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Janith Wanigasekara
Technological University Dublin, janith.manoharawanigasekara@tudublin.ie

Andressa Maria Aguiar de Carvalho
Technological University Dublin

Patrick J. Cullen
University of Sydney

See next page for additional authors

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Converging technologies: targeting the hallmarks of cancer using ultrasound and microbubbles

Janith Wanigasekara,[1,2,3,6] Andressa Maria Aguiar de Carvalho,[1,2,3,6] Patrick J. Cullen,[1,4] Brijesh Tiwari,[2] and James F. Curtin[1,3,5,*]

Various complex biological effects occur when ultrasonic compression waves travel through biological material. The myriad of biological outcomes instigated by ultrasound are evident when viewed through the lens of the hallmarks of cancer. Herein, we summarise the therapeutic potential of ultrasound, enhanced by microbubbles, for the treatment of cancer.

Biological effects of ultrasound

The hallmarks of cancer are a useful framework to distil and understand the underlying changes in this incredibly complex and diverse disease [1]. Six core and two emergent hallmarks underpin tumour development and metastasis. Two enabling hallmarks provide 'functional capabilities that allow cancer cells to survive, proliferate, and disseminate' (Figure 1). Drug development primarily focusses on singular receptors, whereas each hallmark is regulated by semi-redundant pathways allowing tumour adaptation and chemoresistance via mutation [1]. Therapies that broadly target hallmarks of cancer are therefore advantageous to prevent tumour adaptation. Ultrasound imparts focussed energy via ultrasonic compression waves directly to cells and tissues. When used in combination with gas-filled microbubbles, the resultant microbubble oscillation, expansion and contraction, and bursting enhances the antitumour effects of ultrasound, broadly targeting several hallmarks of cancer. Here, we propose that ultrasound therapies can be codified within the hallmarks of cancer framework.

Resisting cell death

Many existing chemotherapies target receptors with the therapeutic intent to overcome or bypass inherent tumour resistance to cell death. Direct application of ultrasound can overcome resistance to cell death, inducing coagulative necrosis, apoptosis, and reduction of tumour growth [2]. However, in glioblastoma and other brain tumours, the blood–brain barrier (BBB) prevents many chemotherapeutic agents from accumulating to effective concentrations in tumours. Two clinical trials used minimally invasive ultrasound to temporarily and repeatedly open the BBB and enhance chemotherapeutic delivery without adverse effects [3,4]. A Phase I/II clinical trial (NCT02253212) enhanced carboplatin uptake without evident neurotoxicity using the implantable ultrasound device, sonocloud-1, combined with microbubbles [3], and a second Phase I trial (NCT02343991) effectively delivered doxorubicin- and temozolomide-loaded liposomes using low-intensity magnetic resonance (MR)-guided focussed ultrasound [4]. These approaches demonstrate the in-human feasibility of micro/nano bubbles and ultrasound to enhance drug delivery, improve penetration and bioavailability in the brain parenchyma, increase local intracerebral drug concentrations, while reducing systemic toxicology. Preclinical development of a sonodynamic therapy complex incorporating chlorin e6 and hydroxychloroquite into liposomes illustrates this point. The complex selectively accumulated in brain tumours during ultrasound-targeted microbubble destruction and hydroxychloroquine was released to glioma cells, inducing reactive oxygen species (ROS) production, mitochondrial dysfunction, and MAPK/p38-PINK1-PRKN-dependent mitophagy [5].

Microbubbles and gene-loaded nanoparticles combined with ultrasound also provides a safe, effective, and targeted delivery system for genes. Microbubbles and a lipid-polymer hybrid nanoparticle complex were used as an ultrasound-mediated gene delivery carrier of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 plasmids, targeting O6-methylguanine-DNA methyltransferase (MGMT), a DNA-repair gene protecting glioma cells from alkylating chemotherapeutics. Ultrasound induced BBB opening, allowing targeted delivery, gene protection from enzyme degradation, biocompatibility, prolonged survival, biosafety, and augmented transfection efficiency, while down-regulating MGMT expression and thereby sensitising cells to temozolomide [6].

