CELLULAR AND MOLECULAR MECHANISMS OF ELECTROCHEMOTHERAPY, SONOPORATION, PHOTODYNAMIC THERAPY AND SONODYNAMIC THERAPY IN TERMS OF ONCOLOGICAL TREATMENT

KOMÓRKOWE I MOLEKULARNE MECHANIZMY ELEKTROCHEMIOTERAPII, SONOPORACJI, TERAPII FOTODYNAMICZNEJ ORAZ TERAPII SONODYNAMICZNEJ W LECZENIU ONKOLOGICZNYM

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Summary: The review focuses on cellular and molecular mechanisms of four novel cancer therapies: electrochemotherapy, sonoporation, photodynamic therapy and sonodynamic therapy. The electrochemotherapy is based on electropermeabilization of cell membranes in the presence of chemotherapeutic drugs in order to increase their uptake by cancer cells. In sonoporation process of propagation of sound wave in a tissue excites microbubbles leading to altered cell membrane permeability. The photodynamic therapy uses reaction between photosensitizer accumulated in tumor cells, light with a specific wavelength that excites the photosensitizer and oxygen in a tissue. The last technique, sonodynamic therapy, likewise photodynamic therapy, creates reactive oxygen species. Although, in this case, the excitation is obtained by ultrasound waves.

Keywords: electrochemotherapy, sonoporation, photodynamic therapy, sonodynamic therapy, free radicals, reactive oxygen species

Streszczenie: Artykuł skupia się na mechanizmie komórkowym oraz molekularnym działania czterech obiecujących terapii przeciwnowotworowych: elektrochemioterapia, sonoporacja, terapia fotodynamiczna i terapia sonodynamiczna. Elektrochemioterapia polega na zwiększaniu prze-

puszczalności błony komórkowej pod wpływem pola elektrycznego. W efekcie leki przeciwnowotworowe docierają do komórek nowotworowych w wyższych stężeniach. W sonoporacji fale dźwiękowe rozchodzące się w tkankach powodują ekscytację mikropęcherzyków prowadząc do zmiany w przepuszczalności błony komórkowej i transporcie leków. Terapia fotodynamiczna wykorzystuje reakcję pomiędzy substancją światłoczułą , skumulowaną w tkance nowotworowej, światłem o długości dobranej tak, aby aktywowała substancję światłoczułą oraz tlenem znajdującym się w tkance. Podobnie jak terapia fotodynamiczna, terapia sonodynamiczna, polega na tworzeniu wolnych rodników. Jednakże, w tym przypadku lek jest aktywowany przez ultradźwięki.

Słowa kluczowe: elektrochemioterapia, sonoporacja, terapia fotodynamiczna, terapia sonodynamiczna, wolne rodniki, reaktywne formy tlenu

INTRODUCTION

There is no doubt that cancer is one of the leading health problems in the world. World Health Organization estimates that it was the cause of around 9.6 million deaths in the year 2018 worldwide, 1 in 6 deaths was due to cancer. Nowadays, there are four main approaches to cancer treatment. First, the most obvious and the less specific, surgery. Second and third, more specific, but on the other hand affecting also non-neoplastic tissue, chemotherapy and radiotherapy. And fourth, that is currently in rapid development, immunotherapy – specific but still not exactly cost efficient and burdened with the risk of cytokine release syndrome (CRS). CRS is rarely fatal for the patient but the risk of CRS significantly rises with augmentation of the dose [20]. The above listed therapies undoubtedly have their advantages and disadvantages. By virtue of those standard, guideline therapies we were able to achieve satisfactory effects against some cancers whereas other types of cancers are still characterized by low survival rate. Worth mentioning is also impact of screening exams in faster recognition and management of neoplasia. Nevertheless, some types of cancers advance extremely fast and are so infrequent that a screening study cannot be implemented. Perfect example of a tumor with both mentioned hindrances is glioblastoma multiforme. Barely none patient survives more than 2.5 years, owing to fast progression and the incidence rate is rather low, 3.19 per 100,000 persons in the United States [65].

Taking everything into account development of novel cancer therapies is crucial. We would like to present an overlook focused on cellular and molecular bases of four approaches that are now rising as alternative or complementary oncological treatments: electrochemotherapy (ECT), sonoporation (SP), photodynamic therapy (PDT) and sonodynamic therapy (SDT). They are characterized by strong selectivity for a cancer tissue due to local character of the treatment. Moreover the maximum tolerated dose of properly chosen, optimised drug is generally higher than in immunotherapy, radiotherapy or chemotherapy on their own.

