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Bioelectrochemistry 57 (2002) 167–172

Bioelectrochemistry

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The effect of pulse repetition frequency on the uptake into electropermeabilized cells in vitro with possible applications in electrochemotherapy

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Received 25 October 2001; received in revised form 24 May 2002; accepted 4 June 2002

Abstract

Electrochemotherapy is a technique where electric pulses in combination with chemotherapeutic agents are applied to tumor cells. In general, patients find electrochemotherapy tolerable, in spite of unpleasant sensations associated with contraction of muscles located beneath or in the vicinity of the electrodes. These contractions are due to the intensity of the electric pulses required for effective electropermeabilization of tumor cell membranes. Since a train of eight electric pulses with repetition frequency of 1 Hz is usually applied to the tumors, each pulse in the train excites underlying nerves and provokes muscle contractions. Therefore, for patients involved in electrochemotherapy, the use of pulses with repetition frequency higher than the frequency of tetanic contraction would represent reduced number of muscle contractions and associated unpleasant sensations. Our results of the uptake of Lucifer Yellow into electropermeabilized cells in vitro show that with increased repetition frequency the uptake stays at similar levels even at frequencies up to 8.3 kHz. On the basis of these results the possibilities for the clinical use of pulses with high repetition frequency in electrochemotherapy are considered.

Keywords: Electrochemotherapy; Pulse repetition frequency; Lucifer yellow; Action potential; Muscle contraction

1. Introduction

Electropermeabilization is a process where a transient, high-permeability state of cell membrane occurs due to cell exposure to high voltage, short-duration electric pulses. Increased membrane permeability allows ions, small molecules and drugs to cross cell membranes, otherwise impermeable for them [1]. In experiments involving electropermeabilization, a single pulse or a train of rectangular or exponentially decaying pulses is most often used. In case of a train, the repetition frequency of the pulses is usually 1 Hz, which is the frequency probably chosen because of the limitations of commercially available electropulsators. To investigate the effect of higher pulse repetition frequencies on electropermeabilization, some authors have used pulse generators to obtain continuous waveforms of various shapes with frequencies from several hundred Hz to 1 MHz [2–6]. Because of the continuity of these waveforms, change in the frequency always resulted in corresponding change of the duration of the pulses.

In contrast, only few authors have attempted to vary the frequency of the pulses by adjusting only the delay between two consecutive pulses in a train, thus keeping the duration and number of pulses constant. One of these studies was performed by Vernhes and co-workers [7], where the effect of the repetition frequency (ranging from 0.5 to 100 Hz) on cell viability and permeabilization was investigated. This study has shown high permeabilization and survival of the cells can be obtained even at 100 Hz. We decided to investigate the effect of different pulse frequencies on the uptake of exogenous molecules at even higher frequencies. For this purpose, at the University of Ljubljana, we developed an electropulsator, which permits to vary the repetition frequency of the pulses keeping the number and duration of pulses constant [8].

The purpose of our study was to examine the possibilities of using pulses with repetition frequencies higher than 1 Hz in electrochemotherapy. Electrochemotherapy is a technique

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1567-5394/02/$ - see front matter
PII: S1567-5394(02)00116-0

where electric pulses in combination with chemotherapeutic agents (e.g., bleomycin, cisplatin [9–13]) are applied to tumor cells. This technique was found to be effective in treatment of cutaneous and subcutaneous tumors in patients [14–17]. In the most comprehensive study [14], the results of five different research groups (France, USA, and Slovenia) were summarized and patient responses evaluated. Objective responses (absence of any trace of tumor, complete response, or more than 50% reduction in tumor volume, partial response, for at least 30 days after the treatment) were obtained in 85.3% of the 273 evaluable tumors. Local clinical complete responses were obtained in 56.4% (154) tumors, and partial responses in 28.9% (79) tumors. A high rate of local objective responses was obtained regardless of the histological type of the treated tumors. In general, the patients found electrochemotherapy tolerable, although some complained about unpleasant sensations associated with the delivery of electric pulses. Because of the relatively low repetition frequency of the pulses used (1 Hz), each individual pulse in the train of pulses (usually 4, 6 or 8 pulses) provokes muscle contraction. Since such individual muscle contractions are disagreeable, any reduction of these sensations would be an improvement for the patients. The use of pulses with repetition frequency exceeding the frequency of tetanic contraction (where successive muscle contractions fuse into smooth motion) would already represent an improvement in sense of reducing the pain associated with electrochemotherapy. To study the possibilities for the use of higher pulse repetition frequencies in experiments in vivo, the first step is to investigate these conditions in vitro. Because electrochemotherapy is based on enhanced uptake of chemotherapeutic agents (e.g., bleomycin, 1500 g/mol) into tumor cells, we examined the effect of repetition frequency of electric pulses on the uptake of Lucifer Yellow, a small nonpermeant hydrophilic molecule (like the bleomycin, but not toxic) into electropermeabilized cells in vitro.

