Review Article

Review of ultrasound mediated drug delivery for cancer treatment: updates from pre-clinical studies

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Abstract: Therapeutic ultrasound has been used to thermally ablate solid tumors since the 90s, and a variety of cancers are presently being treated clinically, taking advantage of ultrasound- or MR-imaging guidance and monitoring. However, an ever-increasing body of preclinical literature demonstrates how ultrasound can achieve bioeffects beyond thermal ablation, including non-invasive drug delivery to target cancer cells. In this review, we will provide a summary of *in vivo* ultrasound-based strategies shown to deliver drug payloads to tumor environments, to enhance permeability of vessel walls and cell membranes, and to activate drugs and genes *in situ*.

Keywords: Drug delivery; ultrasound; blood-brain barrier (BBB); targeted therapy

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Introduction

Since Persian Queen Atossa's breast tumor removal by Demokedes (1,2), surgery has been the only option to fight cancer for two millenaries. Patient survival remained low until the development of chemotherapy drugs that attack rapidly dividing cancer cells (1,3,4). However, efficient drug delivery that also minimizes toxicity to healthy cells remains hampered by the difficult penetration of the drug in the vicinity of the cells that cause the disease. In tumors, the transport of drugs indeed encounters several physical barriers and the penetration of therapeutic molecules is often poor and heterogeneous. The barriers to the natural diffusion and convection of drug molecules from the blood vasculature to the surrounding tissue in tumors are mostly consequences of the characteristics of the angiogenic vasculature [for reviews, see (5-8)]. Angiogenesis is a natural phenomenon that arises during development and wound healing. In these natural phenomena, angiogenesis is tightly regulated (9,10). It can also occur during abnormal processes such as tumor growth. In order to sustain its growth, the tumor needs more nutrients and triggers the formation of new vessels.

But since this angiogenesis occurs in an uncontrolled way in tumors, the newly formed vascular network is often abnormal. At the macroscopic level, the vasculature is tortuous, highly branched and chaotic, with dead ends or loops that impair blood flow (11). Its distribution is spatially heterogeneous, resulting in the coexistence of high and low blood vessel density areas. Angiogenesis is thus a highly inefficient process since the presence of these hypo perfused areas produces hypoxia amongst the tumor cells. Due to the lack of perfusion, drugs are not efficiently delivered to the hypoxic tumor cells; moreover, hypoxic cells are also more resistant to radiotherapy, more likely to develop resistances against chemotherapies and more likely to become invasive (12).

If the macroscopic organization of the angiogenic network is disrupted, the structure of the blood vessel is also abnormal on a microscopic level: the adhesions between endothelial cells are weakened, and the perivascular cells tend to detach from the basement membrane around the

vessel (13-16). All these phenomena result in the leakage of vessels. Plasma proteins can easily enter into the surrounding tissue, increasing the interstitial fluid pressure (IFP) in the surrounding tissues and thus decreasing the pressure gradient between the inside and the outside of the vessel. As a consequence, the convection of fluid that normally moves the drugs molecules from the blood vessels to the surrounding tissue is hindered (17).

The absence of transvascular flow in some areas of the tumor jeopardizes the homogeneous delivery of drugs. New techniques to improve the delivery of drugs within tumors are needed. In this review, strategies based on mechanical and/or thermal effects of non-invasive and non-destructive ultrasound will be described. As will be discussed, versatile ultrasound beams can indeed interact with the cell membranes, the vessel walls, drug carriers and/or the drug itself.

Ultrasonic drug release at targeted sites

Drug-delivery with ultrasound relies on the interaction between a biocompatible carrier and an acoustic wave. The spatial specificity of the release is established by focusing the waves in the zone to be treated using physical principles and technologies developed in the past for diagnostic and therapeutic ultrasound [such as high intensity focused ultrasound (HIFU) or lithotripsy]. The main challenge in ultrasound-triggered therapy is the design of carriers that are both responsive to ultrasound and biologically active. These agents should be able to carry large payloads and have access, or even accumulate preferentially, within the tumor. These challenges have been addressed by early researchers, such as Tacker and Anderson (18), along with wide and recent international collaborations such as Sonodrugs (19-25). In this section, we will first highlight the mechanisms by which ultrasound can release a payload and then describe various drugs, agents or nucleic acids that have been released with ultrasound in pre-clinical studies.

Drug-delivery mechanisms

The field of ultrasound-enhanced drug-delivery has been strongly influenced by the development of microbubbles (MBs) as contrast agents (26) and liposomes as general drug-delivery carriers (27). The mechanisms underlying drug release via ultrasound can be divided into thermal and mechanical processes, and often a combination of both.

Thermal release

Thermal release involves an ultrasound-induced temperature increase in the treated zone, which results from the absorption of acoustic energy at a rate beyond that of diffusion. This usually implies moderate intensities (several W/cm^2), high duty cycles (up to 100%), moderate pressures (100's of kPa to MPa) and long treatment times (several seconds to 30 minutes) with dedicated focused ultrasound (FUS) transducers. To reduce the required acoustic intensity and limit unspecific heating damage, while guaranteeing its specificity, carriers are often designed to deliver their payload at temperatures just a few degrees above physiological temperature (42-43 ℃).

The most common thermally responsive carriers described in the literature are temperature-sensitive liposomes (TSL) (28,29). Liposomes are composed of an aqueous solution inside single or concentric lipid bilayers (30,31). Drugs or agents can be contained within the inner phase (*Figure 1A*). For example, Doxil, an FDA-approved agent, carries doxorubicin (DOX) while reducing the toxicity of the chemotherapeutic agent (32). The liquid-crystalline phase transition of these liposomes can be selected by modifying the content of their lipid shell. TSLs can also be produced by adding leucine-zipper to the membrane of the liposomes (33). As shown in *in-vitro* studies, these agents can release up to 80% of their content after 15 minutes of hyperthermia at 43 ℃ (22). Such TSLs are already well-established because of previous use with other heat sources such as radiofrequency (RF) devices. The primary drawback to both RF and ultrasound hyperthermia remain long treatment times.

