Fizikalni mehanizmi i metode u tumorskim terapijama i prijenosu lijekova do tumora

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Review article

Physical mechanisms and methods employed in drug delivery to tumors

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In addition to several well-known drug delivery strategies developed to facilitate effective chemotherapy with anticancer agents, some new approaches have been recently established, based on specific effects arising from the applications of ultrasound, magnetic and electric fields on drug delivery systems. This paper gives an overview of newly developed methods of drug delivery to tumors and of the related anticancer therapies based on the combined use of different physical methods and specific drug carriers. The conventional strategies and new approaches have been put into perspective to revisit the existing and to propose new directions to overcome the threatening problem of cancer diseases.

Keywords: drug delivery, ultrasound, sonoporation, EPR, magnetoliposomes, magnetic fluid hypethermia, electrochemotherapy, electroporation

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Despite the existence of many modes of therapy that have proved their efficacy, such as chemotherapy, immunotherapy, radiotherapy, thermotherapy or gene therapy, successful treatment of cancer still remains a challenge. Development of new anticancer drugs has not been very successful since it has been found that the administration of drugs alone does not result in their high tumor concentrations and reduction in systemic toxicity. The main hindrances to the distribution of anticancer agents to the tumor site are the highly disorganized tumor vasculature, high blood viscosity in the tumor and high interstitial pressure within the tumor tissue (1–3). In the attempt to improve the anticancer therapy, various delivery systems have been introduced to minimize the systemic drug exposure and bring the anticancer drugs to the site of interest – among them, polymer-drug conjugates (4–6), liposomes (7), polymeric micelles (8, 9), micelles (10), microparticles (11), nanoparticles (12–15), lipid nanoparticles (16, 17) and implants (18).

Blood vessels in tumors greatly differ from the blood vessels in normal tissues, showing abnormal vascular architectures and deficient lymphatic drainage systems (19). It was found that these abnormalities are responsible for enhanced vascular permeability for macromolecules, which are retained in tumors for extended periods (20). This phe-

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nomenon, named enhanced permeability and retention effect, is the hallmark of solid tumor vasculature and represents a key mechanism for solid tumor targeting (21, 22).

Apart from the enhanced permeability and retention effect, another strategy employed to enhance the tumor delivery of anticancer agents is local chemotherapy, which has been recognized as a potential method for delivering high drug doses to the target sites, while minimizing systemic exposure (23). In this type of delivery, drug delivery systems are administered at the local site of the tumor and facilitate more effective exposure of cancer cells to drugs and hence greater antitumor activity (24).

In addition to enhanced permeability and retention effect and local chemotherapy, some special strategies, such as receptor-mediated drug delivery, have been adopted for enhancing the tumor delivery of anticancer agents. Here, the targeting is generally achieved by the mechanism of folate-receptor-mediated endocytosis using folate-conjugated polymeric micelles (25), folate-conjugated polymers (26) and folate-coated solid lipid nanoparticles (27).

The measured pH values of the majority of solid tumors are in the range of 5.7–7.8, which is much lower than the pH values of the surrounding normal tissues (28). This difference in extracellular pH between tumor and normal tissues established a new special strategy for drug delivery named pH-sensitive anticancer drug delivery. Han *et al.* (29) introduced a new pH-sensitive functional group containing water-soluble polymers, modified with sulfonamide self-assembled nanoparticles. Drug transport using such pH--sensitive drug carriers enhances drug release and interaction with cells at tumor pH (30). Also, pH-sensitive polymers showed enhanced cytoplasmic delivery of many therapeutics (31, 32), which is a major advantage compared to other drug delivery systems.

As it can be seen, extensive research performed in the area of drug delivery to tumors based on the principles of biology and chemistry resulted in discovery and development of new drug strategies employing different drug carriers. Although all of these chemical and biochemical strategies have resulted in successful enhancement of drug concentrations at tumor sites and marked anticancer activities, they have not shown any appreciable progress in anticancer therapies and only very few products have actually reached the market. In order to introduce new mechanisms of drug delivery and to develop suitable carrier systems for anticancer agents, new strategies based on application of physical methods and techniques have been established in the last two decades. This paper reviews the mechanistics of these novel strategies with examples of recently reported experimental studies, where these approaches have been successfully implemented.

APPLICATION OF ULTRASOUND IN DRUG DELIVERY TO TUMORS

Encapsulation of the drug into Pluronic micelles

During the last two decades, the drug carriers encapsulated into polymeric micelles, a transport system that offers numerous advantages, have been a constant focus of scientific interest (33, 34). Firstly, micelles are self-assembled spherical nanoparticles that have the appropriate size to avoid renal excretion and at the same time allow extravasation through leaky blood vessels. This results in gradual accumulation of the micellar-

-encapsuled drug in the interstitial space between the tumor cells, which enables passive tumor targeting *via* enhanced permeability and the retention effect. In addition, this drug delivery system is independent of drug character and also provides longer circulation time in the blood (35). Furthermore, incorporation of the drug into micelles is relatively simple compared to covalent binding of the drug to the polymeric carrier (36).

