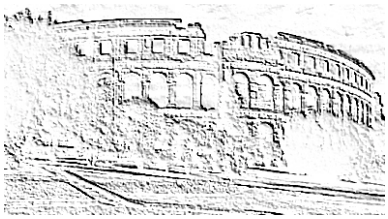

MEDICON 2001
Mediterranean Conference on Medical and Biological Engineering and Computing

Proceedings of the Workshop on

ELECTROPORATIVE ASSISTED DRUG DELIVERY: ELECTROCHEMOTHERAPY AND GENETHERAPY

PULA, CROATIA
June 11, 2001

Edited by
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Welcome note

Dear Colleagues,

In response to kind invitation of Professor Ratko Magjarević, the organizer of the IX Mediterranean Conference on Medical and Biological Engineering and Computing MEDICON 2001, we decided to organize the workshop on Electroporative Assisted Drug Delivery: Electrochemotherapy and Genetherapy. The Workshop is intended for all those interested in application of electroporation/electropermeabilization for experimental use *in vitro* and *in vivo* or clinical applications. Electroporative assisted drug and gene delivery to cells is becoming more and more widely used with clinical electrochemotherapy paving its way to clinical environment. In spite of the fact that exact molecular mechanisms on plasma membrane level are not entirely known, we gathered sufficient knowledge and experience to be able to use it efficiently. We have been fortunate enough to be able to bring together some of the world leading experts in electropermeabilization. We will do our best to provide you with all relevant information to be able to see the potential which is at our hands by electropermeabilization and to use it effectively in your working environment.

The result of exposing cells to electric pulses is transient permeabilization of plasma membrane which enables transmembrane flow of molecules which otherwise can not or difficultly cross plasma membrane. Electropermeabilization can be used in all kinds of isolated cells as well as cells in tissues. The electric field to which we expose the target cell has to be of sufficient value as to induce permeabilization. The magnitude of electric field that we have to use depends on cell type, size, orientation and density, pulse duration and number of pulses. The selection of pulse parameters is influenced also by the size and type of molecule that we want to internalize. Depending on the location and size of the targeted tissue electric pulses will be delivered via electrodes. Geometry and positioning of electrodes affect electric field distribution which is important for effective *in vivo* electropermeabilization.

In the proceedings we have in addition to the papers of all speakers included also the list and description of the key literature and addresses of all speakers. We also need to express our sincere thanks to colleagues working in our and collaborating laboratories, agencies which for years have been sponsoring our research work and to IGEA s.r.l., the European company which is dedicating its resources in order to provide us in near future with adequate clinical instrumentation.

Thank you for participating in our Workshop.

Sincerely Yours,

Damijan Miklavčič

Program

Workshop on Electroporative Assisted Drug Delivery: Electrochemotherapy and Genetherapy

Pula, Croatia, June 11, 2001

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12:30-13:00	Maja Čemažar: A comparison of gene delivery methods using electroporation and lipofectin-based techniques *in vitro* and *in vivo* (page 17)

Electrochemotherapy

Gregor Serša
Institute of Oncology, Ljubljana, Slovenia

Abstract: Electrochemotherapy consists of chemotherapy followed by local application of electric pulses to the tumor to increase drug delivery into the tumors. 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 anti-tumor effectiveness of bleomycin and cisplatin combined with electric pulses has already been demonstrated in experimental and clinical studies. In clinical studies, electrochemotherapy was performed on cutaneous and subcutaneous tumor nodules of malignant melanoma, head and neck carcinomas, basal cell carcinomas, adenocarcinomas, and Kaposi's sarcomas. Objective responses were obtained for the majority of the electrochemotherapy-treated nodules, whereas nodules that were exposed only to electric pulses or treated only with the drugs did not respond. Perspectives of electrochemotherapy are in treatment of various solid tumors. Developments in new electrode design will enable also treatment of tumors seated in internal organs. Electrochemotherapy can be used also in combination with other treatment modalities. Improvement of combined modality therapy with cisplatin and radiation using electroporation of tumors has already been demonstrated. In perspective, reduced tumor perfusion after application of electric pulses could be exploited in combination with bioreductive drugs. All these developments in electrochemotherapy are good basis for its further development, which will lead to broader clinical applicability of electrochemotherapy.

INTRODUCTION

Electroporation is used as drug delivery system for molecules that do not readily pass plasma membrane. It is a physical method, performed by application of high voltage direct current electric pulses to cells, in order to increase cell uptake of molecules such as DNA, antibodies, enzymes, dyes and drugs by permeabilization of plasma membrane [1].

Chemotherapy as treatment modality in cancer treatment is effective for drugs that readily pass plasma membrane and are cytotoxic when reaching their intracellular targets. However, among chemotherapeutic drugs that are very cytotoxic are some that have hampered transport through the plasma membrane. These drugs are good candidates for electrochemotherapy. Electrochemotherapy is a combined modality treatment using chemotherapy and electroporation [1]. In electrochemotherapy, the optimal anti-tumor effectiveness is achieved when treatment is given at the time of the highest extracellular concentration of hydrophilic chemotherapeutic drug, thereby enabling or increasing transport through plasma membrane towards the intracellular targets for cytotoxicity. The chemotherapeutic drugs, bleomycin and cisplatin, have proven to be effective in electrochemotherapy of experimental tumor cells, both *in vitro* and *in vivo* and also in electrochemotherapy of accessible tumour nodules of various malignancies in cancer patients [1-4].

PRECLINICAL DATA

Electroporation proved to be effective to facilitate transport of the molecules across the plasma membrane, thus enabling their cytotoxicity. Increased cytotoxicity by electroporation of cells *in vitro* was shown for several chemotherapeutic drugs. However, the best results were shown for two, bleomycin and cisplatin. Cytotoxicity of bleomycin was shown to be increased for several 100 fold and for cisplatin up to 70 fold after electroporation of cells. Electroporation is performed with 8 rectangular high voltage (800–1200 V/cm) electric pulses with repetition frequency 1Hz, and pulse duration 100μs. Increased cytotoxicity of bleomycin by electroporation is due to its lack for plasma membrane permeability and its high cytotoxicity once reaching intracellular targets. Only several hundred molecules are necessary to kill, once inside the cell. On the other hand only 50% of cisplatin is believed to be transported through the plasma membrane by passive diffusion, the rest is by carrier molecules. Therefore, electroporation increases cytotoxicity of cisplatin to a lesser extent than in bleomycin, but cisplatin by itself without electroporation already exerts considerable cytotoxic effect. These preclinical data *in vitro* have paved the way for testing of these two drugs in electrochemotherapy *in vivo* on tumors in mice, rats, cats and dogs [1].