2. Materials and methods

2.1. Cells

DC3F cells—spontaneously transformed Chinese hamster fibroblasts [18]—were grown in Eagle's Minimum Essential Medium (EMEM) with added 10% Fetal bovine serum (both from Life Technologies, USA). After trypsination, cells were centrifuged for 5 min at 1000 rpm at 4 °C and resuspended in Spinner's minimum essential medium (SMEM, Life Technologies, USA) to obtain 2×10^7 cells/ml.

To determine the uptake of molecules into the permeabilized cells, the nonpermeant fluorescent dye Lucifer Yellow (MW 522 g/mol, Sigma, USA) was added to the cell suspension before electropermeabilization in quantity that led to 1 mM concentration of Lucifer Yellow in the cell suspension. Because Lucifer Yellow can enter the permeabilized cell and stay inside the cell after pores reseal, the quantity of Lucifer Yellow taken up by the cell (the uptake) can be determined by measuring the fluorescence. As Lucifer Yellow is not cytotoxic its presence should not affect cell viability.

Cells were kept at 4 °C until electropermeabilization.

2.2. Electropermeabilization

A 50-μl (± 0.8%) droplet of the cells suspended in SMEM (~ 10^6 cells) was taken and placed between two parallel plate stainless steel electrodes 2 mm (±2.5%) apart. The entrapped droplet was cylindrically shaped with concave sides. In comparison with the cross-section area of the ideal cylinder, the area at the electrode/medium contact was ~ 2% larger, while at the narrowest point of the droplet (in the middle between the electrodes), the actual cross-section area was ~ 15% smaller. In the first experiment, eight rectangular pulses with each 100-μs duration, with amplitudes from 80 to 400 V, and repetition frequencies of 1, 10, 1000, and 2500 Hz were delivered. With our electropulsator, 2500 Hz was the highest frequency that could be generated for 100-μs pulses [8]. To obtain higher pulse repetition frequencies, shorter pulses were used. This was a consequence of the limitation of the electropulsator, where the delay between two consecutive pulses is limited by the duration of the pulse (the shortest delay is three times longer than the duration of the pulse). Therefore, in the second experiment, twenty-six 30-μs rectangular pulses were delivered, with amplitudes from 80 to 400 V and repetition frequencies of 1 Hz and 8.3 kHz. Twenty-six pulses were used in order to have the same cumulative length of the pulses, thus the product $N \times T$ of the number N and duration T of the pulses was constant in both experiments. After 10 min of incubation at room temperature, 950 μl of SMEM was added to prevent drying. After additional 30 min, cells were diluted in 5 ml of phosphate buffer saline (Life Technologies, USA) in order to remove extracellular Lucifer Yellow, and centrifuged at 1000 rpm. The washing procedure was repeated twice, which proved sufficient in preliminary experiments. Cells were then broken down by ultrasonication (Sonifier 250, Branson Ultrasonics, USA) and fluorescence was measured on a spectrofluorometer (SFM 25, BioTek, USA). Excitation was set at 418-nm wavelength and emission was detected at 525 nm. The remnants of the extracellular Lucifer Yellow together with a small amount of Lucifer Yellow taken up by cells with endocytosis represented the background fluorescence, which was measured at 0 V (no pulses). The fluorescence of cell fragments and phosphate buffer saline was considerably lower than the background fluorescence and was thus not taken into consideration.

