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RESEARCH ARTICLE

Synergistic effect of EMF–BEMER-type pulsed weak electromagnetic field and HPMA-bound doxorubicin on mouse EL4 T-cell lymphoma

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Abstract

We have investigated the effects of low-frequency pulsed electromagnetic field (LF-EMF) produced by BEMER device on experimental mouse T-cell lymphoma EL4 growing on conventional and/or athymic (nude) mice. Exposure to EMF–BEMER slowed down the growth of tumor mass and prolonged the survival of experimental animals. The effect was more pronounced in immuno-compromised nude mice compared to conventional ones. Acceleration of tumor growth was never observed. No measurable levels of Hsp 70 or increased levels of specific anti-EL4 antibodies were detected in the serum taken from experimental mice before and at different intervals during the experiment, i.e. before solid tumor appeared, at the time of its aggressive growth, and at the terminal stage of the disease. A significant synergizing antitumor effect was seen when EL4 tumor-bearing mice were simultaneously exposed to EMF–BEMER and treated with suboptimal dose of synthetic HPMA copolymer-based doxorubicin, DOX^{HYD}-HPMA. Such a combination may be especially useful for heavily treated patients suffering from advanced tumor and requiring additional aggressive chemotherapy which, however, at that time could represent almost life-threatening way of medication.

Keywords: EL4 T-cell lymphoma, athymic mice, DOX^{HYD}-HPMA, EMF, anticancer resistance, stimulation of the immune system

Introduction

Over the past three decades, potential health effects of exposure to electromagnetic fields (EMFs) have been extensively investigated in epidemiologic studies. This awareness has been triggered by the growing body of knowledge on how EMFs interact with cellular systems of living organisms. The EMF treatment is widely applied in clinical practice for prevention, diagnosis, and treatment of diseases with various etiologies. The mechanisms of biological and therapeutic effects of EMFs are still not entirely understood (Gapeyev et al., 2011). It was even suggested that low-frequency EMFs may be a risk for human health (Zheng et al., 2000; Erren, 2001; Porock & Gentry, 2002; Scott et al., 2002; Busljeta et al., 2004; Trosic et al., 2004; Chen et al., 2010; de Vocht, 2010).

Widespread concerns about whether EMFs could affect human health have been raised in epidemiologic studies trying to answer the question of their involvement in cancer appearance (Pollan et al., 2001; Weiderpass et al., 2003; Girgert et al., 2005). Low-frequency EMFs were suspected of being involved in carcinogenesis, acting as co-promoters during neoplastic transformation, modifying cell proliferation, and/or signal transduction pathways (Jin et al., 2000; Richard et al., 2002). Experimental findings also suggested that exposure to low-frequency EMFs may affect various cell functions via actions exerted on intracellular and membrane proteins, including ion channels, membrane receptors and enzymes, and cytoskeleton (Grassi et al., 2004; Lange et al., 2004). On the other hand, Scarfi et al. (2005) and Jian et al. (2009)

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report that extremely low frequency (ELF) EMF induces apoptosis only in cancer cell lines which could be even enhanced by low doses of X-ray irradiation. Literature in the area of DNA strand breaks as a consequence of EMF exposure is also contradictory (Ruiz-Gómez & Martinéz-Morillo, 2009). Some investigators report on DNA damage (Vijayalaxmi & Prihoda, 2009), while others deny it (Phillips et al., 2009). So far, the findings gave no support to the hypothesis that EMF exposure increases the risk of cancer (Beniashvilli et al., 2005; Forssén et al., 2005; Sommer et al., 2007; Chen et al., 2010; de Vocht, 2010).

It is the reality that the data from scientific literature as well as from epidemiologic studies are still controversial. While some researches associate ELF-EMF exposure with carcinogenesis, other studies suggest that treatment with selected frequencies is feasible and well tolerated and may have biological efficacy in diseased patients (Lacy-Hubert et al., 1998; Lange et al., 2004; Ronchetto et al., 2004; Chen, 2010).

Here, we aim to study the biological effects of a low-frequency pulsed EMF produced by the BEMER device (EMF-BEMER) (Kafka, 1998) on an experimental cancer model, EL4 T-cell lymphoma (H-2b, Thy-1.2+) growing on normal immunocompetent mice of inbred strain C57BL/6 (B/6) and/or on immunodeficient athymic nu/nu CD-1 mice. It was demonstrated that EMF-BEMER influences microcirculation and the activity of antioxidant enzymes (Kafka & Spodrayk, 2003), especially after chemo- and radio-therapeutic cancer treatment (Gabrys, 2004) and wound healing (Kafka et al., 2005).

A new generation of polymeric anticancer drugs based on N-(2-hydroxypropyl)methacrylamide (HPMA) with improved therapeutic potential considerably decreased nonspecific side effects and the ability to stimulate anticancer immunity is already well documented (Kopecek & Kopeckova, 2010; Lammers & Ulbrich, 2010; Říhová & Kovář, 2010).

