ULTRASOUND AND MICROBUBBLES: A ROLE IN CANCER

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2013
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BIOMEDICAL ENGINEERING GRADUATE PROGRAM

ABSTRACT

Successful cancer treatment relies on the sensitivity to monitor cancer response to therapy and the ability to efficiently deliver drugs to the tumor. Cancer research has continuously improved patient outcomes over the last decades; however, it is still the second leading cause of death in the United States. It is essential to improve methods to progress all areas of cancer treatment, including monitoring and drug delivery. Ultrasound (US) is a popular clinical imaging modality due to its low levels of radiation, portability, and cost effective imaging. These advantageous characteristics have allowed for novel research advancements in the US field including drug delivery, molecular US imaging, and molecular targeted delivery. The addition of microbubbles (MBs) has immensely expanded the potential of US in these areas of imaging and drug delivery. MBs are non-toxic, gas-filled contrast agents which mechanically oscillate, enhancing the signal-to-tissue ratio during imaging of the vasculature. MBs also have the ability to stimulate cellular and vascular permeability when exposed to properly applied low-intensity US fields. This transient increase in membrane permeability introduces a window for more effective localized drug uptake in cancerous masses. This effective increase shows potential to improve tumor delivery of anti-cancer agents, such as chemotherapeutics, targeted antibodies (Ab), and adenovirus (Ad). Evaluation of US parameters further facilitate this technology through the avoidance of potential bioeffects, while maintaining a safe, noninvasive, and effective therapeutic strategy. Functionalizing
MBs, and creating a targeted molecular US approach, demonstrates potential to more efficiently monitor therapeutic response and enhance drug delivery in cancer. This dissertation describes strategies for improving localized therapeutic delivery in cancer and evaluation of response to treatment using ultrasonic techniques. These techniques are explored in preclinical primary cancer animal models and present significant potential for cancer treatment.

Keywords: cancer, contrast-enhanced ultrasound imaging, microbubbles, molecular ultrasound, ultrasound, ultrasound-stimulated drug delivery
DEDICATION

This dissertation is dedicated to my wonderful family who has supported me along the way. To my husband, Tony, thank you for being my rock during this process. To my amazing parents, Tim and Laurie Goblirsch, thank you for the unconditional support and for lending an ear all throughout graduate school. To my siblings, Sara and Matt Campbell, Jenna Goblirsch, and Jack Goblirsch, thanks for the laughs and fun times and for putting up with my craziness. Also thanks for the encouragement and support of my in laws, Ed and Ruth Sorace, Tara and Chad Wooden. Thank you to my family for all the love and support during both the rainy and sunny times! This dissertation is especially dedicated to my grandfather, Popeye, who would have loved to see his granddaughter follow in his engineering footsteps. I know he would be especially proud of the completion of this dissertation.
ACKNOWLEDGMENTS

Thank you to everyone who has helped me throughout this process, especially my committee, who has extended themselves and continued to help better my educational process. Drs. Jack Rogers, Heidi Umphrey, Yogesh Vohra, and Kurt Zinn, thank you for your time and support! To my mentor, Ken Hoyt, thank you for the knowledge and skills you have helped me acquire, and for the continuous help you have given me from start to finish of this dissertation. I am truly grateful for the time you have put into my education and graduate school career. Also, thanks to everyone in the BME department who has helped me in one form or another during this dissertation.

A BIG thanks to all the people who have helped or assisted me over the last years, especially to everyone in the Advanced Medical Imaging Research lab. I couldn’t have completed this without you all and I am glad to have formed some great friendships in the process: Dr. Jason Warram, Sharon Samuel, Karri Folks, Marshall Mahoney, Reshu Saini, Bernice Dunklin, Lee Whitworth, Amber Martin, and Marie Taylor. Thank you for all your help, and good memories of all the fun times in the lab! Thank you to all the coauthors on the manuscripts in this dissertation; this research could not have been completed without you: Bryant Harbin, Dr. Cara Heath, Dr. Kenneth Hoyt, Madura Joshi, Dr. Hyunki Kim, Dr. Joseph Knowles, Dr. Melissa Korb, Marshall Mahoney, Amber Martin, Dr. Eben Rosenthal, Reshu Saini, Sharon Samuel, Dr. Heidi Umphrey, Dr. Jason Warram, Lee Whitworth, Dr. Guihua Zhai, and Dr. Kurt Zinn.
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<td>$\alpha_\text{v}\beta_3$</td>
<td>alpha V integrin</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
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<tr>
<td>Ad</td>
<td>adenovirus</td>
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<tr>
<td>ADC</td>
<td>apparent diffusion coefficient</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BBB</td>
<td>blood-brain barrier</td>
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<td>BLI</td>
<td>bioluminescence imaging</td>
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<td>B-mode</td>
<td>brightness mode</td>
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<td>CEUS</td>
<td>contrast-enhanced ultrasound</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagles medium</td>
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<td>DW-MRI</td>
<td>diffusion weighted magnetic resonance imaging</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
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<tr>
<td>HNC</td>
<td>head and neck cancer</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin &amp; eosin</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<tr>
<td>Luc</td>
<td>luciferase-based</td>
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MB  microbubble
MI  mechanical index
MOI  multiplicity of infection
MRI  magnetic resonance imaging
MVD  microvessel density
NaCl  sodium chloride
OFP  octafluoropropane
PET  photon emission tomography
PFU  plaque forming units
PRF  pulse repetition frequency
PRP  pulse repetition period
PSA  prostate-specific antigen
RBC  red blood cell
RECIST  Response Evaluation Criteria in Solid Tumors
ROI  region-of-interest
SPECT  single-photon emission computed tomography
SVR  angiosarcoma endothelial cells
TGF-α  transforming growth factor alpha
TK  thymidine kinase
US  ultrasound
UST  ultrasound therapy
VEGF  vascular endothelial growth factor
VEGFR2    vascular endothelial growth factor receptor 2
INTRODUCTION

Ultrasound Imaging

Ultrasound (US) in medicine began in 1942 and has continued to evolve and become a progressively popular imaging modality due to its wide range of applications, low exposure to harmful radiation, and temporal imaging strategies (Fass 2008, Goldberg et al. 1994). Other characteristics that identify US as a valuable clinical imaging modality are its inexpensive nature and portability. US is currently used in oncology for detection, monitoring, guided surgeries, and biopsies. Other diagnostic and imaging capabilities include applications in a variety of biological tissues such as tendons, muscles, joints, vessels and internal organs for pathology or lesions. US is also used in obstetrics, musculoskeletal system, anesthesiology, cardiology and cardiovascular system, oncology, gastroenterology, gynecology, neurology, ophthalmology, and urology. These positive characteristics and wide capabilities have established US as an emerging research imaging modality. The progression of US applications over the last decades has greatly increased the potential of the field and broadened it to diverse preclinical and clinical areas of research.

Noninvasive US imaging begins with an external source emitting a signal into the body, which then produces a propagating signal to measure and record the biological response. Sound is mechanical energy that is transmitted by pressure waves from a piezoelectric element transducer. When applied with a voltage, the piezoelectric elements create mechanical vibrations through repeated oscillations, which in turn generate sound waves, or ultrasonic pulses to travel through a medium. As the waves propagate through the tissue, the method in which the waves respond is indicative of the specific medium it
is traveling through. Biological substances differ in the speed at which the US waves travel, averaging speeds of 1540 m/s for soft tissue (Zagzebski 1996). As the waves propagate through tissue, US striking a smooth tissue boundary produces a reflection which is dependent on the acoustical impedance of the two tissues which create the boundary (Zagzebski 1996, Kremkau 2002). The tissue’s acoustical impedance initiates a pulse to return to the transducer as an echo signal. US measures the transmit time for a signal to travel to a reflecting interface and return an echo to the transducer allowing for distance calculations. For US calculations, an assumed biological speed is utilized (1540 m/s); however each tissue’s speed of sound varies. Transducers typically have 128-256 elements depending on the application and desired lateral resolution; however they have been built up to 4000 elements. These piezoelectric probe elements typically pulse in groups of 4 to 32 elements to create pulse sequences in order to combine or steer the direction of the acoustic energy. For weak US reflections, created by structures smaller than the dimensions of the wavelength of the pressure wave, Rayleigh scattering occurs. Specifically, this occurs with rough surfaces or heterogeneous organs within the body, as seen in Figure 1.

Medical US utilizes frequencies around 2-20 MHz, while normal audible sound is between 20 Hz to 20 kHz (Zagzebski 1996, Kremkau 2002). Therefore, US utilizes high frequency sound waves and echoes to create images. Echo images are recorded as a function of depth (A-line) for each group of elements of the transducer. Each of these A-lines is sequentially recorded for all transducer elements in order to create a two dimensional grayscale image brightness mode (B-mode). This B-mode is more typically known as a sonogram. Lateral resolution is dependent on the spacing of the probing
elements. Penetration of an US wave is proportional to the wavelength, therefore resolution and image clarity is dependent on wavelength. At higher frequencies, US images have increased resolution; however the penetration depth of the US imaging is decreased (Zagzebski 1996, Kremkau 2002). A variety of transducers are utilized clinically and the upcoming application directly determines the appropriate transducer.

![US imaging: Reflection and Rayleigh scattering](image)

**Figure 1. US imaging: Reflection and Rayleigh scattering.** On the left exhibits US reflections traveling and reaching a smooth boundary. Z represents the acoustical impedance created between two boundaries. On the right shows Rayleigh scattering which occurs when US waves hit heterogeneous surfaces smaller than the wavelengths emitted.

Ultrasound Contrast Agents

US contrast agents, or microbubbles (MBs), are micrometer-sized gas-filled particles, surrounded by a flexible outer shell composed of albumin, galactose, polymer,
protein, phospholipid, or lipid molecules (Calliada et al. 1998, Cosgrove 2006). MBs internal gas is composed of air or perfluorocarbon, creating a stability to allow for stable resonance between states of compression and rarefaction. Their original designed purpose was to provide US image contrast enhancement for real-time assessment of myocardial perfusion in coronary artery disease. MBs also have the ability to enhance imaging of blood perfusion in the heart and other organs. Their 1-3 µm size allows perfusion within microvasculature in the body. US contrast agents are currently FDA approved for systemic injections, however a limitation of MB contrast agents are their limited half-life in circulation due to filtration from the liver and spleen.

Contrast-Enhanced Ultrasound

The introduction of MBs allowed contrast-enhanced US (CEUS) imaging to emerge, thereby further increasing vascular visualization from traditional grey-scale medical sonography. The high percentages of water within bodily tissues create a practically homogenous acoustical medium for imaging; this can create difficulty when attempting to distinguish degrees of blood flow using traditional sonography. CEUS is the systemic application of MBs, which are highly echogenic and circulate in the blood to better reflect US waves, in order to improve vasculature contrast.

Harmonic or pulse inversion US imaging allows for isolation imaging of the non-linear scattering echoes created from the MBs. Due to the inhomogeneity of compression and rarefaction of the MBs when induced with US pressure, signal from the surrounding tissue can be suppressed, while intensity from the MBs is isolated. This heightens the signal from the vasculature, reduces signal from tissue, therefore improves signal-to-tissue ratio, as seen in Figure 2.
With the help of MBs, US has been used for noninvasive examination of pathologies (Pysz et al. 2010, Pysz et al. 2011). CEUS imaging allows us to increase visualization found with traditional US imaging and distinguish changes in vascularity within tumors. Detection of response with CEUS has been verified with anti-angiogenic drugs, as well as longitudinal monitoring of response to various anti-cancer treatments (Zhou et al. 2011, Hoyt et al. 2010).

**Figure 2. Contrast-enhanced harmonic US imaging.** Shown is pressure versus time of mechanically oscillating MBs between states of compression and rarefaction (top). Pulse inversion, harmonic imaging adds two signals together. Linear signal received from tissues cancel out and equal zero (middle), however the signal received from oscillating MBs create a heightened isolated intensity signal (bottom).
Targeted Microbubbles

MBs have evolved from solely being contrast enhancers in US imaging, to agents that can be used in targeted strategies as pathophysiological markers (Ferrara, Pollard and Borden 2007, Sheffield et al. 2008). This method, known as molecular US imaging, is also an emerging field of interest. This field has been made possible mainly due to the sensitivity and specificity of US improving over the last decade. Functionalizing or targeting MBs to specific receptors create a method for tissue characterization using a noninvasive analysis of fundamental pathways. Targeting MBs to receptors overexpressed in certain diseases has proven to be an effective way to monitor disease states and therapeutic responses. Also, targeting MBs to specific endothelial markers has improved visualization of vascularity through improved contrast enhancement by local accumulation. These targeted MBs have potential in many fields such as inflammation.

Figure 3. Targeted MBs create a molecular US strategy for imaging and drug delivery. Targeted MBs are created by attaching ligands to the outer shell, without compromising the MBs oscillating properties (shown left). MBs flow through the vasculature systemically, and attach to specifically targeted receptors localized on endothelial cells in a region-of interest (ROI) (shown right).

Through targeted strategies, MBs have allowed US to actively progress in the field of molecular imaging. A molecular targeted strategy for imaging and drug delivery can be obtained through attaching antibodies or peptides to the outer surface of the MBs. This can be completed through covalent or non-covalent binding. Targeted MBs are systemically injected, flow through the vasculature similar to non-targeted MBs, and eventually attach themselves to their targeted receptors on endothelial cells, as seen in Figure 3. This allows for a unique signal from a localized region in the vasculature and creates more signal-to-noise in comparison to the surrounding tissue. Molecular US imaging can also be used for receptor profiling for targeted cancer therapies, such as antibodies, and for tracking tumor response to therapies. Molecular imaging is an extremely positive addition in the field of US and oncology for cancer detection, staging, monitoring, and therapy.

Specifically, increasing visualization of tumor angiogenesis can improve detection and monitoring of cancer. MBs with targeted ligands have shown promise in increasing visualization of tumor vascularity through increasing MB accumulation in a targeted region-of-interest (ROI). Molecular US imaging has recently been studied to visualize changes in tumor vasculature and response to drug treatment (Saini et al. 2011a, Hoyt et al. 2012). Multi-targeted MBs have shown an overall synergistic effect to increase tumor vessel visualization compared to single-targeted constituents (Warram et al. 2011a, Willmann et al. 2008). This synergistic effect of targeting multiple receptors
simultaneously enhances the ability of the MBs to attach within the desired region. Using this multi-targeted approach to analyze drug response to therapy allows for increased potential knowledge to determine early response to cancer treatment. Targeted MBs have also been explored as drug delivery vehicles by pre-loading or coating MBs with drugs or molecules (Warram et al. 2012, Eisenbrey et al. 2010). The ability to also use targeted MBs within US therapy (further discussed in the following section) demonstrate potential to create a dual localized and targeted approach promising to further increase delivery of anti-cancer agents to the desired ROI (Phillips et al. 2010, Tsutsui, Xie and Porter 2004b). This concept of targeted MBs for monitoring tumor response and enhancement of drug delivery is further explored in chapters 7 and 8.

Ultrasound-Stimulated Therapy

Due to their small size, MBs are small enough to flow unimpeded through microvasculature (Calliada et al. 1998). This has allowed them to expand from just traditional contrast enhancers to be utilized as drug delivery vehicles and mechanism for enhancement of passive drug delivery. Because of their nontoxic qualities and impressive response characteristics to mechanically oscillate under certain US conditions, MBs have also emerged as a novel therapy to conventional and targeted chemotherapeutics. Systemically circulating MBs can transiently improve localized cellular and vascular permeability under certain US parameters, making it an effective and noninvasive delivery mechanism.

The efficiency of a drug to treat cancer is dependent on its ability to be delivered to a localized ROI, therefore improving drug delivery has become a popular field of interest in cancer research (Orive et al. 2003). Under certain ultrasonic conditions, MBs
have the ability to mechanically oscillate between states of compression and rarefaction as seen in Figure 4. MB contrast agents in the presence of US have been shown to exhibit two mechanisms that complement drug delivery. These include vascular extravasation and temporarily enhancing cell membrane permeability (Dalecki 2004, Kinoshita et al. 2006). US-stimulated drug delivery has become an increasingly attractive field of study due to the ability to noninvasively increase localized passive delivery of chemotherapeutic and other drug therapies. The addition of US therapy to current systemically delivered therapeutics adds no additional toxicity from external agents, as these MBs have been deemed nontoxic when systemically injected. Although not approved for clinical use as therapeutic agents under these conditions; MBs are currently FDA approved for systemic injections for perfusion studies and have undergone successful toxicity studies.

Under the influence of particular US conditions, MBs mechanically oscillate, and when introduced with cells, the oscillating MBs can produce pores in the cellular membrane (van Wamel et al. 2006, Kinoshita et al. 2006, de Jong, Bouakaz and Frinking 2002, de Jong et al. 2009). The concept of modulating membrane permeability has

**Figure 4. US-stimulated therapy in vivo.** MBs are administered systemically in the vasculature of an animal model to transiently enhance localized molecular delivery following exposure to US energy.
become increasingly popular with the intention of introducing active compounds, such as drugs and gene therapy vectors, into diseased cells (Miller, Pislaru and Greenleaf 2002, Tsutsui et al. 2004a). These pores created will open up and react similar to a wound response, healing shortly after physical interaction with the contrast agent and US subsides (Uhlemann, Heinig and Wollina 2003, Breuing et al. 2005). Another report noted that during US-stimulated therapy, cell membrane disruption appears similar to plasma wounds and are actively repaired within minutes after therapy ceases (Schlicher et al. 2006). It has been shown that in the presence of MBs, US pressure (or mechanical index, MI) at low levels can increase the intracellular uptake of chemotherapeutic drugs and genetic materials such as polynucleotides and proteins (Huber and Pfisterer 2000, Hosseinkhani et al. 2003, Zderic et al. 2002, Anwer et al. 2000). This technique has been shown to increase localized delivery of molecules directly to the cytoplasm of cells; drugs, such as paclitaxel and doxorubicin, and molecules, such as lipoplexes for increased transfection, have been demonstrated to have enhanced delivery (Escoffre et al. 2011, Lentacker et al. 2009, Lentacker et al. 2010). In cancer cells, US induced membrane permeability in combination with anti-cancer drugs such as Bleomycin and Adriamycin have been shown to increase drug uptake, demonstrating a promising technique for cancer therapy (Iwanaga et al. 2007, Zhou et al. 2011).

US-stimulated therapy has also been shown to be beneficial in vivo to increase delivery of molecules. Studies have been completed showing US induced MBs can increase extravasation by breaking down gap junctions between cancerous cells and transport molecules across barriers it previously would be unable to cross (Zderic et al. 2002, Miller et al. 2002, Maeda et al. 2009, Zhao et al. 2012). This unique therapy has
been applied to many disease types and molecular agents. Localized cellular delivery of DNA using MB-mediated US therapy has been investigated *in vivo* in areas of enhanced cancer gene therapy, for cardiovascular applications and for bone formation (Sheyn et al. 2008, Miller et al. 2002). Using this technique, US induced MBs have been shown to temporarily open up the blood brain barrier to allow increased delivery of drugs to the brain (O'Reilly et al. 2012, Raymond et al. 2008, Arvanitis et al. 2012, Howles et al. 2012, Park et al. 2012, McDannold et al. 2012, Treat et al. 2012). Molecular extravasation of cytotoxic agents into tumors through MB therapy has also been shown to increase anti-tumor effects and retard tumor growth in mice (Bekeredjian et al. 2007, Iwanaga et al. 2007, Heath et al. 2012, Maeda et al. 2009). US immunogenic therapy for solid tumors has also been evaluated and shown to produce a 55% cure rate in a xenograft tumor model (Casey et al. 2010). Utilizing both of the phenomena that occur in US-stimulated therapy is essential: cell uptake through pore formation and increased vascular extravasation through gaps formed between endothelial cells of the tumor. US-stimulated therapy in combination with chemotherapy has shown potential to enhance drug uptake at US localized cancer sites, showing future potential to decrease systemic toxicity (Phillips et al. 2011).

To improve therapy effectiveness, novel strategies in treatment are needed to overcome the current barriers of poor uptake resulting from tortuous vasculature, limited drug dosages and high tumor interstitial pressure (Jain and Carmeliet 2012, Jain and Carmeliet 2001). To date, research in this field for cancer drug delivery focuses on improving localized delivery of molecules, such as drugs, DNA, or virus, to a primary tumor (Dalecki 2004, Escoffre et al. 2011, Kinoshita et al. 2006, Lentacker et al. 2008).
This relatively noninvasive approach to cancer treatment is considered non-toxic, safe, and effective. US-stimulated therapy has demonstrated a 20-80% improvement in drug delivery compared to drug alone in preclinical murine models of solid tumors (Bekeredjian et al. 2007, Casey et al. 2010, Iwanaga et al. 2007, Park et al. 2012). Current preclinical research and development has shown immense potential in advancing this field; this is further explored in Chapters 1 through 6.

