® Postepy Hig Med Dosw (online), 2020; 74: 171-181
e-ISSN 1732-2693

www.phmd.pl
Review

Received: 08.03.2019
Accepted: 07.11.2019
Published: 02.06.2020

Contemporary directions of application of low power ultrasounds in anticancer therapy

Współczesne kierunki zastosowania ultradźwięków niskiej mocy w terapii przeciwnowotworowej

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Summary

In the recent years, research has been conducted on the role of ultrasounds (US) in anticancer therapy. Although the mechanisms of impact on cancer cells have not yet been fully understood, it is known that the best results are obtained using low power ultrasound. Currently applying ultrasounds to organisms is considered in three areas of influence: thermal (thermic effect), cavitation (cavitation effect), other than thermal and cavitation ones (non-thermal, non-cavitation effect). Under the influence of ultrasonic wave with low power, the absorption of drugs is increased as well as of anti-angiogenic activity. Sonodynamic therapy is aimed at destroying dividing cancer cells through the formation of free radicals in the cavitation mechanism and in the presence of sonosensitizers. At the same time under the influence of US, local hyperthermia is generated. In vivo studies showed a synergistic increase in cytotoxicity due to the effects of ultrasonic hyperthermia and adriamycin. The thermal effect and inertial cavitation are described as two factors induced by US, which may lead to damage to the vascular network within the neoplastic lesion. A proportional increase in tumor echogenicity to the frequency range of the applied ultrasound wave has been demonstrated. The strategy of combining US with photosensitizers, chemotherapeutics or contrast agents is gaining more and more recognition. Obtained results from inter developed studies on antineoplastic sonodynamic therapy indicate that it may become a new additional cancer treatment strategy.

Keywords: **ultrasounds • sonodynamic therapy • sonosensitizers • tumors • thermal effect • inertial cavitation**

GICID 01.3001.0014.1651
DOI: 10.5604/01.3001.0014.1651
Word count: 8065
Tables: 4
Figures: –
References: 62

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INTRODUCTION

It has been over one hundred years since the first experimental use of ultrasounds in humans. This was done by Paul Langevin in 1917 after previous animal trials. He was the first to observe the phenomenon of cavitation waves in water, although several years earlier Richardson proposed the use of ultrasound during the Titanic search. The subsequent years of the scientists' work were focused on creating the possibility of controlling the device's power. The first performed for therapeutic purposes use of ultrasounds was performed by Freundlich in 1939, counting primarily on thermal effect appearing in tissues subjected to surgery. This multiple thermal effect in the 1950s began to be used during surgical procedures in injuries of the vestibular system of the middle ear, alleviation of the symptoms of Meniere's disease, removal of breast tumor and cholelithiasis [52]. Around this time, Wild and Neal developed a method of obtaining images using the ultrasound image (B-mode ultrasound image), which initiated the era of ultrasonography. Subsequent studies from the 1970s and 1980s concerned mainly the parameters of the ultrasound wave, its dosage standards and the phenomena of absorption in tissues. Ultrasounds have found their medical application primarily in physiotherapy and diagnostic imaging. Studies carried out in recent years indicate their important role in anticancer therapy.

BIOLOGICAL EFFECTS OF ULTRASOUNDS

Currently, the application of ultrasounds to organisms is considered in three areas of impact: thermal (thermic effect), cavitation (cavitation effect) and other than thermal and cavitation ones (non-thermal, non-cavitation effect) [21]. The mechanisms of these interactions have not yet been fully understood, although it is known that the best results are obtained when using low power ultrasounds [9, 25]. The therapeutic effect of ultrasounds depends both on the parameters of the applied wave, first of all- on its power, frequency and modulation, but also to a large extent on the ability of tissues to absorb acoustic energy [1]. The propagation of this energy results in the appearance of heat in the tissues. The increase in temperature depends on the dynamic balance between its accumulation and giving it up [2]. Each type of tissue presents a characteristic factor called relaxation time. After passing the impulse of ultrasound energy, tissues with a short relaxation time can return to their original temperature before another impulse arrives. In structures with a long relaxation time, the molecules remain in vibration until the next wave arrives, which ultimately enhances the phenomenon of heat accumulation. The thermal effect is generated primarily in a tissue with a high absorption coefficient. The strength of the ultrasonic wave interaction within the tissues depends on the depth at which we will measure it. The depth at which this value is reduced by half is determined by the half value thickness (HVT) factor. HVT is characteristic of each type of tissue, it is influenced by the density of acoustic structures subjected to acoustic waves, as well as the frequency of the applied stimulus [52]. The HVT factor for the type of tissue and frequency is shown in table 1.