Anti-tumor effectiveness of bleomycin and cisplatin was proved by several groups in numerous experiments. Elaborated were dose and time dependence of drug administration as well as ways of electric pulses application. It was established that maximal concentration of the drugs must be present in the tumors to obtain good anti-tumor effect. Application of 8 electric pulses to solid tumors

(1300V/cm, with repetition frequency 1Hz and pulse duration 100μs) by plate or penetrating electrodes resulted in partial or complete responses of tumors. Sarcomas, carcinomas or melanoma tumors responded with high percentage of complete responses when the drugs were injected either intravenously or intratumorally. All these experiments provided sufficient data to demonstrate that electrochemotherapy with either bleomycin or cisplatin is effective in treatment of solid tumors, with drug concentrations that without application of electric pulses had none or minor anti-tumor effect. Application of electric pulses alone had minor or no effect on tumor growth [1].

Anti-tumor effectiveness of electrochemotherapy is considered to be primarily due to the increased drug uptake into the tumor cells, caused by electroporation. However, several other mechanisms may also be involved in the tumor response to electrochemotherapy: a) prolonged drug entrapment in the tumors as a consequence of decreased tumor blood flow after application of electric pulses, b) vascular targeted effect, and c) involvement of immune response of the host organism [5-7].

CLINICAL DATA

Based on preclinical data, electrochemotherapy with bleomycin and cisplatin entered clinical trials. Both drugs have proved their clinical application in electrochemotherapy protocols. Cutaneous and subcutaneous tumor nodules of different malignancies were treated. Most of the treated nodules responded with objective responses in 60-100% range [1-4].

In clinical protocols both intravenous and intratumoral drug administrations were used. Current knowledge about anti-tumor effectiveness of electrochemotherapy considers electrochemotherapy as a local treatment that is effective on most tumor types tested so far. Electrochemotherapy can be performed as single treatment or as an adjunct to the ongoing chemotherapy. In the latter case anti-tumor effectiveness of systemically given drug is potentiated locally by application of electric pulses where it is needed [1-4].

Comparison between the anti-tumor effectiveness of electrochemotherapy with bleomycin and electrochemotherapy with cisplatin, given intravenously or intratumorally, is possible in treatment of cutaneous tumor nodules of malignant melanoma (Table 1).

The results indicate that electrochemotherapy with bleomycin is equally effective when the drug is given intravenously or intratumorally. However, electrochemotherapy with cisplatin is more efficient when the drug is given intratumorally than when it is given intravenously. The results are comparable to the anti-tumor effectiveness of electrochemotherapy with bleomycin. The advantage of electrochemotherapy with cisplatin is that the drug itself, without application of electric pulses may exert considerable anti-tumor effect.

Table 1: *Summary of electrochemotherapy trials on malignant melanoma*

Treatment	Number of patients	Number of nodules	Objective resp. to treatment (%)
Intravenous			
ECT-BLM	14	94	89
CDDP	9	18	22
ECT-CDDP	9	27	48
Intratumoral			
ECT-BLM	10	71	99
CDDP	10	27	38
ECT-CDDP	10	82	78

BLM- bleomycin
CDDP- cisplatin
ECT- electrochemotherapy

PERSPECTIVES

It is difficult to foresee all the clinical applications of electrochemotherapy. In the first step more controlled clinical trials are needed evaluating treatment response of different tumor types. So far in the clinical trials have been treated only percutaneously accessible tumor nodules, with development of new electrodes it will be possible to treat also tumors seated in internal organs. In its concept electrochemotherapy is local treatment, therefore ways must be exploited to add a systemic component, either by adjuvant immunotherapy or in combination with other systemic treatments.

Some chemotherapeutic drugs interact with radiation therapy. Among the radiosensitizing drugs are also bleomycin and cisplatin. As already indicated in the recent study combined modality therapy with cisplatin and radiation can be improved using electroporation of tumors [8].

Application of electric pulses was shown to modulate tumor blood flow [6]. Reduced tumor blood flow was found in tumors after application of electric pulses, and the latest measurements also demonstrate a reduced pO_2. This reduced oxygen tension could be used to selectively activate bioreductive drugs that exhibit better cytotoxic effect on hypoxic cells than on the cells that are well oxygenated.

Electrochemotherapy can also be effective in treatment of tumors resistant to cisplatin. Recent preclinical data showed that electrochemotherapy with cisplatin highly potentiated the antitumor effectiveness of intravenously injected cisplatin in cisplatin-resistant solid tumors. This may prove useful in clinical chemotherapy for the treatment of tumors with intrinsic or acquired resistance to cisplatin [9].

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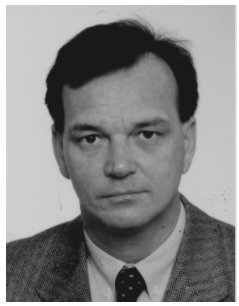

Gregor Serša was born in Ljubljana, Slovenia, in 1956. He received a Masters degree in 1984 and a Doctorate degree in Biomedical Sciences in 1988, both from the Medical Faculty, University of Ljubljana. From 1986 to 1987 he was visiting researcher at MD Anderson Hospital, Department of Experimental Radiotherapy, Texas, USA. He is currently Director of Research at the Institute of Oncology in Ljubljana, and Associate Professor at the University of Ljubljana. His scientific interests are tumor biology, electroporation, electrochemotherapy, electrogenetherapy, and experimental radiotherapy.

Gregor Serša is the author of 98 articles in peer-reviewed journals, 6 chapters in books, and he held 8 invited lectures at international meetings and seminars. He shared the 1995 Award of the Republic of Slovenia for Scientific and Research Achievements.

NOTES

NOTES

Electrodes and Corresponding Electric Field Distribution for Effective *In Vivo* Electroporation

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Abstract: Permeabilizing electric pulses can be advantageously used for DNA electrotransfer *in vivo* for gene therapy, as well as for drug delivery. In both cases it is essential to know the electric field distribution in the tissues: the targeted tissue must be submitted to electric field intensities above the reversible permeabilization threshold and below the irreversible permeabilization threshold (to avoid cytotoxic effects of the electric pulses). A three-dimensional finite element model was built for different electrodes used in various *in vivo* experiments. Electrodes of different geometries were modeled by applying appropriate boundary conditions in corresponding grid points of the model. The anatomically based model was previously validated by comparing our calculations with magnetic resonance current density imaging and electrochemotherapy for specific electrodes. The observations resulting from the numerical calculations, such as mean electric field magnitude within the tumors, are in good correlation with the effectiveness of electrochemotherapy.

INTRODUCTION

In the last two years, promising results for a new non-viral efficient gene therapy have been obtained in *in vivo* DNA electrotransfer studies [1]. It is also important to note that, recently, drug delivery using electric pulses has entered an active period of clinical trials [2,3]. These two new therapeutical approaches are based on cell electropermeabilization, also termed electroporation, a phenomenon where transiently increased plasma membrane permeability is obtained after the cells were exposed to short and intense electrical pulses. Electropermeabilization thus allows otherwise nonpermeant molecules to enter the cytosol.

