mechanism of electrochemotherapy. Br J Cancer *84*: 556-570, 2001.

23 Gehl J, Skovsgaard T and Mir LM: Vascular reactions to *in vivo* electroporation: characterization and consequences for drug and gene delivery. Biochim Biophys Acta *1569*: 51-58, 2002.

24 Cemazar M, Parkins CS, Holder AL, Kranjc S, Chaplin DJ and Sersa G: Cytotoxicity of bioreductive drug tirapazamine is increased by application of electric pulses in SA-1 tumours in mice. Anticancer Res *21*: 1151-1156, 2001.

25 Honess DJ, Hu DE and Bleehen NM: A study of the mechanism of hydralazine enhancement of thermal damage in the KHT tumour. Int J Hyperther *7*: 667-679, 1991.

26 Horsman MR, Sampson LE, Chaplin DJ and Overgaard J: The *in vivo* interaction between flavone acetic acid and hyperthermia. Int J Hyperther *12*: 779-789, 1996.

27 Eikesdal HP, Schem BC, Mella O and Dahl O: The new tubulin-inhibitor combretastatin A-4 enhances thermal damage in the BT4An rat glioma. Int J Radiat Oncol Biol Phys *46*: 645-652, 2000.

28 Murata R, Overgaard J and Horsman MR: Potentiation of the anti-tumour effect of hyperthermia by combining with the vascular targeting agent 5,6-dimethylxanthenone-4-acetic acid. Int J Hyperther *17*: 508-519, 2001.

Received March 5, 2004
Accepted May 10, 2004