Table 1. The values of HVT (cm) coefficient for 2 and 5 MHz frequencies for different tissues

Tissue type	HVT (cm)	
	2MHz	5MHz
Muscle	0.75	0.3
Blood	8.5	3
Brain	2	1
Liver	1.5	0.5
Soft tissues	2.1	0.86
Water	340	54
Bones	0.1	0.04
Air	0.06	0.1

Source: The table was elaborated on [52]

It is worth noting that the lower frequency is able to provide more energy to the deeper regions of the sonificated structure. Another important parameter appearing in the context of the thermal effect is the acoustic impedance factor and it refers to the resistance to sound. The speed of the acoustic wave depends on the density of the carrier and increases with its flexibility. The acoustic impedance (Z) is the density of the carrier multiplied by the speed of the wave propagation in its interior. The flow of the ultrasonic wave depends on the acoustic impedance of the tissue [18]. Each tissue has a unique impedance as shown in Table 2. Similarly as in the case of electromagnetic waves, the ultrasonic stream is reflected at the boundary of two different centers (Table 3).

Table 2. Acoustic impedance (Rayl) value for individual tissues

Carrier	Accoustic impedance (Rayl)
Muscles	1.7
Fat tissues	1.38
Brain	1.58
Kidney	1.62
Liver	1.65
Blood	1.61
Soft tissues (averaged)	1.63
Bone	7.8
Water	1.48
Air (NTP)	0

Source: The table was elaborated on [18]

Table 3. Percentage of reflection at the border.

The border of the media	Rebound effect [%]
Fat tissue/muscle	1.08
Fat tissue/kidney	0.64
Muscles/bone	41.23
Fat tissue/bone	48.91
Soft tissues/water	0.23
Soft tissues/air	99.90

Source: The table was elaborated on [28]

This phenomenon will not occur only in the case of tissues having the same parameters, that is density and impedance [28]. For example, the phenomenon of reflected waves occurs only slightly at the border of fat tissue and muscles due to their very similar impedance. On the other hand, the bones and muscles differ markedly in density. At the interface of these tissues, up to 40% of the delivered ultrasonic waves can be reflected. The strongest effect of rebound in mammals can be observed at the periosteal and bone interface where about 50% of the wave can be reflected [15, 28]. The huge difference in the density of these tissues causes longitudinal waves change to the transverse direction, generating shear forces, which in turn significantly affect the local increase in temperature [15]. In the context of the phenomenon of reflection of waves, and hence losses in their impact, the angle of application of the stream is also important. The most effective seems to be an approach angle of close to 90^0. The higher temperature obtained by means of ultrasound is of great importance for the cellular processes of the human body. At elevated temperatures up to around 40^0C, increased activity of intercellular enzymes and metabolic processes is observed [23]. This effect is desired in biostimulation therapy. However, when the temperature rises to about 45^0C, various types of proteins undergo denaturation and coagulation, so the enzyme activity decreases. This ultimately leads to structural damage and therefore to impairment of cell function. The degree of cell damage also depends on their type. The percentage of irreversibly damaged cells is called LD (lethal dose). For example, in the sarcoma cell line assay, 50% of dead cells were obtained both after 2 hours of exposure at 42^0C and after about 7 minutes at 46^0C. This result can be described as LD50. Because many studies indicate that the denaturation process starts at 43^0C, parameter t43 was created, which determines the time necessary to obtain the above temperature [10].

The term cavitation refers to phenomena related to vibrations and the dynamics of movement of small gas bubbles under the influence of the ultrasonic wave [59]. Cavitation bubbles can grow and pulsate with the flow of subsequent phases of ultrasonic waves. They can also collapse at the moment of wave compaction, making the local phenomenon of the shock wave together with accompanying sonoluminescence flashes and an extremely high local temperature value (even up to several thousand °C) [24, 44]. Thus, cavitation can be divided respectively into inert (transient) and non-inert (permanent), where both types of impact are important for biological processes [3]. Depending on its type, a different type of biological effect should be expected. Non-inertial cavitation is accompanied by various types of behaviors of induced micro bubbles, leading to the formation of micro-jet movements with a high speed gradient. These movements are important for the formation of the phenomenon of sonoporation, in particular when the cell suspended in the liquid is near the pulsating bubble. Sonoporation is a transitory effect of the appearance of channels in the cell membranes allowing the passage of drugs, DNA and antibodies into the cell [35].

Inertial cavitation is a process associated with more sudden phenomena. First and foremost, it appears during the exposure using higher ultrasonic wave intensities. The microbubbles increase in volume and then collapse rapidly. This process is accompanied by the local release of a large amount of energy, an increase in temperature and pressure [32]. In the sonicated inertial area, there is an increase in the number of hydroxyl radicals following the thermal breakdown of water molecules [11]. It is recognized that at the current level of research on the phenomenon of sonoporation it is difficult to clearly determine what kind of cavitation is more important here. Although non-mineral cavitation requires a longer exposure time to achieve biological effects, its advantage is undoubtedly the fact that it is a process easier to control. Inert cavitation brings faster and more reliable results, but there is a greater risk of irreversible damage to normal cells [55].