Polymeric micelles are hydrophobic-hydrophilic block polymers, with the hydrophilic blocks comprising poly(ethylene oxide) (PEO) chains. The dynamic PEO chains prevent particle opsonization and render them unrecognizable by the reticulo-endothelial system (37). This characteristic of PEO chains has promoted development of new techniques for encapsulation of particles (drugs) based on the processes of physical adsorption and chemical conjugation.

The most frequently used copolymer for encapsulation of drugs into micelles is triblock PEO-PPO-PEO Pluronic copolymer [PPO stands for poly(propylene oxide)]. PPO blocks form the core, whereas flanking PEO blocks form the shell (corona) of the micelle (38). The phase state of Pluronic micelles can be controlled using both the variable PPO/PEO block length ratio and concentration (39). The hydrodynamic radii of Pluronic micelles at physiological temperatures range between 10 and 20 nm, which makes them highly suitable for potential drug carriers.

Drug release and uptake mediated by ultrasound

Encapsulation of a drug into micelles decreases systemic concentration of the free drug, which diminishes intracellular drug uptake by normal cells and reduces unwanted side effects caused by drug interaction with healthy tissues. However, encapsulation of the drug into micelles also decreases its uptake by cancerous cells (40, 41). To overcome this problem, it was necessary to find a mechanism that, at the appropriate time, triggers the release of the drug from the sequestering container at the tumor site. Recently, it was shown that such a controlled triggering mechanism could be achieved by the application of focused ultrasound (42, 43). Ultrasound induces formation of cavitation regions in the cell membrane, which results in increased membrane permeability. This process, called sonoporation, is responsible for enhanced intracellular uptake of both the released and micellar-encapsulated drug (44, 45). Therefore, focusing ultrasound on the tumor provides three important advantages: (*i*) ultrasound increases the permeability of blood vessels (46), thus increasing micelle extravasation at the tumor site; (*ii*) sonication (irradiation by ultrasound) enhances drug release from micelles, and subsequently increases the concentration of the free (non-encapsulated) drug at the tumor site; (*iii*) ultrasound-mediated pertubation of cell membrane and other cellular structures results in formation of cavities along the cell membrane (sonoporation), thereby increasing the uptake of micellar-encapsulated drugs. All three factors work in synergy to ensure localized and effective drug uptake at the tumor site.

Although the exact mechanism of sonoporation is still not fully understood, the triggering mechanism that releases the drug from micelles is analogous to the common effect of acoustic cavitation, frequently used in many biomedical applications (*e.g.* for ultrasonic sterilization of the laboratory and medical equipment) (47). Acoustic cavitation refers to the formation, growth by »rectified diffusion« and collapse of gas- and vapor-filled bubbles in liquids exposed to ultrasound above threshold intensity (48). This pro-

cess is based on conversion of the low energy density of the ultrasound field into the high energy density in the interior and the surroundings of the bubbles. Such accumulation of ultrasonic energy induces tremendous internal pressures and temperatures within these small bubbles, which tend to rise until they violently collapse.

Husseini *et al.* (41) showed a strong correlation between the percentage of the released drug and subharmonic acoustic emissions detected by fluorescence measurements. Performed measurements indicated that the drug release is triggered by the cavitation, which perturbs the structure of the micelle and releases the drug.

In the study of Liu *et al.* (49), it is shown that transient acoustic cavitation plays a dominant role in drug release from micelle cores. In the experiments on the effect of ultrasound on the drug uptake from Pluronic solutions they observed a threshold of power densities, below which no cell lysis occurred. However, sonication at power densities above the threshold resulted in pronounced cell lysis. The existence of a threshold suggested the important role of transient cavitation in the perturbation and damaging of the cell membrane and in cell lysis. It was therefore proposed that, in addition to the releasing mechanism, the violently collapsing bubbles or micelles are responsible for mechanical rupturing of the cell membrane, hence inducing sonoporation and allowing entry of molecules (drugs) into the cells (50).

