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Bases and rationale of the electrochemotherapy

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ABSTRACT

Electrochemotherapy (ECT) is a non-thermal tumour ablation modality, safe and effective on any type of solid tumour. Its use is presently standardized to skin and subcutaneous localisations. ECT is based on the use of non-permeant drugs possessing high intrinsic cytotoxicity (such as bleomycin), or low-permeant drugs with known efficacy (such as cisplatin), which act directly on the cellular DNA. ECT is also based on the achievement of in vivo tumor cell electropermeabilization by means of electric pulses locally delivered to the tumors after bleomycin or cisplatin injection. Cell electropermeabilisation, a physical procedure that affects all tumor cell types, allows these anticancer drugs to enter the cells, thus magnifying their cytotoxicity by orders of magnitude. Efficacy is also sustained by a response of the host immune system, probably due to the type of cell death caused by the ECT. At least for the bleomycin injected intravenously, treatment causes a mitotic cell death that rapidly kills the dividing tumor cells and spares the neighboring non-dividing normal cells, explaining selectivity towards the dividing tumour cells and safety of the procedure. Safety is also due to the vascular effects of the electric pulses: ECT provokes a transient vascular lock which prevents further bleeding, and even stops previous bleeding in the case of hemorrhagic nodules. These bases explain why ECT is safe and very well tolerated by the patients, and why its efficacy is very high on the treated nodules, whatever the tumour histological origin.

1. Introduction

Electrochemotherapy is defined as the local potentiation, by means of permeabilizing electric pulses, of the antitumour activity of a non-permeant (or a low-permeant) anticancer drug possessing a high intrinsic cytotoxicity.

This definition reflects the concept as it was initially developed, based on experiments on cells in culture. It remains still valid that the two key points determining the bases as well as the efficacy of the electrochemotherapy are indeed (i) the electropermeabilisation of the cells and (ii) the use of non-permeant anticancer drug (like the bleomycin) or of low permeant anticancer drug (like the cisplatin). However further studies revealed that the interest of the electrochemotherapy is also based on the one hand, on the type of cell death provoked by the bleomycin, that seems to provoke (iii) the emergence of an antitumour immune response and (iv) a selective killing of the tumour cells, and on the other hand on a physiological reaction of the tissues exposed to the electric pulses that results in (v) a transient vascular lock in the tissues exposed to the electric pulses, followed by antivascular effects resulting from death of the endothelial vascular cells.

These different mechanisms impacting on ECT bases and effectiveness are discussed here below, as well as their potential implications in ECT safety and future developments.

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2. Achievement of in vivo cell electropermeabilization

Of course, this is an essential point in ECT efficacy. To stress its importance, the accompanying paper by Miklavcic is entirely devoted to the electrical parameters of the treatment, including the models of electric field distribution in the tissue, the types of electrodes available, the choice of the applied voltage to entirely cover the tumours with appropriate electric fields, etc. The entire coverage of the treated lesions by electric fields of appropriate amplitude is mandatory to ensure the efficacy and safety of the treatment: this condition is satisfied by the electrodes geometry and the corresponding electrical parameters set by the Cliniporator™, provide the whole nodule(s) is covered in one or more applications of the electrodes.

Here, only the general bases of the effects of the electric pulses used in ECT are reported, to emphasize the point that in ECT, the electric pulses are not intended to kill the cells at all. The electric pulses are just a means to transiently permeabilize the cells and to deliver non-permeant or low-permeant drugs inside the cells. Because of its physical nature, the cell permeabilization obtained by electric pulses application is a very elegant way to vectorize drugs. Indeed, contrary to chemical permeabilization, no residues (other than the cytotoxic drugs, of course) are left into the body after the treatment and the treated volume can be controlled by means of the type of electrodes and of their positioning with respect to the tumour nodules. A sort of "dosimetry" is achievable to limit the volume of treatment.[1,2] Moreover, for the safety of the procedure, on line control of the voltage is also possible, to avoid the delivery of excessive voltages (Cukjati et al., personal communication).

Since the initial experiments in the laboratory on cells in culture[3,4] that were rapidly translated into preclinical[5,6] and clinical studies,[7,8] the following parameters have been always used: eight consecutive square-wave electric pulses of 100 μs duration delivered at a repetition frequency of 1 Hz (one per second). The voltages applied depend essentially on the electrode type and distance between the electrodes, and are automatically set by the pulse generator.