The thermal and cavitation effects of ultrasounds are now considered the most important phenomena in the process of obtaining a biological effect. However, there are few reports on the ultrasound bio effect not related to the increase in temperature or the activity of cavitation bubbles. These analyses concern mainly the stresses associated with ultrasonic propagation as a result of the ultrasonic field. The magnitude of stresses and the resulting biological effects induced by these forces depend on the properties of the ultrasonic field as well as the properties of the biological system [8]. Stresses may exert ultrasound pressure on bodies with a density different from the density of the surrounding medium, affect the occurrence of motion between the biological object or other heterogeneous medium, and the surrounding medium and contribute to the change in viscosity, and thus initiate the flow [4].

In recent years, the research on the use of ultrasonic energy in cancer therapy seems to be more and more advanced. Reports on, inter alia, the topic of increasing the absorption of drugs, as well as the action of antiangiogenic ultrasound wave of low power, make it worth investigating the main directions of study on the use of ultrasounds in oncology.

SONODYNAMIC THERAPY

The term sonodynamic therapy (SDT) derives from the term photodynamic therapy (PDT), but unlike the latter in which photosensitizers are activated with light to produce reactive oxygen species, sonodynamic therapy aims to destroy the dividing cancer cells through free radicals created as a result of ultrasonic cavitation in the presence of sonosensitizers [22]. Analysis is available indicating the possible important role of alkoxy and peroxy radicals as cytotoxic agents. They are more permanent compounds that, thanks to longer life and higher selectivity, are able to overcome significant distances inside the body before reaching and reacting with the appropriate organelles in the cell, such as the cell membrane [31]. The reaction of oxygen radicals with phospholipids constituting the cell membrane is the initiation of lipid peroxidation which damages these structures [16]. In the case of PDT therapy, the photosensitizer, which was initially in the basal – resting state (S_0) goes to the active singlet form (S_1). This allows its further electrochemical transformation into the form of a more permanent triplet (T_1) activity status. This form of photosensitizer is necessary to produce enough reactive oxygen species (ROS) to cause cell death. Thus, it is considered that the main activator of ROS PDT is singlet oxygen ($^1O_2$). The most active form of oxygen is also assigned a leading role in the case of sonographic therapy (SDT), although the mechanisms of activation of the photosensitizer are not fully investigated [30].

Inertial cavitation with its collapsing microbubbles and accompanying luminescence flashes seems to be an important activator of the processes occurring during sonodynamic therapy. Confirmation of this thesis may be, among others studies on sonoluminescence of single-bubble character (SBSL) [7]. A high level of intracellular, ROS initiated by SDT can damage the mitochondrial membrane in the lipid peroxidation process, causing depolarization of the membrane potential of the mitochondria, which in turn leads to an increased permeability of this membrane [29]. In an in vivo study carried out on human lung cancer cells (SPCA-1) administered to mice as xenograft, the sole use of 0.4-1.6 W/cm^2 ultrasound or only chlorine (chlorine e6 – Ce6) per dose [10-40 mg/kg] did not show significant anticancer effects. However, the combination of ultrasound [1.6 W/cm^2] with Ce6 significantly inhibited tumor growth. Analysis performed by flow cytometry showed that the sonographic effect mediated by Ce6 was primarily the result of the cell necrosis process caused by free radicals (ROS) [5].

Currently, there is a significant increase in the number of reports on the use and effects of new sonosensitizers. The selection of a suitable preparation for a given type of cancer has become the goal of many studies. One of the most commonly used groups of sonosensitizers are porphyrin-based compounds. In this group, it is worth paying attention to HMME (hematoporphyrin monomethyl ether). It is a photosensitizer associated with hematoporphyrin (HP) which during SDT increases the amount of intracellular ROS and levels of Bax, caspase 3 and caspase 9 [6]. The importance of HMME for the viability and induction of cell apoptosis was also demonstrated [43]. Studies conducted on human leukemia (U937) cells in vitro indicate that the use of HMME in combination with the propagation of ultrasound waves is an effective tool in cancer therapy [41]. Another interesting representative of this group is Photofrin II. With its participation, among others, an attempt to evaluate the treatment options for neoplastic changes in the liver was done. The in vivo experiment concerned the use of the ultrasound wave exposure (6 x 6 mm head) on the proper liver structure of rats with low power (210 kHz, 1.3 W/cm^2, time 3 min) in the presence and absence of Photofrin (at 30 mg dose)/kg) [45]. The results indicated that the mean maximum depth of change in the rats where ultrasound and Photofrin II were used was 5.7 ± 0.9 mm, while in rats treated with only ultrasound this value turned out to be nearly twice lower and amounted to 3.0 ± 0. 4 mm. In the opinion of the authors, this study suggests the possibility of using a photosensitizer as an enhancer in the treatment of liver cancer with ultrasound [45].