Drug targeting by ultrasound requires the use of sharp ultrasound waves focused on the tumor and does not require ultra-high ultrasound energies. Typically, the frequencies of ultrasound used in studies of micellar-encapsulated drug delivery are in the range of 20–90 kHz (41–45). It has been found that drug release was most efficient at ultrasonic frequency of 20 kHz and decreased with increasing frequency despite much higher power densities (41). It has also been shown that at constant frequency drug release increases with increasing power density (41). Optimal power density of ultrasound waves ranges from 1 to 5 W cm^{-2}, depending on the time period of sonication, which is typically 30 s up to a few minutes when continuous wave ultrasound is applied (41–45). Higher ultrasound energies (5–15 kW cm^{-2}) and longer treatment times (a few hours) are used to directly kill the tumor cells by means of hyperthermia and tumor ablation. In addition to the continuous wave ultrasound exposures, scientists are nowadays beginning to use pulsed or high-frequency focused ultrasound. The effect of high-frequency ultrasound on the drug release from Pluronic micelles and intracellular uptake by leukemia HL-60 cells was studied by Marin *et al.* (44). The onset transient cavitation and drug release from micelles were observed at much higher power densities than those measured at low-frequency ultrasound. Although several studies have shown the advantages of pulsed high-intensity focused ultrasound exposures for enhancing the delivery of micellar-encapsulted drugs, the work of Frenkal *et al.* (51) revealed that it was not the case for drugs encapsulated in liposomes, probably because of their inherent ability to preferentially accumulate into tumors on their own. Exposure of micellar-encapsulated drugs to pulsed ultrasound waves showed that the drug uptake increases with increasing pulse duration in the range of 0.1–2 s, and for pulses with longer duration the uptake is close to that under exposure to continuous ultrasound waves (43).

The most widely used chemotherapeutic agent in the ultrasound-mediated drug delivery studies is doxorubicin (DOX). DOX is an intercalating drug that stacks between paired bases in DNA. A strong drug-DNA interaction is critical for the cytotoxic effect. However, like other anticancer drugs of anthracycline family, DOX is cardiotoxic due to

the induced production of active oxygen radicals (52). In the last few years, numerous studies of DOX delivery and action were performed *in vitro* on leukemia HL-60 cells, ovarian carcinoma drug-sensitive and multidrug-resistant cells and breast cancer cells, all of them showing enhanced DOX release from micelles and increased uptake of the free and micellar-encapsulated DOX mediated by ultrasound (41–46).

The most detailed study of ultrasonic enhancement of the uptake of DOX by cancerous cells from Pluronic micelles was performed by Marin *et al.* (43). They employed various techniques, such as electron paramagnetic resonance spectroscopy, fluorescence microscopy and flow cytometry, in order to examine ultrasound-mediated enhancement of both the intracellular uptake of Pluronic micelles and Pluronic trafficking into cell nuclei. A model has been suggested that describes various equilibria controlling drug/cell interactions and the effect of ultrasound on these equilibria. Under the action of ultrasound, the equilibrium between the micellar-encapsulated and free drug is shifted in the direction of the free drug due to the micelle perturbation. The equilibrium between the extracellular and internalized drug is shifted to the intracellular drug because of the ultrasound--induced cellular changes that enhance the accessibility of various cellular structures to the drug. They also showed another important advantage of ultrasound – the same degree of the intracellular drug uptake may be achieved at a substantially lower drug concentration in the incubation medium.

The significant success of *in vitro* studies of ultrasound-mediated drug delivery promoted the experiments on laboratory animals and recently the first *in vivo* experiment on colon cancer in rats was reported (53). *In vivo* results showed that application of low--frequency ultrasound (20–70 kHz) significantly reduced the tumor size compared to non--irradiated controls.

Except in cell cultures, there are very few studies of ultrasound-mediated drug delivery performed on animal models. The knowledge of the mechanism of sonoporation is still very limited, which constitutes a major obstacle in determination of the factors affecting acoustically triggered release and in the development of the standard protocols for successful anticancer therapy. It is to be expected, however, that in the near future a growing amount of experimental data would lead to extensive application of this strategy in various cancer models *in vivo*.

EPR experiments in ultrasound-mediated drug delivery and cell killing

Electron paramagnetic resonance (EPR) spectroscopy is a technique that allows detection of molecules with an unpaired electron (radicals) by measuring the absorption of high frequency microwave energy during the transition of the unpaired electron between two spin levels, which have different energies when exposed to an external magnetic field.

Unfortunately, in the majority of biological systems, radicals have a strong tendency to pair the spin of the lone electron by reacting with another molecule or radical, which results in a lifetime too short to build up sufficient steady-state concentrations that would permit the EPR detection. One of the most useful techniques employed to overcome this limitation is the spin trapping method. In this technique, the reactive radical adds to the double bond of diamagnetic molecule (spin trap) forming a more stable covalent paramagnetic adduct, which is EPR observable. The most frequently used are nitrone and

nitroso spin traps with the unpaired electron located on oxygen atom (N-O), such as *N-tert*-butyl- -phenyl nitrone (PBN), -(4-pyridyl-1-oxy)-*N-tert*-butylnitrone (POBN) or 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). Today, EPR spectroscopy is the most confident technique for both the direct detection and characterization of the free radicals already present in the system and for monitoring the actions of spin labeled particles added in the system. There is a wide range of applications of this physical technique in biological, biochemical and biophysical studies of the free radicals in biologically interesting systems. Some of these applications include studies of free radicals formed in irradiated model systems of DNA bases and their analogues (54, 55), studies of the complexation of the DNA bases with metallic ions (56, 57) and studies of the complexation of the ions of transition metals with biologically important molecules (58, 59).

