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Review

Sonodynamic therapy—a review of the synergistic effects of drugs and ultrasound

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Abstract

Sonodynamic therapy, the ultrasound dependent enhancement of cytotoxic activities of certain compounds (sonosensitizers) in studies with cells in vitro and in tumor bearing animals, is reviewed. The attractive features of this modality for cancer treatment emerges from the ability to focus the ultrasound energy on malignancy sites buried deep in tissues and to locally activate a preloaded sonosensitizer. Possible mechanisms of sonodynamic therapy include generation of sonosensitizer derived radicals which initiate chain peroxidation of membrane lipids via peroxyl and/or alkoxyl radicals, the physical destabilization of the cell membrane by the sonosensitizer thereby rendering the cell more susceptible to shear forces or ultrasound enhanced drug transport across the cell membrane (sonoporation). Evidence against the role of singlet oxygen in sonodynamic therapy is discussed. The mechanism of sonodynamic therapy is probably not governed by a universal mechanism, but may be influenced by multiple factors including the nature of the biological model, the sonosensitizer and the ultrasound parameters. The current review emphasizes the effect of ultrasound induced free radicals in sonodynamic therapy.
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Keywords: Ultrasound; Sonochemistry; Acoustic cavitation; Sonodynamic therapy; Sonosensitizer; Free radical; Cancer

Contents

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1. Introduction

Ultrasound is a mechanical wave with periodic vibrations of particles in a continuous, elastic medium at frequencies equal to or greater than 20 kHz. In liquids, its velocity of about 1000–1600 m/s translates into the wavelength range from micrometers to centimeters. Consequently the acoustic field cannot couple directly with the energy levels of molecules, including the biological milieu at the molecular level. Therefore, this radiation is not only perceived as safe, but has a very good tissue penetrating ability without major attenuation of its energy. This is a quite attractive feature from a clinical point of view and has prompted extensive evaluations of ultrasound for medical purposes [1]. Ultrasound has been in routine use for diagnostic imaging of soft tissue. Other therapeutic applications have been related to the thermal effects caused by ultrasound absorption hyperthermia (rise in tissue temperature to 40–45 °C) and thermal ablation (rise in tissue temperature to 60–85 °C during exposure that lasts a few seconds). The first effect was studied for clinical benefits such as inflammation relief, physical therapy and enhancement of chemotherapy and the latter for non-invasive surgery such as necrosis of solid tumors, sealing of blood vessels and correction of cardiac arrhythmias.

The interaction of ultrasound with bulk liquid is accompanied by the quite unique phenomenon of cavitation that leads to an enormous concentration and conversion of the diffuse sound energy. Historically, cavitation has been classified into two types, non-inertial and inertial. Non-inertial cavitation bubbles oscillate about an equilibrium radius and often persist for many acoustic cycles. As a result of these oscillations, streaming of surrounding liquid occurs and mechanical stresses create mixing of the medium. Inertial cavities are gas bubbles that grow to near resonance size and may expand to a maximum before violently collapsing. The temperature and pressure experienced by the material contained within the imploding cavities can reach values in excess of 5000 K and 800 atm. These extreme conditions may induce a multitude of chemical reactions within and surrounding the bubble, including a concentration of energy sufficient to generate light, an emission known as sonoluminescence.

Unlike thermal applications of ultrasound, the therapeutic use of the non-thermal effects of ultrasound have been less studied, although cavitation causes chemical reactions (sonochemistry) which might have biological implications in vivo. Sonochemical reactions can occur in three different regions. The first is the interior of collapsing gas bubbles in which extremely high temperatures and pressures prevail. Under these conditions typical pyrolysis reactions take place. In aqueous solutions, hydroxyl radicals, hydrogen atoms and oxygen atoms are formed by the thermal dissociation of water. The second region is the interface between collapsing gas bubbles and the bulk solvent, where high temperature and pressure gradients exist. The relative efficiency of non-volatile solutes to decompose thermally at the interface of a collapsing bubble depends on their ability to accumulate at the gas/solution interface of the growing bubble. In the third region, the bulk solution at ambient temperature and pressure, the free radicals formed in the cavitation bubbles react with solutes to produce products similar to those formed in radiation chemistry. Thus sonochemistry, while exhibiting unique features, shares some characteristics with combustion and radiation chemistry [2].

The contemporary oncological arsenal includes strategies based on synergistic (more than additive) interactions of two factors, e.g. ionizing radiation and hyperthermia, ionizing radiation and chemotherapy, or the still experimental photodynamic therapy. The latter is based on the use of an innocuous dye ''hematoporphyrin derivative'' which is taken up preferentially by the malignant tissue and acquires ''drug activity'' only when activated by the correct wavelength of red light. Once in the excited state, the hematoporphyrin derivative initiates a chain of events which ends in tumor necrosis [3]. Sonodynamic therapy is an analogous approach based on the synergistic effect of ultrasound and a chemical compound referred to as ''sonosensitizer''. The effect can be localized by focusing the ultrasound on a defined region and choosing compounds with tumor-affinity [4–6]. The ability to enhance drug cytotoxicity with ultrasound that enables efficient but localized effects on a pathological site with minimal damage to peripheral healthy tissue would be a valuable clinical asset.

The scope of this review is to integrate the information relevant to sonosensitized therapy, excluding the reactions induced by pulse high-energy ultrasound (lithotripsy) in which mechanical stresses seem to play a major role, in the hope to incite interest and further research activity in this domain.

2. Experimental observations in vitro and in vivo

The original approach to sonodynamic therapy can be traced to the non-specific, heating effect of ultrasound [7]. Once it was recognized that hyperthermia enhances cytotoxicity, ultrasound, with its focusing ability, was suggested as an attractive tool to achieve it. Potentiated cytotoxicity by ultrasound was first demonstrated when mouse leukemia L1210 cells were exposed to continuous wave ultrasound (2 MHz, 10 W/cm^2) while suspended in nitrogen mustard solution in vitro. Mice subsequently inoculated with these cells had longer survival times than control animals that received cells exposed to the drug but not ultrasound [8]. A following plethora of data have demonstrated a synergistic increase in cytotoxicity by ultrasound hyperthermia of other alkylating agents [9,10], bleomycin [11], adriamycin [12,13], amphotericin B [12] occasionally the effect was uncertain as for mitomycin C and 1 W/cm^2 continuous wave ultrasound [14], adriamycin and daunomycin and 0.7 W/cm^2 continuous wave ultrasound [15], or absent, as for cis-diaminedichloride platinum [12,16] and etoposide [12].