Another interesting photosensitizing agent is protoporphyrin IX (PpIX) derivative of porphyrin (hematoporphyrin derivative – HPD). The effectiveness of this preparation in the context of SDT has been studied a number of times on various types of cancer cells. Of particular interest is the following evaluation of the sonodynamic induced antitumor effect of (PpIX) in mice with solid liver tumors (H-22). The study also concerned the analysis of possible mechanisms of cell damage in vivo. Pharmacokinetics of PpIX was analyzed in plasma, skin, muscles and tumor of H-22 mice. Tumors were treated with US power of 1.43 MHz, 3 W/cm^2, for 3 minutes in a series of three repetitions: eight, twelve and twenty four hours after administration of 5.0 mg/kg of PpIX. Antineoplastic effects of sonodynamic therapy were estimated based on the tumor inhibition rate (volume-to-weight ratio). SDT biological activity was assessed by hematoxylin and eosin (H & E) staining and Transmission Electron Microscopy (TEM) [51]. Lipid peroxidation (LPO), antioxidant enzymes glutathione peroxidase (GSH-PX), catalase (CAT) were measured. The superoxide dismutase (SOD) test was carried out, as well. In the conducted study evaluating the combined action of US and PpIX, a significant anticancer effect was obtained. Fifteen days after PpIX-SDT therapy, tumor growth and tumor inhibition rates were 53.84% and 45.86%, respectively. In addition, when the PpIX-SDT was used, the biochemical mechanism initiated the destruction of tumor tissue structures and antioxidant enzymes in vivo. In the opinion of the authors, the free radicals produced by the synergistic action of US in the presence of the sonication used are devastating to the antioxidant system of tumor cells in vivo and can play an important role in this action. One cannot exclude a thermal effect in inducing damage to cellular structures, such as destruction of the cell membrane or chromatin condensation [51].

In a study conducted by Haiping Wang et al. [49] to assess the antitumor efficacy of another HPD – chlorine e6 (Ce6), its cytotoxic effect was compared in both SDT and PDT. Ultrasounds were used with a power of 0.36 W/cm^2 and 0.72 W/cm^2 at different concentrations of Ce6 [1, 2, 5, 10 μg/ml]. The test was carried out on MDA-MB-231 cells. The integrity of the cell membrane was assessed using propidium iodide (PI). It was shown that the damage to this structure increased with the increase of ultrasound intensity. The results suggest that ROS can play an important role in both SDT and PDT. In addition, it was assessed that while FDT therapy focuses on the level of mitochondria, SDT can cause damage to many cell organelles of the MDA-MB-231 line [49].

It should be emphasized that HP and HPD exhibit high phototoxicity in the skin cells, which significantly limits their clinical use. The second generation of sonosensitizers whose representative is, among others chlorine e6 overcomes this very important problem. Ce6 is a compound made up of a single monomeric chemical structure. Studies have shown that Ce6 can selectively accumulate in cancerous cells, and at the same time it is rapidly removed from normal tissues [47].

Developed for high singlet oxygen production ability gallium-porphyrin, 7,12-bis (1-decyloxyethyl) -Ga III-3,8,13,17-tetramethylporphyrin-2,18-dipropionyl) - D-aspartic acid, (ATX-70) is one of the newer porphyrin-based sonosensitizers. In mice with colon cancer, the effect of antitumor activity of SDT intensified with the increase of ATX-70 dose. At a dose of 2.5 mg/kg or higher, three days after ultrasound exposure, the average tumor size was reduced by more than half. The use of only ultrasound resulted in a slight reduction in tumor mass, whereas the use of ATX-70 alone did not have a significant impact on the tumor [61]. The study on the pharmacokinetics of this sonosensitizer was performed by intravenous administration of this preparation to CDF1 mice with a colon tumor. Blood and tissue samples were collected up to 72 hours after administration. The concentration of the drug was determined by high performance liquid chromatography (HPLC) with fluorescence detection. Researchers showed that the highest concentration in tumor mass occurred between 2 and 6 hours after administration. However, moderate concentrations of ATX-70 were also maintained in normal tissues for up to 6 hours. The distribution of ATX-70 in the tumor was compared to other tissues to minimize the possible side effects of laser or ultrasound exposure, while maintaining the effect of treatment. 24 hours after the administration, the maximum favorable ratio of tumor/plasma concentration, relatively high tumor/skin and tumor/muscle ratio was observed, which may indicate the optimal time of therapy [38].

Another equally developmental group of sonosensitizers are substances based on Bengali Rose Xanthene (bengal rose - RB), Erythrosine B (EB), or Merocyanin 540 (MC 540). The sarcoma cells exposed to ultrasound 1.93 MHz in continuous wave mode for 60 seconds showed cell damage rate increased 4-5 times using 160 μM/ml) EB, while no cell damage was observed after dye application alone. The measurement was made using electron paramagnetic resonance spectroscopy (ESR) [60]. HL-60 leukemia cells in vitro were exposed to US wave at 255 kHz and power 0.4 W/cm^2 in the presence of MC 540 photo sensitizing agent. The scanning electron microscope showed multiplication of pores on the surface of the cell membrane and significant reduction in the number of leukemia cells. Operation of only ultrasound or the dye itself did not show any specific cytotoxic effect [46].