ELEKTROCHEMOTHERAPY

Electrochemotherapy (ECT) is an antitumor treatment modality in which the local application of electrical field causes electroporation (or electropermeabilization) and thus enables introduction of cytotoxic, nonpermeant drug into the cells. Although the permeability changes induced by electric impulses in vesicular membranes were already reported in 1972 [50]. The first clinical trial of ECT took place in 1991. At the time the researchers from the Institute Gustave Roussy in France demonstrated the antitumor effectiveness of treatment with intravenously injected bleomycin followed by electric pulses in head and neck squamous cell carcinoma patients [45]. Since then the technique gained on importance becoming a widely used procedure in various malignant lesions. On the contrary, the phenomenon of electropermeabilization is not only applied to increase chemotherapeutic drug transport but also enables introduction of genes into cells (gene therapy) as well as DNA vaccine delivery [34]. Moreover, it is widely applied in biotechnology in food processing [43].

ECT consists of intratumoral or intravenous administration of a chemotherapeutic drug, followed by local application of electric pulses to the target tumor, in order to enhance medicaments intracellular transport. According to European standard operating procedures of the electrochemotherapy (ESOPE), published in 2006, the method requires the application of eight, short (100μs), pulses at 1 or 5000 Hz frequency and specified electric field intensity (100V/cm) [46]. Depending on the shape, size and region of the nodule, different sets of electrodes can be chosen. Both plate and needle electrodes are available to provide sufficient electric field, covering the whole tumor volume. The application of sufficiently strong electric field causes redistribution of ions in the liquid environment surrounding lipid cell membrane, which acts as a dielectric. An additional transmembrane voltage is induced, that can be added to normal cell's resting potential. The lipid membrane and adjacent electrolyte solution are equivalent to an electrical condenser, nevertheless, it consist of highly dynamic, mobile molecules. Such a membrane condenser favors the entrance of water molecules to produce localized cross-membrane pores. These transient geometrical changes in the lipid layer make it permeable for charged and larger molecules, for instance cytotoxic drugs [26]. In a short time after delivering the electric pulses cell membrane regenerates and pores disappear. Alternative technique is irreversible electroporation, in which stronger electric field is applied in order to provoke permanent permeabilization and thus cell death due to homeostasis disruption [54]. Both tissue ablation modalities are called non-thermal, since the damage of the cells does not come from the temperature increase. In case of electrochemotherapy, the lethal effects on target tumor cells result from the intrinsic cytotoxicity of chemotherapeutic agents, such as bleomycin, that cross the membrane. Moreover, studies show that

the mentioned drugs are preferably absorbed by the actively dividing cancer cells than by the nondividing population of normal cells in the surrounding tissue. The tumor cell targeting properties of bleomycin are thought to be caused by disaccharide moiety in its structure [58]. Further important effect of ECT on malignant cells is vascular lock, electrical-induced vasoconstriction and collapse of tumor vessels leading to hypoperfusion. Reduced blood flow not only increases drug retaining time and expose cancer cells to lack of oxygen but also prevents bleeding of treated well-vascularized organs [25]. Other research proved that the membrane repairs more effectively in the normal cell line than in cancer cell lines [19], which contributes to conclusion that therapies based on electropermeabilization are more effective on malignant cells compared to normal cells in cancer treatment.

ECT is already a well-established procedure in treatment of cutaneous and subcutaneous metastases located in head or neck as well as primary skin cancer, with objective response ranging from 75% to 99% [17]. The new applications of the method focus on management of large tumors, such as breast cancer metastases to the skin and therapy of deep-seated metastases, located for instance in liver or brain [8, 16, 39]. These techniques require careful 3-D treatment planning, a most important current challenge for ECT. In case of brain metastases, the researchers from the Center for Experimental Drug and Gene Electrotransfer in Copenhagen have made an interesting progress. During ECT procedure a novel expandable brain electrode device was mounted in a stereotactic frame in a specifically developed driver unit, making it possible to carefully reach the correct coordinates, estimated from the MRI scans. Other novel approach was demonstrated by Sersa et al. (2015), whose study presents using current density imaging to monitor electroporation-based therapy [59].