2.1. Anticancer drugs as sonosensitizers

Aiming to increase treatment efficiency and to reduce undesired side effects, it has been hypothesized that ultrasound energy might enhance the cytotoxicity of chemotherapeutic drugs (Table 1). While initial applications have used the thermal effects of ultrasound, research on sonodynamic therapy has evolved to evaluate the non-thermal effects. Two types of low-level ultrasound intensities tone-burst ultrasound (1.765 MHz, $I_{SATA} = 0.25$ W/cm^2, 10% duty cycle), and pulsed ultrasound (2.5 MHz centre frequency, $I_{TA} = 0.031$–0.18 W/cm^2, 1 kHz repetition frequency, MPa-level pressure amplitudes), distributed uniformly over the biological target, were found to potentiate chemotherapeutic cell killing with adriamycin and diaziquone [17] but not of cisplatin or mitomycin C [18]. These ultrasound beams were non-cytotoxic and produced negligible temperature elevation. Statistically significant ultrasound-induced increases in adriamycin and diaziquone cytotoxicity were observed in vitro in Chinese hamster ovary and MCF-7 WT cells, but not in V79 cells. The effects of

Table 1
Anticancer drugs tested as sonosensitizers

Sonosensitizer	Ultrasound[a]		Biological model	Ref.
	W/cm^2	MHz		
Nitrogen mustard	10	2	In vitro (mouse L1210)	[8]
Cyclophosphamide, thiotepa	1.7	2.25	In vivo (TCT-4909 bladder tumor)	[9]
Cyclophosphamide	400	4	In vivo (hepatoma)	[10]
Bleomycin		1	In vivo	[11]
Adriamycin	2	1	In vitro (CHO)	[12]
Adriamycin	2.3	2.6	In vitro (V79 chinese hamster)	[13]
Adriamycin	0.7	1.92	In vivo (Yoshida rat sarcoma)	[15]
Adriamycin	0.25	1.765	In vitro (CHO, MCF-7WT) and in vivo	[17]
	2.5	0.031–0.18 P	(uterine cervical squamous carcinoma)	
Adriamycin	5	0.29–1.62	In vitro (CHO)	[18]
Adriamycin	2	1.733	In vivo (fibrosarcoma RIF-1 and melanoma B-16)	[19]
Adriamycin	3	1.93	In vitro (sarcoma 180)	[21]
Adriamycin	5.12	–	In vitro (human ovarian carcinoma 3AO)	[22]
Adriamycin	3.18	1.92	In vitro (sarcoma ascites 130)	[25]
Adriamycin	6	1.93	In vitro (sarcoma 180)	[99]
Adriamycin	2.5	1.62–1.765	In vitro (CHO)	[109]
FAD104	2.5	1.93	In vitro (sarcoma 180)	[20]
Amphotericin B	2	1	In vitro (CHO)	[12]
Mitomycin C	3	2	In vivo (AH109 sarcoma)	[14]
Mitomycin C (no sensitization)	5	0.29–1.62	In vitro (CHO)	[18]
Daunomycin	0.7	1.92	In vivo (Yoshida rat sarcoma)	[15]
Cisplatin (no sensitization)	2	1	In vitro (CHO)	[12]
Cisplatin (no sensitization)	2	0.87	In vivo (murine renal function)	[16]
Cisplatin (no sensitization)	5	0.29–1.62	In vitro (CHO)	[18]
Etoposide (no sensitization)	2	1	In vitro (CHO)	[12]
Diaziquone	0.25	1.765	In vitro (CHO, MCF-7WT) and in vivo	[17]
	2.5	0.031–0.18 P	(uterine cervical squamous carcinoma)	
Diaziquone	5	0.29–1.62	In vitro (CHO)	[18]
Dihydroxy(oxybi-guanido)boron	18.35	25	In vivo (carcinoma)	[23]
5-fluorouracil	18.35	25	In vivo (carcinoma)	[23]
5-fluorouracil	3	0.8 cw, P	In vivo (Ehrilch carcinoma)	[24]

[a] Continuous wave, unless marked P (pulsed).

combined drug and ultrasound treatments were studied also in vivo by measuring post-treatment volume changes in uterine cervical squamous cell carcinoma implanted in the cheek pouch of the Syrian hamster. Again, a statistically significant ultrasound-drug synergy in reduction of the tumor volume was observed [17]. Mice, bearing either a fibrosarcoma (RIF-1) or a melanoma (B-16) on their thighs, were injected with a single dose of adriamycin (10–20 mg/kg). The tumors were then heated locally to 41–43 °C for 30 min, either by insonation with ultrasound or by immersion of the animals' limbs into hot water baths. Antitumor efficacy was scored by two assays the time for the tumor to double in size, or the X-ray dose required to sterilize 50% of the tumors after the adriamycin-hyperthermia treatment. Ultrasound-induced hyperthermia was substantially more effective in enhancing adriamycin activity than was hyperthermia induced by the water bath. The mean-doubling time was 13 days for tumors treated with the combination of adriamycin and ultrasound and 6 days for tumors heated with a water bath immediately after injection of adriamycin. The X-ray dose required for 50% sterilization was 21.2 ± 0.8 Gy for the combination of ultrasound and adriamycin, and 36.1 ± 0.9 Gy for the water bath heating and adriamycin. The suspicion that ultrasound might enhance metastatic rates, perhaps by mechanically dislodging cells from tumors, was tested in the B-16 melanoma system by quantifying formation of lung colonies but no effects of ultrasound on frequency of metastatic formation were seen [19]. The observed drug potentiation was attributed to increased cellular uptake of adriamycin due to ultrasound, without affecting membrane fluidity [18,19].

Other anthracycline derivatives with cardiotoxicity significantly lower than adriamycin were also tested. Thus, the ultrasonically induced cell damaging effect of fluorine-containing anthracycline derivative (FAD104) was investigated in vitro with sarcoma 180 cells. The rate of inducing cell damage with ultrasound was doubled with 80 μM FAD104, while no cell damage was observed with FAD104 alone [20]. Likewise, the damage to sarcoma 180 cells doubled with 80 μM 4′-O-tetrahydropyranyladriamycin, while no cell damage was observed with the drug alone [21]. Low power ultrasound ($5.12\ W/cm^2 \times 5$ s) which did not cause acute or delayed inhibition in vitro of human ovarian carcinoma cell line 3AO, promoted intracellular accumulation of adriamycin into the cells pretreated with the drug and enhanced its cytotoxicity. When ultrasound exposure preceded the drug application, a smaller effect was observed [22].

The combination of ultrasound with a new tentative anticancer agent, dihydroxy(oxybiguanido)boron(III)-HCl produced an extra tumoricidic action against the mouse ascites tumor as compared to the effect in its absence. However, the drug and ultrasound acted in an additive manner, rather than synergistically [23]. Ultrasound (0.8 MHz, continuous or pulsed, 1–3 W/cm^2) effect on combined treatment with the anticancer drug 5-fluorouracil on Ehrlich ascites tumor in vivo, showed decreased tumor growth, severe damage in cytoplasmic organelles and cytoplasmic vacuoles and severe increase in numbers of pyknotic and apoptotic cells [24].

2.2. Porphyrin sonosensitizers

In addition to the anti-cancer drugs, the tumor-localizing porphyrins which have been traditionally used as sensitizers in photodynamic therapy, have also been evaluated in ultrasound-induced reactions. This followed the assumption that the ultrasound energy which generates sonoluminescence might cause electronic excitation of porphyrins by energy transfer and initiate a photochemical process resulting in the formation of the cytotoxic singlet oxygen. In contrast to anti-cancer drugs, porphyrins are nontoxic in the absence of ultrasound. Table 2 summarizes the reports on the use of porphyrins as sonosensitizers.