Translation Opportunities

Cancer is the second most common cause of death in the United States with a projected 580,000 deaths in 2013. In a generic sense, cancer is the “well-calculated”, out-of-control, growth of abnormal cells within the body. Primary cancer begins a collection of cells which grow into a tumor, and eventually establishes angiogenesis and the formation of its own blood supply. If left untreated, primary tumors will eventually metastasize through delivery of the vasculature to form tumors in other locations in the body. Cancer treatment has greatly evolved over the last decades and now involves a more encompassing approach. Detection, monitoring, and therapy now incorporate both macroscopic and microscopic levels. Cancer treatment incorporates screening and early detection, noninvasive monitoring, surgery, hormone therapy, radiation, chemotherapy, immunotherapy, and targeted therapy (Zaman 2013). Detecting early response to treatment before traditional standards (i.e. tumor size evaluation) allows for more accurate prognosis and determination of treatment options. Current assessment of cancer response to treatment is based on Response Evaluation Criteria in Solid Tumors (RECIST), and utilizes no molecular or internal critique of tumor (Therasse 2000). RECIST is based on evaluation of a single dimension of tumor size, and although this is
the current standard of care technique utilized in the clinic, it has been deemed insufficient for successful evaluation of patient response to treatment and overall patient survival. It has been even less successful for determining responders vs. non-responders in cancer therapy. The ability to monitor drug therapy over time elucidates tumor uptake mechanisms and pending response to treatment for individual patients. Increased demands for new, noninvasive treatments have led to novel modalities to monitor and evaluate early treatment response. Imaging modalities such as positron emission tomography (PET), single-photon emission computer tomography (SPECT), magnetic resonance imaging (MRI), optical, and US are becoming increasing popular in preclinical applications to explore drug delivery and bioeffects. Cost efficiency, spatial resolution, temporal resolution, safety, and specificity are characteristics which determine the appropriate imaging strategy to use. Since the introduction of MBs and targeted strategies, US encompasses all of these ideal characteristics for monitoring response to cancer. Targeted MB strategies, such as molecular US imaging create a noninvasive method which shows potential to evaluate early tumor response to cancer treatment. Determining responders vs. non-responders early in cancer treatment will allow the opportunity to for a clinician to direct patient care to the best method available for that individual patient. These targeted MBs also show potential to be combined with cancer drug delivery to create a targeted, localized technique. Molecular therapy has become an essential component of cancer treatment, and functionalizing MBs creates an improved, additive component to further increase drug delivery.

Currently, the percentage of drug that reaches the tumor in comparison to the total dose is minimal. An insufficient response of many cancers to a multitude of
chemotherapeutic drugs highlights the need for new and improved delivery mechanisms to aid in increased localized drug uptake in cancer cells. The impact of a therapeutic drug depends on the rate and ability to permeate into the desired tissue, which highlights the need for improved drug delivery mechanisms, such as US-stimulated therapy. It is anticipated that creating a universal strategy for improving localized delivery in cancer using US-stimulated therapy has potential to be applied to a variety of anticancer fighting agents such as chemotherapeutics, targeted agents, and gene delivery. Increasing the efficiency of drug delivery to the target tumor will improve antitumor effects, while potentially allowing a dose reduction to minimize systemic toxicity.

Successful research in the field of cancer treatment needs to have translatable opportunities. Due to the appealing characteristics of both MBs and US clinically, there are both novel monitoring and drug delivery approaches that show great potential for cancer treatment. This dissertation describes strategies for improving both monitoring and drug delivery using ultrasonic techniques. These ultrasonic techniques are explored in preclinical animal models of primary tumors and show significant potential for translation clinically for cancer treatment.
CHAPTER 1

MICROBUBBLE-MEDIATED ULTRASONIC TECHNIQUES FOR IMPROVED CHEMOTHERAPEUTIC DELIVERY IN CANCER

by

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ABSTRACT

Background: Ultrasound (US) exposed microbubble (MB) contrast agents have the capability to transiently enhance cell membrane permeability. Using this technique in cancer treatment to increase the efficiency of chemotherapy through passive, localized delivery has been an emerging area of research. Purpose: Investigation of the influence of US parameters on MB mediated drug delivery in cancer. Methods: 2LMP breast cancer cells were used for in vitro experiments and 2LMP tumor-bearing mice were used during in vivo experiments. Changes in membrane permeability were investigated after the influence of MB-mediated US therapy parameters (i.e. frequency, mechanical index, pulse repetition period, US duration, and MB dosing and characteristics) on cancer cells. Calcein, a non-permeable fluorescent molecule, and Taxol, chemotherapeutic, were used to evaluate membrane permeability. Tumor response was also assessed histologically. Results: Combination chemotherapy and MB-mediated US therapy with optimized parameters increased cancer cell death by 50% over chemotherapy alone. Discussion: Increased cellular uptake of chemotherapeutic was dependent upon US system parameters. Conclusion: Optimized MB-mediated US therapy has the potential to improve cancer patient response to therapy via increased localized drug uptake, which may lead to a lowering of chemotherapeutic drug dosages and systemic toxicity.

INTRODUCTION

Cancer is the second most common cause of death in the USA with a projected 570,000 deaths in 2010 (American Cancer Society, 2010). The efficiency of a drug, such as chemotherapy, to be delivered and taken-up by cancerous cells ultimately determines the effectiveness of any systemic treatment (Orive, 2003). The lack of tumor response to
chemotherapy is well documented in many cancer types. Breast cancer studies have shown only 60% response to anthracycline-based chemotherapy with 14% of that being complete response (Carey, 2006). Literature has shown that adjuvant chemotherapy has no benefit to pancreatic cancer patients (Neoptolemos, 2001). Head and neck cancer shows between 12-50% 5-year survival rate using different chemotherapeutic options (Argiris, 2008). In general, the 5-year cancer survival rate in the United States is 68% (American Cancer Society, 2010). This insufficient response of cancer to a multitude of chemotherapeutic drugs highlights the need for improved delivery mechanisms to aid in increased localized drug uptake in the targeted cancer cells.

Diagnostic ultrasound (US) imaging has become a powerful clinical tool due to its real-time capability, portability, minimal exposure to radiation and inexpensive cost. The application of microbubble (MB) contrast agents to traditional US introduces contrast-enhanced US. MBs are flexible polymer, surfactant, or protein shelled gas-filled colloidal particles ranging in size from 1-8 µm (DeJong, 2000). When MBs are exposed to an US field, the mechanical force causes them to oscillate between states of rarefaction and compression. Under certain acoustical conditions MB’s physical interaction with tissue has also been shown to temporarily enhance cellular permeability (Miller, 2000; Ward, 1999). This technique of using US exposed MBs to transiently enhance membrane permeability is an emerging area of research and could lead to improved tumor cell drug internalization (van Wamel, 2006). MB-mediated US therapy is impacted by both US exposure conditions and MB characteristics (Karshafian, 2009). Therefore, optimization of US-based therapy may improve the efficiency of systemic chemotherapeutic delivery and impact cancer treatment.
The concept of modulating membrane permeability has become increasingly popular with the intention of introducing active compounds, such as drugs and gene therapy vectors, into diseased cells (Miller, 2002). It has been shown that in the presence of MBs, US pressure (or mechanical index, MI) at low levels can increase the intracellular uptake of chemotherapeutic drugs and genetic materials such as polynucleotides and proteins (Hueber, 2000; Miller, 1999; Anwer, 2000; Hosseinkhani, 2003; Mukherjee, 2000; Zderic, 2002). During MB-mediated US therapy, cell membrane disruption appears similar to plasma wounds and are actively repaired within minutes after therapy ceases (Schlicher, 2006; McNeil, 2003). In cancer cells, US induced membrane permeability in combination with anti-cancer drugs such as Bleomycin and Adriamycin have been shown to increase drug uptake, demonstrating a promising technique for cancer therapy (Iwanaga, 2007; Wu, 2006). MB-mediated US therapy has also been shown to be beneficial in vivo to increase delivery of molecules. Localized cellular delivery of DNA using MB-mediated US therapy has been investigated in vivo in areas of enhanced cancer gene therapy, for cardiovascular applications and for bone formation (Miller, 2002). These applications have shown increased results using MBs to help penetrate the cellular membrane and/or endothelial barrier. Enhanced delivery of cytotoxic agents to tumors through this therapeutic method has also been used and proven to retard tumor growth in mice (Iwanaga, 2007). MB-mediated US immunogenic therapy for solid tumors has also been evaluated and shown to produce a 55% cure rate in a xenograft tumor model (Casey, 2010). MB-mediated US therapy in combination with chemotherapy has shown potential to enhance drug uptake at US targeted cancer sites, with future potential to decrease systemic toxicity.
In the United States, it is estimated that over 1.5 million new cases of cancer will be diagnosed in 2010 (American Cancer Society, 2010). The impact of a therapeutic drug depends on the rate and ability to permeate into the desired tissue. The effectiveness of a therapeutic drug to resolve the cancer condition is directly dependent upon the amount delivered to the tumor over time. To improve therapy effectiveness, novel strategies in treatment are needed to overcome the current barriers of poor uptake resulting from tortuous vasculature, limited drug dosages and high tumor interstitial pressure (Jain, 2001). Specifically, there is an immediate need for promising strategies such as MB-mediated US therapy that can produce improved chemotherapeutic drug delivery and localized tumor uptake. The objective of this study was to investigate the influence of both US exposure and MB properties on MB-mediated ultrasound cancer drug delivery \textit{in vitro} and \textit{in vivo} and to optimize these conditions to enhance drug uptake.

MATERIALS AND METHODS

Cell lines and culture methods

In this study, 2LMP human breast cancer cells (MDA-MB-231, lung metastatic pooled) were used as a biological model for investigating MB-mediated US therapy as cancer therapy. The 2LMP cell line was maintained in DMEM, 10% FBS, and 1% L-glutamine. All cells were cultured 70% to 90% confluence before passaging. Cells were grown at 37° C and in 5% CO$_2$ and 90% relative humidity. Appropriate cell numbers for all experiments were determined using a hemocytometer and trypan blue dye exclusion.
In vitro ultrasound treatment with fluorescent uptake

2LMP cells (1x10^6) were suspended in cell buffer (PBS with 5% FBS) in 75x12 mm Polystyrene tubes combined with 1 mL of calcein (MP Biomedicals, LLC, Solon, OH), 1x10^{-3} M concentration, and MBs. The brands of MBs studied were Definity (Lantheus Medical Imaging North Billerica, MA), SonoVue (Bracco International BV, Amsterdam, Netherlands), and Levovist (Schering AG, Berlin, Germany). Cells were exposed to US in a water bath of temperature 37° C. During experiment duration, tubes were continuously rotated using a mechanical stepper motor at a rate of 24 degrees per second allowing direct US exposure to the entirety of the cells. The transducer was immersed and stabilized at a far field distance of approximately 12 cm from the cells. Control samples underwent the same procedure, replacing exposure with sham US. The objective of these experiments was to quantify the cellular uptake of low molecular weight fluorescent molecules due to transient enhancements of membrane permeability. Membranes were disrupted using MB-mediated US therapy and a range of exposure conditions. The fluorescent signal from cellular-entrapped calcein was quantified using flow cytometry techniques immediately following therapy and reported as a percentage of signal recorded in control cell populations.

Ultrasound Exposure Parameters

The custom experimental US setup involved single element (0.75 inch) immersion transducer (Olympus, Waltham, MA) in series with a signal generator (AFG3022B, Tektronix, Beaverton, OR) and power amplifier (A075, Electronics and Innovation, Rochester, NY) as illustrated in Figure 1. This study was completed in a
Figure 1. Experimental Design Schematic. (a) Experimental setup using a single element immersion transducer in series with a signal generator and power amplifier.

series of experiments in order to investigate the influence of pertinent pulsed US parameters such as signal frequency (0.5, 1.0, or 2.25 MHz), duration (15, 60, 300, or 600 sec) and magnitude of exposure (MI of 0.1, 0.5, 1.0 or 2.0), pulse repetition period (PRP; 0.01, 0.1, 1.0 sec), MB dosage (10, 50, 250 µL) using a concentration of 14 million MBs/mL and brand (Definity, SonoVue, and Levovist). Concentrations of brands varied, therefore total MB count was held constant at 700,000 MBs (Definity (14 million MBs/mL, Levovist (7 million MBs/mL), SonoVue (7 million MBs/mL)) for those experiments. MB concentration was determined using flow cytometry. Duty cycles for these experiments were fixed at 20%. Unless otherwise stated, default US parameters
were 1.0 MHz frequency, an MI of 1.0, a PRP of 0.01 sec, duration of 300 sec and the default MB brand was Definity using a 50 µL dose.

Intensity measurements

US intensity measurements were performed in a 37°C water bath using a hydrophone (Model HGL-0400, ONDA, Sunnyvale, CA) and preamplifier setup in series with a digital oscilloscope for voltage signal monitoring and recording. Individual immersed transducers were manipulated by a precision stepper motor (Velmex, Inc, Bloomfield, NY) in order to locate the spatial peak pressure maximum. The latter was determined by converting voltage to pressure measurements using hydrophone calibration data.

Flow cytometry

Fluorescent signals from internalized calcein molecules (600 Da) were quantified for each cell population using flow cytometry (Accuri C6, Accuri Cytometers Inc., Ann Arbor, MI). All experimental groups were analyzed in triplicate. Cells were normalized and average fluorescence per cells were calculated. For each experimental variant, data was normalized by control group fluorescence and reported as percent control. Cell viability tests were confirmed using membrane-impermeable propidium iodide (2 µL of 0.5 mg/mL, Fisher Scientific, Waltham, MA).

In vitro drug uptake

2LMP cells (1x10^6) were plated on acoustically transparent flasks (Opticell, Rochester, NY). After a 24 hr period to ensure proper seeding, Definity MBs (50 µL)
were administered and cells underwent combination chemotherapy (Taxol, Parenta Pharmaceutics, Inc, Yardley, Pennsylvania) (100 nM, 0.84 µg) with MB-mediated US therapy. Taxol is a commonly known chemotherapy drug used in breast cancer, and was chosen as the model chemotherapeutic due to its ability to be evaluated *in vitro* and *in vivo*. Opticell flasks were placed in a custom built rack that was positioned attached at the bottom of a water bath of temperature 37° C. The transducer was immersed and stabilized at a far field distance of approximately 12 cm from the cells. The opticell flasks were inverted in order for the MBs to float and physically interact with the cell monolayer. Control cells underwent chemotherapy only, MB-mediated US therapy only or no therapy. Following a 24 hr incubation period, plates were analyzed using either fluorescence microscopy (Olympus 1X70, Olympus American, Inc., Melville, NY) or flow cytometry. For flow cytometry, cells were trypsinized and stained with calcein AM (BD Biosciences, Franklin Lakes, NJ) and propidium iodide for measuring cell viability and death, respectively. Specifically, cells were stained with 1.0 µL of working calcein-AM stock (50 µL) and incubated for 15 min at 37° C. Subsequently, 2.0 µL of 0.5 mg/mL propidium iodide was added. Cells were then analyzed for fluorescence (1x10^3 event counts) using flow cytometry. All experimental groups were analyzed in triplicate. Light microscopy images were acquired and registered to fluorescence images to visibly validate cell viability and death tests.

*In vivo* ultrasound treatment

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Forty-two 4-week-old nude athymic mice (The Jackson Laboratory, Bar Harbor, ME) were implanted
subcutaneously in the flank with 2LMP cells \( (2 \times 10^6) \). Approximately three wks post implantation, animals were sorted by average tumor size and grouped as follows \( (n = 6 \text{ per group}) \): control (no drug or US), drug (Taxol) only, MB-mediated US only \( (MI = 0.5) \), or drug plus MB-mediated US therapy \( (MI = 0.1, 0.5, 1.0 \text{ or } 2.0) \). Grouping was completed by taking caliper measurements of tumor size on Day 0 and sorting mice from smallest to largest tumor and then separating in order. Each group created had the same average size of tumor to ensure no biasing between groups. The US therapy groups were further stratified by the intensity of US exposure and used varying MI values of 0.1, 0.5, 1.0, or 2.0. All drug and MB-mediated US therapies were administered on days 0, 3, 7, 10, 14, and 17 of this study. Drug \( (37 \mu\text{L}, 6\text{ mg/mL}) \) and MBs (Definity, 30 \( \mu\text{L} \)) were administered via tail vein injections after dilution with saline to 100 \( \mu\text{L} \). Drug and MB dosage were determined by the weight range of the animals and specification from respective companies. The remaining control groups received bolus injections of matched drug or MBs doses diluted to 100 \( \mu\text{L} \) with saline. Two min post injection, applicable groups underwent MB-mediated US therapy in a 37°C waterbath for 5 min using a transmit frequency of 1.0 MHz and PRP of 5 sec (20% duty cycle). Animals were weighed and tumors sizes were monitored using both caliper measurements and high-frequency \( (40\text{ MHz}) \) US imaging (Vevo 660, VisualSonics Inc, Toronto, CA) on days 0, 7, 12, 14, and 19. Using a standard normalized tumor size with an ellipse equation for tumor area and ellipsoidal formula for tumor volume, tumor measurements were tracked over time as percent change from day 0. On day 21, animals were humanely euthanized and tumors excised for histological analysis.
Immunohistologic Analysis

Serial sections of 5 μm thickness were cut from formalin fixed, paraffin embedded tissue blocks and floated onto charged glass slides (Super-Frost Plus, Fisher Scientific) and dried overnight at 60º C. An H&E stained section was obtained from each tissue block. All sections subject to immunohistochemistry were de-paraffinized and hydrated with deionized water. The tissue sections were heat treated with 0.01M Tris-1mM EDTA buffer (pH 9) using a pressure cooker (CEPC 800, Cook’s Essentials, China) for 5 min at maximum pressure (15 lb/in²). Following antigen retrieval, all sections were gently washed in deionized water and then transferred to TBST (0.05M Tris-based solution in 0.15M NaCl with 0.1% v/v Triton-X-100, pH 7.6). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. To further reduce non-specific background staining, slides were incubated with 3% normal goat or horse serum for 20 min (Sigma, St. Louis, MO) according to the host where primary antibodies were produced. All slides were then incubated at 4º C overnight with either Ki67 or CD31 antibody. Negative controls were achieved by eliminating the primary antibodies from the diluents. Following washing with TBST, peroxidase-conjugated goat anti rabbit IgG (for CD31 and Ki67) (1:200, Jackson ImmunoResearch, West Grove, PA) was applied to the sections for 30 min at room temperature. Diaminobenzidine (DAB, Scy Tek Laboratories, Logan, UT) was utilized as the chromagen and hematoxylin (7211, Richard-Allen Scientific, Kalamazoo, MI) as the counterstain.

H&E sections were examined for cellular necrosis and reported as percent of the entire tumor cross-section (original magnification x5). Each CD31 section was examined (original magnification x40) to identify five separate areas containing the greatest
microvessel density (MVD). Individual vessels from these five areas were counted (original magnification x200), averaged, and recorded as MVD. Ki67 sections were reviewed to determine level of cell proliferation within the tumors (original magnification x200).

Statistical Analysis

Data was summarized as mean ± SE. Statistical analyses were performed using the software package SAS 9.2 (SAS, Cary, NC). Assessment of in vitro cell death following drug plus MB-mediated US was performed using an analysis of variance (ANOVA) test for cell death. After ANOVA, statistical comparisons between groups were made with Tukey-Kramer multiple comparison procedure. Assessment of in vivo tumor size (both area and volume) was conducted with ANOVA test using day 21 data (percent change). After ANOVA, statistical comparisons between groups were made with Tukey-Kramer multiple comparison procedure. Evaluation of percent necrosis from histological analysis was performed on MB-mediated US therapy groups with ANOVA statistical testing to determine differences in influence of MI. MVD was assessed using ANOVA testing. A p-value less than 0.05 was considered statistically significant.

RESULTS

In vitro fluorescence tracer uptake

Frequency

Within the three US transmission frequencies explored (0.5 MHz, 1.0 MHz and 2.25 MHz), a 1.0 MHz frequency showed the maximum level of extracellular fluorescent
tracer uptake as shown in Figure 2a. At 5 min of exposure, 0.5, 1.0 and 2.25 MHz frequency US resulted in increased tracer uptake levels of $21.36 \pm 1.96$, $60.84 \pm 2.42$ and

*Figure 2. In vitro characterization of US parameters.* (a) Changes in frequency altering fluorescent uptake over a range of MB-mediated US exposure times. (b) Shown are the changes in fluorescent uptake after varying the MI over a range of exposure times. (c) As the MI and the PRF changes, the percent increase of fluorescence signal changes.
20.80 ± 3.60 % respectively. At 5 min exposure, the group with 1.0 MHz frequency presented the greatest increase in uptake level compared to the other frequencies ($p < 0.0001$). MB-mediated US therapy using a transmission frequency of 0.5 MHz only significantly differed from results found using 2.25 MHz at an exposure duration of 1 min ($p = 0.001$). At exposure times of 15 sec, 5 min and 10 min, there were no significant differences in extracellular tracer uptake levels between 0.5 MHz and 2.25 MHz ($p > 0.21$).

**Mechanical Index**

Increasing the magnitude of US exposure does not always translate to greater cellular permeability and extracellular tracer uptake during MB-mediated US therapy as shown in Figure 2b. For the exposure times studied, therapy using very low magnitude US (MI of 0.1) produced a very small increase in extracellular fluorescent tracer uptake that ranged over time from 9.24 ± 1.44 to 21.62 ± 1.38 %. This data set showed a near constant linear trend when compared to other US conditions. Specifically, using an MI of 1.0 produced significantly higher tracer uptake levels ranging from 12.24 ± 1.39 to 60.84 ± 2.42 % ($p < 0.0001$), peaking at an exposure time of 5 min. MB-mediated US therapy exposure at an MI of 0.5 produced a shifted left curve that peaked at an exposure time of 1 min with an uptake of 32.28 ± 4.79 %, while cellular exposure at an MI of 2.0 peaked at 5 min with a corresponding increase in extracellular tracer uptake of 47.24 ± 1.54 %. Importantly, there were no significant differences in the quantity of dead cells found between each control and test group ($p = 0.57$).
**Pulse repetition period**

At lower PRPs (e.g., 0.01 sec), MB-mediated US therapy using higher MI values were more effective at modulating cellular permeability and increasing extracellular tracer uptake as shown in Figure 2c. In total, a PRP of 0.01 sec did not show a difference compared to results using a PRP of 0.1 and 1 sec ($p > 0.07$), but trended toward significance. US exposure using an MI of 1.0 produced a significant increase in extracellular tracer uptake at a PRP of 0.01 compared to 0.1 and 1.0 ($p < 0.001$).