In the experiments of ultrasound-mediated drug delivery, a very frequently used drug is ruboxyl, a paramagnetic analogue of doxorubicin. Ruboxyl has a nitroxide part in its structure and can thus serve as a spin trap, which can be easily monitored by EPR. Ruboxyl molecule is also fluorescent (because of the anthracycline part), which makes it a powerful tool for investigations of the drug uptake, distribution and metabolism. Using the EPR technique, Rapoport *et al.* (60) investigated the effect of the Pluronic micelle structure and ultrasound on the uptake of ruboxyl and the distribution of ruboxyl molecules in Pluronic micelles within various temperature regions. They observed two distinct EPR spectra of ruboxyl in Pluronic solutions with different values of characteristic EPR parameters (hyperfine splittings), which indicated the existence of two drug populations differing in mobility and hydrophilicity of the nitroxide environment – one population located outside the micelles and the other in the micelles' core. From the features of these two EPR spectra, they also concluded that 70% of ruboxyl molecules were solubilized within Pluronic micelles at room temperature. In this manner, the EPR technique can be used for screening various members of the Pluronic family in order to determine their micellization behavior. Moreover, the EPR technique can provide useful information on the structure of Pluronic micellar solutions, such as solubilization efficiency, polarity and microviscosity of the solute environment, critical concentration for the formation of micelles and micelle aggregation number (60).

Several studies have reported that products of ultrasonic cavitation are free radicals, such as hydroxyl, singlet oxygen and hydroperoxyl, indicating that these molecules play an important role in the synergism between ultrasound exposure and drugs (61). EPR spin trapping technique has been used to study these reactive radical intermediates in a promising new approach for cancer treatment termed sonodynamic therapy, which refers to synergistic cell killing by both ultrasound and drugs. In several sonochemical EPR studies, Mišik *et al.* (61, 62) showed that the cavitation-dependent sonodynamic action of different classes of compounds is mediated by the formation of free radicals by pyrolysis inside cavitation bubbles or by hydrogen abstraction by OH radicals and H atoms from these compounds, followed by the formation of peroxyl radicals in the presence of oxygen. These radicals, by virtue of their lower reactivity and hence higher selectivity, are capable of diffusing longer distances and damaging important cellular sites more efficiently than the short living H and OH radicals.

The structure and reactivity of products emerging by interactions of naturally occurring antioxidants (vitamins) with radical intermediates can be easily followed by EPR. In order to determine which free radicals lead to an increase of drug content, effects of

free radical scavengers (antioxidants) on increased intracellular adriamycin accumulation mediated by ultrasound were investigated by Yu *et al.* (63). They showed that the hydroxyl radical is the most important, because it leads to promotion of membrane permeability. Also, the study revealed that vitamin C and vitamin E are very effective in decreasing intracellular uptake of adriamycin, indicating that antioxidants effectively reduce cavitation by rapidly reacting with O_2^- radicals and thus protect cells from damage induced by ultrasound exposure.

The measurement of sonoporation using the voltage clamp technique

Due to the lack of methods for real-time monitoring of sonoporation at the cellular level, the efficiency of drug delivery and sonoporation-associated side effects, such as loss of cell viability and enhanced apoptosis, have been studied only by post-ultrasound exposure analyses. One of the techniques that allows real-time monitoring of the changes in the cell membrane permeability is the voltage clamp technique. The voltage clamp is a current generator with two electrodes. Transmembrane voltage is recorded through a voltage electrode, relative to the ground, and a current electrode passes the current into the cell. The electrodes are connected to an amplifier, which measures membrane potential (V_m) and also gets an input from the signal generator that determines the command potential (V_c). The difference between the command potential and actual voltage of the cell ($V_c - V_m$) is an output to the electrode current. Whenever the membrane potential deviates from the command potential, the amplifier generates an error signal, and the feedback circuit passes the current into the cell to reduce the error signal to zero. Thus, the clamp circuit produces a current equal and opposite to the ionic current. This can be measured, giving an accurate reproduction of the currents flowing across the membrane. Detected changes in the transmembrane current amplitude directly reflect the changes in the cell membrane conductance and can therefore provide sensitive indications of the formation and subsequent resealing of pores in the cell membrane.