Hematoporphyrin, the most common photodynamic sensitizer, enhanced the killing of mouse sarcoma and rat ascites 130 tumor cells exposed in vitro to ultrasound (1.92 MHz) at intensities of 1.27 of 3.18 w/cm^2, from 30% and 50% to 99% to 95% respectively. It was noted however that even after the most severe treatment used, 2–5% of cells remained undamaged [25]. In vitro and in vivo studies with sarcoma 180 cells in mice demonstrate antitumor effect of hematoporphyrin activated by ultrasound irradiation [26,27]. The inhibition of ascitic S180 cells and induced sarcoma 180 tumor in vivo was studied with the combination of hematoporphyrin derivative and ultrasound at the frequency of 1.1 MHz. The injury of ascitic S180 cells increased as time passed and the inhibitory effect was stronger in the ultrasound plus hematoporphyrin derivatives group. Changes in cell structure, cytochrome C oxidase activity and degradation of DNA were the important factors that inhibited the tumor cell growth and even induced cell death [28].

It is however noted that when human colorectal adenocarcinoma cells (HT-29) were used to test the cytotoxicity of hematoporphyrin derivative and ultrasound (2.21 MHz, 3.7 W/cm^2) in vitro, no significant difference was found with or without sonosensitizer. In addition, no structural modification of the hematoporphyrin derivative was detected after ultrasound treatment, as monitored by fluorescence measurements [29]. Chinese hamster ovary cells were also found refractory to the hematoporphyrin-enhancement of sonication (1.955 MHz) [30]. Although these negative results may be due to an unusual resistance of these cell lines, it is more probably the result of the experimental procedure which involved the removal of the extracellular sonosensitizer. Studies with several porphyrins as sensitizers

Table 2
Porphyrins tested as sonosensitizers

Sonosensitizer	Ultrasound		Biological model	Ref.
	W/cm^2	MHz		
Hematoporphyrin	3.18	1.92	In vitro (sarcoma 180 and AH 130)	[25]
Hematoporphyrin	1.7	1.92	In vitro and in vivo (sarcoma 180)	[26]
Hematoporphyrin	1.7	1.92	In vivo (sarcoma 180)	[27]
Hematoporphyrin	–	1.1	In vivo (sarcoma 180)	[28]
Hematoporphyrin (no sensitization)	3.7	2.21	In vitro (human colorectal adenocarcinoma)	[29]
Hematoporphyrin (no sensitization)	9	1.955	In vitro (CHO)	[30]
Hematoporphyrin	1.8	1.92	In vitro (sarcoma 180)	[94]
Hematoporphyrin	4.5	1.92	In vitro (sarcoma 180)	[97]
Diacetylhematoporphyrin-mitomycin C conjugate	–	2.26	In vitro (sarcoma 180)	[54]
Photofrin II	0.45	0.270	In vitro (HL-60)	[33]
Photofrin II	3–5	1.92	In vivo (colon 26 carcinoma)	[34]
Photofrin II	6	1.93	In vitro (sarcoma 180)	[96]
Photofrin II	3	1.92	In vivo (AH130)	[35]
Photofrin	0.3–0.5	0.450	In vitro (MT-2 cells, PMNC from ATL patients)	[37,38]
Mesoporphyrin	4	1.94	In vitro (murine L1210)	[31]
Mesoporphyrin	7.5	1.94	In vitro (murine L1210)	[57]
Protoporphyrin	4	1.94	In vitro (murine L1210)	[31]
Protoporphyrin IX	4.5	1.92	In vitro (sarcoma 180)	[64]
Copper protoporphyrin	4	1.94	In vitro (murine L1210)	[31]
Tetraphenylporphine tetrasulfonate	4	1.94	In vitro (murine L1210)	[31]
ATX-70	4	1.94	In vitro (murine L1210)	[31]
ATX-70	–	0.05	In vitro (HL-525)	[32]
ATX-70	4.5	1.93	In vitro (sarcoma 180)	[39]
ATX-70	3	2	In vivo (colon 26 carcinoma)	[40]
ATX-70	0.51	1	In vivo (squamous cell carcinoma)	[60]
ATX-70	4.5	1.92	In vitro (sarcoma 180)	[97]
ATX-70	8 + 8 = 16	0.5 + 1	In vivo (colon 26 carcinoma)	[65]
ATX-70	12	0.5 + 1	In vivo (Walker 256 tumor)	[66]
ATX-70	4.5	1.93	In vitro (sarcoma 180)	[97]
ATX-70 derivatives	15	0.047	In vitro (HL-525 and HL-60)	[107]
ATX-70/F39 immunoconjugate	1, 2	1	In vitro (KATO-III) and in vivo (xenograft model)	[55]
ATX-S10	3	2	In vitro (sarcoma 180) and in vivo (colon 26 carcinoma)	[42]
ATX-S10	6	2	In vitro (sarcoma 180)	[98]
Pheophorbide-a	4.5	2	In vitro (sarcoma 180)	[43]
Pheophorbide-a	0.51	1	In vivo (squamous cell carcinoma)	[60]
ClAl-phthalocyanine tetrasulfonate	3	1.92	In vivo (colon 26 carcinoma)	[44]
Chlorin PAD-S31	0.3	1	In vivo (neointimal hyperplasia)	[67]

have shown that with ultrasound treatment cell viability was not altered when cells contained only intracellular porphyrins [31,32].

Photofrin II (a commercial, purified version of hematoporphyrin derivative) and low-level ultrasound (270 kHz) at intensities of 0.15, 0.3 and 0.45 W/cm^2, applied for 60 s, enhanced the cell killing of HL-60 cells. Cell survival after treatment, in the presence or absence of Photofrin, was $49.6 \pm 5.1\%$ vs. $92.9 \pm 1.5\%$, $34.5 \pm 3.1\%$ vs. $82.3 \pm 2.2\%$, and $27.4 \pm 3.9\%$ vs. $77.9 \pm 7.2\%$ [33]. The sonodynamically-induced antitumor effect of Photofrin II, was also evaluated in mice bearing colon 26 carcinoma [34] and AH130 solid tumors [35]. Since the highest concentration of Photofrin II in the tumor was observed 24 h after administration, the ultrasonic exposure timing was at peak concentration. The anti-tumor effect, as estimated by measuring the tumor size, became increasingly significant as the dose of Photofrin II was increased. Possible treatment of liver tumors was indicated by a study that measured the decrease in volume of normal rat liver by ultrasound (210 kHz, 1.3 W/cm^2 for a total duration of 3 min) after administration of a Photofrin II. The depth of tissue damage was histologically compared to rats exposed to ultrasound alone. The mean maximum lesion depth on rats applied with ultrasound and Photofrin II was 5.7 ± 0.9 mm whereas in rats treated with ultrasound alone this was 3.0 ± 0.4 mm [36].

In a study on leukemic and normal cells after sonodynamic effects (450 kHz at an intensity of 0.3–0.5 W/cm^2) using Photofrin, cell survival among MT-2 cells, peripheral mononuclear cells in normal and adult T cell

leukemia patients, was compared. The survival rate of MT-2 cells was inversely proportional to the amount of sensitizer. On the other hand, in the normal human peripheral mononuclear cells, no significant differences of cell survival rates were found between ultrasound-treated groups with and without Photofrin. The survival rate of peripheral mononuclear cells in the blood of acute-type adult T cell leukemia patients of $69.4 \pm 22.5\%$ after ultrasound exposure (0.3 W/cm^2, 60 s) alone, was decreased to $30.0 \pm 23.0\%$ when the treatment was repeated in the presence of 100 μg/ml of Photofrin. There were no significant cytotoxicities in all groups treated with Photofrin only. Apparently it was a specific selectivity of sonodynamic effects to MT-2 cell lines and peripheral mononuclear cells in adult T cell leukemia patients, and it was anticipated that this new method of treatment could be used for extracorporeal blood treatment of acute-type adult T cell leukemia patients [37,38].