At the level of the entire cell, the consequences of cell exposure to these type of electric pulses are understood. In the presence of the external electric field, a change in the transmembrane potential difference is generated.[9–11] It superimposes upon the resting transmembrane potential difference and it may be calculated from the Maxwell's equations, **provided** a few approximations were assumed. Above a threshold value of the net transmembrane potential, changes will occur in membrane structure, which will render the cell membrane permeable to otherwise non-permeant molecules.

At the level of the tissues, knowledge in cell electropermeabilization in vivo has recently progressed, particularly in the frame of the CLINIPORATOR project (EU 5th FP, project QLK3-1999-00484). Two-dimensional and three-dimensional models of the electric field distribution in various tissues (muscle, liver,...) have been developed and validated.[1,2,12–14]

However, the description of the phenomena occurring at the cell membrane level is still incomplete. One of the most commonly accepted theories, cell electroporation,[9,10,15] relies on the generation of hydrophilic long-lived pores responsible for the transport of molecules across the membrane. But these pores have never been observed in membranes of cells submitted to "effective" electric pulses (except under conditions in which the observed "pores" were **considered as** experimental artefacts). Another theory supposes that there are no holes: the ordered structure of the lipid bilayer would be lost due to the electrocompressive forces created by the increase in the transmembrane voltage. Then water might enter the membrane through the defects created in the membrane structure, and the hydration of the membrane should increase the permeability coefficient of the hydrophilic molecules **such as bleomycin or cisplatin**.[16]

It is noteworthy that among other new results obtained within the CLINIPORATOR project, it has been shown that electropermeabilisation can also be obtained delivering the pulses at a repetition frequency of 5000 Hz (the eight pulses, and the intervals between them, are delivered in just 1.5 ms).[17,18] This possibility, already offered in the Cliniporator™ device, shortens the treatment duration and makes ECT more comfortable (even though eight pulses are still delivered, there is only one disagreeable sensation that may cause pain, and only one muscular contraction if electrode tips are close to muscles beneath the tumour). The clinical trial reported in this supplemental issue by Marty, Sersa et al. reveals identical efficacy compared to pulse delivery at 1 Hz that resulted in eight sensations and eight muscle contractions.

Finally, as reported in the SOP, ECT can be applied using either invasive electrodes (e.g. needles) or surface electrodes (metallic plates). In the latter case (use of transcutaneous pulses), pulse duration is in fact sufficient to disrupt the complex electrical barrier constituted by the *stratum corneum* and then to permeabilize the subcutaneous tumours. Whatever the electrode used (following the procedures reported in the SOP), there is a simple rule to ensure ECT efficacy: the whole of the tumour must be covered by the electric fields and therefore delivery of the electric pulses must be repeated if necessary to avoid the possibility to leave part of the tumour untreated. Moreover, at each place where the electrodes are inserted, enough current must be delivered: significantly ($p = 0.01$) higher objective response rate (100%) was achieved for hexagonal needle electrodes when electric current exceeded the value of 1.5 A, compared to 73.3% OR rate with currents below 1.5 A (Marty M, Sersa G, et al., this issue).

3. The anticancer drugs suitable for electrochemotherapy

3.1. *Non-permeant drugs possessing high intrinsic cytotoxicity: bleomycin*

3.1.1. *The origin of the electrochemotherapy*

First of all, in 1986, using a square-wave electric pulse generator we determined electropermeabilization conditions under which a vast majority of the cells exposed to the pulses was simultaneously permeabilized and alive.[3] A new cell biology and pharmacology was then possible, using non-permeant

molecules that could enter the transiently permeabilized cells and act on intracellular targets.[10] Combining bleomycin and cell electropermeabilization we found in vitro an increase of the toxicity of this anticancer drug by hundreds of thousands times.[4,19] Trying to understand the reasons of this incredible synergy, we revisited the pharmacology of the bleomycin.[20] We showed that bleomycin, a currently used anticancer drug, was in fact a non-permeant molecule (a non-permeant drug is a molecule that cannot diffuse through the plasma **membrane because** of its size and physico-chemical properties) and that is not actively transported across the plasma membrane. We described the actual mechanism of internalisation of the bleomycin in the cells not exposed to the electric pulses, which needs the binding of the bleomycin to a membrane protein. This protein carries the bleomycin inside the cells through the endocytotic pathway. Internalisation is limited by the number of carrier proteins exposed at the cell surface and by the speed of proteins withdraw from the membrane, that is linked to the endocytosis rate of the cell.[21–23] This mechanism of uptake actually limits the bleomycin toxicity.