To investigate the dynamics of sonoporation, Deng *et al.* (64) employed the two-electrode voltage clamp technique to obtain a real-time measurement of the transmembrane current of a single *Xenopus* oocyte during ultrasound irradiation in the presence of albinum-shelled gas bubbles. During measurements, the membrane potential of the oocyte was clamped at –50 mV by two microelectrodes inserted into its membrane. Ultrasound activation and duration were synchronized using trigger signals from the voltage clamp for recording the transmembrane current and thus permitting capture of the cell membrane status before, during and after ultrasound irradiation in real-time. The observed characteristic stepwise transmembrane current changes during the voltage clamp measurements are ascribed to an ultrasound-mediated pore formation (increasing of transmembrane current) and subsequent recovery of the cell membrane (decreasing of transmembrane current). They also showed that the inward transmembrane current increases after a delay of ultrasound activation and that the current reaches a maximum value at the end of ultrasound duration. For the ultrasound with the duration of 1 s and pressure amplitude less than 1 MPa, the observed recovery time is in the order of 4 to 10 s. For higher ultrasound pressures (greater than 1 MPa), they observed an irreversible current increase, which indicates spontaneous cell death, confirmed also by an immediate microscopic examination.

In a similar study using *Xenopus* oocyte as a model system, Zhou *et al.* (65) employed the voltage technique to investigate the pore membrane formation, extension and resealing by measuring the transmembrane current as a direct result of decreased membrane resistance due to the pore formation induced by ultrasound application. They observed that the transmembrane current amplitude increases with increasing duration of the applied ultrasound waves, while the recovering mechanism of membrane pores and cell survival rate decreases with longer duration.

These studies demonstrate that the novel application of electrophysiology methods, such as the two-electrode voltage clamp techniques, allows to study the cell response to ultrasound exposure in real-time. Monitoring and characterization of the dynamic cell sonoporation process provides information essential for better understanding of the sonoporation mechanism, which can then serve as a guidance for optimal design of ultrasound protocol applied in drug delivery to tumors.

APPLICATION OF MAGNETIC FIELDS IN DRUG DELIVERY TO TUMORS

Drug delivery by magnetoliposomes

Magnetoliposomes are liposomes with ferromagnetic particles embedded into their lipid bilayer (66). Magnetic particles functionalized with the drug can serve as potential drug carriers in a new drug delivery strategy based on the application of external magnetic fields. The principle of drug delivery by magnetoliposomes is based on the use of both constant and high-frequency oscillating magnetic fields. A constant magnetic field provides targeted drug delivery, while a high-frequency oscillating magnetic field is responsible for the controlled release of encapsulated drug. Since the magnetic particles in magnetoliposomes are made of ferromagnetic materials (iron, cobalt, nickel as well as alloys containing these elements), they are attracted to high magnetic flux density and thus can be targeted to specific areas (cancer tissues) by external constant magnetic fields. Releasing mechanism is evoked by thermal excitations of these biocompatible magnetic particles induced by an external high-oscillating magnetic field (67). Magnetic-field-induced excitations produce heat that increases the temperature of magnetoliposomes. Such specific heating represents a triggering mechanism for controlled release of conjugated therapeutic compounds from magnetoliposomes when they are heated to sufficiently high temperatures (around 40 °C) (68).

It has been shown that by using this kind of transport, the drug may be applied very selectively at a particular site of the organism and this procedure may be repeated several times using stealth magnetoliposomes, which are circulating in the blood-stream for several days (69).

Moreover, magnetoliposomes concentrated by the external constant magnetic field in tumor vasculature may lead to embolic lesions and necrosis of a tumor body. In addition, the heat produced for thermal activation of the drug enhances the effect of chemotherapy by means of local hyperthermia of tumor cells (see next chapter).

Zhang *et al.* (70) reported that lyophilized negatively charged magnetoliposomes could be used as paclitaxel carriers in the treatment of breast carcinoma. Pharmacokinetic stu-

dies showed that encapsulation of paclitaxel in magnetoliposomes produced a much higher concentration of paclitaxel at the tumor site than the use of lyophilized conventional liposomes.

Babincova *et al.* (71) developed magnetoliposomes encapsulated with doxorubicin for site-specific anticancer therapy in response to an externally applied oscillating magnetic field. The results revealed that specifical heating of magnetoliposomes to 42 °C resulted in a massive release of encapsulated doxorubicin.

Another *in vivo* study with doxorubicin showed that administration of magnetoliposomes under an applied external magnetic field produced an approximately four times higher doxorubicin concentration in the tumor compared to the doxorubicin solution applied (72). These results suggest that systemic chemotherapy could effectively control the primary tumor without significant side effects due to the specific targeting of magnetic doxorubicin liposomes.