However, it is believed that while RB acting on tumor cells in vitro has a devastating effect, there are some significant difficulties in vivo leading to the inability to apply them to organisms, e.g. mammals. The reason for this is primarily the hepatotoxicity of these compounds, their relatively slow removal from the body, and the low accumulation within the tumor may cause the need for higher doses of the preparation. In the case of porphyrins, the key to accumulating of these substances in the tumor mass is their amphiphilicity. Sugita et al [42] have attempted to modify the RB sensitizer to maximize its amphiphilicity. As a result of these studies, a compound called Bengal rose derivative (RBD) was formed. In experiments on sarcoma cells, the ability of RB to RBD in the presence of US was tested. The result of this work was to double the effect of cell damage using RBD. The measurement was done using trypan blue. In addition, the authors noted that the level of cell damage was inhibited by the addition of active oxygen scavengers such as histidine, tryptophan and N-acetyl-L-cysteine [42].

Studies on the applicability of other compounds in sonodynamic therapy are still underway. The most frequently tested ones include non-steroidal anti-inflammatory drugs (NSAIDs). In in vitro studies, the anti-cancer effects of non-steroidal anti-inflammatory drugs, tenoxicam and piroxicam were tested on sarcoma cells cultured in 7-week-old male mice. The survival rate of sarcoma cells in vitro exposed to the tenoxicam or piroxicam in the presence of US was significantly lower than when using ultrasound alone. Furthermore, when L-histidine, a singlet oxygen scavenger and a hydroxyl radical were used, the survival of tumor cells was significantly higher. From the above findings it follows that tenoxicam and piroxicam increase the antitumor effect of ultrasound by increasing the production of singlet oxygen and its other active forms [37]. Quinolone compounds, a type of clinically used anti-infective drugs with a broad spectrum of activity, show significant photosensitivity. As many of the sonosensitizers originate from photosensitizers, quinolone compounds are suspected to have sonodynamic effects. For example, fluoroquinolone antibiotics have been used with positive effect as sonosensitizers in order to obtain an antitumor effect in an in vitro study. The tests were carried out on sarcoma cells in the presence of US 2 W/cm^2 during 30 and 60 sec [17].

Methylene blue (MB) is another test agent showing significant efficacy. An in vitro test using MB showed positive results. In the experiment, tumor cells of the HO-8910 ovary were subjected to an ultrasonic wave with a power of 0.46 W/cm^2 for 5 sec. The MB concentration was consistently kept at 100 μM/ml. An attempt was also made using the MB itself and only US. Analysis made by flow cytometry, 24 hours after SDT showed that a significant increase in early and late apoptosis in tumor cells was obtained. In addition, a high concentration of intracellular ROS has been demonstrated. The authors pointed to the phenomenon of apoptosis as an important mechanism of damage to tumor cells HO-8910 in the therapy of SDT-MB [57].

Curcumin is an active compound of plant origin derived from the rhizomes of *Curcuma longa* presenting photosensitizing properties. However, it has been difficult to find research for its anti-cancer application so far. In contrast, attempts have been made to assess the effect of this sonosensitizer on macrophages, the key inflammatory cells in the atherosclerotic plaque. THP-1 derivatives of macrophages were incubated with curcumin for 2 hours at a concentration of 40.7 μmol/l, and then subjected to a pulsed US wave 2 W/cm^2, 0.86 MHz for 5–15 minutes. Six hours after the application of sonodynamic therapy the number of apoptotic and necrotic cells in the SDT group was higher than in the group in which only ultrasound was used, and the number of apoptotic cells was higher than that of necrotic cells. Both loss of mitochondrial membrane potential and morphological changes of the cytoskeleton were visible already 2 hours after the use of curcumin-SDT. The study confirms the fact that curcumin exhibits a sonodynamic potential compared to THP-1 derivatives of macrophages and that therapy with the use of this sonosensitizers may be one of the methods of atherosclerosis in the future [48].

The search for optimal substances that can effectively cooperate with the US wave continues, especially for those which have high aggregation abilities in the tumor tissue, and at the same time are neutral to normal tissues, especially located between the source of the ultrasound wave and the target site of the cancer to be treated. Recently, it has been shown that some nanoparticles possess spontaneous sonodynamic properties, which is why they are sometimes referred to as nanosensitizers [33]. For example, titanium dioxide (TiO_2) nanoparticles can strongly absorb light, including ultraviolet, and then generate free radicals.

Silicon dioxide SiO_2 nanoparticles have the same properties as TiO_2. Studies from 2014 assessing the cytotoxicity and the sonodynamic abilities of silicon nanoparticles (SiNp) have shown inhibition of cancer cell growth. These results open a new perspective on the use of biocompatible compounds in the treatment of cancer [33, 34].

ULTRASOUNDS IN COMBINATION WITH CHEMOTHERAPY

Chemotherapy is a very important method in the clinical treatment of cancer, while resistance to anticancer drugs, both natural and acquired, significantly limits its effective clinical use.

Study on sensitizing cancer cells to chemotherapeutics has long been a key issue in the field of research related to the treatment of patients.