**Duration of US exposure**

Cells were exposed to MB-mediated US therapy for a range of duration (0.25, 1, 5, and 10 min) in addition to a variety of MI values, transmission frequencies, MB doses, and MB brands. Varying the duration of US exposure demonstrated that simply increasing the duration of US exposure does not always lead to increased cellular permeability and subsequent extracellular tracer uptake. As US parameters change, exposure time must be dictated for that specific constraint. Note that 5 min of US exposure resulted in the greatest increase in extracellular tracer uptake under the experimental conditions specified (figures 2a, 2b and 2c).

**MB dose**

A trend emerged for MB-mediated US therapy, revealing as the quantity of MBs dose increased, the subsequent level of extracellular tracer increased as seen in Figure 3a. At an exposure time of 1 min, cells demonstrated a $5.47 \pm 2.72 \%$, $23.28 \pm 4.87 \%$ and $62.21 \pm 1.41 \%$ increase in fluorescent tracer uptake as the MB dose went from low (10µL), medium (50µL), to high (250 µL), respectively. At an US exposure time of 5
**Figure 3. In vitro characterization of MB parameters.** (a) Shown are changes in fluorescent uptake from varying MB dosing amounts over time. As the amount of MB administered increased, fluorescent uptake increased. (b) Shown are the changes in fluorescent signal uptake after varying MB brands over a range of exposure times. Definity resulted in the highest molecular uptake.

\[ \text{min, increases in tracer uptake were found to be } 1.14 \pm 2.11\% , \ 60.84 \pm 1.42\% , \ \text{and} \ 88.81 \pm 3.02\% \ \text{for the low, medium and high MB doses, respectively. It is shown that MB dose is associated with increased uptake level at both 1 min (} p < 0.0001 \text{) and 5 min (} p < 0.0001 \text{). Within each time period, the uptake resulting from different doses was significant from each other (} p < 0.05, \ p < 0.05 \text{).} \]
**Figure 4. In vitro characterization of combination US MB mediated therapy and chemotherapy.** (a) A viability stain of dead cells (stained in red with propidium iodide) and viable cells (stained in green with calcein AM) were imaged using fluorescent microscopy viability stain 24 hrs after combination MB-mediated US therapy. (b) Shown are quantifications of cell death (% control) using flow cytometry 24 hrs following combination MB-mediated US therapy.

**MB brand**

Definity MBs were shown to have the greatest enhancement effect for modulating cellular membrane permeability and subsequent extracellular tracer uptake as seen in Figure 3b. At 15 sec, there were no significant differences ($p = 0.09$) between results obtained using the three MB brands, while at 1 and 5 min, extracellular tracer
uptake using Definity was significantly increased compared results obtained using Sonovue and Levovist ($p < 0.001$).

**In vitro drug uptake**

Combination drug and MB-mediated US therapy studies showed that the percentage of cell death increased by 120% as compared to control data ($p < 0.001$) (Figure 4b). Combination drug and MB-mediated US therapy increased the percentage of cell death by approximately 50% when compared to results obtained using drug alone ($p < 0.05$), as seen in Figure 4b. MB-mediated US therapy alone (no chemotherapeutic drug) showed no significant difference in the amount of cell death compared to control data ($p > 0.05$). Fluorescent images allowed qualitative analysis of viable and non-viable cells, showing an increased amount of cell death when comparing drug to MB-mediated US drug therapy. Representative pictures are shown in Figure 4a.

**In vivo drug uptake**

Evaluation of MB-mediated US therapy in a tumor-bearing mouse model using various magnitudes of US pressure (i.e., MI values of 0.1, 0.5, 1.0, and 2.0) consistently resulted in decreased tumor growth over groups receiving: chemotherapeutic drug alone, MB-mediated US therapy alone, or controls. MB-mediated US therapy using an MI value of 0.5 resulted in the highest impediment in tumor growth over the three week treatment period confirming that optimization of US parameters can further enhance antitumor drug effects (see figures 5, 6 and 7). As shown in figure 5a, terminal tumor area calculations via caliper measurements indicated no significant difference between any groups that were administered drug ($p = 0.74$) or between the control and US therapy alone groups.
Figure 5. In vivo characterization of tumor size after combination MB-mediated US therapy. (a) Control groups of saline treatment and US treatment only showed exponential growth of tumors (measurements of tumor area was performed using calipers). (b) Variations of tumor volume (measurements from high frequency US) are shown for chemotherapy and MB-mediated US treatment with chemotherapy groups. A MI of 0.5 depicted the best results with the smallest percent of growth of tumor.

\( p = 0.49 \). However, there was a significant difference in terminal tumor sizes of non-drug groups (US alone and control groups) and those that received drug treatment \( p < 0.001 \). Tumor assessment using high-frequency US imaging, shown in figure 5b, allowed for more precise tumor size measurements, particularly in the animal groups receiving drug therapy due to their smaller tumor sizes. Mean tumor volumes measured via high-frequency US for MB-mediated US drug therapy group animals (MI of 0.5) was
195.79 ± 53.01 mm$^3$, which was significantly lower than that found in drug alone, 337.06 ± 21.36 mm$^3$ ($p = 0.037$). MB-mediated US therapy using an MI of 1.0 also showed a significant enhancement of antitumor effects on tumor volume (221.61 ± 45.12 mm$^3$) compared to the therapy with drug alone group ($p < 0.05$). There were no significant differences found between the MB-mediated US therapy groups with varied MI values as all groups demonstrated comparable antitumor effects. However, there was a discernible trend in therapeutic response and tumor growth retardation as MB-mediated US therapy and the magnitude of US exposure alternated from MI values of 0.1, 2.0, 1.0, to 0.5. The latter therapeutic condition demonstrated the smallest terminal group tumor size.

As figure 6a details, MB-mediated US therapy using an MI value of 0.5 in combination with drug therapy also produced the highest degree of necrosis per tumor volume (40.72 ± 15.8 %), followed by therapy using an MI of 1.0 (24.92 ± 17.31 %), MI of 0.1 (15.31 ± 10.76 %) and MI of 2.0 (3.21 ± 1.10 %). MB-mediated US therapy using an MI value of 0.5 resulted in 17 and 3.5 times higher tumor necrosis levels than therapy with an MI values of 2.0 ($p = 0.12$) and 0.1 ($p = 0.21$), respectively. Due to tumor ulcerations in control group animals, they were excluded from necrosis percentages. These intratumoral necrosis levels suggest that MB-mediated US combination drug therapy using an MI value of 0.5 produces the greatest antitumor effect ($p = 0.21$), which coincides with tumor growth retardation. Representative pictures are shown in Figure 6b-e. The light pink area shown is necrotic tissue, where the deep purple area shown is viable cancerous tissue. There was no significant weight loss observed between day 0 to day 21 ($p > 0.11$) for each animal group. In addition, there were no observed differences in grooming and diet throughout the study duration indicating there was minimal adverse
Figure 6. In vivo characterization of tumor necrosis per volume after combination MB-mediated US therapy. (a) MB-mediated US treatment with various MIs was investigated in combination with chemotherapy. Shown are necrosis and tumor size from the varying treatments on the last day of experimentation. Representative histology slide are shown for each varying MI: (b) MI=0.1, (c) MI=0.5, (d) MI=1.0 and (e) MI=2.0. Control tumors are shown in (f) drug alone, (g) ultrasound alone, and (h) saline control. The control tumors were excluded from calculation due to ulcerations. MB-mediated US treatment with an MI of 0.5 resulted in the highest percentage of tumor necrosis.

effects from the treatment. Immunohistologic cross-sectional slides of CD31 stained tumor tissue showed no significant differences in MVD counts between any of the four MB-mediated US combination drug therapy groups (p > 0.09). However, there was a noticeable decrease in MVD counts that trended towards significant in the MB-mediated US drug therapy group using an MI of 2.0. There were no differences in MVD counts between the control group animals (p = 0.18). Significant differences in MVD counts were found between MB-mediated US drug therapy groups using an MI of 2.0 (38 ± 2.57
Figure 7. In vivo characterization of Ki67 staining. MB-mediated combination therapy shows greatest effect of lowering levels of cellular proliferation localized to the tumor peripheral. Representative images are shown between varying MI values: (a) MI=0.1, (b) MI=0.5, (c) MI=1.0 and (d) MI=2.0.

counts) and both the drug only (55 ± 4.79 counts) and control (51 ± 3.26) animal groups (p < 0.05). Qualitative analysis of Ki67 staining confirmed results from the tumor necrosis analysis, exhibiting non-proliferative regions of tumor necrosis. Ki67 staining also revealed that throughout viable tumor regions, markedly lower levels of cellular proliferation were localized to the tumor peripheral and the greatest effect was found in the MB-mediated US combination drug therapy group animals exposed to US using an MI of 0.5 (Figure 7).

DISCUSSION

Improving cellular and vascular permeability to enhance chemotherapeutic drug uptake in cancer yields the potential to improve drug delivery and treatment efficacy. Development of more effective strategies for systemic delivery of these agents could permit lower drug dosing sessions throughout a therapeutic regimen, thereby, decreasing patient toxicity. MB-mediated US combination drug therapy is a promising method for addressing these concerns.

The in vitro component of our experimental study demonstrated that as the MB-mediated US therapeutic parameters were varied, the transient extent of cellular
permeability and subsequent extracellular molecular uptake varied. Monitoring distributions of the membrane impermeable fluorescent tracer calcein allowed the effects of MB-mediated US therapy to be analyzed. US exposure parameters such as magnitude, duration, and pulse repetition period were shown to influence membrane permeability. Note that normalization by sham US (control) data was necessary since cell populations were not washed following incubation with the fluorescent dye. This leads to surface accumulation of fluorescent molecules and a weak fluorescent background signal. Viability studies showed there were no significant differences in the percentage of dead cells after receiving MB-mediated US therapy or sham US, suggesting that the incorporation of MBs or calcein had no net impact on cell viability. Increasing the exposure duration of MB-mediated US therapy was not shown to directly correlate with increased membrane permeability as determined by fluorescent tracer uptake. Interestingly, a previous study determined that cellular membranes can remain porous throughout a MB-mediated US therapeutic session (Pan, 2005), but decreasing MB concentrations toward the end of exposure can possibly decrease affects or allow molecules to flow back out of the cell. Extended periods of US exposure can also cause prolonged cell membrane damage (without cell death) leading to insufficient results considering the molecules are unconstrained and free to diffuse back outside the cells.

The in vitro studies revealed that maximal uptake of extracellular molecules occurred at an US transmission frequency of 1.0 MHz, which parallels findings by other groups (Hwang, 2005; Rahim, 2006). Stable cavitation of MBs is known to be dictated by, and proportional to, US wavelength (Krebs, 2004). For the transmission frequencies investigated and the size of the MBs, the optimal frequency is closest to 1.0 MHz. Since
this US frequency was found optimal for our setup, it was used extensively throughout the duration of the experiments. A PRP of 0.01 seconds (PRF of 100 Hz) exhibited the greatest increase in membrane permeability and fluorescent uptake. Shorter pulse periods excite MBs more often, thus, leading to enhanced uptake. An MI of 1.0 showed the greatest increase in cellular permeability. At an MI of 0.1, it is inferred that the pressure amplitude is not sufficient to drive cavitation and induce molecular-level permeability effects in cell suspensions. At an MI of 0.5, stable cavitation occurs, which will increase cell membrane permeability, however it does not utilize the full MB potential. At an MI of 2.0, it is concluded that inertial cavitation dominates MB response, which minimizes mechanical interaction with cellular membranes due to MB destruction. The effects of increasing membrane permeability through MB-mediated US therapy have been shown to occur while MBs are still intact (Forbes, 2011). Therefore, these US parameters appear to lean towards stable MB cavitation. Stable cavitation has been shown to increase the effects of MB-mediated US therapy without inertial cavitation (Kamaev, 2004; Datta, 2008; Forbes, 2008). Given that an MI of 1.0 showed the greatest extracellular fluorescent tracer uptake, results suggest that stable MB cavitation was the dominant mechanism, thereby creating optimal US conditions for maximizing MB interaction with the cell suspensions. The duty cycle was purposely fixed throughout the entirety of the study so the time-average intensity of US exposure to the various cell groups was constant. MB resonance generated by the US parameters and interaction with cells ultimately leads to increased cellular permeability during MB-mediated US therapy. Limitations of the described *in vitro* studies could relate to the use of cell suspensions in comparison to cell monolayers. Cell monolayers exhibit more realistic conditions when
comparing to \textit{in vivo} work, yet cell suspensions were chosen for these experiments to allow for more parameters to be investigated. Having the cells suspended allows for immediate transition to quantitative analysis using flow cytometry. Another limitation of the study was that MB destruction analysis was not performed, which may have permitted complete differentiation between stable and inertial cavitation, yet literature sources allow us to point to stable cavitation as the positive affect of increased cellular membrane permeability.

MB properties influence the effectiveness of MB-mediated US therapy. Differences in MB brands have been previously studied and MB shell integrity and stability are important factors to consider before their use in MB-mediated US therapeutic applications (Dalecki, 2004). Definity was used for the majority of the study because of availability and approval for use in the United States for echocardiographic applications. Sonovue and Levovist are both lyophilized, dry powder MBs that are reconstituted in saline to enclose sulfur hexafluoride (SF6) gas and air, respectively, while Definity MBs are non-lyophilized encapsulating octafluoropropane (OFP) gas. OFP gas has a compressibility factor of 0.975, while air has a compressibility factor of 0.999 (Fowler, 1947; Vassernan, 1966). Definity and Sonovue both exhibit a lipid-shelled membrane, while Levovist contains a galactose-based shell (Bracco Diagnostics, 2001; RxMed, 1999; Lantheus Medical Imaging, 2008). Sonovue MBs has a mean diameter of 2.5 µm and 90% are smaller than 8 µm (Schneider, 1999). Definity MBs have a mean diameter between 1.1 to 3.3 µm with 98% less than 10 µm. Levovist MBs measure 2-8 µm in diameter, with 95% less than 10 µm. MB stability and ability to create stable cavitation may result from combinations of a shell, gas and lyophilized state. Definity proved to
have the greatest enhancement effect of membrane permeability, but optimization for each MB type may need to occur. By adjusting ultrasound parameters such as frequency, concentration and attenuation, other types of MBs may also be able to improve membrane permeability. At 15 sec, there were no significant differences ($p = 0.09$) between the three MB brands, yet at 1 and 5 min, there was a significantly increased cell membrane permeability using Definity MBs, i.e. fluorescent uptake ($p < 0.001$ and $p < 0.001$, respectively). This could be due to increased stability of this MB composition from the lipid shell and the OFP gas, allowing greater mechanical oscillations of the MBs and interactions with the cells before dissipating. Sonovue and Levovist were both lyophilized MBs, required reconstitution in the presence of liquid. However, Definity was non-lyophilized which may have contributed to the increased stability and performance during MB-mediated US therapy. Five min duration of US exposure had the greatest cell membrane permeability. At 5 min duration, a 5-fold increase in MB dose (from 10 µL to 50 µL) produced a 61.98% increase in membrane permeability when combined with US exposure, yet an additional 5-fold increase in MB dosing (250 µL) showed only a 26.83% increase in membrane permeability. This trend indicates that there is a diminishing marginal response of cell membranes (permeability) exposed to increasing concentrations of MBs and US therapy. Because MBs are more stable at higher concentrations during cavitation (Calliada, 1998), increasing MB dosing may in fact hinder membrane permeability modulation and extracellular molecule uptake.

During *in vitro* experiments conducted with pre-determined optimized parameters, MB-mediated US therapy in combination with chemotherapy produced significant increases in cell death when compared to chemotherapy alone ($p = 0.003$).
This difference in cell death is attributed to an increase in cellular permeability and intracellular drug loading. Noteworthy, there were no differences in cell viability levels when comparing cell groups exposed to sham US and MB-mediated US therapy alone ($p = 0.30$). This confirms again that MB-mediated US therapy alone is a mechanism that does not pose any additional biological effects over sham US. MB-mediated US therapy improves cell membrane permeability through generating small pores to allow for increased passive drug delivery. This is the source of enhanced cancer necrosis: MB-mediated US therapy produced a $50\%$ increase in cell death \textit{in vitro}. Increasing cancer cell death without increasing drug dose could be an important attribution to overall patient care. One study limitations from the \textit{in vitro} combination chemotherapeutic MB-mediated US therapy was that only a portion (estimated $25\%$) of the cell monolayer was directly exposed to the US beam. Given the analysis is conducted on cells from the entire monolayer, increasing the effective treatment area would increase the already positive response.

\textit{In vivo} results showed that increasing drug delivery of Taxol to cancerous cells can improve tumor response through passively increasing drug uptake. These \textit{in vivo} results showed a relationship between the terminal tumor size and terminal tumor necrosis from treatment. Tumor size decreases parallel tumor necrosis increases when optimal MB-mediated US parameters were used. Limitations of this study were the minimal number of parameters investigated \textit{in vivo}. It was necessary to minimize changes in parameters to accurately determine which alterations were critical to improve therapy. Further investigations should include using the optimal MI found for drug uptake and varying the PRPs, followed by altering the drug concentration levels to determine the
minimal dose possible to achieve desired affects while decreasing systemic toxicity. Another limitation to the study is there was not a biodistribution comparing drug accumulation in tumors. An MI of 0.5 had the greatest anti-tumor effect determined by both inhibition of tumor growth over the period of the study and end point tumor necrosis levels in vivo. An MI of 1.0 was favorable during the in vitro optimization studies. This slight variation between in vitro and in vivo is to be expected because temporarily opening cancer cells directly in vitro in suspension has less outside influences than within a murine model. There was no significant difference between the in vivo therapy with a MI of 0.5 and that using 1.0 and further studies will have to be done to conclude the differences between them in vivo. An MI of 2.0 exhibited very little tumor necrosis, 17x less than that with an MI of 0.5. Excessively high MI values have been shown to cause bursting of capillaries (Dalecki, 2004; Shi, 2006; Yeh, 2008; Miller, 2008), which in turn could decrease drug delivery to the tumor cells. Conversely, when a low MI is used, there is thought to be little MB cavitation, creating no additional increased drug delivery. An MI of 0.1 produced less tumor necrosis when compared to an MI of 0.5. It is hypothesized that the low pressure amplitude is not intense enough to sufficiently drive cavitation and induce cellular-level permeability effects, therefore showing no noticeable results. A PRP of 5 sec (PRF of 0.2 Hz) was chosen to allow sufficient time for MB recirculation. It is hypothesized that increasing drug delivery to endothelial cells is the chief mechanism to inhibit cancer growth.

Increases in endothelial cell death have been shown to reduce tumor size and increase necrotic activity within the tumor, yet they are extremely resistant cells (Cameron, 2005; Carmeleit, 2000; Karson, 1996; Haran, 1994). Overexpression of endothelial growth
factor receptors have been found in many cancers such as head and neck, breast, pancreatic, colorectal, ovarian, and lung carcinomas. These growth factor receptors are an important factor in regulating cellular proliferation, differentiation and survival (Bo, 2008). A neoplastic tumor cannot grow beyond millimeters in size without recruitment of endothelial cells and new blood vessels to supply nutrition and oxygen for tumor cell survival (Folkman, 1975; Folkman, 1985). Increasing the delivery of Taxol, an anti-proliferation drug, to endothelial cells through MB-mediated US therapy will reduce the cell proliferation, resulting in starvation of the tumor and prohibiting increased tumor growth. Specifically, Taxol inhibits microtubule dynamics of cytoskeletal elements resulting in mitotic arrest (Subramanian, 2011). Additional positive antitumor effects of MB-mediated US therapy is the ability to increase vascular permeability. Significant results have shown the ability to increase tumor perfusion by degrading junctions between the endothelial cells causing increased flow of drug to the cancer cells (Shang, 2011). This in vivo phenomenon of enhancing angiogenesis has been shown on several tissue and cancer cells lines, such as colon cancer and skeletal muscle (Kodama, 2010; Zhao, 2010). The combination of increasing drug flow to induce endothelial cell death and increasing extravasation explains how MB-mediated US therapy can non-invasively and successfully improve cancer response to therapy. Future directions should include investigating the specific mechanisms of MB-mediated US therapy and conclusively determine whether endothelial cell uptake, extravasation, or a combination of the two is occurring under each chosen parameter.
CONCLUSION

Optimizing parameters of MB-mediated US therapy to improve cellular permeability is essential to enhance therapeutic delivery through increased uptake level in cancer cells. Combination chemotherapy and MB-mediated US therapy with optimized parameters increased cancer cell death by 50% over chemotherapy alone. Using a non-invasive approach to enhance effects of chemotherapy treatment in cancer patients is a novel mechanism for improving patient response to cancer treatment. Increasing the effectiveness of drug delivery is critical for further explorations in decreasing chemotherapy dosage amounts, leading to decreased systemic toxicity.