SONOPORATION

While carrying out ECT it is crucial to use electrodes near the tumor, in the case of sonoporation (SP) no invasive equipment is needed to reach deeper layers of tissues. First attempts to use ultrasound (US) as a facilitating factor for anticancer drug delivery were made in the second half of 90’ last century. High US intensities were set to observe cavitation within tissues. Such intense exposure to acoustic waves could cause temperature increase and mechanical damages to the tissues. Consequently, in following experiments, intravenous addition of microbubbles significantly reduced the required level of US intensities and abolished any similar side effects [48]. The present parameters of the acoustic wave in SP are the same or even lower than those used in common medical US examination. Preferable frequency is localized in the range of about 0.5 to about 5.0 MHz [28].

SP effect of short-lasting, forced by pressure waves permeabilization of cells membrane enables high efficiency transport of genes and medicaments into the cancer cells, lowering drugs dosage needed to obtain planned effect and therefore, overall toxicity to the human body [61, 64]. The permeabilization is obtained by: creation of pores in a cell membrane, opening of intercellular junctions, promotion of endocytosis, transcytosis and/or exocytosis [63]. There are several movements of the microbubbles responsible for creating forces enhancing the drugs transportation.

First of all sustained bubble radius oscillations (expansion and shrinkage movement of the bubbles membrane) in the ambience of stable oscillations which generate a circulating fluid flow that provokes shearing flow near no-slip boundaries and microstreaming [6, 13, 23].

Second of all microbubbles that are close enough to the cell membrane can push and pull on it while oscillating [29]. The pulling movement has been proposed as one of the most significant promoters of pores formation [69].

Third of all Fan et al. (2012) observed under ultrafast video microscopy that creation of pores was also induced by inertial cavitation. There expansion of microbubbles to twice their original radii and then drastic contraction and implosion near the cell created a hole in the cell membrane [36, 24]. Microjetting flow that is excited when the bubble collapses aspherically is the most important in this mechanism [71]. Although cavitation of the bubbles also creates shock waves that are said to disturb the tissue and likewise enhance drug transportation [47].

Fan et al. (2012) assumed two reparation types of the disruption in the integrity of the membrane. The slow reparation, represented by extracellular Ca2+-triggered membrane-fusion phenomena and the fast recover, associated with facilitated self-sealing caused by depletion of cell membrane tension by exocytosis. Fast recover is believed to be associated with small membrane disruptions and determined by physical property of membrane of each cell type [18]. The creation of pores causes immediate influx of Ca2+ ions since under normal conditions the intracellular concentration of the ion is strictly controlled reaching inside a cell sub-micromolar (μM) concentration whereas outside Ca2+ concentration is mostly in the mM range. Li et al. (2017) described second, slow calcium influx after the SP in the mechanism of stretch-activated ion channels [38, 61]. The influx can modulate various signaling pathways regulated by calcium ions [18, 55]. The most desirable in the cancer therapy is programmed cell death – apoptosis. Honda et al. (2004) discovered that the mitochondria-caspase pathway and the Ca2+-dependent pathway play cardinal roles in apoptosis induced by US. Moreover, they conclude that intracellular ROS originated from mitochondria, rather than extracellular ROS (from inertial cavitation phenomenon), are responsible for apoptosis induced by US. Furthermore, SP changes membrane potential [67], and induces

biochemical changes both on the level of cellular membrane and nucleus [10, 56]. Additionally, recent studies revealed that microbubbles can augment the cytotoxic effect of the US [15, 57, 62, 70].

In terms of clinical trials, astonishing effects were obtained in SP treatment of inoperable pancreas tumor with gemcitabine enhanced by microbubbles. In the clinical trials the patients' median survival rate was doubled [15, 70]. Similar results were observed in patients with glioblastoma multiforme where blood-brain barrier has been disturbed by US combined with microbubbles without detectable adverse effects on radiologic (MRI) or clinical examination whatsoever [7]. Additionally, SP in vitro studies have shown enhancement of induction of apoptosis, oxidative stress and modulation of activity of MDR proteins, especially when microbubbles were applied [2, 12, 14, 27, 33, 62].

Further research on adequate parameters for SP and microbubbles may contribute to broaden its applications – cancer treatment, influencing blood-brain barrier, neurological diseases treatment or immunological therapy [4, 29, 35, 68].