Sonodynamic therapy as a new method supporting cancer treatment has aroused great interest in recent years. In the face of this, many reports and studies have been made presenting possible mechanisms and their consequences in the context of improving the effectiveness of chemotherapy coupled with low frequency ultrasound. As a result of studies on the impact of low frequency ultrasound on PC-3R cells Paclitaxel (PTX) resistant, it was shown that US can induce apoptosis of tumor cells, inhibiting MDRP3 multidrug resistance protein, MDRP7 multidrug resistance protein (MDRP) and P-glycoprotein expression. Moreover, the presented results show that low frequency ultrasound induces autophagy in PC-3R tumor cells by inhibiting the PI3K/AKT/mTOR pathway (phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin) [56]. The cellular autophagy induced by US was correlated with the stress level of the endoplasmic reticulum. The trial using 4-phenylbutyric acid (4-PBA), which was used to protect the effect of damage to the endoplasmic reticulum, clearly indicates the role of the latter in the activation of autophagy and cellular apoptosis. In addition, the results showed that it is a superior effect of the PI3K/AKT/mTOR pathway effector [56, 62].

Some of the achievements of nanotechnology, such as the previously mentioned TiO_2 nanoparticles, have the potential to be used for the transfer and directed delivery of cytostatics to improve the effectiveness of chemotherapy in cancer patients. The experiment in which nanoparticles (NPs) of iron oxide (Fe_3O_4) encapsulated in a titanium dioxide (Fe_2O_4 @ TiO_2 NPs) capsule were found to show interesting results [49]. The Fe_3O_4 @ TiO_2 nanoparticles produced show pH-dependent release of this cytostatic in vitro. After the incubation period with tumor cells and application of the ultrasound wave, effective generation of ROS was observed. Experiments based on in vivo biodistribution proved very high accumulation as well as long-term retention of nanoparticles (NPs) Fe_3O_4 @ TiO_2 compared to chemotherapy alone or only sonodynamic therapy [40]. The combined effect of doxorubicin from SDT showed a synergistic effect, resulting in stronger cytotoxicity, and thus higher therapeutic efficacy. The nanoparticles constructed in this way are endowed with multifunctionality, which enables them to provide highly effective and selective delivery of a combined therapeutic preparation to the tumor site, with minimized side effects [40].

When assessing the mechanisms of support for chemotherapy with US, one should not forget about their ability to generate local hyperthermia. In vivo studies showed a synergistic increase in cytotoxicity caused by the action of ultrasound hyperthermia and Adriamycin (ADR). Fibrosarcoma (RIF-1) or melanoma (B-16) mice were injected with a single dose of Adriamycin [10-20 mg/kg]. The tumors were then heated locally to 41°C - 43°C, either by the US wave, or by immersing the limbs of animals in a hot water bath [36]. The temperature was maintained for 30 minutes. The antitumor efficacy was assessed by two methods. The tumor volume was measured and measured to determine the doubling time of its size or by determining the X-ray dose (TCD50). Both tests gave similar results. Hyperthermia induced by ultrasound was much more effective in increasing the activity of the tested cytostatics than the hyperthermia induced by the water bath. An almost double therapeutic effect was observed in the ADR-SDT group. Due to concerns that US may increase the likelihood of metastases, perhaps by a mechanical effect on tumor cells, this effect was tested on B-16 melanoma cells by quantifying the possible formation of tumor foci in the lungs. There was no effect of SDT on the frequency of metastasis [36]. In another study, the thermal effect of US was excluded from the assumption by using two low US wave parameters having a small effect on the local temperature rise. The waves were used with the frequency 1.765 MHz, power 0.25 W/cm^2, the fill factor 10% and pulsating with the average frequency 2.5 MHz, 0.031-0.18 W/cm^2, with the frequency of 1 kHz repetitions. After exposure to the drug for 1 hour, at several different concentrations, a statistically significant US-induced increase in cytotoxicity was observed in Chinese hamster ovary (CHO) and breast cancer (MCF-7) cells, but not Chinese hamster lung fibroblasts (V79). The effect of combined treatment in vivo was examined by measuring volume changes during the treatment of cervical squamous cell carcinoma implanted in the tissue of the Syrian hamster's cheek. Statistically significant synergy between US and drug leading to tumor volume reduction was observed in the case of Adriamycin and Diaziquon [13].