ACKNOWLEDGEMENTS

The authors are grateful for all the helpful suggestions and feedback from Drs. Eben Rosenthal and Kurt R. Zinn. This research was supported in part by NIH grant UL1RR025777, EP50CA089019-09 and NCI grant CA13148-38.

DECLARATIONS OF INTEREST

Authors state they have no declarations of interest.

REFERENCES


CHAPTER 2

MICROBUBBLE THERAPY ENHANCES ANTI-TUMOR PROPERTIES OF CISPLATIN AND CETUXIMAB IN VITRO AND IN VIVO

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Otolaryngology- Head and Neck Surgery
June 146 (6): 938-945, 2012

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Format adapted and errata corrected for dissertation

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ABSTRACT

Objective: To determine if microbubble-mediated ultrasound therapy (MB-UST) can improve cisplatin or cetuximab cytotoxicity of head and neck squamous cell carcinoma (HNSCC) in vitro and in vivo by increasing tumor specific drug delivery by disruption of tumor cell membranes and enhancing vascular permeability. Study Design: In vitro and in vivo study. Setting: University Medical Center. Subjects: Immunodeficient mice (6 wks old) and 4 HNSCC cell lines. Methods: Changes to cell permeability were assessed in vitro after MB-UST. Cellular apoptosis resulting from adjuvant MB-UST with sub-therapeutic doses of cisplatin or cetuximab was assessed by cell survival assays in vitro. The in vivo effect of adjuvant MB-UST in flank tumors was assessed in vivo with histological analysis and diffusion weighted MRI (DW-MRI). Results: In vitro results revealed that MB-UST can increase cell permeability and enhance drug uptake and apoptosis in four HNSCC cell lines. In vivo adjuvant MB-UST with cetuximab or cisplatin showed a statistically significant reduction in tumor size when compared to untreated controls TUNEL analysis yielded a larger number of cells undergoing apoptosis in tumors treated with cetuximab and adjuvant MB-UST than cetuximab alone, but was not significantly greater in tumors treated with cisplatin and adjuvant MB-UST compared to cisplatin alone. DW-MRI analysis showed more free water, which corresponds to increased cell membrane disruption, in tumors treated with MB-UST. Conclusion: MB-UST promotes disruption of cell membranes in tumor cells in vitro which may be leveraged to selectively improve uptake of conventional and targeted therapeutics in vivo.
INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the 5th most common cancer worldwide with an estimated global incidence of 533,100 new cases including over 40,000 people in the United States diagnosed annually\textsuperscript{1-3}. During the past 20 years, treatments for HNSCC have gradually evolved to combining surgery, radiotherapy, and chemotherapy. Cisplatin is one of the most frequently used chemotherapeutic agents in the treatment of HNSCC and the antitumor activity is dependent on its ability to cross the cell membrane. A combined multi-modality approach, including the addition of conventional chemotherapy (e.g., cisplatin) to radiation has improved disease outcomes but with significant patient morbidity and increase in treatment related deaths\textsuperscript{4,5}.

More recent advancements in therapies for HNSCC have emerged since the identification of new molecular targets that are specific for head and neck carcinomas. One of these new targets includes the epidermal growth factor receptor (EGFR). EGFR is a tyrosine kinase receptor that has been found highly expressed in HNSCC and has been associated with more advanced disease and less favorable outcomes\textsuperscript{6,7}. These discoveries led to the development of novel therapeutics like cetuximab, which is a monoclonal antibody that enters the tumor and targets the extracellular domain of EGFR. Study results have shown modest outcome improvements in HNSCC treated with cetuximab, especially when used in combination with radiotherapy\textsuperscript{8}.

Despite advancements in the treatment of HNSCC with novel targeted therapeutics and combined treatment regimens, toxicities still contribute significantly to patient morbidity. The toxic drugs pose limitations on treatment by exerting their effects on normal tissue resulting in toxicities and dose-limiting side effects\textsuperscript{9,10}. There is a need
for combined treatment regimens with non-overlapping toxicities or treatment regimens that increase drug delivery and uptake in diseased tissues while sparing normal tissue.

Recently, a technique known as microbubble-mediated ultrasound therapy (MB-UST) has been explored in other cancer types as a potential modality to locally enhance drug delivery at the tumor site\textsuperscript{11-13}. Microbubbles are ultrasound contrast agents that function as intravascular tracers. They are made of a protein, starch, lipid, or polymer shell filled with inert gas. Definity\textregistered MBs are commercially available and approved by the United States FDA for cardiac outflow applications. It is known that exposure of MBs to a properly timed ultrasound field results in mechanical oscillations that can disrupt nearby endothelial cell membranes and temporarily enhance vascular permeability. It is hypothesized that MB-UST increases permeability of cells by the membrane disruption induced by MB-UST thereby allowing intracellular uptake of exogenous membrane impermeable molecules. The treatment of HNSCC with adjuvant MB-UST may subsequently enhance the anti-tumor effectiveness of chemotherapeutic and biologically targeted treatment by increasing cell permeability and tumor uptake of chemotherapeutic agents resulting in enhanced drug delivery to diseased tissue while minimizing the harmful systemic side effects of the toxic drugs.

MATERIALS AND METHODS

Cell lines, culture, and transformation methods

This work was approved by the University of Alabama at Birmingham Institutional Review Board. The head and neck cancer cell lines SCC-1, SCC-5, Cal27 and FaDu were grown and maintained under appropriate culture conditions using proper
aseptic techniques\textsuperscript{14}. Cell lines were maintained in DMEM, 10\% FBS, 1\% L-glutamine, and 1\% Pen/Strep (Sigma, St. Louis, MO). Cells were grown at 37\°C, with 5\% CO\textsubscript{2} and 90\% relative humidity. All cells were cultured to 70\% to 90\% confluence before passage. Appropriate cell numbers for \textit{in vitro} assays were determined using flow cytometry (Accuri C6, Accuri Cytometers Inc., Ann Arbor, MI). The FaDu cell line was established from a squamous cell carcinoma of the pharynx (ATCC, Manassas, VA). The SCC-1 cell line was established from a squamous cell carcinoma from the floor of the mouth and the SCC-5 cell line was established from a primary tumor of the supraglottis. The SCC-1 and SCC-5 cell lines were provided by Thomas Carey, PhD, University of Michigan, Ann Arbor.

The SCC-1 cell line was chosen for transfection with a Lentivirus containing both puromycin resistance and Luciferase genes\textsuperscript{15}. Briefly, SCC-1 cells were plated in a 24-well plate 24 hours prior to viral infection at a density of 0.5×10\textsuperscript{5} cells per well in 0.5 ml of complete DMEM medium (with serum and antibiotics). Lentivirus was thawed in a 37\°C water bath and prepared in a mixture of complete medium with Polybren (concentration of 5\(\mu\)g/ml). Media was aspirated from plate wells and replaced with 0.5 ml of this prewarmed Polybrene/media mixture per well (for 24-well plate). Cells were infected by adding 10 \(\mu\)l of viral stock. The infected target cells were selected for stable expression using puromycin. Luciferase expression was assessed by Xenogen 200 series IVIS photon counter (Caliper Life Sciences, Hopkington, MA) after adding 150 \(\mu\)g/ml D-luciferin (Gold Biotechnology, St. Louis, MO) in the culture medium. Puromycin selection pressure was used to generate stable SCC-1 cell lines.
In vitro ultrasound therapy and Luciferin uptake

Microbubble-mediated ultrasound therapy (MB-UST) was administered in vitro under conditions previously described by Sorace et al. Briefly, 50 μl of activated microbubbles (concentration of 14 million MBs/mL) (Definity, Lantheus Medical Imaging, Billerica, MA) were added to cells grown in acoustically transparent flasks (Opticell, Rochester, NY). Immediately after administration of MBs, flasks were inverted and immersed in a water bath at 37° C opposite an 0.75 inch immersion transducer (Olympus, Waltham, MA) in series with a signal generator (AFG3022B, Tektronix, Beaverton, OR) and power amplifier (A075, Electronics and Innovation, Rochester, NY). Cells were exposed to an ultrasound field for 5 minutes at a transient frequency of 1.0 MHz, a mechanical index of 0.5, a pulse repetition period of 0.01 seconds, and a duty cycle of 20%.

To look at Luciferin uptake, SCC-1 cells expressing the Luciferase gene were grown on acoustically transparent flasks. After 24 hours, 30 μg Luciferin was added. MB-UST was administered to half of the flasks. Control flasks were administered MBs and subjected to the same conditions but ultrasound was not used. Luciferase expression was assessed again using the Xenogen photo counter.

In vitro cellular uptake and viability

Cisplatin (NovaPLUS, Irving, TX) was conjugated to an Alexa680 fluorophore (Invitrogen, Carlsbad, CA) by separation column chromatography, while cetuximab (Imclone, New York, NY) was conjugated to Cy5.5. MBs were administered to plated cells (1x10^6) using combination MB-UST and 10 μM cisplatin or 10 μM cetuximab
therapy. Control cells underwent no therapy, monotherapy with cisplatin, cetuximab or UST alone. Following a 24 hr incubation period, cells were washed using PBS and assessed for fluorescence using an Olympus 1X70 microscope (Olympus American, Inc., Melville, NY).

After treatment SCC-1, SCC-5, FaDu and Cal27 cells were trypsinized and stained for viability and death using calcein AM and propidium iodide (BD Biosciences, Franklin Lakes, NJ). Cells were then stained with 1.0 μL of working calcein-AM stock (50μL) and incubated for 15 min in 37ºC. After incubation, 2.0 μL of 0.5 mg/mL propidium iodide was added. Cells were analyzed for viability by fluorescence counts (10k event) using flow cytometry. All experimental groups were analyzed in triplicate.

In vivo ultrasound therapy

Athymic female nude mice (6 wks old) were obtained (Jackson Laboratories, Bar Harbor, ME). SCC-5 cells (2x10^6) were implanted in the left flank of 30 mice. Three
weeks post implant, mice were sorted into the following 6 groups (N=5): no treatment (control), UST alone, 10 uM cisplatin, 100µM cetuximab, 10 µM cisplatin + UST and 100 µM cetuximab + UST. All reagents were delivered by intravenous tail vein injection. MB-UST consisted of administering 100 µl of MBs (Definity®) followed by US exposure in a 37°C waterbath using the set-up described in the in vitro methods but with a post repetition period of 5 seconds (Figure 1). The control group received 100 µl saline injections. Mice remained under isoflurane anesthesia for the entirety of the experiment. Therapy occurred twice weekly for 4 weeks, and was followed by weight and digital caliper measurements of tumor size. On days 0, 14 and 28, tumors were assessed by diffusion weighted magnetic resonance imaging (DW-MRI) (Bruker 9.4T MR, Bruker corp, Billerica, MA). On day 28, animals were euthanized and tumors excised for histological analysis.

Immunohistologic Analysis

Serial sections of 5 µm thickness from tumor samples were cut from formalin fixed, paraffin embedded tissue blocks and floated onto charged glass slides (Super-Frost Plus, Fisher Scientific). Antigen retrieval was performed with 0.01M Tris-1mM EDTA buffer (pH 9) using a pressure cooker. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. After blocking, all slides were then incubated at 4°C overnight with either Ki67, TUNEL or CD31 antibody. Negative controls were achieved by eliminating the primary antibodies from the diluents. Following washing with TBST, peroxidase-conjugated goat anti rabbit IgG (for CD31, TUNEL and Ki67) (1:200,
**Figure 2:** A) Bioluminescence of luciferase-positive SCC-1 cells after MB-UST. SCC-1 cells showed increased fluorescence following MB-UST when compared to controls with no MB-UST \((p<0.001)\). B) Fluorescently labeled drug uptake with cetuximab and cisplatin following MB-UST. SCC-1 cells treated with MB-UST and fluorescently labeled cetuximab showed a statistically significant increase in drug uptake as compared to controls receiving MB-UST alone \((p=0.02)\). There was also an increase in cisplatin uptake although not statistically significant \((p=0.3)\). Error bars represent standard error.

Jackson ImmunoResearch, West Grove, PA) were applied to the sections for 30 min at room temperature. Diaminobenzidine (DAB, Scy Tek Laboratories, Logan, UT) was utilized as the chromagen and hematoxylin (7211, Richard-Allen Scientific, Kalamazoo, MI) as the counterstain.

**Statistical Analysis**

Data was summarized as mean ± SE. Statistical analyses were performed using JMP 7.1 (SAS, Cary, NC) software. Analysis of *in vitro* cell death, fluorescent uptake and histological staining was performed using a chi-square ANOVA, while Luciferase expression, tumor size and DW-MRI were analyzed using a two sample t-test. A \(p\)-value less than 0.05 was considered statistically significant.
RESULTS

MB-mediated ultrasound therapy disrupts cell membranes *in vitro*

Transmembrane migration of a small molecule, luciferin (the substrate for luciferase), was used to determine the amount of disruption of cell membranes. Enhanced luciferin uptake following MB-UST was demonstrated with a luciferase positive SCC-1 head and neck cancer cell line. Comparison of luciferase expression between the control and therapy groups showed a 41% increase in luciferin uptake in cells receiving MB-UST as compared to control (*p*<0.001) (Figure 2A). *In vitro* results also demonstrated that adjuvant MB-UST can increase intracellular drug concentrations of fluorescently-labeled cisplatin and cetuximab. Cetuximab with adjuvant MB-UST demonstrated a 28% intracellular increase compared to treatment with cetuximab in the absence of MB-UST (*p*=0.01). Cisplatin with adjuvant MB-UST revealed a 9% increase over control counterparts without MB-UST (*p*=0.67) (Figure 2B).

After determining that MB-UST can disrupt cell membranes and the cytotoxic effects of cisplatin and cetuximab with adjuvant MB-UST by examining cellular apoptosis in four HNSCC cell lines *in vitro* (SCC-1, SCC-5, FaDu, and Cal27), a dose curve for one cell line was established. Results showed that adjuvant MB-UST enhances the cytotoxic effect of cisplatin and cetuximab in all four head and neck cancer cell lines (Figure 3A). Fluorescent images of propidium iodine stained SCC5 cells showed qualitative analysis of viable and dead cells. The representative images showed increased fluorescent red expression (dead cells) signifying an increased amount of apoptosis when using adjuvant MB-UST (Figure 3B). Enhanced drug uptake in SCC5
Figure 3: A) Quantitative analysis of apoptosis as percent of untreated control in HNSCC cell lines (SCC-1, SCC-5, FaDu, and Cal27). Cell were treated with ultrasound only (UST), cisplatin (1 µM) only, cisplatin plus UST, cetuximab (10 µM) only, cetuximab plus UST. In all four cell lines treated with combination drug and MB-UST there was statistically more apoptosis (*, \(p<0.05\)). B) Quantitative analysis of apoptosis using propidium idodide (PI) in SCC-5 cells. Co-treatment with MB-UST significantly increased cell death with both cetuximab (\(p<0.05\)) and cisplatin (\(p<0.05\)). C) Dose response curves for SCC-5 cells in the presence of cetuximab and UST and cisplatin and UST.
cells resulted in a significant leftward shift in the dose response curves. This shift was
greatest at the 1µM dose for cisplatin and 10µM dose of cetuximab (Figure 3C).

*In vivo* drug uptake

*In vivo* treatment of xenografted tumors using MB-UST demonstrated an
inhibition of tumor growth in comparison to animals receiving chemotherapeutic drug
(cisplatin or cetuximab) alone, MB-mediated UST alone, or no treatment (*p*<0.05). Mice
that underwent MB-UST alone revealed no significant difference in tumor size following
treatment when compared to untreated controls (*p*=0.81). Also, groups treated with either
cetuximab or cisplatin alone showed a reduction in tumor size, but the difference was not
statistically significant different compared with controls (*p*=0.15 and *p*=0.06,
respectively). However, tumors treated with adjuvant MB-UST in addition to cetuximab
or cisplatin were significantly smaller than control (*p*=0.02 and *p*=0.01, respectively).
Although the addition of MB therapy to cetuximab or cisplatin did reduce tumor size, this
effect was not significant (*p*=0.19, *p*=0.20). Tumors treated with cetuximab and adjuvant
MB-UST exhibited a 26% decrease in tumor size at termination compared to cetuximab
alone (*p*=0.05) (Figure 4A). Tumors treated with cisplatin and adjuvant MB-UST
presented a 21% decrease in tumor size when compared to cisplatin alone (*p*=0.24)
(Figure 4B). There was no tissue damage observed in any of the surrounding tissues in
any of the mice treated with MB-UST.

Histologic analysis using TUNEL, CD-31, and H&E staining

TUNEL analysis yielded a larger number of cells undergoing apoptosis in tumors
treated with cetuximab and adjuvant MB-UST than cetuximab alone (55% vs. 44%,

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Figure 4: A) *In vivo* MB-UST and cetuximab and cisplatin treatment on SCC-5 tumors. Tumors treated with cetuximab and adjuvant MB-UST exhibited a decrease in tumor size at termination compared to cetuximab alone (*p*=0.05) (Figure 4A). Tumors treated with cisplatin and adjuvant MB-UST decreased in tumor size when compared to cisplatin alone (*p*=0.24). Error bars represent standard error.
Figure 5: A) CD31 microvessel staining in SCC-5 flank tumors with no treatment (A), MB-UST alone (D), cetuximab (B), cetuximab + MB-mediated UST (C), cisplatin (E), and cisplatin + MB-mediated UST (F). B) CD31 staining of SCC5 tumors. C) TUNEL staining of SCC5 tumors. Error bars represent standard error.

$p=0.015$), but was not significantly greater in tumors treated with cisplatin and adjuvant MB-UST compared to cisplatin alone (84% vs. 77%, $p=0.24$) (Figure 5C). Though H&E
staining did not show changes in vascular morphology, there was a positive correlation between microvessel density and reduction in tumor size ($p=0.05$). Comparisons between drug and drug + MB-UST group data revealed no statistically significant differences in tumor vascularity (CD31) measurements ($p > 0.05$). This indicates that existing tumor vascularity did not impact UST results. (Figure 5A,B).

Diffusion weighted MRI

Intratumoral apoptosis and early phase cell wall disruption was assessed per tumor volume from the apparent diffusion coefficient (ADC) values obtained from DW-MRI on days 0, 10, and 20. ADC values are known to correspond to disruption of cell membranes and release of free water into the tumor mass and can be used as an early predictor of response to therapy in HNSCC$^{5,16}$. Significant increases in ADC values were found in tumors treated with cetuximab and adjuvant MB-UST from cetuximab alone ($p=0.001$) and cisplatin and adjuvant MB-UST and cisplatin alone ($p=0.001$) (Figure 6). These changes in the ADC values suggest an increase in cell wall rupture associated with MB-UST therapy.

DISCUSSION

Currently the overall effectiveness of chemotherapeutic agents is determined by their ability to penetrate tissues and cross cell membranes. This effectiveness is compromised by insufficient delivery of drug to the tumor microenvironment and by the side effects of delivering the drug to normal tissues. Our current studies show that adjuvant MB-UST can increase the efficacy of HNSCC chemotherapeutics, such as cetuximab and cisplatin. This technique may be leveraged to adjust dosing of
conventional and targeted therapeutics to allow treatment with subtherapeutic drug concentrations and therefore decrease toxicity. As MB-UST is a localized treatment like radiation and surgery, it can be used on any disease that is localized. The effects of MB-

Figure 6: DW-MRI ADC values of SCC5 flank tumors before and after ultrasound treatment. Tumors treated with cisplatin + MB-UST and cetuximab + MB-UST had an increase in free water content, reflected as an increase in the ADC ($p<0.001$ and $p=0.002$, respectively). Error bars represent standard error.
UST allow a site-specific method to increase drug uptake and the localized nature of treatment also means localized toxicity.

MB-UST may increase the therapeutic efficacy of these agents by causing a transient increase in tumor permeability and therefore increase the uptake of exogenous molecules into the tumor \textsuperscript{13,17}. This application has also been shown effective in the delivery of small molecules and genetic material into cells.\textsuperscript{13,18,19} The proposed mechanism of action is through mechanical vibration of MBs using US, whereby membrane disruption and tumor permeability is temporarily induced.\textsuperscript{20} This effect has been demonstrated previously by increased cellular uptake of small fluorescent molecules and labeled chemotherapeutic agents \textsuperscript{19}. In the current \textit{in vitro} experiments we were able to use a membrane impermeable fluorescent molecule to demonstrate the effects that MB-UST has on extracellular small molecule uptake. Results show that MB-UST was sufficient to increase molecule uptake by showing a 41% increase in luciferase expression secondary to an increase in membrane permeability.

\textit{In vitro} results also show that MB-UST increases the uptake of two fluorescently labeled drugs, cisplatin and cetuximab resulting in an increase in drug delivery and apoptosis. The \textit{in vivo} results suggest that adjuvant MB-UST has an additive effect with cetuximab and cisplatin as demonstrated by a statistically significant reduction of tumor size in these treatment groups. The enhancement of cisplatin uptake with adjuvant MB-UST is possibly attributed to the transient porous membrane created by MB-UST thereby facilitating more drug entry into the nucleus. The increased cytotoxicity seen with cetuximab and adjuvant MB-UST may be secondary to increased uptake in the tumor from the MB-UST -induced increase in vascular permeability or by changes in cell
signaling. In order to assess response to MB-UST we investigated the use of DW-MRI as an imaging technique to assess tumors. *In vivo* data from diffusion weighted MRI analysis showed a statistically significant increase in ADC values associated with MB-UST treatment. These ADC values correlate with an increase in free water in the tumor secondary to a loss of cell wall integrity.

