SELECTIVE NEURO-ONCOLOGICAL THERAPIES USING FOCUSED ULTRASOUND TO DISRUPT THE BLOOD-BRAIN BARRIER

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Ultrasound in clinical medicine is most commonly associated with imaging, but can be harnessed to yield an array of bioeffects. Of particular interest in neuro-oncology is the interaction of ultrasound with pre-formed ultrasound contrast agents, the combination of which can be used to increase the permeability of the cerebrovasculature in a reversible manner [blood-brain barrier (BBB) disruption]. Many promising therapeutic agents have in vitro efficacy but fail in vivo due to their inability to accumulate to any significant degree in the central nervous system (CNS). While primary brain tumours alone do not impose an enormous healthcare burden compared with many other cancers, as many as 40% of all cancer patients will eventually develop brain metastasis, making the potential impact of drug delivery with ultrasound significantly larger.

We focused particularly on therapeutic agents that, once in the brain, selectively targeted malignant cells, as existing therapies cause much morbidity in their wake secondary to collateral neuronal injury. We investigated the effects of BBB disruption on boron neutron capture therapy with the delivery agent boronophenylalanine. BBB disruption increased the absolute boron concentration, important for tumour killing, by almost 150%, and improved the ratio between normal brain and tumour, minimizing radiation to healthy cells. It also increased the uptake in infiltrating cells, particularly important for tumours like glioblastoma. We then demonstrated that
an even more selective agent - engineered, targeted immune cells - could be delivered to a brain tumour in a viable state. Furthermore, we demonstrated improved survival in a brain metastasis model of HER2 amplified breast cancer. Both of these targeted therapies have been investigated in early clinical trials, and with the mounting evidence for the safety of BBB disruption with ultrasound, there exists real potential to translate these promising therapies into the clinical realm and offer new treatments to patients with little hope for a cure.
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## CONTENTS

**LIST OF FIGURES** ......................................................................................................................... vii

**LIST OF TABLES** .......................................................................................................................... xi

**LIST OF ABBREVIATIONS** ............................................................................................................ xi

1 Introduction ..................................................................................................................................... 1
   1.1 Motivation and significance ................................................................................................. 1
   1.2 The brain ............................................................................................................................... 2
      1.2.1 Structure and function ................................................................................................. 2
      1.2.2 The blood-brain barrier and factors influencing its permeability ............................. 3
   1.3 Cancer in the brain ................................................................................................................ 8
      1.3.1 Primary .......................................................................................................................... 8
      1.3.2 Secondary ...................................................................................................................... 9
      1.3.3 Existing treatments for malignant brain tumours ....................................................... 9
   1.4 Circumventing the blood-brain barrier in cancer therapies ............................................. 11
      1.4.1 Bypassing the BBB ...................................................................................................... 11
      1.4.2 Disrupting the BBB ...................................................................................................... 12
      1.4.3 Shortcomings of current therapies ................................................................................ 12
   1.5 Blood-brain barrier disruption with ultrasound and microbubbles ............................... 13
      1.5.1 Introduction and history .............................................................................................. 13
      1.5.2 Ultrasound as a compressional wave ........................................................................... 15
      1.5.3 Ultrasound interactions with tissue .............................................................................. 18
         1.5.3.1 Reflections ............................................................................................................... 18
         1.5.3.2 Attenuation: absorption and scattering ................................................................. 19
         1.5.3.3 Thermal effects ....................................................................................................... 21
         1.5.3.4 Cavitation ................................................................................................................ 22
         1.5.3.5 Acoustic radiation force .......................................................................................... 26
         1.5.3.6 Other interactions with tissue .................................................................................. 27
      1.5.4 Microbubble ultrasound contrast agents and their interactions with ultrasound .......... 28
      1.5.5 BBB-Disruption ............................................................................................................. 29
      1.5.6 Ultrasound technology for clinical treatments ........................................................... 32
         1.5.6.1 Transducer design ................................................................................................... 32
      1.5.7 Treatment guidance and monitoring ............................................................................ 36
         1.5.7.1 MR thermometry ..................................................................................................... 36
         1.5.7.2 Ultrasound-based treatment and cavitation monitoring ........................................ 37

2 Enhancing Drug Delivery for Boron Neutron Capture Therapy of Brain Tumours .............. 38
   2.1 Introduction .......................................................................................................................... 38
   2.2 Materials and Methods ......................................................................................................... 40
      2.2.1 Tumour Implantation ..................................................................................................... 40
      2.2.2 Animal Preparation, Focused Ultrasound Setup and MRI Imaging .......................... 41
      2.2.3 Treatment Groups ........................................................................................................ 42
      2.2.4 Specimen Preparation .................................................................................................. 43
      2.2.5 Imaging Mass spectrometry ......................................................................................... 43
      2.2.6 Inductively-Coupled Plasma Atomic Optical Emission Spectroscopy (ICP-AES) and semi-quantification of ToF-SIMS data ........................................................................... 44
      2.2.7 Statistical Analysis ......................................................................................................... 45
LIST OF FIGURES

Figure 1.1: Mid-sagittal drawing of the human brain. The forebrain makes up most of the brain and consists of the gyrated neocortex, its associated white matter tracts, and a number of deeper structures including the thalamus, hypothalamus, and basal ganglia. The midbrain sits at the rostral portion of the brainstem, the remainder of which forms the hindbrain and includes the pons, medulla and cerebellum. Image from Gray’s Anatomy of the Human Body available at http://www.bartleby.com/107 ................................................................. 3

Figure 1.2: Schematic representation of the structural elements of the BBB. Endothelial cells are interconnected by tight junctions. Pericytes play a regulatory role in endothelial cell function. Astrocyte foot processes play an integral role in homeostasis in the brain. The astrocyte foot processes encircle the capillaries, anchored to the basement membrane. ........................................... 4

Figure 1.3: Transport mechanisms across the cerebral endothelium. A, the passage of small hydrophilic molecules is greatly restricted by tight junctions. B, small lipophilic drugs can diffuse across the cell membrane. C, specific transport proteins allow glucose, amino acids, nucleosides and other necessary compounds to reach the brain. D, proteins such as insulin and transferrin reach the brain via receptor-mediated endocytosis and transcytosis. E, some plasma proteins such as albumin can be delivered via absorptive-mediated endocytosis, but this is not a major pathway in the brain. ..................................................................................................................... 6

Figure 1.4: Schematic diagram depicting a sinusoidally varying longitudinal ultrasound wave and its effects on the propagating medium. An observer would perceive the pressure amplitude (top graph, y axis) varying in the same manner both at a fixed point in time while traversing the medium, or at a fixed location in real time. The lower graph illustrates the behavior of the particles in the medium as the longitudinal pressure wave passes. The trough or low pressure portion of the wave causes rarefaction, while the peak or high pressure phase causes compression ........................................................................................................................................... 16

Figure 1.5: Block diagram of the basic components of a therapeutic ultrasound system. RF, radiofrequency. In the case of phased arrays used for transcranial applications, each element requires an independent driving circuit like the one shown. ............................................................................................................. 35

Figure 2.1: Representative images of the rodent brain before and after BBBD with focused ultrasound. A schematic diagram is shown in A, with the positioning of the animal relative to the ultrasound transducer. The 3-axis positioning system allows the transducer to be positioned so that the focal spot can be targeted anywhere in the brain. B and C show Omniscan® enhanced pre- and post-BBBD T1-weighted MR images, respectively. D and E show the corresponding T2-weighted MR images. The tumour is indicated by the single arrow in panels B and D prior to BBBD. The 4 sonication foci (arrows) can be identified in C and E encircling the tumour following BBBD. The square pattern is slightly distorted as a result of transmission through the skull. The increase signal on the T2-weighted images corresponds to edema induced by the disruption of the vasculature. There also appears to be some BBBD towards the midline resulting from the postero-medial focus, which has resulted in contrast enhancement periventricularly in the left frontal lobe, in the anterior commissure and in the anterior portion of the left thalamus. This is due to the 3 mm pressure full width half maximum (FWHM) lateral beam width, which combined with the skull distortion, would overlap these structures ............................................................................. 42

Figure 2.2: Optical imaging within the vacuum chamber of the ToF-SIMS instrument was used to locate the tumour (indicated by the arrow) on the lyophilized specimens (A). Following
collection of the secondary ion spectra, the signal from $^{10}\text{B}^+$ was easily distinguishable due to the lack of interferences in that atomic mass range (B). ................................................................. 46

**Figure 2.3:** Representative secondary ion images taken on the brain-tumour interface. The tumour can be identified in the lower left of each image, with an obliquely oriented island of tumour in the centre. A shows the corresponding H&E stained frozen section adjacent to that used for ToF-SIMS analysis. B-F were acquired simultaneously and are representative of the data sets collected. D shows the relatively uniform signal obtained from $^{12}\text{C}^+$ both in tumour and normal brain tissue. For this reason it is used as a reference element. Both $^{23}\text{Na}^+$ and $^{24}\text{Mg}^+$ can provide significant contrast between tumour and brain tissue (B and E), as the concentrations of both are higher within malignant cells. The boron content of the tumour is noticeably higher compared with the brain (F). ................................................................. 48

**Figure 2.4:** ToF-SIMS imaging of a 150 x 200 µm region in the rat brain following infusion of BPA-f. The image on the left serves as a schematic of the relative locations in the brain from which the secondary ion signals were acquired. The adjacent 4 columns depict, from left to right, a tumour treated with ultrasound, a control tumour, the ipsilateral peri-tumoural normal brain, and the contralateral untreated brain. A-D show the $^{10}\text{B}^+$ signal normalized by the $^{12}\text{C}^+$ signal. E-H show the signal in A-D overlaid on the sum of the remaining secondary ion signal, where cellular detail can be appreciated. A, C, and D are all from the same animal. The quantitative results are presented in Table 2-1. .................................................................................. 51

**Figure 2.5:** MR imaging of BBBD without tumour implantation. Representative axial gadolinium-enhanced T1-weighted (A) and T2-weighted (B) MRI of BBBD without implanted tumours. The $^{10}\text{B}$ secondary ion signal obtained with ToF-SIMS from the sonicated (C) and unsonicated (D) frontal lobe is shown. The yellow and white boxes are not to scale but denote the approximate regions of analysis. The corresponding yellow and white stars denote the appropriate ToF-SIMS image. There was no statistically significant difference in the concentration in the normal brain tissue exposed to ultrasound (p=0.39)............................................................................. 51

**Figure 3.1:** The experimental MRI-guided FUS setup. The anesthetized animal was positioned supine over an MRI RF surface coil and coupled to the piezo-ceramic focused transducer through a bath of de-gassed water. The transducer was repositioned with the aid of a computer-controlled three-axis positioning system, allowing the ultrasound focus to reach any point within the brain. The entire setup fits in the bore of the 1.5T MR scanner, allowing for coordinate co-registration, targeting and confirmation of BBBD. .................................................................................. 62

**Figure 3.2:** Experimental timeline for the three groups. All groups initially underwent baseline imaging to assess the tumour size and location. In group 1 (control) this was followed by injection of SPIO nanoparticle labeled HER2-specific NK-92 cells. Follow-up imaging was performed at 16 hours following cell injection and immediately prior to sacrifice. Group 2 underwent BBBD, followed 5 minutes later by T1-weighted MRI with contrast to assess the change in contrast extravasation. Cells were then injected approximately 30 s after the completion of the imaging. In group 3, cells were injected via the tail vein and BBBD initiated 30 s after the injection. T1-weighted MRI was performed 5 minutes post-BBBD again to assess the change in contrast extravasation. Both groups 2 and 3 were imaged at 16 hours following the cell injection and immediately euthanized. .................................................................................. 64

**Figure 3.3:** The results of blood-brain barrier disruption with FUS. Representative T1-weighted MR images with Omniscan contrast of the tumour before and after BBBD, A and B respectively. The average enhancement of untreated tumours was 17 ± 8 % but increased to 34 ± 10 % following exposure to ultrasound and microbubbles (mean ± SD, p<0.05). A small region of
erythrocyte extravasation (arrow) was seen in one of the treatment animals, C. There was no further evidence of tissue injury. 66

**Figure 3.4:** HER2-specific NK-92 cell detection with 7T MRI. A baseline axial T2*-weighted MR image from group 3 is shown with the tumour identified in the left frontal striatum (white arrow). A. The corresponding post-treatment image demonstrates a signal reduction at the tumour site (white arrow). B. C shows the average signal intensity change (mean ± SEM) at the tumour site following treatment. A negative change suggests the accumulation of iron-labelled effector cells. There was a statistically significant difference between group 3 and group 1. 68

**Figure 3.5:** Histological quantification of HER2-specific NK-92 cells accumulating at the tumour site. Effector cells were co-localized with CD45 IHC (upper panel) and Prussian blue histochemistry (lower panel) in the three experimental groups, A. HER2-specific NK-92 cells reaching the tumour were quantitatively assessed (mean ± SEM), B. When NK-92 cells were injected prior to BBBD, the number reaching the tumour was significantly higher than if they were injected following or without BBBD (group 3 vs groups 1 and 2: 0.95 ± 0.23 vs 0.09 ± 0.11, 0.21 ± 0.15, p < 0.01). There was no statistical difference between groups 1 and 2. These results are in agreement with the iron-sensitive MR imaging in Figure 4. 69

**Figure 3.6:** FUS causes the translocation of HER2-specific NK-92 cells from the vasculature into the brain and tumour when they are present in the circulation at the time of BBBD. A, CD45 IHC depicting a vessel from which a large number of cells have extravasated and appear to track to the tumour (indicated by the star). B, the corresponding Prussian blue stained section is shown, colocalizing the HER2-specific NK-92 cells. C, a normal capillary adjacent the tumour but within the sonicated region, shows HER2-specific NK-92 cells forced to the adluminal surface of the vessel. FUS results in HER2-specific NK-92 cells circumventing both the BBB and BTB. D, the corresponding Prussian blue section. These cell distributions were seen exclusively in group 3 animals. 70

**Figure 3.7:** HER2-specific NK-92 cells accumulate at the tumour and have preserved function. 73

**Figure 4.1:** Schematic depiction of BBB disruption with FUS in the rodent brain using encapsulated perfluorocarbon microbubbles (commercially available as ultrasound contrast). A) A focused ultrasound transducer delivers sub-megahertz pressure waves to a precise location within the brain. Under the influence of these waves, the microbubbles undergo stable cavitation and result in NK-92 translocating into the tumour. B) Images from Alkins et al. 2013b showing the NK-92 cells on the abluminal surface of vessels following exposure to FUS and ultrasound contrast. The cells are identified with CD45 (above) and Prussian blue (following transfection with iron nanoparticles, below). The cells can be seen tracking to the tumour following egress from the vasculature. 76

**Figure 4.2:** Schematic representation of the two treatment arms. Within each arm there was a group receiving cells only, a group receiving FUS only and a group receiving combined treatment with FUS and cells. The distributed arm received an evenly distributed treatment over a 4-week period while the front-loaded arm received aggressive upfront therapy. Each animal, regardless of the arm, underwent a total of 8 treatment sessions. They were then followed with imaging twice weekly until they exited the study. 79

**Figure 4.3:** Representative images of the evolution of tumor size measured on axial T1-weighted gadolinium-enhanced MRI. The tumor can be readily identified in the right frontal lobe due to the extravasation of gadolinium contrast and corresponding increased signal on T1 MRI. At day 1 the tumors are relatively consistent in size, but by 2 weeks there has been significant interval growth, with some tumors showing non-enhancing regions likely representing cystic components seen on
post-mortem histology. The only group that showed a significant treatment effect was the FUS+Cells group of the front loaded arm.

**Figure 4.4:** The two final T2-weighted MR imaging studies of the long-term survivors, showing the structural impact of the treatment on the right frontal lobe. The animal in A was euthanized at 90 days due to pyelonephritis while the animal in B was healthy at the time of euthanasia at 150 days. Both animals showed some volume loss in the treated (right) frontal lobes, evidenced by the increased prominence of the corresponding frontal horn of the lateral ventricle. The treated region is highlighted by the dashed circle (not to scale).

**Figure 4.5:** The effect of the therapy in the distributed treatment arm. In A the survival appears to be slightly greater in the FUS+Cells group but this was not statistically significant. The tumor volumes for the first 4 weeks are shown in B. While it looked as though the curves began to diverge into the 3rd treatment week, there was only a small difference by the end of the 4th week.

**Figure 4.6:** The effect of therapy in the front-loaded treatment arm. In A and B, the normalized and absolute tumour volumes are shown over the first 4 weeks of treatment. The FUS only and Cell only curves are roughly overlapping, highlighting the anticipated ineffectiveness of either one of those therapies alone. In C, the normalized body weights decline over the first week during the intensive treatment schedule, but universally increase beyond that. As might be expected, the Cell group appears to have less weight-loss than the two ultrasound groups. In D, the statistically significant increase in survival can be appreciated in the FUS+Cells group. In the latter group, 2 of the 4 animals had long-term survival. One animal died at 90 days due to pyelonephritis, while the second survivor was euthanized after 150 days but was in good health and showed no ill effects of the treatment.

**Figure 4.7:** Representative distributed arm post-mortem histology. A, B, and C show a representative axially cut H&E section of the tumor at the time of euthanasia for the Cell, FUS, and FUS+Cells groups. At the time of death, the tumors were very similar in size, confirming that our study exit endpoints were relatively robust. However, the mean survival times were quite different, suggesting some benefit for FUS+Cells even in the distributed arm. D shows a section adjacent the H&E shown in C with IHC for CD45. There were no targeted NK-92 cells seen at the time of sacrifice in any of the animals, although these had last been injected at least a week prior to sacrifice.

**Figure 4.8:** Heterogeneity of HER2 expression at the time of animal sacrifice. A and B are representative H&E sections of the tumors from the two short-term survivors in the FUS+Cells group of the front-loaded arm. C is an animal from the Cell-only group of the front-loaded arm. D-F show higher magnification views of the tumours with IHC for the HER2 protein. A/D and C/F show no HER2 presence despite very different treatments. The tumor in B/F, which was treated with FUS+Cells, unfortunately developed leptomeningeal spread. Interestingly, possibly due to the treatment, the parenchymal portion of the tumour targeted by the treatment shows a drastically lower expression of HER2 compared to the leptomeningeal component. Regions of necrosis, as well as cystic components, are identifiable in the tumors shown in A and C.
LIST OF TABLES

Table 1-1: Cytoplasmic tight junction-associated proteins .......................................................... 7
Table 1-2: Soluble factors affecting the permeability and function of the BBB ............................. 8
Table 2-1: Main tumour (MT):brain and infiltrating tumour (IT):brain 10B ratios and tissue concentrations following BBBD with focused ultrasound and BPA-f infusion, compared with the control group receiving infusion alone.................................................................................. 47

LIST OF ABBREVIATIONS

BBB       Blood-Brain Barrier
BBBD      Blood-Brain Barrier Disruption
BNCT      Boron Neutron Capture Therapy
BTB       Blood-Tumour Barrier
CNS       Central Nervous System
CSF       Cerebrospinal Fluid
CT        Computer Tomography
Da        Dalton
FUS       Focused Ultrasound
GBM       Glioblastoma Multiforme
IA        Intra-arterial
IT        Infiltrating Tumour
IHC       Immunohistochemistry
IV        Intravenous
MRI       Magnetic Resonance Imaging
MRIGFUS   MRI-guided Focused Ultrasound
MT        Main Tumour
NK        Natural Kill
1 INTRODUCTION

1.1 Motivation and significance

The treatment of tumours in the brain remains a great challenge and despite many advances in oncology, the outcome of patients with brain tumours is very poor. Full surgical resection in the brain is often impossible and leaves behind microscopic and sometimes macroscopic residual, while the toll of current adjuvant chemo- and radiotherapy is significant (Verstappen et al., 2003; Sioka and Kyritsis, 2009). Many therapeutic agents, despite possessing in vitro cytotoxicity against malignant cell lines, do not have the same activity against tumours in vivo due to the blood-brain barrier (BBB) (Muldoon et al., 2007). Given that cancer treatments should aim not only to prolong survival, but also to preserve quality of life, new therapies should make every attempt to spare healthy tissue, preferably by acting solely upon malignant cells, or at least selectively on malignant cells while being directed to the vicinity of the pathology.

The overarching hypothesis in the present thesis work was that the combination of focused ultrasound and injectable microbubbles to disrupt the blood-brain barrier, could improve upon targeted brain tumour therapies with pre-existing human experience, in order to facilitate the translation to clinical adoption. We first examined the combination of focused ultrasound with boron neutron capture therapy (BNCT) in a rodent model of glioblastoma. In order to determine the benefits of ultrasound on BNCT, we developed a technique using imaging mass spectrometry to quantify the concentration and distribution of boron in tumour cells, both within the main tumour and along the infiltrating border. The infiltrating cells are clinically the most important target of the therapy, while the improved homogeneity that we postulated would occur has been blamed for failures in early human trials. Survival studies were not possible due to the difficulty in accessing a thermal neutron source for animal research. As a result, we next focused our attention on an even more targeted therapy in a rodent breast cancer model, given that breast cancer affects up to 1 in 8 women. We hypothesized that we could deliver targeted immune cells to the brain using focused ultrasound and microbubbles in a rodent model of HER2-amplified metastasis. During this phase of the thesis, we used both immunohistochemistry as well as iron-
sensitive MRI to detect the cells in the brain, and subsequently investigated various delivery strategies in order to identify one which resulted in a survival benefit.

1.2 The brain

1.2.1 Structure and function

The brain is the most complex human organ. While it represents only about 2% of our body weight, it consumes 20-25% of our metabolic demands (Mink et al., 1981). The brain is responsible for centralized control of the body, allowing the integration of sensory information and the subsequent control of complex responses and behaviours, as well as forming the physical basis for the mind. Structurally the brain has three major divisions: the forebrain, midbrain and hindbrain (Kiernan 2005). The midbrain and hindbrain collectively form the brainstem and cerebellum. The brainstem plays an integral role in bi-directional signal conduction between the brain and the body. It also houses the nuclei of most of the cranial nerves (III-XII, but plays a role in all “special senses” but smell), supplying most of the innervation to the head as well as the body’s organs. As a result, the brainstem is also critical in respiratory control, the cardiovascular system, pain, alertness and consciousness. The cerebellum is primarily responsible for coordination, motor learning, posture and equilibrium, but may also play roles in language and attention. The largest part of the brain, that which most differentiates mammals from other animals, is the forebrain. The forebrain consists on the surface of multiple folds of neural tissue (neocortex, grey matter, containing the cell bodies of neurons), giving rise to the characteristic appearance of the human brain. The forebrain has been divided into two hemispheres, each with four lobes (frontal, parietal, temporal and occipital). Below its convoluted surface are numerous white matter (neuronal axons) tracts, and other crucial structures such as the basal ganglia (voluntary motor control), thalamus (“gateway” to the brain), hypothalamus (hormonal control of the body, homeostasis) and hippocampus (memory). Also associated with the forebrain are three cerebrospinal fluid (CSF)-filled cavities called ventricles. There is a smaller fourth ventricle associated with the brainstem and cerebellum. The forebrain gives rise to the higher functions including language and thought.
Figure 1.1: Mid-sagittal drawing of the human brain. The forebrain makes up most of the brain and consists of the gyrated neocortex, its associated white matter tracts, and a number of deeper structures including the thalamus, hypothalamus, and basal ganglia. The midbrain sits at the rostral portion of the brainstem, the remainder of which forms the hindbrain and includes the pons, medulla and cerebellum. Image from Gray’s Anatomy of the Human Body available at http://www.bartleby.com/107

1.2.2 The blood-brain barrier and factors influencing its permeability

Because the brain is so critical to the function of the organism as a whole, it has developed a specialized vasculature to maintain a milieu optimal for neuronal function. In general, blood vessels are broadly classified as arteries, veins or capillaries. Capillaries are the smaller vessels responsible for the bidirectional chemical exchange between blood and tissues. Vessels typically consist of three layers: an outer supportive layer (adventitia), a middle layer (media), containing circularly oriented elastic fibres as well as smooth muscle (particularly in arteries), and an inner (intima), consisting of a single layer of endothelial cells with a basement membrane and small
amount of connective tissue (an internal elastic lamina is also present in arteries, but is very thin in veins). In some organs the endothelial cells are loosely connected or even fenestrated to facilitate the passage of substances into or out of the blood. In stark contrast, endothelial cells in the brain are connected by specialized protein complexes called tight junctions, and possess specialized transport systems that tightly regulate traffic into and out of the brain.