The same authors in a later study confirmed the enhancement of the action of Adriamycin using ADR-SDT, visible even when using very low US power. Testing simultaneously Cisplatin and Mitomycin C showed no significant additional effect in SDT with the ultrasonic wave parameters used [14]. The potential clinical application of ultrasound with their ability to regulate the parameters of the emitted wave has not been fully understood. For example, the doxorubicin (DOX) test calls into question the use of power below 0.2 W/cm^2 in increasing the effect of the given anti-cancer drug. The combined effect of ultrasounds generating a low power wave and doxorubicin on the damage and induction of apoptosis of human tumor effusions of pleural effusion (U937) and associated mechanisms have been tested here. For the purposes of the experiment, 4 groups were distinguished: control, treated with doxorubicin (DOX), treated with US and combined DOX-SDT. In the latter group, cells were exposed to 5 μM doxorubicin for 30 minutes and subjected to a pulsed ultrasonic wave with the parameters 0.2–0.5 W/cm^2, 1 MHz (PRF, pulse repetition frequency 100 Hz), a fill factor of 10% over 60 seconds. The synergistic effect of cell necrosis and additive apoptosis induction was observed at and above 0.3 W/cm^2. Importantly, there was no enhancement of the therapeutic effect when using 0.2 W/cm^2. The formation of hydroxyl radicals was detected at and above 0.3 W/cm^2. The radicals were produced to a greater extent during the action of DOX-SDT than when using US alone. The absorption of cytostatics by the cells increased by 13% in a combination therapy of 0.5 W/cm^2 in relation to the drug itself. The phenomenon of sonoporation considered important in the process of increasing the uptake of the drug was suggested by an experiment using fluorescein isothiocyanate (FITC). The authors hypothesized that treatment with DOX causes cell sensitization to US, although DOX itself did not show any effect on lipid peroxidation and cell membrane continuity. Only higher concentrations and longer treatment induced a significant change in these processes [58]. Scutellarin 7-O-β-D-glucuronide has enormous potential as a chemotherapeutic agent in the treatment of cancer, however it requires the use of high doses. The possibility of low-power ultrasound was investigated to optimize the amount of Scutellarin needed to apply. Ultrasonic intensities of 1.0 W/cm^2 and 0.05 W/cm^2 were used in in vivo and in vitro experiments respectively. In the experiment, very low doses of Scutellarin were given - 15 nM Balb/c tumor-bearing mice and squamous cell carcinoma of human language in vitro (SAS cells) cultured in suspension. The material was divided into the following groups: control, ultrasound itself, with Scutellarin alone, and Scutellarin-SDT treatment group. With the chemotherapy dose used, only the combined treatment showed a strong anticancer effect. In the case of an in vivo study, combined therapy significantly delayed tumor growth and initiated fragmentation of condensed nuclear chromatin. In addition, it inhibited tumor angiogenesis and tumor lymphangiogenesis, arrested proliferation of tumor cells. It also reduced the expression levels of MMP-2 and MMP-9 (matrix metalloproteinase) and initiated apoptosis. In the case of in vitro tests, the combined treatment caused changes in the shape of cells and a significant degree of microvilli damage. The migration and invasive activity of cancer cells has been inhibited. The therapy induced apoptosis. However, the combined treatment did not increase the production of intracellular free radicals. Scutellarin is not a sensitiser susceptible to ultrasound, and the obtained anti-cancer effect is not a positive effect of sonodynamic therapy. Ultrasounds of low intensity only increase the permeability of Scutellarin to the interior of tumor cells. The collected results indicate that using low-power US can be performed localized chemotherapy using significantly reduced doses of the drug, which significantly reduces the unwanted side effects [26].

In recent years, the analysis of the possibilities of improving the impact of ultrasounds in the treatment of cancer has resulted in further attempts to enhance its effectiveness also through the parallel use of a chemotherapeutic agent and a sonosensitizer. Adriamycin in the action of SDT-dependent showed a significant synergistic effect in the inhibition of the proliferation of human breast cancer cells (MDA-MB-231). However, these effects were dependent on the parameters tested and proved to be the strongest when Adriamycin was added after SDT with Ce6 [12].

Wang et al. [50] evaluated the efficacy of the use of Doxorubicin in combination with PpIX and low-power ultrasound on multidrug-resistant myeloid leukemia in vitro (K562). Under optimal conditions, the combination treatment significantly increased the death of K562 myeloid leukemia cells compared to monotherapy. The synergistic effects of DNA damage, the formation of intracellular ROS and the inhibition of P-glycoprotein (the transporter of the ATP-binding cassette) have been clearly demonstrated here [50].

ULTRASOUND ANTIANGIOGENIC THERAPY

Research on sonodynamic therapy in the antitumor aspect focuses primarily on its direct cytotoxic activity. The influence of SDT on the tumor microenvironment, especially on its vascularization, remains less understood. Currently, the thermal effect and inertial cavitation are described as two factors induced by ultrasounds, which can lead to damage to the vascular network within the neoplastic lesion. In a mouse model with implanted melanoma cells, a study was conducted to determine whether the echogenicity within the tumor changes as a result of US low power and whether such changes may be associated with antiangiogenic activity. It was estimated that the increase in tumor echogenicity was proportional to the frequency range of the ultrasound wave used. The frequency in the range of 1–3 MHz was tested and the effect obtained was at least partially related to the heterogeneity of tissues resulting from tumor vessel damage [53].