Microbubbles loaded with the halogenated xanthene contrast agent rose bengal conjugated via stable amine linkages to dihexadecylamine combined with ultrasound has been used as a novel sonosensitiser delivery system in a preclinical *in vivo* study. Ultrasound converted loaded microbubbles into nanoparticles, resulting in enhanced drug accumulation in the tumour and ROS generation, leading to tumour growth inhibition with minimal side effects [7]. Ultimately, microbubbles combined with ultrasound can overcome cell death resistance. Future progress will be dependent on further development of materials that increase tumour cell death while minimising damage to surrounding cells. While not yet undergoing clinical trials, nanomaterials combined with ultrasound have potential to selectively ablate tumour tissue deep within the body.

Sustaining cell proliferation

Cancer cells deregulate the normal signals that control entry and progression through

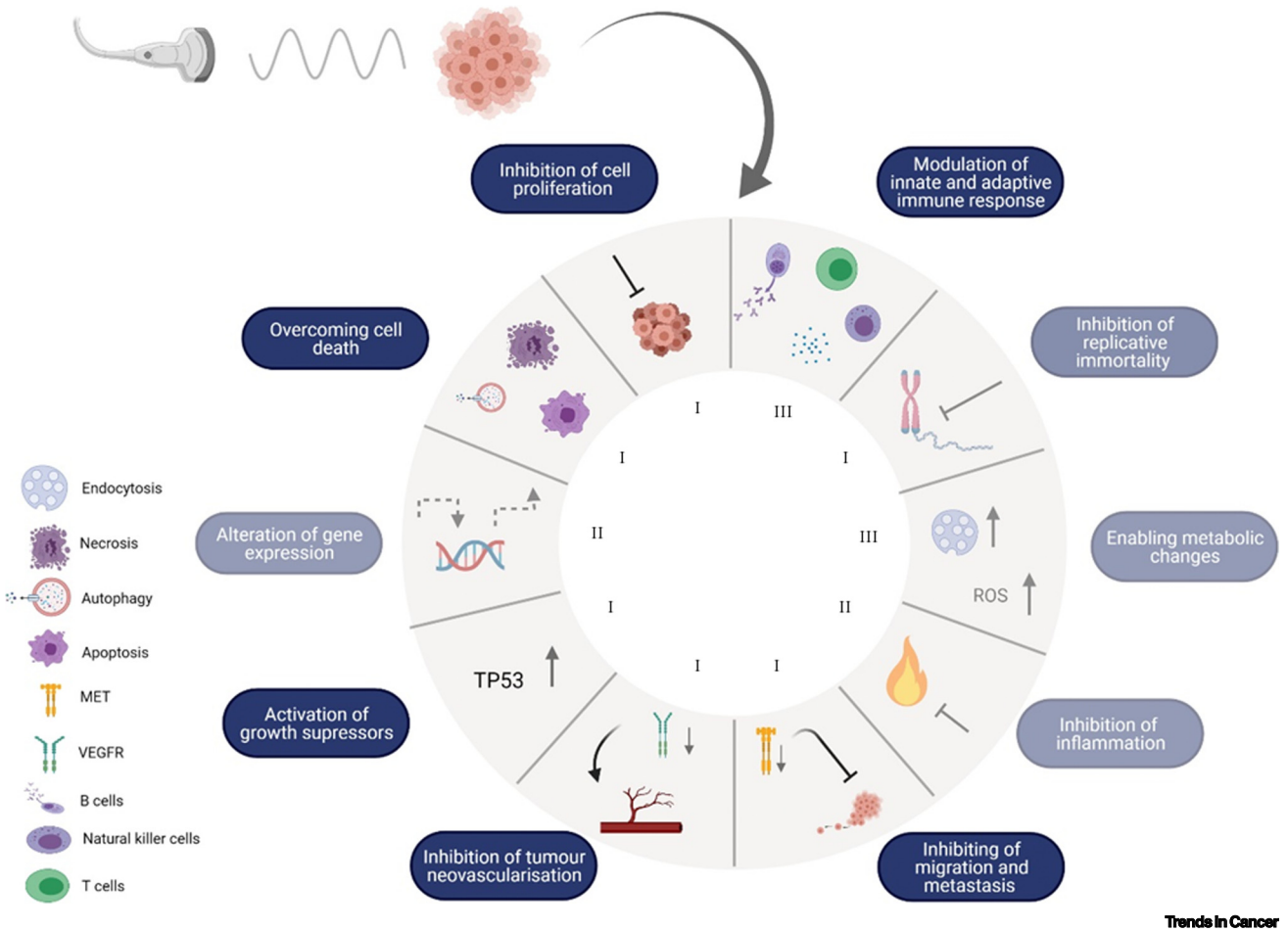

Figure 1. Ultrasound targets the hallmarks of cancer. Ultrasound directly modulates five core hallmarks and one emerging hallmark of cancer. Figure created with BioRender. Abbreviations: I, core hallmarks; II, enabling hallmarks; III, emerging hallmarks; ROS, reactive oxygen species; TP53, tumour protein P53.