**Figure 1.2:** Schematic representation of the structural elements of the BBB. Endothelial cells are interconnected by tight junctions. Pericytes play a regulatory role in endothelial cell function. Astrocyte foot processes play an integral role in homeostasis in the brain. The astrocyte foot processes encircle the capillaries, anchored to the basement membrane.
Cerebral endothelial tight junctions force most molecules to take a transcellular route; only soluble gas and small lipophilic molecules diffuse freely across the endothelial cell membrane (Abbott et al., 2006). For small hydrophilic molecules, the luminal and abluminal membranes have specific transport systems that facilitate the uptake of required nutrients, while excluding or effluxing undesirable compounds (Begley and Brightman 2003). There are also intra-(monoamine oxidase, cytochrome P450) and extra-cellular (nucleotidases, peptidases) enzymes capable of inactivating or metabolizing signaling or toxic compounds (El-Bacha and Minn 1999). Large hydrophilic molecules typically do not enter the CNS unless there is a specific mechanism for endocytosis/transcytosis; however, this occurs less frequently in the CNS than elsewhere in the body (Abbot et al., 2006). This combination of structural and functional barriers is referred to as the BBB. The BBB does not consist solely of endothelial cells but also involves pericytes, known to contribute to the regulation of endothelial cell function (Kim et al. 2006); astrocytes, whose foot processes encircle the capillaries; and the intervening basement membrane. The aquaporin water channel AQP4 and the Kir4.1 potassium channel are found on perivascular astrocyte foot processes, and anchored to the basal lamina via agrin (Abbot et al., 2006). Agrin accumulates in the cerebral microvasculature during BBB tightening, while AQP4 and Kir4.1 are involved in water and ion homeostasis in the brain (Simard and Nedergaard, 2004), suggesting important roles for the basal lamina and astrocytes at the BBB, and their association with endothelial cells.

The tight junctional protein complexes in the brain are so resistive to para-cellular traffic that they even impede small ions such as Na\(^+\) and Cl\(^-\), resulting in up to a 500-fold increase in the transendothelial electrical resistance compared with that in peripheral endothelium (Butt et al., 1990). Tight junctions are composed of transmembrane proteins, with extracellular domains linked to the corresponding domain on adjacent endothelial cells, and a series of cytoplasmic adaptor and signaling proteins that anchor the transmembrane protein to the actin cytoskeleton and mediate control of this interaction. Adherens junctions are closely associated with tight junctions in the brain, providing a structural link between cells, and are located more basely at the intercellular junction. The two main transmembrane proteins are occludin and claudin. Ocludin is thought to be responsible for regulation of tight junctions (Yu et al., 2005), while claudin 3, 5 and 12 contribute to the high endothelial impedance (Abbot et al., 2006). The junctional adhesion molecules (JAM-1, JAM-2, JAM-3) are glycoproteins of the
immunoglobulin superfamily that appear to serve multiple functions, including permeability of the BBB. Their extracellular domains are involved in leukocyte-endothelial cell interactions,

**Figure 1.3:** Transport mechanisms across the cerebral endothelium. A, the passage of small hydrophilic molecules is greatly restricted by tight junctions. B, small lipophilic drugs can diffuse across the cell membrane. C, specific transport proteins allow glucose, amino acids, nucleosides and other necessary compounds to reach the brain. D, proteins such as insulin and transferrin reach the brain via receptor-mediated endocytosis and transcytosis. E, some plasma proteins such as albumin can be delivered via absorptive-mediated endocytosis, but this is not a major pathway in the brain.

while their cytoplasmic domains associate with many other tight junctional proteins, including those involved in cell polarity (Johnson- Léger et al., 2002; Chavakis et al., 2004; Naik et al., 2003). The cytoplasm of brain endothelial cells contains large plaques of proteins with which the transmembrane proteins interact. Adaptor proteins with multiple domains for protein-protein interaction, such as ZO-1, help organize the regulatory and signaling molecules, which include regulators of transcription such as ZO-1-associated nucleic acid-binding protein (ZONAB) (Table 1, Abbott et al., 2006). Both JAM’s and occludin are known to directly associate with ZO-1. Finally, there are proteins that serve to anchor the entire complex to the actin cytoskeleton.
Table 1-1: Cytoplasmic tight junction-associated proteins

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<tr>
<th>Adaptor</th>
<th>Regulatory and Signalling</th>
<th>Anchor</th>
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<tbody>
<tr>
<td>ZO-1, ZO-2, ZO-3</td>
<td>RGS5</td>
<td>JACOP</td>
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The BBB is not a single structure, and manipulation of any single component can affect the function as a whole. A number of soluble factors exist which can alter the permeability of the BBB. The release of many of these factors is triggered or exaggerated during certain disease or pathological states. IL-1, IL-6 and TNF-α act on endothelial cells to cause opening of the BBB; this is in part thought to be due to the recruitment and extravasation of leukocytes (Veldhuis et al., 2003; Persidsky et al., 2006). Elevated levels of interleukins are seen in trauma, infection, and inflammatory conditions including HIV, dementia, Alzheimer’s disease and multiple sclerosis. In some experimental models interferon-β can counteract this BBB-opening (Veldhuis et al., 2003). Astrocyte dysfunction can significantly impact the integrity of the BBB and lead to alteration of the tight junction. When astrocytes are lost from the BBB, the permeability increases within hours and there is re-arrangement of occludin, claudin 5 and ZO-1 (Willis et al., 2004). In tumours of astrocytes, a reduction in claudins 1,3,5 and occludin is seen as well as redistribution of AQP4 and Kir4.1 (Liebner et al., 2000; Papadopoulos et al., 2001). While the BBB is impaired in malignant astrocytomas, the dysfunction is heterogeneous and thus results in non-uniform uptake of systemically administered therapeutic agents (Yamada et al., 1982). Tight junction protein expression can be more directly affected by the transcription factor NF-κβ, which is known to alter claudin 3 expression (Brown et al., 2003). Bradykinin can increase intracellular calcium concentrations and lead to opening of tight junctions, as well as activating NF-
κβ and triggering IL-1β release (Schwaninger et al., 1999). Damage or alteration of the basal lamina occurs in brain tumours (downregulation of agrin), stroke (proteolysis), Alzheimer’s dementia (agrin and β-amyloid), and multiple sclerosis (downregulation of laminin) and also contributes to BBB dysfunction. Finally, the permeability of the BBB can be modulated on a short time-scale, and in focal regions, by the neurovascular unit through the release of compounds such as histamine, bradykinin and glutamate (Abbott et al., 2006). In health the BBB protects the brain and maintains tight homeostasis, but in disease it can sometimes greatly hinder therapeutic intervention.

**Table 1-2:** Soluble factors affecting the permeability and function of the BBB

<table>
<thead>
<tr>
<th>Down-Regulation/Opening</th>
<th>Up-Regulating/Tightening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin, histamine, serotonin, glutamate</td>
<td>Steroids</td>
</tr>
<tr>
<td>ATP, ADP, AMP</td>
<td>cAMP</td>
</tr>
<tr>
<td>Adenosine, platelet-activating factor</td>
<td>Adrenomedullin and noradrenergic agents</td>
</tr>
<tr>
<td>Phospholipase A-2, arachidonic acid, prostaglandins, leukotrienes</td>
<td></td>
</tr>
<tr>
<td>IL-1α, IL-1β, IL-6</td>
<td></td>
</tr>
<tr>
<td>TNF-α, VEGF</td>
<td></td>
</tr>
<tr>
<td>Free radicals, nitric oxide</td>
<td></td>
</tr>
</tbody>
</table>

### 1.3 Cancer in the brain

#### 1.3.1 Primary

Malignant brain tumours portend a terrible prognosis regardless of whether they are of primary or metastatic origin (Nussbaum et al., 1996; Hall et al., 2000; Ohgaki et al., 2004; Stupp et al., 2005; Miralbell et al., 1999; Patchell, 2003). Glioblastoma is the most aggressive glial tumour, accounting for more than half of gliomas and 17% of all primary brain and CNS tumours, with an annual incidence of 3-5 cases per 100,000 (Dolecek et al., 2012). While not the most common
human malignancy, it deserves attention on the basis of its universally poor prognosis. Glioblastoma is a tumour of glial cells, a group of non-neuronal cell types with supportive roles in the brain. The most abundant glial cell in the brain is the astrocyte, which is intimately related to the BBB (Section 1.2.2). Glioblastoma is classified as a World Health Organization (WHO) grade IV astrocytoma, and its hallmark features include hypercellularity, nuclear atypia, mitoses, pseudopallisading necrosis and microvascular proliferation (Louis et al., 2007). It occurs most frequently in the subcortical white matter of the frontal lobe (40%), followed by temporal (29%), parietal (14%), and occipital (3%), with 14% involving deeper structures (Larjavaara et al., 2007). The appearance on contrast-enhanced magnetic resonance imaging (MRI) is typically that of a single, stellate, ring-enhancing lesion centered in the white matter, with a necrotic core. T2-weighted MRI often shows significant peritumoural vasogenic edema. It is known that malignant cells reside in the peri-tumoural region (Halperin et al., 1989), and because of its diffusely infiltrating nature glioblastoma may be regarded as a whole brain disease (Halperin et al., 1988). Without treatment, the median survival is less than 3 months.

1.3.2 Secondary

The burden of metastatic disease is not precisely known, but it is estimated to be ten-fold that of primary tumours, occurring in 20-40% of cancer patients (Patchell 2003). Metastatic tumours are most commonly due to lung, breast and melanoma carcinomas (Patchell 2003). In a large prospective study, the median duration from presentation with a single brain metastasis to death was 5 months while only 3 months for patients with multiple lesions (Nussbaum et al., 1996). A similar study found the two year survival to be only 8.1% (Hall et al., 2000). Metastatic tumours can occur anywhere in the CNS, and are classically described to originate at the grey-white interface in the brain. While metastatic disease is heterogeneous, metastatic lesions often have a more defined margin, but with an advancing front consisting of clusters or islands of cells at the brain-tumour interface when examined microscopically.

1.3.3 Existing treatments for malignant brain tumours

There is no cure for primary malignant brain tumours, and until recently the treatment consisted of surgical resection followed by radiation therapy, yielding a median survival between 9 and 12 months, with 17-34% and 3-5% of patient alive at 1 and 2 years, respectively (Miralbell et al.,
While there are no randomized trials examining no surgery versus surgery in glioblastoma, there is evidence to suggest a modest benefit in survival with gross total resection (Sanai and Berger, 2008). The introduction of the oral alkylating agent temozolomide resulted in the most significant recent improvement in survival; a large randomized trial by the European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups and the National Cancer Institute of Canada Clinical Trials Group (EORTC and NCIC) showed that the addition of temozolomide to surgical resection and radiation resulted in an increase in median survival to 14.6 months and two year survival to 27.2% (Stupp et al., 2005).

The treatment of metastatic brain tumours can be more variable due to the frequent finding of multiple lesions, leptomeningeal involvement, or the burden of extra-cranial disease. When the extra-cranial disease is stable and there are fewer than three lesions, surgery improves survival when compared with adjuvant therapy alone (Patchell 2003). The particular adjuvant therapies depend on the chemo- and radio-sensitivities of the primary malignancy.

Compared with other sites in the body, the brain requires more selective and precise therapies owing to difficult surgical access and the impossibility of wide resection margins. Surgery is a mainstay of brain tumour therapy; a complete resection is desired but this goal is frequently unattainable due to the proximity to, or involvement of, eloquent structures. As a result, maximal safe resection is undertaken with the intent of debulking, cyto-reduction, and tissue diagnosis. Even if gross total resection is achieved, microscopic residual remains. To address this, following surgery a combination of chemo- and radio-therapy is typically prescribed. Despite this multi-modality approach, life expectancy following the diagnosis of a malignant brain tumour is significantly reduced (Nussbaum et al., 1996; Hall et al., 2000; Stupp et al., 2005). While surgery and radiotherapy have seen a number of improvements over the past decades, arguably there is little further that these two therapies can contribute to significantly increasing survival. Surgery is a local therapy directed towards, in glioblastoma, a whole brain, and in metastasis, a whole body disease. On the other hand, localized radiotherapy has the same limitations as surgery, while the irradiation of larger brain regions causes significant long-term
collateral injury. The optimization of chemical and biological therapies is thus where the greatest potential lies.

1.4 Circumventing the blood-brain barrier in cancer therapies

A number of pre-clinical and clinical studies have investigated methods of circumventing the BBB to augment the accumulation of therapeutic molecules in the brain. The strategies can be broadly divided into those which act to bypass the BBB and cerebrovasculature altogether, and those that aim to increase its permeability.

1.4.1 Bypassing the BBB

Local interstitial therapies have been developed to avoid the barriers of the cerebrovasculature. These require application at the time of surgery, or a procedure specifically to gain cranial access. The most straightforward interstitial therapy involves depositing the therapeutic agent directly onto the raw surface of the resection cavity and allowing diffusion to permeate the agent through the peri-tumoural parenchyma. Gliadel® (Kunwar et al., 2010) has been used as a strategy with modest success, using drug-eluding wafers or polymers placed into the resection cavity at the time of the tumour resection. Another technique, convection enhanced delivery (CED), aims to improve upon the simple action of diffusion by placing a catheter into the resection cavity or adjacent tissue to infuse a therapeutic agent under a gentle and constant pressure gradient (Sampson et al., 2006). The most familiar local therapy for neurosurgeons is the placement of an Ommaya reservoir for cystic tumours, lymphoma and leptomeningeal metastasis (Elder and Chen, 2006; Sandberg et al., 2000). This involves the placement of a catheter into a fluid space, either a lateral ventricle or cyst, with a connecting silicone reservoir under the scalp into which a therapeutic agent can be injected. This can be repeatedly accessed by needle injection, allowing for multiple treatments.
1.4.2 Disrupting the BBB

BBBD can be accomplished with the intravascular administration of hyper-osmolar agents and pharmacological agents. These agents are typically delivered via intracarotid (IC) injection to maximize the concentration in the desired arterial distribution and minimize the effects elsewhere. By far the most common osmotic agent is hypertonic mannitol, which is thought to temporarily destabilize tight junctions both by shrinking endothelial cells and altering intracellular calcium concentrations leading to cytoskeletal changes (Dobrogowska and Vorbrodt, 2004) (Kroll and Neuwelt, 1998). The effects of mannitol on the BBB are immediate and brief (Wang et al., 2007). Labradimil (Cereport), a bradykinin agonist whose mechanism is described in 1.2.2, has also been used to cause BBBD (Dean et al., 1999; Emerich et al., 2001). In theory, any of the agents listed in Table 1-2 could be administered to disrupt the BBB, but at the cost of uncertain and possibly life-threatening systemic complications.

1.4.3 Shortcomings of current therapies

A number of the techniques to circumvent the BBB have enjoyed modest success in small clinical trials, often in conjunction with existing chemotherapeutic agents (Kunwar et al., 2010; Sampson et al., 2007). While this helps affirm that removing the BBB as a barrier could offer significant therapeutic gains, there remain a number of limitations with the methods discussed. While localized delivery places chemotherapeutic agents at the site of the pathology, it is often limited to the time of surgery or requires an invasive procedure if used repeatedly or in a delayed treatment schedule. Furthermore, the diffusion or convection of the therapeutic agent may be quite heterogeneous or anisotropic, as there is little control over the agent once it is deployed or released in the brain. The dose of chemotherapeutic agent required locally to obtain a sufficient gradient for diffusion in the peripheries of the treatment volume may also be neurotoxic locally. Indwelling catheter-based therapies have a risk of infection, blockage, accidental removal or migration. Intracarotid osmotic BBBD in combination with chemotherapy has had some promise in early clinical trials but requires cannulation of the internal carotid artery via endovascular techniques, which is not without some risk. Furthermore, in one pre-clinical study up to 10% of animals undergoing BBBD with IC mannitol developed fatal cerebral edema (Barth et al., 1997).
One of the more recent methods developed for reversible BBBD is the combination of focused ultrasound with microbubble ultrasound contrast agents. This technique has been shown to facilitate the entry of a number of therapeutic agents into the brain, focally and without significant adverse effects, and is both reversible and repeatable (Kinoshita et al., 2006; Treat et al., 2007; McDannold et al., 2012). The occurrence of massive cerebral edema with focused ultrasound is far less likely, as it can be restricted to the tumour and surrounding brain, rather than encompass an entire hemisphere or arterial distribution. Compared with IA administration and interstitial therapies, the main advantages of ultrasound as an alternative for BBBD are the non-invasive nature, and a more controlled, uniform and focal disruption of the cerebrovasculature. The latter may serve not only to reduce the risk of massive cerebral edema, but also to spare healthy tissues and reduce neurological side effects.

### 1.5 Blood-brain barrier disruption with ultrasound and microbubbles

#### 1.5.1 Introduction and history

Observations of blood-brain barrier disruption (BBBD) as a consequence of ultrasound in the brain began as early as the 1950’s. Bakay et al. (1956), in response to a paper by Barnard et al. (1955) that reported vessels in the brain exposed to ultrasound appeared structurally unaltered, described that the vessels were functionally very different. Using trypan blue and radioactive phosphate they found that the permeability of the BBB was significantly increased in the periphery of lesions created with high-intensity focused ultrasound (Bakay et al., 1956). Ballantine et al. (1960), using a high-power unfocused ultrasound beam, were the first to achieve BBBD without the formation of an associated discrete lesion. Although there were regions of hemorrhage within the treated volumes, the authors postulated that ultrasound parameters could be tuned to produce BBBD without any permanent tissue injury (Ballantine et al., 1960). Vykhodtseva (1981) also noticed BBBD without the formation of a permanent lesion (albeit unintentionally), which was visualized with trypan blue and attributed to cavitation. Later, regions of BBBD were generated in the rabbit brain without any evidence of permanent damage on histological analysis, but the set of parameters required to do so was found to be quite variable (Vykhodtseva et al., 1995). Further parameter studies by Hynynen, Vykhodtseva, and
McDannold in the late 1990’s to establish consistent BBBD with ultrasound were largely unsuccessful (Vykhodtseva et al., 2008).

The modification that solidified ultrasound as a safe, reliable and practical means of BBBD was the introduction of, and combination with, commercially available ultrasound contrast agents, first reported by Hynynen et al. (2001). Furthermore, the authors were able to semi-quantitatively assess the degree of BBBD using gadolinium contrast and MRI. While the underlying mechanism of BBBD was still cavitation, the high pressures needed to incite bubble formation in tissue (Vykhodtseva et al., 1995; Section 1.5.3.4) were no longer required as the pre-formed microbubbles were introduced directly into the circulation. Microbubbles simultaneously decreased the burden of manufacturing high-powered ultrasound systems, vastly reduced the risk of complications related to skull heating, and restricted the effects of cavitation to the vasculature (Vykhodtseva et al., 2008). In contrast to historical experiments where BBBD tended to be associated with tissue injury, even when specifically attempting to avoid it, the modern technique has been shown to generate very few permanent effects, even with long-term follow-up in rodents and non-human primates (McDannold et al., 2005; McDannold et al., 2012).

In the same vein, while studies using ultrasound alone for BBBD have found the set of reliable parameters elusive, those combining ultrasound and microbubbles have found a wide range of successful parameters. Ultrasound frequencies selected are generally those in the 0.1-1 MHz range, striking a balance between transmission through the human skull and focal spot size. As in the original study by Hynynen et al. (2001), sonications are typically pulsed with a low duty cycle (0.5-5 Hz) and total duration on the order of seconds to minutes. Within this clinically applicable frequency range, the BBBD threshold (pressure amplitude) has been found to increase with frequency and decrease with pulse duration (McDannold et al., 2008a). The magnitude of BBBD increases with both pulse duration and acoustic power (McDannold et al., 2008b). While McDannold et al (2008b) reported that the magnitude of BBBD was not significantly affected by PRF or ultrasound contrast agent dose, as measured by contrast enhanced MRI, the same group found the concentration of the chemotherapeutic agent doxorubicin increased linearly with microbubble dose (Treat et al., 2007). They also achieved higher drug concentrations with prolonged sonication times (up to 40 min) (Treat et al., 2007).
Patrick et al. (1990) were the first to propose that, using ultrasound to disrupt the BBB, the delivery of anti-neoplastic agents into the brain could be increased. Since that time, the delivery of a myriad of therapeutic agents has been enabled using variations of the method first described by Hynynen et al (2001), ranging from small therapeutic molecules such as the alkylating agent doxorubicin (580 Da) to large molecules such as the HER2 antibody trastuzumab (145 kDa), and more recently stem cells (Treat et al., 2007; Kinoshita et al., 2006, Burgess et al., 2011). The development of highly focused ultrasound beams, CT and MRI have allowed ultrasound to be applied transcranially with millimetre accuracy (Jeanmonod et al., 2012). The potential therapeutic benefits of using ultrasound to disrupt the blood-brain barrier have been recognized and there are now more than 110 in vivo studies indexed on MEDLINE® investigating ultrasound-mediated BBBD for a variety of clinical problems. This increased interest in BBBD with ultrasound, and its practical and safe application, has in large part been propelled by the enormous technological advances over the 57 years since its first observation.

1.5.2 Ultrasound as a compressional wave

Ultrasound is a form of mechanical energy that propagates through a medium in the form of a pressure wave. The most familiar pressure waves are those discernable as sound by the human ear; ultrasound waves by definition occur with a frequency above the audible range (> 20 kHz). A material with elasticity (compressibility $\kappa$) will have a restoring force when distorted from its equilibrium state that will tend to overcorrect due the inertia of the material (mass density $\rho$); therefore materials with these properties allow propagating phenomena. In the case of ultrasound, when a medium is compressed or expanded due to a pressure wave, the molecules within the medium oscillate back and forth due to the distorting and restoring forces, resulting in adjacent regions of rarefaction and compression (Figure 1.4). Typically this results in particle oscillations along the direction of propagation, or longitudinal waves; however, in some instances shear waves occur, where the motion is orthogonal to the direction of propagation. For the most part shear waves are rapidly attenuated in biological soft tissues; they propagate more readily in solid materials, such as bone. As a result, most medical ultrasound applications assume that tissue behaves as a fluid and consider only longitudinal waves.
Figure 1.4: Schematic diagram depicting a sinusoidally varying longitudinal ultrasound wave and its effects on the propagating medium. An observer would perceive the pressure amplitude (top graph, y axis) varying in the same manner both at a fixed point in time while traversing the medium, or at a fixed location in real time. The lower graph illustrates the behavior of the particles in the medium as the longitudinal pressure wave passes. The trough or low pressure portion of the wave causes rarefaction, while the peak or high pressure phase causes compression.

Ultrasound waves carry energy just as electromagnetic waves do. The intensity of an ultrasound wave is the rate of energy transmission per unit area, orthogonal to the direction of wave propagation, in Watts·m$^{-2}$. The time-averaged intensity is proportional to the square of the pressure amplitude. The spatial and temporal pattern of ultrasound waves, or the ultrasound field, generated by a given source, at an arbitrary point in space, is of importance in a number of practical applications; as a result, a number of theoretical models exist. Ultrasound waves are described by solutions to an appropriate wave equation, with suitable boundary conditions and parameters describing the propagating medium. An example of a one-dimensional linear wave
equation is shown in Equation 1.1, solutions of which are plane harmonic waves with phase speed given by Equation 1.2.

$$\frac{\partial^2 p}{\partial x^2} = \rho_o \kappa \frac{\partial^2 p}{\partial t^2} \quad \text{(Equation 1.1)}$$

$$c = \pm \sqrt{\frac{1}{\rho_o \kappa}} \quad \text{(Equation 1.2)}$$

More generally, the solution for longitudinal waves propagating in an inviscid medium is given by the Rayleigh-Sommerfeld diffraction equations (O’Neal, 1949). More complicated source geometries can often be more easily handled using the impulse response method, based on linear systems theory (Cobbold, 2006). The impulse response method breaks the source up into small elements, and combines the contribution from each of these elements that arrives at a given point in space with the same time delay. Approximate methods also exist including the Fresnel approximation for the near-field and the Fraunhofer approximation for the far-field. A detailed description of the solution to these problems is beyond the scope of this thesis, but the reader is referred to the textbook by Wells or Cobbold for more details (Wells, 1977; Cobbold 2006).

For purposes of illustration, consider two common source geometries: the piston and the spherically curved concave source. First consider the ultrasound field generated by a uniformly vibrating planar disk of radius A, or piston, in simple harmonic motion with wavelength $\lambda$. For $A \leq \lambda$, a diverging hemispherical wave is generated. As the radius A gets large compared to the wavelength, the acoustic field becomes a complex series of intensity minima and maxima in the near-field. The last maximum occurs a distance $A^2/\lambda$ from the piston face, and past this point the intensity of the acoustic field decays smoothly as the inverse square of the distance. The width of the beam at the last maximum can be described by the full width at half maximum (FWHM), which is approximately equal to $0.7 \cdot A$, significantly narrower than the diameter of the piston. A spherically curved source can be used to generate a more focused ultrasound field than that which is achieved with a piston of radius $A \gg \lambda$. The near field is similar in form to that generated by a piston, with successive intensity maxima and minima, although the intensities of the maxima tend to increase toward the geometric focus; past the focus the beam diverges. The focus created by a concave spherical source has an ellipsoid shape, with the major axis aligned in the axial direction. The intensity maximum actually occurs slightly closer to the source than the
geometric focus (O’Neal, 1949; Cobbold 2006). Its theoretical shape can be characterized by the diameter, 2A, and radius of curvature (also equal to the geometric focal length), R, of the source, as well as the wavelength, \( \lambda \). A common parameter used to describe a spherical source is the f-number, given by \( R/2A \). We can then express the lateral extent of the focus as \( 1.22 \cdot \lambda \cdot (f\text{-number}) \), and the axial FWHM of the beam (depth of field) as \( 7.2 \cdot \lambda \cdot (f\text{-number})^2 \) (Cobbold 2006). By varying the f-number of a spherically focused source it is possible to target virtually any location in the human body; however, the further a source is reduced from a complete hemisphere, the longer the focus becomes in the axial direction, approaching that of a planar disk.