The main purpose of this study was to test (i) the effect of an exposure to EMF-BEMER on growth an experimental cancer model (EL4 T-cell lymphoma) and (ii) a possible synergizing effect of suboptimal treatment with HPMA copolymer-based doxorubicin (DOX^{HYD}-HPMA) as an anticancer agent and EMF-BEMER.

Materials and methods

Polymers conjugate DOX^{HYD}-HPMA

Polymers conjugate DOX^{HYD}-HPMA was prepared according to Etrych et al. (2008). It is a doxorubicin bound to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer carrier through a hydrazone bond with a MW ~34,000 (Figure 1).

EMF source and exposure

The BEMER device is a certified medical instrument. The control unit works with an operating voltage from 12 V to 15 V. In the connected coil mat the special multidimensional pulsating current generates a weak, pulsating EMF. The basic BEMER impulse starts at a frequency of 0 Hz and constantly increases and within 30 ms reaches its maximum of 2 kHz. From there it falls back to 0 Hz and the impulse starts again. Parallel the magnetic flux intensity begins at 0 μT and pulses upward until it reaches its maximum, according to the chosen level. From there, like the frequency, it falls back to 0 μT and the impulse starts again (Figure 2). For the experiment were chosen maximum levels of 3.5 μT, 10.5 μT, 21 μT, and 35 μT. The BEMER device neither offers the choice of only one constant frequency nor only one constant intensity.

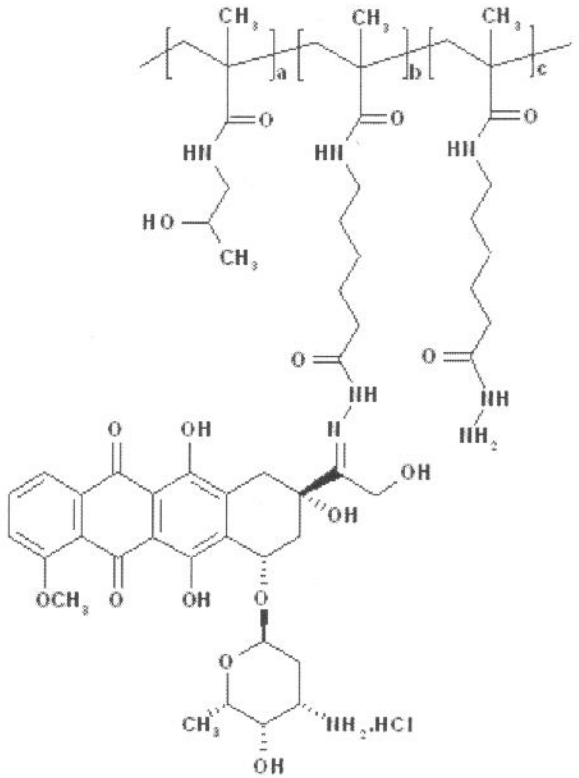

Figure 1. Structure of the DOX^{HYD}-HPMA conjugate.

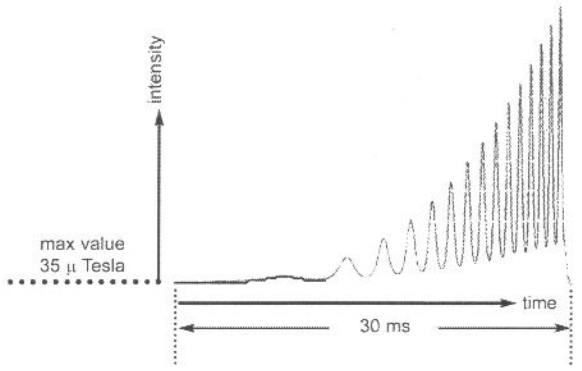

Figure 2. Typical form of electromagnetic impulse generated by the BEMER device.

Cancer cell line

Mouse T-cell lymphoma EL4 cells were obtained from American Type Culture Collection (ATTC).

Culture conditions

The EL4 cells were grown at 37°C with 5% CO_2 in RPMI 1640 medium (Gibco BRL) supplemented with heat-inactivated 10% v/v fetal calf serum (FCS) selected for low mitogenicity, 4 mM L-glutamine (Gibco BRL), 1 mM Na-pyruvate, 50 mM 2-mercaptoethanol, 4.5 g/L glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma).

Animals

All experiments were done either on conventional 8-week-old female mice of inbred strain C57BL/6 (H-2^b) purchased from the Animal Center of the Institute of Physiology, Academy of Sciences of the Czech Republic, V.V.I. or on 8-week-old female immunodeficient athymic nu/nu CD-1 mice obtained from AnLab Ltd., Prague. The mice were randomly assigned to either experimental or control groups and housed in accordance with approved guidelines. Food and water were given *ad libitum*. The animal room was maintained at 22°C. The experimental designs were in accordance with the Czech Republic Act for Experimental Work with Animals (Decrees No.311/97, 117/87, and Act No. 246/96), which is fully compatible with the corresponding European Community Acts.