the cell cycle, resulting in sustained cell proliferation. Antineoplastic agents have been developed, such as gemcitabine, to inhibit DNA synthesis in cancer cells. Moreover, high expression of some miRNAs, such as miR-21, has been shown to downregulate tumour suppressors and modulate Akt phosphorylation and others, such as miR-122, have tumour suppressor effects and are downregulated in various cancers, suggesting that targeting miRNAs could inhibit cancer cell proliferation. Ultrasound-targeted microbubble destruction can enhance clathrin-mediated endocytosis of nanoparticles and therefore enhance delivery of antineoplastic agents. A preclinical pancreatic cell study using ultrasound-targeted microbubble disruption promoted uptake of dendrimer-entrapped gold nanoparticles loaded with gemcitabine or a miR-21 antisense inhibitor, increasing cytotoxicity by more than 80-fold and 10-fold, respectively. This approach reduced tumour volume and increased blood perfusion *in vivo* [8]. Similar effects were seen in hepatocellular carcinoma (HCC) when sense miR-122 and antisense anti-miR-21 were encapsulated into poly (lactic-co-glycolic acid) nanoparticles and delivered by ultrasound-mediated microbubble disruption. This approach induced dose-dependent decreases of antiapoptotic proteins CD-320 and IGFR-1 and dose-dependent increase of the proapoptotic protein Programmed Cell Death Protein 4, along with enhanced HCC death in doxorubicin-resistant and nonresistant human xenografts *in vivo* [9]. In a Phase I clinical trial (NCT01674556), ultrasound combined with microbubbles and gemcitabine was demonstrated to be clinically feasible and safe, while enhancing intratumoural drug delivery. This combination treatment also doubled median survival of patients with inoperable pancreatic cancer [10].

Epidermal growth factor receptor (EGFR) also promotes cell proliferation and is commonly overexpressed in cancers.

Permeabilization of intact BBB using ultrasound in combination with microbubbles was applied before injecting ^{89}Zr-labeled anti-EGFR monoclonal antibody (cetuximab). This allowed prolonged exposure of the brain parenchyma to cetuximab with slow diffusion and clearance, allowing for high and localised exposure to monoclonal antibodies [11].

Inducing angiogenesis

Angiogenesis is required to sustain tumour growth and much effort has been devoted to inhibiting blood vessel growth to reduce tumour burden. Ultrasound inhibits tumour neovascularisation, inducing thrombosis and preventing metastasis. Moreover, heat caused by microbubble oscillation results in selective damage in tumour endothelial cell linings due to slower blood flow and a higher retention of microbubbles in the tumour neovasculature. This effect leads to inhibition of tumour neovascularisation and potentially necrosis, apoptosis, reduced tumour growth, and improved survival [12]. We expect to see a future shift away from microbubbles towards nanobubbles that damage tumour endothelial cell linings more effectively.

Tissue invasion and metastasis

Invasion and metastasis are commonly associated with poor tumour prognosis. Tumour cells leave the primary tumour site and enter the vascular system before forming distal metastases. MET activation is involved in epithelial to mesenchymal transformation, which is necessary for invasion and metastasis. Ultrasound induces gas accumulation within cell membranes, altering cell membrane micro-morphology, thereby interrupting MET-induced cell motility [13].

Additional efforts are underway to target tumour cells from entering the bloodstream or inhibiting blood vessel cells from allowing cells to pass through. Microbubbles can cause capillary damage, activating coagulation and inducing thrombosis, thereby limiting or preventing metastasis [12]. Moreover, as EGFR is also involved in cell migration, the combination of cetuximab with ultrasound and microbubbles increases permeability of cetuximab into the brain, thereby leading to reduced migration in a preclinical study [11]. While promising preclinical results have been demonstrated, clinical effects of ultrasound on invasion and metastasis remain unclear and further exploration will allow a more complete understanding of whether ultrasound can inhibit this hallmark.