As a final comparison, consider a hemispherical and a circular planar source (piston) of equal radii, oscillating at the same frequency and with equal source pressures measured at the face. It can be shown that the maximum pressure achieved at the focal spot of the hemispherical source is \( 2\pi \cdot A/\lambda \) times that at the last axial maximum of the planar source (Cobbold, 2006). As we have chosen \( A/\lambda \) to be large, this quantity is always much greater than 1, so that the gain at the focus is greatly enhanced with spherical focusing compared with a piston transducer (Hynynen et al., 1981). Practically this is highly advantageous for intracranial applications, where the focusing gain can be used to overcome attenuation losses due to the skull, and achieve a small focal spot, with a resulting therapeutic effect at depth without affecting the surrounding and intervening tissues.

### 1.5.3 Ultrasound interactions with tissue

#### 1.5.3.1 Reflections

The acoustic impedance, \( z \), of a material is the ratio of acoustic pressure to the associated particle speed (Kinsler et al., 2000). It can be shown that for plane waves this is equal to the product of the density and the speed of sound in the medium. The acoustic impedances at 20°C for air, distilled water and bone are 415 Pa\( \cdot \)s\( \cdot \)m\(^{-1} \), \( 1.48 \times 10^6 \) Pa\( \cdot \)s\( \cdot \)m\(^{-1} \) and \( 5.3 \times 10^6 \) Pa\( \cdot \)s\( \cdot \)m\(^{-1} \), respectively (Hatakeyama et al., 2000). Given that the bulk of soft tissue is water, it stands to reason that the acoustic impedances of soft tissues in the body differ little from that of water. Adipose tissue does have a slightly lower acoustic impedance due to both its lower density and speed of sound. The major exceptions are the lungs, which have a much lower acoustic impedance due to the vast number of air spaces \( (0.1 \times 10^6 \) Pa\( \cdot \)s\( \cdot \)m\(^{-1} \) inflated, \( 1.4 \times 10^6 \) deflated Pa\( \cdot \)s\( \cdot \)m\(^{-1} \) \) (Oelze et al., 2008),
and bone, with a higher density and speed of sound and thus a higher impedance \((4.8 \times 10^6 \text{ Pa} \cdot \text{s} \cdot \text{m}^{-1})\) (Laugier and Haïat, 2011).

For a normally incident ultrasound wave to an interface between materials of acoustic impedance \(z_1\) and \(z_2\), it can be shown that the intensity reflection and transmission coefficients, \(R\) and \(T\), are

\[
R = \frac{(z_2 - z_1)^2}{(z_2 + z_1)^2} \quad \text{(Equation 1.3)}
\]

\[
T = \frac{4z_2 z_1}{(z_2 + z_1)^2} \quad \text{(Equation 1.4)}
\]

where \(R+T=1\). It can be seen then that if \(z_1=z_2\), \(R=0\) and all of the wave’s energy is transmitted; this is essentially the case in soft tissues, where the acoustic impedances are similar and losses from reflections are small. At tissue-air interfaces, however, virtually the entire incident wave is reflected back into the tissue. At soft tissue-bone interfaces, about 60-70\% of normally incident waves are transmitted, depending on the frequency. For obliquely incident waves at a soft tissue-bone interface, the transmitted wave decreases up to the critical angle of 25-30°, at which point all of the wave is reflected.

The above is true for longitudinal waves beyond Snell’s critical angle, but in fact at tissue bone interfaces there is mode conversion of the incident longitudinal wave to a shear wave. The speed of a longitudinal wave in tissue is similar to that of a shear wave in bone, and as a result there is better acoustic impedance matching between these modes (Clement et al., 2004). This is of practical significance primarily for transcranial imaging; although there tends to be less distortion with shear wave propagation through the skull, there is more attenuation and heating of the skull (White et al., 2006), which could lead to complications in high power therapeutic applications.

1.5.3.2 Attenuation: absorption and scattering

The attenuation of ultrasound waves in tissue is the result of dissipation of energy due to both absorption and scattering. For a given frequency, attenuation dictates the depth of penetration. The contribution of scattering to the total attenuation is relatively small; the scattered energy is eventually also absorbed, but results in acoustic energy being absorbed over a larger region than
that which would have otherwise been predicted (Mahoney et al., 2001). Absorption of ultrasound energy can occur as a result of intrinsic properties of the medium or its boundaries; eventually all acoustic energy is converted to thermal energy. Losses take the form of viscous losses, heat conduction losses, and losses due to internal molecular processes. Viscous losses occur when there is relative motion between parts of the medium, as occurs with the compression and rarefaction associated with the propagating pressure wave. Losses due to heat conduction occur via the transfer of thermal energy between higher temperature compressive regions to lower temperature regions of expansion. Losses at the molecular level that occur may be a result of the conversion of kinetic energy to potential energy, absorption into rotational or vibrational energy, and even the association and dissociation of different ionic species and complexes in solution (Kinsler et al., 2000). In effect, with every cycle of the ultrasound wave, energy is exchanged between the wave and the medium, but tends to return to the wave out of phase due to the finite time required for this transfer to occur, resulting in absorptive losses (Wells, 1977; Mortimer, 1982).

Experimentally it has been found that the absorption and the total attenuation are both approximately linearly proportional to the frequency of the propagating wave. The attenuation coefficient $\alpha$ for a given tissue is frequency-dependent and can be modeled by Equation 1.5, where $a_0$ is the attenuation in dB•MHz$^{-1}$•cm$^{-1}$, $f$ is the frequency in MHz, and $m$ is an experimentally measured parameter, found to be between 1 and 1.2 (Goss et al., 1979). With the exception of a few tissue types, the attenuation coefficients for soft tissue are relatively well preserved across mammalian species (Goss et al., 1979).

Attenuation can be greatly enhanced in fluids and tissues by the presence of gas bubbles, which expand and collapse under the influence of the ultrasound wave. The scattered ultrasound waves from an interaction with bubbles have a broadened frequency content, and correspondingly some energy is transferred into higher frequency components that are more readily absorbed (Equation 1.5). Furthermore, the induced temperature variations of the oscillating bubbles cause significant energy losses. For an ultrasound wave propagating in tissue with bubbles present, multiple scattering and absorption events result in more concentrated dissipation and absorption of the
ultrasound energy. High peak rarefaction pressures can actually cause the formation of bubbles in soft tissue, and this interaction between a gas bubble and a sound wave is called cavitation, a phenomenon which is at the heart of BBBD.

Nonlinear effects must also be taken into account when considering attenuation in soft tissue. As shown in Equation 1.2, the speed of sound is dependent on the density of the medium in which it is propagating; however, this assumes a uniform density. In reality, because the ultrasound wave causes adjacent regions of compression and rarefaction, the wave travels faster in the regions of compression compared with regions of rarefaction, resulting in distortion of the wave. This process transfers energy into higher harmonics of the fundamental frequency, which from Equation 1.5 will be more highly attenuated (Carstensen et al., 1981, 1982; Duck 2002). Nonlinear effects increase with pressure amplitude, distance travelled, viscosity, density and frequency (Watkin et al., 1996). As a result, for focused acoustic fields the distortion is greatest just in front of the geometric focal spot, where the pressure amplitude is the greatest, resulting in increased absorption and heating of the tissue (Watkin et al., 1996; Goss and Fry 1981; Swindell 1985; Hynynen 1987, 1991; Connor and Hynynen 2002). As the pressure amplitude increases, this region of enhanced energy deposition has been found to move increasingly close to the transducer face; however, the cavitation threshold in tissue limits the extent to which nonlinear effects can be exploited.

1.5.3.3 Thermal effects

It has long been know that focused ultrasound can be used to create thermal lesions in tissue (Lynn 1942). As described in Section 1.5.3.2, the attenuation of ultrasound as it propagates through tissue results in the conversion of mechanical energy into thermal energy. If this deposition of energy is concentrated and sustained for a suitable duration, an elevation of tissue temperature can be achieved sufficient to yield a biological effect. Thermal tissue injury has been extensively studied and cytotoxicity tends to be observed above a threshold temperature of 41-42°C; beyond this, the fraction of surviving cells decreases exponentially with increasing exposure time and temperature. Thermal dose is often expressed as the equivalent time at 43°C to produce the same biological response. It has been shown experimentally that the relationship between the temperature at which tissue necrosis occurs and the logarithm of the duration of
exposure is linear, so that a decrease in tissue temperature of 1°C requires twice the exposure time to achieve the same effect. While this relationship holds true for all tissues, the threshold and sensitivity to thermal injury varies with tissue type, pH, hypoxia, and nutrient availability (Sapareto and Dewey 1984; Dewhirst et al., 2003).

The actual temperature reached in tissue for a given ultrasound field depends on the attenuation coefficient of the tissue, the regional blood flow (convective heat losses) and the thermal conductivity – parameters that are not easily determined in vivo. For this reason, it is not possible to exactly calculate the temperature elevation that will be produced by a given ultrasound field in living tissue; this is the impetus for real-time thermometry in modern therapeutic ultrasound systems. With detailed temperature mapping, the temperature within a target volume can be confirmed to have reached that required for cell death in virtually all cells. However, the goal of heating with ultrasound is not always to cause cell death, and thus careful tissue temperature monitoring can be used to guide a number of thermal therapies. For example, non-lethal hyperthermia of tumours may be used to increase perfusion and thus drug delivery, as well as to increase the sensitivity to subsequent radiation and chemotherapy treatments (Dewhirst et al., 2003). It can also be used to focally release drug from heat-sensitive liposomal formulations (Magin and Niesman 1984; Needham and Dewhirst 2001; McDannold et al., 2004a; Staruch et al., 2011), as well as to activate drug and gene therapies (Moonen et al., 1997; Bednarski et al., 1998). Hyperthermia can also cause BBBD, but not without the simultaneous occurrence of permanent tissue injury (McDannold et al., 2004b).

1.5.3.4 Cavitation

Cavitation is one of the best-studied non-thermal mechanisms by which ultrasound can induce biological effects. Rayleigh first published on cavitation in the early 1900’s, noting that collapsing bubbles could inflict damage to nearby objects (Rayleigh 1917). Rapid pressure variations in a liquid cause a void, or bubble, to form and subsequently collapse; given that ultrasound waves are rapidly varying pressure waves, cavitation may result. The process is similar to boiling but through an alternate thermodynamic path; rather than the vapor pressure of the liquid rising above the ambient pressure, the ambient pressure is lowered below the saturated vapor pressure and a vapor forms in the low-pressure region of the fluid. Acoustic cavitation
refers generally to the interaction between sound waves and bubbles in liquids, and can be broadly divided into non-inertial, or stable, and inertial, or transient cavitation. Non-inertial cavitation results in stable oscillation of the bubble size under the influence of the ultrasound field, with microstreaming and shear stresses in the surrounding liquid (Dalecki, 2004). Inertial cavitation takes place at high pressure amplitudes in the form of bubble formation and collapse, the latter resulting in high temperatures within the bubble, shock wave formation, the emission of light (sonoluminescence) and the formation of fluid jets and turbulent flow (Suslick and Hammerton, 1986; Ashokkumar and Grieser, 2006; Ashokkumar et al., 2007).

In order for cavitation to occur, stabilized cavitation nuclei, or microscopic gas bodies, are required. These cavitation nuclei may occur naturally within fluids of the body, and it is thought that hydrophobic impurities act to stabilize them (Flynn, 1964; Crum, 1982). Cavitation nuclei are on average much smaller than the resonant size corresponding to typical therapeutic ultrasound frequencies (Shi et al., 2000), but in the presence of a high-amplitude acoustic field, begin to oscillate and approach the resonant volume by rectified diffusion and bubble-bubble coalescence (Crum, 1984; Margulis, 1995; Lee, 2005). Rectified diffusion is the net flow of gas into a bubble over the course of a number of cycles of expansion and contraction; bubble expansion during the rarefaction phase of the pressure wave is a slower process than compression, giving more time for volatiles to accumulate in the bubble than for them to return to the surrounding fluid (Ashokkumar et al., 2007). The larger bubble surface area during expansion also allows more gas to diffuse into the bubble compared with the compression phase. Alternatively, microbubbles may be deliberately introduced intravenously into the body using preformed gas microbubble ultrasound contrast agents.

As previously noted, inertial cavitation in the absence of pre-formed bubbles requires ultrasound fields with relatively high pressure amplitudes. Experimental data suggest that the threshold intensity for inertial cavitation in tissue is roughly proportional to the frequency and decreases with increasing pulse repetition frequency and pulse length (Fry et al., 1970; Hill, 1972; Hynynen, 1999). It also tends to decrease with decreasing viscosity, and with increasing cavitation nuclei concentration (Deng et al., 1996). The Mechanical Index is an approximation of the cavitation threshold for a short pulse based on theory and experiments in an aqueous medium. It assumes all bubble diameters are present in an incompressible fluid, with isothermal
growth, adiabatic collapse, and disregards rectified diffusion (Apfel and Holland, 1991). For soft tissues, it is defined as

$$MI = \frac{P_{opt}}{\sqrt{f}} \quad \text{(Equation 1.6)}$$

where $P_{opt}$ is the derated peak rarefaction pressure in MPa, in the fluid of interest, and $f$ is the center frequency of the ultrasound source in MHz. This theoretical parameter is used to characterize diagnostic ultrasound systems to attempt to avoid cavitation related bioeffects. However, theory predicts that the cavitation threshold will be higher in tissue, a viscoelastic material, than in a fluid (Yang and Church, 2005). There is some evidence that the MI may nevertheless be used to predict the frequency dependency of the threshold for ultrasound-induced vascular permeability in the presence of microbubble contrast agents (McDannold et al., 2008b).

In vivo experiments using continuous wave sonications in canine muscle have shown that a pressure of 5-6 MPa·MHz$^{-1}$ is required for inertial cavitation to occur, however, there are large variations between tissue types and even locations within the same tissue (Hynynen, 1991). In the same study, there was a sudden increase in the tissue attenuation with the onset of inertial cavitation due to the large scattering cross-section of bubbles, and a corresponding significant increase in temperature. With the introduction of microbubble ultrasound contrast agents into the vasculature, the inertial cavitation threshold has been shown to be as low as 0.5 MPa at 1.5 MHz in the rabbit brain, with a corresponding rise in temperature at the focal spot higher than that achieved with sonications without the contrast agent (Umemura et al., 2005 McDannold et al., 2006a).

Below the inertial cavitation threshold, non-inertial cavitation results in stable expansion and compression of bubbles. The maximum expansion of bubbles in this regime is typically no greater than double the equilibrium radius; however, these oscillations lead to a number of physical effects including heating (Klotz et al., 2010), microstreaming of fluid near the bubble, and localized shear stresses (Dalecki, 2004). Once the inertial cavitation threshold is reached, bubble behavior in an ultrasound field becomes highly non-linear, with bubbles expanding then collapsing violently to a fraction of their initial size. Furthermore, the response of bubbles to increases in pressure near the collapse threshold is non-linear, so that small increases in the
pressure amplitude for a bubble undergoing stable cavitation may result in inertial cavitation. Inertial cavitation may require as little as a single microsecond ultrasound pulse under suitable conditions (Flynn, 1982). During the collapse of a bubble undergoing inertial cavitation, simulations and theoretical models predict temperatures in the thousands of Kelvin and pressures of thousands of atmospheres (Dalecki, 2004). This rapid collapse may result in a spherically diverging shock wave, or if the bubble is near a boundary, asymmetric collapse resulting in a fluid jet. Shockwave formation appears to be a prominent mechanism in the destruction of kidney stones with ultrasound (Bailey et al., 1999). In soft tissues, inertial cavitation may cause complete disintegration of the tissue with a resultant debris- or fluid-filled cavity (McDannold et al., 2006b; Lele, 1977; Vykhodtseva et al., 1994; Roberts et al., 2006), and is distinguishable from thermally induced injury. The high temperatures and pressures may also result in the formation of free radicals, which may go on to further interact or damage tissues (Verral and Sehgal, 1988).

Cavitation has a number of possible therapeutic applications, many of which have been investigated in pre-clinical studies. It increases the volume of coagulation at the focus, which could decrease the time needed for ablative treatments of larger targets (Umemura et al., 2005; Sokka et al., 2003), and reduces transmission beyond the focus, effectively shielding non-target tissues (Hynynen, 1991; Lele, 1977; Holt and Roy, 2001). Cavitation also decreases the threshold for thermal tissue damage, and decreases the power required for a given treatment (Hynynen, 1991). Cavitation has been shown to have important effects in ischemic stroke, where it accelerates intra-arterial clot disruption (Hitchcock and Holland, 2010). On a microscopic level, the shear stresses generated by cavitation are thought to be in part responsible for increasing the permeability of cell walls and blood vessels. Cavitation as it pertains to blood-brain barrier disruption will be discussed in depth in 1.5.5. Sonoporation exploits cavitation to increase transfection in vitro, for example of DNA fragments (Fechheimer et al., 1987; Miller et al., 1996; Kim et al., 2002), but has also been shown to occur in vivo (Huber et al., 2000, 2003; Casey et al., 2010). One of the main challenges of harnessing cavitation for therapeutic ultrasound, in addition to the variability of the cavitation threshold in living tissue, is that the effects are not always seen at the focal spot, and may occur along the path of the beam, particularly at tissue interfaces (Hynynen, 1991).
1.5.3.5 Acoustic radiation force

As an ultrasound wave travels through a medium and undergoes scattering and absorption, it transfers momentum to the medium and its component particles, resulting in a time-averaged force called the acoustic radiation force (Rooney and Nyborg, 1972). In the case of a plane wave incident on a perfectly absorbing object, the force, $F_{rad}$, is equal to

$$F_{rad} = \frac{P_a}{c} \text{ (Equation 1.7)}$$

where $P_a$ is the total acoustic power and $c$ is the speed of sound in the coupling medium. The radiation force is double this for a completely reflecting object. Although most therapeutic ultrasound fields are not infinite plane waves, and may be focused and susceptible to non-linear effects, the plane wave is often a sufficient approximation of the force experienced. This principle has been used for years to characterize ultrasound systems; using springs or scales, the force generated by a particular ultrasound source can be measured, and used to determine the acoustic power delivered, as well as the efficiency of the source when the electrical power is known (Jolesz and Hynynen, 2008).

The acoustic radiation force has demonstrable effects in living tissues. In a chick embryo, a standing wave field has been shown to cause red blood cells within the blood vessels to align in bands at half-wavelength intervals (Dyson et al., 1971). The radiation force of a travelling ultrasound wave has been used to slow intravascular contrast agents in murine vasculature and concentrate them around the periphery of the vessel (Dayton et al., 1999). Ultrasound pulses delivered to frogs during systole of the cardiac cycle have been shown to transiently modulate the contractility of the heart (Dalecki et al., 1993), and appear to be attributable to the radiation force applied (Dalecki et al., 1997). High-amplitude pulses have been shown to cause premature ventricular contractions (PVCs) when delivered during diastole of the cardiac cycle (Dalecki et al., 1993; MacRobbie et al., 1997). In laboratory animals, ultrasound pulses have even been used to capture and pace the heart (Dalecki et al., 1991). The pressure amplitude required to cause PVCs is reduced if ultrasound contrast agents are present within the circulation (Li et al., 2003; Zachary et al., 2002; van der Wouw et al., 2000), and in fact PVCs have been reported in humans during diagnostic ultrasound procedures (van der Wouw et al., 2000). The acoustic
radiation force from an ultrasound field has also been reported to stimulate the auditory system (Foster and Weiderhold, 1978; Tsirulnikov et al., 1988), as well as tactile receptors in the human hand (Dalecki et al., 1995).

The acoustic radiation force is also the underlying mechanism for radiation torque and acoustic streaming. Radiation torque can cause symmetric particles to rotate in an acoustic field, while it may cause asymmetric particles to assume a preferential orientation. Rotatory motion of small clusters of cells has been reported as a result of radiation torque in vivo (Martin et al., 1983). Acoustic streaming occurs when an ultrasound field causes a bulk flow of fluid in a single direction secondary to the radiation force. This is different than microstreaming, which is thought to be associated predominantly with volume oscillations generating flow around the bubbles’ surface (Elder, 1959; Nyborg, 1982; Wu and Du, 1997).

1.5.3.6 Other interactions with tissue

A number of tissue- and organ-specific effects have been reported with ultrasound exposure, the underlying mechanisms of which are generally poorly understood. Lung hemorrhage has been extensively studied, and cavitation and thermal effects have been ruled out as etiologies. It appears to occur with a similar pattern in all species investigated, regardless of size, in the intensity range of diagnostic ultrasound, and independent of frequency and pulse repetition frequency (O’Brien, 2007). In bone, low-intensity pulsed ultrasound exposures have been shown to accelerate fracture healing and new bone growth in a number of animal models (Dyson and Brookes, 1982; Duarte, 1983; Pilla et al., 1990; Lai et al., 2011; Tobita et al., 2011); given the intensities used, the mechanism is unlikely to be thermal or cavitation related.

There are a number of reports of ultrasound causing effects at the cellular level not attributable to thermal- or cavitation-related mechanisms. Some authors have suggested that ultrasound exposure can increase angiogenesis; however, the experimental results are inconsistent (Young and Dyson, 1990; Rubin et al, 1990). It has also been reported that low-intensity pulsed ultrasound has effects on gene expression both in vivo and in vitro through interactions with various receptors, leading to stimulation or inhibition of a number cellular pathways. It has been shown to both alter the expression of a number of genes, and trigger apoptosis in human lymphoma cell lines via stimulation of the mitochondrial pathway (Tabuchi et al., 2008; Feril et
In cultured fibroblasts it has been shown to activate receptors leading to increased cell proliferation (Zhou et al., 2004) and expression of structural proteins (Oliveira et al., 2009). Upregulated expression of a number of proteins associated with bone formation has recently been reported (Bandow et al., 2007; Olkku et al., 2010; Fávaro-Pípi et al., 2010); this is thought to be an underlying molecular mechanism for the improved bone healing seen with ultrasound treatments of fractures.

1.5.4 Microbubble ultrasound contrast agents and their interactions with ultrasound

Ultrasound contrast agents are typically on the order of 1-10 µm in diameter (mean 2-3 µm), consisting of a gas core stabilized by a protein, lipid, or polymer shell (de Jong et al., 2009). Higher molecular weight gases tend to dissolve less quickly (compared to air), and thus perfluorocarbon gases are a common choice because of their low solubility in water. However, even un-encapsulated perfluorocarbon microbubbles persist in the blood on time-scales too short to be of any practical clinical use. A gas bubble is under the influence of the surrounding hydrostatic pressure, the acoustic pressure due to the ultrasound field and the surface tension at the gas-fluid interface, which results in an opposing gas pressure within the bubble greater than the partial pressure of gas within the surround fluid, leading to the rapid dissolution of the bubble (de Jong et al., 2009). The addition of a shell further slows this process by, in the case of surfactants such as phospholipids, the reduction of surface tension or in the case of polymers, the shell rigidity that is able to support a strain (Dalecki, 2004; de Jong et al., 2009). The shell also maintains a more uniform size distribution by deterring coalescence.

The acoustic emissions from unencapsulated bubbles exposed to increasing ultrasound intensities have been studied since the 1950’s, when it was first noted that a strong peak was observed at half the fundamental (transmitted) frequency, along with its first few harmonics (ultraharmonics), prior to the onset of inertial cavitation and the broadband noise that accompanied it. Later, the harmonics and sub-harmonics were postulated to be the result of non-linear bubble oscillations during stable cavitation (Neppiras 1980). The behaviour of encapsulated microbubbles is more complex than that of a simple gas bubble due to the additional structure. The shell increases the resonant frequency of individual bubbles due to its stiffness and also increases the dampening of the oscillations (de Jong et al., 2009). As one
would expect however, the acoustic emissions of coated bubbles have similar signatures corresponding to different regimes of oscillation. At very low excitation amplitudes bubbles undergo volume oscillations and emit at the fundamental frequency (de Jong et al., 2009). As the intensity increases, non-linear volume oscillations generate emissions at the second and higher harmonics, as well as the half- and ultraharmonics (de Jong et al., 2009; Dollet et al., 2008). Non-spherical volume oscillations (surface modes) also give rise to the half-harmonic and ultraharmonics but are driven by the radial oscillations (Dollet et al., 2008). Inertial cavitation results in a broadband signal and is associated with destruction of the bubble. The behaviour of a bubble may be further modulated by the proximity to a rigid or semi-rigid boundary, such as a vessel wall. The scattered signal from bubbles is an important tool for the control of BBBD, as will be discussed later in 1.5.7.2.