Mechanical release

Drug-delivery can also be performed by inducing high mechanical stresses on drug carriers using short ultrasound pulses. Ultrasound drug-delivery through non-thermal processes requires the presence of micelles (*Figure 1B*), MBs (*Figure 1C-F*) or liquid perfluorocarbon droplets (*Figure 1G*). MBs are used in the clinic for diagnostic ultrasound because of their high echogenicity, their nonlinear scattering and propensity to disrupt under sufficient acoustic pressures (34). Because of their high compressibility, the radius of MBs can vary by a factor of two, leading to important mechanical effects in the surrounding environment (35) that can modify the shell of the microbubble itself. For instance, their oscillations during an ultrasound cycle can cause shedding of its shell. The motion of the shell can move and propel content that was adsorbed on its surface (36). The disruption

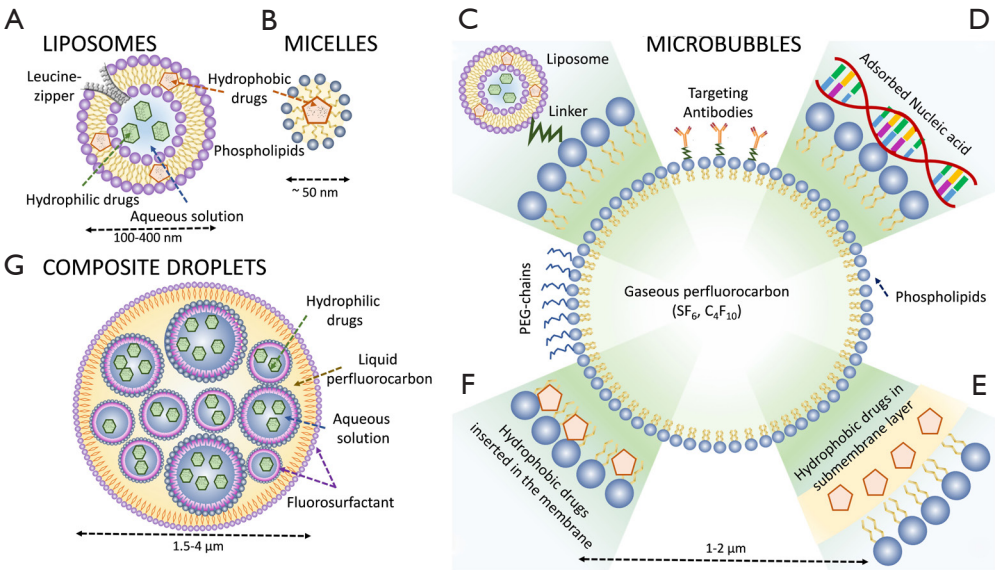

Figure 1 Several ultrasound-sensitive carriers have been designed. For example, heat-sensitive liposomes (A), generated with specific doses of phospholipids, can deliver hydrophobic or hydrophilic drugs after heating with ultrasound. Micelles (B) can carry hydrophobic drugs within their core, which can then be released with ultrasound. Microbubbles used as contrast agents can also be exploited as ultrasound-induced drug carriers. For instance, drug-loaded liposomes can be covalently-linked to their membrane (C) or nucleic acids can be adsorbed on the surface of the microbubbles (D). Their membrane can be underlaid with a hydrophobic shell (E) or doped with drugs (F). Finally, composite-droplets (G) are multiple emulsions (water or oil-in perfluorocarbon-in water) that can be converted with an imaging scanner and can transport large payloads.

of the bubble wall can also lead to the release of agents that were bound within the shell or at the core of the bubble. Moreover, the motion of the surrounding fluid induced by the large oscillations of the bubbles facilitates further convection and penetration of the drug. These effects do not depend on temperature, but rather on the peak acoustic pressure and the frequency. Short pulses of a few MPa with total intensities within diagnostic limits (a few hundred mW/cm^2 or less) are sufficient, compared to several mW/cm^2 of continuous sonication for temperature-induced release. Therefore, microbubble based drug-delivery can be achieved with low-intensity ultrasound systems, often unfocused, or even diagnostic scanners.

These physical phenomena have led to the development and investigation of various types of MBs for drug-delivery. For example, FDA-approved Doxil can be attached on the surface of MBs with the help of molecular linkers, allowing the release of the liposomes during the ultrasound-induced oscillations of the contrast agent (37,38) (*Figure 1C*). Drugs or DNA can also be adsorbed on the charged surface of the shell (*Figure 1D*). Moreover, hydrophobic drugs can be inserted below (*Figure 1E*) or into (*Figure 1F*) the membrane of the MBs (39) or even added to a polymeric shell (40). The primary drawback of these methods is that most of the content of MBs is gas, rather than drug or gene payload. Moreover, the size of MBs resonating at diagnostic frequencies (3-15 MHz) is close to 1 micrometer, which prevents certain tumor-specific accumulation. To alleviate these issues, liposomes containing gas bubbles have been proposed (41). Polymeric micelles (42), a hydrophobic carrier similar in size to liposomes, were also shown to release their content through stable cavitation (42).

Spontaneous cavitation can be induced in tissue with sufficient peak-negative pressure (tens of MPa). As demonstrated by its effect on kidney stones and tissue, cavitation can disrupt surrounding interfaces, including liposomes or micelles. For instance, short pulses with large pressure were used to deliver the content of liposomes in Somaglino *et al.* (43). Additionally, mechanical stresses exploited for drug-delivery can also be generated through radiation pressure (44).