Combining different treatment modalities has been shown to improve locoregional control of HNSCC, however at the same time this can increase overall toxicity. In particular, it has been shown that the addition of cetuximab to either radiotherapy or cisplatin will potentiate the toxic effects of either therapy. We can overcome this limitation by delivering the drug only to the tumor itself by using site specific MB-mediated ultrasound therapy to enhance drug delivery into HNSCC cells. It has been shown in previous studies that MB-UST can be effective at delivering drugs and transferring genetic material into cells for potential treatment in breast, prostate, and colon cancer\textsuperscript{13,21-23} and we are now able to demonstrate this effect in HNSCC. The effect cannot be described as synergistic as MB-UST is not applicable to the synergy effects as described by Chou\textsuperscript{24}. We are outside of this statistical analysis since MB-UST alone without drug has no effect. However, MB-UST can produce an additive effect with non-overlapping toxicity. *In vitro* studies show that we can use lower doses of a therapeutic agent and see a cytotoxicity profile similar to higher doses.

In conclusion, we show that MB-UST can be effective in increasing drug uptake and thereby apoptosis in HNSCC *in vitro* and *in vivo*. This application can allow the use sub-therapeutic doses of cetuximab and cisplatin without reducing the clinical efficacy of
the drugs. We also conclude from our imaging results that DW-MRI may be a potential tool for monitoring response to combination MB-UST and drug therapy.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Health (2T32 CA091078-06).

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CHAPTER 3

OPTICAL FLUORESCENT IMAGING TO MONITOR TEMPORAL EFFECTS OF MICROBUBBLE-MEDIATED ULTRASOUND THERAPY

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*IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*

60(2):281-289, 2013

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ABSTRACT

Microbubble-mediated ultrasound therapy can non-invasively enhance drug delivery to localized regions in the body. This technique can be beneficial in cancer therapy, yet currently there are limitations in tracking the therapeutic effects. The purpose of this experiment was to investigate the potential of fluorescent imaging for monitoring the temporal effects of microbubble-mediated ultrasound therapy. Mice were implanted with 2LMP breast cancer cells. The animals underwent microbubble-mediated ultrasound therapy in the presence of Cy5.5 fluorescent-labeled IgG antibody (large molecule) or Cy5.5 dye (small molecule) and microbubble contrast agents. Control animals were administered fluorescent molecules only. Animals were transiently imaged in vivo at 1, 10, 30 and 60 min post therapy using a small animal optical imaging system. Tumors were excised and analyzed ex vivo. Tumors were homogenized and emulsion imaged for Cy5.5 fluorescence. Monitoring in vivo results showed significant influx of dye into the tumor ($p < 0.05$) using the small molecule, but not in the large molecule group ($p > 0.05$). However, after tumor emulsion, significantly higher dye concentration was detected in therapy group tumors for both small and large molecule groups in comparison to their control counterparts ($p < 0.01$). This paper explores a non-invasive optical imaging method for monitoring the effects of microbubble-mediated ultrasound therapy in a cancer model. It provides temporal information following the process of increasing extravasation of molecules into target tumors.

Index Terms: fluorescence, optical imaging, ultrasound contrast agents, microbubble-mediated ultrasound therapy, cancer
INTRODUCTION

The ability to monitor drug therapy over time elucidates tumor uptake mechanisms and pending response to treatment for individual patients. Cancer is the second most common cause of death in the United States with a projected 572,000 deaths in 2011 [1]. The efficiency of drug delivery to a target tumor ultimately determines the effectiveness of the systemic treatment [2]. Therefore, increasing the amount of drug localized and taken up by the tumor will improve antitumor effects, while potentially allowing a dose reduction in order to minimize systemic toxicity. The demands for new, non-invasive treatments have led to novel modalities to monitor and evaluate early treatment response.

In combination with chemotherapy, antibody therapy has been an emerging field in many cancer types including breast, pancreatic, head and neck, lung, colon and esophageal. Antibody-based therapies are a positive addition to chemotherapy because they use humanized or human antibodies that have high specificity and low toxicity values [3, 4]. The current difficulties with antibody therapy are the large size of the antibody and its ability to extravasate from the blood stream to the tumor region of interest. Although antibodies circulate for weeks, if blood flow to the tumor is limited, those that do not reach their intended target are metabolized by the liver prior to reaching the cancer [3]. While targets for antibody therapy are extracellular, previous in vitro studies have shown that using microbubble-mediated ultrasound therapy can increase localized effectiveness of cancer therapy [5].

Imaging modalities such as PET, SPECT, MRI and ultrasound are becoming increasing popular in preclinical applications to explore drug delivery and bio effects.
Although this is true, optical imaging to monitor drug delivery gives an inexpensive, noninvasive approach to efficiently analyze longitudinal studies of molecular delivery [6]. Advantages of optical imaging include safety and immediate analysis (compared to secondary analysis such as perfusion, tumor size, etc). One limitation to optical imaging is the ability to clinically translate. Unless the drug is already fluorescently labeled, optical imaging is most effectively used in preclinical applications to study and learn about new drugs, drug delivery vehicles and drug delivery methods [6, 7].

Microbubbles are micrometer-sized gas-filled particles, surrounded by a flexible outer core composed of polymer or lipid molecules [8-10]. Their original purpose was to provide ultrasound image contrast enhancement for real-time assessment of myocardial perfusion in coronary artery disease [11]. Yet due to their impressive response under certain ultrasound conditions, microbubbles have emerged as a novel adjuvant therapy to conventional and targeted chemotherapeutics. Microbubble contrast agents in the presence of ultrasound have been shown to exhibit two mechanisms that complement drug delivery. These include vascular extravasation and temporarily enhancing cell membrane permeability [12, 13]. Under the influence of particular ultrasound conditions and in the presence of cells, microbubbles mechanically oscillate, which can produce pores in cellular membranes in the localized area of interest [13-15]. Using this technique, microbubbles have been shown to temporarily open up the blood brain barrier to allow increased delivery of drugs to the brain [13, 16]. Molecular extravasation into tumors through microbubble therapy has also been shown to increase anti-cancer effects [17]. This technique can increase localized delivery of molecules directly to the cytoplasm of cells; drugs, such as paclitaxel and doxorubicin, and molecules, such as
lipoplexes for increased transfection, have been demonstrated to have enhanced delivery [18-19]. These pores created will react similar to a wound response, healing shortly after physical interaction with the contrast agent and ultrasound subsides [20, 21]. Microbubbles have also been shown to increase extravasation by breaking down gap junctions between endothelial cells and transport molecules across a cell membrane barrier through the temporary pores [22-26]. Two phenomena occur in microbubble-mediated ultrasound therapy: increased vascular extravasation through gaps formed between endothelial cells of the tumor and cell uptake through pore formation. Microbubble-mediated ultrasound therapy has become an increasingly attractive field of study due to the ability to non-invasively increase localized passive delivery of chemotherapeutic and other drug therapies.

With the emergence of combination cancer therapies, it has become essential to understand the mechanics behind the therapy. The ability to non-invasively monitor effectiveness of drug uptake from microbubble-mediated ultrasound therapy is beneficial in gaining further understanding on the mechanisms of the treatment. In this paper, we explore the potential of optical fluorescence imaging techniques for monitoring the temporal effects of microbubble-mediated ultrasound therapy in a preclinical breast cancer animal model.

MATERIALS AND METHODS

Cell Culture

2LMP human breast cancer cells (MDA-MB-231, lung metastatic pooled) were used as a biological model for investigating microbubble-mediated ultrasound therapy in
cancer therapy applications. The 2LMP cell line was maintained in Dulbecco’s Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% L-glutamine. All cells were cultured 70% to 90% confluency before passaging. Cells were grown at 37° C and in 5% CO₂ and 90% relative humidity. Appropriate cell numbers for all experiments were determined using hemocytometer and trypan blue dye exclusion.

Animal Preparation

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham. Twenty-three 6-week-old nude female athymic NCr-nu/nu mice (Jackson Laboratory, Bar Harbor, ME) were implanted subcutaneously with 2x10⁶ 2LMP breast cancer cells in the left flank. Implanted tumors were allowed to grow approximately three weeks before beginning experimental studies. Mice were randomly sorted into four groups: small molecule with microbubble-mediated ultrasound treatment (N = 6), small molecule control with no additional treatment (N = 6), large molecule with microbubble-mediated ultrasound treatment (N = 6) and large molecule control with no additional treatment (N = 5). Tumor area was measured from images acquired using a small animal optical imaging system (Pearl Impulse, LI-COR Biotechnology, Lincoln, NE).

*In vivo* Microbubble-mediated Ultrasound Therapy

Cy5.5-labeled IgG antibody (SouthernBiotech, Birmingham, AL) and Cy5.5 dye (GE Healthcare, Piscataway, NJ) were analyzed in this experiment for their variation in size and representation of antibody and chemotherapeutic drugs in cancer. Cy5.5-labeled IgG (MW = 150 kDa) is a larger molecule, similar in size to antibodies used in cancer
Fig. 1. The therapeutic setup for in vivo microbubble-mediated ultrasound is shown and involves a single element immersion transducer in series with an oscilloscope, a signal generator and power amplifier. Animals were intravenously injected with microbubbles and fluorescent tracers and then underwent therapy in a water bath with constant flow of isoflurane anesthesia.

therapy, while Cy5.5 dye (MW = 1.1 kDa) is a smaller molecule, comparable to chemotherapeutics used in cancer treatment. Hereafter, these fluorescent tracers will be referred to as either small (Cy5.5 dye) or large (Cy5.5- labeled IgG) molecular agents. The blood half-life of these Cy5.5 molecules are estimated at 20 min and 11 days for the small and large molecule, respectively [27, 28]. Optimized parameters from previous microbubble-mediated ultrasound therapy studies were used as a baseline to induce fluorescent tracer tumor uptake in vivo [26]. The custom experimental ultrasound setup involved an unfocused single element (0.75 inch/ 19 mm) immersion transducer (Olympus, Waltham, MA) in series with an oscilloscope (TDS 2014B, Tektronix,
Ultrasound parameters included: 1.0 MHz transmit frequency, 5 min duration of exposure, 5 sec pulse repetition period, 20% duty cycle and a mechanical index (MI) of 0.5 (22 mW/cm²). Mice were submerged in a custom-built 37°C water bath and remained under isoflurane gas anesthesia for the entirety of the US imaging. Each mouse was initially anesthetized, then strapped on a custom board. This board has a port to keep constant flow of isoflurane to the animal for the entirety of placement on the board. The board was submerged at a 30 degree angle in the water bath (entire body of the mouse, except the head is submerged) and the transducer was placed perpendicular to the tumor, as seen in Figure 1. Mice underwent microbubble-mediated ultrasound therapy in the presence of small (25 µL, 10⁻³ M) or large (25 µL dye-labeled antibody, 10⁻⁷ M) fluorescent molecules and uncoated MBs (Definity, Lantheus Medical Imaging North Billerica, MA) in a 37°C water bath. Cy5.5 labeled IgG molecules were purchased pre-conjugated from SouthernBiotech in a working dilution of 10 µL per 1x10⁶ cells. Amount of fluorescence on the IgG molecule was calculated with a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE), as this was not information given in the product sheet. Although molarity concentration was not identical for small or large molecule group, therapy was completed using the same batches during the experiment and all therapy advancements were compared to a control injection of the same amount, allowing appropriate standardization to calculate therapeutic gains. Combination microbubbles (30 µL) and fluorescent molecules (25 µL) were mixed and diluted with saline to a total volume of 100 µL. This mixture (100 µL) was delivered simultaneously
Fig.2. *In vivo* temporal fluorescent imaging setup shows fluorescent optical imaging system for monitoring molecular uptake in live animals. Imaging near-infrared fluorescent tracers reduces background signal and improves dye-to-tissue contrast levels.

intravenously via tail vein bolus injections. Control mice underwent sham therapy (i.e., receiving no ultrasound exposure, yet kept under the same conditions as therapy groups, including injection of microbubbles and fluorescent molecules). Animals were controlled under isoflurane anesthesia throughout the microbubble-mediated ultrasound therapy process.

*In vivo* Optical Fluorescent Imaging

Immediately after completing microbubble-mediated ultrasound or sham therapy, each mouse was transiently imaged at 1, 10, 30 and 60 min post therapy using a dedicated small animal optical imaging system (Pearl Impulse, LI-COR Biotechnology, Lincoln, NE). The Pearl Impulse optical imaging system is a closed configuration with a cooled charge-coupled camera that achieves deep target imaging with laser excitation, and near-
infrared fluorescent detection. Imaging was performed using a near-infrared window 700-nm channel, exciting at a wavelength of 685 nm and emitting at 720 nm (Fig. 2). Animals were maintained under isoflurane anesthesia for each imaging time point. Each animal was positioned on its side, with the tumor side facing upwards in the chamber. One image for each time point was retrieved (grey scale image of the mice and fluorescence intensity were taken simultaneously). The mice were awakened between each imaging sequence.

The ability to image tumors progressively over time allowed measurement of local fluorescent tracer uptake and any therapy-induced effects. Fluorescent intensity was measured using Pearl Impulse Software Version 2.0 (LI-COR Biotechnology, Lincoln, NE) region-of-interest (ROI) analysis of the tumors. An ROI was drawn around each tumor (this was done separately for each image, as there might be slight differences in mouse positioning). Then using Pearl Impulse Software, total fluorescent intensity within that ROI was measured. Each ROI was then normalized by its total pixel counts to quantify mean intratumoral fluorescence. All measurements from small and large molecule groups were compared to control counterparts to quantitatively determine fluorescent tracer uptake and retention. Qualitative analysis of spatial variations in tumor samples was observed.

*Ex vivo* Optical Fluorescent Imaging

Animals were humanely euthanized immediately following the last optical imaging time point (1 hour post therapy) and tumors were extracted. Excised tumors were rinsed with saline, weighed (AX105 DeltaRange, Mettler Toledo, Columbus, Ohio) and imaged for fluorescent signal detection using the Pearl Impulse. Tumors were then sectioned into quarters for fluorescent dye extraction.
Fluorescent Dye Extraction

Each of the quarters was weighed and 250 µL of a 100 mM of sodium hydroxide (NaOH) solution (Sodium Hydroxide Molecular Biology Grade, Fisher Scientific, Fair Lawn, New Jersey) was added. The tumor samples were digested in the NaOH solution for 2 hrs with periodic vortexing. Samples were centrifuged (10 min at $16.1 \times 10^3$ g) and the supernatant (50 µL) was analyzed for dye extraction using a fluorescent plate reader with a 694 nm filter (Victor$^2$ 1420 Multilabel counter, PerkinElmer Life Sciences, Waltham, MA), exciting at a wavelength of 675 nm. The tumor samples continued to incubate in NaOH solution for the remainder of 48 hours to extract the remaining dye. Supernatant was analyzed again using previously described methods at 24 and 48 hrs after tumor extraction. Forty-eight hrs post extraction, the remaining supernatant was removed from the tumor pellets and the samples were again imaged using the small animal imaging system to determine presence of any residual Cy5.5 dye.

Statistical Analysis

All statistical data was analyzed using SAS software (SAS, Cary, NC). Unpaired two-sample $t$-tests were used to assess differences between the small and large molecule group data, as well as control and microbubble-mediated ultrasound therapy exposed group data. For $in vivo$ experiments of sequential fluorescent testing, analysis of variance (ANOVA) statistical testing was used to test difference in groups. Data was recorded at mean ± SEM. A $p$-value of less than 0.05 was considered statistically significant.
Fig. 3. Optical monitoring allows intratumoral analysis of fluorescent tracer accumulation. The mean fluorescent signal from each tumor location is normalized by tumor size. (a) Representative images of therapy and control animals at 1, 10, 30 and 60 min after microbubble-mediated ultrasound therapy exposure and/or fluorescent tracer dosing. Summary of fluorescent image temporal measurements from therapy and control tumors for the (b) small molecular tracer and (c) large molecular tracer dosed animals.

RESULTS

In vivo Optical Fluorescent Imaging

In vivo optical imaging following application of microbubble-mediated ultrasound allows monitoring of local tumor uptake of systemically circulating fluorescent tracers. Average tumor size was 113.8 ± 10.6 mm². There was no significant differences between tumor sizes, between small molecule therapy and control group (p = 0.32), as well as large molecule therapy and control group (p = 0.26). Temporal measurements showed a 70.1% increase in small molecule fluorescent tracer accumulation over control group.
measurements at 1 min, followed by increases of 45.1%, 25.5% and 18.6% at 10, 30 and 60 min, respectively ($p = 0.02$). In the large molecule group, *in vivo* data demonstrated tracer accumulation increases of 9.6%, -4.3%, -2.6%, and -2.8% over control at 1, 10, 30 and 60 min, respectively, albeit the data was not significant ($p = 0.51$) as shown in Fig. 3. Fig. 3a depicts representative images of intratumoral fluorescent tracer uptake for the small molecule group at 60 min. Fig. 3b and 3c shows uptake trends for the small and large molecule groups, respectively. Qualitative analysis of tumor samples showed spatial

**Fig.4.** Tumor samples analyzed *ex vivo* for fluorescent signal detection. (a) Representative tumor images are shown for the small (Cy5.5) and large (IgG-labeled Cy5.5) molecular tracer exposed samples. (b) Differences in fluorescent signal from *ex vivo* samples (normalized by pixels in region-of-interest).
variations throughout the tumor. Compared to control data, significant increases in small molecular tracer uptake were observed up to 10 min following microbubble-mediated ultrasound exposure ($p < 0.05$). However, this trend subsided and no significant differences in tracer accumulation were found at 30 or 60 min ($p > 0.05$). No such trend was observed in the large molecule group data. Tumors were quantitatively analyzed for fluorescent signal as a whole, yet increased signal levels were frequently noted in the tumor periphery where vascularity is most dense in these preclinical animal models. Although this is true, accumulation in blood cannot be distinguished from uptake in tissue, therefore variation is unknown to be linked to vascularization.

Ex vivo Optical Fluorescent Imaging

Tumors ($N = 23$) were imaged ex vivo with the Pearl Impulse and exhibited no significant differences during intra-group comparisons ($p > 0.44$), although fluorescent signals from Cy5.5 therapy samples were 19% greater than control (Fig. 4). Fig. 4a shows representative images of ex vivo tumors depicting detection of intratumoral fluorescence after microbubble-mediated ultrasound therapy using either the small or large molecular tracers. Fig. 4b shows fluorescent signal therapy and control groups, normalized by pixels in the region-of-interest. Tumor weights were $0.312 \pm 0.052$ grams. No differences were found during analysis of intragroup tumor weights between small molecule therapy and control group ($p = 0.50$) and large molecule therapy and control groups ($p = 0.92$), which allows direct comparison between therapy and control groups.
Fig. 5. (a) Fluorescent signal analyzed in supernatant after tumor extraction. (b) Analysis of pellet fluorescence following completed dye expulsion reveals no significant differences remain between control and therapy exposed groups, indicating accurate measurements can be made from the supernatant solution and extracted dye.

Fluorescent Molecule Extraction

In order to quantify the amount of intratumoral dye (following microbubble-mediated ultrasound therapy exposure), excised tumor samples were homogenized, fluorescent dye released from within the tumor and the supernatant was imaged for fluorescent tracer presence. Fluorescent dye in the supernatant extracted from the tumors
was used to determine the total amount of dye that was taken up or extravasated into the tumor. At 2 hrs of being in NaOH, no significant difference in supernatant fluorescence of degraded tumors was found in comparison to the counterpart control samples ($p > 0.61$). At 24 hrs of degrading in NaOH, sample analysis revealed a significant increase (10.9%, $p = 0.003$) for the large molecular tracer group compared to control samples. At 24 hrs, no significant differences in the small molecular tracer group were found, yet there was a 5.5% increase in detected fluorescence signals for the samples exposed to microbubble-mediated ultrasound therapy compared to control tissue ($p = 0.09$). However, after 48 hours of continuous NaOH treatment on ex vivo tumors to destroy cell barriers and allow fluorescent dye release, there was significantly higher Cy5.5 signal concentration in samples receiving microbubble-mediated ultrasound therapy with both small molecular ($p = 0.01$) and large molecular ($p = 0.002$) tracers, Fig. 5a. Specifically, the microbubble-mediated ultrasound therapy exposed samples exhibited increases in fluorescent signal of 9.3% and 14.2% using small and large molecular tracers, respectively. The tumor pellet samples were imaged to determine presence of any residual fluorescent Cy5.5 signal. No significant difference between residual amounts of fluorescent tracer in pellet samples post liquid removal were found ($p > 0.10$), Fig. 5b. These findings suggest that the majority of fluorescent dye was extracted out of all the excised tumor samples analyzed.

**DISCUSSION**

Microbubble-mediated ultrasound therapy has considerable potential for increasing localized drug uptake in cancerous tissue in addition to a host of other clinical applications [10, 15-19]. As this novel therapy continues to be studied, it becomes
increasingly important to elucidate the effects and mechanisms of this therapeutic adjunct. In this study, we investigated a non-invasive optical imaging method for monitoring the effects of microbubble-mediated ultrasound therapy in living animals. This strategy provides mechanistic insight into the process of increasing the localized delivery of molecules (such as chemotherapeutic drugs and therapeutic antibodies) to tumors and may prove useful for optimizing this ultrasound-based technology for in vivo applications.