Evading immune destruction

Evasion of the immune system and defective or suppressed immune responses against tumour antigen are common features of cancer. These immune responses can be (re)activated under certain conditions, making them an ideal target to fight cancer more effectively. Recent efforts to counter tumour-induced immune suppression has led to the development of various immune-based therapies, including immune checkpoint inhibitors and adoptive cellular therapies. The feasibility of ultrasound-triggered transfection of bone marrow-derived dendritic cells (BM-DC) was demonstrated in preclinical models. Primary murine C57BL/6 BM-DC cultures were generated and ultrasound-triggered transfection of BM-DC cultures was achieved using microbubbles loaded with both antigen ($OVA_{257-264}$ SIINFEKL) mRNA as well as immunomodulating TriMix mRNA. This enabled potent $OVA_{257-264}$ antigen-specific immune responses *in vivo* against MO4 melanoma cells and E.G7-OVA T lymphoma cells, resulting in a significant reduction of tumour growth, increased survival, and long-lasting antigen-specific protection against tumour recurrence [14]. By releasing damage-associated molecular patterns (DAMPs), increasing immune cell infiltration, decreasing immunosuppressive cytokine release from tumour cells, and releasing cell debris, including tumour antigen through the above-mentioned mechanisms [2], the potential for ultrasound to augment immune responses is clear. Further studies should evaluate the combination of ultrasound with immune-based therapies.

Deregulated metabolism

Cancer cells adapt their metabolism to support biomass production, ATP generation, and maintain redox state. Disrupting these processes can interfere with tumour growth and metastasis. Ultrasound enhances endocytosis and exocytosis, thereby modifying nutrient, micronutrient, and ion uptake [15] and, potentially, cancer cell metabolism. Ultrasound induces cavitation, enhancing cell and tissue membrane permeability, and creates free radicals. Direct biological effects and bystander effects of cavitation and free radical production can be further enhanced using microbubbles and sonosensitisers and controlled by adjusting duration, amplitude, intensity, frequency, shape, focus, and type of ultrasound transducer used [2,12].

Concluding remarks

Ultrasound elicits a range of biological changes in a tumour with minimally invasive technology. Clinical trials demonstrate safe delivery of a range of therapeutic agents across the BBB and into cancer cells, along with *in situ* heat-dependent cytotoxicity to the tumour. Advances in microbubble and nanobubble technologies will further improve combinational therapy approaches. A wide array of clinical trials are planned or underway using chemotherapy, sonodynamic therapy, immunotherapy, and overcoming BBB, many incorporating microbubbles or nanomaterials. However, there are no reported clinical trials currently planned or underway using ultrasound and microbubbles to enhance gene delivery, despite successful preclinical studies, including the above-mentioned CRISPR-Cas9 studies [6], miRNA studies [8,9], and BM-DC transfection studies [14]. These findings demonstrate the potential of nucleic acid delivery by ultrasound and microbubbles and as