A Gallium-porphyrin, 7,12-bis (1-decyloxyethyl)-Ga (III)-3,8,13,17-tetramethylporphyrin-2,18-dipropionyl-diaspartic acid, (ATX-70), developed for a high yield of singlet oxygen generation and presumed efficiency in photodynamic therapy, was found to induce damage to isolated sarcoma 180 cells in air-saturated suspension at four times higher rate vs. only twice by the same concentration of hematoporphyrin, in combination with 2 MHz ultrasound [39]. In mice bearing colon 26 carcinoma, the antitumor effect became increasingly significant as the dose of ATX-70 was increased. At an ATX-70 dose of 2.5 mg/kg or higher, the average tumor size decreased by more than a half, three days after the ultrasonic exposure. This was less than a decrease of a third of the untreated tumors on the same day. When used alone, ultrasound shrunk the tumor slightly, while use of ATX-70 alone had no significant effect on the tumor [40]. A pharmacokinetic study of ATX-70 showed that about 24 h after administration the tumor/plasma concentration ratio peaked and relatively high tumor/skin and tumor/muscle concentration ratios were seen, pointing to the best treatment time [41]. The sonodynamically induced antitumor effect of a chlorin derivative, 4-formyloximethylidene-3-hydroxy-2-vinyl-deuterio-porphynyl (IX)-6,7-diaspartic acid (ATX-S10) which is significantly less toxic than ATX-70 and it's long phosphorescence lifetime can be an advantage in generation of singlet oxygen, was investigated in vitro with sarcoma 180 cells, and in vivo with implanted colon 26 tumor in mice. The rate of ultrasonically induced damage to isolated sarcoma 180 cells in air-saturated suspension was enhanced two-fold with 80 μM ATX-S10. The co-administration of 25 mg/kg ATX-S10 followed by ultrasonic exposure at 2 MHz stopped the growth of implanted colon 26 tumor in mice at an intensity at which ultrasound alone showed only a slight antitumor effect [42].

A valuable mechanistic insight was provided when mesoporphyrin, protoporphyrin, copper protoporphyrin, tetraphenylporphine tetrasulfonate and ATX-70 were evaluated for synergism with ultrasound or light, on the murine leukemia L1210 cell line in culture. Loss of cell viability was associated with inhibition of amino acid transport and cell fragmentation, suggesting disruption of the integrity of the cell membrane. In contradistinction to the photodynamic reaction, all porphyrins tested enhanced the ultrasound-induced cell damage only when present in the extracellular environment and not in the intracellular space. Furthermore, it was noted that there was no correlation between the efficacy of a given porphyrin for light- vs. ultrasound-induced cytotoxicity [31].

Likewise, the rate of ultrasonically induced cell damage to sarcoma 180 cells in an air-saturated suspension was enhanced two times by the presence of a chlorophyll derivative, pheophorbide-a, over treatment in its absence. In mice injected with 5 mg pheophorbide-a/kg before the insonation, the tumor growth was stopped at an intensity at which ultrasound alone showed only a slight antitumor effect [43].

Chloroaluminum phthalocyanine tetrasulfonate, a second generation sensitizer developed for photodynamic therapy, was also evaluated for sonochemical activation in mice bearing subcutaneously colon 26 carcinoma. 24 h after intravenous administration, when the drug reached maximum concentration in the tumor tissue, the animal was exposed to ultrasound at 2 MHz in a standing wave mode and free-field intensity of at least 3 W/cm^2. The results for the combined treatment showed a significant antitumor effect as evaluated by the decrease in the tumor size. It is however noted that no total eradication was achieved [44].

The question of the integrity of the sensitizer after ultrasonic irradiation (20, 40 and 540 kHz at 25 °C) was addressed for 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrin. The concentration of the porphyrin, as estimated spectrophotometrically, decreased in a first order reaction at a rate depending on the output power of the ultrasonic generator. The degree of decomposition was correlated with the concentration of NO_3^- and H_2O_2 produced in water by the ultrasonic irradiation [45,46].

2.3. Other sonosensitizers

In addition to chemotherapeutic drugs and photodynamic porphyrins, various compounds have also been tested for sonodynamic activity (Table 3). The ultrasound potentiation of the anti-inflammatory drugs tenoxicam and piroxicam against sarcoma 180 cells was observed in vitro [47] and in vivo (2 MHz, 10 W, 120 s) in a mouse air pouch model [48]. The mortality rate of tumor cells immediately after the irradiation and the survival rate of mice were significantly higher than those

Table 3
Other sonosensitizers

Sonosensitizer	Ultrasound		Biological model	Ref.
	W/cm^2	MHz		
Tenoxicam	1.3–3	2	In vitro (sarcoma 180)	[47]
Piroxicam	1.3–3	2	In vitro (sarcoma 180)	[47]
Piroxicam	10	2	In vivo (sarcoma 180)	[48]
Rose bengal	0.8–5.9	1.93	In vitro (sarcoma 180)	[49]
Erythrosine B	5.9	1.93	In vitro (sarcoma 180)	[50]
Merocyanine 540	0.4	0.255	In vitro (HL-60)	[51]
Dimethylformamide	0.5–2.5	0.985	In vitro (HL-60)	[52]
Cytosine arabinoside	0.3	0.48	In vitro (HL-60)	[53]
Pyridocarbazole	–	2.26	In vitro (sarcoma 180)	[54]
2,2′-azobis(2-amidinopropane)	2	1	In vitro (U937)	[57]
5,5-dimethyl-1-pyrroline-*N*-oxide	–	0.05	In vitro (HL-525)	[32]
4-pyridyl-1-oxide-*N*-*t*-butylnitrone	–	0.05	In vitro (HL-525)	[32]

when ultrasound alone was applied and these effects of piroxicam were dose-dependent.

In an extension to dye-sensitized photooxidation reactions which proceed via electronically excited triplet state and the singlet oxygen mechanism, the use of xanthene photosensitizers was suggested also for sonodynamic therapy. The rate of inducing cell damage on sarcoma 180 by ultrasound was enhanced two to three times with 160 μM rose bengal, while no cell damage was observed with rose bengal alone [49]. Likewise, sarcoma 180 cells exposed to ultrasound at 1.93 MHz in a standing-wave mode for up to 60 s, showed a cell damage rate enhanced by 4–5 times with 160 μM erythrosine B, while no cell damage was observed with dye alone [50]. HL-60 cells in suspension exposed to continuous wave ultrasound (255 kHz) in the presence of the photosensitizing drug merocyanine 540, at an intensity of 0.4 W/cm^2 showed multiple surface pores as observed with a scanning electron microscope, and significant reduction in the number of colonies. Ultrasound alone and dye alone had no cytotoxic effects [51].