For effective drug delivery and gene transfection *in vivo*, the knowledge of electric field distribution is of utmost importance, to obtain an effective permeabilization as well as to maintain the viability of the electropermeabilized cells. Indeed, in order to achieve electropermeabilization in the tissue of interest, the magnitude of electric field intensity has to be above a critical threshold value, i.e. the reversible threshold. Furthermore, the magnitude of electric field intensity should not exceed the value which would produce irreversible damages to the plasma membrane, i.e. the irreversible threshold. Thus, the magnitude of electric field intensity should be high enough to cause reversible electropermeabilization but lower than the value causing irreversible damage [4]. The latter is the most critical for *in vivo* gene transfer but is also desirable in electrochemotherapy in order not to produce large instantaneous necrosis, which could result in exulceration and wound appearance. Moreover, for gene therapy, it has been recently reported that, under relatively homogeneous exposure conditions, the optimal conditions for gene transfer correspond to the use of long pulses (20 milliseconds) at a voltage just necessary to obtain cell electropermeabilization, i.e. just above the reversible permeabilization threshold. Above the irreversible permeabilization threshold, when permanent damages are inflicted to the plasma membrane, viability is lost and efficacy of the DNA transfer is severely impaired [1]. Therefore it is necessary to determine (i) the electric field distribution in the target tissues, and (ii) the reversible as well as (iii) the irreversible permeabilization thresholds in order to use voltages and electrode geometries resulting in optimal exposure of the targeted tissue to electric fields intensities comprised between the two thresholds. Very few studies have dealt with these questions. In *ex vivo* experiments, using two parallel plates separated by 2mm, which represents a rather homogeneous exposure system, a variable threshold (ranging from 300 V/cm to 500 V/cm) was found for a fibrosarcoma tumour exposed to 8 pulses of 100 μs at a frequency of 1 Hz. Recently, using a numerical two-dimensional model for electric field distribution, parallel plates as electrodes, and a quantitative Cr^{51}-EDTA uptake assay, threshold for reversible *in vivo* permeabilization of mouse skeletal muscle was found at 450 V/cm for the same type of pulses. Our work [4] using a three-dimensional finite element model in which needle electrodes of different diameters were modeled and compared to appropriate experiments in rabbit liver tissue showed excellent agreement between numerical predictions and experimental observations. By this approach it was possible to make the first precise determination of the magnitude of the electric field intensity for reversible (362±21 V/cm, avg±std) and for irreversible (637±43 V/cm) permeabilization thresholds of rabbit liver tissue *in vivo*.

A variety of electrodes have been used in *in vivo* electrochemotherapy and gene delivery by electroporation (Fig. 1). The first electrodes and the most widely used are plate electrodes (Fig. 1 A) which are used as plate electrodes of fixed distance or variable distance

between the electrodes as conductive parts of electrodes are mounted on caliper. This type of electrodes is used for electrochemotherapy of cutaneous and subcutaneous experimental tumors, smaller tumors in patients and gene delivery in rat mouse and subcutaneous tumors. Other types of electrodes (Figs. 1 B to E) have been used and compared in electrochemotherapy of experimental subcutaneous mice tumors with variable response [5]. Another type of electrodes – "honeycomb" has been constructed and used for treatment of larger volumes. In this electrode (Fig. 1 F) a division of volume to smaller fractions is introduced as pairs of needle electrodes are sequentially fired in a way that eventually the whole (arbitrarily large) volume is being permeabilized. Since it is difficult to compare the "effectiveness" of all these electrodes due to different voltages applied by different electrodes we compared electric field distribution in the tumor obtained by numerical modeling with electrochemotherapy efficiency using different electrodes and corresponding voltages in *in vivo* electrochemotherapy experiments reported previously [5-8]. In addition to correlation between mean magnitudes of electric field in the tumor and electrochemotherapy effectiveness we also report the minimum and maximum electric field magnitudes in tumors for all electrodes analyzed. This measure of inhomogeneity of electric field distribution is important especially for gene delivery, where cell have to maintain their viability.

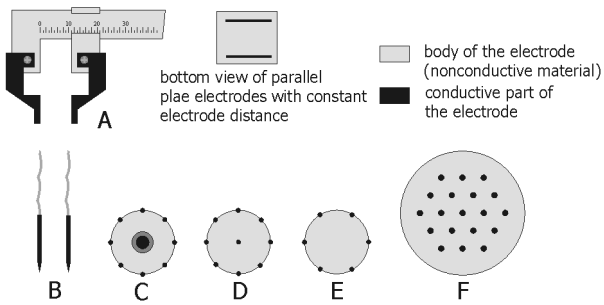

Figure 1: Various electrodes used for *in vivo* electropermeabilization for electrochemotherapy and gene delivery.

MATERIALS AND METHODS

Electrodes and Electrochemotherapy

The exact protocols for electrochemotherapy and electrodes used were previously reported [5,6,8]. Briefly, electrochemotherapy consisted of application of electric pulses to the tumor 3 minutes after intravenous injection of bleomycin. The dose used was sufficient for killing the cells once being permeabilized and was well tolerated by mice. Electric pulses were delivered by either pressing electrodes on the skin or inserting the electrodes through the skin and applying voltage as reported in Table 1. Additionally to the electrodes presented in Fig. 1, specific electrodes with known distribution of electric field were also used for electrochemotherapy [6].

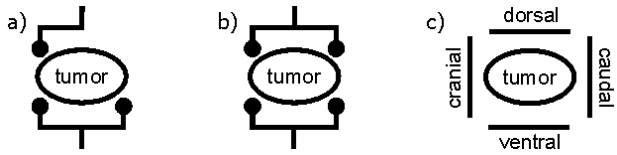

Figure 2: Electrodes used in electrochemotherapy (top view). Electrodes 2+1 (left) and 2+2 (middle) were needle electrodes which were pressed against the skin. Plate electrodes (right) were used as two plate electrodes pressed against the skin in either dorsal-ventral direction or cranial-caudal direction. In addition, plate electrodes were also used to apply four electrodes in one direction and the other four electrodes in perpendicular direction (4+4).

Eight square-wave pulses of 100 μs and repetition frequency of 1 Hz were delivered in one direction unless otherwise specified in Table 1. Current and voltage were monitored during pulse delivery. Control groups included mice without treatment, mice treated with electric pulses and bleomycin as single treatment. Electrochemotherapy was performed on tumors when they reached approximately 50 mm^3 in volume (day 0). Tumor volume was estimated by measuring three main mutually perpendicular tumor diameters.

Mean tumor volume and standard error of the mean were then calculated for each experimental group and presented as a tumor growth curve. For each tumor individually tumor doubling time was determined, i.e. the time tumor needed to double its initial volume of day 0. For each experimental group mean and standard error of the mean was calculated.