The combination of thermal and mechanical stresses can be used to induce the vaporization of gas-precursors and perform drug-delivery without microbubble

injection. This often requires the use of perfluorocarbon (PFC) in various forms, from decafluorobutane (45) and perfluoropentane (46) to perfluorohexane (47). These PFC can be confined as liquids within micro or nano droplets thanks to Laplace pressure. When insonified, liquid rapidly converts into gas, leading to disruption of the droplets and rapid expansion of their content. The mechanism of acoustic droplet vaporization was recently explained by Shpak *et al*. as a form of superfocusing of the acoustic wave within the agent (48). This phenomenon can be triggered by pressures compatible with a diagnostic scanner [3 MPa at 5.5 MHz for few cycle pulses in (49) and 3.5 MPa at 8 MHz for few cycle pulses in (50)], especially with droplets made of PFC, with low-boiling point (49,50).

This concept was used to create nanodroplets (hundreds of nm) that are also effective as ultrasound contrast agents (51). For drug-delivery, they can be mixed in various ways with payload. A nanodroplet of liquid PFC can be added to the content of a liposome to trigger the disruption of the carrier under high pressure ultrasound exposure (52). Other agents can be designed by creating a nanoemulsion of PFC and a hydrophilic (53,54) or hydrophobic solution (46) containing the payload, itself encapsulated in a larger droplet (*Figure 1F*). The vaporization of the PFC by low-intensity pulses releases the nanoemulsion. This construct allows larger payloads since up to ⅔ of the volume of the droplets can be used to contain drugs. One potential application for these droplets is tissue tattooing for surgical guidance (50).

Liposomes, micelles, MBs and liquid-PFC particles not only need to react specifically to ultrasound, but they should also deliver sufficient payload at the appropriate site. MBs are intravascular agents and do not extravasate. Their circulation lasts several minutes after intravenous bolus injection. Sonication must be performed during the passage of the agents within the target. Longer retention within a tumor can be attained by targeting MBs with tumor-specific antibodies attached on their surface (55). Also, magnetic (56) and radiation forces (57) can be used to slow their progress through the tumor and increase drug-delivery. Because of their size (about 4 microns in diameter), composite droplets behave similarly to MBs and circulate within a restricted amount of time (58). Liposomes and micelles, on the other hand, can be produced to be retained specifically in cancer cells (59,60). Indeed, when their size is below 200 nm, the porous vascular structure of angiogenic tumors allows their passage to the extravascular space. These agents are thus injected several hours, or days, before sonication to allow preferential accumulation in the tumor.

Drugs and targets

Several groups have used these carriers (liposomes, micelles, MBs, liquid droplets) and mechanisms (thermal, mechanical, vaporization) to perform *in vivo* drug-delivery. Only a fraction of several hundred articles on the subject can be discussed in this review. After the discovery of thermosensitive liposomes, Tacker and Anderson (18) rapidly performed ultrasound experiments on tumor-bearing mice. By heating the tumor with ultrasound beyond the 42 °C transition of their methotraxate-filled liposomes, they showed an elevated accumulation of the chemotherapeutic drug in the tumor in this bladder-cancer model. The same group demonstrated the encapsulation of cisplatin (61). More recent studies on ultrasound-released liposomal cisplatin showed cancer regression in mice (29,62).

Paclitaxel was also inserted in ultrasound-sensitive agents. For instance, Rapoport *et al*. (63) observed the regression of pancreatic-tumors in mice after administration of an ultrasound-sensitive PFC nanoemulsion.

DOX, a cancer chemotherapeutic, was encapsulated in ultrasound-sensitive carriers by several groups (64-66). In 1994, Ning *et al*. (67) showed a 10-fold increase in the release of DOX using stealth liposomes. More recently, temperature-sensitive liposomal DOX were tested in the context of magnetic resonance guided HIFU (MRgHIFU) highlighting *in vivo* increased accumulation of the drug within tumor models in rabbits (68). In another study, the specific release of DOX from liposomes during ultrasound treatment lead to complete regression of tumors in mice (69). DOX was included within liposomes attached to the surface of MBs (70), and lead to a reduction of tumor growth in rats (71). DOX was also confined within micelles (72) and polymeric nanoparticles, but Cochran *et al*. (73) obtained better encapsulation of the hydrophobic molecule paclitaxel in this later construct.

Thirty-two years after the first ultrasound drug-delivery in animals, clinical trials are approved to begin in Oxford to release DOX from liposomes with the help of a HIFU system for the treatment of liver metastasis (74).

In parallel with the delivery of conventional chemotherapeutic agents, other paths are being explored where the advantages of MBs and ultrasound are more specifically exploited. For instance, MBs and liposomes, in conjunction with ultrasound, can deliver nucleic acids for gene therapy (75,76). Indeed, not only can ultrasound-sensitive carriers enable the passage of nucleic acids in specific zones (77), but they can also protect this genetic material from enzymatic activity in the blood. Initially proposed with liposomes (78), ultrasound gene delivery was

rapidly performed with cationic lipid transfection complex showing a 270-fold increase in DNA expression (79). Negishi *et al*. (80) achieved gene silencing effects by introducing small interfering RNA (siRNA) within cells using bubbles, liposomes and ultrasound. Anti-cancerous effects were obtained with EGFR-directed siRNA or thymidine kinase and ganciclovir in murine carcinoma, hence reducing tumor growth (81,82). Plasmid which can trigger the expression of reporter genes were also targeted to tumor cells in mice using cationic bubbles (83).

Interestingly, ultrasound-mediated delivery was also performed with oncolytic viruses. These viruses can kill and transfect tumor cells, but can also self-replicate. However, their passage into the extravascular space remains limited. The use of MBs and ultrasound can increase the expression of these viruses by a factor of 50 (84).