The ability to enhance local drug cytotoxicity with ultrasound while minimizing effects peripheral to the treatment site, has prompted the investigation of polar solvents such as dimethylformamide which had been considered as anticancer drugs but their potential usefulness is constrained by hepatoxic side effects. Thus, the lysis of HL-60 human promyelocytic leukemia cells were significantly increased when sonicated with a noncytotoxic dose of dimethylformamide [52].

Significant differences were obtained between ultrasound treated (48 kHz, 0.3 W/cm^2) and untreated HL-60 cells in the presence of various concentrations of cytosine arabinoside (10^{-7}–2×10^{-9} M). Morphological evaluation of ultrasound irradiated cells with scanning electron microscopy showed minor disruption of the cell surface. These observations suggest that low intensity ultrasound altered the cell membrane, possibly resulting in changed sonosensitizer uptake [53].

Some strategies to increase the efficiency of sonodynamic therapy have focused on the sensitizer, particularly since at this stage the molecular structures and features to identify useful sonosensitizers are not yet defined. Hence, diacetylhematoporphyrin-mitomycin C conjugate was synthesized and was found to show excellent cell-killing effect in vitro in combination with ultrasound, as compared to metal free-, Co-, or Fe-hematoporphyrin, diacetylhematoporphyrin, Acrinol or mitomycin C. Very good results were obtained also with two pyridocarbazole derivatives [54]. In another approach, an immunoconjugate was prepared between ATX-70 and a high affinity monoclonal antibody against carcinoembryonic antigen which is often overexpressed in various carcinoma cells (F11-39). This conjugate, designated F39/ATX-70, was shown to retain immunoreactivity against carcinoembryonic antigen-expressing cells. When applied in combination with ultrasound irradiation, the cytotoxicity of the conjugate against carcinoembryonic antigen-expressing human gastric carcinoma cells in vitro was found to be greater than that of ATX-70. When anti-tumor effects were assessed in vivo in a mouse xenograft model, intravenous administration of F39/ATX-70 followed by ultrasonic irradiation produced a marked inhibition of tumor growth as compared with ultrasound exposure alone or applied after administration of ATX-70. These results suggest that this immunoconjugate may have applications in sonodynamic therapy where destruction of carcinoembryonic antigen-expressing tumor is required [55].

Water-soluble azo-compounds have also been considered as sonosenzitizers. These are thermally labile molecules which decompose to form carbon-centered radicals following the release of molecular nitrogen, and ultimately peroxyl radicals if the decomposition is carried out in the presence of oxygen. These latter radicals are cytotoxic and, therefore, the feasibility of using focused ultrasound for site-specific decomposition of azo

compounds was studied by spin trapping and ESR spectroscopy. Tertiary carbon centered radicals were detected in argon-saturated solution, and the corresponding oxygen-centered radicals in aerated sonicated solutions. Experiments using scavengers of hydroxyl radicals and hydrogen atoms which are produced by sonolysis in aqueous solutions, demonstrated that these radicals are not involved in the radical production from azo compounds. The most likely mechanism of decomposition of azo compounds by ultrasound is their thermolysis in the heated shell of liquid surrounding cavitating bubbles and/or by pyrolysis inside these bubbles [56].

Indeed, ultrasound-induced lysis and apoptosis of human monocytic leukemia cells (U937) were enhanced in the presence of 2,2′-axobis(2-amidinopropane)·2HCl at a concentration that is nontoxic to the cells [57].

2.4. Physical means to improve sonodynamic therapy

Kessel et al. [58], found that the sequential application of ultrasound and mesoporphyrin followed by photodynamic treatment with the same sensitizer, decreased the viability of cells which had survived ultrasonic treatment. Photodynamic damage to cells before exposure to ultrasound potentiated cell breakage but did not affect the clonogenicity of the surviving cell population. Differences were noted between photo- and ultrasound-induced treatments. The photodynamic effects mediated by mesoporphyrin caused a delayed toxic reaction, the presence of a ''shoulder'' on the dose-response curve, indicating the capacity for limited repair of photodamage. In contrast, ultrasound-induced loss of viability resulted from rapid cell destruction and was proportional to the time of sonication. In the context of potential repair of an ultrasound-induced damage, it is however noted that ultrasound irradiation alone can induce reversible cell-membrane modifications, as occurred in sonoporation. During this process, exogenous molecules, such as plasmids [59] or drugs [60], can enter the cells through transient pores formed in the membrane by ultrasound which can reseal following the uptake.

In another attempt to improve the tumoricidal effect in a transplantable mouse squamous cell carcinoma model, ultrasound therapy was combined with photodynamic therapy. ATX-70 and pheophorbide-a were tested as photosonosensitizers. The separate or combined (sono- and photo-) treatments were applied at the time of maximum drug retention in the tumor. The combination of these two modalities, which was found to have an additive rather than synergistic effect, resulted in a significantly improved inhibition of tumor growth (92–98%), as compared to either single treatment (27–77%) due to a deeper tumor necrosis by 2–3 times. Moreover, the median survival period (>120 days) was significantly greater than in single treatment groups (77–95 days) [61].

Umemura and colleagues [62,63] have demonstrated that sonochemically active cavitation can be enhanced by an order of magnitude by superimposing the second harmonic on the fundamental wave. With this method the cavitation can be controlled with relative ease even in a progressive field and the optimum phase can be maintained in the target tissue, thus promising improvement in localized therapeutic intervention. The tumor growth of sarcoma cells inhibited by hematoporphyrin and protoporphyrin and ultrasound at 2 MHz is greatly accelerated by superimposing the second harmonic onto the fundamental [64]. The antitumor effects of focused ultrasound at superimposed 500 kHz and 1 MHz in a progressive wave mode was investigated in vivo on colon 26 carcinoma implanted in mouse [65] and on Walker 256 tumors implanted in rat kidneys [66] after administration of ATX-70. Histological observation 7 days after the exposure revealed the destruction of tumor tissue with the ultrasonic treatment in combination with ATX-70, while the treatment with ATX-70 or ultrasound alone did not cause any necrosis.

2.5. Applications of sonodynamic therapy for non-cancer disorders

A pivotal problem in the treatment of cardiovascular diseases by intravascular interventions for enlargement of blood vessels, such as balloon angioplasty and stenting, is the production of a tissue matrix defined as neointimal hyperplasia and restenosis. This growth is initiated by activated smooth muscle in response to the irritation of the inner wall and since cell culture studies have showed that low-frequency ultrasound may impact smooth muscle cell proliferation, therapeutic ultrasound catheters are currently being developed. Since it is known that the activation of sonochemical sensitizers by ultrasound inhibits tumor growth, the efficiency of a water-soluble chlorin-derivative (PAD-S31) activated by transdermally delivered ultrasound energy (1 MHz, 0.3 W/cm^2) was tested on neointimal hyperplasia in a rabbit stent model. The arteries treated with ultrasound and PAD-S31 showed only mild neointimal hyperplasia, whereas in all control experiments the growth was significantly larger. All rabbits tolerated the combined treatment well and it was concluded that the treatment has clinical potential [67]. Likewise, ultrasound is emerging as a promising modality for recanalization of thrombosed blood vessels. In most cases, application of low frequency (20–27 kHz) ultrasound has an additive effect to antiplatelet, antithrombotic and fibrinolytic drugs [68]. However, in combination with streptokinase it had a synergistic effect on disruption of both fresh and aged blood clots in vitro [69].