On the contrary, after cell electropermeabilisation, the molecules of bleomycin diffuse almost freely inside the cell. We demonstrated that bleomycin molecules enter the cell and cut the DNA already in 30 s[24,25] even though non-permeant molecules may enter the cells by diffusion during all the lifetime of the permeabilized regions, for example during several minutes, in vitro for cells incubated at 25° or in vivo for muscle fibres, after exposures to eight reversibly permeabilizing pulses of 100 μs.[12] Interestingly, diffusion may thus occur for periods of time which are orders of magnitude greater than the pulse duration.

Moreover we also revealed the huge intrinsic cytotoxicity of this molecule: **several hundred molecules** of bleomycin inside the cell are sufficient to kill the cell.[19] Bleomycin causes single- and double-strand DNA breaks, but we could show that the double-strand DNA breaks are intrinsically 300 times more toxic than the single-strand breaks.[26] Cells are actually killed because some of the double-strand breaks remain unrepaired: cytotoxicty is revealed when cells try to divide because their chromosomes are fragmented, while quiescent non-dividing cells remain alive (metabolically stable) because the probability that these DNA breaks affect the expression of an essential housekeeping gene is almost null. This mitotic cell death process results in a selective killing of the dividing (tumour) cells that actually spares the non-dividing (normal) cells around the treated tumour. An interesting difference in the biological response in mice between an actively growing tissue (tumours) and a normal tissue (liver) has actually been observed (H. Mekid and L. M. Mir, unpublished results). Thus differential effects are obtained between tumour and normal tissues.

3.1.2. *Administration routes for the bleomycin*

Two different ways to **administer** bleomycin have been explored in preclinical and clinical trials, namely the systemic or the local delivery. The latter (intratumoural injection of the bleomycin)[27–29] was developed after the preclinical work of Heller and colleagues.[30,31] The former corresponds essentially to the intravenous route,[7,8,32–34] but, in patients, at least two cases of intra-arterial delivery have also been reported,[32] and the intraperitoneal route has also been tested in preclinical studies.[35]

The amount of bleomycin required for efficient antitumour effects in the presence of the electric pulses delivery are low enough as to be injected systemically without the need to take safety procedures. The only precaution may be the administration of an anti-histaminic drug to prevent the light febrile allergic reactions that can occur 2 h after the bolus administration of the bleomycin. The advantage of the systemic injection lies in the possibility to treat several nodules at a time, or large nodules, within the therapeutic window determined in humans (from 8 to 28 min[32] after the intravenous bolus injection of 15 000 IU/m^2, that is 15 mg/m^2). It is important to recall that the growing parts of the tumours are well vascularised. Therefore, they will be well perfused by the drug, ensuring electrochemotherapy efficacy. Nevertheless, it is also important to note that bleomycin must be administered in a bolus and not in a long perfusion, because it is necessary to reach a minimal concentration in the tissue.

Obviously, the intratumoural injection of bleomycin requires, in principle, less bleomycin. The amounts of bleomycin recommended in the SOP depend on the tumour volume. Therefore, total injected amount depends on nodule volume, and on the number of nodules to be treated. It is obvious that, for the treatment of large tumour masses or of a large number of nodules, the advantage of a reduction in the dose, with respect to the systemic injection, may be lost. On the contrary, the need to inject each nodule separately makes the procedure longer, and also less secure because it is very difficult to reliably perfuse the whole tumour mass. Moreover, in some cases (e.g. pancreatic carcinomas), direct injection into the tumour mass is almost impossible.