In the case of infiltrating or invading cancers, the localized approach described before might be insufficient, even with an efficient drug-delivery device. To encourage the immune system to identify and destroy remote tumor cells, several approaches of cancer vaccination were suggested with ultrasound (85). Not only can HIFU inherently cause the immune system to recognize tumor cells (86,87), but delivery of mRNA, plasmid DNA and cancer antigens can also be promoted via ultrasound-sensitive agents (88). For instance, by introducing plasmid DNA to antigen presenting cells using MBs, lipoplexes and ultrasound, Un *et al.* (89) demonstrated encouraging antitumor effect and improved survival rate.

Despite these advances, ultrasound-induced drug delivery is not a "magic bullet", mainly because most of the injected drugs still accumulate in non-cancerous tissue. Recently, Bezagu *et al*. (90) proposed to produce the drugs in-situ by inducing a chemical reaction through ultrasound release of composite droplets. By exploiting the strong hydrophobicity and lipophobicity of the PFC composing the droplets, two prodrugs can be isolated from each other until ultrasound-induced release, which becomes a condition for the very existence of the active drug. Such a concept guarantees that any pharmaceutical effect will be isolated within the specific zone of the ultrasound-induced delivery.

Ultrasound-enhanced permeability of biological barriers

Once the payload has been delivered intravenously, the permeability of the vascular walls is critical to deliver the drug to pathological tissues.

The particular case of the brain: blood-brain barrier (BBB) disruption

Specific organs such as brain and retina present much more impermeable vasculature which acts as a barrier blocking almost all therapeutic access. The main example is the BBB which consists of tight junctions between endothelial cells in the brain (91). This barrier is a complex biological system involving a large number of specific proteins and receptors and is therefore difficult for large and hydrophilic molecules to cross (92,93). In its normal state, it protects the brain from infections as well as ensures its homeostasis (92).

Ultrasound combined with the injection of MBs has been shown to safely and reversibly disrupt the BBB in a multitude of studies since 2001 (94). Since then, as recently reviewed by Aryal *et al*. (95), a large number of animal studies have been conducted to determine the optimal acoustic parameters and timings and the optimal properties for MBs. Studies have also measured the obtained enhanced permeability in an effort to understand the mechanisms of interaction of sonicated MBs with endothelial cells and the induced bio-effects, and to assess the potential tissue damage and its time course for this reversible process (*Figure 2*). Under proper conditions, it was possible to reach locally and reversibly the same vascular permeability as the one measured in peripheral organs without any adverse effects for several hours (97). It is thus a very promising tool for targeted drug delivery.

Regarding cancer treatment, ultrasound was shown to enhance delivery of several chemotherapy agents across the BBB in healthy brain, including: Herceptin (98), liposomal DOX (99), methotrexate (100), cytarabine (101), and DOX (102). Gene delivery and transfection was also demonstrated in normal brain and ultrasound-aided gene therapy was achieved for cancer therapy (103-106).

However, it is well documented that neoangiogenic vascular networks present vessels with already altered BBB (the so-called blood-tumor barrier or BTB) as well as longer residence times for drugs. Therefore, the potential benefit of ultrasound was not obvious. Nevertheless, it was recently shown that tumor vascular endothelium becomes more permeable after ultrasound. As a result, since 2010, many preclinical studies have evaluated the therapeutic gain of using ultrasonic BTB disruption for brain tumor treatments. Liu *et al*. recently reviewed the current status of drug delivery to brain tumors using ultrasound (107). *Table 1* summarizes the current status of the animal cancer models that were used and the anti-tumor drugs that were injected as well as the main acoustic parameters used. As illustrated in *Figure 3*, these studies demonstrated that