In vivo tumor growth

Exposure to EMF only

On day 0, 1×10^5 EL4 T-cell lymphoma cells in 0.1 mL RPMI 1640 medium were injected subcutaneously (s.c.) on right back of C57BL/6 or nu/nu CD-1 mice. The experimental animals were exposed to low-energy EMF-BEMER. Controls were transplanted with cancer cells but were not exposed to EMF. At least 10 mice were used for each experimental group. The animals were observed daily for signs of tumor progression. The survival time, size of tumor, and the number of long-term survivors (LTS) were determined.

Exposure to EMF and DOX^{HYD}-HPMA conjugate

Mice were exposed to EMF-BEMER similarly as described above. The mice that developed palpable tumors reaching 5–8 mm^3 in diameter within 8 to 9 days after the implantation of cancer cells were intravenously treated with DOX^{HYD}-HPMA (15 mg of DOX eq./kg) diluted in PBS. Those surviving at least 60 days without any signs of a tumor were considered as LTS, and they were re-transplanted with a lethal dose (1×10^5) of the same tumor cells and left without treatment to determine the therapy-induced tumor resistance.

Hsp 70

Quantitative heat-shock protein analysis was performed in serum samples using StressXPress (Stressgen Biotechnologies) Hsp70 ELISA Kit for the detection and quantitation of Hsp70 in serum according to the manufacturer's manual.

ELISA detection of anti-EL4 antibodies

Mice were bled from the tail veins and the separated serum was stored at –80°C until analyzed. The antibody level was estimated by an indirect ELISA method (double layer). Serum taken before the transplantation with cancer cells was used as a negative control. The sera of mice immunized five times with 5×10^5 dead EL4 cells incorporated in complete Freund's adjuvant (CFA) were taken as a positive control. Detection was carried out as reported previously (Říhová et al., 2002). Briefly, NUNC Immunoplate MaxiSorp F 96 microplates were coated either with EL4 cells (10^5 cells/well) or with EL4 cell lysate (25 μg of protein/well). Two plates were prepared simultaneously. After overnight incubation at 4°C, the plates were washed three times with PBS and PBS/0.2% Tween 20 and blocked with 1% BSA/PBS/0.02% gelatin at 37°C for 2 h. After five more washings with PBS, and PBS with 0.2% Tween 20, the microplate wells were filled with 100 μL of serial dilutions of tested sera and the plates were kept overnight at 4°C to allow quantitative antigen-antibody binding. The next day, the microplates were washed as described above and horse-radish peroxidase-conjugated, affinity-purified porcine anti-mouse Ig was diluted 1: 500 and added at 37°C for 1 h. The conjugate with the enzyme was removed, and the plates were developed with 0.015% H_2O_2-o-phenylenediamine for 10–20 min at 22°C in the dark. The reaction was stopped by the addition of 20 μL of 1M H_2SO_4 and the absorbance of the colored product was measured using an automatic ELISA reader (Tecan) at a wavelength of 492 nm against a series of wells treated only with substrate. The results were calculated as the arithmetic mean of the titer detected in three individual wells on each plate (altogether six individual wells/sample). Every assay included a negative control (serum tested alone, antigen without horse-radish peroxidase-conjugated anti-mouse Ig, or antigen without tested antisera) as well as a blank control of the specificity of the reaction.

Statistics

The statistical significance ($p < 0.05$) of the differences between volumes of tumors in the various groups was assessed by applying a two-sided Student's *t*-test. For each approach, three independent experiments were conducted and differences between exposed and control animals with an error probability of $p < 0.05$ were considered to be statistically significant.

Results and discussion

Conventional C57BL/6 mice were first exposed to pulsed EMF-BEMER (30 min every 4 h with the 10.5 μT, 21 μT, or 35 μT intensity or permanently with the intensity 3.5 μT on day 4 before s.c. transplantation of cancer cells and then every day until the end of the experiment.

Figure 3A illustrates mean tumor volume change for each of the four treatment groups and Figure 3B documents survival of experimental animals. Both figures show significant retardation of tumor growth and prolongation of lifespan in mice exposed to EMF–BEMER with an intensity of 21 μT. The experiment was repeated three times with similar results.

The slight antitumor activity demonstrated in the experimental group exposed to EMF–BEMER of the intensity of 21 μT could be, among others, related to the activation of the immune system. To elucidate a tentative involvement of innate (natural or native) and/or adaptive (specific) immunity in the mechanism of action of EMF–BEMER on tumor growth, we used for further experiments immuno-compromised athymic nude (nu/nu) mice. Athymic mice suffered from an extremely limited number of T-cells, which is the reason why they have only marginal specific immunity and are routinely used to define the role of T/B lymphocytes in immunity and disease.

Similarly as in conventional mice, we have repeatedly observed in nude mice that EMF–BEMER to which the animals were exposed slowed down the growth of experimental EL4 T-cell lymphoma (Figure 4A) and significantly extended their average lifespan (Figure 4B). Interestingly enough, the exposure to EMF–BEMER gave a better result in terms of the tumor growth retardation and prolongation of survival time in immuno-compromised nude mice, where the effect was more pronounced than in conventional animals. This suggests that either innate immunity, that is strong in athymic mice, or absence of T-suppressive activity may contribute to the protective effect of EMF–BEMER.