Kullberg *et al.* (73) used magnetoliposomes to target the epidermal growth factor (EGF) receptors of the tumor cells. Combining the transport of toxins by magnetoliposomes with the increased level of specificity originating from additional EGF receptor molecules, they showed that a tumor cell endosome has a 25 times greater chance to receive the toxin in comparison with normal cells.

All of these studies promote a new strategy for drug delivery to tumors based on specifically developed drug carriers, consisting of magnetic particles that can be targeted by an external magnetic field. The application of external magnetic fields to these functionalized magnetic particles with their unique features further improves the drug delivery to tumor tissues and establishes a new anticancer treatment, generally based on the physical principles of magnetism.

Magnetic fluid hyperthermia

Hyperthermia is the use of therapeutic heat to treat various cancers on and inside the body. The purpose of this anticancer therapy is to shrink and hopefully destroy cancer without harming noncancerous cells. It can be used to treat cancer in many areas of the body, including brain (74), thyroid (75), lungs (76), breast (77), and prostate (78). It is thought that high temperatures, up to 40 °C, can help shrink cancerous tumors. Hyperthermia is now being used more widely, because it does not have as many negative side effects as conventional forms of cancer treatment such as radiation or chemotherapy. In some instances, hyperthermia is used in combination with other forms of cancer therapy (79).

Hyperthermia can be used on very small areas of the body to the entire body itself. Local hyperthermia refers to heating only one body area, usually where the tumor is located. The heat can be applied using microwaves (80), high-frequency radio waves (81) or magnetic fields (82). The heating mechanism achieved by the combined use of magnetic fields and specifically developed hyperthermia-causing anticancer agents will be discussed in detail.

Magnetic fluid hyperthermia (MFH) is a novel anticancer therapy, based on the use of magnetic fluids composed of ferromagnetic nanoparticles coated with biocompatible materials (83). These magnetic particles can be targeted to specific areas of a human body by focused external magnetic fields or by simple intratumoral injection (83).

Since magnetic fluids have a specifically high absorption rate per mass, by the use of external oscillating magnetic field, it is possible to selectively excite areas containing fluids without interference with different neighboring tissues that normally have a lower absorption rate (67). Such magnetic-field-induced excitation produces heat absorbed mostly by magnetic fluids and thus increasing the temperature only in regions with accumulated magnetic nanoparticles.

The main objectives in providing a successful MFH treatment are: (*i*) to create stable magnetic fluids, (*ii*) to develop an apparatus that is capable of producing oscillating magnetic fields and heating the magnetic fluids, (*iii*) to determine the range of field strengths and frequencies suitable for particular fluids used in MFH therapies, and (*iv*) to estimate the specific absorption rate of magnetic fluids accumulated at tumor sites as well as the number, size and position of magnetic nanoparticles depots required for sufficient heat deposition. The results of electron microscopy, X-ray spectroscopy and calorimetric measurements in several studies have confirmed that the use of magnetic fluids with strengths between 4–12.5 kA m^{-1} and frequencies in the region 500–800 kHz (depending on the used magnetic nanoparticles) is optimal for producing heating of magnetic fluids up to 42 °C, which is required for successful MFH treatments (83–86).

Nanoparticles found to be very promising for the use in magnetic fluids for MFH treatments are superparamagnetic nanoparticles with the core consisting of iron oxides that can be targeted through external magnetic fields (85). In several studies, the application of Fe_2O_3 nanoparticles in MFH treatments has been investigated *in vivo* on patients with hepatocellular carcinoma (86, 87). The use of iron oxide particles inhibits proliferation and induces apoptosis of SMMC-7731 tumor cells and thus has a significant inhibitory effect on the mass and volume of treated cancer, superior to the effects produced by conventional anticancer therapies.

Johannsen *et al.* (83) evaluated the potential of MFH as a minimally invasive treatment for prostate cancer by carrying out a systematic analysis of the effects of MFH in the orthotopic Dunning tumor model of the rat. Rats received two MFH treatments following a single intratumoral injection of magnetic fluid. Treatments were carried out for 10 days after tumor induction using an oscillating magnetic field. The rats were sacrificed after 20 days, and the tumor mass was determined and compared with the control. The results indicated that the MFH treatment led to significant growth inhibition in the orthotopic model of this tumor.

In another study, this group of authors reported on the evaluation of the effect of MFH therapy combined with external radiation in the Dunning tumor model of prostate cancer induced in rats (88). They showed an additive effect demonstrated for the combined treatment at a radiation dose of 20 Gy, which was equally effective in inhibiting tumor growth as 60 Gy radiation alone.

Recently, the same group of authors presented the first clinical application of interstitial hyperthermia treatment using magnetic nanoparticles in locally recurrent human prostate cancer (89). The study provided preliminary results necessary for the evaluation of feasibility, toxicity and quality of life during MFH in patients with biopsy-proven local recurrence of prostate cancer following radiotherapy.