Finite Element 3D Model

The model has been described *in extenso* in our previous publications [6,8]. Briefly, a three-dimensional (3D) anatomically based finite element (FE) model of the mouse with injected subcutaneous solid tumor was built using MSC/EMAS (Electro-Magnetic Analysis System) software package (trademark of The MacNeal-Schwendler Corporation, USA). The geometry of the model was based on the 14 cross section scans of one typical animal with a subcutaneous tumor, obtained by magnetic resonance imaging. The geometry of the model was described with 1390 points, which defined 3859 curves/lines. A total of 1379 3D geometric bodies were defined using those curves. The resulting three-dimensional geometric structure was built of eleven different tissues (organs), i.e. skin, fat, skeletal and heart muscles, bone, connective tissue, intestine, kidney, liver, lung and tumor. Anisotropic characteristics were considered for skeletal and heart muscles, while all other tissues (organs) were modeled as isotropic. The values of the electric conductivity of tissues (organs) used in the model were collected from

literature and used in one of the previous studies where similar model was verified with the measurements of electric potential in the 5 points in the tumor and surrounding tissue. In addition, the model was validated by comparing numerical results with current density magnetic resonance imaging [6].

Different electrode sets were modeled by applying appropriate boundary conditions in the grid points corresponding to each of the electrodes (Figs. 1 and 2). Increased area with the same electric potential under each electrode resulting from the use of conductive gel was also taken into consideration. Fixed values of scalar electric potential, i.e. Dirichlet boundary conditions, were assigned to grid points in the regions where electrodes were placed. All electrode sets were modeled according to the position of the electrodes with respect to the tumor. Potentials of 0 V and 1300 V (or other, as specified in Table 1) for electrochemotherapy were assigned to groups of appropriate grid points of the FE mesh corresponding to each of the electrodes. On the remaining outer surfaces of the model, a Neumann boundary condition was applied. This boundary was considered as the interface between a conducting medium and air (assimilated to an ideal dielectric). Since the conductor (skin layer) was linear and isotropic, the usual Neumann condition was applied i.e. the normal derivative of the electric potential on the interface between the model and surrounding air was zero. Distribution of electric field intensity was then calculated from the values of the scalar electric potential in the grid points of the model. The distribution of the electric field was more precisely studied for 48 elements representing subcutaneous tumor since we were most interested in the electrical phenomena inside tumor tissue. Mean magnitudes of electric field inside 48 elements representing the tumor were calculated at appropriate voltages used in electrochemotherapy with different electrodes. For electrode configurations (e.g. 4+4) where for the last 4 pulses, the electrodes were oriented perpendicularly with respect to the position of the electrodes for the first 4 pulses, electric field distribution was determined as a combination of the results for the cranial-caudal and dorsal-ventral electrode configurations. Since electroporation is a threshold phenomenon, it can be assumed that in the 4+4 electrode configuration the effective magnitude of electric field intensity in each finite element of the model is the highest of the magnitudes for cranial-caudal and dorsal-ventral electrode configurations in that particular element. The same approach was used for 2x2 electrodes (Fig. 1 E) where two by two opposite electrodes were connected to the opposite poles of the generator and electric pulses were delivered in six directions, and for 3x3 electrodes (Fig. 1 E), where three by three electrodes were connected to the opposite poles of the generator and six pulses were applied altogether by rotating the electric field in the tumor. Minimal and maximal values in the tumor are reported as well.

RESULTS

For all electrodes used in electrochemotherapy and presented in Figs. 1 and 2, electric field was calculated in all 7089 elements of the mouse model corresponding to experimentally applied voltage (Tables 1 and 2). The mean, standard deviation, minimum and maximum of electric field magnitudes in 48 elements representing the tumor are given in Tables 1 and 2. The mean values of electric field magnitudes are presented in Figs. 3 and 4 in the form correlation diagrams for each of the electrode orientations and antitumor effectiveness of electrochemotherapy. The summary of the data together with experimental results, i.e. tumor doubling

Table 1: Electrodes with resulting electric field and effectiveness of electrochemotherapy for studies reported in [6,8]

Electrode type with figure reference	Applied voltage (V)	Number of pulses	E (V/cm) mean±std	Emin (V/cm)	Emax (V/cm)	max/min	doubling time (days) mean±std
2+1 Fig 2a	1300	8	202±61	115	317	2.7	11.4±0.5
2+2 Fig 2b	1300	8	251±60	166	408	2.4	17.8±1.4
CC Fig 2c	1040	8	327±93	210	518	2.5	20.9±1.0
DV Fig 2c	1040	8	406±65	327	643	2.0	25.1±1.4
4+4	1040	8*	425±64	333	643	1.9	30.0±1.4

*pulses were delivered 4 in direction caudal-cranial and 4 in dorsal-ventral direction

Table 2: Electrodes with resulting electric field and effectiveness of electrochemotherapy for study reported in [5]

Electrode type with figure reference	Applied voltage (V)	Number of pulses	E (V/cm) avg±std	Emin (V/cm)	Emax (V/cm)	max/min	doubling time (days)
caliper Fig 1a	700	8	629±204	373	1048	2.8	19.5±1.6
s.c. needles Fig 1b	330	8	189±47	129	291	2.2	11.7±1.3
8+1 needle Fig 1d	750	8	644±362	339	1350	4.0	20.6±1.4
8+ cilinder Fig 1c	700	8	351±164	191	842	4.4	15.0±1.1
6needle 2x2 Fig 1e	1300	6*	696±107	550	909	1.6	30.9±1.5
6needle 3x3 Fig 1e	705	6*	435±61	349	542	1.5	19.9±1.2

*single pulses were delivered in six different orientations

times are listed in Table 1 and 2. Good agreement between the level of anti-tumor effectiveness and the mean value of electric field intensity for each particular electrochemotherapy treatment regime was obtained.

The results of electrochemotherapy with all electrode sets are given in Figs. 3 and 4. Tumor growth was most retarded in experimental groups of electrochemotherapy where the electric field magnitude in tumor was the highest. Tumor growth delay in electric pulses alone and chemotherapy alone was negligible.

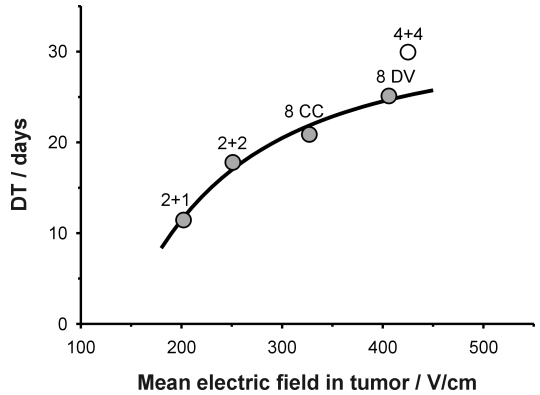

Figure 3: Correlation graph between effectiveness of electrochemotherapy (DT-tumor doubling time) and mean electric field in tumor for various electrodes used in the experiments [6,8].

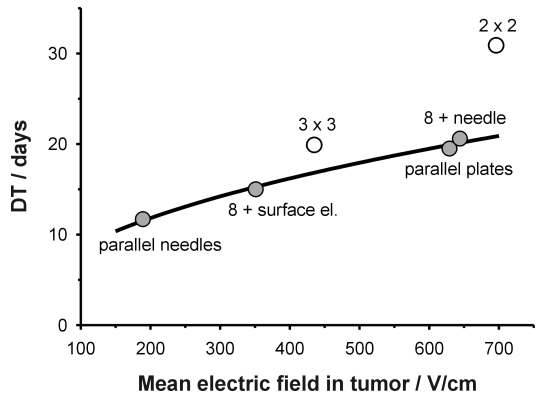

Figure 4: Correlation graph between effectiveness of electrochemotherapy (DT-tumor doubling time) and mean electric field in tumor for various electrodes used in the experiments [5].