3.2. *Low-permeant drugs with known efficacy: cisplatin*

The studies that were performed by Sersa and colleagues are very interesting because they demonstrated that electrochemotherapy can also be performed using cisplatin instead of bleomycin at the preclinical[36,37] and clinical stages.[38,39] The increase in cisplatin efficacy due to cell electropermeabilization is much lower than that of bleomycin.[40,41] However **cisplatin delivered as a single agent is active on many** tumour types, while bleomycin alone is a quite inefficient drug in the absence of the electric pulses. Thus any increase in cisplatin efficacy is welcome since it directly results in an increase in the antitumour effects. It was shown that the increase in efficacy was actually due to the increase in cisplatin uptake and in adducts generation on the DNA.[37]

In the treatment of cutaneous metastases in humans, cisplatin can be used alone, by means of intratumoural injections, with some antitumour activity. This administration route has also been used in electrochemotherapy, with interesting results. A clinical study showed an increase of the objective response rate of human in transit melanoma nodules from 19% with cisplatin alone to 82% with cisplatin and electric pulses.[39] The use of cisplatin is an interesting alternative to the use of bleomycin, in particular to reduce the cumulative dose of bleomycin in the case of repeated treatments:

bleomycin (delivered intravenously) can then be reserved to sessions devoted to the treatment of patients with a high number of nodules or with large nodules (as recommended in the SOP), while cisplatin could be given **for the treatment of a few small nodules**.

Because of its limited increase in efficacy,[38] electrochemotherapy with intravenous cisplatin did not seem interesting. Thus the use of cisplatin in electrochemotherapy has been restricted to the intratumoural route of administration, as clearly specified in the SOP.

3.3. *Other drugs*

It is important to note that, in spite of several attempts to find more appropriate cytotoxic molecules, bleomycin and cisplatin are still the most adequate candidates for the combined use with electric pulses. In vitro, several studies looked for potential increases in the toxicity of anticancer drugs due to cell electropermeabilization. All showed a several hundreds to thousands fold increase in bleomycin toxicity and, in vitro a several tens fold increase in that of cisplatin.[4,40,41] None of the other drugs of the usual armamentarium of the chemotherapy showed an increase in its toxicity, probably **because these drugs easily enter cells by diffusion** through the plasma membrane or by means of transporters at the cell membrane (like the 5 FU or the methotrexate). Of course, cell permeabilisation is not of interest for molecules acting on the cell surface and that does not need to enter the cells.

However, electrochemotherapy might find in the future an extension through the use of very active short sequences of nucleic acids (either oligonucleotides or SiRNAs) directed to specific DNA sequences responsible for the disease (not only cancer). Another possibility, of course, would be to reconsider molecules that did not show high efficacy in vivo, and thus did not reach the clinical level, for **use in association with electric pulses**.

4. Selectivity towards the dividing tumour cells

The normal tissues located just around the tumour nodule are often already infiltrated by tumour cells that can originate disease relapse after an unpredictable period of time. Because of the aggressiveness of classical tumour treatments, margins are not extensively treated. In the case of the intravenous delivery of the bleomycin, the amounts of bleomycin in the interstitial fluids of the tumour, and thus the amount of bleomycin entering the cells, provoke the mitotic cell death process described above. This situation opens the possibility of a safe treatment of large margins around the treated nodules, an approach already performed in humans, particularly in the case of chest wall carcinoma recurrences. Interestingly, necrosis is restricted to the tumour tissue and few crusts are provoked, particularly in the case of subcutaneous nodules, confirming that in humans as well, differential effects are obtained between tumour and normal tissues.

In the case of the intratumour injection of the cytotoxic drugs, the high local drug concentration, associated to the potentiation of the uptake and of the activity consecutive to the electric pulse delivery results in a marked necrosis of the cells within the volume of tissue exposed to the electric pulses. Cisplatin is known to produce the apoptosis of the treated cells and bleomycin, entering the cells in high amounts is a pseudoapoptotic drug: all the morphological and biochemical characteristics of the apoptosis are observed because the bleomycin itself plays the role of the endonucleases activated during the apoptotic process.[24,25] Under these conditions, the selective sparing of the non-dividing cells (a consequence of the mitotic cell death process) is lost and more necrosis and crusts are provoked.

5. Participation of the host immune response

Very soon after the initial preclinical trials on electrochemotherapy,[5,6] it was shown that the host immune response was participating in the achievement of the cures after electrochemotherapy.[42] At that time, immunotherapy was essentially based on the administration of biological response modifiers like cytokines or lymphokines. Low doses of interleukin-2 were injected in order to enhance mice immune responses. Several other preclinical trials have shown that the combination of electrochemotherapy with immunotherapy results (i) in the enhancement of the local effects and (ii) in the achievement of systemic antitumour effects in different experimental models (for example, a metastazing murine tumour model,[43] a non-metastazing murine tumour model,[44] a carcinoma model transplanted in the liver of rabbits).[45] Therefore this combination is an attracting possibility that requires further experimental and clinical developments. Other immunostimulatory approaches can be considered: for example the transfer of the IL-12 gene in combination with bleomycin delivery has already been tested in mice,[46] and the same gene, in combination with electrochemotherapy with cisplatin, is being tested in horses (Dr. J. Teissié, personal communication). Other combinations with chemokines have been already explored like the association of electrochemotherapy and TNFα administration.[47]