Taken together, the results point to slight but clear-cut antitumor effects of low-frequency EMF–BEMER on EL4 mouse T-cell lymphoma or at least could be taken as a proof that exposure to EMF–BEMER is not a risk factor intensifying the development of experimental mouse T-cell lymphoma EL4.

There are numerous data confirming not only the safety but also certain antiproliferative effects of EMF

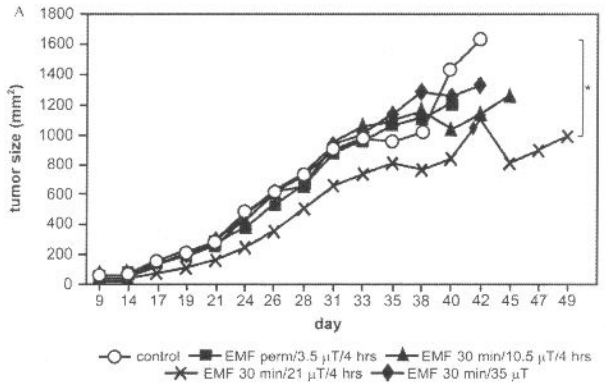

Figure 3A. Effect of pulsed EMF–BEMER on the growth of EL4 mouse T-cell lymphoma in conventional C57BL/6 mice exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT, or 35 μT) or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation; $*p < 0.05$.

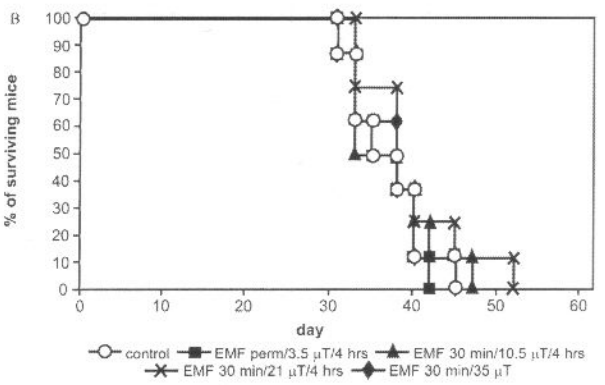

Figure 3B. Effect of pulsed EMF–BEMER on the survival of C57BL/6 mice bearing EL4 mouse T-cell lymphoma and exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT, or 35 μT) or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation.

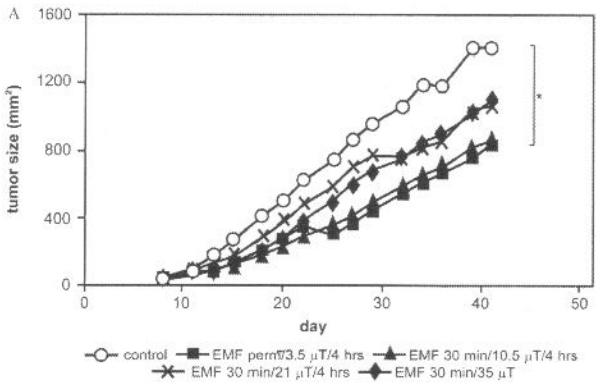

Figure 4A. Effect of pulsed EMF-BEMER on the growth of EL4 mouse T-cell lymphoma in nu/nu CD-1 mice exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT, or 35 μT) or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation; $*p < 0.05$.

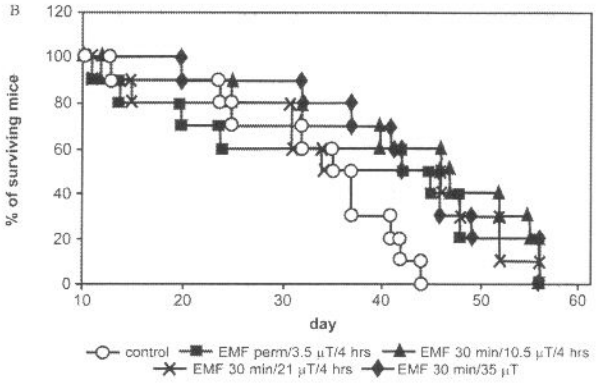

Figure 4B. Effect of pulsed EMF-BEMER on the survival of nu/nu CD-1 mice bearing EL4 mouse T-cell lymphoma and exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT, or 35 μT) or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation.

treatment (Beneducci et al., 2005; Jiménez-García et al., 2010). Williams et al. (2001) were the first to report the reduction of tumor angiogenesis after exposure of mice with experimental cancer to pulsating EMFs. As a result, tumor growth was significantly reduced in female C57H/HeJ mice bearing mammary adenocarcinoma. Tofani et al. (2002) documented that the treatment of tumor-bearing nude mice with daily exposure to ELF-magnetic fields for 4 weeks caused significant tumor growth inhibition. Mice suffering from cancer xenograft had significantly fewer lung metastatic sites, slower tumor growth, and reduced vascularization, which together resulted in an increased survival time compared to untreated controls. Similar data were obtained with AKR/J mice suffering from spontaneous lymphoblastic lymphoma. The EMF exposure did not alter malignacy or the progression of the disease and lymphatic infiltration did not occur more often in EMF exposed than in control mice (Sommer et al., 2004; 2007). Cameron et al. (2005) report a decreased growth and reduced vascularization of human breast cancer xenografts in female athymic (nude) mice exposed to EMF either alone or in combination with gamma radiation. Similarly, a slight inhibition of the formation of chemically induced neoplastic foci in rat livers was observed when the animals were exposed to the EMF (Rannug et al., 1993).