CONCLUSIONS

Mathematical modeling for *in vivo* electroporation has proven to be relatively simple and efficient tool for the analysis of electrical phenomena inside biological tissue. It is very useful for the explanation of experimental results and analysis of different electroporation regimes. We demonstrated that better coverage of tumors with sufficiently high electric field is necessary for improved effectiveness of electrochemotherapy and so this approach can be very useful in further search for electrodes which would make electrochemotherapy and *in vivo* electroporation in general more efficient. The objective of such studies would be to optimize electrode configuration in order to obtain electric fields over threshold value in the whole selected tissue, e.g. in the tumor.

Mathematical modeling can thus be used in the transfer of the knowledge gained in experimental work into clinical practice. The long-term perspective of mathematical modeling is to contribute to understanding and wider clinical applicability of electrochemotherapy in treatment of cancer and of electro gene transfection as a future treatment for various diseases.

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electromagnetic fields with biological systems. In the last years he has focused on the engineering aspects of electropermeabilization as the basis of drug delivery into cells in tumor models *in vitro* and *in vivo*. His research includes biological experimentation, numerical modeling and hardware development for electrochemotherapy.

Damijan Miklavčič is the author of 75 articles in peer-reviewed journals. He received the MAPHRE Award at the 2nd European Congress of Physical Medicine and Rehabilitation in Madrid in 1989 and the National Industrial Award from Krka Pharmaceuticals in 1993. With Lojze Vodovnik and Gregor Serša he shared the Award of the Republic of Slovenia for Scientific and Research Achievements in 1995.

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The Role of Pulse Parameters in the Efficiency of Electropermeabilization *In Vitro*

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Abstract: The paper summarizes the studies of the role of pulse parameters in the efficiency of cell membrane electropermeabilization *in vitro*. Permeabilization becomes detectable if the pulse amplitude exceeds a certain critical value. Above this critical value, which depends considerably on the type of cells used in the experiment, the percentage of permeabilized cells increases, and the percentage of cells surviving the treatment decreases with further increase of pulse amplitude. In general, increase of pulse duration or number of pulses lowers the critical pulse amplitude of electropermeabilization and – within a limited range – also augments the uptake of molecules per cell. For efficient uptake of smaller molecules, typical pulse durations are in the range of hundreds of microseconds, while for macromolecules, durations from several milliseconds to several tens of milliseconds are usually required. Several studies imply that bipolar pulses and waveforms are more efficient with respect to their unipolar analogues, while pulse rise- and falltimes do not seem to be of importance. These studies offer some general advice in the design of studies involving electropermeabilization, but for best possible results, pulse parameters should be optimized under specific experimental conditions before the actual study is performed.

INTRODUCTION

For the large majority of applications *in vitro*, the efficiency of electropermeabilization is determined by the fraction of reversibly permeabilized cells with respect to the whole treated cell population. In the optimization of electropermeabilization, one thus searches for pulse parameters and other experimental conditions that yield the highest fraction of permeabilized cells that survive the treatment. In addition, for the treatment to serve its purpose, it is often necessary that a certain quantity of exogenous molecules enters into each cell, and in these cases optimal pulse parameters should also ensure a sufficient molecular uptake per cell.

For these reasons, the role of pulse parameters and experimental conditions is usually investigated using a combination of tests, estimating the fraction of permeabilized cells, the fraction of cells surviving the treatment, the average amount of exogenous molecules introduced into the cell, and sometimes also the time of recovery of the cells back into the nonpermeabilized state.

THE ROLE OF THE AMPLITUDE, DURATION, AND NUMBER OF PULSES

The role of parameters of rectangular pulses in the efficiency of electropermeabilization was investigated in a number of studies [1-6]. These studies show that permeabilization becomes detectable as the pulse amplitude exceeds a certain critical value. Above this value, with further increase of pulse amplitude the percentage of permeabilized cells increases, while the percentage of cells surviving the treatment decreases. As a function of pulse amplitude, the percentage of permeabilized cells approximately follows an ascending sigmoidal curve, while the percentage of viable cells resembles a descending sigmoidal curve (Fig. 1). Similar results have been obtained with exponentially decaying pulses [8], where the time constant of pulse decay was used instead of pulse duration.

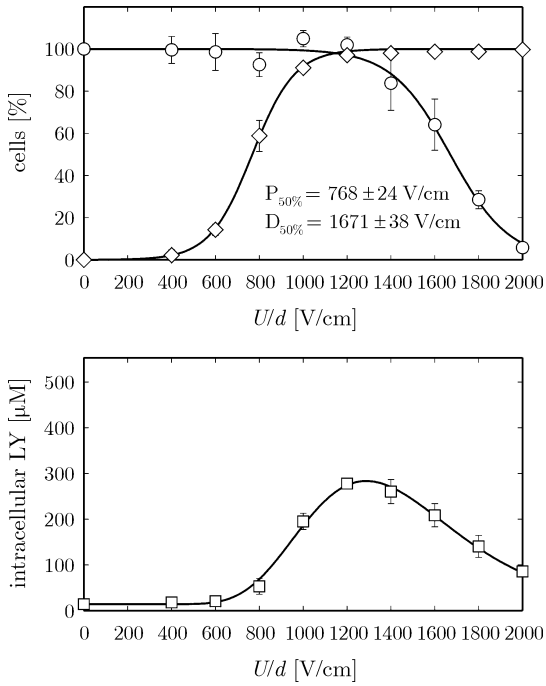

Figure 1: Top: Percentages of permeabilized (diamonds) and viable cells (circles) as functions of pulse amplitude (the ratio between the voltage and the electrode distance). Bottom: uptake of lucifer yellow (LY) into the cells. DC-3F cells (spontaneously transformed Chinese hamster fibroblasts) were permeabilized with eight unipolar rectangular 100-μs pulses delivered in 1-s intervals. $P_{50\%}$ and $D_{50\%}$ denote pulse amplitudes which lead to permeabilization and death, respectively, of 50% of the cells. Extracellular concentration of LY was 1 mM.

In a study performed on six different cell lines, Čemažar and co-workers have shown that both permeabilization and cell survival as functions of pulse amplitude vary significantly between various types of cells [4]. Though some of the observed differences can be attributed to differences in cell size, these results imply that the differences in membrane composition and structure also play an important role.

Experiments show that the critical pulse amplitude of electropermeabilization is lowered if the number and/or duration of the pulses is increased [1,5,6] (*cf*. Figs. 1 and 2). If the values of these two parameters are not too large, the average amount of molecules introduced into a cell also increases with an increase of the number of pulses. Using four or more pulses, a pronounced peak of molecular uptake is obtained (see Figs. 1 and 2, bottom).