6. Vascular effects of the electric pulses and electrochemotherapy

Blood flow changes occur after the delivery of electric pulses in vivo.[45,48,49] In the case of normal tissues, these effects appear as a transient hypoperfusion of not only the area defined by the electrodes but also distally thereof.[49] It is a physiological reaction the duration of which, 1–2 min in the case of the skeletal muscle, is **independent of the electric pulses parameters. The mechanism behind this has been** proposed to be a reflexory vasoconstriction of afferent arterioles mediated by the sympathetic nervous system. The effect is most pronounced when high voltage pulses (leading to irreversible electroporation) are used.[49] In the case of the electrochemotherapy, this is used advantageously. The vascular effect implies that just at the time when the cell is permeabilised, the drug is withheld within the electroporated area, by the so-called "vascular lock". Interestingly, it has been shown that in tumours vascular lock is much more longer than in the normal tissues. Restoration of the initial blood flow levels may take hours. These modifications in blood flow could be

particularly advantageous for intratumoural drug injection, as this would decrease drug washout.[49] There is also an impact on the systemic route for bleomycin administration since, due to this vascular lock, bleomycin injection must precede the beginning of the electric pulses delivery even if cells remain permeabilized for a time sufficient to allow, in principle, a protocol following the reverse order, pulses first and injection second.

These short-term vascular effects caused by the electric pulses are amplified in the case of the antitumour electrochemotherapy by the fact that the tumour endothelial cells are also dividing cells that can be killed by the combination of the drugs and the electric pulses. A particular sensitivity of some endothelial cells strains has even been shown in vitro.[50] The mid-term and long-term antivascular effects of the electrochemotherapy (as well as its permanent antihaemorrhagic effects, see below) could thus result from the killing of the tumour endothelial cells that would prevent the rapid reorganization of the tumour vasculature. As a consequence, an almost permanent, extremely hypoxic situation is created after nodule treatment by electrochemotherapy, which can also contribute to the highly efficient antitumour effects observed.

Vascular lock has another practical consequence. The treatment of heamorraging nodules by electrochemotherapy has shown an immediate and lasting interruption of tumour bleeding.[33,51] Thus treatment of haemorrhagic and painful nodules appears as a very interesting indication of the electrochemotherapy.

7. Conclusion

In summary, the bases of the electrochemotherapy and the reasons to use bleomycin and cisplatin in association with specific electric pulse are fully known. The physico-chemical characteristics of these two drugs explain why they can be used in association with permeabilizing electric pulses, while their biological effects explain the efficacy of the electrochemotherapy. ECT is a very general treatment for tumours of any origin. Indeed all living cells are permeabilized by the electric pulses, and both bleomycin and cisplatin interact directly with the DNA molecules, whatever the set of (onco- or antionco-)genes expressed by the tumour cells. Treatment is efficient and safe, particularly if the recommended SOP are followed. Drug dosages, timing for electric pulses delivery and the other treatment details are now well established.

A convenient coverage of the tumour nodules by appropriate non-deleterious electric fields is mandatory. It must be added that too much intense electric pulses can irreversibly damage the tissues. This is not at all desired in electrochemotherapy but could be used as a sort of electroablative treatment[52,53] comparable to ablathermia (heating of tumours at 90° by means of radiofrequency electromagnetic fields) or its opposite cryosurgery. However, none of these physical-only treatments may produce the exquisite selective killing of the tumours cells observed with the physico-chemical electrochemotherapy, particularly using intravenously delivered bleomycin.

Electrochemotherapy is indeed a local treatment but in many cases, efficient local control of tumour growth is important in cancer treatment. Moreover most of cancer cures are still achieved by local treatments (surgery and/or radiotherapy). As already shown at the clinical stage, electrochemotherapy could be used as an adjuvant cytoreductive treatment facilitating ulterior (and non-devastating) surgery,[54] and potential combination with other approaches, like radiotherapy[55] or boron neutron capture,[56,57] have also been foreseen. Finally we also showed here that the bases exist for a future combination of electrochemotherapy with immunotherapy (whether through the administration of immunomodulatory agents or the combination with the (electro)transfer of genes coding for immunoregulatory proteins) that could potentially result in a safe and systemic cancer treatment devoid of severe side effects. The indications of the electrochemotherapy will thus extend in the future.