Another therapy-related application of ultrasound is improved drug delivery, based on the observation that ultrasound may induce reversible membrane permeabilization. This application is however beyond the scope of this review.

3. The mechanism of sonodynamic therapy

Excluding, a priori, the thermal effects as a mode of action of ultrasound in biological systems, ultrasonic waves of sufficient intensity are capable of initiating changes in biological systems through mechanisms involving acoustic cavitation and subsequent sonomechanical and/or sonochemical processes. While both non-inertial and inertial cavitation can generate mechanical forces, only inertial cavitation can produce chemical effects.

Reviewing the widely structurally different compounds which have been reported to have sonodynamic activity it is difficult to expect a universal mechanism for the synergism between ultrasound and drugs. Experimental evidence indicates that ultrasound-increased cellular uptake may contribute to the enhanced cytotoxicity of sensitizers such as adriamycin [18,19,22]. Exogenous additives, such as ascorbic acid, inhibited the ultrasound-induced intracellular accumulation of adriamycin in human ovarian carcinoma cells 3AO in vitro [70].

On the other hand, the activation of sensitizers which are innocuous in the absence of ultrasound activation, is poorly understood.

3.1. Hydrodynamic stress

Apart from the potential sonochemical effects on cells, ultrasound irradiation can create damage to a biological milieu by hydrodynamically shearing the cells when fast moving bubbles pass by the cells on their way across the medium (acoustic microstreaming), erosion due to pressure pulses on rebound or bubble involution and jetting. Even the streaming of fluid around oscillating bubbles can result in shear stresses sufficient for cell destruction. It should be noted that changes in certain parameters during sonolysis such as ambient temperature and pressure, composition of the dissolved gas, frequency and intensity of the acoustic wave, and solution viscosity result in significant changes in the cavitation phenomenon. The viscosity is particularly relevant since the effective viscosity of the contents of disrupted cells is not known and the radial motion of bubbles and their ability to damage cells, declines as the viscosity of the surrounding fluid increases.

Attributing the cell-damaging effects of ultrasound in the absence of sonosensitizers to either mechanical stress and/or sonochemical effects of acoustic cavitation is a challenging quest. Miller and Miller [71] and Clarke and Hill [72] showed that cell lysis is closely linked to cavitation and suggested that ultrasound induced shear forces primarily disrupt cellular membranes [71,72]. Recently, comprehensive studies on the lysis of red blood cells by ultrasound in the MHz frequency were conducted by Miller et al., who investigated the effects of cell size [73], dissolved oxygen concentration [74], tonicity of the medium [75] and pulse length dependence [76]. The conclusion from these studies was that shear induced cytolysis was the dominant mechanism under the conditions of these studies.

Since ultrasound can induce the mechanical lysis of cells, it follows that these mechanical effects could be enhanced by the addition of membrane destabilizing molecules that can physically reduce the strength of the cell membrane. For example, unlike porphyrins which have been shown to sensitize ultrasound induced cell damage only when present in the extracellular medium [31,32], the addition of trolox, a water-soluble derivative of vitamin E, renders human erythrocytes more fragile to mechanical stress due to the inclusion of this molecule in the cell membrane, resulting in enhanced cytolysis [77]. On the other hand, in a study of dimethylformamide-enhanced lysis by ultrasound (985 kHz, 0.5–2.5 W/cm^2) of cultured HL-60 human promyelocytic leukemia cells, evidence was presented to support the hypothesis that cell damage is due to a sonochemical rather than a sonomechanical process. Thus, cellular response to shear without ultrasound, as evaluated using a Couette flow chamber, showed that dimethylformamide did not increase cell susceptibility to shear stress, and the toxic effect was attributed to unknown short lived reactive species produced from dimethylformamide by acoustic cavitation [52].

3.2. Sonochemical effects via hydroxyl radicals

Inertial cavitation is an extremely violent process of bubble activity on microsecond and nanometer scales that can result in pyrolysis of the water vapor inside the bubble, generating the very reactive hydroxyl radical and hydrogen atom. Using the spin trapping technique, the ESR spectra characteristic of the hydrogen atom and hydroxyl radical adducts were observed following sonolysis of water [78]. These primary free radicals may recombine or react with volatile solute molecules on the inside of the bubble to generate new free radicals. Thus for example, hydrogen atoms can react with oxygen in the bubble to produce hydroperoxide radical which dissociates at biological pH to the superoxide radical anion. The primary radicals can also react with non-volatile solutes surrounding the bubble, e.g. sonolytically-generated hydroxyl radical can modify purine and pyrimidine bases of DNA as shown by ESR and spin trapping [79,80].

Longer-lived, residual chemical compounds may be produced in sonicated media and have been reported to generate bioeffects in cells not directly exposed to ultrasound. Clarke and Hill found that insonation of cell-free culture medium reduced its ability to support cells [72]. Single strand breaks could be induced in Chinese hamster ovary cells not only during continuous-wave insonation [81] but also after sonolysis has ceased, presumably through the action of residual H_2O_2 [82]. It is however noted that the yield of hydroxyl radical and H_2O_2 was significantly reduced in a sonicated culture medium as compared to exposure in phosphate-buffered saline, primarily due to the scavenging effect of glucose and hydrophobic amino acids (Trp, Phe, Tyr, Leu, Val, Met) [83].

When rare gases (Xe, Kr, Ar, Ne, He) were employed to modify the final temperature of collapsing cavitation bubbles, a direct relationship between the amount of hydroxyl radicals and the thermal conductivity of the rare gas, hence the collapse temperature, was observed. However, for 50 kHz ultrasound, shearing stress causes lysis of Chinese hamster ovary cells independent of the final temperature of the cavitation bubbles [84]. These observations were explained by referring to a model proposed by Flynn which predicted that pressure dependent phenomena such as cell lysis, were fairly constant as a function of the thermal conductivity of the rare gas [85]. These studies suggest that the physical effects of ultrasound on cells are more important then the chemical effects of ultrasound, in the absence of sonosensitizers.

In biological systems, much of the genotoxic free radicals in vivo are related to the transition metal-ion assisted conversion of superoxide radical anion and H_2O_2 to hydroxyl radicals which in turn almost indiscriminately react with the nuclear material. However, a GC/MS-SIM assay of products following sonolysis of DNA solutions suggested a direct action of hydroxyl radicals and other unknown cavitation produced sonochemicals, rather than a reaction with H_2O_2 [86].

Continuous-wave ultrasound (1 MHz, 1 W/cm^2) was found to significantly enhance the hydroxyl radical production from two clinically employed redox cycling drugs, adriamycin (doxorubicin) and mitomycin C, with respect to the control drug-free insonicated phosphate buffer suspension. Identical ultrasound treatments on non-redox cycling clinical drugs, 5-fluorouracil and methotrexate, did not yield any significant enhancement in the production of hydroxyl radical. Under identical ultrasound treatments at 3 MHz no hydroxyl radical was produced in the presence or absence of these four anti-cancer drugs. Benzoic acid which is initially non-fluorescent and upon aromatic hydroxylation becomes permanently fluorescent, was employed as a sensitive chemical probe to detect hydroxyl radicals and free radical scavengers such as mannitol, superoxide dismutase, catalase and a transition metal chelating agent were employed independently to elucidate the chemical species and pathways involved in its production. The findings strongly implicate an active role of acoustically induced cavitation in potentiating redox cycling drugs via chemical reduction and production of the hydroxyl radical via Fenton's reaction [87]. Normally, a Fenton reaction might be limited in biological tissues by the low availability of free iron. Therefore, it was suggested that exposure to ultrasound-generated (2 MHz, 35 W) superoxide radical ions could augment release of iron from ferritin and provide a pool of active Fe^{2+} to catalyze the Fenton reaction [88].