PHOTODYNAMIC THERAPY (PDT)

The history of phototherapy reaches to antiquity. Starting from ancient Greek, the way of treating known as heliotherapy, caused human concern in healing properties of light. Evolution of biophysics knowledge, years of scientific and biomolecular analyses led to clarification of modern photodynamic therapy (PDT). Niels Ryberg Finsen is considered as a pioneer of this branch present-day shape. His research showed a big amount of undiscovered PDT capabilities that currently are discussed on the biomolecular level [1]. Dynamically developing PDT, with his specify and selectivity, became a strong alternative for conventional oncological treatment. The concept of PDT depends on three inseparable subunits: photosensitizers (PSs), visible light and oxygen in tissue. PSs are activated by absorption of visible light to initially form the excited singlet state, followed by a transition to the long-lived excited triplet state. This triplet state can undergo photochemical reactions in the presence of oxygen to form reactive oxygen species (ROS) [53]. The cascade of energy transfer ends up with two main destructive reactions: directed lesion or undirected biomolecular process that results in the transcription of genes. Tissue reaction to PDT can be systemized as immediate, early and late.

Immediate reaction consists of processes that are directly caused by the presence of singlet oxygen in place of action (10-20nm), lipid peroxidation and protein crosslinks. Main specific proteins modified in this phase are epidermal growth factor receptor (EGFR), anti-apoptotic protein Bcl-2 and signal transducer and activator of transcription STAT-3.

Early molecular response refers to multilevel signal transduction and genes expression. In this state takes place reactions that we can divide into two types. First helps tumor survival, second direct cellular metabolism into death pathway. Knowledge of PDT action during this phase gives us possibilities to implement adjuvant medicaments that will help to avoid tumor resistance to PDT. Local hypoxia and the presence of ROS in the cellular space are the primary triggering factor for this effect. NF-κB is involved in the regulation of proinflammatory responses (IL-1β, IL-6, IL-8, G-CSF, ICAM, VCAM, E-selectin) and apoptotic pathways, but also modulates expression of genes that are involved in tumor survival and regrowth, such as COX-2 and MMP-9. Hypoxia occurs due to use of molecular oxygen in order to produce singlet oxygen. This effect is responsible for overexpression of hypoxia-induced factor (HIF)-1α that participate with increased expression of VEGF causing reduced PDT response. To prevent this outcome, it is necessary to combine PDT with anti-VEGF medication. Dependent the PS, PDT induces cytokines, such as TNF-α, a versatile cytokine involved in inflammatory disorders and in cell death signalling.

The late reaction leads to cell death in three possible pathways: most desirable apoptotic, autophagic and necrotic. Apoptosis can be activated by an extrinsic pathway, involving receptor signalling, and by an intrinsic pathway with a central role for mitochondria. Both pathways converge at the critical step of caspase activation. Autophagy has also recently been demonstrated in response to PDT and may represent an attempt by the cell to remove organelles damaged by photosensitized ROS generation. The most important differential effect of necrosis versus autophagy and apoptosis is that necrosis causes tissue inflammation and this sequel is suspected to be induced by cytokines interactions [5].

Presently main attention is focused on PSs, their chemical structure, way of administration and the affinity to the target cells. The most effective PSs tend to be relatively hydrophobic compounds that rapidly diffuse into tumor cells and localize in intracellular membrane structures such as mitochondria and endoplasmic reticulum. PSs used in cancer therapy are based on the tetrapyrrole backbone, a structure similar to that contained in the protoporphyrin prosthetic group contained in hemoglobin [53]. Currently the challenge for the scientists is to develop PSs delivery systems that may significantly improve targeting in PDT, reducing side effects and amplifying advantages. This is followed by nanoparticles investigations. Nanoparticles represent an emerging technology in the field of PDT that can overcome most of the limitations of classic PS [42]. Very interesting amplification showed the trial of perfluorocarbon nanoparticles enhanced by reactive oxygen levels (oxy-PDT agent). PS loaded to nanoparticle filled by oxygen delivered a high dose of the substrate to photodynamic reaction regardless of local hypoxia [11]. The Killer Red molecule is the well-known protein, present in cell