Fortunately, the obtained data clearly indicated that the administration of biocompatible magnetic nanoparticles excited by oscillating external magnetic fields in the mag-

netic fluid hyperthermia anticancer therapy is a promising new approach for anticancer treatment. Also, there is no doubt that rapidly growing development of new drug carrier systems containing magnetic nanoparticles, along with the huge amount of new biological data on heat response of cells and tissues, will further improve this anticancer treatment.

APPLICATION OF ELECTRIC FIELDS IN DRUG DELIVERY TO TUMORS

Electrochemotherapy

The combined treatment consisting of a chemotherapeutic agent and pulsed electric fields has been termed electrochemotherapy (ECT). This relatively new treatment relies on the physical effect of locally applied electric fields to destabilize cell membranes in the presence of a drug. The destabilization of cell membranes has been described as a dielectric breakdown due to an induced transmembrane potential that results from electrical treatment (90). When the transmembrane voltage induced by the external electric field exceeds a certain threshold (normally 0.2–1 V), a rearrangement of the molecular structure of the membrane occurs, leading to pore formation and a considerable increase in the cell membrane permeability to ions, molecules and even macromolecules. This physical phenomenon was termed electroporation or electropermeabilization, because it was observed that molecules that do not normally pass the membrane gain intracellular access after the cells were treated with electric fields. In the case of small molecules, such as anticancer drugs, transfer into the cell interior occurs through simple diffusion, and in the case of macromolecules, such as DNA, transfer is accomplished by a multi-step mechanism that involves electrophoretically driven processes allowing passage of the macromolecules through the destabilized membrane (91). Porous state of the cell membrane was noted to be temporary. Typically, upon the application of electric pulses, cells remain in a destabilized state for several minutes.

In the ECT treatments, the electric-field-mediated permeabilized state of membranes is used to load tumor cells with anticancer drugs that do not easily enter the cells through intact membranes (non-permeant drugs) (92) or partially enter the cells by diffusion (low-permeant drugs) (93).

There are four parameters to be taken into account when administering the electric component of ECT: electric field strength, pulse width (duration of the pulse), number of pulses and duty cycle. The intensity of electrical treatments is described by the magnitude of the applied electric field. Electric field strengths ranging from 1100 to 5000 V cm^{-1} have been used for ECT and are also specific to the cells (tissues) under investigation. Field strengths below 1250 V cm^{-1} were found to be less effective in most ECT treatments (94). Fields between 1250 and 1500 V cm^{-1} gave similar results, providing excellent delivery and marked antitumor effects (94). Pulses are usually rectangular in shape; however, exponentially decaying pulses have also been used. ECT has been performed with the pulse widths ranging from microseconds to milliseconds. The number of delivered pulses can range from one to eight pulses per second. Typically, multiple pulses are utilized during the ECT treatment, allowing the use of much lower doses of anticancer agents (up to 20-fold decrease) in ECT treatments that still yield results equivalent to

a single treatment (95). Also, it was noted that the maximum antitumor effects were obtained when pulses were administered between 8 and 28 min after injection of the anticancer agents (96). This indicated the time when the drug concentrations were highest in the interstitial fluid surrounding the tumor cells.

Thus far, only two drugs have been used in electrochemotherapy – non-permeant bleomycin and low-permeant cisplatin. The anticancer effect of other currently used drugs is not increased by the electric pulses because these drugs easily enter the cells by diffusion or are actively pumped inside the cells (93). The drug most often used in ECT protocols and the only one used in clinical trials is bleomycin.

Bleomycin is a non-permeant hydrophilic drug and normally almost no bleomycin molecules enter the intact cells. However, once inside the cell, bleomycin acts as an enzyme, creating single- and double-strand DNA-breaks and the uptake of 500 molecules is sufficient to kill the cells (97). When the cells are electroporated, bleomycin at an external concentration as low as 10^{-9} mol L^{-1} can enter the cells (91). Consequently, cytotoxicity of bleomycin can be augmented hundreds or thousands times by electroporation.

Jordan *et al.* (98) performed an experiment to enhance cell-killing effects of bleomycin using electric field pulses with 50–200 kV cm^{-1} peak electric field strength, 150 ns duration and nanosecond rise time. Dramatic increases in cell killing (approximately factor 1000) were observed with a low dose of bleomycin after treatment with trains of ten or more pulses at all electric field strengths tested, compared to pulse-only or drug--only treatments.

Byrne *et al.* (99) performed a study on nineteen patients with metastatic melanoma to compare the anticancer activity using bleomycin together with ECT treatment with bleomycin alone. Over a period longer than 12 weeks, they showed a 78% objective response rate in the patients treated with bleomycin/ECT, which was significantly higher than the 32% response rate observed in the patients treated with bleomycin alone.