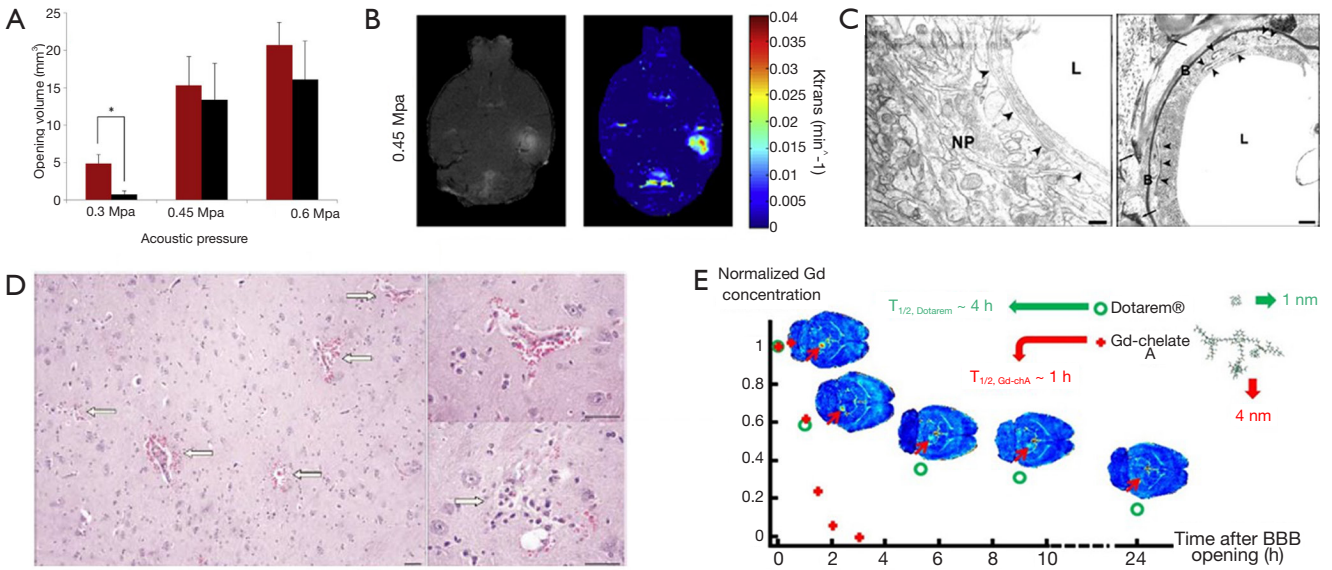

Figure 2 Some results of ultrasound induced BBB disruption reproduced with permission. (A) Influence of acoustic pressure and microbubble size (different size distribution for each bar color) on BBB opening volume (96); (B) contrast-enhanced T1 weighted MRI and permeability maps obtained after injection of Gd chelate to localize and quantify BBB disruption (96); (C) photomicrographs of cross-sectioned microvessels without ultrasound (upper frame) and after sonication (lower frame) to understand cellular mechanisms responsible for enhanced permeability (48); (D) H&E histological staining to assess tissue damage after disruption showing limited petechia hemorrhage with no effect on surrounding neurons (49); (E) follow up of BBB closure dynamics by the quantification of an MRI contrast agent as a function of injection time after disruption showing complete recovery of impermeability after 24 hours (original figure from author's results).

ultrasound can enhance delivery of a wide variety of drugs to tumors [for example, +200% BCNU delivery to C6 glioma bearing rats (119)]. Most studies showed significant control of tumor growth or even full regression (*Figure 3B*). This indicates that therapeutic levels of drugs were delivered in targeted regions and that drugs sufficiently diffused inside tumors. When measured, the median survival time was improved as well (*Figure 3C*). In addition, two interesting concepts were successfully tested. First, encapsulating the drug payload into magnetic nanoparticles, liposomes, or MBs enables less peripheral toxicity. This method limits the required dose, increases drug lifetime in the circulation and achieves high local concentration after drug release (113,117,119,122,123). Second, biologically targeting theses drug cargos by grafting specific proteins on their surfaces enables further increase of the local concentration before sonication (113,114,124).

Although qualitatively consistent with each other and very encouraging, these studies present significant discrepancies in their quantitative outcomes (tumor growth, mean survival gain). Indeed, they are difficult to compare since they were conducted under very different experimental conditions. Namely the acoustic frequency ranged from 400 kHz to 1.7 MHz and the size of the therapeutic agents that have been tested ranges from less than 1 nm molecules (200 Da) to cells of several microns in size. The spatial extent of BTB disruption, depending on acoustic frequency, peak negative pressure, geometry of the transducer and number of sonication points, will affect the efficiency of drug delivery. The number of treatment sessions and their timings are also important to consider (110). The influence of key parameters such as anesthetics used during BBB disruption should also be considered (125).

To date, all brain tumor models were orthotopic murine models which are much less infiltrating than human gliomas. Data obtained on tumor models that better mimic human pathology are needed to ensure translation of these promising results. In order to extrapolate to humans, safety data is critical. Although several histological studies have been conducted in healthy brain after BBB disruption with a wide number of acoustic parameters, only few papers have studied tissue damage and repair in tumors. In particular, since tumor vasculature might be easier to disrupt, it would be of great importance to determine whether or not

Table 1 Summary of preclinical brain cancer treatments studies after blood-tumor barrier permeabilization using ultrasound

Reference	Tumor model, animal strain	Injected drug	Acoustic parameters: Freq., PN pressure; duration, pulse length, PRF	Microbubbles	Treatment planning	Outcome
Kovacs *et al*. 2014 (108)	Glioblastoma multiforme (GL261) , female B6-albino mice	Doxorubicin	612 kHz, 0.4 MPa*; 180 s, 10 ms, 1 Hz	BG6895, Bracco	Single session at day 11	68% IMST versus no treatment
Kovacs *et al*. 2014 (108)	Glioblastoma multiforme (SMA-560), female VM/Dk mice	Doxorubicin	500 kHz, 0.4 MPa*; 180 s, 10 ms, 1 Hz	BG6895, Bracco	Single session at day 5	12% IMST versus no treatment
Burke *et al*. 2014 (109)	Glioma (C6), Rag-1(-/-) mice (outside brain)	5FU-loaded nanoparticles linked to MBs	1 MHz, 1.2 MPa*; 3,600 s, 0.1 ms, 0.2 Hz	5FU-loaded nanoparticles linked to MBs	Single session at day 7-12	67% reduction of tumor volume after 7 days; increase survival
Aryal *et al*. 2013 (110)	Gliosarcoma (9L), male SD rats	Liposomal doxorubicin	690 kHz, 0.55-0.81 MPa; 60 s, 10 ms, 1 Hz; 5-20 sonic. points	Definity, Lantheus Medical Imaging	3 weekly sessions from day 7-8	100% IMST versus untreated; 40% long term survivors
Wei *et al*. 2013 (111)	Gliosarcoma (9L), male Fisher 344 rats	Temozolomide	500 kHz, 0.8 MPa*; 60 s, 10 ms, 1 Hz	Sonovue, Bracco	2 sessions at day 11 and 13	37% IMST
Fan *et al*. 2013 (112)	Glioma (C6), male SD rats	VEGF-targeted BCNU-loaded MBs	1 MHz, 0.7 MPa; NA, 10 ms, 5 Hz; 2 sonic. Points	VEGF-targeted BCNU-loaded MBs	Single session at day 9-10	Enhanced drug delivery; 100% IMST
Fan *et al*. 2013 (113)	Glioma (C6), male SD rats	Magnetic Doxorubicin-loaded MBs	400 kHz, 0.32 MPa; 90 s, 2.5 ms, 1 Hz; 4 sonic. points	Magnetic Doxorubicin-loaded MBs	Single session at day 10	2-fold increase of drug delivery in the tumor
Alkins *et al*. 2013 (114)	Breast cancer brain metastasis (MDA-MB-231-HER2), male athymic nude rats	HER2-targeted immune cells (NK-92)	551 kHz, 0.33 MPa; 120 s, 10 ms, 1 Hz	Definity, Lantheus Medical Imaging	Single session	5-fold increase of cell delivery
Alkins *et al*. 2013 (115)	Gliosarcoma (9L), Fisher 344 male rats	^{10}B enriched boronophenylalanine: Boron neutron capture therapy agent	558 kHz, 0.4 MPa*; 30 s, 10 ms, 1 Hz; 4 sonic. points	Definity, Lantheus Medical Imaging	Single session at day 8-9	Increased accumulation of ^{10}B in tumors