membranes, that may act as ROS donor under the excitation of light. Affecting this molecule expression is possible to create endogenic PSs [53]. Big amount of newly designed PSs delivery systems gave the possibility to target the ligand that may directly attack desirable neoplastic cells. Example of this connection is targeted photodynamic therapy of breast cancer, using lactose-phthalo-cyanine functionalized gold nanoparticles, which delivers PSs directly to breast cancer cells via galactin-1 receptor expressed on the cellular surface [21]. Vascular-targeted-PDT (VT-PDT) becomes a method with widest clinical application. VT-PDT is based on intravenous PSs administration with subsequent irradiation of the tumor region. It leads to release of ROS in the nearest tumor venous area causing local thrombus and afterwards break of cancer blood supply. This technique showed great results in low-risk prostate cancer and with patented Padeliporfin is successfully used in modern treatment [3]. Beside oncology, PDT has satisfying results in dermatology, ophthalmology and antimicrobial therapy. In the era of bacterial multidrug resistance, PDT seems to be a promising practice. Studies on amino-functionalized antimicrobial nitrogen-doped graphene quantum dots for eliminating multidrug-resistant species in dual-modality photodynamic therapy showed high efficiency of bacteria elimination in low energy light irradiation [30]. All of these points that coming years are going to prove PDT clinical value with the possibilities of bypass side effects of conventional therapies.

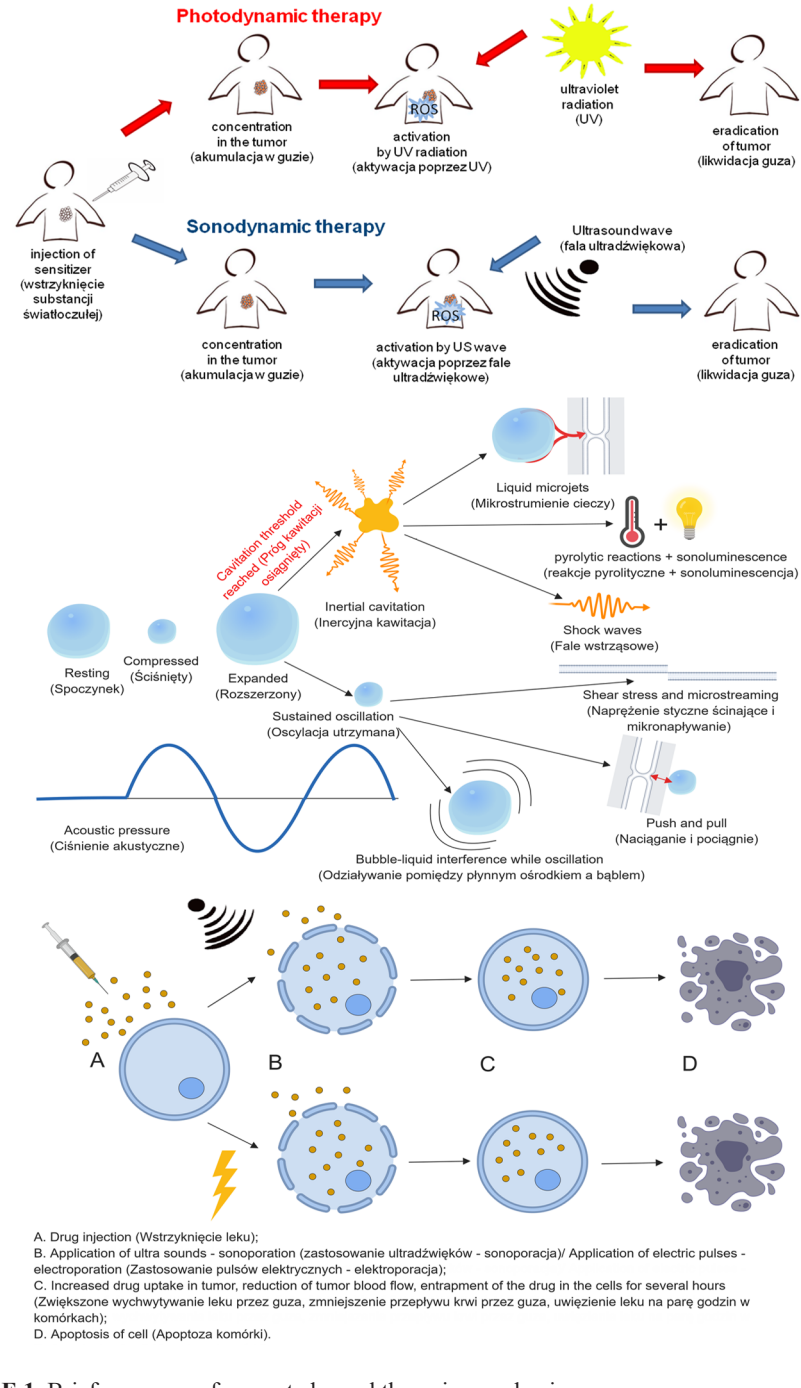
SONODYNAMIC THERAPY (SDT)