Ultrasounds with low power, 1–2 W/cm^2, 0.2–0.3 MPa were used to more accurately analyze the thermal effects of antiangiogenic therapy. The research was carried out on melanoma mice (K1735). Ultrasound therapy antivascular ultrasound (AVUS) was used at frequencies of 1 and 3 MHz, AVUS reduced tumor vasculature at both 1 MHz and 3 MHz, reduction of vascularization at 3 MHz was twice as high as with 1 MHz wave therapy. Reinforced antiangiogenic effect at 3 MHz was significant. Based on the conducted experiment, the authors suggest that ultrasound anti-angiogenic activity is primarily of a thermal nature, although it does not explicitly exclude the effect of inertial cavitation [39].

Microbubbles (ultrasound microbubble USMB), such as Optison, or Definity significantly improves the antivascular effect of US. Clinically available microbubble formulas mainly consist of bubbles in the range of 2 to 8 microns, making these preparations able to easily resonate with typical diagnostic ultrasonic frequencies 2–10 MHz. At sufficiently high pressures generated by the US wave, the interaction of oscillating microvesicles and microvascular walls can induce a spectrum of bioeffects, ranging from a temporary increase in permeability to persistent vascular damage. In preclinical studies, it was found that ultrasound-stimulated microbubbles are capable of closing blood flow through the tumor and causing its growth delays [27]. In vivo studies have shown that microvascular hemorrhages and changes in endothelial permeability can be produced in tissues containing these ultrasound contrast agents when these tissues are subjected to ultrasound with appropriate pressure amplitudes [20].

In another study conducted in mice, US 3 MHz, 2.4 W/cm^2 and 0.2 ml ultrasound contrast media were used. Antiangiogenic ultrasound therapy reduced the rate of growth of implanted melanoma and increased the survival time in relation to the untreated control group [54].

In another study, which aimed to assess the effectiveness of the ultrasound wave in anti-vascular therapy, the effects were analyzed using dynamic magnetic resonance imaging (MRI), contrast ultrasonography, histopathological analysis and immunohistochemistry. The studies were carried out on a mouse model of subcutaneous melanoma (K1735). Quantitative tumor perfusion characteristics were measured before and after treatment. Tumors were subjected to 1 or 3 minute exposure of low power, continuous US wave, after intravenous administration of ultrasound contrast agent. Both in 1 and 3 minute groups there was a strong reduction in tumor perfusion. A decrease in the vascular area was observed by approximately 40% and 70%, respectively. Pathological and histological changes correlated spatially with the regions of reduced perfusion revealed in contrast sonography with contrastive dynamic MRI. A significant increase in hypoxia-inducible factor 1α (HIF 1α and an increase in CD45/CD3 surface receptors in tumors in hypoxia-induced T lymphocytes indicates tumor-induced abnormalities. Therefore, the effects of antiangiogenic treatment with ultrasound go beyond direct cytotoxicity, including ischemic-dependent cytotoxicity, increased retention of molecules, and activation of the immune response within the tumor [19].

CONCLUSIONS

The analysis shows that SDT can be effective in the treatment of cancer with no significant adverse effects. Due to the significant depth to which the ultrasounds penetrate the tissue, SDT therapy provides an advantage over FDT, in which stimuli with lower penetration abilities are used. However, more studies are needed, especially in vivo, before SDT is accepted as an additional treatment method in selected oncological cases. Conducting research on the mechanisms of US operation, in the con-

Table 4. List of discribed substances used in SDT method.

Group	Subgroup	Substance
Sonosensitizers	hematoporphyrin (HP)	hematoporphyrin monomethyl ether (HMME)
		Photofrin II
	hematoporphyrin derivative (HPD)	protoporphyrin IX (PpIX)
		chlorine e6 (Ce6)
		Gallium-porphyrin (ATX-70)
	Bengali Rose Xanthene	rose bengal (RB)
		Erythrosine B (EB)
		Merocyanin 540 (MC 540)
		Bengal rose derivative (RBD)
non-steroidal anti-inflammatory drugs (NSAIDs)		tenoxicam
		piroxicam
anti-infective drugs		Quinolone
phenothiazine derivative		methylene blue (MB)
Nanoparticles		titanium dioxide (TiO_2)
		silicon dioxide ($SiO_{2)}$
		iron oxide (Fe_3O_4)
chemotherapeutics		Paclitaxel (PTX)
		Adriamycin (ADR)
		Doxorubicin (DOX)
ultrasound microbubble (USMB)		Scutellarin
		Optison
		Definity
Others		curcumin

text of modern design solutions of devices emitting ultrasonic waves, will help to control the parameters of the ultrasound wave, so as to minimize the risk of their application at the maximum possible therapeutic response. A better understanding of the mechanisms underlying the generation of ROS induced by SDT will enable the design of more effective and more effective sonosensitizers. The strategy of combining ultrasound with photosensitizers, chemotherapeutics or contrast agents is gaining more and more recognition, and it seems that it can be successful in the treatment of cancer. Conducting further research on antineoplastic sonodynamic therapy may in the future answer whether SDT will become a new additional cancer treatment strategy.

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The authors have no potential conflicts of interest to declare.