Table 1 *(continued)*

Table 1 *(continued)*

Reference	Tumor model, animal strain	Injected drug	Acoustic parameters: Freq. PN pressure; duration, pulse length, PRF	Microbubbles	Treatment planning	Outcome
Park *et al*. 2012 (116)	Breast cancer brain metastasis (BT474), male (nu/nu) rats	Trastuzumab (Herceptin)	690 kHz, 0.69 MPa*; 60 s, 10 ms, 1 Hz; 1-27 sonic. points	Definity, Lantheus Medical Imaging	6 weekly sessions from day ~14	>32% IMST; no more tumor in 40% of treated animals
Treat *et al*. 2012 (117)	Gliosarcoma (9L), male SD rats	Liposomal doxorubicin	1.7 MHz, 1.2 MPa; 60-120 s, 10 ms, 1 Hz; 5-9 sonic. points	Definity, Lantheus Medical Imaging	Single session at day 8	Reduced tumor size; limited IMST
Yang *et al*. 2012 (118)	Glioma (GBM8401), male NOD-scid mice	IL-4-receptor-targeted liposomal doxorubicin	1 MHz, 0.7 MPa; NA, 50 ms, 1 Hz	Sonovue, Bracco	2 sessions at day 5 and 9	Enhanced accum; drug in tumor cells. Tumor growth inhibition; 67% IMST
Ting *et al*. 2012 (119)	Glioma (C6), male SD rats	BCNU loaded MBs	1 MHz, 0.5 MPa (cranio); 120 s, 10 ms, 5 Hz; 4 sonic. Points	BCNU loaded MBs	2 sessions at day 4 and 5	Increased drug half-life in blood; controlled tumor growth; 12% IMST
Yang *et al*. 2012 (120)	Glioma (GBM8401), male NOD-scid mice	^{10}B enriched boronophenylalanine: Boron neutron capture therapy agent	1 MHz, 0.7 MPa; 60 s, 50 ms, 1 Hz	Sonovue, Bracco	Single session at day 8	82% increased accumulation in tumors. Modest treatment improvement at day 20
Liu *et al*. 2010 (121)	Glioma (C6), male SD rats	BCNU	400 kHz, 0.62 MPa; 30 s, 10 ms, 1 Hz	Sonovue, Bracco	Single session at day 10	Doubling drug release in tumor; 72% IMST versus drug alone
Liu *et al*. 2010 (122)	Glioma (C6), male SD rats	Epirubicin-loaded magnetic nanoparticles	400 kHz, 0.62 MPa; 120 s, 10 ms, 1 Hz	Sonovue, Bracco	Single session at day 10	Enhanced drug delivery; tumor growth slowed down
Chen *et al*. 2010 (123)	Glioma (C6), male SD rats	BCNU-loaded magnetic nanoparticles	400 kHz, 0.7 MPa; 30 s, 10 ms, 1 Hz	Sonovue, Bracco	Single session at day 17	Enhanced drug release; tumor growth control; improved survival

*, as measured in free water; NA, not available; IMST, Increase of Median Survival Time; MBs, microbubbles.

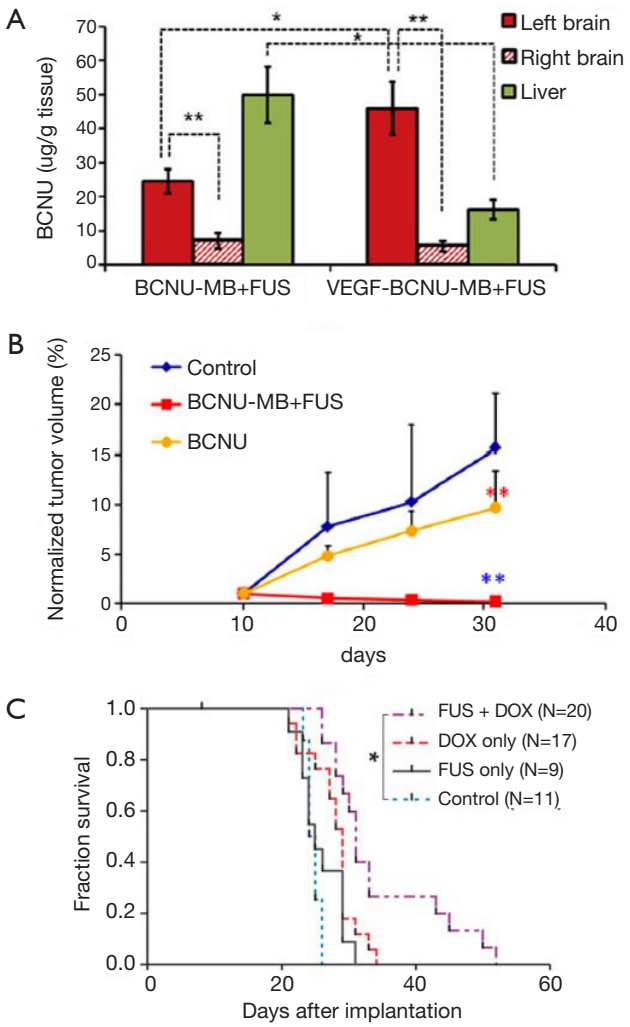

Figure 3 Some results of targeted drug delivery to tumor models in rodents reproduced with permission. (A) Increased BCNU release at sonicated sites and further enhancement of drug delivery by additional targeting of drug cargos as well as protection of peripheral organs (117); (B) tumor size reduction after BBBD enhanced chemotherapy (105); (C) improved median survival time after treatment of gliomas with doxorubicin (106).

hemorrhage results at lower acoustic thresholds than in the rest of brain parenchyma. The propagation of hemorrhage through blood leakage could jeopardize the advantages of this technique. The safest parameters showing no occurrence of hemorrhage should be identified.