2.3. Data processing

All experiments were repeated at least three times on different days. Due to scattering of the measured fluorescence, the results were normalized to the value of the uptake

at 8 × 100 μs, 1 Hz, $U = 200$ V in each experiment, which represented 100 arbitrary units of fluorescence. Results from different repetitions of experiments were pooled together and are presented as the mean and standard deviation of the mean (S.D.). On these data, three-parameter Gaussian peaks were fitted,

$$y(u) = y_{max}\exp(-(u_C - u)^2/2b^2) \quad (1)$$

where y is the uptake, u is the pulse amplitude, y_{max} is the maximum concentration of Lucifer Yellow in cell suspension, u_C denotes the value of u, corresponding to $y = y_{max}$, and b determines the width of the peak. All fits were obtained by least-squares nonlinear regression using SigmaPlot 5.0.

3. Results

The results of the uptake of Lucifer Yellow into electropermeabilized cells as a function of pulse amplitude and repetition frequency of electric pulses are shown in Figs. 1 and 2. As the figures show, increasing the amplitude of the pulses results in an increase of the uptake of Lucifer Yellow, which reaches its maximum value at a certain pulse amplitude. With further increase of the pulse amplitude the uptake decreases. The decrease in the uptake induced by higher amplitudes is a result of irreversible cell electropermeabilization. Cells either disintegrate or do not reseal, therefore allowing the leaking of Lucifer Yellow from the cells after dilution with SMEM (see M&M above).

In the first experiment, where eight 100-μs pulses were used, the highest uptake was obtained at the frequency of 10 Hz, while no significant difference was obtained between the maximum uptake values at 1 Hz, 1 kHz, and 2.5 kHz (Fig. 1). However, higher pulse amplitudes seem to be required to obtain the maximum uptake with an increase of the repetition frequency of electric pulses. For frequencies 1 Hz, 10 Hz, 1 kHz, and 2.5 kHz, these pulse amplitudes (u_C) are 234, 273, 304, and 313 V, respectively. Also, with an increase of the repetition frequency of electric pulses, the uptake peaks are wider.

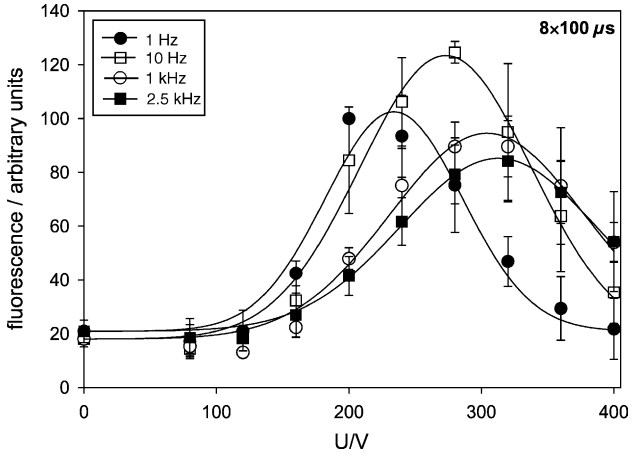

Fig. 1. The uptake of Lucifer Yellow (LY) as a function of pulse amplitude U at pulse repetition frequencies of 1 Hz (●), 10 Hz (□), 1 kHz (○), and 2.5 kHz (■) (8 pulses of 100-μs duration). Each point on the figure represents the mean of three values ± S.D.

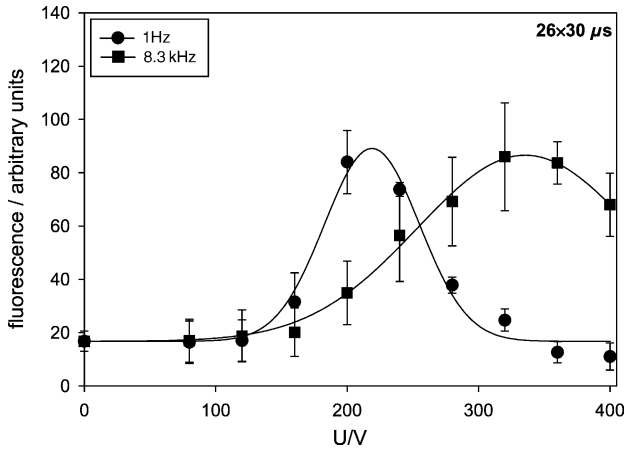

Fig. 2. The uptake of Lucifer Yellow (LY) as a function of pulse amplitude U at pulse repetition frequencies of 1 Hz (●) and 8.3 kHz (■) (26 pulses of 30-μs duration). Each point on the figure represents the mean of three values ± S.D.