Sersa *et al.* (100) reported that a metastasis of hypernephroma could be successfully treated with ECT with bleomycin. ECT treatment resulted in stabilization of the tumor volume for 12 months, whereas the subcutaneous metastasis next to the ECT-treated one that did not receive electric pulses progressed immediately.

Applications of ECT with bleomycin are widely used nowadays. A large number of animal models and recent clinical trials have been conducted for the treatment of various types of tumors, such as head and neck squamos cell carcinoma, melanoma, hypernephroma and basal cell carcinoma. Treatments of nonmetastatic cancers, such as basal cell carcinoma, are ideal applications for ECT. The advantage of ECT in comparison with other available therapies is the minimal scaring produced by a single treatment, for which ECT can be used as a tissue sparing treatment. The use of ECT for extensive metastatic diseases is not practical, except for palliative measures.

Recently, extensive research has been performed on the combined use of ultrasound of various intensities (for both thermal effects and enhancement of drug delivery) with permeabilizing effects of electric pulses on cell membranes. It was demonstrated in several studies that tumor cells may be sensitized and subsequently destructed by this joint treatment (101, 102). Obtained results suggest that the combined electric field and ultrasound therapy may provide a novel, drug-free modality for cancer treatment that might have a great perspective in the near future.

The latest modeling and experimental studies have shown that pulsed electric fields of nanosecond duration (typically 10 ns) and megavolt per meter amplitude affect subcellular structures, but do not lead to formation of large pores in the outer membrane (103). Such short, highly powerful electric pulses can penetrate into the interior of tumor cells and cause tumor cell nuclei to rapidly shrink and tumor blood flow to stop. Nuccitelli *et al.* (104) showed that this »intracellular electromanipulation« could shrink melanomas by 90% within two weeks following a cumulative exposure to electric fields with strengths greater than 20 kV cm^{-1} and 300 ns durations. This new therapy for treating solid skin tumors provides a highly localized targeting of tumor cells with only minor effects on the overlying skin. Typically, each pulse deposits energy of 0.2 J and 100 pulses increase the temperature of the treated region by only 3 °C, which is still lower than the minimum temperature required for hyperthermia effects (104).

ECT is a noninvasive therapy for treatment of solid tumors that has proven its efficacy in many different tumor types, both in preclinical and clinical trials. Investigations of ECT in a multicenter study, called European Standard Operating Procedures for Electrochemotherapy, demonstrate that over 80% of cutaneous and subcutaneous metastatic nodules can be healed by ECT (105). Also, the physical nature of electroporation allows the technique to be applied to any tissue. Therefore, a logical step forward for this drug delivery method would be to adopt protocols for the treatment of internal tumors. This includes treating nonresectable tumors and reducing large tumors during standard chemotherapy treatments or prior to surgical excision. An additional investigation for translating ECT to internal tumors is currently in progress. All these facts, together with continuous development of appropriate equipment and efforts to prepare standard optimized operating procedures, make the ECT a real alternative offered to treat solid tumors.

CONCLUSIONS

During the last 20 years, it was shown that there are unprecedented opportunities to employ the mechanisms and principles of physics in order to gain more information about the tumor cell behavior and to introduce and develop new mechanisms of drug delivery and new carriers for anticancer agents. As described in this review, there are plenty of possibilities of engaging the knowledge of physicists to acquire new insights into the drug delivery to tumors and anticancer treatments. The proven efficacy of the physical mechanisms for drug targeting and delivering to tumor cells, such as sonoporation or electroporation, indicates that future success in developing new and improving the existing effective anticancer therapies will require a coordinated, multidisciplinary team approach, which must involve the knowledge of physicists.

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S A Ž E T A K

Fizikalni mehanizmi i metode u prijenosu lijekova do tumora

ERIM BEŠIĆ

Osim dobro poznatih metoda prijenosa lijekova u kemoterapijskom pristupu liječenja tumora, nedavno su otkriveni novi načini prijenosa koji se zasnivaju na specifičnim mehanizmima uzrokovanim upotrebom ultrazvuka, magnetskih i električnih polja. U članku se daje prikaz fizikalnih mehanizama na kojima se temelje te nove metode, pregled novootkrivenih prijenosnika lijekova (Pluronske micele, magnetoliposomi, magnetski fluidi), novih načina terapije tumora (magnetska hipertermija, elektrokemoterapija) i najnovijih istraživanja temeljenih na fizikalnom pristupu ovoj problematici.

Ključne riječi: prijenos lijekova, ultrazvuk, sonoporacija, EPR, magnetoliposomi, magnetska hipertermija, elektrokemoterapija, elektroporacija

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