Sonodynamic therapy (SDT) is a sprouting treatment that combines ideas from two above mentioned approaches, PDT and SP. It is based on acoustic field activation of a sonosensitizer, whereas the mechanism is alike PDT, mostly ROS mediated. SDT had been already observed and described in 1989 by Yumita et al. and later on was named by Umemura et al. (1992). Both scientists worked together on stimulation of hematoporphyrin with acoustic field, before known for its PS nature. Nowadays, still most of sonosensitizers besides being sensitive to acoustic field are also used as PS. The parameters needed to excite a sonosensitizer with an US strongly resemble those used in SP. Frequencies of the waves range from 0.4 to 3 MHz [45].

As mentioned before, ROS creation is the most important factor of cellular damage in SDT and it was broadly described [31, 73]. Nevertheless, the mechanism behind ROS creation in the ambience of acoustic field is still fairly uncertain. Sonosensitizer could be activated by light through the sonoluminescence process or with pyrolytic reactions (inertial cavitation implosions of bubbles forming hydroxyl radicals [•OH] and hydrogen atoms [•H] in aqueous solutions), or upon the increase of other acoustic cavitation effects like bubble-liquid interference while

oscillation [6, 32]. Interestingly enough, some scientists state that the idea of sonoluminescence as sonosensitizer activation factor is just an oversimplification and an attempt to describe sonodynamic phenomenon with the PDT mechanism. They suggest that sonoluminescence could be just an irrelevant outcome of the recombination of ROS, not their genesis factor [32]. Supportive for the statement are studies on two sonosensitizers 13,17-bis (1-carboxyethyl)-8-[2(2,4-dichlorophenyl-hydrazono)ethylidene]-3-ethenyl-7-hydroxy-2,7,12,18-tetramethylchlorin, disodium salt [DCPH-P-Na(I)], a novel porphyrin derivative, and titanium deoxide (TiO2), a photocatalyst. They show a very different reactivity in light and in US irradiation, on the favor of US [60]. The cell molecular response to the excessive levels of ROS caused by SDT is alike PDT. Cascade of events eventually leads to apoptosis. Due to interactions between ROS and cellular biomolecules, Bax, caspase 3 and CytC expression, and reduced Bcl-2 expression rises intracellularly. Bax activation or Bcl-2 degradation activates the mitochondrial apoptotic pathway to induce CytC release into the cytosol. CytC functions with Apaf-1 activate caspase-9, which in turn activate the apoptotic caspase cascade such as caspase-3 [74]. Recently Li et al. (2019) studied SDT on hepatocellular carcinoma with sinoporphyrin as a sonosensitizer and they observed the p53-caspase-3 pathway as a primary mechanism of apoptosis and G2/M phase as a cell cycle blockage point [37]. Chen et al. (2012) carried out SDT on glioma cells with Zn-PcS2P2 as a sonosensitizer and proved that in their case the different apoptosis pathway, extrinsic pathway (receptor dependent) was also initiator of apoptosis after exposition to the acoustic field [9]. Additionally, ROS promote other proteins degradation and peroxidation of cell membrane lipids [22, 72]. Levels of vascular endothelial growth factor are also said to be much lower after the SDT [40]. Apart from ROS mediated reaction, sonosensitizers are thought to disturb the structure of mitochondria and change mitochondrial membrane potentials, which could lead to release of cytochrome C, Smac/Diablo and other apoptosis-inducing factors. Since the soundwave specificity is almost equal with the one used in SP, here also occurs augmentation of calcium ions concentration which can inevitably trigger cells to undergo apoptosis or necrosis [37]. TEM revealed more macroscopic damage done to the cell organelles after SDT such as membrane destruction, mitochondria swelling and chromatin condensation [40].