In the second experiment where 30-μs pulses were used, the maximum uptake at the frequency of 8.3 kHz is comparable with the uptake at 1 Hz (Fig. 2). Again, higher pulse amplitudes are required to obtain maximum uptake values at the higher frequency ($u_C = 219$ V for 1 Hz and $u_C = 335$ V for 8.3 kHz) and the uptake peak is wider at the higher frequency.

4. Discussion

4.1. Electropermeabilization in general

Increased efficiency of high frequency unipolar or bipolar waveforms compared to a single rectangular pulse was already reported for fusion and gene transfection by different authors. For example, Tekle and co-workers examined the transfection efficiency of NIH 3T3 cells electropermeabilized by a single rectangular pulse and a high frequency unipolar or bipolar rectangular waves (60 kHz, 250 kHz, 1 MHz) [3]. They reported increased transfection efficiency and also higher survival of the cells with high frequency bipolar and unipolar waves with respect to a single rectangular pulse. Chang studied poration of COS-M6 cells and fusion of human red blood cells by radio frequency (RF) sinusoidal waves (several kHz–1 MHz) superimposed onto a rectangular pulse [4]. He found that pulsed RF field is more efficient in both cell fusion and cell poration than a DC rectangular pulse. He also found that electropermeabilization with RF pulses results in higher percentage of cells surviving the exposure to electric field compared to a DC rectangular pulse. With the

same type of pulses (40-kHz frequency), Chang and co-workers have also reported increased efficiency of gene transfection of COS-M6 cells by electropermeabilization [5].

According to the results of the above-mentioned studies, high-frequency waveforms seem to be more efficient in cell electropermeabilization and also less damaging to the cells than a single rectangular pulse. Because these authors were using amplified signals from common pulse generators, continuous waveforms were generated instead of a train of consecutive pulses that is typically used in electrochemotherapy and electrogenetherapy. This resulted in a frequency-dependent number and duration of the pulses in a train. On the contrary, in our study we varied the frequency of the pulses by changing the delay between two consecutive pulses in a train, thus keeping both the duration and number of pulses constant (except for 8.3 kHz, see below). The effect of different pulse repetition frequencies (from 1 Hz to 8.3 kHz) on the uptake of Lucifer Yellow into electropermeabilized cells was therefore examined. Because 2.5 kHz was the maximum frequency generated by our electropulsator at 100-μs pulses, we shortened the duration of the pulses to 30 μs, increased the number of pulses (to keep the total cumulative length of the pulses $N \times T$ constant) and therefore increased the maximum generated frequency to 8.3 kHz.

Our results (Figs. 1 and 2) show that the increase in the repetition frequency of the pulses does not significantly reduce the maximum value of the uptake even at the highest frequencies applied (8.3 kHz), while different voltages correspond to the maximum uptake at a given frequency. The frequency of 10 Hz seems to be the optimum frequency for the maximum uptake, which we are not able to explain at this time.

A study where the duration and the number of consecutive unipolar rectangular pulses in a train were kept constant regardless of the pulse frequency was performed by Vernhes and co-workers [7]. The frequency effect on cell viability and permeabilization of Chinese hamster ovary cells was investigated in the range from 0.5 to 100 Hz. Their results show biphasic dependence of cell viability on pulse frequency, viability increased from 0.5 to 10 Hz and then decreased, while the percentage of permeabilized cells increased with the frequencies above 10 Hz. If we consider that the increasing part of the uptake curves in Fig. 1 corresponds to an increase in the fraction of the permeabilized cells, while the decreasing part corresponds to increased fraction of irreversibly electropermeabilized cells, our results are in agreement with these results, at least up to 10 Hz frequency. In both studies, the 10 Hz frequency seems to be the optimum frequency for the highest viability of the cells (Vernhes), or the highest uptake (our study).

4.2. Prospects of using high frequency pulses in electrochemotherapy

The main purpose of our study was to investigate the possibilities of the use of pulses with higher frequencies in electrochemotherapy in order to reduce the painful sensations caused by low-frequency (1 Hz) electrochemotherapy. For efficient electrochemotherapy, electric pulses of appropriate amplitude must be delivered to the electrodes. Typically, 1000-V pulses are applied to the electrodes having a distance of 8 mm. Besides electropermeabilization of tumor cell membranes, these pulses also excite the nerve fibers located beneath or in the vicinity of the electrodes. In the form of action potential, the excitation is then carried along the nerve fiber to neuromuscular junctions to cause muscle contraction.