1.5.5 BBB-Disruption

The primary mechanism responsible for BBBD is cavitation. While BBBD is also possible via thermal mechanisms, this has been shown to occur above the threshold for tissue damage (McDannold et al., 2004c). To disrupt the BBB reversibly and without injury, the desired mechanism is stable cavitation, where gas bodies undergo sustained volume oscillations rather than violent collapse. However, to generate gas bodies at the focus but avoid inertial cavitation, all the while restricting the effects to the vasculature, and to do so in a controlled manner, is extremely challenging. Correspondingly, injectable microbubbles are needed to consistently achieve stable cavitation. The microbubbles now used for BBBD were originally developed as ultrasound contrast agents due to their high scattering-cross section. The shell can also provide a substrate for attaching ligands or antibodies that can be used to target the bubbles to specific tissues. Because the circulation half-life is short, the microbubbles are injected intravenously with the onset of each sonication. Injectable microbubbles require an ultrasound intensity for stable cavitation orders of magnitude less than that needed to generate inertial cavitation in their absence (Vykhodtseva et al., 2008), while also restricting the effects to the cerebrovasculature.

It is well known that BBBD can be elicited with inertial cavitation (section 1.5.1), but at the expense of variable permanent tissue destruction. Several studies using passive cavitation detection to investigate the acoustic emissions from microbubbles during BBBD (see section
1.5.4) have confirmed that BBBD can occur without inertial cavitation (harmonics without detectable broadband emissions), but with a significantly reduced incidence of tissue injury (Tung et al., 2010; McDannold et al., 2006a). During stable cavitation, microbubbles oscillate within the vessels and it is postulated that the combination of mechanical and sheer stresses result in the histological findings of endothelial tight junction disruption and increased para- and trans-cellular transport (Sheikov et al., 2004; Sheikov et al., 2008; Deng et al., 2012). These bubble oscillations, and the associated deformations of the endothelium (invagination into the lumen and expansion) have been observed with high-speed photomicroscopy in ex vivo vessels (Chen et al., 2011). In larger vessels and at higher amplitudes fluid jets were also seen, directed away from the vessel wall (Chen et al., 2011). In the studies by Sheikov et al. (2004), disruption of endothelial tight junction integrity was not a homogeneous finding within the sonicated tissue volume. Furthermore, an increased number of vacuoles were observed, as well as an upregulation of caveolin (Sheikov et al., 2004; Deng et al., 2012), which is involved in receptor-independent transcytosis (Figure 1.3). Trans-endothelial channel formation has also been described. Sheikov et al. (2008) found that there was a decrease in the immunohistochemical staining of the major tight-junctional proteins (section 1.2.2) in the brain following BBBD, suggesting that they were either damaged or conformationally altered. The protein staining returned to baseline over a period of approximately 6 hours (Sheikov et al., 2008). Whether any of the pathways or soluble factors listed in Table 1-2 is also involved is unknown presently.

With regards to how exactly the spectrum of structural changes comes about from the stable cavitation that has been implicated, several observations have been made that contribute to the understanding; however, the exact interaction remains unknown. Two photon microscopy experiments of the dynamics of BBBD have revealed two leakage patterns after exposure to ultrasound and microbubbles, namely a fast (immediate) and a slow (delayed) pattern, with the former occurring more frequently in smaller vessels (Cho et al. 2012). Simulations of bubble oscillations in a vessel predict the greatest shear stresses on the vessel wall in this same scenario (Hosseinkhah and Hynynen 2012). It has also been noted that the vesicular transport is more pronounced in arterioles compared with capillaries (Sheikov et al., 2006). Synthesizing all of the above, it is postulated that the high vessel wall stresses generated in smaller vessels (where the vessel and bubble diameter are near the same size, given that typical bubbles are <10 microns) is responsible for the fast leakage that is seen and is the result of immediate disruption of the tight
junctional proteins. In the larger vessels where the slow mechanism is more common, the interaction of the cavitating bubbles may be more likely to induce downstream pathways and increase the expression of caveolin-1 with induction of vesicular transport, resulting in a delayed and sustained increase in uptake.

In practical applications of BBBD, the most convenient method of assessing the change in BBB permeability is to calculate the change in contrast extravasation measured on contrast-enhanced MRI sequences following a sonication (Treat et al., 2007; Yang et al., 2010). Variations of this technique have been widely used for more than a decade to explore BBBD with a variety of microbubbles, ultrasound parameters and therapeutic agents, with MR contrast extravasation as a surrogate of the biological effect (Hynynen et al., 2005; McDannold et al., 2005; McDannold et al., 2006; McDannold et al., 2007; Howles et al., 2010). The most pertinent ultrasound parameters available for manipulation are the frequency, amplitude, pulse length, total sonication time, and pulse repetition frequency (PRF). Parameters related to the microbubbles include the dose, size and size distribution, shell properties, and infusion method. The frequency selected should be based on the experimental model used, with consideration to the depth of the intended target and the size of the focus required. McDannold et al. (2008b) showed that the 50% threshold for BBBD could be characterized by an MI of 0.47 (PRF 1 Hz, frequency range 0.26-1.63 MHz; see Equation 1.6), thus roughly defining a parameter space for the amplitudes and frequencies necessary for successful BBBD. Increasing the amplitude for a fixed frequency results in an increase in enhancement, but also an increase in the probability of permanent tissue injury (Hynynen et al., 2001; McDannold et al., 2006; Chopra et al. 2010; Tung et al., 2011). Increasing the pulse length decreases the amplitude needed for BBBD (McDannold et al., 2008a), while also increasing the degree of enhancement (McDannold et al., 2008a; Choi et al., 2011). Similarly the enhancement increases with increasing PRF only up to a few Hz with pulse lengths in the millisecond range (McDannold et al., 2008; Choi et al., 2011). Both pulse length and PRF are related and plateau, likely due to the lack of recovery of microbubbles between pulses as proposed by Goertz et al. (2010). Increasing the total length of the sonication also increased enhancement, but this effect also appeared to plateau after about 600s (Chopra et al, 2010); whether this holds true only for MR contrast, compared with other molecules, is unknown. For otherwise fixed parameters, increasing the microbubble dose results in an increase
in enhancement (McDannold et al., 2008; Choi et al., 2011). As one might expect, microbubble diameters much smaller than the average diameter of capillaries require higher amplitudes for BBBD (and more likely through inertial cavitation), while those with diameters closer to the vessel size require a lower amplitude (Tung et al., 2011).

Finally, numerous studies have found no long-term ill effects (up to 5 weeks) (McDannold et al., 2005; 2006). There is a safe window in which BBBD can be achieved without any tissue damage, the boundaries of which are likely those corresponding to stable cavitation. Beyond the upper threshold of this safe window, the first sign of damage and the most commonly identified post-mortem finding is erythrocyte extravasation or hemoglobin breakdown products. Further increases to the intensity of the treatment result in progressively more tissue injury. Generally subjects of BBBD display a relative absence of adverse effects.

1.5.6 Ultrasound technology for clinical treatments

1.5.6.1 Transducer design

The piezoelectric effect was first demonstrated in 1880 by Pierre and Jacques Curie and forms the modern basis for the generation of ultrasound. It was discovered that mechanical strain in certain crystals resulted in an electric potential. Soon afterwards, the converse was also shown to be true; application of an electric potential to piezoelectric crystals resulted in a mechanical deformation. A number of piezoelectric materials have since been found or manufactured, including naturally occurring substances like Quartz and Topaz, and man-made crystals (langasite), ceramics (lead zirconate titatanate PZT) and polymers (polyvinylidene fluoride PVDF). The most commonly used piezoelectric materials used in medical ultrasound applications are from a group of man-made materials called polarized polycrystalline ferroelectrics, such as PZT.

In order to produce a continuous ultrasound wave, an oscillating potential is applied to electrodes on the front and back face of a slab of piezoelectric material. The fundamental resonant frequency of a piezoelectric slab occurs at a frequency at which the wavelength is equal to twice the thickness of the slab. At clinically relevant frequencies, the thickness of these transducers is
on the order of millimeters. When the transducer is driven at this frequency, the maximum pressure amplitude and acoustic power are obtained. The transducer can also be driven at odd harmonics of the fundamental, although the conversion efficiency of electric to acoustic power is reduced. Because therapeutic ultrasound treatments often require high power, in addition to designing a transducer that will be operated at its fundamental frequency, it is desirable to have all of the acoustic power directed forward through the face of the transducer to the target. By examining Equations 1.3 and 1.4, it can be seen that this is achieved if the acoustic impedance of the backing material is either much higher or much lower than that of the transducer. In practice, air backing results in virtually all of the backward power being reflected into the forward direction. Furthermore, to ensure that as much of the forward directed beam reaches the intended target, a coupling medium is required between the transducer and the subject. For complete transmission, a coupling medium is chosen with the same or similar acoustic impedance to soft tissue, such as degassed water or polyacrylamide gels.

Clinical ultrasound treatments require the accurate delivery of energy to the target tissue volume. Higher frequencies yield a smaller focus, but at the expense of increased attenuation and therefore decreased depth of penetration. Although the axial dimension of the focal spot in the near field is similar for both a planar and spherical source, the intensity at the focus is much greater for a spherical source; consequently, for an equivalent electrical power source, more power can be delivered deeper into the tissue. There are a number of ways a focused transducer can be constructed. A single-element spherically curved piezoelectric transducer is the simplest design but has a number of limitations; the transducer has a fixed focus and therefore treatment over larger or multiple regions requires repositioning. Furthermore, the focal spot may be severely distorted if the ultrasound beam must pass through bone, as is required in transcranial applications. Acoustic lenses are constructed from a material with a different speed of sound than the coupling medium, so that the ultrasound beam exiting the lens converges to a focal spot similar to that generated from a spherical source. The focal depth may be adjusted in some lens designs, but phase correction and steering are not possible (Foster and Hunt, 1979).

Multi-element arrays allow the individual transducer elements to be driven independently, allowing the possibility electronic beam focusing, steering, and phase correction. As a result of this increased flexibility, it is the most commonly used design in current therapeutic systems.
The first electronically focused ultrasound array for hyperthermia consisted of circular transducer elements arranged in concentric rings, allowing the focus to be adjusted in the axial direction (Do-Huu and Hartemann, 1981). Presently, small transducer elements may be arranged in virtually any configuration. For planar arrays, the elements can be driven with amplitudes and phase delays such that the ultrasound waves add constructively at the desired focal spot (Figure 3). If the centre-to-centre spacing of the elements is half the wavelength or less, the focal spot can be chosen anywhere in front of the array without secondary foci (Wooh and Shi, 1999). This allows for larger volumes to be treated without the need to reposition the patient or transducer. The amplitude and phase from individual elements can also be adjusted to correct for aberrations resulting from the beam path, as is done for transcranial treatments (Clement et al., 2000; Hynynen et al., 2004). Similar advantages are achieved with multi-element spherically shaped arrays; where phase corrections can be applied to reduce distortion of the focus and electronic steering allows the efficient treatment of multiple or larger targets (Fjeld and Hynynen, 1997). As discussed in Section 1.5.3.1 for therapeutic ultrasound frequencies, incidence angles beyond approximately 30° are reflected, so that concave transducers are ideal for the human skull as they tend to maximize the proportion of elements oriented orthogonally to the skull surface (White et al., 2006). Such a transducer design is currently used in a commercially available therapeutic ultrasound system (Clement et al., 2000). Other specialized configurations have been developed for various access routes including trans-urethral (Chopra et al., 2005; Ross et al., 2005), trans-rectal (Hutchinson and Hynynen, 1998), trans-esophageal (Melodelima et al., 2005; Werner et al., 2010), and endovascular (He et al., 1994; Hynynen et al., 1997).
Figure 1.5: Block diagram of the basic components of a therapeutic ultrasound system. RF, radiofrequency. In the case of phased arrays used for transcranial applications, each element requires an independent driving circuit like the one shown.

Typical treatment configurations involve a transducer coupled to the patient via a coupling medium such as degassed water or ultrasound gel. In clinical treatments through the skull, the coupling water is circulated and cooled to avoid temperature elevations of the skin and scalp (McDannold et al., 2004b). Any hair in the beam path is typically removed either mechanically or chemically to reduce attenuation, but this may be less of a concern with low frequencies and intensities (Raymond and Hynynen, 2005). Rigid fixation of the patient to the treatment assembly and transducer is required for intracranial therapies (McDannold et al., 2010). For single element, fixed-focus transducers, a computer controlled 3-axis positioning system may hold the transducer and allow it to be positioned throughout the treatment volume (McDannold et al., 1998). In order to generate ultrasound, the transducer is excited with a radiofrequency (RF) signal, generated by a signal generator or oscillator and amplified with an RF amplifier (Figure 1.5). The total RF-power is measured following amplification, and if the transducer has been previously calibrated so that the efficiency is known, then the transmitted acoustic power can be calculated. Finally, the input signal to the transducer passes through a matching circuit to couple the impedances of the amplifier and the transducer. The output acoustic power from the transducer can then be adjusted by either adjusting the duty cycle, the amplitude of the input signal to the amplifier, or the amplifier itself. For multi-element arrays, a separate driving system is typically required for each element.
1.5.7 Treatment guidance and monitoring

Therapeutic ultrasound applications rely on image guidance and in some cases, real-time feedback, to deliver the ultrasound energy to the correct location in the body and to prevent adverse events. For most non-invasive, intra-cavitary, and interstitial applications, this is done with MRI. Currently, computed tomography is used only for obtaining skull morphology for phase correction in transcranial treatments, but may in the future be adapted for use in thrombolysis. Many catheter-based ultrasound therapies rely on fluoroscopy for navigation, as is done in conventional endovascular procedures. Ultrasound imaging has insufficient soft tissue contrast for planning most clinical treatments, but it is used for treatment guidance in some anatomical locations, and various ultrasound-based technologies are being investigated for real-time treatment monitoring, even for intracranial treatments through the intact skull (Jones et al., 2013).

1.5.7.1 MR thermometry

The most well established quantitative monitoring for thermal ultrasound therapies exploits the proton resonance frequency shift detected with MRI to measure temperature changes. In essence, MR imaging exploits differences in the proton environment to give image contrast and, in the case of thermal ultrasound therapies, this change is temperature. As the tissue temperature increases, so does Brownian motion, leading to the stretching and disruption of hydrogen bonds between water molecules. This net decrease in the hydrogen bond strength leads to an increased strength of the covalent bond between hydrogen and oxygen, with a resultant increase in the shielding of the proton from the external magnetic field of the MRI (Hindman, 1966). The increase in shielding, and subsequent change in resonant frequency, as a function of increasing temperature, has been shown to be approximately linear (Hoffman and Conradi, 1997). In practice, most MR thermometry sequences consist of a gradient-echo type sequence where the accumulated phase change over time in each voxel, due to the local proton resonance frequency shift, is compared to a reference image in order to generate a spatiotemporal temperature map (Ishihara et al., 1995; Kuroda et al., 1996). MRI thermometry is sufficiently fast that it can be used for real-time feedback of thermal ultrasound treatments. The MRI environment is also used for BBBD, as it allows structural imaging with high soft-tissue contrast and the ability to perform
contrast-enhanced imaging to assess the degree of BBBD. Furthermore, it can be used to verify co-registration of the ultrasound focus and the MRI coordinate systems for image-guidance; confirmation of the ultrasound focus in MRI coordinates can be done by heating a small volume of tissue to sub-lethal temperatures, and making any corrections if needed (Hynynen et al., 1997).

### 1.5.7.2 Ultrasound-based treatment and cavitation monitoring

Given the high cost of clinical MRI systems, efforts have been made to design purely ultrasound-based treatment systems, with real-time monitoring and feedback. In ablative thermal ultrasound applications, the temperature dependence of a number of tissue properties has been investigated including the attenuation and absorption coefficients (Damianou et al., 1997; Zhong et al., 2007), backscattered energy (Arthur et al., 2003; Zhong et al., 2007), speed of sound (Varghese et al., 2002a; Varghese and Daniels, 2004; Seip and Ebbini, 1995), and harmonic content, relative to the fundamental frequency, of non-linearly propagating waves (van Dongen and Verweij, 2011). These can then be monitored as a surrogate of the temperature change occurring in the target tissue.

For BBBD applications, MRI provides the best structural imaging for treatment planning, and is used to assess the degree of opening following a sonication, but it does not provide any real-time information and thus no opportunity to intervene if inertial cavitation develops during a sonication. An increasing number of therapies are attempting to harness cavitation, and correspondingly efforts are being made to characterize and map cavitation events in order to guide therapy at the focus and avoid undesirable cavitation events elsewhere within the beam path. Both active and passive cavitation detection systems have been described (Madanshetty et al., 1991), the latter of which use the scattered ultrasound, along with an arrangement of one or more passive receivers, to characterize cavitation events. In cases where inertial cavitation is unwanted, one or more passive detectors may be used to detect the broadband emissions associated with inertial cavitation and terminate the treatment prior to inadvertent tissue injury. Such a system is now in place in a commercially available clinical ultrasound system (ExAblate®, Insightec, Haifa, Israel). Where stable cavitation is desired, particularly for BBBD with intravascular ultrasound contrast agents, a similar system may be used to monitor the
acoustic emissions from the microbubbles, and with post-processing, perform real-time adjustments of the transmitted acoustic power in order to achieve the desired treatment effect (O’Reilly and Hynynen, 2012). It has been shown in pre-clinical studies of BBBD with ultrasound contrast agents that the acoustic emissions correlate with the degree of opening (McDannold et al., 2006). Furthermore, it has been shown possible to control therapy and titrate the bioeffect by real-time analysis of the pattern and amplitudes of the sub- and ultra-harmonics in the scattered ultrasound signal (O’Reilly and Hynynen, 2010; Arvinitis et al., 2012). As described previously in 1.5.4, the harmonic emissions from sonicated microbubbles can be used to characterize the particular oscillation regime.

Clinical applications will require monitoring the harmonics and mapping their locations, not just at the focus but in the near- and far-field as well. Currently, passive cavitation mapping to guide therapy is still in its infancy. Most experimental setups have employed single-element fixed-focus receive transducers, with limited coverage of the treatment volume (Farny et al., 2009). More recently, several groups have described passive cavitation mapping in vitro using multi-element arrays (Farny et al., 2009; Salgaonkar et al., 2009; Gyöngy and Coussios, 2010), with slightly greater coverage. Recently a method to image microbubble oscillations using a sparse array of receive transducers within a treatment array has been described by Jones et al. (2013). In the future this work may eventually allow the desired level of control in clinical applications.

2 ENHANCING DRUG DELIVERY FOR BORON NEUTRON CAPTURE THERAPY OF BRAIN TUMOURS

2.1 Introduction

The dismal prognosis associated with glioblastoma is attributable not only to its aggressive and infiltrative behavior, but its location typically deep within the parenchyma of the brain. Surgical corridors and extent of resection are limited by the potential for further neurological injury. Photon-based radiation therapy is a mainstay of treatment, but causes significant collateral injury
to the brain that worsens over time (Szerlip et al., 2011; Crossen et al., 1994). Finally, the endothelial cells of the brain’s microvasculature are interconnected by tight junctions, forming the blood-brain barrier (BBB) between the circulating blood and the interstitial fluid space of the brain (Abbot et al., 2006). The BBB tightly regulates the passage of molecules into the brain, limiting the accumulation of many therapeutic agents, particularly in infiltrating cells advancing beyond the tumour margin (Muldoon et al., 2007). The blood-tumour barrier (BTB) is variably more permeable, but remains a significant obstacle to therapy (Lockman et al., 2010).

Boron neutron capture therapy (BNCT) restricts the effects of ionizing radiation to malignant cells by exploiting the high thermal neutron capture cross-section of $^{10}$B, but requires the preferential delivery of a $^{10}$B-containing carrier molecule to the tumour. We investigated focused ultrasound in combination with ultrasound contrast agents to increase the uptake of $^{10}$B in malignant cells by focally increasing the permeability of the BBB and BTB.

BNCT is a binary treatment whereby a compound containing $^{10}$B is formulated such that it is taken up preferentially by tumour cells following administration into the blood. The tumour and surrounding tissue are then irradiated with a thermal or epithermal neutron beam, wherein $^{10}$B captures a neutron to form $^{11}$B and then rapidly undergoes alpha decay (Equation 2.1). The high linear energy transfer radiation produced in this reaction by the recoiling alpha particle and lithium ion has a range of approximately 5-9 µm in tissue, or on the order of a single cell, and

$$^{10}\text{B} + n_{th} \rightarrow [^{11}\text{B}] \rightarrow ^4\text{He}(\alpha) + ^7\text{Li} + 2.79 \text{ MeV} \text{ (Equation 2.1)}$$

results in DNA damage that is irreparable and potentially lethal (Coderre and Morris, 1999). Only $^{10}$B-containing cells, given a sufficient intracellular concentration, will be eradicated while other cells will be relatively spared. Successful BNCT thus requires an adequate concentration of $^{10}$B within target cells, estimated to be on the order of 20 µg/g, and a high tumour-to-brain ratio of $^{10}$B (Coderre and Morris, 1999; Barth et al., 2005). This high ratio is desirable as it increases the relative dose to the tumour, while minimizing the number of hydrogen, nitrogen and boron capture reactions that occur in non-target tissues.

One of the two most commonly studied $^{10}$B delivery agents is L-4-boronophenylalanine (BPA), which is complexed with fructose (BPA-f) to increase its solubility (Yoshino et al., 1989). As an
amino acid analogue, it accumulates preferentially in malignant cells due to comparatively higher metabolism. Early clinical trials of BNCT for glioblastoma used 2-hour intravenous infusions of BPA-f, at doses ranging from 100-400 mg/kg body weight (b.w.) (Barth et al., 2005; Skold et al., 2010; Diaz 2003). In animal glioblastoma models however, significant improvements have been obtained by dose escalation, prolonged infusions, intracarotid injection, and pharmacological or osmotic BBB-disruption (BBBD) prior to drug administration (Barth et al., 2003; Smith et al., 2001; Joel et al., 1999). In a rodent model of glioblastoma, we postulated that increasing the vascular permeability in and around the tumour (BBB/BTB-disruption, henceforth referred to as BBBD) with MR-guided focused ultrasound would result in an increased and more uniform uptake of intravenously administered BPA-f into the tumour (Alkins et al., 2013a).

2.2 Materials and Methods

BPA (98.4% purity, Interpharma Praha, Prague, Czech Republic) was prepared without the use of glass labware to avoid contamination from borosilicate glass (Green et al., 1976). The $^{10}$B enrichment was confirmed by ToF-SIMS. The BPA-f complex was prepared as described previously in the literature (Yoshino et al., 1989; Shibata et al., 2004). It was refrigerated in a polypropylene vessel overnight, and passed through a 0.22 µm filter prior to administration.