Perfect sonosensitizers are being sought. The fact that most of the sonosensitizers are also PSs is a downside owing to patients sunlight photosensitivity after the administration of sonosensitizer that can last even 30 days [60]. Biodistribution is an essential feature for a perfect sonosensitizer. It should be accumulated long enough in a tumor tissue and promptly cleared form the tissues that would lie between the US source and the target cancer. Therefore, analogically to PDT, scientist tent to develop drug delivery platforms that could be the solution to the delivery difficulty [44]. Two modalities seem to have primacy. First, locking a son-



A. Drug injection (Wstrzyknięcie leku);
B. Application of ultra sounds - sonoporation (zastosowanie ultradźwięków - sonoporacja)/ Application of electric pulses - electroporation (Zastosowanie pulsów elektrycznych - elektroporacja);
C. Increased drug uptake in tumor, reduction of tumor blood flow, entrapment of the drug in the cells for several hours (Zwiększone wychwytywanie leku przez guza, zmniejszenie przepływu krwi przez guza, uwięzienie leku na parę godzin w komórkach);
D. Apoptosis of cell (Apoptoza komórki).

FIGURE 1. Brief summary of presented novel therapies mechanisms
RYCINA 1. Podsumowanie mechanizmu działania prezentowanych terapii

osensitizer in a lipid-shelled microbubble has an advantage of enhanced ROS production in the presence of an acoustic field [51]. Second modality exploits nanoparticles loaded with sonosensitizer and has a great advantage of reaching deepest layers of a tumor by passing through leaky tumor vasculature [66].

In vitro and in vivo studies are being procced. 5-aminolevulinic acid (5-ALA) is a metabolic precursor of porphyrin IX (PpIX). It has been already successfully tested as a PS for glioblastomas in preclinical studies and has been widely used in fluorescence-guided resection of malignant glioma. In the study of Ohmura et al. (2011) a selective tumor destruction and growth inhibition were achieved using 5-ALA-SDT, without damaging the normal tissues. Treated rat models tumors had sizes 18.32±5.69 mm2 comparing to 29.94±10.39 mm2 with only sham operation. Because 5-ALA has already been applied clinically as a PS, SDT with this sensitizer is expected to be applied clinically to treat deep-seated tumors in humans in the future [52]. Logan et al. (2019) evaluated the potential of US targeted microbubble destruction (UTMD) to deliver Rose Bengal (sonosensitizer) SDT in combination with paclitaxel and doxorubicin chemotherapy as a potential treatment for breast cancer. This approach is called UTMD-mediated chemo-sonodynamic therapy and in animal models gave satisfactory results. The tumor after 25 days with the treatment was 11.44% smaller than before applying the therapy. In contrast, tumors in animals treated with the same formulation but without US, increased volume by 40.47% over the same time period [41]. Other study showed that SDT with HiPorfin induced systemic antitumor immunity. SDT was proven to induce vaccine-like immune responses that could be combined with immune adjuvants and immune checkpoint inhibitors to design efficient cancer immunotherapy [75].

There are also some modalities from ‘standard’, if such exists, SDT. For example Sono-Photo Dynamic Therapy which uses additive effect of PDT and SDT or chemosonodynamic therapy profiting from conjugated cytotoxicity of chemotherapeutic drug and US induced ROS creation [49].

CONCLUSIONS

Taking everything into account, all mentioned cancer treatment methods are effective in the therapy of wide range of cancer types. All the modalities exhibit relatively low toxicity to the human body and are nonspecific to the subtypes of a tumor cells. ECT stands out from three other methods, in terms of mechanism, but its purpose is similar to SP when combined with chemotherapeutics. The aim of these two novel techniques involves the facilitation of drugs delivery and consequently, improvement of local treatment as well as decrease of dosage resulting in the diminution of overall toxicity for the human organism. PDT and SDT are methods where drug excitation must be induced or by proper wavelength or by

ultrasound. PDT is well characterized method, and applied in clinical practice. The knowledge about PDT was the basis to understand some of SDT mechanism including ROS generation. SDT is relatively new method but it seems to be the most prospective one. Unfortunately, it is still not explained how sonosensitizers promote ROS release. Nonetheless, novel therapies do not attempt to replace existing methods, rather augment their effectiveness and lower side effects.

Summarizing, we can assume that the combination of standard (CT, PDT) and novel anticancer approaches as SDT, ECT including also immune adjuvant therapy, nanotechnology, and specific inhibitors may be promising to eradicate primary and metastatic tumors.

Undoubtedly, the understanding of molecular mechanism of the utilized anticancer protocol boosts clinical implication of a treatment. On the other hand, the lack of this knowledge is one of the main hindrances to accept, for instance, SDT as a guideline treatment.

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