Both IgG-labeled Cy5.5 (large molecules, MW = 150 kDa) and Cy5.5 dye alone (small molecules, MW = 1.1 kDa) were analyzed in this experiment due to the matched size relationship to antibody and chemotherapeutic cancer drugs, respectively. Exposure parameters can influence the impact of microbubble-mediated ultrasound therapy through size, location and number of the pores developed; therefore molecular size is an important consideration. IgG molecules are composed of four peptide bonds [29]. These IgG molecules are similar in size to drugs such as cetuximab (MW = 152 kDa), which targets the epidermal growth factor (EGF) receptor and is used to treat cancers of the head and neck, lung, colon and esophageal [30]. Bevacizumab (MW = 148 kDa) has been used in cancer treatment and is an IgG molecule [31]. Although, these antibodies are the ones mentioned for comparison of size, there are many other that are currently used in cancer treatment. The molecular weight of Cy5.5 dye alone is similar to the size of current breast cancer chemotherapeutics such as paclitaxel (MW = 0.9 kDa) [32, 33]. The large molecular tracer group had fluorescent molecules directly conjugated to the antibody, while the small molecule group was the same fluorescent molecules alone. Note the total dye amount injected was not the same between the small and large
molecular tracer groups. Because the amount of dye differed between the small and large molecules, it was necessary to analyze each group separately and use their control counterparts as direct comparison for quantitative analysis of fluorescent uptake. Although there were significant differences between the therapy and control groups in the supernatant analyzed, during the in vivo optical imaging analysis, only the small molecular tracer group data revealed significant increases in tumor uptake when comparing the microbubble-mediated ultrasound therapy exposed and control samples throughout the study. This is most likely due to the smaller size and ease of extravasation during microbubble-mediated ultrasound therapy exposure. It is currently unknown whether intracellular delivery of all antibodies would present an additive beneficial treatment or if it would decrease the effectiveness of some antibody therapy because of their targeting to extracellular markers. However, Maeda et al. showed that sonoporation of anti-EGFR antibody in vitro makes it possible to administer bleomycin into cells more efficiently and specifically, showing an application for cancer therapy in squamous cell carcinoma [5]. It could be possible to decrease effectiveness of the antibody when internalized if that particular antibody requires an extracellular interaction to begin its cascade of events (ex. TRA8 and targeting the DR-5 receptor to kill tumor cells) [34]. In vivo, total delivery of antibody to the tumor may be improved by endothelial cell disruptions to improve extravasation of antibody from the circulation. This data supports the need for increased research on in vivo longitudinal studies of sonoporation using both small and large molecules. Our in vivo data however, did not support this hypothesis. Further optimization of parameters might allow only increased cellular delivery of small
molecules (e.g., chemotherapeutics), while allowing larger molecules (e.g., antibody treatments) to only target extracellular ligands.

When exploring *in vivo* microbubble-mediated ultrasound therapy, previously reported methods analyzed secondary effects, such as tumor response to chemotherapy, by measuring size and evaluating histological results post mortem [35]. Secondary effects, such as MRI signal enhancement have shown to be effective in correlating drug delivery from microbubble-mediated ultrasound therapy through the blood-brain barrier [13]. There has yet to be an established method for directly analyzing temporal affects *in vivo*. Optical fluorescent imaging has been used to look at investigating biological systems in many areas of interest and is extremely popular because imaging can be accomplished with native, unaltered cells [36-38], while still remaining non-invasive. Another option includes bioluminescent imaging; however, it is limited by genetic alteration of cells (e.g. luciferase-positive cells) [39]. One limitation to this study is the imaging system only reads near-infrared wavelengths of 700 nm or higher, therefore Cy5.5, Alexa fluor near-IR spectra and IR-dye are among the only fluorescent dyes available. Although this is a limitation, it is also advantageous because it limits the amount of background from surrounding tissue and is optimized for high-performance optical imaging. Without utilizing near-infrared dye, intrinsic autofluorescence from animal tissue can be high and mask the signal from optical probes [40, 41]. Another limitation to this study is there was no control group of molecules plus ultrasound treatment alone (without microbubbles). This was done due to limitations in animal numbers. However, this limitation does not take away from the discoveries of the experiments, but shows that more extensive studies will need to be completed in the
future to further explore the mechanisms of this therapy. This study demonstrates feasibility for a novel application for fluorescent optical imaging. Future studies will consist of combination microbubble-mediated ultrasound and fluorescently-labeled anticancer drug therapy with longitudinal optical monitoring.

This serial quantification allowed monitoring direct temporal effects of microbubble-mediated ultrasound therapy on tumor tissue \textit{in vivo}. This information also gives insight into biological effects into recovery time of the cellular and vascular membranes post microbubble-mediated ultrasound therapy. \textit{In vivo} results revealed significant differences over time in tumor uptake of the small molecular tracer when comparing therapy to control group data, yet no difference was seen when using the large molecular tracer. The ultrasound parameters used in this study have been previous explored \textit{in vivo} with the delivery of paclitaxel [17]. The parameters may not have been optimal for antibody therapy and larger molecules, such as IgG. However, it was shown that dye was accumulated inside the tumor. When analyzing the small molecule group, there was initially a 70\% increase of dye accumulation compared to control, which progressively decreases over time before plateauing at approximately 20\%. There was no significant difference in fluorescent uptake at time point 30 and 60 min within the therapy group ($p = 0.12$), yet there was significant differences when compared to time point 10 min ($p = 0.02$). This is important because it may reveal significant insight regarding cell wound healing responses after microbubble-mediated ultrasound therapy. When examining this study, it appears that after 30 min post microbubble-mediated ultrasound therapy, there is no additional uptake of molecules. At that point, it is suspected that the fluorescent molecules, or chemotherapeutics, will remain entrapped in the cells or
extracellular space within the solid mass tumor. It is hypothesized that the first thirty minutes of microbubble-mediated ultrasound therapy will have the greatest enhanced effect given the conditions used for this study. When monitoring the ex vivo supernatant fluorescent release from the tumors, the initial release of fluorescence (at 2 hrs) is believed to be released from the vascularity. After 48 hrs of sodium hydroxide treatment, supernatant from lysed tumor cells presented significant levels of Cy5.5 in both the small and large molecular tracer dosed tumors. This additional fluorescent signal from time point 2 hrs to 48 hrs is hypothesized to be from the trapped fluorescent molecules within the cell membranes because it would take longer for the cells to lyse and release their fluorescence compared to the tumor vascularity. It is hypothesized that there was not enough of an increase to visualize using optical imaging for the large molecule group. In general, this experiment illustrates that it takes 48 hrs to completely extract the dye from the excised tissue samples. The ability to track each phenomenon is necessary to learn more about the in vivo mechanistic action of microbubble-mediated ultrasound therapy. Although both were significant when compared to their control counterpart, the small molecular tracer group produced higher levels of fluorescent signal than the large tracer group. This indicates that, in general, more fluorescent dye is being accumulated within the tumor when using the smaller size particles. There was no significant difference in any of the pellet samples, showing that the majority of Cy5.5 was extracted in the supernatant for both groups, small and large molecule. This allowed an accurate reading on fluorescent levels and comparison between groups. Although tumors were quantitatively analyzed for fluorescent signal as a whole, increased fluorescence was most frequently observed around the tumor periphery. This is important to note because
increased angiogenesis/vascularity in tumors is most commonly detected in animal models around the rim of the tumor. Using a near-IR dye, the amount of background from surrounding tissue is minimized, although it’s important to note there is still some signal quantified from imaging the tissue itself. A limitation to the study is a lack of knowledge in whether extravasation or intracellular delivery is the dominant mechanism for the observed increased molecular uptake. We hypothesize that it is a combination effect, yet the exact mechanism still remains elucidated. For the study, it was necessary to use the whole tumor to directly analyze amount of fluorescent dye released from the tumors, therefore no histological evidence is available to analyze what cells the chromophore is located in and whether it is truly intracellular. Although there is not conclusive information showing where the chromophores are residing after uptake by the tumor, we hypothesize that the initial release of fluorescence signal from the excised tumor (2 hrs of NaOH immersement) was from the vasculature of the tumor, while it took 48 hrs to completely degrade the cell membranes in NaOH in order to release the molecules that were entrapped intracellularly. Future studies will include histological analysis of tumors post microbubble-mediated ultrasound therapy to determine which cells within the tumor were involved within the molecular uptake. This study shows that microbubble-mediated ultrasound treatment may be more effective if in cancer types that have increased vascularity or blood flow, and not hypo vascularized tumors, such as pancreatic cancer.

Near-infrared fluorescent optical imaging has proven useful for detecting cancer and monitoring cancer growth [42, 43]. The dye acts as a surrogate for drugs, and presents potential in analyzing uptake in preclinical studies. The ability to monitor the
effectiveness of increased tumor perfusion from microbubble-mediated ultrasound therapy is extremely beneficial and will lead to increased understanding of systemic drug delivery. The capability to both monitor cancer treatment and evaluate pharmacological distribution will help us gain further understanding on the mechanisms of the treatment. As we further explore mechanisms of localized and targeting tumors in cancer therapy, optical fluorescent imaging could continue to improve our capabilities. This understanding and potentials are important as cancer research continues to progress and introduce combination therapy to current clinical practices.

CONCLUSIONS

A non-invasive optical imaging method for monitoring the effects of microbubble-mediated ultrasound therapy provides insight into the process of increasing extravasation of molecules into target tumors. Preliminary results are encouraging and fluorescent optical imaging may prove useful for optimizing therapy and drug delivery monitoring in preclinical cancer models.

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CHAPTER 4

ENHANCEMENT OF ADENOVIRUS DELIVERY AFTER ULTRASOUND-STIMULATED THERAPY IN A CANCER MODEL

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Accepted to Journal of Ultrasound in Medicine and Biology

Format adapted for dissertation
ABSTRACT

Improving the efficiency of adenovirus (Ad) delivery to target tissues has the potential to advance the translation of cancer gene therapy. Ultrasound (US)-stimulated therapy utilizes microbubbles (MBs) exposed to low-intensity US energy to improve localized delivery. We hypothesize that US-stimulated gene therapy can improve Ad infection in a primary prostate tumor through enhanced tumor uptake and retention of the Ad vector. In vitro studies were performed to analyze the degree of Ad infectivity after application of US-stimulated gene therapy. A luciferase-based Ad on a ubiquitous cytomegalovirus (CMV) promoter (Ad5/3-CMV-Luc) was used in an animal model of prostate cancer (bilateral tumor growth) to evaluate Ad transduction efficiency after US-stimulated therapy. Bioluminescent imaging was employed for in vivo analysis to quantify Ad infection within the tumor. In vitro studies revealed no difference in Ad transduction between groups receiving US-stimulated therapy using high, low, or sham US intensity exposures at various multiplicity of infections (MOIs) (p = 0.80). In vivo results showed that tumors receiving US-stimulated therapy after intratumoral injection of Ad5/3-CMV-Luc (10^6 plaque forming units) demonstrated a 73.6% enhancement in tumor delivery and retention compared to control tumors receiving sham US (p = 0.03). US-stimulated therapy has significant potential to immediately impact Ad-based cancer gene therapy by improving virus bioavailability in target tissues.

INTRODUCTION

There is an urgent need to improve delivery of recombinant adenovirus (Ad) in order to advance cancer gene therapy. Ad vectors show immense potential in cancer therapy due to their ability to selectively target and destroy specific tumor cells, while not
injuring healthy tissue. Multiple applications using Ad vectors in cancer have been explored. Therapeutic strategies often involve the triggering of cell death via a death-inducing reporter that is specifically driven by a cancer promoter. Other utilities involve immunotherapeutic approaches aimed at inducing host antitumor immune responses (Lupold and Rodriguez 2005). Direct cancer cell death can be accomplished through delivery of replication oncolytic viruses or non-replicating vectors encoding tumor suppressor genes, suicide genes, or antiangiogenic genes (Kaplan 2005). Tumor cells can be destroyed at both primary and metastatic locations through induction of host antitumor immune responses. Although gene therapy has advanced in the last decade, there are many limitations that prevent routine applications. The effectiveness of gene therapy is directly dependent on successful site-specific delivery. Limitations of delivery include anti-Ad host immune response, tumor cell transduction, extravasation of the large molecules to their intended site, and the ubiquitous Ad receptor can lead to virus uptake in cell types other than the targeted region. Additional hurdles include the inability to overcome filtration from the liver, and the limited infectivity of Ad serotype 5 (Ad5). These limitations lead to necessary advancements in the field of Ad delivery.

US-stimulated therapy provides a localized technique to enhance agent delivery. MBs are clinically used US contrast agents have proven to be non-immunogenic and non-toxic in nature (Calliada, Campani 1998, Cosgrove 2006). This unique therapy uses US-exposed MBs to both increase cell membrane permeability and induce localized molecular extravasation (Dijkmans, Juffermans 2004, Ferrara, Pollard 2007, Lindner 2004, Song, Chappell 2002, Sorace, Warram 2012). Although there is some disputing evidence between the exact duration of this effect, this therapeutic stimulation has been
shown to last up to 30 minutes post US exposure (Sorace, Saini 2013). US-stimulated therapy has been increasingly popular in preclinical models because it is generally noninvasive and has significant potential for translation. US-stimulated therapy has been shown to increase cytotoxic agent delivery to cancer to improve response by greater than 50% compared to drug alone (Casey, Cashman 2010, Heath, Sorace 2012, Sorace, Warram 2012). It has also been demonstrated that positive effects can be achieved after only a single dose of treatment (Sorace, Saini 2013). Other applications of this therapy include delivery of drugs through the blood-brain barrier and enhancing delivery of DNA (Klibanov 2006, McDannold, Arvanitis 2012, Sirsi and Borden 2012, Treat, McDannold 2012). Studies have also shown that Ad particles can be safely delivered to a localized region by MB packaging to avoid ubiquitous uptake in other cells and liver filtration (Warram, Sorace 2012). To the best of our knowledge, applying US-stimulated therapy to enhanced Ad delivery is a novel application of this US technology.

In order to establish genetic-based therapeutics as a routine treatment option for cancer patients, delivery barriers must be overcome (de Vrij, Willemsen 2010). In the current study, US-stimulated therapy is evaluated for the potential to improve Ad vector transduction in an animal model of prostate cancer. Considering the relatively safe properties associated with US-stimulated therapy, positive evaluation of this technique could immediately impact Ad-based therapy trials leading to improved treatment success and patient survival.
MATERIALS AND METHODS

Adenovirus Preparation

A non-replicative, luciferase-reporter based, serotype 5 Ad on a ubiquitous cytomegalovirus (CMV) promoter (Ad5/3-CMV-Luc) was used to evaluate Ad transduction due to the ease of bioluminescence imaging for evaluation of response. In order to improve infectivity and ablate coxsackie Ad receptor (CAR) mediated infection, the Ad5/3-CMV-Luc contains a serotype chimeric infectivity motif where the serotype 3 Ad (Ad3)-specific tropism conferred to the Ad serotype 5 fiber by its knob domain replacement with that of the Ad3 fiber. For the study, particle amplification was performed in HEK-293 cells and purified using cesium chloride centrifugation gradients. A standard agarose-overlay plaque assay was conducted with HEK-293 cells to determine a viral titer of 1.1x10^{11} plaque forming units (PFU) per mL.

Cell Culture

PC3 human prostate cancer cells were purchased from the American Tissue Type Collection (Manassas, VA). The cell line was maintained in DMEM media with 10% FBS and 1% L-glutamine. All cells were grown to 80 to 90% confluence before passaging. Cell numbers were determined using a standard hemocytometer and cell viability was measured by trypan blue dye exclusion.

In vitro Experimentation

Experiment 1: Aliquots (0.1 mL in PBS) of Ad5/3-CMV-Luc were placed in 1.5 mL polypropylene microcentrifuge tubes at various PFU amounts (0, 3.5, 3.5 x 10^1, 3.5 x 10^2, and 3.5 x 10^3 infectious virus particles). Groups were then subdivided into low-
pressure US, high-pressure US, and sham US exposure (control) groups. All groups were evaluated in triplicate. To each tube, 10 μL of MBs (Definity, Lantheus Medical Imaging, North Billerica, MA) was added immediately prior to US-stimulated therapy. US exposure was performed on the groups in a 37°C water bath with the following acoustic parameters: 1.0 MHz transmit frequency, peak negative pressures of 0.85 MPa (high pressure condition) or 0.1 MPa (low pressure condition), a pulse repetition period of 0.1 sec, 10% duty cycle, and a 5 min duration of US exposure. An unfocused single-element (0.75 inch) immersion transducer (Olympus, Waltham, MA) was placed in series with a signal generator (AFG3002B, Tektronix, Beaverton, OR) and power amplifier (A075, Electronics and Innovation). Ad groups were then added at various multiplicity of infection (MOI) ratios (0, 0.01, 0.1, 1, and 10) to PC3 cells (3.5 x 10^2 cells per well in a 24 black well plate) plated 24 hr prior. Note that an MOI value is defined as the ratio of Ad particles to infectious targets (i.e., number of cells per well). Virus aliquots were allowed to incubate for 1 hr then removed and replaced with complete media. Following a 24 hr incubation period, plates were imaged using an IVIS-100 CCD imaging system (Caliper Life Sciences, Mountain View, CA) and the bioluminescence signal (photon counts) were quantified for each group and well using equipment software.

Experiment 2: Aliquots (0.1 mL in PBS) of PC3 cells (3.5 x 10^2) and Ad5/3-CMV-Luc (3.5 x 10^2 PFU, MOI = 1) in polypropylene microcentrifuge tubes underwent high pressure US treatment (N = 13) or sham US treatment (N = 13) in the presence of MBs in a 37°C water bath using identical high pressure US parameters as experiment 1. After applying US therapy, cells and Ad were plated in a 24 well plate, and then rinsed
and replaced with complete media after 1 hr. Twenty-four hr thereafter, plates were imaged for presence of bioluminescence as described in the previous section.

In vivo Experimentation

Animal studies were approved by the Institute of Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham. PC3 cancer cells (2 x 10^6 cells per 100 µL DMEM media without FBS) were implanted subcutaneously in bilateral flanks of five-week-old nude athymic male mice (Jackson Laboratory, Bar Harbor, ME) (N = 24 animals, N = 48 tumors). Tumors were allowed to grow approximately five weeks to equal tumor size according to caliper measurements (final tumor size of 34.1 ± 2.8 mm^2). Each animal received a 30 µL tail vein injection of MBs (Definity) diluted to a final volume of 100 µL with saline. Animals were then submerged in a custom-built 37°C water bath and remained under isoflurane gas anesthesia for the entirety of the US-stimulated therapy. Two min post MB injection, US exposure was applied to the left flank tumor. Right tumors did not receive US treatment. US-stimulated therapy was performed using the previously detailed parameters. This setup allowed exposure of the entire target tumor with US energy while the contralateral tumor was outside the path of US transmission. Immediately following US, the Ad5/3-CMV-Luc vector was injected intratumorally in both bilateral flank tumors. Animals were divided into three groups and dosed with different Ad concentrations: 1 x 10^6 PFU (N = 12), 1 x 10^7 PFU (N = 5), and 1 x 10^9 PFU (N = 7). For the remainder of the manuscript, 1 x 10^6, 1 x 10^7, and 1 x 10^9 will be referred to as the “low”, “medium”, and “high” concentrations of Ad injection, respectively. Intratumoral doses were administered in total volume of 50 µL. Animals were imaged for bioluminescence expression before
therapy on day 0 (baseline) and again on day 2 (48 hours post therapy) using the following methods. Animals were injected intraperitoneal (IP) with firefly luciferin. After a 15 min period allowing for systemic circulation, all animals were oriented so both tumors were visualized and then imaged for bioluminescence expression using a Xenogen small animal imaging machine (BioSciences, Cranbury, NJ, USA) and established data acquisition protocols. Five animals were imaged simultaneously using a 300 see exposure, f/stop of 1, binning of 8, and fixed stage height. Standardized region-of-interests (ROIs) were generated using instrument software to analyze photon counts.

Statistical Analysis

All experimental data was summarized as mean ± SE and reported as percent change or bioluminescence counts. A 2-sample paired t-test was used to calculate statistical difference between control and US-stimulated tumors within each group. No comparisons were done directly between the various Ad concentration groups. A p-value of less than 0.05 was considered statistically significant. Analyses were completed using the SAS statistical software package (Cary, NC).

RESULTS

During in vitro experiment 1, various concentrations of Ad particles were exposed to US-stimulated therapy in the absence of cells to determine the effect of US treatment on the infectivity potential of the virus. After US exposure, the Ad was added to plated cells at various MOIs and allowed in incubate. Bioluminescence imaging resulting from successful Ad infection demonstrated that there is no significant difference in virus infectivity or vector transduction at high, low, or no (sham) exposure to US pressures (p = 0.80), Figure 1. The importance of this finding is that it confirms that exposing the Ad
Figure 1. Representative bioluminescence images of plated cancer cells incubated with Ad vectors after exposure to high pressure US-stimulated therapy or sham (control) US (top left). Detailed analysis of the bioluminescent signal (counts) representing Ad infection efficiency after the Ad vector was exposed to acoustic conditions akin to that used during US-stimulated therapy (bottom left). Sham or US exposure at low or high acoustic pressures did not produce any differential effects or alterations in Ad infectivity potential. The application of US-stimulated therapy to cancer cell cultures incubated with an Ad vector resulted in no significant differences in bioluminescence images (top right) or the Ad infectivity rate (bottom right), which again indicates US had no negative effects on Ad vector infectivity or the transduction pathway.