2.2.1 Tumour Implantation

All procedures were approved by the institutional animal care committee and conformed to the guidelines set out by the Canadian Council on Animal Care. General anesthesia was induced in male Fisher 344 rats weighing 250-300g with isofluorane and maintained with a combination of ketamine (90 mg/kg), xylazine (10 mg/kg) and isofluorane. Tumours were implanted in the left frontal striatum using a stereotactic frame, at a depth of 4 mm from the cortical surface. The 9L gliosarcoma cell line was selected as it has been extensively used in BNCT studies (Barth 1998). Using a 5 µL Hamilton syringe, $2.5 \times 10^5$ 9L gliosarcoma cells were injected in 2 µL of media. Animals were imaged and treated 8-9 days after implantation, once the tumours had reached 1.5-2 mm in diameter.
2.2.2 Animal Preparation, Focused Ultrasound Setup and MRI Imaging

For ultrasound treatments, animals were anesthetized with ketamine and xylazine at the same dose previously detailed. A 22-gauge tail vein cannula was inserted and the head of the animal shaved and chemically depilated. Definity® ultrasound contrast (Lantheus, North Billerica, MA) was prepared as directed and diluted 1:10 in normal saline. Animals were positioned supine and fixed in place with a bite bar and lower extremity restraints. The head was coupled to a single element concave ultrasound transducer (centre frequency 558 kHz, F=0.8, R=10 cm) through a bath of degassed water. The free-field peak rarefaction pressure was measured to be 0.4 MPa. Following MRI co-registration, a computer-controlled 3-axis positioning system allowed the focal spot of the transducer to be positioned at any location within the brain. All treatments began with baseline T2- and T1-weighted MR imaging, including a T1-weighted sequence with gadolinium contrast (0.2 ml/kg; Omniscan, GE Healthcare, Milwaukee, WI), to identify the tumour. A single sonication was performed on each animal, each consisting of ultrasound bursts of 10 ms in length, repeated once every second (pulse repetition frequency of 1 Hz) for each corner of a 1.5 mm square in the axial plane (Figure 2.1). The total treatment duration was 120 s. With the initiation of each sonication, animals were given a 0.02 ml/kg Definity® bolus into the tail vein catheter, followed by a 0.5 ml saline flush. The same MR imaging sequences performed pre-treatment were then repeated following the sonication to confirm the extent and location of BBBD. Contrast enhancement (CE) on T1-weighted images was calculated as CE=(I\text{post} – I\text{pre})/I\text{pre}, where I\text{pre} and I\text{post} are the pre- and post-contrast average signal intensities of the tumour, each normalized by the intensity in the contralateral hemisphere (where the post-contrast scan was taken 5 minutes after BBBD). The CE was then used to characterize the increase in vascular permeability due to the sonication.
Figure 2.1: Representative images of the rodent brain before and after BBBD with focused ultrasound. A schematic diagram is shown in A, with the positioning of the animal relative to the ultrasound transducer. The 3-axis positioning system allows the transducer to be positioned so that the focal spot can be targeted anywhere in the brain. B and C show Omniscan® enhanced pre- and post-BBBD T1-weighted MR images, respectively. D and E show the corresponding T2-weighted MR images. The tumour is indicated by the single arrow in panels B and D prior to BBBD. The 4 sonication foci (arrows) can be identified in C and E encircling the tumour following BBBD. The square pattern is slightly distorted as a result of transmission through the skull. The increase signal on the T2-weighted images corresponds to edema induced by the disruption of the vasculature. There also appears to be some BBBD towards the midline resulting from the postero-medial focus, which has resulted in contrast enhancement periventricularly in the left frontal lobe, in the anterior commissure and in the anterior portion of the left thalamus. This is due to the 3 mm pressure full width half maximum (FWHM) lateral beam width, which combined with the skull distortion, would overlap these structures.

2.2.3 Treatment Groups

There was one tumour treatment group (n=3) and a control tumour group (n=4). All animals were delivered a BPA-f dose of 250 mg/kg body weight (b.w.) via tail vein catheter over a two hour period, and sacrificed 1 hour after the conclusion of the infusion (3 hours after the initiation
of the treatment). In both groups, 25% of the total dose was delivered as an initial bolus, followed by a single sonication to induce BBBD. The remaining BPA-f was delivered over a 2-hour infusion. The control group received only the 2-hour infusion following the bolus. As an additional control two animals (n=2) without tumour implantation underwent BBBD with BPA-f infusion to evaluate the effect on normal brain, with the contralateral unsonicated hemisphere serving as a matched control.

2.2.4 Specimen Preparation

Animals were sacrificed by IV injection of euthanyl. Specimen preparation was similar to that described in the literature for ion imaging (Ausserer et al., 1989; Benabdellah et al., 2010). Animals were not perfused as any $^{10}$B present within the vasculature at the time of irradiation would contribute to the overall radiation dose delivered to the respective tissue. Brains were removed and flash-frozen in isopentane cooled in liquid nitrogen, then maintained at -80°C until the time of histological preparation. Ten µm thick coronal sections, at 250 µm levels, were cut using a cryostat (Leica Microsystems GmbH, Wetzlar, Germany) with the chamber held at -20°C, and immediately mounted on 50 mm N-type semiconducting silicon wafers (Wafer World, West Palm Beach, FL) maintained at the same temperature. The silicon-mounted sections were then lyophilized for 24 hours and stored at room temperature in hermetically sealed containers with Drierite (W.A. Hammond Drierite Co., Xenia, OH) until analysis by ToF-SIMS. An adjacent frozen section at each level was mounted on a glass slide and stained with hematoxylin and eosin (H&E) for light microscopy.

2.2.5 Imaging Mass spectrometry

Mass spectrometry was performed with a ToF–SIMS IV time-of-flight mass spectrometer (Ion-Tof GmbH, Munster, Germany), taking measurements at the tumour-brain interface, where both the main tumour (MT), islands of infiltrating tumour (IT) and normal brain could be analyzed. Samples were pre-sputtered for 60 s with oxygen (36 nA) over a 400 x 400 µm area centered on the region of interest. Positive secondary ions were analyzed within 200 x 200 µm regions (128 x 128 pixels, 1 shot per pixel, 550 scans, 100 µs cycle time) in imaging mode with a Bi$^+$ liquid metal ion analysis gun (200 ns pulse) and non-interlaced oxygen sputtering (0.2 s sputter, 0.1 s
Imaging parameters were kept constant for all measurements. Regions for analysis were identified using a combination of optical imaging of cryosections and the corresponding H&E stained slides. On average, 9 measurements were performed on 2-3 separate levels for each animal. To account for differences in secondary ion intensities between samples, the secondary ion intensity of $^{10}\text{B}$ was normalized by the intensity of $^{12}\text{C}$, as the latter is a relatively uniform matrix element (Ausserer et al., 1989). This same normalization was used for all other elements investigated. For the analysis of the IT regions, because they were not present in all sections and a particular island of cells was typically only visible in a single level, 2-3 IT regions were selected for each animal and the average normalized $^{10}\text{B}$ intensity was compared with that of the MT in the same section. The average IT:MT ratio of $^{10}\text{B}$ was then calculated for the BBBD and control groups.

2.2.6 Inductively-Coupled Plasma Atomic Optical Emission Spectroscopy (ICP-AES) and semi-quantification of ToF-SIMS data

Boron concentrations were measured with a Perkin-Elmer Optima 7300 DV ICP-AES using the 249.7 nm line of boron. Whole blood samples were collected at the time of sacrifice, digested with trichloroacetic acid (Sigma Aldrich), centrifuged, and the supernatant analyzed for total boron concentration as described elsewhere (Laakso et al., 2001). To convert the secondary ion intensities obtained with ToF-SIMS to concentration values (ppm), 9L cell pellets were enriched with BPA-f, homogenized and frozen. These were digested using a combination of concentrated double distilled nitric and hydrochloric acid stored in Teflon (Veritas, GFS Chemicals, Columbus, OH), and the concentration of total boron measured (Ausserer et al., 1989). Magnesium was also measured in these specimens with both ToF-SIMS and ICP-AES (285.2 nm line) to help confirm uniform tissue processing between samples. The boron concentrations in the samples were then calculated using a relative sensitivity factor as shown in Equation 2.2, where $I_B$, $I_C$, and $[B]$ are the secondary ion intensities of

$$[B]_{\text{sample}} = \frac{I_{B, \text{sample}} I_{C, \text{control}}}{I_{C, \text{sample}} I_{B, \text{control}}} [B]_{\text{control}} \quad \text{(Equation 2.2)}$$
\(^{10}\)B and \(^{12}\)C, and the concentration of \(^{10}\)B in ppm wet weight, respectively, for the sample of interest and the control samples digested and analyzed by ICP-AES (Ausserer et al., 1989).

2.2.7 Statistical Analysis

GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for statistical analysis. ToF-SIMS spectrums were analyzed and poisson corrected with SurfaceLab6 (Ion-Tof GmbH, Munster, Germany) prior to analysis with GraphPad. Ratios were calculated as the mean \(\pm\) SE of the individual tumour:brain boron ratios for each animal. Boron concentrations (mean \(\pm\) SD) were estimated from the average secondary ion intensity for each of the ultrasound-treated tumour, control tumour and normal brain using Equation 2.2 and the ICP-AEOS measurements of the doped cell pellets. Student’s t test was used to compare the means \(\pm\) SD and means \(\pm\) SEM from two groups.

2.3 Results

All animals tolerated the drug infusion and ultrasound treatment without complication, and recovered from general anesthesia to their pre-treatment condition prior to sacrifice.

Figure 2.1 depicts representative T2-weighted and contrast enhanced T1-weighted MR images before and after BBBD. The increase in contrast extravasation following treatment with ultrasound was relatively uniform; the average contrast enhancement of the tumour region without BBBD was 12.5 \(\pm\) 0.7 \%, while post BBBD it was 46 \(\pm\) 4 \%(p < 0.01). Although the BTB is permeable, these results demonstrate that it can be made much more so with the combination of ultrasound and microbubbles. T2-weighted imaging shows an increase in edema following BBBD as a result of the increased capillary permeability (Figure 2.1E). All animals received the same weight-based dosing of BPA-f, and whole blood samples obtained from both sonicated and control animals, analyzed by ICP-AES, showed no statistically significant difference in total boron concentration at the time of sacrifice.
Figure 2.2: Optical imaging within the vacuum chamber of the ToF-SIMS instrument was used to locate the tumour (indicated by the arrow) on the lyophilized specimens (A). Following collection of the secondary ion spectra, the signal from $^{10}\text{B}^+$ was easily distinguishable due to the lack of interferences in that atomic mass range (B).

On thorough histological review of the brain, no tissue injury was identified as a result of the ultrasound exposure; specifically, there were no regions of red blood cell extravasation, vacuolation, or abnormal neurons (pyknotic or karyorrhexic). Light microscopic review of H&E sections was used to select regions of interest for ToF-SIMS analysis on the corresponding adjacent silicon wafer-mounted sections. The tumour was easily differentiated from the normal brain tissue using optical imaging within the vacuum chamber of the ToF-SIMS instrument (Figure 2.2A). A ToF-SIMS method was developed for the measurement of boron in cryogenically prepared specimens, which increased the sensitivity to elemental boron more than forty-fold compared to the initial imaging parameters, using bismuth as a primary ion source without oxygen sputtering. To our knowledge this is the first use of a time-of-flight SIMS instrument to measure boron in the brain relating to BNCT. Although the secondary ion signals due to sodium and potassium saturated the detector, which precluded accurate quantitative comparisons of these particular ions, the $^{39}\text{K}/^{23}\text{Na}$ ratio ranged between 2:1 and 5:1 and did not correlate with the $^{10}\text{B}$ distribution. These results suggest that the cryoprocessing of the samples was acceptable (Chandra et al., 1987). Imaging of sodium and potassium also helped delineate
structural details of the tumour. As has been similarly reported in $^{23}$Na MR imaging of human brain tumours, the secondary ion intensity of $^{23}$Na was higher within the tumour compared to the adjacent normal brain tissue (Ouwerkerk et al., 2003). Representative secondary ion images of $^{23}$Na$^+$, $^{39}$K$^+$, $^{12}$C$^+$, and $^{10}$B$^+$ are shown at the tumour-brain interface in Figure 2.3. Within the tumours, $^{10}$B$^+$ appeared to be distributed without any appreciable pattern relative to cellular structure. As reported in previous mass spectrometry studies of rodent tumours, a higher concentration of $^{25}$Mg$^+$ was detected in 9L tumours compared with the surrounding brain, and served to further differentiate tumour from non-tumour regions (Smith et al., 2001).

**Table 2-1:** Main tumour (MT):brain and infiltrating tumour (IT):brain $^{10}$B ratios and tissue concentrations following BBBD with focused ultrasound and BPA-f infusion, compared with the control group receiving infusion alone.

<table>
<thead>
<tr>
<th>Group</th>
<th>MT:Brain $^{10}$B Intensity$^a$</th>
<th>MT $[^{10}$B] (ppm)$^b$</th>
<th>MT $[^{10}$B] Range (ppm)</th>
<th>IT:MT $^{10}$B Intensity$^a$</th>
<th>IT $[^{10}$B] (ppm)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB-D</td>
<td>6.7 ± 0.5**</td>
<td>123 ± 25</td>
<td>118-126</td>
<td>0.86 ± 0.10**</td>
<td>106 ± 25</td>
</tr>
<tr>
<td>Control</td>
<td>4.1 ± 0.4</td>
<td>85 ± 29</td>
<td>51-117</td>
<td>0.29 ± 0.08</td>
<td>25 ± 11</td>
</tr>
<tr>
<td>Normal Brain</td>
<td>-</td>
<td>21 ± 5</td>
<td>13-26</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Mean±SE  
$^b$Mean±SD  
** Indicates p < 0.01
Figure 2.3: Representative secondary ion images taken on the brain-tumour interface. The tumour can be identified in the lower left of each image, with an obliquely oriented island of tumour in the centre. A shows the corresponding H&E stained frozen section adjacent to that used for ToF-SIMS analysis. B-F were acquired simultaneously and are representative of the data sets collected. D shows the relatively uniform signal obtained from $^{12}\text{C}^+$ both in tumour and normal brain tissue. For this reason it is used as a reference element. Both $^{23}\text{Na}^+$ and $^{24}\text{Mg}^+$ can provide significant contrast between tumour and brain tissue (B and E), as the concentrations of both are higher within malignant cells. The boron content of the tumour is noticeably higher compared with the brain (F).

Representative images of the normalized $[^{10}\text{B}]$ in ultrasound-treated tumour, control tumour, peri-tumoural healthy brain, and contralateral brain, and these respective concentrations overlaid on the sum of the remaining secondary ion signal, are depicted in Figure 2.4. Measurements of $^{10}\text{B}$ near the core of the tumour were compared with measurements taken at periphery (ROIs in the respective regions were selected with the optical imaging of the spectrometer prior to spectroscopic measurements). There was no statistically significant difference between these measurements, likely in part explained by the lack of a necrotic core in these relatively small 9L tumours, so that the entire volume was relatively well vascularized. Measurements of $^{10}\text{B}$ in normal brain tissue were also compared to those in the untreated contralateral hemisphere.
(Figure 2.4 C and D). Again, there was no statistically significant difference found (p=0.98), suggesting that ultrasound does not augment the baseline uptake of BPA-f by non-malignant cells in the brain. This was confirmed by the control group without tumour implantation, where we found no significant difference between the $^{10}$B concentration within the sonicated region and the contralateral hemisphere (21.3 ± 8.7 versus 17.8 ± 3.9 ppm, p = 0.39, Figure 2.5).

The average ratio of $^{10}$B within the tumour to that in the adjacent brain was 6.7 ± 0.5 in the group that underwent BBBD, while only 4.1±0.4 in the control group (p < 0.01). The average $[^{10}\text{B}]$ in the BBBD tumours was 123 ± 25 ppm, compared with 85 ± 29 ppm in the control tumours, and 21 ± 5 ppm in the normal brain (Table 2-1). The range of $[^{10}\text{B}]$, and the standard deviations of both the normal brain and tumours having undergone BBBD are proportionally smaller, suggesting less variation in $^{10}$B distribution both within and between animals. This variability was very striking in the secondary ion intensities measured with ToF-SIMS, but once converted to concentrations using ICP-AES, the measurement uncertainty from the latter dominated the error term. The range in all groups is shown in Table 2-1, and varies considerably more in the control tumour group (51-117 ppm). Although the 9L gliosarcoma does not exactly duplicate the infiltrative pattern of glioblastoma in humans (Barth et al., 2003), the normalized $^{10}$B intensity from small infiltrating islands of cells (IT) adjacent to the main tumour mass (IT), was measured. The $[^{10}\text{B}]$ in the IT, of which a representative image is shown in Figure 2.3
was found to be on average 0.86 ± 0.1 of the MT $[^{10}\text{B}]$ in the BBBD group, compared with 0.29 ± 0.08 in the control group (Mean ± SE). This difference was found to be highly statistically significant (p < 0.01) and suggests that BBBD with focused ultrasound can improve the accumulation of $^{10}\text{B}$ in infiltrating tumour cells.
**Figure 2.4:** ToF-SIMS imaging of a 150 x 200 µm region in the rat brain following infusion of BPA-f. The image on the left serves as a schematic of the relative locations in the brain from which the secondary ion signals were acquired. The adjacent 4 columns depict, from left to right, a tumour treated with ultrasound, a control tumour, the ipsilateral peri-tumoural normal brain, and the contralateral untreated brain. A-D show the $^{10}\text{B}^+$ signal normalized by the $^{12}\text{C}^+$ signal. E-H show the signal in A-D overlaid on the sum of the remaining secondary ion signal, where cellular detail can be appreciated. A, C, and D are all from the same animal. The quantitative results are presented in Table 2-1.

**Figure 2.5:** MR imaging of BBBD without tumour implantation. Representative axial gadolinium-enhanced T1-weighted (A) and T2-weighted (B) MRI of BBBD without implanted tumours. The $^{10}\text{B}$ secondary ion signal obtained with ToF-SIMS from the sonicated (C) and unsonicated (D) frontal lobe is shown. The yellow and white boxes are not to scale but denote the approximate regions of analysis. The corresponding yellow and white stars denote the appropriate ToF-SIMS image. There was no statistically significant difference in the concentration in the normal brain tissue exposed to ultrasound ($p=0.39$).
2.4 Discussion

Prolonged remission has been achieved with BNCT in rodent models of glioblastoma using prolonged infusion times, escalated doses (typically above 500 mg/kg), and intracarotid administration with osmotic BBBD. By comparison in humans, drug doses have been in the range of 100-400 mg/kg following the dose escalation study conducted at Brookhaven National Laboratories (Diaz 2003). BPA-f was found to precipitate in the urine at doses above 330 mg/kg (delivered over 2 hours), raising the concern of renal injury at higher doses (Diaz 2003; Barth et al., 2005). Furthermore, infusion times have also been relatively short (2 hours), resulting in outcomes no different than those achieved with the current standard of care (Barth et al., 2005). A recent exception was a Swedish phase II trial using a prolonged infusion time of 6 hours and a dose of 900 mg/kg in an attempt to improve the efficacy of BNCT. Although higher blood levels were achieved at the time of treatment compared to lower dose regimens, the average survival was 14.2 months, similar to that achieved with current standard therapy (Henriksson et al., 2008). All patients had recurrent disease within 10 months of follow-up, and it was postulated that, as in prior studies, these early recurrences are secondary to heterogeneous drug uptake in the tumour, resulting in sub-therapeutic radiation dosing in portions of the tumour (Smith et al., 2001; Henriksson et al., 2008).

Pre-clinical studies have demonstrated increased accumulation of a number of therapeutic molecules, including BPA-f, within tumours of the brain following osmotic and pharmacological disruption of the BBB/BBT (Smith et al., 2001; Kroll and Neuwelt, 1998; Dean et al., 1999). Local interstitial therapies have also been developed to circumvent these barriers, including drug-eluding wafers positioned in the resection cavity, and catheter-based convection-enhanced delivery of targeted toxins and chemotherapeutic agents (Kroll et al., 1998; Rainov et al., 2006; Sampson et al., 2007; Kunwar et al., 2010). More recently, focused ultrasound has been shown to facilitate the entry of a number of therapeutic agents into the brain, focally and without significant adverse effects (Kinoshita et al., 2006; Treat et al., 2007; Liu et al., 2010; Escoffre et al., 2011). While intra-arterial (IA) chemotherapy in combination with osmotic BBBD has had promising results in early clinical trials, Barth et al. (1997) reported 10% of animals undergoing BBBD with IA mannitol died of cerebral edema. The occurrence of massive cerebral edema with
focused ultrasound is far less likely, as it can be restricted to the tumour and surrounding brain, rather than encompass the entire hemisphere. Furthermore, realizing intracarotid drug administration in humans requires cannulation of the internal carotid artery via endovascular techniques, which is not without risk. Compared with IA administration and interstitial therapies, the main advantages of ultrasound as an alternative for BBBD are its non-invasiveness, and a more controlled and focal disruption of the cerebrovasculature. The latter may serve not only to reduce the risk of massive cerebral edema, but also to reduce the accumulation of BPA-f in healthy tissues and thus spare radiation exposure during BNCT, and particularly with more neuro-toxic chemotherapeutic agents, to reduce neurological side effects.

With ongoing advancements in the field of focused ultrasound, as well as improved ultrasound contrast agents, safe and reproducible BBBD is achievable. Pre-clinical studies in rodents have progressed to the investigation of BBBD in larger mammals, and BBBD has now been performed safely in large mammals and non-human primates, even through the intact skull (Xie et al., 2008; McDannold et al., 2012). The preliminary safety of transcranial therapeutic ultrasound in humans has been demonstrated with the successful treatment of more than 30 patients for chronic pain and tremor using similar techniques and treatment infrastructure (Jeanmonod et al., 2012; Elias et al., 2013).

In the present study we show that using a relatively low dose of BPA-f, administered intravenously, and delivered in combination with BBBD performed non-invasively with transcranial focused ultrasound, that therapeutic tumour levels of $^{10}$B can be achieved not only in the main tumour but also in infiltrating clusters of cells, without increasing the accumulation in healthy brain tissue (Table 2-1). These measurements were taken at 1 hour following the termination of the BPA-f infusion, which we believe would reflect the concentrations at the time of treatment, allowing for patient transfer from the MRI environment to one suitable for neutron irradiation. Our results compare rather favorably to BPA-f distribution studies in the literature, and although we did not perform survival studies, we anticipate that similar gains in survival would be achieved. Smith et al. reported their findings in 9L-bearing Fisher 344 rats using 250 mg/kg b.w. BPA-f over 2-, 3-, and 6-hour intravenous infusions. The $^{10}$B concentrations after a 2-hour infusion, measured with a dynamic SIMS technique, were $83 \pm 23$ ppm in tumour and $20 \pm 8$ ppm in normal brain, comparing very closely with our control group ($85 \pm 29$ ppm and $21 \pm$
5 ppm, tumour and brain respectively, Table 2-1) (Smith et al., 2001). Only after a 6 hour infusion did the tumour concentration reach 90 ppm, with less variability compared with the shorter infusion time and a tumour:brain ratio of 5.0; however, the ratio and concentration are still slightly lower than what was achieved with ultrasound-mediated BBBD following a 2-hour infusion (Smith et al., 2001).

We were not able to detect any change in the $[^{10}\text{B}]$ in the normal brain tissue owing to BBBD. This was thoroughly examined, comparing the peri-tumoural sonicated region with the contralateral hemisphere (Figure 2.4), as well as sonicated normal brain with the un-sonicated contralateral hemisphere in animals without tumours following BPA-f infusion (Figure 2.5). These findings are supported by those of Smith et al. (2001) and Barth et al. (1997) neither of whom detected a statistically significant difference between the ipsilateral and contralateral $[^{10}\text{B}]$ with osmotic BBBD even with intracarotid administration. This is likely due to the constant metabolic demand of the normal brain tissue, so that increased bioavailability due to a more permeable BBB alone does not increase the uptake of amino acids (of which BPA is an analogue) into the cell by the L-Amino Acid Transporter-1 (Detta and Cruickshank, 2009). Put differently, the normal brain appears to be adequately supplied through the intact BBB and therefore does not change its demand by an increased interstitial availability, particularly when the intracellular concentrations are governed by active transport rather than diffusion alone. The interstitial concentration then begins to fall immediately following the termination of the infusion, as does the concentration in blood, giving in the present study a blood concentration of 15-20 ppm at 1 hour post-infusion (Laakso et al., 2001). Measurements from human glioblastoma have found that the normal brain concentrations at similar time points were equal to or less than the blood concentrations, which is consistent with our findings within measurement error (Elowitz et al., 1998). There is, however, some suggestion that reducing the peri-tumoural edema pharmacologically with dexamethasone can reduce the uptake of BPA-f in peri-tumoural brain compared with the contralateral hemisphere, but this resulted in only a 14% difference, a change that is likely too small to detect with the present technique (Morris et al., 2004).

Because many resections of glioblastoma are subtotal, the uptake of BPA-f into the main tumour mass is still of considerable importance. However, there is justifiable concern that infiltrating cells will not accumulate BPA-f to the same extent as the main tumour where the BTB is more
permeable than the BBB. We evaluated the [\textsuperscript{10}B] in small infiltrating clusters of tumour cells separated from the main tumour mass and found that BBBD with ultrasound resulted in a significantly improved [\textsuperscript{10}B] compared with infusion alone. In the BBBD group the IT [\textsuperscript{10}B] was more than 80% of the MT concentration, while in the control group it was only approximately 30% (p < 0.01). This latter result in the control group is comparable to results previously reported in the literature for a 2-hour infusion of BPA-f (Smith et al., 2001). The improved BPA-f uptake in infiltrating cells following BBBD with ultrasound is an important finding as these cells are the most likely source of residual tumour following resection and likely the cause of most eventual recurrences.