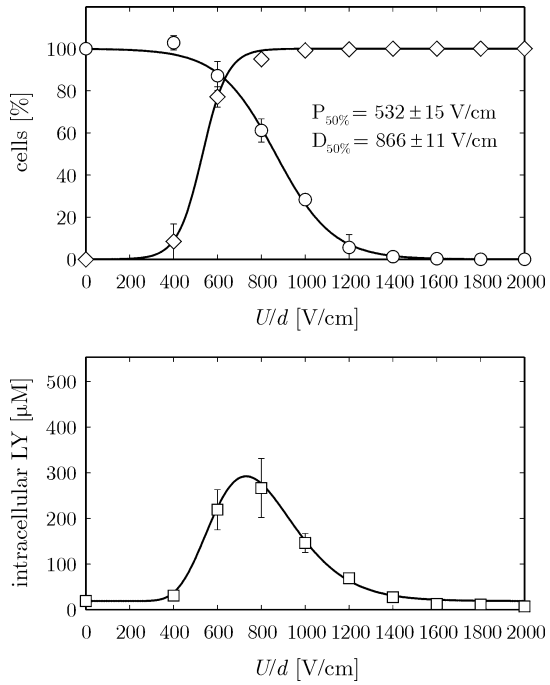

Figure 2: Electropermeabilization of DC-3F cells and uptake of LY with eight unipolar rectangular 1-ms pulses delivered in 1-s intervals. For legend and experimental details, see the caption of Fig. 1.

Several studies have demonstrated that in the case of macromolecules, electrophoresis plays an important role in the transport of molecules across the membrane, and sufficiently long pulse duration is crucial for adequate uptake [9,2,5]. Typically, pulse durations for the uptake of smaller molecules are in the range of hundreds of microseconds, while for macromolecules, durations from several milliseconds to several tens of milliseconds are usually required.

In a study utilizing a broad range of rectangular pulse parameters, Maček-Lebar and co-workers have shown that the total energy of a train of pulses is not a crucial parameter in either drug uptake or cell survival. On the contrary, a significant difference was observed in the uptake induced by different trains of the same total energy [3].

THE ROLE OF PULSE SHAPE

Because commercially available pulse generators with sufficient voltages for electropermeabilization of cells in suspension are mostly limited to rectangular and exponentially decaying pulses, relatively few studies have dealt with the role of pulse shape in the efficiency of cell electropermeabilization. Chang and co-workers have reported that the efficiency of permeabilization was increased when a sine wave of 30-200 kHz amplitude was superimposed onto a rectangular pulse, though the amplitude of the sine wave was only about 5% of the total pulse amplitude [10,11]. Tekle and co-workers found that the efficiency of DNA transfection *in vitro* was significantly higher with a bipolar 60 kHz square wave of 400 μs duration than with a unipolar wave of the same frequency and duration [12]. Schoenbach and co-workers have reported on electropermeabilization with ultrashort (60 ns) pulses [13]. In a study comparing unipolar and bipolar rectangular pulses, we have shown that with bipolar pulses, the critical voltage of electropermeabilization is lowered considerably, while cell viability remains practically unaffected (*cf*. Figs. 2 and 3); at the same time, the peak of the uptake increases with respect to the one obtained by unipolar pulses [7].

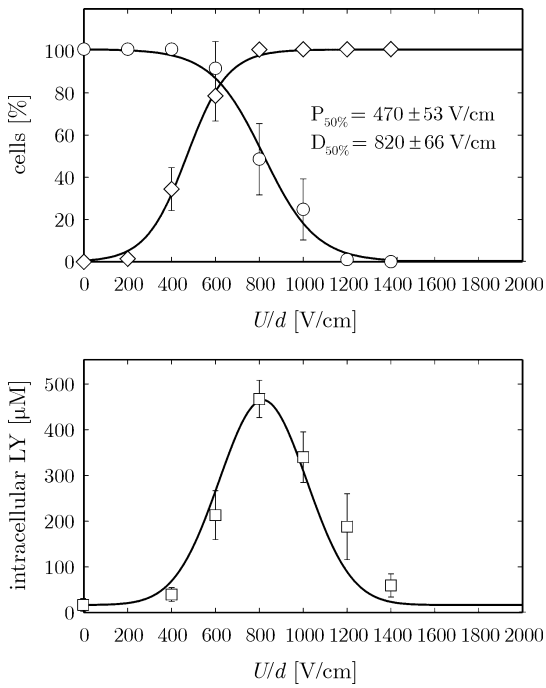

Figure 3: Electropermeabilization of DC-3F cells and uptake of LY with eight bipolar rectangular 1-ms (500 μs+500 μs) pulses delivered in 1-s intervals. For legend and experimental details, see the caption of Fig. 1.

In addition, we have shown that the release of metal ions from the electrodes into the cell suspension is reduced by more than an order of magnitude if bipolar pulses are used [14]. This reduces the electrolytic contamination of the cell suspension, and also prolongs the lifetime of the electrodes.

It seems that the rise- and falltimes of the pulses do not play a significant role in electropermeabilization. Comparing the results obtained with pulses having 1-ms amplitude duration, but with rise- and falltimes ranging from 2 μs to 100 μs, we found no detectable differences between the efficiencies of these pulses.

CONCLUSIONS

Based on the studies discussed above, some general advice in the design of experiments involving electropermeabilization can be made. Pulse amplitude (voltage-to-distance ratio) should typically be in the range from 200 V/cm up to 2000 V/cm. Pulse durations should be in the range of hundreds of microseconds for smaller molecules, and from several milliseconds up to several tens of milliseconds for macromolecules such as DNA fragments (in the latter case, due to the very long pulse duration, optimal pulse amplitude can even be lower than 100 V/cm). If the equipment allows, bipolar pulses should be used instead of unipolar ones. Bipolar pulses yield a lower permeabilization threshold, higher uptake, and an unaffected viability compared to unipolar pulses of the same amplitude and duration.

These guidelines should provide a starting point for a design of experiments involving electropermeabilization. Still, the optimal values of pulse parameters strongly depend on the cell type used, on the molecule to be introduced, and on specific conditions under which the experiment is performed. Therefore, for best possible results, pulse parameters should be optimized under specific experimental conditions before the actual study is initiated.

The purpose of this paper was to summarize the studies that have investigated the role of pulse parameters in the efficiency of cell membrane electropermeabilization *in vitro*. For completeness, it must be noted that the efficiency of permeabilization also depends on many physical and chemical parameters. An important role is played by the properties of the extracellular medium: its ionic strength [15], osmotic pressure [16,17], and its temperature before and after permeabilization [18]. In addition, for successful electropermeabilization *in vivo*, homogeneity of the distribution of the electric field in the tissue is also important [19]. The references [15-19], as well as other contributions in this book of proceedings should provide some informative material for the interested reader.