After the first pulse, the membrane of the nerve axon cannot be excited for the refractory period of the axon membrane [19–21]. If a train of pulses is used, with the delay between two consecutive electric pulses shorter than the combined duration of the action potential and the refractory period, each pulse in the train will not be able to initiate a new action potential. In addition, if the duration of the whole train of pulses is shorter than the duration of the action potential including the refractory period, only a single action potential will be generated (Fig. 3). Skeletal muscles are mostly innervated by myelinated nerve fibers for which the maximum frequency of generated action potentials (inverse value of the duration of the action potential and the refractory period) ranges from 400 to 2500 Hz [19–21], depending on the diameter of the nerve axon.

As mentioned above, after excitation of the nerve, the action potential is carried to neuromuscular junctions to provoke muscle contraction, which typically lasts for tens (e.g., ocular muscle) up to hundreds of miliseconds (e.g., soleus). Since the pulse repetition frequency of 1 Hz is most

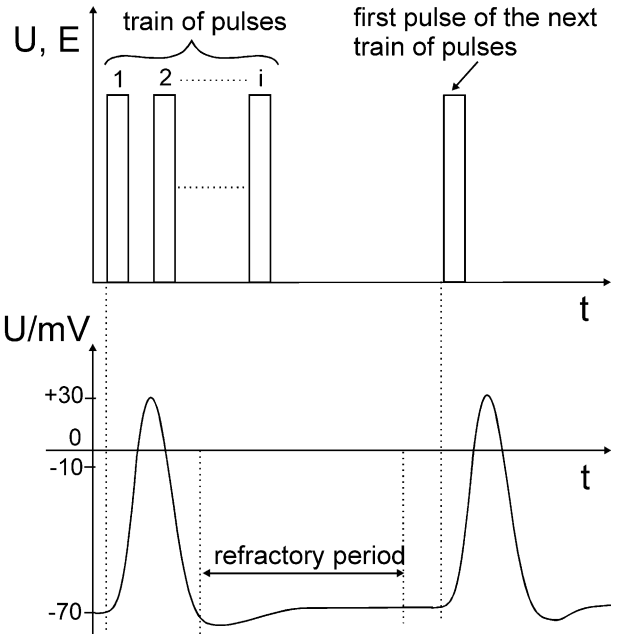

Fig. 3. The duration of the whole train of pulses in comparison with the duration of the action potential considering refractory period.

often used in electrochemotherapy [11–14,22,23], each individual pulse in the train of pulses causes the contraction of muscles innervated by the excited neurons. Increasing the frequency of electric pulses would increase the frequency of generated action potentials in the excited nerve, shorten the delay between two consecutive muscle contractions, and eventually increase the force of muscle contraction (Fig. 4). At a certain frequency of excitation (~ 40 Hz [19,20]) successive muscle contractions will fuse into smooth motion-tetanic contraction (Fig. 4). Electrochemotherapy with pulse repetition frequencies above the frequency of tetanic contraction would therefore reduce the number of individual muscle contractions but also increase the force of muscle contraction, while at frequencies lower than tetanic, the muscle response to the excitation will not be smooth (Fig. 4).

Suppose the pulses are delivered at the frequency for which the duration of the whole train of pulses is shorter than duration of the action potential with the refractory period (Fig. 3), the response of the muscle to the high frequency of excitation would probably be the same as if the muscle were excited by a single pulse. Considering the train of eight pulses with duration of 100 μs, the repetition frequency of electric pulses generating a single action potential, and thereby a single muscle contraction, is expected to be in the range of a few kHz, but not higher than 20 kHz even for the fastest muscles.

Although many electropulsators are unable to generate 100-μs pulses at this frequencies, the use of pulses with considerably lower repetition frequencies would still result in a decreased muscle response with respect to a typical 1 Hz exposure because: (a) the refractory period of the axon membrane consists of the absolute refractory period (during which it is impossible to initiate a new action potential) and the relative refractory period (during which a stronger stimulus can initiate a new action potential). Because the data on the maximum frequency of generated action potentials mentioned above were calculated regarding to the absolute refractory period only, the maximum frequency of generated action potentials considering both refractory periods can be lower; (b) the pulses with repetition frequencies higher than the frequency of tetanic contraction (~ 40 Hz) will already reduce the number of consecutive muscle contractions and thereby, reduce the number of unpleasant sensations; (c) the pulses with frequencies higher than the maximum frequency of generated action potentials (>400 Hz) will reduce both, the force and the number of muscle contractions, considering finite duration of the train of pulses (filled circles in Fig. 4). For example, the total duration of a pulse train (eight 100-μs pulses) at 1 Hz repetition frequency is 8 s, while at 1 kHz pulse repetition frequency the total duration of the train is 8 ms.