Another important finding is that the [\textsuperscript{10}B] within the tumours treated with focused ultrasound were more uniform than those in the control group, as evidenced by the wider range of measured concentrations and greater standard deviation of the concentrations in the latter (Table 2-1). This is perhaps not unexpected, as the increased capillary permeability of the tumour vessels is unlikely to be uniform throughout the tumour given that this is due to a variety of vascular phenotypes (some deficient in tight junction proteins) (Rascher et al., 2002) as well as the influence of soluble factors (causing opening of “normal” tight junctions) (Schneider et al., 2004). The combination of microbubbles and ultrasound, on the other hand, should cause a similar effect in the tumour and peri-tumoural vasculature as they are wholly encompassed within the sonicated volume. The exact mechanisms through which cavitation disrupts the blood-brain barrier are unknown, but they ultimately result in alterations in tight-junctional proteins and increased paracellular and transcellular permeability (Sheikov et al., 2004). While at higher sonication pressures bubbles may collapse, generating fluid jets or shock waves (1.5.3.4), at the pressures used in the present study we expect that stable cavitation is the dominant mechanism, whereby BBBD is likely achieved via direct mechanical effects or microstreaming due to the oscillating microbubbles (1.5.4). Regardless of the mechanism, this improved uniformity is an important finding, as there is no satisfactory \textit{in vivo} assessment of the tumour boron concentration prior to neutron irradiation; thus a more uniform BPA-f uptake would allow more reliable use of weight-based dosing or of a surrogate measurement (e.g. blood-boron levels), and lead to fewer patients being undertreated with BNCT. As previously mentioned, it has been theorized that this heterogeneous boron uptake with BPA-f infusions alone in human tumours may be responsible for the disappointing results in clinical trials (Smith et al., 2001).
The present study is not without limitations. We have not conducted survival experiments as we do not have access to a neutron source at our institution. However, the survival benefit of increased BPA-f accumulation in the 9L gliosarcoma and other rodent brain tumour models has been well studied (Barth et al., 2003; Smith et al., 2001; Joel et al., 1999; Barth 1998; Barth et al., 1997). We would expect similar improvements in survival when these improvements in BPA-f uptake are realized with ultrasound. Furthermore, there is recent evidence that ultrasound and microbubbles enhance the effect of radiation on endothelial cells (Czarnota et al., 2012), so that in theory there could be a synergistic tumouricidal effect of BNCT and ultrasound-mediated BBBD. For acute studies such as this, the 9L gliosarcoma cell line is acceptable but had we done survival studies it would not have been a suitable choice. Additionally, this cell line does not exactly mimic the infiltrative nature of glioblastoma in humans as previously discussed. Another limitation is the small blood volume of rodents compared to humans, so that the number of sonications was limited by the risk of volume overload; optimization of the BPA-f infusion protocol used in conjunction with focused ultrasound could potentially be improved further in humans compared to rodents. Drug penetration through the BBB/BTB with ultrasound was found to be most effective when BPA-f was in the circulation at the time of the sonication. However, uptake of BPA-f into cells is not only a function of the concentration to which they are exposed, but also the duration of the exposure. As a result, a bolus was delivered prior to BBBD and then an infusion continued for two hours afterwards, knowing that the BBB remains open for approximately 6 hours (Hynynen et al., 2001). In theory, additional sonications could be performed during the infusion which might serve to further augment the accumulation of BPA-f; however, in the present rodent model the added fluid volume required would be injurious. A longer infusion time could potentially improve the accumulation of $^{10}$B in infiltrating cells within the sonicated region.

Translating this treatment technology into humans requires careful consideration. A more sophisticated ultrasound setup than the one described here is required, as aberrations due to human skull cannot be neglected as they are in rodents. In humans the skull is much thicker, but also heterogeneous, necessitating large phased arrays to maintain a tight focus (Thomas and Fink, 1996; Hynynen and Jolesz, 1998). Fortunately, there exists a commercial transcranial system manufactured by InSightec® (Tirat-Carmel, Israel), which fits many of the requirements for BBBD to succeed in humans, and has previously been used for transcranial applications in
phase I human trials (Jeanmonod et al., 2012; Elias et al., 2013). To improve transmission through bone, the system is available in a lower frequency (220 kHz) than the one used in the present study. This system is used in conjunction with MR-guidance, to assess the degree of BBBD as we have done. While some pre-clinical systems now use acoustic emissions to monitor BBBD (O’Reilly and Hynynen, 2012), this is not yet available in a clinical system although would likely serve to improve the safety of the treatment. A facility attempting to deliver this treatment would require an MRI suite accompanied with a hemispherical phased-array ultrasound system, as well as a nearby neutron source; these requirements would limit this therapy to only a handful of sites in the world at present.

In summary, the current state of BNCT in humans requires further optimization in order to yield an appreciable benefit over conventional therapy. We have demonstrated that BBBD with MR-guided focused ultrasound in combination with IV microbubbles safely augments BPA-f tumour uptake compared to infusion alone. Not only does it increase the tumour:brain $^{10}$B ratio for the main tumour, but also for infiltrating cells, with $^{10}$B concentrations that compare very favorably with published results in the literature using higher doses and longer infusion. The narrower range of tumour $^{10}$B concentrations in the tumours undergoing BBBD with ultrasound, in combination with higher accumulation of $^{10}$B in infiltrating cells along the tumour-brain interface, may substantially reduce under-treatment with BNCT. As with BPA-f studies achieving a similar [$^{10}$B] in tumours, we would expect a similar improvement in survival, and with expanding clinical investigations of therapeutic ultrasound, translation of these results to the clinical domain may soon be possible.
3 DELIVERY OF TARGETED IMMUNE CELLS TO HER2-AMPLIFIED BRAIN TUMOURS

3.1 Introduction

The HER2 receptor is thought to be involved in signalling pathways regulating cell growth and differentiation (Slamon et al., 1987). It is expressed in a number of epithelial tumours including glioblastoma and breast cancer (Mineo et al., 2007; Slamon et al., 1987). Breast cancers with HER2 amplification are more aggressive, have a higher risk of CNS metastasis, and poorer prognosis (Slamon et al., 1987; Lin et al., 2004). As many as 30% of breast cancer patients develop CNS metastasis (Lee 1983), for which current breast cancer therapies are largely unsuccessful (Lin 2004). As described in Section 1.2.2, systemic therapies are hindered by the cerebral capillary endothelium and the BBB. Within the tumour there is a heterogeneously permeable BTB, which while less of an obstacle than the healthy BBB, still manages to significantly hinder treatment efforts (Lockman et al., 2010). Furthermore, P-glycoprotein, expressed by cerebral capillaries and tumour cells, results in the efflux of many chemotherapeutic agents that would otherwise have therapeutic activity (Linn et al., 1995). As a result, intravenously delivered agents currently have limited efficacy in the treatment of brain metastasis.

Antibodies targeted to HER2 have resulted in improved tumour control and survival in HER2 amplified breast cancers (Slamon et al., 2001), while the HER2 inhibitor lapatinib has shown improved progression-free survival in advanced HER2-positive breast cancers (Geyer et al., 2006). Similar targeted treatments have been investigated in pre-clinical models using natural killer (NK) cells, the cytotoxic lymphocytes involved in the innate immune response to malignant cells (Smyth et al., 2002). The HER2-specific NK-92-scFv(FRP5)-zeta cell line is a human NK-92 cell line modified to express a chimeric HER2 antigen receptor (Uherek et al., 2002). It has been shown to localize to extracranial HER2 amplified breast cancers in animal models and cause selective tumour cell death (Uherek et al., 2002; Daldrup-Link et al., 2005; Meier et al., 2008). However, neither HER2-targeted antibodies nor NK-92 cells are able to cross
the BBB to a significant extent (Pestalozzi et al., 2000; Stemmler et al., 2007), while lapatinib is susceptible to P-glycoprotein and breast cancer resistance protein (Polli et al., 2008).

We investigated the feasibility of delivering HER2-specific NK-92 cells to the brain with focused ultrasound (FUS) because in contrast to antibodies and inhibitors, these cells are simultaneously targeted, possess direct cytolytic activity, and are unaffected by efflux channels (Smyth et al., 2002). Cavitation of microbubbles under the influence of ultrasound causes temporary disruption of the BBB with increased para- and transcellular passage of molecules from the blood into the brain (Sheikov et al., 2004, 2008). This technique has been shown to increase the penetration and accumulation of a number of therapeutic agents into the brain in rodent models, including BPA-f as we demonstrated in Chapter 2 (Kinoshita et al., 2006; Liu et al., 2010; Burgess et al., 2011; Alkins et al., 2013b). We examined whether HER2-specific NK-92-scFv(FRP5)-zeta cells injected intravenously could be delivered to the brain using MRI-guided FUS in a xenograft HER2-expressing breast metastasis model. By transfecting the HER2-specific NK-92 cells with superparamagnetic iron oxide (SPIO) nanoparticles, we were able to track their accumulation in the brain. Furthermore, we quantified the density of effector-to-tumour cells in order to confirm that the number was sufficient to successfully treat a tumour, and assessed the cytolytic function of HER2-specific NK-92 cells following exposure to ultrasound in vivo.

3.2 Materials and Methods

3.2.1 Cell Lines and Culture

Human HER2-expressing MDA-MB-231 breast tumour cells were cells were isolated from brain metastases, secondary to a primary tumour implanted in a mouse mammary fat pad, and transfected to express HER2 as previously reported (Francia et al., 2009). MDA-MB-231-HER2 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Wisent, St. Bruno, QC Canada). For implantation, cells were collected, centrifuged and resuspended in Hanks’ balanced salt solution (HBSS; Wisent) at a concentration of $10^3$ cells/μl. HER2 expression was confirmed using immunocytochemistry. MDA-MB-231-HER2 cells were plated on 8 well chamber slides (Nunc, Roskilde, Denmark) for 24 hrs and then fixed with 4%
paraformaldehyde for 10 min at room temperature. Cells were treated with blocking solution containing 1% donkey serum for 1 hr followed by incubation with primary rabbit anti-HER2 (Thermo Fisher Scientific, Nepean, ON Canada) overnight. Slides were washed and incubated with donkey anti-mouse Cy3 for 1 hr, and coverslipped. Fluorescent images were captured with a Zeiss Axiovert microscope.

The human cell line, NK-92 (ATCC, Manassas, VA), was virally transduced to stably express a chimeric antigen receptor specific to HER2 (Uherek et al., 2002). The antigen receptor expression was confirmed by fluorescence activated cell sorter (FACS) analysis (Uherek et al., 2002). HER2-specific NK-92-scFv(FRP5)-zeta cells were maintained in X-VIVO 10 medium (Lonza, Basel, Switzerland) supplemented with 5% heat- inactivated human serum (Cedarlane, Burlington, ON Canada), 0.6 mg/ml G418 (Wisent, St. Bruno, QC Canada) and 100 μg /ml IL-2 (RnD systems, Minneapolis, MN USA). Expression of CD45 was evaluated on a cell smear of targeted cells using mouse anti-human CD45 (RnD systems, Minneapolis, MN USA) and the procedures described above. Prior to use in vivo, HER2-specific NK-92 zeta cells were labeled with SPIO nanoparticles (diameter 10-50 nm) as MRI contrast. 100 μg Fe₃O₄ (Sigma-Aldrich, Oakville, ON Canada) was combined with Lipofectamine transfection reagent (Invitrogen, Burlington, ON Canada) for 30 min and subsequently added to 10⁶ NK-92 cells in normal media for 24 hrs at 37°C (Daldrup-Link et al., 2005). Cells were collected, centrifuged and re-suspended in sterile, physiological saline at a concentration of 10⁷ cells/ml for injection in vivo. Transfection efficiency was estimated using light microscopy to identify SPIO nanoparticles within the cell cytoplasm.

3.2.2 Tumour Implantation

All procedures were approved by the Sunnybrook Research Institute Animal Care and Use Committee and conformed to the guidelines set out by the Canadian Council on Animal Care. General anesthesia was induced and maintained with isoflurane in 250-300 g male athymic nude rats (Charles River, Sherbrooke, QC Canada). A burrehle was fashioned in the skull and cells were implanted in the left frontal striatum with a Hamilton syringe guided by stereotactic frame, at a depth of 4 mm from the cortical surface. MDA-MB-231-HER2 cells were combined with a 1% agar solution at 37°C to a final density of 10⁵ cells/μL, and 10 μL were injected
(Kobayashi et al., 1980). Animals underwent serial MRI imaging and the tumours treated once they had reached 1.5-2 mm in size.

### 3.2.3 MRI-guided Focused Ultrasound

In preparation for BBBD, animals were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). A 22-gauge cannula was inserted into the tail vein and the head of the animal shaved and chemically depilated. Definity® ultrasound contrast (Lantheus Medical Imaging, North Billerica, MA) was prepared as directed by the manufacturer by warming to room temperature and activating in the proprietary agitator (VIALMIX®, Lantheus Medical Imaging). It was then diluted 1:10 in 0.9% saline. Animals were positioned supine on the in-house designed 3-axis positioning system (Chopra et al., 2009) and fixed in place with a bite bar and lower extremity restraints. The head was coupled to a 551.5 kHz single element focused ultrasound transducer (F=0.8, R=10 cm) through a bath of degassed water and surrounded by an in-house manufactured 3x5 cm (inner dimensions) rectangular RF surface coil (Figure 3.1). The focal spot size for this transducer, characterized by the full-width at half maximum of the beam pressure profile, corresponded to a diameter of 3.0 mm in the axial plane (x-y plane in Figure 3.1) by 12.5 mm in the beam direction (z direction in Figure 3.1). The tumours were therefore completely encompassed by the focus.
Figure 3.1: The experimental MRI-guided FUS setup. The anesthetized animal was positioned supine over an MRI RF surface coil and coupled to the piezo-ceramic focused transducer through a bath of de-gassed water. The transducer was repositioned with the aid of a computer-controlled three-axis positioning system, allowing the ultrasound focus to reach any point within the brain. The entire setup fits in the bore of the 1.5T MR scanner, allowing for coordinate co-registration, targeting and confirmation of BBBD.

Targeting and registration images were acquired with a 1.5T clinical MRI scanner (Signa, GE Healthcare, Milwaukee, WI). All animals underwent baseline T2- (FOV 6 cm, 128x128 matrix, slice thickness 1 mm, NEX 2, ETL 4, TR 2000 ms, TE 75ms) and T1-weighted (FOV 6 cm, 128x128 matrix, slice thickness 1 mm, NEX 6, ETL 4, TR 500, TE ~10ms) MR imaging, including a contrast enhanced (0.2 ml/kg; Omniscan, GE Healthcare, Milwaukee, WI) sequence,
to identify the tumour. Following MRI and ultrasound co-registration, the computer-controlled 3-axis positioning system allowed the focal spot of the transducer to be positioned at any location within the brain. BBBD was performed (10 ms pulses, 1 Hz pulse repetition frequency, 120 s total duration) using a controller to modulate the acoustic power (0.33 MPa average estimated peak rarefaction pressure in the brain; range 0.32-0.35 MPa) (O’Reilly and Hynynen, 2012). The estimated in-situ pressure was determined assuming 73% transmission through the rat skull (Goss et al., 1979) and an attenuation coefficient of 5.0 Np/m/MHz in brain (Hynynen et al., 2001) with an acoustic path length of 5 mm.

A 60 s infusion of diluted Definity® (0.2 ml/kg) was administered via the tail vein catheter simultaneously with the onset of the sonication. Post-treatment proton-density and contrast-enhanced T1-weighted sequences were acquired to assess the location and degree of BBBD. Contrast enhancement (CE) on T1-weighted images was calculated as CE=(I_{post} – I_{pre})/I_{pre}, where I_{pre} and I_{post} are the pre- and post-contrast average signal intensities of the tumour, each normalized by the intensity in the contralateral hemisphere (where the post-contrast scan was taken 5 minutes after BBBD). The CE was then used to characterize the increase in vascular permeability due to the sonication. To detect iron oxide-labelled cells, imaging was conducted on a 7T MRI (BioSpec 70/30 USR, Bruker BioSpin, Billerica, MA) fitted with gradient and shim inserts (BGA-S, Bruker BioSpin) and using a circularly polarized rat brain RF coil (Bruker BioSpin). Both proton density (TR=3000 ms, TE=37.2 ms, flip angle=180°) and T2*-weighted (8 echo train, TR=1000 ms, TE=24 ms, flip angle=30°) sequences were performed. The change in signal intensity within the tumour on T2*-weighted images (19 ms echo) following treatment with SPIO-labelled cells, compared with pre-treatment, was quantified in order to ascertain whether this was a feasible means of monitoring the therapy.

Contrast enhancement (CE) on T1-weighted images was calculated as CE=(I_{post} – I_{pre})/I_{pre}, where I_{pre} and I_{post} are the pre- and post-contrast average signal intensities of the tumour, each normalized by the intensity in the contralateral hemisphere (where the post-contrast scan was taken 5 minutes after BBBD). The CE was then used to characterize the increase in vascular permeability due to the sonication.
3.2.4 Treatment Groups

Three treatment arms received intravenous injection of \(10^9\) targeted NK-92 cells/m\(^2\) body surface area (estimated from body weight), on the order of the number of cells delivered in previous phase I/II trials (Tonn et al., 2001; Arai et al., 2008). Group 1 (n=4) received HER2 specific NK-92 cells but no BBBD. Group 2 (n=4) received HER2 specific NK-92 cells following BBBD. Group 3 (n=5) had HER2 specific NK-92 cells injected immediately prior to BBBD. The experimental timeline for the three treatment groups is shown in Figure 2.

![Figure 2: Experimental timeline for the three groups. All groups initially underwent baseline imaging to assess the tumour size and location. In group 1 (control) this was followed by injection of SPIO nanoparticle labeled HER2-specific NK-92 cells. Follow-up imaging was performed at 16 hours following cell injection and immediately prior to sacrifice. Group 2 underwent BBBD, followed 5 minutes later by T1-weighted MRI with contrast to assess the change in contrast extravasation. Cells were then injected approximately 30 s after the completion of the imaging. In group 3, cells were injected via the tail vein and BBBD initiated 30 s after the injection. T1-weighted MRI was performed 5 minutes post-BBBD again to assess the change in contrast extravasation. Both groups 2 and 3 were imaged at 16 hours following the cell injection and immediately euthanized.](image)

3.2.5 Histological Analysis

Animals were sacrificed by euthanyl injection 12-16 hrs following treatment. Brains were removed, fixed in 10% neutral buffered formalin, and serial 4 \(\mu\)m coronal sections cut at 250 \(\mu\)m levels. All sections were stained with Hematoxylin & Eosin (H&E) for morphological analysis,
and Prussian blue for the detection of SPIO nanoparticles (Polysciences, Inc., Warrington, USA). IHC using the polymerized reporter enzyme staining system (Vector Laboratories, Burlingame, USA) was used to detect CD45 (mouse anti-human, 1:200, R&D Systems, Minneapolis, USA), perforin (mouse anti-human, 1:10, Abcam, Cambridge, USA) and granzyme B (rabbit anti-human, 1:100, Abcam, Cambridge, USA). Sections were digitized with a Mirax Scanner (Zeiss, Germany) and analyzed using Pannoramic Viewer (v.1.15, 3DHISTECH, Budapest, Hungary). For each animal, 4 sequential levels were analyzed by two blinded investigators. HER2-specific NK-92 cells were identified by CD45 expression and intra-cellular iron on Prussian blue histochemistry performed on serial sections at each level. Effector cells within the tumour boundary were counted and expressed as a percentage of the number of tumour cells in each section. Effector cells outside of the tumour region were ignored for this analysis. The average ratio (effector per 100 tumour cells, mean ± SEM) was then calculated for each treatment group.

### 3.2.6 Cytotoxicity Assay

Cytotoxicity assays were performed to determine the extent of MDA-MB-231-HER2 cell death achieved using differing effector-to-tumour cell ratios. $10^6$ MDA-MB-231-HER2 cells were plated and maintained for 2 hrs at 37°. Serial dilutions ($10^6$, $10^5$, $10^4$) of HER2-specific NK-92 cells were prepared in fresh medium and added to the MDA-MB-231-HER2 cell layer (corresponding to effector:tumour cell ratios of 1:1, 1:10 and 1:100). At 2 and 24 hrs, the cells were rinsed, collected and analyzed using Trypan blue cell exclusion dye. Results were expressed as the ratio of dead cells to the total number of cells counted. Similar experiments were completed after iron transfection.

### 3.2.7 Statistical Analysis

GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for statistical analysis. Student’s t test was used to compare the means ± SEM of two groups and the means ± SEM of three or more groups were compared with one-way ANOVA and Tukey’s post-test. Statistical significance was noted if $p < 0.05$. 

65
3.3 Results

Prior to implantation, HER2 expression in MDA-MB-231-HER2 cells was determined to be 80% using immunohistochemistry. CD45 expression by HER2-specific NK-92 cells was found to be 100% using IHC. The average transfection efficiency of HER2-specific NK-92 cells with SPIO nanoparticle was 80% and did not affect their cytolytic function.

Figure 3.3: The results of blood-brain barrier disruption with FUS. Representative T1-weighted MR images with Omniscan contrast of the tumour before and after BBBD, A and B respectively. The average enhancement of untreated tumours was 17 ± 8 % but increased to 34 ± 10 % following exposure to ultrasound and microbubbles (mean ± SD, p<0.05). A small region of
erythrocyte extravasation (arrow) was seen in one of the treatment animals, C. There was no further evidence of tissue injury.

Representative pre- and post-treatment contrast-enhanced T1-weighted MR images are shown in Figure 3.3 A and B. The degree of contrast enhancement following BBBD was relatively uniform and was double that of the tumour region prior to treatment (34 ± 10% versus 17 ± 8%, p=0.01). This represents the relative increase in extravasation of gadolinium contrast following BBBD and confirms the anticipated effect of the ultrasound and microbubbles on the vascular permeability. All animals tolerated the cell injection and BBBD without complication, and recovered from general anesthesia to their pre-treatment condition. No abnormal behavior was noted in the 16 hour interval between treatment and sacrifice. Post-treatment 7T MR imaging detected HER2-specific NK-92 cells at the tumour site in group 3 by a -17 ± 4% change in the signal intensity versus a 14 ± 7% change in group 1 (p < 0.001). A decreased signal is expected with increasing SPIO concentration (and thus NK-92 cell density) because of the paramagnetic effect of the SPIO nanoparticles which results in shortening of the T2 relaxation time of the surrounding water molecules and a corresponding decrease in the signal intensity. Representative pre- and post-treatment T2*-weighted sequences are shown in Figure 3.3 A and B. The differences between other groups were not found to be statistically significant (p > 0.05, Figure 3.4C).

Following animal sacrifice, H&E stained sections were examined for tissue injury. One FUS-treated animal had a small region of erythrocyte extravasation adjacent the tumour (Figure 3C). HER2-specific NK-92 cells were counted using both CD45 expression and the presence of intracellular iron on Prussian blue stained sections, and expressed as number of effector cells per 100 tumour cells. Without BBBD, a very small number of HER2-specific NK-92 cells localized to the tumour (group 1, 0.09 ± 0.11 NK-92 cells per 100 tumour cells, Figure 3.5), and almost equally few reached the tumour when BBBD was done prior to the injection of cells into the circulation (group 2, 0.21 ± 0.15 NK-92 cells per 100 tumour cells, Figure 3.5). In contrast, the ratio of HER2-specific NK-92-to-tumour cells was increased five-fold when the former were circulating at the time of sonication compared to injection afterward (group 3, 0.95 ± 0.23 NK-92 cells per 100 tumour cells, Figure 3.5). The number of effector cells accumulating in the
sonicated tumours in group 3 corresponds to approximately 0.34±0.09% of the total number of cells injected. This increase was statistically significant when compared to the former two groups (p < 0.01, Figure 3.5B). There was no statistical difference between group 1 and 2. More dense accumulations of cells were observed around arterioles and venules in group 3, with an effector:tumour ratio significantly greater than the volume-averaged value (Figure 3.6 A and C). Furthermore, cells were identified outside of the tumour boundary in a number of sections in group 3 but not counted in the analysis; theoretically these cells could migrate to the tumour and contribute to a higher effector to tumour cell ratio.

![Figure 3.4](image)

**Figure 3.4:** HER2-specific NK-92 cell detection with 7T MRI. A baseline axial T2*-weighted MR image from group 3 is shown with the tumour identified in the left frontal striatum (white arrow), A. The corresponding post-treatment image demonstrates a signal reduction at the tumour site (white arrow), B. C shows the average signal intensity change (mean ± SEM) at the tumour site following treatment. A negative change suggests the accumulation of iron-labelled effector cells. There was a statistically significant difference between group 3 and group 1.

We also examined whether HER2-specific NK-92 cells circulating prior to BBBD would be negatively affected by interactions with oscillating microbubbles at the ultrasound focus. Perforin and granzyme B IHC demonstrated that HER2-specific NK-92 cells retained their function following translocation into the brain. Figure 3.7 illustrates a targeted NK-92 cell within the tumour having released granzyme B, which can be identified within the cytoplasm of surrounding tumour cells. Perforin was similarly seen in the extra-cellular space surrounding
effector cells and within the cytoplasm of tumour cells. Tumour cells undergoing apoptosis following interaction with HER2-specific NK-92 cells are seen in Figure 3.7 A and B.

**Figure 3.5:** Histological quantification of HER2-specific NK-92 cells accumulating at the tumour site. Effector cells were co-localized with CD45 IHC (upper panel) and Prussian blue histochemistry (lower panel) in the three experimental groups, A. HER2-specific NK-92 cells reaching the tumour were quantitatively assessed (mean ± SEM), B. When NK-92 cells were injected prior to BBBD, the number reaching the tumour was significantly higher than if they were injected following or without BBBD (group 3 vs groups 1 and 2: 0.95 ± 0.23 vs 0.09 ± 0.11, 0.21 ± 0.15, p < 0.01). There was no statistical difference between groups 1 and 2. These results are in agreement with the iron-sensitive MR imaging in Figure 4.