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Tadej Kotnik is the author of 12 articles in peer-reviewed journals, one chapter in a book, and he held one invited lecture at an international conference. He received the Galvani Prize of the Bioelectrochemical Society in 2001.

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DNA Electrotransfer for Nonviral Gene Therapy

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Abstract: DNA electrotransfer is based: (i) on the use of electric pulses to permeabilize the target cells, which makes possible the interaction between the DNA and the cell membrane, and therefore the internalization of the DNA into the cells; (ii) on the electrophoretic displacement of DNA (a highly charged molecule) under the influence of the external electric field, which approaches the DNA towards the cell membrane (therefore also favoring DNA interaction with cell membrane and DNA internalization). Because of the importance of the electrophoretic component in DNA uptake, longer pulses (tens of milliseconds) seem to be more effective. However, electropermeabilization must not be excessive (i.e. irreversible) to allow a good cell recovery and efficient expression of the genes of the electrotransferred DNA molecules. Consequently, the best conditions that have been described until now correspond to the use of pulses of long duration (20 ms or more) and moderate electric field intensity (for example 200 – 250 V/cm for the skeletal muscle).

INTRODUCTION

Cell electropermeabilization [1] is an efficient way to transfer DNA to cells in tissues. While in electrochemotherapy the uptake of molecules proceeds mainly by diffusion, electrophoresis plays a very important role in DNA electrotransfer. Because of this, longer pulses (tens of milliseconds) are more effective [2,3]. However, electropermeabilization should be reversible to allow for cell recovery and efficient expression of the genes of the electrotransferred DNA molecules. Consequently, the best conditions that have been described until now correspond to the use of pulses of long duration (20 ms or more) and moderate electric field intensity (e.g., 200 - 250 V/cm for the skeletal muscle) [2-4].

THERAPEUTICAL PERSPECTIVES OF DNA ELECTROTRANSFER FOR GENE THERAPY

While electrochemotherapy has already entered clinical trials, DNA electrotransfer has only been performed nowadays in animals (mice, rats, primates...). Nevertheless, DNA electrotransfer for gene therapy is a field rapidly expanding because it may constitute a real alternative to the need of viruses in the use of DNA for correcting genetic diseases.

Electrotransfer in the skeletal muscle [2-4] is really promising because it had already been shown that muscle is a secretory organ and because injections of naked DNA resulted in the expression of the injected DNA for long periods (months). However, injections alone result in a low and moreover extremely variable level of the foreign gene expression. In spite the fact that injection of naked DNA is a very attractive approach (stability of the molecule, easiness of the procedure), these two restrictions make this approach untranslatable to biomedical applications because the result of DNA injection is not predictable and, usually, not efficient enough. This situation is improved considerably by the concomitant delivery of appropriate electric pulses, which increases gene expression by two to three orders of magnitude and significantly reduces its variability [2].

Consequently, the DNA electrotransfer method has properties that make it appropriate for the correction of genetic diseases, vaccination, cancer treatment, ... In fact, expression of therapeutic genes has already been reported in animals. For example, after the electrotransfer of the gene coding for the erythropoietin, animals have shown elevated hematocrit values for long periods after the gene transfer [5,6].

Many parameters for the achievement of efficient drug and DNA delivery in normal tissues are presently known [7-9]. Thus, due to the problems linked to the use of viruses (whatever the type of virus) for gene therapy, it can be expected that an efficient and easy way to perform non viral gene therapy, like DNA electrotransfer, will led to the rapid development of a number of studies and to the generation of new results in the very near future [10].

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Lluis M. Mir was born in Barcelona, Spain, in 1954. He received a Masters in Biochemistry in 1976 from Ecole Normale Supérieure, Paris, and a Doctorate (D.Sc.) in Cell Biology in 1983. In 1978 he entered CNRS as Attaché de Recherches in the Laboratory of Basic Pharmacology and Toxicology, Toulouse. In 1983 he was promoted to Chargé de Recherches at CNRS, and in 1985 he moved to the Laboratory of Molecular Oncology, Institut Gustave-Roussy. In 1989 he moved to the Laboratory of Molecular Pharmacology, Institut Gustave-Roussy. In 1999, he was promoted to Directeur de Recherches at CNRS.

Lluis M. Mir was one of the pioneers of the research of electropermeabilization (electroporation) and the applications of this technique for antitumor electrochemotherapy and DNA electrotransfer. He is the author of 84 articles in peer-reviewed journals, 8 chapters in books, and over 200 presentations at national and international meetings, invited lectures at international meetings and seminars. He received the Award for the medical applications of electricity of the Institut Electricité Santé in 1994, the Annual Award of Cancerology of the Ligue contre le Cancer (committee Val-de-Marne) in 1996, and the Award of the Research of Rhône-Poulenc-Rorer in 1998.

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A Comparison of Gene Delivery Methods Using Electroporation and Lipofectin-Based Techniques *In Vitro* and *In Vivo*

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Abstract: We have compared electroporation, lipofectin and integrin-targeted lipofectin to deliver the marker gene green fluorescent protein (GFP) to cells *in vitro* and to tumours *in vivo*. *In vitro* experiments in rodent and human tumour cell lines showed a variation in transfection efficiency depending on the method and cell line. Integrin-mediated transfection and electroporation were superior with up to 66% and 57% of cells transfected, respectively. Our *in vivo* data showed that different tumours have different susceptibility to transfection. Electroporation showed a higher efficiency than lipofectin-based methods, with up to 6% of cells transfected in the tumours. Transfection efficiency was very heterogeneous and GFP fluorescence varied both qualitatively and quantitatively between tumour models and treatments. GFP expression in tumours growing in window chambers was observed already 5 h following transfection, reaching maximal value by 2 days and stayed at this level throughout the observation period.

INTRODUCTION

The main stumbling block for the gene therapy of cancer remains the delivery of genetic material to tumours. Non-viral delivery methods are still plagued by poor transfection efficiencies *in vivo*. However, the advantages regarding safety and patient confidence, as well as the possibility of targeted delivery, make non-viral methods attractive for further development. Electric pulses have been used successfully for drug and gene delivery *in vitro*, and recent *in vivo* results are promising [1-3]. Antitumour effects of electrochemotherapy have been demonstrated in clinical studies [4]. Lipofectin-based gene delivery is widely used *in vitro*, and recent *in vivo* results reported around 1% of tumour cells transfected [5]. Integrin-targeted delivery has proven very effective in a range of *in vitro* cell cultures, and recent *in vivo* results demonstrated transfection efficiencies in healthy rat lungs similar to those achievable using adenovirus [6,7].

In this study, we have compared electroporation, lipofectin and integrin-targeted lipofectin, as methods of delivering the marker gene green fluorescent protein (GFP) to cells *in vitro* and to tumours *in vivo*.

MATERIALS AND METHODS

Transfections using pEGFP-N1 were performed in B16 mouse melanoma, P22 rat carcinosarcoma, SaF mouse sarcoma and T24 human bladder carcinoma.