The anticancer effects of EMF could result from inhibition of cell proliferation, targeted apoptosis induction, regulation of cellular homeostasis, affecting pathways associated with heat stress and/or activation of the immune system.

Tokalov & Gutzeit (2004) demonstrated the expression of heat-shock genes, in particular *Hsp70 (A, B, and C)* in human cells in response to ELF-EMFs alone and in combination with thermal stress. Since EMFs interact with moving charges, it is generally accepted that such treatment could stimulate the stress response by interacting directly with moving electrons in DNA (Blank & Goodman, 1999). The events mediating the EMF-stimulated stress response appear to be similar to those reported for other physiological stresses (e.g. hyperthermia, heavy metals, oxidative stress) and could well constitute the general mechanism of cell response to EMF (Lin et al., 1999). Detailed mechanisms of the processes of transduction of the electromagnetic signals into biological responses, especially changes in biosynthesis, are however still unknown. The full understanding of complicated mechanisms of action of LF-EMFs could take years. But meantime, the LF-EMF could be and has already been widely explored as a noninvasive way to treat cancers where a multimodality therapy is urgently needed.

We used an ELISA test to quantify the release of Hsp 70 into the serum of cancer-bearing mice and to test whether heat-shock proteins are involved in the positive anticancer reaction of mice exposed to EMF–BEMER. The level of Hsp 70 in the serum taken 4 days before the experiments represented a control. Serum samples were then taken from individual mice on day 0, i.e. before transplantation of malignant EL4 cells, on day 9 after the transplantation, i.e. at the time when solid cancer is already palpable, on day 16, i.e. at the time of aggressive growth of the tumor, and on day 30, i.e. in the terminal state of the disease. Using sensitive ELISA test, we repeatedly failed to determine *in vivo* measurable levels of Hsp 70 in serum samples. The reason could be quantitative as the positive effect of EMF on the expression of the heat-shock protein genes *HSP27, HSP60,* and *HSP70* was documented *in vitro* in tissue culture of human cells, malignant as well as normal, exposed to a wide range of environmental stimuli, including EMFs alone or in combination with thermal stress (Dressel & Günther, 1999; Lin et al., 1999).

Using a combination of low electric field cancer treatment and chemotherapy with 5-FU, Plotnikov et al. (2004) reported a significant tumor size reduction and a prolongation of survival time in mice bearing murine colon carcinoma CT-26. Tumor growth inhibition was accompanied by an initiation of antitumor immune reaction probably due to the antigenic material released from the deteriorating cancer cells.

Thus, we tested the formation of specific anti-EL4 antibodies in mice suffering from EL4 T-cell lymphoma and exposed or non-exposed (controls) to EMF–BEMER. The antibodies were detected by indirect ELISA and their maximal level was seen between days 9 and 16 (Tables 1 and 2). Then the free antibodies from serum disappeared, probably due to their binding to solid tumor and to metastasizing cells in peripheral blood and solid lymphatic tissues, such as lymph nodes and spleen (Říhová et al., 2002). Anti-EL4 antibodies detected in experimental animals before their first contact with EL4 cells (negative control) represent cross-reacting natural antibodies taken as a baseline. The sera of mice immunized five times with 5×10^5 dead EL4 cells incorporated in a CFA were used as a positive control. In the end, no difference was recorded between experimental groups exposed to EMF–BEMER and that without EMF intervention.

In several studies, the exposure to EMF was combined with different anticancer drugs, such as 5-FU (Plotnikov et al., 2004), anthracyclines (Liang et al., 1997; Orel et al., 2005), or methotrexate (Laqué-Rupérez et al., 2003). Liang et al. (1997) report the enhancement of direct *in vitro* cytotoxicity of daunomycin by a pulsed magnetic field using multidrug resistant subline KB-ChR-8-5-11, while no such effects were seen by Laqué-Rupérez et al. (2003) in MCF-7 breast cancer cells treated with methotrexate. The rare animal studies explain a positive effect of EMF given simultaneously with anticancer drugs by enhancing the drug delivery across biological barriers (Murthy, 1999).

The original reason for the conduction of this study was to document the effect, if any, of EMF–BEMER on the growth of cancer cell line EL4 *in vitro* and on experimental EL4 cancer model *in vivo*. The data presented in

Table 1. Serum level of anti-EL4 antibodies; antigen = EL4 cells.