Cytotoxicity assays were performed to estimate whether a sufficient number of HER2-specific NK-92 cells accumulated at the tumour sites following BBBD for efficient tumour cell lysis. At the 2-hr time point there was a higher proportion of dead MDA-MB-231-HER2 cells in the 1:10 and 1:1 NK-92:tumour groups, but at 24 hrs there were no statistically significant differences between the three different starting ratios (p > 0.05, **Figure 3.7C**).
Figure 3.6: FUS causes the translocation of HER2-specific NK-92 cells from the vasculature into the brain and tumour when they are present in the circulation at the time of BBBD. A, CD45 IHC depicting a vessel from which a large number of cells have extravasated and appear to track to the tumour (indicated by the star). B, the corresponding Prussian blue stained section is shown, colocalizing the HER2-specific NK-92 cells. C, a normal capillary adjacent the tumour but within the sonicated region, shows HER2-specific NK-92 cells forced to the adluminal surface of the vessel. FUS results in HER2-specific NK-92 cells circumventing both the BBB and BTB. D, the corresponding Prussian blue section. These cell distributions were seen exclusively in group 3 animals.

3.4 Discussion

HER2-specific immune cells have demonstrated in vivo efficacy against HER2-amplified extracranial tumours when injected into the blood stream (Uherek et al., 2002; Daldrup-Link et al., 2005; Meier et al., 2008). Intracranially, however, successful treatment has necessitated direct injection of targeted immune cells into the tumour through a burrhole in the skull (Ahmed et al., 2007, 2010). In the present study, we have demonstrated for the first time the feasibility of
using MRI-guided FUS to allow intravenously injected HER2-specific NK-92 cells access to tumours in the brain. SPIO-laden effector cells were detected at the tumour site with T2*-weighted MRI in group 3. SPIO nanoparticles are paramagnetic, resulting in shortening of T1 and T2 relaxation times, and thus an increase in signal on T1-weighted imaging (similar to the paramagnetic MRI contrast gadolinium) and a decrease in signal on T2*-weighted sequences. Although an average signal decrease over the tumour volume was detected, quantitative measurements are challenging with paramagnetic iron in the human body because iron is at the core of the heme molecule, the key subunit of hemoglobin in the blood and thus ubiquitous in the body. Deoxyhemoglobin and methemoglobin, formed as biproducts of hemoglobin metabolism after extravasation, are both paramagnetic and generate similar shortening of both T1 and T2 relaxation times. Therefore, extravasated erythrocytes from the time of the tumour implantation could have resulted in paramagnetic heme breakdown products at the tumour site that could have interfered with the analysis. That being said, no significant increased T1 signal was appreciated within the tumours at the first imaging session to suggest the presence of blood products. Furthermore, the effect if any would be seen in all groups. A similar factor that could affect the analysis is the extravasation of erythrocytes due to BBBD itself. The presence of intra-tumoural heme products in human patients, either due to BBBD, or due to spontaneous hemorrhage within the tumour, could compromise the in vivo tracking of SPIO nanoparticle-labeled effector cells and may not be the optimal method for monitoring the accumulation of targeted cells in the tumour.

The order of BBBD and cell injection was found to have a significant impact on the number of HER-specific NK-92 cells reaching the tumour (Figure 3.5 A and B). The exact mechanisms underlying this increase in HER2-targeted NK-92 cell accumulation in the sonicated tumour volume are unknown. Despite identical ultrasound exposures, the presence of HER2-targeted NK-92 cells in the circulation at the time of BBBD resulted in an almost five-fold increase in the ratio of effector-to-tumour cells. One possibility is that inter-endothelial clefts may be larger during the sonication, facilitating the extravasation of effector cells present during BBBD. It is uncertain whether the effect of BBBD on endothelium stimulates or inhibits diapedesis, but if priming of the endothelium was the sole mechanism responsible for effector cell extravasation at the tumour site, the order of cell injection should have made little difference. It has been
previously reported that FUS stimulates the release of nitric oxide (NO) from endothelial cells \textit{in vitro} (Atland et al., 2004; Hsu and Huang, 2004), as well as increases the expression of caveolin-1 \textit{in vivo} (Deng et al., 2012), and thus it is conceivable that BBBD could affect the expression of proteins involved in leukocyte adhesion. Furthermore, the interaction of ultrasound and microbubbles with the HER2-targeted NK-92 cells may play a role. Microbubbles undergoing stable cavitation are known to generate shear stresses (Dewitz et al., 1977; Ward et al., 2004; Wu 2007), and increased fluid shear stresses have a number of \textit{in vitro} effects on activated leukocytes including increased adhesion (Okuyama et al., 1996; Moazzam et al., 1997), deformability (Kitayama et al., 2000), motility (Cinamon et al., 2001), and transmigration (Cinamon et al., 2001; Marty et al., 2012). Although speculatory, it is possible that circulating HER2-specific NK-92 cells are more likely to adhere to endothelium and extravasate following exposure to fluid shear stresses generated by microbubbles at the ultrasound focus. These \textit{in vitro} studies typically required fluid shear stresses in the setting of an activated endothelium, and therefore from the group 2 results (where the endothelium was exposed to FUS but the HER2-specific NK-92 cells were not) the effect of BBBD on the endothelium alone is one that at least partially promotes leukocyte extravasation, either by disrupting endothelial tight junctions or through some unknown mechanism. Finally, there may be direct mechanical effects of the oscillating microbubbles on the effector cells that have yet to be elucidated.
Figure 3.7: HER2-specific NK-92 cells accumulate at the tumour and have preserved function. A, IHC for granzyme B highlights an NK-92 cell releasing granzyme B into the surrounding extra-cellular space (white arrows). An adjacent apoptotic tumour cell can be seen (black arrow). B, granzyme B-containing NK-92 cell (white arrow) causing apoptosis in a tumour cell (black arrow). At 24 hrs, a 1:100 ratio of effector:tumour cells is statistically no different (p > 0.05) in causing tumour cell lysis than higher starting ratios, C. This is the ratio of targeted NK-92-to-tumour cells that was achieved in vivo in group 3.

In addition to the increased accumulation of HER2-specific NK-92 cells in group 3 tumours, dense accumulations of HER2-specific NK-92 cells around arterioles and venules in group 3 (Figure 3.6 A and C). Larger vessels may have permitted greater microbubble activity (decreased bubble damping compared to capillary sized vessels, or a bubble population with a
mean size closer to the resonant frequency used), or possibly was able to support larger inter-
endothelial openings. Along capillaries, the effector cells were seen to line the adluminal surface,
even in the absence of nearby tumour cells (Figure 3.6 B and D). We did not see any of these
features in group 1 or 2 animals. Of particular note in both ultrasound-treated groups is the
absence of erythrocytes amongst extravasated HER2-specific NK-92 cells (there was a small
region of erythrocyte extravasation adjacent the tumour absent effector cells). It is believed this
is in keeping with the postulated mechanism for the increased leukocyte extravasation with
BBBD. The inter-endothelial openings, only studied post-BBBD, have been shown to be on the
order of tens to hundreds of nanometers, and thus much smaller than the diameter of an
erthrocyte (2002; Sheikov et al., 2004). Erythrocytes are typically dispersed with little
adherence to endothelial cells in the face of fluid shear stresses (Yedgar et al., 2002), whereas the
reverse is true for leukocytes (Okuyama et al., 1996; Moazzam et al., 2000; Kitayama et al.,
2000; Cinamon et al., 2001).

Of significant concern was whether HER2-specific NK-92 cells circulating at the time of the
BBBD would maintain their cytolytic function. Figure 3.6 A and C show a large group of
extravascular HER2-specific NK-92 cells surrounding an extra-tumoural venule. In such a vessel
(outside the tumour but within the ultrasound focus), the direction of extravasation should be
random, but the preferential clustering of cells toward the tumour suggests preserved bioactivity
of the cells. Furthermore, perforin and granzyme B IHC provided evidence that these mediators
of apoptosis were being released into the intercellular space, and a number of apoptotic cells
were identified in close association with HER2-specific NK-92 cells (Figure 3.7 A and B). In
addition to preserved biological function, we estimated that the effector-to-target cell ratios
realized would be sufficient to result in efficient tumour cell lysis (Figure 3.7C). Because the
SPIO nanoparticle transfection efficiency was less than 100%, the calculated densities likely
underestimate the actual number of HER2-specific NK-92 cells reaching the tumours. Although
free SPIO could have potentially accumulated at the tumour site and resulted in an
overestimation, the SPIO-transfected cells were washed prior to injection to reduce the presence
of any extracellular iron oxide.

We have demonstrated for the first time the feasibility of using targeted immune cells combined
with MRI-guided FUS to treat tumours in the brain. This treatment holds significant promise as it
is specific not only to the environs of the tumour, but also to the malignant cells themselves, and can be performed non-invasively under MRI guidance. Furthermore, theoretically the NK-92 cells could be re-targeted to any number of cancer-associated antigens to allow the treatment of a variety of human malignancies. Future studies will aim to show a reduction in tumour volume and an improved survival in brain metastasis-bearing rats, as these are real-world indicators of treatment success. The NK-92 cell line has already been used in early clinical trials (Tonn et al., 2001; Arai et al., 2008) and with the expanding clinical testing of FUS, and also the recent demonstration of the safety and repeatability of BBBD in rhesus macaques (McDannold et al., 2012), this treatment has the potential to benefit patients with brain metastasis in the foreseeable future.

4 EARLY TREATMENT OF HER2-AMPLIFIED BRAIN TUMOURS WITH TARGETED NK-92 CELLS AND FOCUSED ULTRASOUND IMPROVES SURVIVAL

4.1 Introduction

As previously discussed in section 1.3, malignant brain tumours affect a significant number of North Americans, with both primary and metastatic tumours having a dismal prognosis. Macroscopic or microscopic residual tumour often remains after surgical resection, necessitating adjuvant chemo- and/or radiotherapy. Many chemotherapeutic agents are hindered by the cerebral capillary endothelium and BBB. The BBB normally allows passage of only small lipophilic compounds into the CNS, while efflux channels, expressed by both malignant and endothelial cells, limit the accumulation of many agents that would otherwise have therapeutic activity. Adjuvant therapies that successfully reach the brain often result in varying degrees of injury to healthy tissues (Kannarkat et al., 2007). As a result, intravenously delivered agents currently have a modest efficacy in the treatment of brain tumours as has been previously discussed in section 1.3.
In the previous chapter we investigated whether targeted natural killer (NK) cells could be delivered to focal regions of the brain, harbouring a metastatic tumour, using focused ultrasound combined with microbubbles (Alkins et al., 2013). As discussed in Section 3.1, targeted NK cells have advantages over other systemic therapies including their very specific cytotoxicity to malignant cells, which is further heightened when encountering those malignant cells expressing the target antigen. As such, once these cells gain access to the CNS, they can track to their relatively defenseless target tissues without any known impact on the healthy brain. In the current chapter, we investigate whether multiple combined treatments of targeted NK cells and FUS could slow tumour growth and improve survival in a HER2-amplified brain tumour model, using a human breast cancer line as a prototype. Many tumours express proteins that could be exploited for targeted therapies. As previously described in Section 3.1, one such protein is the HER2 receptor, thought to be involved in signalling pathways regulating cell growth and differentiation, and expressed by a number of epithelial tumours including breast and glioblastoma (Slamon et al., 1987 Mineo et al., 2007). Breast cancers with HER2 amplification are more aggressive, have a higher risk of CNS metastasis, and poorer prognosis (Slamon et al., 1987; Lin et al., 2004). Antibodies targeted to HER2 have resulted in improved tumour control

![Figure 4.1: Schematic depiction of BBB disruption with FUS in the rodent brain using encapsulated perfluorocarbon microbubbles (commercially available as ultrasound contrast). A) A focused ultrasound transducer delivers sub-megahertz pressure waves to a precise location within the brain. Under the influence of these waves, the microbubbles undergo stable cavitation and result in NK-92 translocating into the tumour. B) Images from Alkins et al. 2013b showing the NK-92 cells on the abluminal surface of vessels following exposure to FUS and ultrasound contrast. The cells are identified with CD45 (above) and Prussian blue (following transfection with iron nanoparticles, below). The cells can be seen tracking to the tumour following egress from the vasculature.](image-url)
and survival in HER2 amplified breast cancers, but along with NK cells, do not normally cross the BBB (Abbott et al., 2006).

NK cells are cytotoxic lymphocytes involved in the innate immune response to malignant cells (Smyth et al., 2002). The HER2-specific NK-92-scFv(FRP5)-zeta cell line is a human NK-92 cell line modified to express a chimeric HER2 antigen receptor (Uherek et al., 2002). It has been shown to localize to extracranial HER2 amplified tumours and cause selective tumour cell death (Uherek et al., 2002; Daldrup-Link et al., 2005). We have demonstrated that it is possible to deliver this targeted NK-92 cell line to tumours in the brain using MRI-guided FUS, in combination with microbubble ultrasound contrast agents, to disrupt the BBB (BBBD, Figure 4.1; Chapter 3; Alkins et al., 2013b). The stable cavitation of microbubbles under the influence of sub-megahertz ultrasound frequencies temporarily alters the permeability of the cerebrovasculature in focal regions by disrupting endothelial tight junctions as well as increasing para- and transcellular transport as described in Section 1.5.

We hypothesized that HER2-specific NK-92 cells injected IV and delivered to the brain using MRI-guided FUS, in a xenograft HER2-expressing breast metastasis model, would reduce tumour progression and prolong survival. Based on our previous observations, we anticipated that the effect would be more pronounced with a smaller tumour burden. With the recent demonstration of safe BBB-disruption with FUS in non-human primates (McDannold et al., 2013) potential exists for clinical translation if successful.

4.2 Methods

4.2.1 Study Design

This was a prospective, randomized, blinded study in nude rats to determine if targeted NK-92 cells delivered to the brain could prolong survival in a breast metastasis model. All procedures were approved by the Sunnybrook Research Institute Animal Care and Use Committee and conformed to the guidelines set out by the Canadian Council on Animal Care.
4.2.2 Cell Lines and Tumour Implantation

Human HER2-expressing MDA-MB-231 breast tumor cells were isolated from brain metastases as previously reported and transfected to express high levels of the HER2 receptor (Francia et al., 2009). HER2 expression was confirmed by immunocytochemistry with primary rabbit anti-HER2 (Thermo Fisher Scientific, Nepean, ON Canada). For tumour implantation, MDA-MB-231-HER2 cells were combined with BD Matrigel (BD Biosciences) at a density of $10^3$ cells/µL. 5 µL were injected stereotactically in the right frontal striatum of 200-250 g male athymic nude rats (Charles River, Sherbrooke, QC Canada). Treatments began exactly one week following cell implantation.

The human cell line, NK-92 (ATCC, Manassas, VA), was virally transduced to stably express a chimeric antigen receptor specific to HER2 (Uherek et al., 2002). The antigen receptor expression was confirmed by fluorescence activated cell sorter (FACS) analysis. Expression of CD45 was evaluated on a smear using mouse anti-human CD45 (R&D systems, Minneapolis, MN USA). HER2-specific NK-92-scFv(FRP5)-zeta cells were maintained in X-VIVO 10 medium (Lonza, Basel, Switzerland) supplemented with 5% heat-inactivated human serum (Cedarlane, Burlington, ON Canada), 0.6 mg/ml G418 (Wisent, St. Bruno, QC Canada) and 100 µg/ml IL-2 (RnD systems, Minneapolis, MN USA). Cells were collected, centrifuged and resuspended in sterile, physiological saline at a concentration of $10^7$ cells/ml for injection in vivo.

4.2.3 Treatment Arms

A total of 31 animals each underwent 8 treatment sessions, entered into one of two arms each with a different schedule. Within each arm, animals were randomly assigned to receive combined FUS and targeted NK-92 cells (FUS+Cells), FUS alone (FUS) or cells alone (Cells). Cell injection consisted of intravenous injection of $10^9$ targeted NK-92 cells/m² body surface area (estimated from body weight), on the order of the number of cells delivered in previous phase I/II trials. The first arm (distributed) received treatments twice per week for four weeks (Figure 4.2; FUS+Cells N=7, FUS N=7, Cells N=6). The second arm (front-loaded) received 5 treatments the first week, 2 the second and 1 the third (Figure 4.2; FUS+Cells N=4, FUS N=3, Cells N=4). The order in which animals were treated at each of the 8 sessions was randomly
assigned.

**Figure 4.2:** Schematic representation of the two treatment arms. Within each arm there was a group receiving cells only, a group receiving FUS only and a group receiving combined treatment with FUS and cells. The distributed arm received an evenly distributed treatment over a 4-week period while the front-loaded arm received aggressive upfront therapy. Each animal, regardless of the arm, underwent a total of 8 treatment sessions. They were then followed with imaging twice weekly until they exited the study.

### 4.2.4 MRI-guided Focused Ultrasound

Definity® ultrasound contrast (Lantheus Medical Imaging, North Billerica, MA) was activated at room temperature with the VIALMIX® (Lantheus) agitator, diluted 1:10 in 0.9% saline, and a 20μl/kg dose injected with the onset of the sonication. Animals were positioned supine on an in-house fabricated sled and transferred between the 7T MRI and an ultrasound system. The latter consisted of a 551.5 kHz single element focused ultrasound transducer (F=0.8, R=10 cm) submerged in a bath of degassed water and combined with a three-axis positioning system. The focal spot size for the transducer, characterized by the full-width at half maximum of the beam pressure profile, corresponded to a diameter of 3.0 mm in the axial plane by 12.5 mm in the beam direction. BBBD was performed (10 ms pulses, 1 Hz pulse repetition frequency, 120 s total duration) using a controller to monitor the acoustic emissions and modulate the acoustic power to pre-determined ultraharmonic signatures (O'Reilly and Hynynen, 2012). The power was increased incrementally until this threshold was reached and then decreased to 60% of the peak power for the remaining duration of the sonication, which we previously found to result in successful translocation of NK-92 cells into the CNS (Alkins et al., 2013b).

All imaging was performed with a 7T MRI (BioSpec 70/30 USR, Bruker BioSpin, Billerica, MA) fitted with gradient and shim inserts (BGA-S, Bruker BioSpin). MR imaging consisted of
T2-weighted and contrast enhanced T1-weighted sequences (0.2 ml/kg; Omniscan, GE Healthcare, Milwaukee, WI) in at least two perpendicular planes for treatment planning, tumor volume estimate and quantification of BBB. Follow-up imaging performed beyond the treatment period (4 weeks for Group I and 3 weeks for Group II) consisted of axial and coronal T2-weighted MRI done twice weekly.

4.2.5 Criteria for Study Exit

Animals were sacrificed by euthanyl injection when they showed signs of pain, distress or when their tumor dimensions or mid-line shift exceeded predetermined threshold values. Animals were monitored on a daily basis by veterinary technicians unaffiliated with the study, who were blinded to the study groups. They were also assessed and weighed by study personnel at the beginning of each treatment day, prior to any imaging. Signs of distress were defined as weight loss greater than 20% in one week, lethargy, failure to groom or the presence of neurological deficits. Tumor dimensions were followed with serial 7T MR imaging, and a maximal tumor diameter of 10 mm, and/or midline shift greater than 2 mm, were taken as cutoffs for tumor burden and mass effect. This was consistent with previous studies where animals harboring tumors greater than these thresholds become rapidly symptomatic (Aryal et al., 2013).

4.2.6 Histological Analysis

Once sacrificed, the brain was removed, fixed in 10% neutral buffered formalin, and sectioned in 4 μm coronal sections cut at 250 μm levels. All histological analysis was blinded. A section at each level was stained with Hematoxylin & Eosin (H&E) for morphological analysis. At each level through the tumor a section was also stained for CD45 to survey for any remaining HER2-specific NK-92 cells. IHC using the polymerized reporter enzyme staining system (Vector Laboratories, Burlingame, USA) was used to detect CD45 (mouse anti-human, 1:200, R&D Systems, Minneapolis, USA). Sections containing the tumor were also stained for HER2 to examine whether the HER2-specific NK-92 changed or eradicated the HER2-amplified cells. Sections were digitized with a Mirax Scanner (Zeiss, Germany) and analyzed using Pannoramic Viewer (v.1.15, 3DHISTECH, Budapest, Hungary).
4.2.7 Statistical Analysis

Graph Pad Prism 6 (Graph Pad Software, San Diego, CA) was used for statistical analysis. The enhancement data was analyzed using student’s t-test. Survival data was compared using Kaplan-Meier analysis with the Mann-Whitney U test. The average survival ± standard deviation for each group was also tabulated and these were compared using a one-way ANOVA and Tukey’s post-test. Statistical significance was noted if p < 0.05.

4.3 Results

Prior to the initiation of the longitudinal experiments, the cytolytic activity of the targeted NK-92 cell line was evaluated using flow cytometry. The efficacy of tumor cell lysis was found to be proportional to the ratio of effector-to-target cells consistent with both our prior findings (Alkins et al., 2013b) and previous experience with this cell-line in the published literature (Uherek et al., 2002).

The success rate for establishment of a tumor following implantation was 100%. Tumor progression was rapid as quantitatively assessed on serial 7T MRI (Figure 4.3). For both treatment arms, the contrast enhancement measured on T1-weighted sequences following BBBD was not statistically different between FUS only versus combined FUS and targeted NK-92 cells (distributed arm, p=0.10; front-loaded arm, p=0.76; data not shown).

4.3.1 Tumor Surveillance

Tumors began to cause severe mass effect with brain distortion, and surrounding vasogenic edema at sizes nearing that pre-established for sacrifice. The ipsilateral frontal horn of the lateral ventricle showed progressive effacement, with relative enlargement of the remainder of the ventricular system, as the tumors progressively enlarged. Tumors showed solid growth for the first two to three weeks (as seen on MRI) but often began to develop cystic components, seen in serial MRI (Figure 4.3) and subsequently confirmed on histological examination. All animals except for one met the criteria for sacrifice based on size or mass effect rather than on the appearance of behavioral symptoms.
Figure 4.3: Representative images of the evolution of tumor size measured on axial T1-weighted gadolinium-enhanced MRI. The tumor can be readily identified in the right frontal lobe due to the extravasation of gadolinium contrast and corresponding increased signal on T1 MRI. At day 1 the tumors are relatively consistent in size, but by 2 weeks there has been significant interval growth, with some tumors showing non-enhancing regions likely representing cystic components seen on post-mortem histology. The only group that showed a significant treatment effect was the FUS+Cells group of the front loaded arm.

The two long term survivors in the up-front arm were followed with MRI (Figure 4.4) and did not show any evidence of tumor progression. A decrease in the volume of the frontal lobe in the region of the treated tumor was observed, as evidenced by the increased prominence of the ipsilateral frontal horn of the ventricle. It was impossible to distinguish whether the etiology of this tissue loss was due to the tumor or the treatment, or a combination of the two. The animal depicted in Figure 4.4B had a more prominent “inflammatory” reaction surrounding the tumor, with significant surrounding edema and increased signal intensity on T2-weighted MRI, which occurred beyond the end of treatments and resolved spontaneously with the disappearance of the tumor, but with more atrophy than was seen in the other animal.
Figure 4.4: The two final T2-weighted MR imaging studies of the long-term survivors, showing the structural impact of the treatment on the right frontal lobe. The animal in A was euthanized at 90 days due to pyelonephritis while the animal in B was healthy at the time of euthanasia at 150 days. Both animals showed some volume loss in the treated (right) frontal lobes, evidenced by the increased prominence of the corresponding frontal horn of the lateral ventricle. The treated region is highlighted by the dashed circle (not to scale).

4.3.2 Tumor Progression

Kaplan-Meier analysis of the distributed arm did not demonstrate a statistically significant difference in the median survival times (Figure 4.5); the log-rank test for trend gave a p-value of 0.075, which did not reach statistical significance. Tumor volumes in the group receiving both FUS and targeted NK-92 cells appeared to have a slower growth rate initially, but this was not statistically different than the other groups, and in particular by the completion of the 4 weeks of treatment the volumes were roughly equivalent (Figure 4.5B).
Figure 4.5: The effect of the therapy in the distributed treatment arm. In A the survival appears to be slightly greater in the FUS+Cells group but this was not statistically significant. The tumor volumes for the first 4 weeks are shown in B. While it looked as though the curves began to diverge into the 3rd treatment week, there was only a small difference by the end of the 4th week.