Vector to US intensity levels necessary for inducing membrane permeabilization during US-stimulated therapy has no negative effect on the infectivity potential of the Ad. The various MOIs tested confirm that this observation was consistent across various concentrations of Ad.
For *in vitro* experiment 2, Ad particles received US-stimulated therapy in the presence of PC3 cells (MOI = 1) to determine effects of US treatment on cellular response to infection. The control group contained Ad5/3-CMV-Luc, cancer cells, and MBs without US treatment. Figure 1 demonstrates that there was no difference in bioluminescence expression when measured after Ad infection in the therapy group (5.4 x 10^5 ± 1.9 x 10^4 counts) and control group (5.3 x 10^5 ± 2.0 x 10^4 counts) (p = 0.92). These results suggest that there was no cellular internalization of the Ad. US-stimulated therapy induced neither negative nor positive effects when applied to a combination of MBs, cells and Ad.

Analysis of US-stimulated gene therapy effects on Ad5/3-CMV-Luc transduction in an *in vivo* model of prostate cancer was also performed. Bilateral flank tumors were used in order to provide *in situ* control tumors that did not receive US exposure. After US-stimulated therapy was applied to treatment tumors, Ad5/3-CMV-Luc was immediately administered to the three animal groups via an intratumoral injection using low, moderate, and high Ad concentrations. Forty-eight hr post treatment, the low concentration therapy group showed a 95.1 ± 35.1% increase in bioluminescence expression compared to control (p = 0.03). At the moderate concentration, the therapy group exhibited a 12.1 ± 6.4% increase compared to control, trending towards significance (p = 0.06). Finally, at the high concentration, the therapy group exhibited no difference compared to control group showing a 10.1 ± 22.8% increase (p = 0.09), Figure 2. A qualitative analysis shows the visual differences in overall bioluminescence expression between therapy and control tumors of low, moderate, and high Ad concentration groups at baseline and 48 hours. Figure 2 also shows representative
bioluminescence images exhibiting enhancement of infectivity at 48 hr in the tumors receiving US-stimulated therapy compared to the contralateral control tumor and baseline

**Figure 2.** US-stimulated therapy improves delivery of the Ad vector to the target flank tumors especially at low Ad concentrations (top). Representative bioluminescence images at baseline and 48 hours after US-stimulated therapy for various Ad concentrations denoted as low, moderate, and high (bottom). US-stimulated therapy produced an increase in bioluminescence signal measurements (via increased Ad infection) over contralateral control tumors at various Ad concentration levels (bottom).
images. Further analysis of the low Ad concentration group revealed that at baseline, there were no statically significant differences between the control and US therapy.

![Bar chart](image.png)

**Figure 3.** Analysis of individual animals in the low Ad concentration group which demonstrated the greatest enhancement after receiving US-stimulated therapy. Of the animals investigated, 75% of the tumors produced increased bioluminescence signals compared to contralateral tumor measurements. These findings were attributed to improved Ad retention and infectivity in the target tumor receiving US-stimulated therapy.

Exposed tumors ($p = 0.27$). Individual analysis of the animals in the low concentration Ad group demonstrates that 75% of the animals studied revealed increased infectivity, 17% showed relatively no change, and one animal exhibited a negative response, Figure 3.

**DISCUSSION**

Enhanced tissue-specific delivery of Ad-based vectors has the potential to make significant improvements to current cancer gene therapy protocols. The strategies investigated here provide original advances in Ad delivery to the intended region to assist virus retention within the target tumor tissue. The *in vitro* studies confirmed that Ad
infectivity was not affected by either the high or low US pressure conditions (1.0 MHz frequency). While the approach in this study was to inject the virus post US-stimulated therapy, evaluating the effects of US-stimulated therapy directly on Ad particles was necessary for future translation and investigations. For future work utilizing either a multi-dose study or intratumoral injections administered prior to therapy, we can be confident that we are not altering the gene therapy vector during exposure to US energy levels detailed in this article.

Internalization of the Ad vector, which ultimately leads to tissue infection, is triggered by interaction of the viral penton with epithelial integrins. From there it is processed to the nucleus and is eventually expressed (Lupold and Rodriguez 2005). The Ad requires receptor-mediated internalization therefore intracellular delivery through membrane permeabilization could decrease expression. In vitro experiments demonstrated that increased Ad transduction did not occur and therefore was not due to increased infection by altering the mechanisms of the adenoviral process or internalization. This is consistent with previous studies analyzing gene transfer with US-stimulated therapy (Miller and Quddus 2000, Price, Skyba 1998). Bioluminescence signal measurements between in vitro experiments shows precision of infection at an MOI of 1. US alone has the capacity to increase the surrounding medium’s temperature, which could potentially alter infection rate however there was no change in water bath temperature as monitored throughout the entirety of each US treatment session. No differences were found in Ad infectivity in vitro when US-stimulated therapy was applied directly to cells. Previous experiments have shown that no decreases in cell viability occurs during similar US-stimulated conditions (Sorace, Warram 2012). The MOI of 1
was chosen for this experiment to not oversaturate the cells in order to more accurately evaluate and quantify interactions between cells and Ad. Considering receptor-mediated internalization required for successful virus infection, US-induced internalization would not lead to reporter transduction. Therefore, if a decrease in bioluminescence signal was observed within the US group, it would indicate the viral particles were sequestered inside the cell and not available for traditional infection. Since this was not observed, the conclusion is that US-stimulated internalization did not occur. It is proposed that the large size of the Ad vector compared to drug molecules decreases its ability to be internalized through a membrane permeabilization effect from US-stimulated therapy.

Detailed *in vivo* studies evaluated enhancement of Ad delivery after applying US-stimulated therapy in an animal model of prostate cancer. The greatest enhancement of Ad transduction was observed at the lowest vector dose, while little or no change was observed at the highest doses. At the highest dose, Ad availability at the cancer cell level was not a boundary for transduction due to the high concentration (tissue saturation) of the Ad vector. Considering the purpose of the study was to highlight enhanced transduction from US-stimulated therapy, the lowest dose provided the greatest potential for improvement. Although 75% of the animals studied demonstrated a positive outcome when administered a low concentration of Ad in combination with US-stimulated therapy, there was one animal which demonstrated a decrease in Ad tumor delivery. The animal showing a negative response could potentially be a result of a poor intratumoral injection. Notwithstanding, the utilization of this promising US technology would improve the bioavailability of low Ad vector doses and allow a lower dose to achieve the same therapeutic effect as high dose administrations. This outcome could help reduce
patient toxicity, which currently hinders widespread use of gene therapy techniques in the clinic. As opposed to intravenous injections, intratumoral injections can offset the limitations of adenoviral gene delivery such as the anti-Ad host immune response and the ubiquitous Ad receptor leading to adenoviral uptake in all cell types.

Several studies have been detailed in the literature that investigated gene transfection efficiency in various tissue types using Ad vectors enhanced by US-stimulated delivery techniques. Specifically, the efficacy and safety of multidrug resistance 1 (MDR1) gene transfer into bone marrow cells was recently shown in a series of in vitro experiments to be enhanced using US-stimulated therapy (Guo, Hong 2011). Another research group integrated plasmid DNA into a MB shell once the injected agents reached the target tumor tissue, high-intensity US energy was used to destroy the MBs and trigger localized payload (DNA) delivery (Sirsi, Hernandez 2012). Using bioluminescence imaging techniques, this group was able to detect a significantly higher region of expression within the tumor compared to normal tissue. Research analyzing the longitudinal effects of antiangiogenic gene therapy on hepatocellular carcinoma demonstrated a significant decrease in microvessel density and increase in apoptosis using US-stimulated therapy with plasmid compared to plasmid alone (Nie, Xu 2008). Various other studies have investigated US-stimulated delivery of genetic material in the heart (Bekeredjian, Grayburn 2005, Shohet, Chen 2000, Tsunoda, Mazda 2005), pancreas (Chen, Ding 2006), skeletal muscle (Wang, Liang 2005, Zhang, Wang 2006), kidney (Koike, Tomita 2005), central nervous system (Shimamura, Sato 2005), and solid tumors (Nie, Xu 2008, Wang, Wu 2009, Warram, Sorace 2012).
There are phase I and II trials currently ongoing to evaluate Ad vector delivery in human. Ad vectors are being explored due to their high transduction efficiency compared to a retrovirus or lentivirus. Phase II clinical studies in Ad-based prostate-specific antigen (PSA) vaccine are also being conducted. The PSA vaccine has been deemed safe with minimal toxicity side effects compared to more conventional anticancer drugs and the investigators hope that the Ad vector will produce immunity to the PSA and destroy cancer cells producing PSA (NCT00583024) (Department of Defense 2007 [cited from 2013]). Along with the vaccines that are being studied, there is also a phase I trial utilizing an Ad5.SSTR/TK.RGD gene therapy vector, which is an infectivity-enhanced Ad that expresses a therapeutic thymidine kinase (TK) suicide gene and a somatostatin receptor (SSTR) for imaging patients with recurrent gynecologic cancer. When used in combination with a chemotherapeutic drug (ganciclovir), this novel Ad vector has been shown to induce cancer cell apoptosis (Kim, Dmitriev 2012). This particular study utilized an Ad for imaging gene transfer and monitoring therapeutic response and represents one of the first studies of its kind to prove safety and efficacy in human. Notwithstanding, these authors noted that further refinements in enhancing Ad vector infectivity are needed. Incorporation of US-stimulated gene therapy may help overcome this problem.

The ability to protect Ad vectors from systemic clearance and liver retention while enhancing its bioavailability within the tumor are important advancements in gene delivery to the target tumor. Previous research has shown that US-stimulated therapy can enhance delivery of both drugs and plasmids for cancer treatment. To the best of our knowledge, this article details the first study utilizing US-stimulated therapy to improve
Ad delivery to the target tumor. Our results illustrating that Ad infection can be considerably enhanced after a single session of US-stimulated therapy is a significant finding in the field of cancer gene therapy and warrants further investigation.

ACKNOWLEDGMENTS

The authors would like to thank Anton Borovjagin, PhD, from the School of Dentistry at the University of Alabama at Birmingham (UAB) for the generous donation of the Ad vector used in this article. This research project was supported in part by grant number PC111230 from the Prostate Cancer Research Program of the Department of Defense and a pilot award from the Department of Radiology at UAB.

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Enhanced gene delivery into skeletal muscles with ultrasound and microbubble
CHAPTER 5

BIOLOGICAL EFFECTS FOLLOWING ULTRASOUND-STIMULATED THERAPY IN AN ANIMAL MODEL OF BREAST CANCER

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Submitted to IEEE Transactions on Biomedical Engineering

Format adapted for dissertation
ABSTRACT

Objective: Ultrasound (US)-stimulated therapy has been shown beneficial for improving drug delivery in solid tumors; however there are potential bioeffects that are not fully understood. This study evaluates the presence of bioeffects associated with US-stimulated therapy through the use of multimodal methods. Tumor bearing mice (N = 32) underwent US-stimulated therapy by exposing systemically circulating microbubbles to various acoustic pressures of 0.75 MPa (low) or 3.75 MPa (high) US treatment. Mice were imaged at baseline, day 1, or day 2 with quantitative multimodal methods to evaluate tumor response to US-stimulated therapy. Contrast-enhanced T1-weighted MR imaging evaluated vascular permeabilization. Contrast-enhanced US imaging calculated temporal changes in tumor perfusion. Immunohistochemistry was utilized to evaluate microvessel density, red blood cell (RBC) extravasation, inflammation, apoptosis, and stress. At high acoustic pressures, US-stimulated therapy resulted in a 41.1% decrease in tumor perfusion evaluated with contrast-enhanced US imaging (P < 0.001), while low pressure US-stimulated therapy demonstrated no changes in perfusion (P = 0.74). High pressure US-stimulated therapy also revealed a 136.4% decrease in MR contrast agent accumulation (P = 0.03) at the tumor site. Histological analysis of CD31 confirmed decreased microvessels in the high pressure US-stimulated therapy group compared to low pressure (P = 0.02). Elucidating the bioeffects of US-stimulated therapy is critical for the successful translation of this technique to improve drug delivery in cancer. US-stimulated therapy utilizing high pressure US pulse sequences produced a decrease in vascular perfusion that inhibited molecular delivery; however optimized US-stimulated therapy using low intensity pressures demonstrated no such bioeffects.
Index Terms- bioeffects, cancer, drug delivery, microbubble contrast agent, ultrasound- stimulated therapy, vascular disruption

INTRODUCTION

Ultrasound (US) contrast agents are non-toxic, traditionally polymer or lipid gas-filled contrast agents, that mechanically oscillate under the influence of US waves[1, 2]. Augmentation of cellular and vascular permeability can be induced with microbubble (MB) contrast agents exposed to low-intensity US fields. This unique, noninvasive US-based therapy (referred to as US-stimulated therapy) was first studied for improving macromolecule delivery through the reversible opening of the blood-brain barrier (BBB). US-stimulated therapy has since been investigated to introduce a temporal window for more effective delivery of therapeutic drugs, DNA, or gene therapy vectors to a restricted or challenging region-of-interest (ROI)[3-9]. Other studies have demonstrated that US-stimulated therapy can significantly improve systemic delivery of anticancer drugs to cancerous tissue[10-12].

Ultrasound is considered a relatively safe and inexpensive imaging modality due to the absence of ionizing radiation. However, with research endeavors aimed at the development of US-based strategies for drug delivery augmentation, it is important to study the potential bioeffects[13, 14]. Bioeffects resulting from US-stimulated therapy have been revealed following an assortment of US-stimulated therapeutic approaches. For example, US-stimulated therapy was shown to cause vascular disruption and ceramide-related endothelial cell apoptosis[15]. This high pressure US-induced vascular disruption was confirmed by histology and Doppler US. US contrast agents targeted to endothelial receptors have also demonstrated the ability to collapse tumor vascularity in a
cancer-bearing mouse model after exposure to clinically relevant US parameters[16]. For improved cancer treatment utilizing US-stimulated drug delivery, it is essential to both increase vascular permeability while minimizing any disruption in tumor perfusion and the delivery pathway.

The ability to monitor changes in tumor vascularity validates contrast-enhanced magnetic resonance (MR) and US imaging as potential tools to assess tissue response to US-stimulated therapy. When exploring in vivo applications of US-stimulated therapy, previously reported methods analyzed secondary effects, such as tumor response to chemotherapy, by measuring size and evaluating histological results post mortem[17, 18]. Contrast-enhanced US is an ideal tool to evaluate changes in tumor perfusion as the contrast delivery is self-contained within the vascularity. This technique has been previously successful in longitudinal studies to evaluate tumor response to drug intervention[19-24]. Contrast-enhanced US has also emerged as a noninvasive imaging tool for evaluation of vascular response after US-stimulated drug delivery revealing significant improvements in antitumor response when assessed in combination with chemotherapy[25, 26]. MR imaging has emerged to monitor long-term response to treatment enhanced by US-stimulated drug delivery in a murine model of cancer through evaluation of permeability and cellular apoptosis. Our group has previously shown that we can use diffusion-weighted MR imaging to monitor longitudinal response through secondary analysis of tumor death from combination chemotherapy plus US-stimulated therapy in vivo in SCC5 head and neck squamous cell carcinoma[11]. MR imaging has also been utilized to both guide delivery and evaluate enhanced delivery and bioeffects.
associated with a US-stimulated therapy to temporarily induce BBB disruption in a nonhuman primate model[27-31].

Although US-stimulated therapy and enhanced drug delivery has been shown effective preclinically for cancer treatment, there are certain biological characteristics that remain unidentified. Contrast-enhanced US imaging in cardiac applications has been approved by the FDA for certain indications and is in general deemed safe. As US-stimulated therapy becomes more popular for drug delivery, more research has developed to explore potential bioeffects in tissue following application of US-stimulated treatment[16, 32]. The potential of US-stimulated therapy has been established for improved cancer therapy in preclinical animal models, however to help predict drug dosing and for design of individualized treatments, further analysis is essential. In this study, multimodal imaging techniques (i.e., contrast-enhanced MR and US) were utilized to evaluate the potential bioeffects associated with US-stimulated therapy in a murine model of cancer as confirmed by immunohistologic analysis.

MATERIALS AND METHODS

Tumor implants

All animal work was reviewed and approved by the Institute of Animal Care and Use Committee at the University of Alabama at Birmingham. 2LMP (a lung metastasis subclone of MDA-MD-231) breast cancer cell line was maintained in DMEM media with 10% FBS and 1% L-glutamine. All cells were grown to 80-90% confluency before passaging. Cells were implanted in four-week-old nude athymic female mice (Frederick National Laboratory, Frederick, ME) (N = 32). Tumor implantation used a 27.5 gauge needle and 2x10^6 cells/100µl DMEM media (without FBS) were injected subcutaneously
into the right flank of the mouse. Tumors were allowed to grow approximately three weeks and then randomly assorted into groups.

**Ultrasound-stimulated therapy**

Ultrasonic energy was transmitted across the tumor with a single element 2.25 MHz (0.75 inch) frequency unfocused US immersion transducer (Olympus, Waltham, MA), in series with a signal generator (AFG3002B, Tetronix) and power amplifier (A075, Electronics and Innovation). US-stimulated therapy was applied using the following parameters: a pressure of 0.75 MPa (low) or 3.75 MPa (high) MPa, a pulse repetition period of 15 sec, a 5% duty cycle, and 5 min duration of treatment. For the remainder of the manuscript, these groups will be referred to as low or high pressure groups (N = 4 - 5 animals per group). MBs (Definity, 30 μl containing 9.3x10⁷ MBs diluted with saline to 100 μl) were systemically injected via the tail vein. Following a two min period to allow for adequate MB systemic circulation, mice were submerged in a custom-built 37°C water bath while still under isoflurane gas anesthesia. Mice were then exposed to a single session of US-stimulated therapy. Mice were imaged for day 0 (baseline), day 1 (20 min post-therapy), and day 2 (24 hr post therapy) with contrast-enhanced US imaging, MR imaging, or using optical microscopy for immunohistologic analysis.

**Contrast-enhanced US imaging**

Mice were imaged at baseline, day 1, and day 2 with contrast-enhanced US imaging. Contrast-enhanced US imaging was utilized to evaluate low and high pressure therapy for changes in tumor perfusion and vascular disruption, with baseline data
serving as controls. To monitor response of therapy the following imaging methods were utilized: mice were systemically injected with MBs (identical dose utilized during therapy) via tail vein. US imaging was performed using a SONIX RP research US scanner (Ultrasonix Medical Crop, Richmond, BC, CA) equipped with a L12-5 linear array transducer. Following a 2 minute period for systemic circulation, each mouse was imaged at the largest cross section of the tumor in a MB-sensitive harmonic imaging mode (5 MHz transmit and 10 MHz receive). Imaging parameters are as follows: gain = 90%, depth = 2.5 cm, MI = 0.1, dynamic range = 70 dB, frames per second = 13 Hz. Animals were maintained under isoflurane anesthesia while imaging was performed in a water bath setup at a temperature of 37 °C. The transducer was fixed (Assist Positioning Arm, CIVCO Medical Solutions, Kalona, IA) to minimize motion and registration artifacts when processing. Prior to contrast-enhanced US imaging, mice underwent traditional greyscale US imaging for anatomical mapping.

Quantitative intratumoral intensity was calculated from the mean pixel intensity within the tumor using ImageJ software. Percent change of intratumoral intensity from baseline was analyzed. Greyscale imaging was completed for qualitative analysis of background signal from the tissue, and reveals any artifacts that would appear during future contrast-enhanced US imaging.

Magnetic Resonance Imaging

Mice were imaged on day 0, 1, and 2 with T1- and T2-weighted MR imaging. T1-weighted MR imaging was used to conduct contrast-enhanced MR imaging to evaluate changes in molecular delivery and evaluate vascular permeability after low or high US pressure. T2-weighted MR imaging was conducted for anatomical visualization of the
tumor. Small-animal imaging was performed on a 9.4T MR imaging system (BioSpec; Bruker BioSpin, Billerica, Mass). An MR imaging compatible respiratory gating device (SA instrument, Stony Brook, NY) was used during imaging for continued anesthesia.

Anatomic imaging was acquired with a T2-weighted fast spin echo sequence (rapid acquisition with relaxation enhancement). The parameters were as follows: TR/TE = 3000/32 ms, rare factor = 4, FOV = 30×30 mm, matrix size = 256×256, NEX = 2, and slice thickness = 1 mm for 25 slices. Contrast-enhancement from permeability and delivery was acquired with a T1-weighted imaging FLASH sequence. The imaging parameters were as follows: TR/TE = 122/3 ms, FOV = 30×30 mm, matrix size = 256×256, NEX = 4, with three flip angles 10°, 30°, and 50°, and a slice thickness = 1 mm to cover the tumor region. An MR contrast agent (0.027 mmol/ml, Gadoteridol, Bracco Diagnostics Inc., Princeton, NJ) was injected systemically via tail vein for each mouse (0.2 mmol/kg). T1-weighted imaging was acquired pre- and post-contrast injection for each day. For each of the image sequences a standard of 70% ethanol in a plastic eppendorf tube was placed within the image to verify any discrepancies between MR signal intensity heterogeneity.