Table 1. Clinical trials combining ultrasound and microbubbles/nanomaterials

Ultrasound device	Combination	Condition/disease	Phase	Hallmark[a]	Status	Identifier (NCT number)
Chemotherapy						
Focused ultrasound	Lyso-thermosensitive liposomal doxorubicin	Liver tumour	I	I	Completed	NCT02181075 [16]
	FOLFIRINOX regime, microbubbles	Pancreatic ductal adenocarcinoma	II	I	Recruiting	NCT04146441
	Panobinostat, microbubbles	Diffuse intrinsic pontine glioma	I	I	Recruiting	NCT04804709
	Conventional chemotherapy, microbubbles containing sulphur hexafluoride stabilised by phospholipids	Colorectal neoplasms, breast neoplasms	I/II	I	Recruiting	NCT03477019
	Platinum, gemcitabine, microbubbles	Gastrointestinal neoplasms	I/II	I	Recruiting	NCT02233205
Contrast-enhanced ultrasound	Gemcitabine hydrochloride, nab-paclitaxel, fluorouracil, irinotecan hydrochloride, leucovorin calcium, oxaliplatin, perflubutane microbubble	Metastatic pancreatic ductal adenocarcinoma	I/II	I	Not yet recruiting	NCT04821284
	Perflutren protein-type A microspheres, microbubble	Hepatocellular carcinoma	II	I	Recruiting	NCT03199274
	Gaseous microbubbles, systemic chemotherapy, sonoporation	Liver metastases of colorectal cancer	II	I	Recruiting	NCT03458975
	Neoadjuvant chemotherapy, sulphur hexafluoride microbubbles	Breast cancer	N/A	I	Completed (no results posted)	NCT00245869
	Gemcitabine, microbubbles	Pancreatic adenocarcinoma	I	I	Completed	NCT01674556 [10]
Magnetic resonance-guided high-intensity focused ultrasound	Lyso-thermosensitive liposomal doxorubicin, cyclophosphamide	Metastatic breast cancer	I	I	Recruiting	NCT03749850
	Lyso-thermosensitive liposomal doxorubicin	Refractory solid tumours	II	I	Not yet recruiting	NCT04791228
	Lyso-thermosensitive liposomal doxorubicin	Paediatric cancer	I	I	Recruiting	NCT02536183
Blood–brain barrier disruption						
NaviFUS system	N/A	Recurrent glioblastoma	N/A	I	Completed (no results posted)	NCT03626896
	Bevacizumab, microbubbles	Recurrent glioblastoma	N/A	I	Recruiting	NCT04446416
ExABlate system	Carboplatin	Recurrent glioblastoma	I/II	I	Recruiting	NCT04440358
	Microbubbles	Brain tumour	N/A	I	Active, not recruiting	NCT02343991 [4]
	Carboplatin	Recurrent glioblastoma	I/II	I	Recruiting	NCT04417088
	N/A	Brain metastases of breast cancer	N/A	I	Recruiting	NCT03714243
	Temozolomide	Glioblastoma	N/A	I	Recruiting	NCT03712293
SonoCloud-9 system	Albumin-bound paclitaxel, microbubbles	Glioblastoma	I/II	I	Recruiting	NCT04528680
	Carboplatin	Recurrent glioblastoma	I/II	I	Completed	NCT02253212 [3]
	Carboplatin	Recurrent glioblastoma	1/2	I	Active, not recruiting	NCT03744026
	Nivolumab injection	Brain metastases of melanoma	1/2	I	Recruiting	NCT04021420

Table 1. (continued)

Ultrasound device	Combination	Condition/disease	Phase	Hallmark[a]	Status	Identifier (NCT number)
Focused ultrasound	N/A	Glioblastoma	N/A	I	Recruiting	NCT03616860
	N/A	Glioblastoma	N/A	I	Recruiting	NCT03551249
Immune therapy						
Focused ultrasound ablation (Echopulse)	PD-1 antibody blockade	Advanced solid tumours	I	III	Recruiting	NCT04116320
High-intensity focused ultrasound	PD-1 antibody blockade (pembrolizumab)	Metastatic breast cancer	I	III	Recruiting	NCT03237572
Low-intensity pulsed ultrasound (SONOCLOUD)	PD-1 antibody blockade (nivolumab and pembrolizumab)	Brain metastases of melanoma	I/II	I	Recruiting	NCT04021420
Sonodynamic therapy						
Magnetic resonance-guided high-intensity focused ultrasound	SONALA-001(5-aminolevulinic acid)	High grade glioma	I	I	Recruiting	NCT04559685
	5-Aminolevulinic acid	Glioblastoma	N/A	II	Not yet recruiting	NCT04845919

[a]Abbreviations: I, core hallmarks; II, enabling hallmarks; III, emerging hallmarks; N/A, not applicable.

a clinical research gap worth exploring (Table 1).

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Author contributions

J.F.C. designed the study. J.W. and A.M.A.dC. collated and reviewed literature with support from J.F.C., B.T., and P.J.C. J.W., A.M.A.dC. and J.F.C. wrote the manuscript with support from B.T. and P.J.C.

Declaration of interests

No interests are declared.

[1]BioPlasma Research Group, School of Food Science and Environmental Health, Technological University Dublin, Dublin, Ireland
[2]Department of Food Biosciences, Teagasc Food Research Centre, Ashtown, Dublin, Ireland
[3]Environmental Sustainability & Health Institute (ESHI), Technological University Dublin, Dublin, Ireland
[4]University of Sydney, School of Chemical and Biomolecular Engineering, Sydney, Australia
[5]FOCAS Research Institute, Technological University Dublin, Dublin, Ireland
[6]These authors contributed equally to this work

*Correspondence:
james.curtin@tudublin.ie (J.F. Curtin).

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