	EMF				
Day	30 min/10.5 μT/4 h	30 min/21 μT/4 h	30 min/35 μT/4 h	perm/3.5 μT	0
−4	6.0[a,b]	4.5	3.0	7.0	6.0
0	6.0	4.5	6.5	6.0	6.0
9	8.5	7.5	8.5	8.0	7.5
16	10.5	10.5	10.0	9.0	10.0
30	5.5	5.0	5.0	7.5	5.0

[a]The numbers represent $\log_2$ of serum dilution.
[b]Positive control > 24, negative control (natural antibodies) 4.5–6.0.

Table 2. Serum level of anti-EL4 antibodies; antigen = EL4 cell lysate.

	EMF				
Day	30 min/10.5 μT/4 h	30 min/21 μT/4 h	30 min/35 μT/4 h	perm/3.5 μT	0
−4	9.5[a,b]	8.0	7.0	10.0	9.5
0	9.5	8.5	9.5	10.0	10.0
9	10.5	10.5	9.0	9.5	10.5
16	12.5	13.5	11.5	14.5	13.0
30	8.5	8.0	7.0	9.5	8.5

[a]The numbers represent $\log_2$ of serum dilution.
[b]Positive control > 22, negative control (natural antibodies) 8.5–10.5.

Figures 3A,3B,4A, and 4B, which document slight but undoubted anticancer effect of EMF, substantiated the study of a hypothetical combinatorial effect of EMF-BEMER and a cytostatic drug. We decided to use its polymeric form, as anticancer drugs bound to different polymeric carriers represent an advanced approach for anticancer treatment. Such derivatives have long-term peripheral blood circulation, increased tumor accumulation, a decrease of side-toxicity (Kopecek 2010; Kopecek & Kopeckova, 2010), and have also been extensively used for combination therapies (Krinick et al., 1994; Greco & Vicent, 2009; Lammers, 2010). In addition, in those based on *N*-(2-hydroxypropyl)methacrylate (HPMA) carrier was repeatedly documented therapy-dependent activation of the immune system (Říhová & Kovář, 2010).

We used a suboptimal dose (15 mg of Dox eq./kg) of doxorubicin bound to N-(2-hydroxypropyl)methacrylamide (HPMA) carrier through a hydrazone bond (DOX^{HYD}-HPMA; Figure 1). It is a formulation which was repeatedly shown to have an exceptional anticancer effect based on the direct cytotoxicity and therapy-activated anticancer-immune response (Říhová & Kovář, 2010; Šírová et al., 2010). The decreased growth of tumor was recorded in all experimental groups. About 60% (5/8) of cured mice, when treated with DOX^{HYD}-HPMA only, correspond to the fact that a suboptimal dose of the drug derivative was used. Similar percentage of LTSs was seen when mice were simultaneously exposed to EMF of the intensity of 10.5 μT (30 min every 4 h; 60% of LTS; 6/10) or permanently to 3.5 μT (70% of LTS; 7/10). Results definitely proving the effect of EMF were obtained in mice exposed to EMF-BEMER of the intensity of 21 μT (30 min every 4 h; 80% of LTS; 8/10) or 35 μT (30 min every 4 h; 80% of LTS; 8/10) (Figure 5A and B). The survival of athymic nude (nu/nu) mice was also

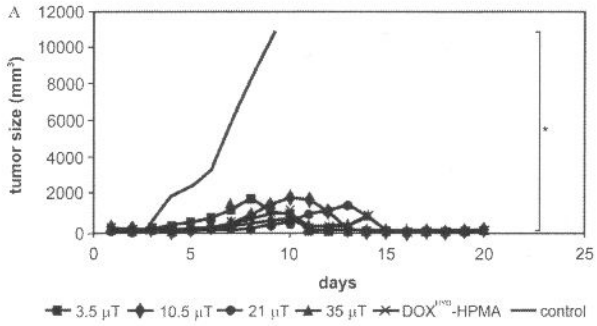

Figure 5A. The combinatory effect of pulsed EMF-BEMER and DOX^{HYD}-HPMA (15 mg DOX eq./kg) on the growth of EL4 mouse T-cell lymphoma in conventional C57BL/6 mice exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT, or 35 μT) or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation; $*p < 0.001$.

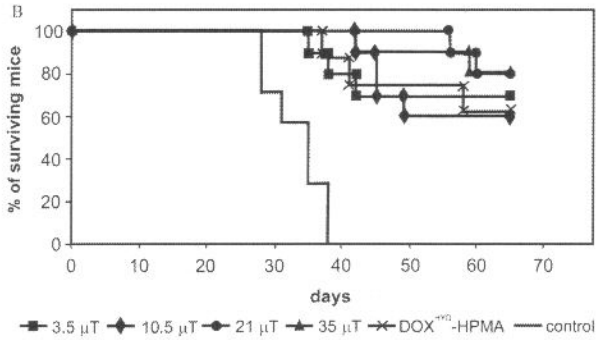

Figure 5B. The combinatory effect of pulsed EMF-BEMER and DOX^{HYD}-HPMA (15 mg DOX eq./kg) on the survival of conventional C57BL/6 mice exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT or 35 μT), or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation.

prolonged when the animals were treated with the same dose of DOX^{HYD}-HPMA as conventional animals (15 mg of Dox eq./kg) and exposed to the EMF-BEMER. In addition, the higher intensities (21 μT and 35 μT) were more efficient (Figure 6). Unexpectedly, one mouse survived more than 4 months. It could not be recorded as a LTS as tumor, even if considerably shrunken, was still there (see "The case report").