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REFERENCES

1. Miklavcic D, Beravs K, Semrov D, Cemazar M, Demsar F, Sersa G. The importance of electric field distribution for effective in vivo electroporation of tissues. *Biophys J* 1998;**74**:2152–8.
2. Miklavcic D, Semrov D, Mekid H, Mir LM. A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. *Biochim Biophys Acta* 2000;**1523**:73–83.
3. Mir LM, Banoun H, Paoletti C. Introduction of definite amounts of nonpermeant molecules into living cells after electropermeabilization: direct access to the cytosol. *Exp Cell Res* 1988;**175**:15–25.
4. Orlowski S, Belehradek Jr J, Paoletti C, Mir LM. Transient electropermeabilization of cells in culture: increase of the cytotoxicity of anticancer drugs. *Biochem Pharmacol* 1988;**37**:4727–33.
5. Mir LM, Orlowski S, Belehradek Jr J, Paoletti C. Electrochemotherapy: potentiation of antitumour effect of bleomycin by local electric pulses. *Eur J Cancer* 1991;**27**:68–72.
6. Belehradek Jr J, Orlowski S, Poddevin B, Paoletti C, Mir LM. Electrochemotherapy of spontaneous mammary tumours in mice. *Eur J Cancer* 1991;**27**:73–6.
7. Mir LM, Belehradek M, Domenge C, et al. Electrochemotherapy, a new antitumor treatment: first clinical trial. *C R Acad Sci III* 1991;**313**:613–8.
8. Belehradek M, Domenge C, Luboinski B, Orlowski S, Belehradek Jr J, Mir LM. Electrochemotherapy, a new antitumor treatment: first clinical phase I-II trial. *Cancer* 1993;**72**:3694–700.
9. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J* 1982;**1**:841–5.
10. Orlowski S, Mir LM. Cell electropermeabilization: a new tool for biochemical and pharmacological studies. *Biochim Biophys Acta* 1993;**1154**:51–63.
11. Pucihar G, Kotnik T, Valic B, Miklavcic D. Numerical determination of transmembrane voltage induced on irregularly shaped cells. *Ann Biomed Eng* 2006;**34**:642–52.
12. Gehl J, Sorensen TH, Nielsen K, Raskmark P, Nielsen SL, Skovsgaard T, et al. In vivo electroporation of skeletal muscle:

threshold, efficacy and relation to electric field distribution. *Biochim Biophys Acta* 1999;**1428**:233–40.
13. Sersa G, Cemazar M, Semrov D, Miklavcic D. Changing electrode orientation improves the efficacy of electrochemotherapy of solid tumors in mice. *Bioelectrochem Bioenerg* 1996;**35**:61–6.
14. Belehradek Jr J, Orlowski S, Ramirez LH, Pron G, Poddevin B, Mir LM. Electropermeabilization of cells in tissues assessed by the qualitative and quantitative electroloading of bleomycin. *Biochim Biophys Acta* 1994;**1190**:155–63.
15. Weaver J, Chizmadzhev YA. Theory of electroporation: a review. *Bioelectrochem Bioenerg* 1996;**41**:135–60.
16. Teissié J, Golzio M, Rols MP. Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge. *Biochim Biophys Acta* 2005;**1724**:270–80.
17. Pucihar G, Mir LM, Miklavcic D. The effect of pulse repetition frequency on the uptake into electropermeabilized cells in vitro with possible applications in electrochemotherapy. *Bioelectrochem* 2002;**57**:167–72.
18. Miklavcic D, Pucihar G, Pavlovec M, Ribaric S, Mali M, Macek-Lebar A, et al. The effect of high frequency electric pulses on muscle contractions and antitumor efficiency in vivo for a potential use in clinical electrochemotherapy. *Bioelectrochemistry* 2005;**65**:121–8.
19. Poddevin B, Orlowski S, Belehradek Jr J, Mir LM. Very high cytotoxicity of bleomycin introduced into the cytosol of cells in culture. *Biochem Pharmacol* 1991;**42**(S):67–75.
20. Mir LM, Tounekti O, Orlowski S. Bleomycin: revival of an old drug. *Gen Pharmacol* 1996;**27**:745–8.
21. Pron G, Belehradek Jr J, Mir LM. Identification of a plasma membrane protein that specifically binds bleomycin. *Biochem Biophys Res Commun* 1993;**194**:333–7.
22. Pron G, Belehradek Jr J, Orlowski S, Mir LM. Involvement of the membrane bleomycin-binding sites in the bleomycin cytotoxicity. *Biochem Pharmacol* 1994;**48**:301–10.
23. Pron G, Mahrour N, Orlowski S, Tounekti O, Poddevin B, Belehradek Jr J, et al. Internalisation of the bleomycin molecules responsible for bleomycin toxicity: a receptor-mediated endocytosis mechanism. *Biochem Pharmacol* 1999;**57**:45–56.
24. Tounekti O, Pron G, Belehradek Jr J, Mir LM. Bleomycin, an apoptosis-mimetic drug that induces two types of cell death depending on the number of molecules internalized. *Cancer Res* 1993;**53**:5462–9.
25. Tounekti O, Belehradek Jr J, Mir LM. Relationships between DNA fragmentation, chromatin condensation and changes in flow cytometry profiles detected during apoptosis. *Exp Cell Res* 1995;**217**:506–16.
26. Tounekti O, Kenani A, Foray N, Orlowski S, Mir LM. The ratio of single- to double-strand DNA breaks and their absolute values determine cell death pathway. *Br J Cancer* 2001;**84**:1272–9.
27. Heller R, Jaroszeski MJ, et al. Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. *Cancer* 1998;**83**:148–57.
28. Panje WR, Hier MP, Garman GR, Harrell E, Goldman A, Bloch I. Electroporation therapy of head and neck cancer. *Ann Otol Rhinol Laryngol* 1998;**107**:779–85.
29. Glass LF, Jaroszeski M, Gilbert R, Reintgen DS, Heller R. Intralesional bleomycin-mediated electrochemotherapy in 20 patients with basal cell carcinoma. *J Am Acad Dermatol* 1997;**37**:596–9.
30. Heller R, Jaroszeski MJ, Perrott R, Messina J, Gilbert R. Effective treatment of B16 melanoma by direct delivery of bleomycin using electrochemotherapy. *Melanoma Res* 1997;**7**:10–8.
31. Hyacinthe M, Jaroszeski MJ, Dang VV, et al. Electrically enhanced drug delivery for the treatment of soft tissue sarcoma. *Cancer* 1999;**85**:409–17.
32. Domenge C, Orlowski S, Luboinski B, et al. Antitumor electrochemotherapy: new advances in the clinical protocols. *Cancer* 1996;**77**:956–63.
33. Kubota Y, Mir LM, Nakada T, Sasagawa I, Suzuki H, Aoyama N. Successful treatment of metastatic skin lesions from bladder cancer by electrochemotherapy. *J Urology* 1998;**160**:1426.
34. Kubota Y, Tomita Y, Tsukigi M, Kurachi H, Motoyama T, Mir LM. A case of perineal malignant melanoma successfully treated with electrochemotherapy. *Melanoma Res* 2005;**15**:133–4.
35. Tada T, Matsumoto K, Suzuki H. Electrochemotherapy significantly inhibits the growth of colon 26 tumors in mice. *Surg Today* 1997;**27**:506–10.
36. Sersa G, Cemazar M, Miklavcic D. Antitumor effectiveness of electrochemotherapy with *cis*-diamminedichloroplatinum(II) in mice. *Cancer Res* 1995;**55**:3450–5.
37. Cemazar M, Milacic R, Miklavcic D, Dolzan V, Sersa G. Intratumoral cisplatin administration in electrochemotherapy: antitumor effectiveness, sequence dependence and platinum content. *Anti-Cancer Drugs* 1998;**9**:525–30.
38. Sersa G, Stabuc B, Cemazar M, Miklavcic D, Rudolf Z. Electrochemotherapy with cisplatin: the systemic antitumour effectiveness of cisplatin can be potentiated locally by the application of electric pulses in the treatment of malignant melanoma skin metastases. *Melanoma Res* 2000;**10**:381–5.
39. Sersa G, Stabuc B, Cemazar M, Miklavcic D, Rudolf Z. Electrochemotherapy with cisplatin: clinical experience in malignant melanoma patients. *Clin Cancer Res* 2000;**6**: 863–7.
40. Gehl J, Skovsgaard T, Mir LM. Enhancement of cytotoxicity by electropermeabilization: an improved method for screening drugs. *Anti-Cancer Drugs* 1998;**9**:319–25.
41. Jaroszeski MJ, Dang V, Pottinger C, Hickey J, Gilbert R, Heller R. Toxicity of anticancer agents mediated by electroporation in vitro. *Anticancer Drugs* 2000;**11**:201–8.
42. Mir LM, Orlowski S, Poddevin B, Belehradek Jr J. Electrochemotherapy tumor treatment is improved by interleukin-2 stimulation of host's defenses. *Eur Cytokine Netw* 1992;**3**:331–4.
43. Orlowski S, An DJ, Belehradek Jr J, Mir LM. Antimetastatic effects of electrochemotherapy and of histoincompatible interleukin-2 secreting cells in the murine Lewis lung tumor. *Anti-Cancer Drugs* 1998;**9**:551–6.
44. Mir LM, Roth C, Orlowski S, et al. Systemic antitumor effects of electrochemotherapy combined with histoincompatible cells secreting interleukin-2. *J Immunother* 1995;**17**:30–8.
45. Ramirez LH, Orlowski S, An DJ, et al. Electrochemotherapy on liver tumors in rabbits. *Br J Cancer* 1998;**77**:2104–11.
46. Heller LC, Pottinger C, Jaroszeski MJ, Gilbert R, Heller R. In vivo electroporation of plasmids encoding GM-CSF or interleukin-2 into existing B16 melanomas combined with electrochemotherapy induces long-term antitumour immunity. *Melanoma Res* 2000;**10**:577–83.
47. Sersa G, Cemazar M, Menart V, Gaberc-Porekar V, Miklavcic D. Anti-tumor effectiveness of electrochemotherapy with bleomycin is increased by TNF-α on SA-1 tumors in mice. *Cancer Lett* 1997;**116**:85–92.
48. Sersa G, Cemazar M, Parkins CS, Chaplin DJ. Tumor blood flow changes induced by application of electric pulses. *Eur J Cancer* 1999;**35**:672–7.
49. Gehl J, Skovsgaard T, Mir LM. Vascular reactions to in vivo electroporation: characterization and consequences for drug and gene delivery. *Biochim Biophys Acta* 2002;**1569**:51–8.
50. Cemazar M, Parkins CS, Holder AL, Chaplin DJ, Tozer GM, Sersa G. Electroporation of human microvascular endothelial cells: evidence for an anti-vascular mechanism of electrochemotherapy. *Br J Cancer* 2001;**84**:565–70.