Two different conditions of transfection were tested *in vitro*; either optimised *in vitro* (Fig. 1a) or simulated *in vivo* (Fig. 1b) conditions. The optimised *in vitro* conditions were as follows: 1.5µl lipofectin and 2.0µg DNA (LD), or 1.5µl lipo, 3.4µg peptide [6] and 2.0µg DNA, in 1ml OptiMEM (LPD); per 1-3x10^5 pre-plated cells; transfection 5h/ 37^{o}C; analysed by fluorescent activated cell sorting (FACS) after 24h. *In vitro* simulated *in vivo* conditions: 10µg DNA (D10); 50µg DNA (D50); 7.5µl lipo and 10µg DNA (LD); or 7.5µl lipo, 19µg peptide and 10µg DNA (LPD); in Hanks balanced salt buffer; with or without electroporation (8 electric pulses, electric field intensity 600V/cm, pulse length 5msec, frequency 1Hz; Ep) using two flat parallel stainless steel electrodes with 2mm gap; per 1-2x10^6 cells in suspension; analysed by FACS after 24h.

In the *in vivo* experiments, transfection efficiencies in subcutaneous tumours in mice and window chamber preparations in rats were tested. DNA complexes were injected i.t. with or without subsequent electroporation. Presented in Fig. 2 is the summary of 4-6 tumours per treatment: DNA only at 50µg per tumour (D50); 50µg DNA with Ep1 (8 electric pulses, voltage/electrode distance ratio 600 V/cm, pulse length 5msec, frequency 1 Hz; D50Ep1); 50µg DNA with Ep2 (8 electric pulses, voltage/electrode distance ratio 1300 V/cm, pulse length 0.1msec, frequency 1 Hz; D50Ep2); 14µl lipo and 19µg DNA (LD); 7.5µl lipo, 19µg peptide and 9µg DNA (LPD) in 70µl PBS per tumour; without or with Ep (see Ep1). To visualise GFP fluorescence in solid tumours, frozen sections of the tumours were cut at different depths throughout the tumour. A fluorescence microscope with a narrow band filter (500–510 nm) equipped with an imaging system was used to quantify the transfection efficiency. Intravital microscopy set-up using an inverted Nikon Diaphot 200 fluorescence microscope, with a stage modified in-house for taking rats was used to direct visualization of GFP. Tumour preparations were alternatively viewed under transmitted visible light for measurement of tumour diameter and under fluorescence epi-illumination using a 100W

mercury arc lamp, for visualisation of GFP fluorescence. Prior to transfection two different region of interest (ROI) were selected, one in the centre of the tumour and one in the tumour periphery. At each time point the ROI's were monitored for 15 sec using transmitted or epi-illumination. Observations were recorded in digital format, using Sony DSR-30P digital videocassette recorder, for off-line analysis. Multiple frames were captured onto computer and the images averaged for the analysis of the fluorescence intensity using the Visilog Image Processing package (Noesis, France).

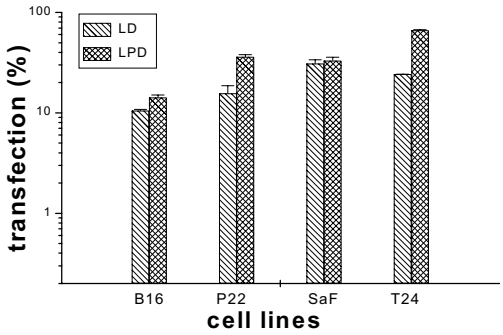

***Figure 1a*:** *In vitro* transfection efficiencies in rodent and human cell lines using lipofectin-based methods; optimised for *in vitro* conditions.

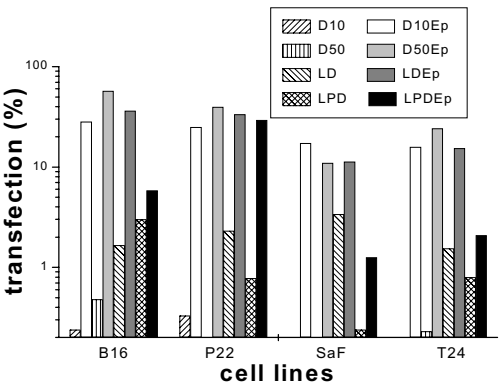

***Figure 1b*:** *In vitro* transfection efficiencies using lipofectin-based methods and/or electroporation using simulated *in vivo* conditions.

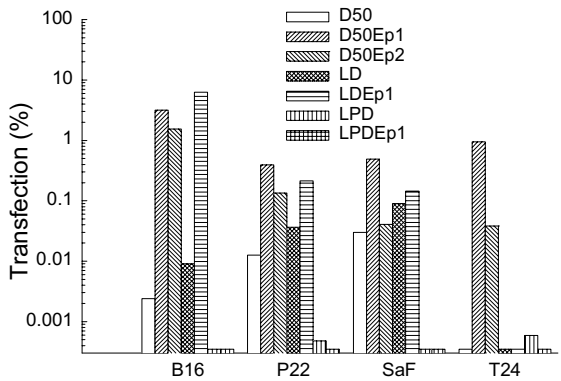

Figure 2: *In vivo* transfection efficiencies in rodent and human xenografts using lipofectin-based methods and/or electroporation.

RESULTS

Transfection efficiencies *in vitro* varied depending on the protocol and the cell line used (Fig. 1a/b). Peptide-mediated transfection under *in vitro* conditions, and electroporation with or without lipofectin under simulated *in vivo* conditions were equally effective. Up to 66% of the cell population expressed GFP 24h following transfection. An MTS proliferation assay showed that electroporation generally reduced survival by 80%, however survival of SaF cells was reduced by 50%. Lipofectin-based methods had no effect on cell survival.

Transfection of solid tumour *in vivo* was far less efficient than under *in vitro* conditions, with at best 6% of cells transfected. Electroporation was superior to lipofectin-based methods, except in the case of B16 tumours (Fig. 2). In window chamber preparations electroporation yield better transfection efficiency compared to lipofectin-DNA method. GFP expression in tumours growing in window chambers was observed already 5 h following transfection, reaching maximal value by 2 days and stayed at this level throughout the observation period.

CONCLUSIONS

These results show that gene transfer into solid tumours *in vivo* is possible, especially using electroporation with or without lipofectin-DNA complexes. Time dependence studies showed early onset of GFP expression with constant levels of expression throughout observation period. With further optimisation these conditions may form the basis for future therapeutic gene delivery.

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Maja Čemažar is the author of 49 articles in peer-reviewed journals, 2 chapters in books, and she held 3 invited lectures at international meetings and seminars. She received the Award for Research from Krka Pharmaceuticals in 1996, and the Young Investigator Award at the 11th International Conference Chemical Modifiers Treatment Series, Tumour Physiology and Cancer Treatment, in 2000.

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Suggestions for further reading

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M.J. Jaroszeski, R. Heller, R. Gilbert (eds.). *Electrochemotherapy, Electrogenetherapy, and Transdermal Drug Delivery* (*Methods in Molecular Medicine, vol. 37*). New Jersey, Humana Press, 2000.

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