Enhanced permeability of cell membranes: sonoporation

A large number of *in vitro* studies have demonstrated that ultrasound alone or combined with MBs can efficiently increase cell membrane permeability in cell cultures (126). This technique has been used intensively for the last 20 years as a competitor to electroporation to deliver chemical substances, a majority of them being DNA plasmids. However, like for BBB disruption, quite a wide range of acoustic conditions were proposed. Moreover, the mechanisms of interaction of ultrasound, MBs and cell membranes and their bio-effects are not fully elucidated as recently reviewed by Mullin *et al.* for nanoparticle delivery (127). In a recent paper, Lentacker *et al.* categorized the experiments that have been published to date according to the probable mechanisms they exploit (128): bio-effects induced (I) by stable cavitation of MBs; (II) by inertial cavitation of MBs; or (III) by ultrasound without MBs. It is hypothesized that in the latter case, ultrasound-induced cavitation of tissue dissolved gases or acoustic microstreaming or shear stresses could be responsible for the observed bio-effects. Studies have also reported different bio-effects responsible for drug uptake in cells: direct pore formation in the membrane or activation of repair mechanisms aiming at replacing portions of the phospholipid membrane and thus stimulating endocytosis through different biological signaling pathways.

In vivo gene delivery and transfection for cancer treatment

This review paper focuses on *in vivo* preclinical studies related to cancer. Around half of the currently published papers in this area concern non-viral gene delivery and gene transfection. Early work included proofs of concept for increased gene delivery in tumors using ultrasound alone, but under very strong acoustic conditions i.e., for example using shock waves (129-135). As an example, Anwer *et al.* (132) obtained without MBs a major increase of IL-12 gene transfer (up to 270-fold increase) limited to tumor endothelial cells. It was sufficient to inhibit tumor growth in mice. Hayashi *et al.* showed that drug-encapsulating liposomes could enhance gene transfection and chemotherapy after sonication better than independent MBs and drug injections (134).

Most of the recent *in vivo* studies used MBs and more modest acoustic conditions (i.e., lower mechanical index and lower duty cycle) while showing higher gene expressions (136-146). Sakakima used IFN-γ plasmid cDNA to treat human hepatic cancer (SK-Hep1) in mice with a reduction of tumor size (137). They injected

the MBs mixed together with the plasmid directly in the tumor right before sonication. Using intravenous injections and a standard diagnostic ultrasound scanner, Hauff *et al*. increased tumor doubling time in capan-1 tumor mice treated by p16 tumor suppressor gene for 5 weekly sessions (138). The originality of their approach is the encapsulation of the plasmid DNA into the ultrasound contrast agent. Li *et al*. and Tsai *et al*. optimized the ultrasound parameters to enable prolonged gene expression in muscles and tumors (139,140). For instance, Li *et al*. found the best transfection efficiency for an acoustic frequency of 1 MHz, an intensity of 4 W/cm^2 and a duty cycle of 25%. More recent work demonstrated promising treatment outcomes for various genes in different murine models (141,142,144). Liao *et al*. (144) combined anti-angiogenic gene therapy with either chemotherapy or immunotherapy. Interestingly, they performed ultrasound aided gene transfection in muscles distant from the tumors and observed an additional therapeutic effect due to gene therapy.

Rychak and Klibanov recently published a review paper on DNA delivery using MBs and ultrasound (147). Although it would be the easiest protocol to implement in clinics, very few studies have injected the MBs into the blood stream so far (138,143). The therapeutic gain reported in these studies is likely to be due to an enhanced vascular permeability rather than to cancer cell sonoporation. Indeed, due to their size, MBs are likely to stay in the vasculature. To a smaller extent than in the brain, the permeability of vessel walls in peripheral organs can be increased as well. When genes and MBs are directly injected into the tumors, it is not clear how far they can diffuse from injection points to reach a maximum of cancer cells. Obtaining efficient gene transfection at a distance of blood vessels after systemic injection would be critical to the clinical translation of the technique.

Chemotherapies

Twenty years ago, it was observed in rodents and patients that shock waves were potentiating concomitant chemotherapy, likely due to enhanced permeability of biological membranes (148,149). In 2007, Iwanaga showed massive tumor regression in Ca9-22 tumor bearing mice after sonoporation and treatment by either bleomycin or a toxin-expressing plasmid (150). Similarly, Matsuo *et al*. demonstrated increased tumor regression after sonoporation of melanoma treated with melphalan (151). In these studies, MBs and therapeutic agents were both directly injected into the tumor thus the protocol remained invasive.

Recently, Yamatomo *et al*. demonstrated that boron neutron capture therapy of squamous cell carcinoma benefited from sonoporation of the tumor, with the drug injected intraperitoneally (152). Sato *et al*. compared intravenous and intralymphatic administration of MBs and cisplatin to treat lymph node metastasis (153). They only found significant improvements when using intralymphatic injections. Kotopoulis *et al*. showed an enhanced effect of gemcitabine after sonoporation on a mouse model of human pancreatic adenocarcinoma (154). The same authors recently published the first clinical results of sonoporation enhanced drug delivery in pancreatic cancer patients (155). The co-injection of Sonovue® MBs in the blood stream with gemcitabine followed by sonication with a clinical ultrasound scanner resulted in a slowdown of the tumor growth and a significant extension of the healthy period of life of these patients bearing a very aggressive cancer. The question remains whether cancer cells really experienced sonoporation or whether therapeutic gain came from a MB-induced increase of vascular permeability and thus enhanced gemcitabine delivery.