The front-loaded treatment arm was more successful, and in comparison with the distributed arm (Figure 4.5B) where the tumor volumes were nearly identical at the completion of 4 weeks of therapy, the tumor growth curves in the former (Figure 4.6 A and B) showed a marked divergence in the FUS+Cells group compared with both the FUS or Cell groups. Kaplan-Meier analysis showed a statistically significant difference in survival between the FUS+Cells group compared to the other two (p<0.05, Figure 4.7D). There was a statistically significant difference in the mean survival times between the FUS+Cells group of the front-loaded arm compared with the remaining groups (p<0.05). There was no survival difference between the FUS or Cells groups. The long-term surviving animals continued to be asymptomatic and were sacrificed at 150 days after the study onset.

This upfront treatment regimen was intensive and stressful for the animals, resulting in an arrest in weight gain for the Cell group, and a weight loss in the two groups receiving ultrasound and BBBD (Figure 4.7C). However, no animal had an adverse event related to the treatment schedule and by 14 days all animals demonstrated parallel increasing growth trajectories. Similar to what was seen in the distributed arm, approximately half of the animals appeared to have little to no response to the treatment, with survival times similar to the animals in the FUS and Cell alone groups.
Figure 4.6: The effect of therapy in the front-loaded treatment arm. In A and B, the normalized and absolute tumour volumes are shown over the first 4 weeks of treatment. The FUS only and Cell only curves are roughly overlapping, highlighting the anticipated ineffectiveness of either one of those therapies alone. In C, the normalized body weights decline over the first week during the intensive treatment schedule, but universally increase beyond that. As might be expected, the Cell group appears to have less weight-loss than the two ultrasound groups. In D, the statistically significant increase in survival can be appreciated in the FUS+Cells group. In the latter group, 2 of the 4 animals had long-term survival. One animal died at 90 days due to pyelonephritis, while the second survivor was euthanized after 150 days but was in good health and showed no ill effects of the treatment.

4.3.3 Histological Analysis

Levels through the brain were stained with H&E and reviewed for all animals. Representative histological images are depicted for the distributed arm in Figure 4.7 and for the front-loaded arm in Figure 4.8. There were no signs of hemorrhage or erythrocyte extravasation within the tumor itself or surrounding brain; however, most animals were sacrificed a week or more following the last sonication. Despite the differences in survival times for the animals depicted,
the appearance of the tumor at the time of sacrifice is similar, suggesting that our study exit criteria were relatively robust. Many of the tumors developed large cystic components that were visible on MRI surveillance. The brains uniformly showed dilatation of the ventricular system with significant midline shift and mass effect due to the large tumors. IHC for CD45 at the time of animal sacrifice did not reveal any surviving targeted NK-92 cells in the brain (Figure 4.7 D).

IHC for the HER2 protein was performed on levels through the tumor at the time of post-mortem examination. Despite confirming the parent tumor cell line to be 90% positive for HER2 by IHC prior to tumor implantation, there heterogeneous expression in both treatment arms, irrespective of treatment modality (Figure 4.8). In some cases it did appear as though the treated tumor volume contained a paucity of HER2 expression compared with the untreated tumor volume (Figure 4.8 B and D).
Figure 4.7: Representative distributed arm post-mortem histology. A, B, and C show a representative axially cut H&E section of the tumor at the time of euthanasia for the Cell, FUS, and FUS+Cells groups. At the time of death, the tumors were very similar in size, confirming that our study exit endpoints were relatively robust. However, the mean survival times were quite different, suggesting some benefit for FUS+Cells even in the distributed arm. D shows a section adjacent the H&E shown in C with IHC for CD45. There were no targeted NK-92 cells seen at the time of sacrifice in any of the animals, although these had last been injected at least a week prior to sacrifice.
Figure 4.8: Heterogeneity of HER2 expression at the time of animal sacrifice. A and B are representative H&E sections of the tumors from the two short-term survivors in the FUS+Cells group of the front-loaded arm. C is an animal from the Cell-only group of the front-loaded arm. D-F show higher magnification views of the tumors with IHC for the HER2 protein. A/D and C/F show no HER2 presence despite very different treatments. The tumor in B/F, which was treated with FUS+Cells, unfortunately developed leptomeningeal spread. Interestingly, possibly due to the treatment, the parenchymal portion of the tumour targeted by the treatment shows a drastically lower expression of HER2 compared to the leptomeningeal component. Regions of necrosis, as well as cystic components, are identifiable in the tumors shown in A and C.

4.4 Discussion

The therapeutic effect of delivering NK-92 cells in the present study could best be described as preventing progression rather than reducing tumor-burden once established. In its current state, this treatment scheme would best serve patients as a component of adjunct therapy to prevent recurrence following a resection or ablative procedure. The treatment effect was not uniform amongst animals in the treatment groups. Even in the front-loaded arm, where we saw our most
promising results, roughly half of the animals experienced a treatment failure relatively early on. This could be appreciated to a lesser extent even in the distributed arm. Interestingly, a similar phenomenon has been observed in other studies of FUS and brain tumors, where two distinct groups emerge – one with a promising response to the therapy and the other behaving similarly to the controls (Aryal et al., 2013). In the present study, these failures are unlikely to represent more resistant tumor phenotypes given that all the tumors were derived from the same parent cell line. One possible contribution could simply be the difficulties associated with scaling BBBD to the rodent. Our results suggest that the treatment is more effective when delivered to animals with a small tumor burden, so that an ineffective treatment early in the course of the study would have a pronounced effect. Unfortunately with the transducer geometry and ultrasound frequency in conjunction with the rat skull, standing waves are present in up to 25% of animals (O’Reilly et al., 2010). At a small tumor size, much of the tumor could be near a node, thus significantly reducing the volume receiving effective therapy. If this occurred early in course of treatment, it could have significantly impacted the overall response. Other features that could have contributed to a reduction of the treatment efficacy as the study progressed include the development of cystic compartments, as well as necrosis, both of which negatively affect vascular access of the targeted NK-92 cells to the tumor.

Prior to commencing our longitudinal studies, we examined the cytolytic function of the HER2-specific NK-92-scFv(FRP5)-zeta cell line. Consistent with work previously published using these cells, we found that the killing efficiency increased proportionally with an increasing ratio of effector-to-target cells (Uherek et al., 2002; Alkins et al., 2013b). Extending these in vitro results to the in vivo scenario, it should not be surprising that as the tumors got progressively larger, and the delivery of targeted NK-92 cells became more variable (for reasons previously postulated), a decreasing ratio of targeted NK-92-to-tumour cells contributed to the waning efficacy of the treatment.

Given that NK-92 cells do not persist long in the absence of IL-2, which is required for their successful culture, it is also not surprising that none were seen on post-mortem examination; because of the treatment and surveillance schedule, the most recently a treatment could have been given was three days prior to sacrifice, and in the case of the FUS+Cells treatments, typically more than one week. Concerns have previously been raised in the literature of the
possibility of an unabated clonal expansion of NK-92 cells but we did not detect any clinical evidence of this in the brain, nor did it impact the endpoints in the current study.

The tumor cell line used was known from our previous work to be approximately 90% positive for HER2. Post-mortem IHC for HER2 showed that the proportion of HER2-positive tumors cells was quite variable, regardless of the treatment group (Figure 4.8). In some Cell animals, where we expected virtually no targeted NK-92 cells to reach the tumor and therefore no selective pressure on HER2-positive cell proliferation, the tumor was virtually HER2-negative by the time it was examined histologically. On the other hand, in some cases it appeared as though the treated regions harbored fewer HER2-positive tumor cells, and those areas not accessible to ultrasound more uniformly expressed the protein. In one of the two FUS+Cells animals that died early on, leptomeningeal disease was seen on histological assessment (Figure 4.8E). Interestingly, this region of tumor was much more uniformly HER-positive compared with the parenchymal component that was targeted by the ultrasound; however, given that this was only seen in a single animal it is merely an observation. Regardless, despite verifying that a high percentage of cells over-expressed HER2 at the onset of experiments, the population evolved with considerable variability.

It might be expected that because the tumors were seeded from a cell line not homogeneously comprised of HER2-positive cells that effective therapy would select for a HER2-negative population and lead to the eventual progression of a more resistant HER2-negative tumor. This was not seen to be the case, as 50% of the FUS+Cells group was found to have no tumor at the time of post-mortem examination, and there remained HER2-positive cells in variable proportions in all groups. This may be explained by the fact that the HER2-specific NK-92 cells maintain cytotoxicity toward HER2-negative tumor cells as well, albeit with less affinity, so if they managed to reach the tumor they did not discriminate between HER2-positive and HER2-negative cells to an appreciable extent. Furthermore, if it is accepted that treatment is most effective when the tumor burden is small as our data suggest, by the time most tumors were analyzed they would have been growing unabated for weeks and therefore any changes might have been obscured.
We have demonstrated for the first time the modest but statistically significant survival advantage achieved using targeted immune cells combined with MRI-guided FUS to treat tumors in the brain. In its current formulation, this treatment paradigm might best be used in combination with other modalities, or for the treatment of residual tumor following a more cytoreductive procedure, as it does not appear to be an effective therapy when the tumor burden is large. The current pilot study was limited by the small sample size, owing to the highly resource intensive nature of these experiments. Future work might include optimization and augmentation of effector cell delivery, both in terms of overall numbers but also with a more uniform spatial distribution so that all portions of the tumor are treated. Other avenues of future research might include the investigation of the concomitant administration of systemic IL-2 to prolong the viability of the NK-92 cells, as well as a better understanding of the mechanisms by which ultrasound enhances the extravasation of NK-92 cells.

5 SUMMARY, DISCUSSION AND CONCLUSIONS

5.1 Introduction and Discussion

It has been known for more than 50 years that ultrasound can influence the permeability of the BBB, but since its combination with ultrasound contrast agents a little more than a decade ago, stemming from the groundwork laid by Hynynen et al., (2001), the number of studies has increased considerably. In the present body of work we have attempted to harness this highly promising technique of targeted, reversible BBBD to deliver targeted therapeutics to the brain with the eventual goal of improving survival in patients with intracranial malignancies, while sparing the normal brain and avoiding the significant morbidity associated with chemoradiotherapy. To this end, in chapter 2 we explored the advantages of combing FUS-mediated BBBD with BNCT. While we demonstrated significant improvements in the concentration of BPA-f delivered, there are inherent limitations with the agent and techniques that lead to irradiation of a relatively large volume of healthy brain. The first trials of BNCT in the early 1950’s at the Brookhaven and Massachusetts Institute of Technology reactors were discouraging, as the prototype boron delivery agents were boric acid and borate. The boron-
carrying agents used showed little predilection for malignant cells over the healthy brain, while also being present in the normal brain and blood at concentrations sufficient to cause significant off-target tissue damage and patient morbidity (Diaz, 2003). Even with a perfect delivery vehicle, there are capture reactions in tissue which result in a non-zero dose to the brain within the neutron beam path. While we have demonstrated that the uptake of BPA-f can be increased quite significantly with FUS-mediated BBBD, efforts might first be better directed towards engineering a boron delivery agent with higher specificity for malignant cells, after which time the combination with ultrasound might yield even more impressive gains. This is particularly true given the recent findings from Czarnota et al. (2012) that show a synergistic killing effect of ultrasound and microbubbles combined with radiotherapy. Finally, the infrastructure required for a longitudinal study of BNCT is highly limited at the present time. We were unable to find a collaborator within the province able to provide a suitable neutron source for the necessary treatments. The clinical centres capable of delivering such a treatment would similarly be quite limited, requiring both a clinical ultrasound device and slow neutron source in very close geographical proximity.

In chapter 3 we introduced the modified Natural Killer cell line, NK-92, as our BBB payload - a delivery vehicle with the utmost specificity for malignant cells particularly when targeted toward a particular cancer antigen (Uherek et al., 2002). These targeted NK-92 cells are in a perpetually activated state, immune to the inhibitory and immune-modulatory effects of malignant cells, with preserved cytoxicity to “non-self” cancer cells and enhanced activity against those cancer cells possessing the targeted antigen. When combined with FUS to enable CNS access, these cells appear to track towards the tumour once outside of the vasculature (3.3), thus having potential advantages over antibodies, in addition to being directly cytotoxic. After demonstrating that the cells could be successfully delivered to the brain in a viable state (Figure 3.7), in chapter 4 we initiated a long-term survival study to elucidate whether they would have any appreciable effect on tumour progression and survival, given the encouraging pre-clinical results in extra-cranial malignancies (Uherek et al., 2002; Daldrup-Link et al., 2005).

We found that on average there was a significant difference in the median and mean survival when therapy was delivered aggressively before animals harboured a large tumour burden (Figure 4.6). This result was at least partially attributable to an insufficient ratio of NK-92 to
tumour cells as the tumour increased in size. The latter could have been influenced by a number of factors including necrosis in the larger tumours leading to large poorly perfused regions, the development of relatively avascular cystic components, heterogeneity of the HER2 expression resulting in less efficient killing by the targeted NK-92 cells, and heterogeneous BBBD coverage of the tumour (for example secondary to standing waves). Furthermore, in small tumours where the disrupted volume was larger than the tumour, NK-92 cells from adjacent brain could possibly track to the tumour and increase the relative density of effector:tumour cells, as we believe may have occurred in previous experiments (Section 3.3, Figure 3.6).

We attempted to elucidate some of the mechanisms by which ultrasound and microbubbles facilitate the transmigration of NK cells into the brain. It is unclear whether attributing this process to BBBD alone is justified. In chapter 3, we showed a striking difference between the number of cells accumulating in the CNS when they were in the circulation at the time of the ultrasound exposure compared to when they were delivered afterwards. This suggests some combined interaction between the ultrasound, microbubbles and NK cells. We postulated that this might be due to shear stresses generated by the oscillating microbubbles.

The ultimate goal of these studies is to eventually translate the benefits to the clinical realm. With this in mind, it is useful to discuss the limitations of our pre-clinical models specifically, to hope to understand where they approximate the human condition and where the two diverge. The major clinical results of therapeutic ultrasound, both in the brain and elsewhere in the body, are reviewed to illustrate how the field is rapidly expanding, and doing so without major adverse events in patients. The pre-clinical safety evidence for BBBD with ultrasound and microbubbles is then reviewed, which at this point in time is adequate to make a compelling argument that the technique is ready for a pilot trial in humans. Finally, the future clinical and pre-clinical work stemming from this body of research will be briefly discussed.

5.2 Limitations of Pre-Clinical Models

5.2.1 Biological

Pre-clinical models are exactly that – models – which only approximate the disease process in humans. Orthotopic tumour implantation was done in all experiments to best approximate the
corresponding clinical pathology. In Chapter 2 we used the 9L gliosarcoma cell line, which is a rodent-derived brain tumour that attempts to approximate human glioblastoma. This is a reasonable approximation for acute studies as we have conducted, but could potentially have more serious limitations for a longitudinal study as it has been noted to be immunogenic. Also, it does not infiltrate along its borders to the same degree that glioblastoma does. The MDA-MB-231-HER2 cell line used in chapters 3 and 4 is a human cell line, but it is derived from brain metastases in a murine model which were then propagated and altered to express elevated levels of the HER2 protein. Wild-type tumours certainly will have variable degrees of HER2 expression that will not be perfectly approximated by our model. Finally, the rodents required to implant a cell line from a foreign species must be immunocomprised, and while these are frequently studied animals, the applicability of the results obtained to the human problem is not fully known.

5.2.2 Physical

Another large contribution to experimental variability, and its applicability to the clinical problem, is the scale of the animals compared with humans. The dose of microbubbles, as well as that of other drugs used, are small and must be diluted in order to yield an administrable volume. Despite this, the volume is not very large compared with the dead space in the IV’s and syringes available, introducing error in the dose delivered. Furthermore, the microbubbles are available in a human-scale dose, which must be activated at the beginning of an experiment and therefore some animals receive more recently activated microbubbles than others. To minimize this effect the order in which animals were treated on a given day was randomized, but even if the differences between individual animals evened out, the overall effect was likely to reduce the treatment efficacy.

In terms of scale differences, the fact that the rodent skull is obviously much smaller than that of a human, and correspondingly much thinner, must be discussed. A single element transducer operating at approximately 0.5 MHz was used for all of the experiments. This requires a frequency that is then not heavily distorted or attenuated when applied transcranially as there is no means of aberration correction with a single element. However, this must be balanced with the focal spot size for the particular transducer, which was relatively large compared to the
rodent brain (see sections 2.2.2 and 3.2.3). Even at the lower frequency (230 kHz) available with a commercially available clinical system (which produces a larger focus due to the longer wavelength; section 1.5.2), the focal spot size is comparatively much smaller in the human brain. The more concerning consequence of a relatively large focal size relative to the intracranial cavity is that standing waves are often seen within the rodent brain (O’Reilly et al., 2010), increasing the risk of tissue damage while simultaneously having little to no therapeutic effect at the nodes. While we used a short duty cycle in the experiments, which is known to reduce standing waves (O’Reilly et al., 2010), this was frequently visible on MR imaging done in a parallel plane to the beam direction, manifested as bands of enhancing and non-enhancing brain. The effect of this in the experiments would be to leave portions of the tumour virtually untreated, which would have the largest impact on the longitudinal experiments conducted in chapter 4. In fact, the early failures in the FUS+cells groups could have been in part due to this phenomenon, particularly if the tumours weren’t effectively treated early in the course of therapy, given that the treatment seemed to be much less effective once the tumours were large. Other factors which contributed to error in the treatments, and possibly untreated regions of tumour, included variability in the incidence angle of the ultrasound to the skull (which for lateral tumours occasionally resulted in a more medial region of BBBD due to a lensing effect), initial registration error between the MRI and ultrasound coordinates, positioning error due to transport of the animals between the ultrasound system and MR environment, and error due to the ultrasound positioning system itself.

5.3 Pre-Clinical Evidence of BBBD Safety

There are clearly a number of promising clinical applications of BBBD with FUS, including those examined in Chapters 2, 3, and 4, but as of yet BBBD has not made the transition to human subjects. In the following chapter we review the pre-clinical safety of BBBD with FUS, again in the vein of translation to the clinical domain. The technique first described by Hynynen et al., (2001) has been successfully adapted using a variety of microbubbles, ultrasound parameters and therapeutic agents. The recurring finding in these pre-clinical investigations, aside from the increased accumulation of dozens of agents that would otherwise have little access to the brain, is the relative absence of adverse effects. There is a safe window in which BBBD can be
achieved without any tissue damage (Hynynen et al., 2001; 2005; McDannold et al., 2005; 2006a; 2007; Howles et al., 2010). Furthermore, numerous studies have found no long-term ill effects (McDannold et al., 2005; Hynynen et al., 2006). Beyond the upper limit of this window, the most commonly identified post-mortem finding is erythrocyte extravasation or hemoglobin breakdown products. Published research from our laboratory, with an in-house manufactured MRI-guided focused ultrasound system looking at BBBD at 86 sites in 27 rodents, supports that BBBD can be achieved consistently without tissue damage, either at the focus or elsewhere in the beam path (O’Reilly and Hynynen, 2012). The aforementioned study revealed that ultrasound intensity sufficient to produce MRI contrast enhancement of 20% or less resulted in no evidence of histological tissue injury. In the proposed trial we will begin with low-intensity sonications and gradually increase the intensity until BBBD is detected by contrast-enhanced MRI to avoid the erythrocyte extravasation seen at higher intensities. Unpublished large mammal studies (N=13 swine) in our laboratory using the study device (ExAblate 4000 @ 230 kHz) did not reveal tissue injury however the flat geometry of the porcine skull is not ideal when paired with a hemispherical array. Another group investigating BBBD in swine similarly reported no adverse effects but used an unfocused ultrasound transducer through the temporal bone generating in situ pressures well below what is needed to achieve stable cavitation in injectable microbubbles (Xie et al., 2008).

5.3.1 Pre-Clinical Cancer Therapy

There are several studies investigating the use of doxorubicin paired with focal BBBD using ultrasound. The first showed that ultrasound significantly increased the accumulation of doxorubicin at the focus in healthy rodent brain tissue (Treat et al., 2007). Furthermore, it was shown that the tissue level could be varied greatly by the manipulation of the ultrasound parameters. A subsequent study by the same group showed a modest survival advantage with a single treatment of pegylated liposomal doxorubicin and BBBD with ultrasound and Definity. The most recent study showed that three weekly treatments doubled the median survival time (compared to no treatment) with 50% (4 of 8) animals being cured (Aryal et al., 2013). None of these studies revealed any untoward or unexpected off-target effects, and the findings in the multiple treatment study at the tumour site were largely consistent with the side-effects of doxorubicin treatment in extensive tumours. The tumour cell line used in both survival studies
was the 9L gliosarcoma, which is a well-studied rodent brain tumour cell line, intended to mimic human glioblastoma. However, this cell line does not have the same infiltrative potential as glioblastoma, but has behaviour somewhere in between the typical glioblastoma and metastatic brain tumour. A number of other studies have been conducted in rodents showing the potential of augmenting chemotherapeutic agents into the brain and brain tumours: BCNU in a C6 glioma model (Liu et al., 2010; Chen et al., 2010), methotrexate in the rabbit brain (Mei et al., 2009), cytarabine in the rodent brain (Zeng et al., 2012), and herceptin in the rodent brain (Park et al., 2012). We published our results of borono-phenylalanine-fructose complex (BPA-f) augmentation with BBBD for boron neutron capture therapy in 9L gliosarcoma-bearing rodents and found that focused ultrasound resulted in not only increased BPA-f concentrations but also increased inter- and intra-animal drug homogeneity (Alkins et al., 2013b). Heterogeneous chemotherapy uptake is thought to play a large role in tumour recurrence. Together these studies show that the concentrations of a large number of therapeutic agents can be augmented both in tumour and brain tissue using BBBD with focused ultrasound, without significant adverse effects.

5.3.2 Primate Studies

The best pre-clinical data to date is from a non-human primate study conducted at Brigham and Women’s Hospital in Boston by McDannold et al. (2012). The authors used the proposed study device and ultrasound contrast (ExAblate 4000 in combination with Definity) in 7 primate subjects and again found that BBBD could be consistently and safely achieved in normal brain tissue, both in single and volumetric sonications (1 cm³), in a window within which there was no tissue damage. The authors performed repeated sonications to the visual pathway over a period of more than a month and were able to show through functional evaluation of the animals that visual acuity and performance of visual tasks was unaffected by BBBD with ultrasound. Furthermore, 72 of 75 target regions showed no abnormalities on heme-sensitive MRI (T2*-weighted sequences). In those where an abnormality was detected, the histological evaluation showed a small amount of petechial hemorrhage. This supports that in a model most similar to human patients, that BBBD, even in eloquent brain, can be performed without functional impairment and with a very low risk of tissue injury at the focus, without any sign of collateral tissue injury. In the current study, eloquent cortical regions will not be targeted. However, we
offer that the low risk and minor severity of petechial hemorrhage seen with ultrasound-mediated BBBD would, in the future, be justifiable if it meant a significantly prolonged progression-free survival. This is no different than accepting a non-zero risk at present for surgery and chemotherapy.

5.4 Conclusion

The ability to open the BBB in discrete regions, in a controlled, reversible and safe manner using ultrasound and microbubbles has now been well established in pre-clinical studies. This paradigm offers new therapeutic avenues not only for patients with intracranial malignancies, but also for patients with neurodegenerative and genetic diseases, and for patients with cerebrovascular disease and ischemia. In the current work the goal was to build upon focal brain tumor treatments designed to minimize as much as possible deleterious effects to healthy tissue. Thanks to BBBD with ultrasound, many previously abandoned therapeutic agents can be re-examined. One such agent that we explored is the well-established boron-containing amino acid analogue, boronophenylalanine, used for BNCT, to establish if there was a benefit to the marriage of these two focal modalities. The results were promising, but the practical implementation, and the ability to pursue further studies, is limited by the availability of nuclear reactors for medical research.

We then moved to study an engineered cell line targeted to the HER2 receptor, used in early clinical trials with some promise and, importantly for the brain, which restricts its cytotoxic effects to malignant cells. A cell line targeted to particular cell-surface proteins which is able to home in on malignant cells once set loose in the brain, while respecting normal cells, is in theory an optimal therapy, if a sufficient number of cells can be delivered. We were able to first demonstrate that NK-92 cells could be delivered to the brain with ultrasound and microbubbles with preserved cytolytic function, and subsequently show that early treatments with targeted NK-92 cells are capable of improving survival in a HER2 amplified orthotopic breast metastasis model. HER2 is expressed by a number of epithelial tumours, and future studies might employ a combination of targets to broaden the cytolytic effect. Furthermore, the mechanisms via which ultrasound enhances the transmigration of NK-92 cells into the brain has not been elucidated,
however we postulated this may be related to shear stresses on the cerebral endothelium and the subsequent production of nitrous oxide.

Ultimately, the goal of this work is to improve survival in patients with brain tumours by providing novel treatments that minimize morbidity. Both BNCT and NK-92 cells were selected because there is previous experience in human trials. Currently work is underway for the first trial of BBBD with ultrasound in humans. The hope is thus that the translation of these combined treatments can be streamlined and reach real patients in the foreseeable future.

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