We have seen a clear EMF dose-response, which implies that higher doses are more effective. Barbault et al. (2009) suggest that tumor-specific frequencies have to be used for the treatment of patients with advanced tumors. Such studies could be the basis for the design of strategic and clinical application of selected EMF sources for the treatment of different diseases.

Immunocompetent cells involved in the defense mechanisms are those preferentially acting in native (natural) immunity, such as macrophages and natural killer (NK) cells, and those effective in acquired (specific) immunity, such as NKT, and different subpopulations of T- and B-cells. The NK cells have an important role, though not decisive, in anticancer response where CTL cells are the major player in the game. The possibility of

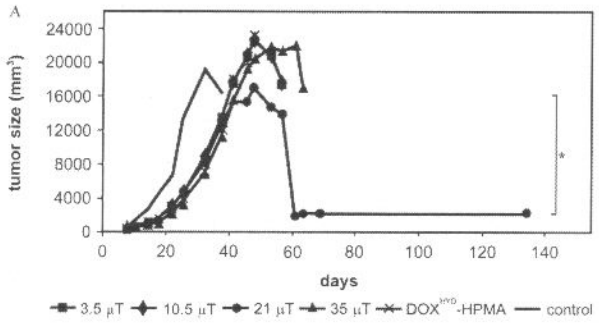

Figure 6A. The combinatory effect of pulsed EMF-BEMER and DOX^{HYD}-HPMA (15 mg DOX eq./kg) on the growth of EL4 mouse T-cell lymphoma in nu/nu CD-1 mice exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT, or 35 μT) or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation; *$p < 0.001$.

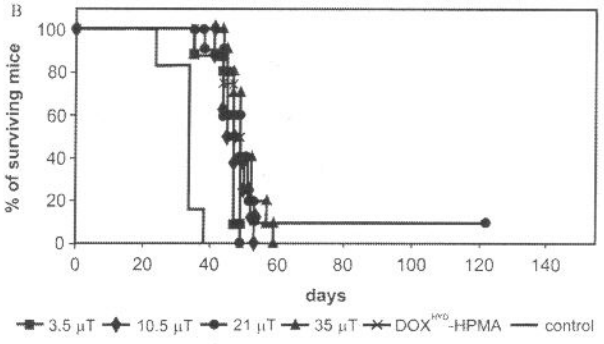

Figure 6B. The combinatory effect of pulsed EMF-BEMER and DOX^{HYD}-HPMA (15 mg DOX eq./kg) on the survival of nu/nu CD-1 mice exposed to EMF for 30 min every 4 h (intensity of 10.5 μT, 21 μT, or 35 μT) or permanently (intensity of 3.5 μT). The exposure to EMF started 4 days before cancer cell transplantation.

the effects of EMF on activity of the immune functions in living organisms has already been hypothesized and tested (Arafa et al., 2003; Di Giampaolo et al., 2006; Tuschl et al., 2006; Boscolo et al., 2007; Akan et al., 2010; Kleijn et al., 2011) but never directly demonstrated *in vivo*. For instance, Rossi et al. (2007) report that ELF-EMFs (source SEQEX) reduce the oxidative stress and the side effects of chemotherapy, and specifically myelodepression (myelotoxicity), in patients with Hodgkin's lymphoma. As oxidative stress may be, at least in part, responsible for secondary malignancies, they conclude that SEQEX with its ability to reduce an oxidative stress induced by treatment with chemo-radiotherapy may reduce the risk of late toxicities. The EMF was reported as both increasing and decreasing the activity/number of circulating NK cells or no effect at all (Gobba et al., 2009a; 2009b). However, it has to be stressed, that serious scientific data are so far still extremely limited.

In all our experimental systems, we routinely proved the activation of the immune system during anticancer therapy by re-transplantation of LTS with a lethal dose of cancer cells. As no therapy is provided after such a re-transplantation, the only explanation for the eventual eradication of re-injected cancer cells is the activation of defense mechanisms of the cancer-bearing host during the primary treatment (Říhová & Kovář, 2010). Figure 7A and B document a high cancer resistance in experimental

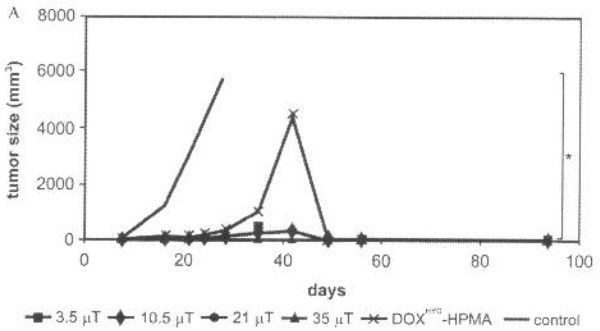

Figure 7A. The growth of EL4 mouse T-cell lymphoma in EL4-cured LTS (see Figure 5B) re-transplanted with a lethal dose (1×10^5) of EL4 cancer cells; *$p < 0.001$.