51. Gehl J, Geertsen PF. Efficient palliation of hemorrhaging malignant melanoma skin metastases by electrochemotherapy. *Melanoma Res* 2000;**10**:585–9.
52. Davalos RV, Mir IL, Rubinsky B. Tissue ablation with irreversible electroporation. *Ann Biomed Eng* 2005;**33**:223–31.
53. Edd JF, Horowitz L, Davalos RV, Mir LM, Rubinsky B. In vivo results of a new focal ablation technique: irreversible electroporation. *IEEE Trans Biomed Eng* 2006;**53**:1409–15.
54. Snoj M, Rudolf Z, Cemazar M, Jancar B, Sersa G. Successful sphincter-saving treatment of anorectal malignant melanoma with electrochemotherapy, local excision and adjuvant brachytherapy. *Anticancer Drugs* 2005;**16**:345–8.
55. Sersa G, Kranjc S, Cemazar M. Improvement of combined modality therapy with cisplatin and radiation using electroporation of tumors. *Int J Radiat Oncol Biol Phys* 2000;**46**:1037–41.
56. Ceberg CP, Brun A, Mir LM, Persson BR, Salford LG. Enhanced boron uptake in RG 2 rat gliomas by electropermeabilization in vivo – a new possibility in boron neutron capture therapy. *Anticancer Drugs* 1994;**5**:463–6.
57. Cemazar M, Skrk J, Mitrovic B, Sersa G. Changed delivery of boron to tumors using electroporation for boron neutron capture therapy with BSH. *Br J Radiol* 2000;**73**:195–200.