The free radical scavenger cysteamine, which penetrates the cell wall, and cystamine, which does not, have been used to determine where cavitation may occur. Fu et al. [89] reported that cysteamine promotes cell survival in vitro following exposure to 1 MHz ultrasound. Further investigation confirmed that cysteamine has a protective effect, in contrast to the absence of protective effect from cystamine [90]. These results support the idea that inertial cavitation produces free radicals, and that their biological efficiency depends on their diffusion into, or presence in, the cell. Intracellular cavitation has been proposed to explain how short-lived free radicals reach internal components of cells [91]. Alternatively, it was suggested that the protective effect of cysteamine may be due to protection from DNA damage by removing the cell-penetrating H_2O_2, an extracellular product of radical recombination, with no necessity of invoking intracellular cavitation [71], and indeed this suggestion was experimentally confirmed. High concentrations (>10 mM) of the thiol cysteamine effectively lower H_2O_2 yields following ultrasound exposure (47 kHz) in argon- and air saturated phosphate-buffered saline, while cystamine is less effective under argon and practically without effect in air-saturated buffer. Direct removal of H_2O_2 by cysteamine is the dominant mechanism while scavenging of the H_2O_2 precursors, hydroxyl and superoxide radicals plays a lesser role [92].

3.3. Sonochemical effects via singlet molecular oxygen

By analogy to photodynamic therapy where the cytotoxicity results from the production of reactive oxygen species following excitation of the photosensitizer by light, it has been frequently suggested that active/singlet molecular oxygen plays a primary role in ultrasonically induced cell damage in the presence of hematoporphyrin [25,93–95]. Electronic excitation of the sensitizer during sonolysis by sonoluminescent light, followed by energy transfer to oxygen to generate the highly reactive singlet molecular oxygen has been considered as possible mechanisms also for photofrin [96], ATX-70 [97], ATX-S10 [42,98], pheophorbide-a [43], adriamycin [21,99], rose bengal [49] erythrosine B [50],

anti-inflammatory drugs tenoxicam and piroxicam [47,48], and diacetylhematoporphyrin-mitomycin C conjugate [54]. In these studies, several diagnostic tools have been used to substantiate ultrasonic-generation of active oxygen. The most common was the observed sonoprotective effect of histidine, a singlet oxygen and hydroxyl radical scavenger, as contrary to the absence of any effect induced by mannitol or superoxide dismutase, which are scavengers of hydroxyl radicals and superoxide radical, respectively. Furthermore, the detection of ESR signals of 2,2,6,6,-tetramethyl-4-piperidone-N-oxyl yielded by oxidation of 2,2,6,6-tetramethyl-4-piperidone added to the sonicated mixture, was considered an indication of singlet oxygen.

Miyoshi et al. have shown that 2,2,6,6-tetramethyl-4-piperidone can react with singlet oxygen or hydroxyl radicals to give the EPR detectable nitroxide and is therefore not a specific test for the generation of singlet oxygen by the porphyrin ATX-70 [100]. It is also noted that during a hematoporphyrin study, singlet oxygen fluorescence could be easily detected when produced by laser excitation, but was not detected in the same system when using the same ultrasound exposure that produced rapid disruption of the cells [30]. Finally, it is noted that some sonosensitizers claimed to generate singlet oxygen such as piroxicam and adriamycin have been reported to have extremely low singlet oxygen yields, $<2\times10^{-3}$ and 0.02 in D_2O, respectively [101]. Also, the ultrasound-induced cytotoxicity of leukemia L1210 cells enhanced by an array of porphyrins does not show the expected structure-activity relationship as related to the photodynamic efficiency of these agents known to proceed by a singlet oxygen mechanism. In particular, the observation that copper protoporphyrin was an effective sonosensitizer excluded a singlet oxygen involvement since this compound contains a paramagnetic metal ion and therefore is unable to generate singlet oxygen due to the very short lifetime of the triplet state [31].

3.4. Sonochemical effects via other free radicals

Although cavitation-producing ultrasound can generate hydroxyl radicals and hydrogen atoms, in contrast to ionizing radiation, there is only limited evidence about the genotoxic potential of ultrasound, most probably because of the site of production. Unlike ionizing radiation or photodynamic exposure where free radicals and singlet oxygen respectively can be produced intracellularly, exposure of cells to ultrasound results in extracellular production of free radicals. There is no conclusive evidence so far that cavitation can also occur intracellularly, but such an event would lead to immediate cell destruction because the resonant size of the cavitation bubble in the low MHz range is comparable to the size of cells [71]. Hence, there would be no role for intracellular free radical damage, and therefore if free radicals are involved in biological damage by ultrasound, is likely to depend on extracellularly produced reactive intermediates. Indeed, Kessel et al. found that porphyrins enhanced ultrasound-induced cell damage in vitro only when present in the extracellular space [31] and also in a study of ATX-70 sonosensitization of human leukemia HL-525 cells exposed to 50 kHz ultrasound, the requirement of extracellular localization of ATX-70 molecules has again been established. Short-lived toxic intermediates produced from ATX-70 by ultrasound are implicated in the mechanism, since no cytotoxicity was found when medium-containing ATX-70 was sonicated and subsequently added to the cells [32]. However, hydroxyl radicals either formed primarily during collapse of cavitation bubbles in aqueous media or secondarily by Fenton conversion of H_2O_2, are unable to cause distant or specific cellular damage because of their high reactivity and very short lifetime which limits the migration range to only 1.5–9 nm. Alternatively, Mišik and Riesz have suggested that alkoxyl and peroxyl radicals could be cytotoxic factors [102]. These are sluggish radical species which by virtue of their longer lifetimes and higher selectivity, are able to migrate significant distances through the biological milieu before reaching and reacting with critical cellular sites, such as the cell membrane. For example, the rates of hydrogen atom abstraction from saturated alkanes by hydroxyl radical and alkylperoxyl radicals are $\approx10^9$ and 1 $M^{-1}\,s^{-1}$, respectively. However, once alkylperoxyl radicals reach the cell membrane, they may abstract bis-allylic hydrogen atoms from polyunsaturated fatty acid chains of the membrane lipids, relatively rapidly ($\approx10^4$ $M^{-1}\,s^{-1}$). Among the likely consequences of the reactions of oxy radicals with membrane phospholipids is initiation of lipid peroxidation, which has deleterious consequences in cell membranes. Indeed, the level of lipid peroxidation was increased by sonication (150 W, 22 kHz) in homogenates from Ehrlich ascitic tumor cells [103] as shown by monitoring the oxidation products and the change in composition of fatty acids [103], and in membranes of ghost erythrocytes in the presence of hematoporphyrin and ultrasound [104].