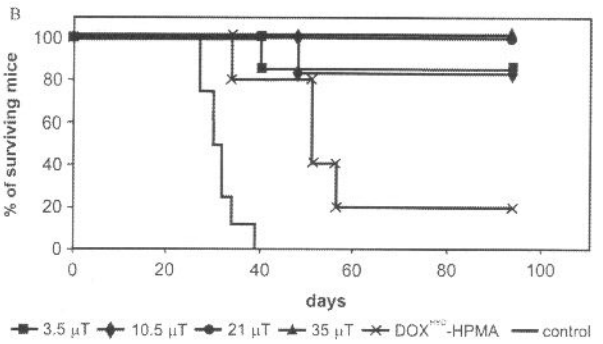

Figure 7B. Survival of EL4-cured LTS (see Figure 5B) re-transplanted with a lethal dose (1×10^5) of EL4 cancer cells.

groups exposed simultaneously to DOX^{HYD}-HPMA and EMF-BEMER. While 20% of re-transplanted LTS (1/5) survived when treated with DOX^{HYD}-HPMA only, up to 100% of them survived if simultaneously exposed to EMF-BEMER. Around 86% (6/7) of primary LTS survived, when permanently exposed to 3.5 μT; 84%; 5/6 when exposed to 10.5 μT, and 100% (8/8) when exposed either to 21 μT or to 35 μT given 30 min every 4 h. To our knowledge, it is the first direct *in vivo* documentation the immune-stimulating effect of EMF.

The case report

One nude mouse treated with DOX^{HYD}-HPMA and exposed to EMF-BEMER of an intensity of 21 μT survived more than 4 months, which is quite exceptional (Figure 6). As a rule for conventional or nude mice, immediately after the treatment with polymeric drugs the growth of experimental cancer stops. In a week or so the cancer shrinks. After another few days the tumors disappear (mice are cured) or their aggressive growth starts again. However, in that one mouse the size of the tumor (about 15 mm^3) and health condition stayed unchanged for more than 4 months. About 140 days from the beginning of the experiment and 122 days after "stabilization" of the cancer size it was decided to re-transplant the mice with a lethal dose of cancer cells similarly as we have routinely done for conventional mice to test the mechanisms responsible for the control of cancer growth. Here, mainly innate immunity could be involved in cancer eradication as the number of T-cells responsible for adaptive anticancer immunity in nude mice is very limited. Rather surprisingly, no cancer growth was observed at the site of secondary re-injection, i.e. on the left side on the back of mice. However, immediately after such "a second cancer cell attack" we have detected aggressive growth of previously stabilized primary cancer (solid EL4 thymoma) on the right side of the mouse back. The growth was almost exponential until day 38 (Figure 8). Then, from day to day, a substantial decrease in the size of tumor was observed which is usual in tumor-exhausted experimental models. We decided to end the experiment and to test (i) the sensitivity/resistance of EL4 cells isolated from the tumor to original DOX^{HYD}-HPMA conjugate, (ii) the ability of spleen cells to respond to activation with Con A (T-cell response), LPS (B cell response), and anti-CD3 plus IL-2, (iii) different immune cell subpopulations in blood, and finally (iv) to perform histopathological examination of different organs (tumor, liver, spleen, lung, heart, and bone marrow). The drug sensitivity of EL4 cancer cells isolated from the tumor was comparable with that of original cancer cell line EL4 (IC_{50} = 0.44 μg/mL versus 0.53 μg/mL) and so was the ability of spleen cells to respond to different activation stimuli. Histopathological analyses did not reveal substantial metastatic cancer cell infiltration. Unfortunately, there was not enough material to precisely determine the immune cell subpopulations in the blood. However, we consider the case interesting enough to share it with others as hypothetical documentation of "immunoediting" (Dunn et al., 2004; Prestwich et al., 2008).

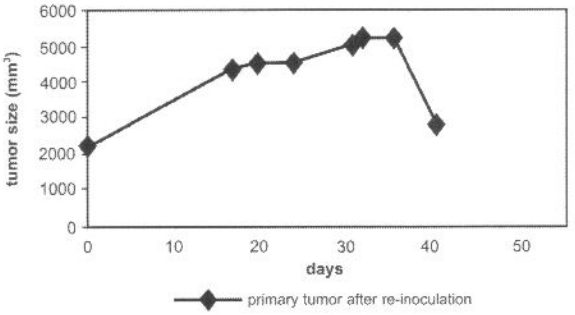

Figure 8. The growth of primary solid EL4 T-cell lymphoma injected s.c. on the right back of experimental mouse (see Figure 6B); after its s.c. re-transplantation with a lethal dose (1×10^5) of the same cancer cells on the left back.

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Declaration of interest

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