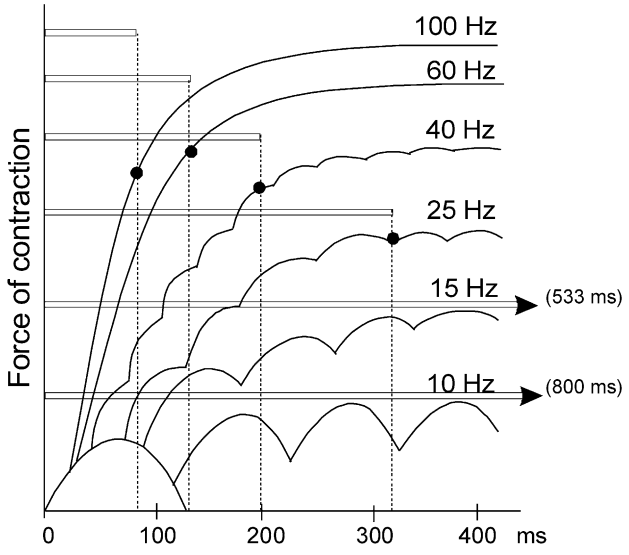

Fig. 4. The dependence of the force of muscle contraction on the pulse repetition frequency and the occurrence of tetanic contraction for continuous muscle stimulation. The filled circles (●) represent the force of contraction due to excitation with a pulse train (8 × 100 μs) at a given frequency (partially adapted from Ref. [24]). ▭ duration of a pulse train (8 × 100 μs) at a given repetition frequency.

Besides the nerves that innervate the muscles, electric pulses could also excite pain receptors or nerve endings located nearby. An increased pulse repetition frequency could also eliminate these side effects.

In a recent study by Daskalov and co-workers [25], electrochemotherapy with high frequency pulses was performed on basal cell and spin cell carcinoma and on melanoma metastases in patients. They have compared 8 × 100 μs, 8 × (50 + 50 μs), both 1-Hz rectangular pulses and a burst of eight bipolar rectangular pulses (50 + 50 μs, pulse repetition frequency 1 kHz). No difference between tumor responses on treatment protocols was observed. However, electrochemotherapy with higher pulse frequencies was better accepted by the patients, because of only one electrical excitation instead of eight. Our theoretical considerations are in agreement with these results, while results of our in vitro study further substantiate that electrochemotherapy could be effectively performed with higher pulse repetition frequencies. In addition, the total duration of the pulse train is important in the case of treating large tumors with arrays of multiple electrodes, since the total treatment duration can be reduced.

In summary, the uptake at the highest repetition frequencies examined (2.5 kHz for 100 μs and 8.3 kHz for 30-μs pulses) stays at similar levels as the uptake at 1 Hz, while different voltages correspond to the maximum value of the uptake at the specific frequency. However, with pulses of longer duration or increased number of pulses, an additional increase in the uptake can be obtained [26,27]. If we refer to the previously mentioned study [14] where muscle contractions and painful sensation were presented as the most disagreeable side effects during electrochemotherapy, on the basis of our theoretical considerations and in vitro results, we suggest the use of pulses with higher frequencies as an improvement in a sense of reduced force and number

of muscle contractions for the patients. Certainly, before clinical applications, the results obtained in our study in vitro should be verified on animal tumor models in vivo.

Acknowledgements

G.P. wishes to thank Dr. Tadej Kotnik for valuable help and instructions while the experiments were conducted and for reading the manuscript. This research has been supported through various grants by the Ministry of Education, Science and Sports (MESS) of the Republic of Slovenia. Experimental work at Villejuif (France) has been supported by grants of CNRS and Institut Gustave-Roussy, while visits and stays have been made possible by the Proteus program of scientific, technological and cultural co-operation between CNRS (project no. 5386), France and MESS (Proteus program), Slovenia. G.P. and D.M. would also like to acknowledge the partial support of IGEA Carpi (Modena), Italy.

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