The experimental support for the "alkoxyl and peroxyl radical mechanism" stems from the ESR detection of carbon-centered and alkoxyl and peroxyl radicals derived from the sonosensitizer, the latter being formed by addition of oxygen to the former. Experimental evidence suggests that sonosensitization follows the chemical activation of sonosensitizers inside or at the gas/solution interface of hot collapsing cavitation bubbles to form sensitizer-derived free radicals either by pyrolysis-induced hemolytic bond cleavage or due to reactions with hydrogen atoms and hydroxyl radicals formed by pyrolysis of water [105]. Thus in nitrogen-saturated aqueous solutions of dimethylformamide, methyl formamide, or dimethylsulfoxide exposed to 50 kHz

ultrasound, methyl and ·CH2N(CH3)CHO radicals for dimethylformamide, mostly ·CH2NHCHO radical for methyl formamide, and methyl radicals for dimethylsulfoxide were detected by spin trapping and ESR. These radicals were formed either by reactions of the solutes with ultrasound-generated hydrogen atom and hydroxyl radical (such as ·CH2R-type radicals in dimethylformamide and methyl formamide, and methyl radicals in dimethylsulfoxide), or by direct pyrolysis of the weak bonds in the solute molecules (e.g. methyl radicals from dimethylformamide). In air-saturated sonicated solutions these carbon centered radicals were converted by reaction with oxygen to the corresponding peroxyl radicals [106].

Support for the relevance of chemical activation of sonosensitizers at the gas/solution interface of hot collapsing cavitation bubbles was provided by an in vitro study of cell killing of human leukemia cell lines (HL-525 and HL-60) exposed to 47 kHz ultrasound in the presence of ATX-70 with two n-alkyl side chains of various lengths from 2 to 12 carbon atoms. Since a strong correlation for the yield of carbon-centered radicals and cell killing was observed, cytolysis was directly correlated to the extent of accumulation of surface active ATX derivatives at the interface of cavitation bubbles and subsequent production of sensitizer radicals. Increasing the hydrophobicity by increasing the carbon chain length of the ATX derivative from 2 to 7 increased the sonosensitizing efficiency. However, a further increase in hydrophobicity, from 7 to 12 carbon atoms resulted in a decrease [107]. Therefore, nonequilibrium adsorption dynamics appear to affect the concentration of ATX derivatives at the gas/solution interface of cavitation bubbles, analogous to a previous study on surfactant-derived radicals in aqueous solutions exposed to ultrasound [108].

The direct formation of sensitizer radicals by pyrolysis-induced hemolytic bond cleavage and not secondary to reactions with hydrogen atoms and hydroxyl radicals, may explain why no correlation was found between production of hydroxyl radicals, as measured by ESR, and cytotoxicity in an in vitro study of adriamycin-enhanced lethality of Chinese hamster ovary cells exposed to continuous-wave ultrasound (intensities ranging between 1 and 2.5 W/cm^2) [18,109]. In an attempt to reveal whether the yield of hydroxyl radicals and hydrogen atoms produced by pyrolytic homolysis of water could explain the killing of cells in vitro with and without sonodynamic agents, the correlation between inertial cavitation, free radical production, and cytotoxicity was measured. It was concluded that cytotoxicity is not linked to attack from free radicals formed outside the cells, since the yield of free radicals was much too small to explain the cell killing observed, as estimated from a model of DNA as the primary and sensitive target in cellular death [30,110]. However, this evaluation does not contradict the suggested involvement of peroxyl and alkoxyl radicals in the damage of cell membranes as the underlying mechanism of cytotoxicity since the oxidation of the lipid membrane of cells is a chain reaction that can be initiated by a relatively small number of peroxyl and alkoxyl radicals.

Conversely, in a study using 2,2′-azobis(2-amidinopropane), a compound for which carbon-centered and oxyl radicals have also been detected by ESR [56], the amount of free radicals did not correlate with the data on cell killing, including apoptosis, following sonolysis. In this case, the increased uptake of the azo sensitizer by the cells exposed to ultrasound, rather than its ability to generate extracellular free radicals, has been shown to cause sensitization [57]. In the first study of its kind, it was found that cytolysis of HL-525 cells by ultrasound and ATX-70 could not be prevented to any degree by spin traps (68 mM DMPO or 70 mM POBN) capable of trapping reactive radical intermediates [32]. Although no protective effect was afforded to the cells by these spin traps, it was shown that the spin traps acted as sonosensitizers when added to the cells without ATX-70, during sonolysis [32]. In an EPR/spin trapping study (using DBNBS as a spin trap) on the sonolysis of aqueous solutions of a number of spin traps, it was shown that spin traps themselves decompose to produce carbon-centered radicals [111]. This supports the hypothesis that short-lived radical intermediates could be responsible for enhanced cell killing observed in the presence of DMPO and POBN [32].

Seemingly, the sonosensitizer, the ultrasound exposure parameters, and the type of biological system being irradiated are determinant factors for the specific mechanism of sonosensitization.

3.5. Future research directions

It is tempting to speculate on the factors which have stalled clinical applications of sonodynamic therapy over the 25-year period of laboratory studies. First of all, it is still difficult to predict how laboratory experiments extrapolate to clinical conditions. The human body possesses an excellent filtration system, and the nucleation sites may be found in relatively small quantities and only in specific sites, such as the lung and intestines. Therefore, in vivo, cavitation is more difficult to produce than in an in vitro system and acoustic streaming effects may be atypical [112].

The acoustic pressure, frequency of the applied ultrasound, pulse duration and, most importantly, the extent of nucleation of the exposed medium, influence the occurrence of inertial cavitation. The likelihood of nucleation increases as the ultrasonic pressure amplitude increases. In vitro, as the pressure amplitude is raised, significant thermal damage may occur at amplitudes well below the cavitation threshold. It was noted that

biological significant tissue heating is expected at intensities above approximately 1 W/cm^2 at MHz frequencies [113]. Although heating has been considered the only appreciable biological effect of ultrasound without cavitation, recently it was shown that streaming flow around a gently and linearly oscillating single bubble may exert large enough shear forces to deform and lyse unilamellar lipid vesicles [114].

One way to minimize heating is by using pulsed, rather than continuous ultrasound. Cavitation activity sufficient to produce reactions was detected even when diagnostic pulse durations are used [115,116]. Addition of sensitizers, such as xanthene dyes—rose bengal, phloxin, erythrosine B and tetrachlorofluorescein—were shown to reduce the intensity threshold for inducing cavitation, as correlated with their ability to stabilize foams [117]. Thus therapeutic conditions may not require extremely high intensity in order to induce cavitational effects. Evidence for cavitation nuclei in vivo, in hind limb of a guinea pig, was provided with ultrasound at 0.75 MHz and 80 mW/cm^2 intensity [118]. Subsequent theoretical studies suggest these bubbles were formed by rectified diffusion [119].

Another determinant are the technical aspects of ultrasound exposure such as ultrasound sources, calibration equipment and exposure systems. New focal acoustic fields such as second-harmonic superimposed focal fields promise a better localization of sonochemical activity. Other topics of investigation include making more effective but biocompatible agents for cavitation nuclei, and shaping the acoustic waveform to enhance cavitation. A better understanding of the mechanism, followed by the conception of tailor-made sonosensitizers would aid in the challenge of determining how these model processes are assembled into a working therapeutic modality.

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