Several other molecules have been successfully tested *in vitro* but not yet *in vivo*.

Combined sonoporation and local drug release

Interestingly, Yudina *et al*. combined cavitation-induced sonoporation and thermally induced drug release from thermosensitive liposomes. In tumor bearing mice, they achieved significant cellular internalization of TO-PRO-3, a cell impermeable molecule after intravenous injection of MBs and drug-loaded thermosensitive liposomes (156).

Ultrasonic activation of drugs (sonodynamics)

The needs to enhance the effectiveness and reduce the toxicity of chemotherapy are major drivers for development of new drug delivery techniques. To this end, photodynamic therapy (PDT) and more recently sonodynamic therapy (SDT) have been shown to enable precise destruction of tumor cells without damaging adjacent normal cells.

Certain drugs, including chemical agents such as hematoporphyrin (HDT) and 5-aminolevulinic acid (5-ALA), are known to preferentially accumulate in tumors cells. On their own, these agents are inert and non-toxic. However, the agents can be activated by light of a certain wavelength, in a technique known as PDT, to induce apoptosis of the tumor cells. The process of PDT generates

oxygen free radicals that result in DNA damage and ultimately apoptosis (157). PDT is used clinically to treat various cancers although it has inherent qualities that could limit its clinical utility (158,159). Light cannot penetrate deep within tissue and therefore it can only reach superficial tumors and still be non-invasive. If deep tumors are the target, the procedure will require inserting a fiberoptic probe into the tissue, raising the risk of potential infection. Furthermore, the light may diffuse irregularly throughout the tumor and result in an incomplete treatment (160,161).

With recent advancements in the field of SDT, the technique has shown potential to overcome the limitations of PDT (96). SDT employs ultrasound rather than light to activate many of the same chemical agents. Focused ultrasound energy has been observed to excite agents including HDT, Rose Bengal or 5-ALA, after accumulation in tumor cells, to induce apoptosis of the targeted cells (162,163). Although the mechanism is not widely understood, it may be similar to that for PDT including the involvement of reactive oxygen species to lead to apoptosis. Alternatively, the shear forces generated by FUS of appropriate parameters could potentially induce damage sufficient to trigger cell death (164-167). FUS of low to moderate intensity (e.g., 1.0 MHz, 10 to 25 W/cm^2), and applied continuously for 5 min, has been shown to be effective in a rat intracranial tumor model at inducing apoptosis and reducing the overall tumor size (162,163).

As compared to PDT, SDT could provide a true noninvasive option even for deep seated tumors. Ultrasound energy does not have limited depth penetration or irregular diffusion within the tumor tissue, issues common for light. Therefore, FUS could provide a more conformal treatment of the tumor, via homogeneous delivery of energy and apoptosis throughout the entire tumor. FUS is also more highly focused than light, thus minimizing potential damage or toxicity to intervening or adjacent tissue (168,169).

The field of SDT is still early stage, and clinical utility has yet to be realized. Early research suggests low-power ultrasound to induce non-thermal effects is most effective. While further research must be conducted on the mechanisms responsible for this phenomenon, and the development and optimization of sonosensitizers and ultrasound parameters, SDT holds promise in non-invasive cancer treatment (169,170).

Potential role of combination therapy

The standard practice for treatment of many cancers today involves multiple different treatment modalities, such as the combination of surgery, radiation and chemotherapy. The development of a technique such as FUS to enable more efficient and localized drug delivery could reduce the need for standard systemic chemotherapy. However, optimal treatment for some cancers may still require a combination therapy approach.

There are many different combinations that could be envisioned that will require further investigation before clinical practice is altered. One such combination therapy could include FUS hyperthermia of a tumor followed by FUS-enhanced delivery of chemotherapy drugs to the tumor bed and a margin of adjacent tissue (171,172). The hyperthermia would aim for bulk necrosis of the tumor whereas the localized chemotherapy could protect against any tumor cells not identified and targeted via hyperthermia. Similarly, traditional surgical removal of the tumor could be followed by FUS-enhanced chemotherapy.

Therapies to combine FUS-enhanced drug delivery with radiation therapy may also prove beneficial. These therapies combined with FUS ablation could also prove synergistic. There has been research indicating that hyperthermia can make tumors more sensitive and receptive to radiation (173). FUS-enhanced drug delivery methods often employ the hyperthermia capabilities of FUS. In this case, hyperthermia can stimulate blood flow to the tumor, increasing its oxygenation, enhancing its metabolic rate and increasing the effectiveness of radiation therapy. This is particularly useful in the case of hypoxic tumors that are ordinarily difficult to treat via radiation. This sensitization via FUS can enable treatment using lower doses of radiation and thus more minimal side effects (174,175). Furthermore, FUS ablation could be added to this treatment regimen.

The ability of FUS in combination with ultrasound contrast agents (MBs) to temporarily and reversibly open the BBB is a promising technique enabling more effective delivery of drugs to the brain. Oftentimes, getting through the BBB is only one issue, and the tumor barrier itself may prevent effective delivery of therapies. Therefore, it could be useful to combine FUS-enhanced BBB opening with FUS-enhanced drug delivery at the site of the tumor via drug-loaded liposomes (e.g., Thermodox) or nanoparticles (176). These carrier vehicles, designed to respond to a specific threshold of heat or pressure produced by FUS, could then release drugs locally at the tumor for more effective uptake. Furthermore, FUS-induced sonoporation at the brain/tumor barrier could enhance permeation of drugs into the tumor.

Conclusions

Ultrasonic drug delivery has been limited to *in vitro* experiments for decades. Promising *in vivo* results have accumulated in the past ten years and this field is now nearing clinical trials. Ultimately, FUS-enhanced drug delivery is one tool in the armamentarium for optimal treatment of cancer. It may be enough on its own in some cases, but in other more complex cases, a combination therapy approach may be more effective.

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