Using the anatomical T2-weighted MR imaging, tumors were segmented and an ROI mask of the tumor was created using ImageJ (Softonic, San Fransisco, CA). T2-weighted MR imaging was also utilized for qualitative analysis of edema. The ROI mask was then was applied to each of the T1-weighted imaging slices for the three different flip angles: 10°, 30° and 50° using custom software in MATLAB (MathWorks, Natick, MA). Changes in intratumoral intensity based from mean voxel counts were also calculated using MATLAB. Intratumoral intensity was calculated through evaluation of
voxel intensity over each tumor volume per day. Tumor signal was standardized by dividing mean signal of the tumor by the mean signal from the eppendorf tube for each pre- and post- contrast imaging. Post-contrast images were normalized (post/pre) by their corresponding pre-contrast images for each day and percent change was calculated from baseline. Percent change of intensity was then evaluated with respect to baseline for the whole tumor over days 1 and 2.

Immunohistochemistry

Immunohistology was utilized to evaluate microvessel density, red blood cell (RBC) extravasation, inflammation, apoptosis, and stress. Mice underwent US-stimulated therapy at sham, low or high pressure US-stimulated therapy. Histology requires post-mortem analysis; therefore this modality required a sham/control US-stimulated therapy group. Sham US-stimulated therapy incorporated saline injection with five minutes duration in the water bath (without US exposure: 0 MPa pressure). At 24 hr post-therapy, mice were euthanized and tumors were extracted. Tumors were placed in formalin for 24 hr, and then switched to 70% ethanol. Serial sections of tumor were prepared as previously described[10]. Antibodies utilized were anti-ICAM (Abcam, Cambridge, MA), anti-IL-13 (Abcam, Cambridge, MA), anti-Caspase 3 (Cell Signaling Technology, Danvers, MA), and anti-CD31 (Abcam, Cambridge, MA). Diaminobenzidine (DAB; Scy Tek Laboratories, Logan, UT) was used as the chromagen and hematoxylin (no. 7211, Richard-Allen Scientific, Kalamazoo, MI) for H&E staining.

H&E sections were examined for cellular necrosis and reported as percent of tumor cross-section. Necrotic tissue within the tumor was calculated through pixel analysis and compared to total tumor size.
Microvascular hemorrhage, or total red blood cell extravasation, was also calculated as a percentage for each tumor and compared to total tumor size. Viable tissue was calculated identically and utilized for the remainder of the histological analysis.

Microvessel density was examined through analysis of CD31 staining (original magnification x40) to identify dense regions of staining within the tumor. Caspase 3 staining was determined by calculating total regions of apoptosis in comparison to the negative controls for each tumor. ICAM and IL-13 were quantified by calculating percent expression of staining within the tumor. Total pixels within the dense region were calculated and compared to total pixels within the viable tumor cells using ImageJ. Microvessel density, Caspase-3, ICAM, and IL-13 values reported are calculated as percent of viable tumor.

Statistical Analysis

All experimental data was summarized as mean ± SE. Analysis of variance (ANOVA) assessed differences within the groups for the MR imaging evaluation, contrast-enhanced US evaluation and histology. Linear regression analysis was conducted to correlate RBC extravasation and pressure values of US-stimulated therapy. A paired t-test was used to compare the initial baseline values with their subsequent values to assess changes within individual groups. A P-value of less than 0.05 was considered statistically significant. This analysis was completed using statistical software (SAS Institute Inc, Cary, NC).
Fig. 1. Contrast-enhanced US analysis of changes in tumor perfusion after US-stimulated therapy. (a) Percent change in US enhancement (intratumoral intensity) is quantitatively represented after various US-stimulated therapy at low (0.75 MPa) and high (3.75 MPa) pressure on days 0, 1, and 2. On day 2 (24 hrs post-therapy), high pressure US-stimulated therapy showed a 41% decrease in intratumoral intensity ($P < 0.001$). (b) Representative images show intratumoral intensity at greyscale, baseline, day 1, and day 2. High pressure US-stimulated therapy demonstrated significant decrease in vascularity after US-stimulated therapy as represented through a decrease in intensity in both (a) and (b) (qualitative analysis shows a decrease in intensity within the tumor).
RESULTS

Contrast-enhanced ultrasound imaging

Contrast-enhanced US imaging is a proven tool for monitoring changes in vascular perfusion. Tumors that received low pressure US-stimulated therapy resulted in an initial 36.5 ± 21.6% increase in intratumoral image intensity on day 1 ($P = 0.15$). On day 2, these same tumors exhibited a slight decrease of 4.5 ± 13.0% in image enhancement when compared to baseline images ($P = 0.74$), demonstrating there was no detectable changes in overall tumor perfusion at 24 hrs. Tumors receiving high pressure US-stimulated therapy experienced an initial change of -2.1 ± 9.6% from baseline imaging on day 1 ($P = 0.20$). On day 2, there was a significant decrease of -41.1 ± 2.1% from baseline measures of image enhancement ($P < 0.001$). An overall decrease in intratumoral intensity demonstrates a decrease in vascular perfusion within the tumor at 24 hrs post therapy, which is hypothesized to be due to vascular disruption. Longitudinal changes in intratumoral perfusion are depicted in Fig. 1a. Although not statistically significant compared to baseline measures, an increase in contrast intensity at 20 min after therapy in the low pressure US-stimulated therapy groups was unexpected and a high standard error associated with these perfusion estimates could be due to the increased circulating blood volume in the mouse due to the multiple contrast injections within 20 min. By 24 hr, the additional volume is expected to have sufficient time for secretion. Representative images are shown in Fig. 1b showing qualitative changes with visible decreases in intratumoral intensity decrease in the high pressure group on day 2.
Fig. 2. MR imaging of changes in tumor permeability with US-stimulated therapy. Changes in contrast delivery from T1-weighted imaging allowed qualitative analysis of tumor permeability as demonstrated with (a) representative images of high vs. low pressure groups showing changes in contrast delivery to the tumor at 24 hr post US-stimulated therapy. A decrease in contrast was revealed in the high pressure US-stimulated therapy group, demonstrating decreased permeability and delivery to the tumor. Arrows indicate areas of decreased contrast intensity. Changes in tumor permeability were evaluated with (b) quantification of MR intratumoral intensity through mean contrast enhancement after MR contrast injections on days 0, 1, and 2. Shown is the change compared to baseline imaging demonstrating permeability significantly decreased in the high pressure US-stimulated group on day 2.
Magnetic resonance imaging

T2-weighted MR imaging allowed anatomical imaging of the tumor prior to T1-weighted MR imaging. T2-weighted MR imaging alone revealed no conclusive evidence of edema in either the low or high pressure US-stimulated therapy groups. When analyzing the MR signal after contrast agent injections, image intensity is a function of vascular permeability (leakage) and the progressive accumulation and clearance of contrast agent in the interstitial tumor tissue. Fig. 2a shows representative images of changes in T1 contrast-enhancement from baseline to day 2. Tumors that received high pressure US-stimulated therapy exhibited an $44.9 \pm 22.9\%$ decrease in T1-weighted MR image enhancement on day 1 compared to baseline imaging ($P = 0.06$). On day 2, this amount was decreased by $136.4 \pm 49.4\%$ compared to baseline ($P < 0.001$), Fig. 2b. The MR contrast agent is a relatively small molecule; therefore this significant decrease in image enhancement demonstrates a fundamental decrease in agent delivery to the tumor. Conversely, there was no change in contrast agent delivery at 24 hr after tumor exposure to low pressure US-stimulated therapy. However, low pressure US-stimulated therapy produced a decrease of $24.9 \pm 9.0\%$ in T1-weighted MR image enhancement by day 2 albeit not statistically significant ($P = 0.08$).

Immunohistology

Histological analysis showed that there was no significant differences in tumor necrosis ($P = 0.90$), Fig. 3a. This revealed no internal differences in the tumor viability. Increased central tumor necrosis can reveal that a tumor has outgrown its blood supply; therefore no differences in necrosis help assure a similar initial angiogenic activity and
Fig. 3. Histological analysis of necrosis and RBC release. H&E staining shows (a) graph showing percent necrosis for the different groups ($P = 0.90$), and (b) percent microvascular hemorrhage or RBC extravasation ($P = 0.06$, $R^2 = 0.20$). (c) Representative images are shown with arrows pointing at examples of necrosis (pink), viable tissue (purple), and microvascular hemorrhage (red). These ROI are also zoomed in for clarification. RBC extravasation is a marker for vascular disruption, and increases as pressures increases in the US-stimulated therapy groups.

growth. When analyzing red blood cell release, there was a positive linear relationship between vascular hemorrhage and pressure used during US-stimulated therapy exposure ($P = 0.06$, $R^2 = 0.20$). There was a significant 1.8% increase in the amount of RBC extravasation in the high pressure US groups compared to control ($P = 0.05$), Fig. 3b. This increase in RBC extravasation reveals microvascular hemorrhage and a disruption of vascularity within the tumor. Representative H&E images are shown in Fig. 3c. The H&E slides show viable tissue (purple), tumor necrosis (light pink) and RBC extravasation (red). Arrows detail out these important features. The remaining histological analysis demonstrated no significant changes between the groups in ICAM ($P = 0.77$), IL-13 ($P =$
Fig. 4. Histological analysis of Caspase3, IL-13 and ICAM. Shown is quantification of inflammation (ICAM staining), IL-13 (marker of heat-shock stress), and caspase 3 (apoptosis). Each was determined and analyzed as percent of viable tumor. No significant changes were revealed between the groups for any of these markers ($P > 0.77$).

0.96), and apoptosis ($P = 0.88$), Fig. 4. This reveals important characteristics involving US-stimulated therapy, as the pressure levels of US-stimulated therapy did not reveal significant changes in inflammation (ICAM), stress (IL-13), or initial cell apoptosis (caspase 3). CD31 analysis demonstrated a significant difference between the high pressure US group and the low pressure US group ($P = 0.02$), as seen in Fig. 5a. Areas of high microvessel density were filled in black for clarity in Fig. 5b. A significant decrease in CD31 values reveals destruction of microvessels within the tumor following high pressure US-stimulated therapy. It is also important to note that the low pressure US-
Fig. 5. Histological staining of CD31 for microvessels. Changes in tumor microvessel density after US-stimulated therapy at various pressures is represented with (a) quantitative comparison of control, low, and high pressure US-stimulated therapy groups and their respective representative images. The representative images show filled in areas (black) representing regions of high microvessel density. High pressure US-stimulated therapy demonstrated a significant decrease in microvessel density, further confirming vascular disruption ($P = 0.02$).

DISCUSSION

The purpose of this study was to analyze the bioeffects on the microscopic and macroscopic level (i.e. tumor vascularity, histology) during US-stimulated therapy at various US pressure levels. High pressure US-stimulated therapy was confirmed to trigger biological changes within the tumor; this analysis was completed utilizing three stimulated therapy demonstrated no changes in microvessel density, which allows us to conclude that there would be no vascular restriction for delivery.
clinically relevant modalities: MR imaging, US imaging, and histology. A decrease in intratumoral intensity measured with contrast-enhanced US imaging, as shown in our high pressure US group, demonstrates that intratumoral perfusion and blood flow have been compromised. MR imaging also confirmed a significant decrease in tumor permeability, determined by changes in intratumoral contrast agent delivery. Histology confirmed a decrease in microvessel density at 24 hr after high pressure US-stimulated therapy, as well as indicated an increase presence of microvascular hemorrhage and RBC extravasation. It is especially important to note that the low pressure US-stimulated therapy parameters demonstrated no decrease in perfusion (contrast-enhanced US imaging), permeability (MR imaging), microvessel density, or RBC extravasation (histology) compared to baseline readings, suggesting no negative bioeffects after treatment.

There has been increased exploration analyzing bioeffects from MBs under various US conditions. It has been shown that targeted MBs decrease tumor vascularity and perfusion after exposure to high pressure US fields[16]. Our study revealed a 41% reduction in intratumoral perfusion following use of US-stimulated therapy, which was almost identical to the previously discussed model that showed a 40% decrease using targeted MBs at similar peak pressures. Our study utilized an unfocused transducer, which allows the ability to excite more MBs within the tumor simultaneously in comparison to a focused transducer. Due to the localization of targeted MBs to the endothelium, targeted MBs are more likely to induce endothelium changes than freely flowing MBs when injecting the same total amount. Increased US exposure on the MBs can directly impact the overall biological influence to tumor vascularity, explaining how
we demonstrated similar effects using non-targeted MBs. Goertz et al.[25] demonstrated an 11-fold decrease in intratumoral perfusion as demonstrated with a decrease in contrast-enhancement during US imaging after two treatments of a high pressure US-stimulated therapy alone (US pressure utilized was 2.2 fold greater than our low pressure value). Studies have also utilized post-mortem imaging analysis to assess potential damage from US-stimulated therapy, showing vascular damage and disruption results. To that end, it was shown that US-stimulated therapy using high pressure (9.0 MPa) US exposure produced microvascular hemorrhage and decreased vascular integrity[33]. Although these results were found using a higher US pressure (2.4 fold greater than used in our study for high pressure), it supports the evidence that high pressure US-stimulated therapy can introduce bioeffects and microvascular changes.

It has been well documented that low-intensity US-stimulated therapy can be used in conjunction with gene therapy vectors and chemotherapeutic drugs to improve localized delivery and uptake in the target tissue[4, 8, 10, 34-36]. In a previous study analyzing tumor response immediately following low-pressure US-stimulated therapy, increased tumor uptake occurs immediately following US-stimulated therapy and continues to progress for 10 min post-therapy. It was revealed that after 10 min post therapy there was no additional improvement in tumor uptake, suggesting conclusion of the biological effects creating additional vascular extravasation[6]. This biological response of increased extravasation after US-stimulated therapy at low pressures is considered temporary and has been compared to a wound healing process after injury. The MR imaging contrast injection in this study was 20 min post US-stimulated therapy, therefore we would not expect to see an improvement in permeability of MR imaging
contrast agent at the low pressure levels as previously shown in other studies following therapy. However in the high pressure group, which demonstrated a pronounced vascular effect, we were able to visualize decreases in permeability at both 20 min and 24 hr. During perfusion analysis of contrast-enhanced US imaging, we did not visualize a decrease in tumor perfusion until 24 hr post therapy. Although it is expected that initial endothelial cell membrane injury occurred during US exposure, complete endothelial cell death has been previously determined to require hours to occur, explaining why tumor perfusion was not immediately affected at the 20 min time point after US exposure[37, 38]. Once the endothelial cells are undergoing cell injury and death there will be a down-regulation of CD31 expression, decreasing the staining of these microvessels. Our histology confirms a decreased microvessel density in the high pressure US-stimulated therapy group. One group in particular has utilized high pressure US-stimulated therapy for disintegration of vasculature in order to enhance treatment of radiation in solid tumors[15, 39]. Immediately following therapy they presented a slight decrease in vascular function, however heightened vascular disruption occurred at 6 hours after US-stimulated therapy, with sustained vascular disruption at 24 hr after therapy. This data supports our study visualizing significant bioeffects at 24 hr post high pressure US-stimulated therapy. The group which utilized targeted MBs visualized an immediate vascular reaction after US-stimulated therapy with restored vascularization and recovery after 40 min[16]. This immediate reaction within the vasculature is hypothesized to be a direct result from the targeted MBs interfering with the endothelium, which was not evaluated in our study or the previously compared study.
US-stimulated therapy for BBB disruption has visualized edema in the brain through T2-weighted MR imaging\cite{30}, however this was not possible to conclude with the tissue type we investigated. Further explorations of MR imaging to monitor improved delivery would need to utilize an US therapeutic device integrated with an MR imaging system to simultaneously induce drug delivery and monitoring. A limitation during the MR imaging component of our study was slight changes in mouse positioning (with respect to the magnet) before and after injection of the contrast agent. This decreased the sensitivity of our MR image quantification, but was controlled for by the inclusion of the 70% ethanol standard.

**CONCLUSIONS**

It was revealed through all three modalities that the use of high pressures in US-stimulated therapy can create vascular disruption and a decrease intratumoral perfusion. Notwithstanding, low pressure US-stimulated therapy was not seen in our study to generate any negative bioeffects within the tumor, which is essential for drug delivery. Some evidence of vascular disruption has been previously confirmed; however this study validates safety within the low level pressures using clinically relevant modalities. This study helps further validate the use of low-pressure US-stimulated therapy for the effective and safe delivery of systemic drugs as well as confirming the limitations of this technology when using higher US pressures for the same therapeutic goal.

**ACKNOWLEDGMENTS**

The authors would like to thank investigators in the Small Animal Imaging Shared Facility of the Comprehensive Cancer Center and the Center for Metabolic Bone
Disease at the University of Alabama at Birmingham (UAB) for assistance with the imaging studies and immunohistology, respectively. This research was supported through a pilot award from the Department of Radiology at UAB.

REFERENCES


CHAPTER 6

ULTRASOUND-STIMULATED DRUG DELIVERY FOR TREATMENT OF RESIDUAL DISEASE FOLLOWING INCOMPLETE RESECTION OF HEAD AND NECK CANCER

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Submitted to *Journal of Ultrasound in Medicine and Biology*

Format adapted for dissertation
ABSTRACT

Microbubbles triggered with localized ultrasound (US) can improve tumor drug delivery and retention. Termed US-stimulated drug delivery, this strategy was applied to head and neck cancer (HNC) in a post-surgical tumor resection model. Nude athymic mice ($N = 24$) were implanted in the flank with luciferase-positive HNC squamous cell carcinoma (SCC) and underwent various degrees of surgical tumor resection (0%, 50% or 100%). Following surgery, animals received adjuvant therapy with cetuximab-IRDye alone, cetuximab-IRDye in combination with US-stimulated drug delivery, or saline injections (control) on days 4, 7, and 10. Tumor drug delivery was assessed on days 0, 4, 7, 10, 14, and 17 with an in vivo fluorescence imaging system, while tumor viability was evaluated at the same time points with in vivo bioluminescence imaging. Tumor caliper measurements occurred two times per week for 24 days. Optical imaging demonstrated that in the 50% tumor resection group, US-stimulated drug delivery resulted in a significant increase in cetuximab delivery compared to drug alone on day 10 (day of peak fluorescence) ($p = 0.03$). Tumor viability decreased in all groups receiving US-stimulated drug delivery plus drug compared to the drug alone group. After various degrees of surgical resection, this novel study demonstrates positive improvements in drug uptake in the residual cancer cells when combined with US-stimulated drug delivery.

Key words: adjuvant therapy, drug delivery, cetuximab, head and neck cancer, optical imaging, microbubbles, ultrasound

INTRODUCTION

Tumor retention and vascular permeability can be reversibly enhanced using mechanically oscillating microbubble (MB) contrast agents exposed to a low-intensity
ultrasound (US) field in a process known as US-stimulated drug delivery. This strategy was first introduced to augment delivery of molecules through the blood-brain barrier (Marty 2012, McDannold 2012), and has since been investigated for multiple other purposes. This transient increase in membrane permeability introduces a therapeutic window where enhanced drug uptake occurs, thereby improving the anticancer effects. This relatively noninvasive approach to cancer treatment is generally considered non-toxic, safe, and effective. US-stimulated drug delivery has demonstrated a 20 to 80% improvement in tumor response to drug treatment compared to drug alone in preclinical murine models (Bekeriedjian 2007, Casey 2010, Heath 2012, Iwanaga 2007, Park 2012, Sorace 2012). To improve therapeutic effectiveness, novel treatment strategies are needed to overcome the current barriers of poor drug uptake resulting from tortuous vasculature, limited drug dosages and high tumor interstitial pressure (Jain and Carmeliet 2012, Jain and Carmeliet 2001). Preclinical in vivo cancer research traditionally evaluates treatments through a neoadjuvant animal model by treating primary solid tumors. To date, research in the US-stimulated drug delivery field for cancer treatment has focused on improving localized delivery of molecules, such as drugs, DNA, or virus (Dalecki 2004, Escoffre 2011, Kinoshita 2006, Lentacker 2008).

In most cancer types, including head and neck cancer (HNC), treatment strategies involve surgery followed by chemotherapy or radiation. Residual disease is common in HNC and systemic therapy is delivered to further regress tumor size or treat margins that were unable to be surgically resected (Vermorken and Specenier 2010). Currently, HNC patients who undergo surgical resection of a localized tumor have an 80% likelihood of disease recurrence within two years (Ridge 2013). To improve overall HNC patient
survival, it is critical to improve delivery of adjuvant therapy to any residual disease to help improve the therapeutic outcome and reduce cancer reoccurrence. The devascularized wound bed remaining after surgical removal of a tumor can hinder drug extravasation, thereby further heightening the difficulty of systemic adjuvant treatment.

US-stimulated drug delivery has the potential to improve adjuvant chemotherapy delivery to residual disease and reduce recurrence in HNC. The effectiveness of a therapeutic drug is directly dependent upon the amount delivered to the tumor. In this paper, we investigate the effects of US-stimulated drug delivery on residual disease in a preclinical animal model of HNC. The model system detailed provides the basis for advancing preclinical surgical models of adjuvant US-stimulated drug delivery in various cancer types with a range of chemotherapeutics. Findings from this study may help advance US-stimulated drug delivery towards clinical translational.

MATERIALS AND METHODS

Cell preparation

Luciferase-positive HNC cells (squamous cell carcinoma (SCC) 1, provided by Thomas Carey, PhD, University of Michigan, Anna Arbor, MI) were maintained in DMEM media, supplemented with 10% Fetal Bovine Serum and 1% L-glutamate. Cells were passaged at 90% confluency and stored at 37°C and 5% CO₂. Cell counts and viability was determined through hemocytometry and trypan blue dye exclusion.

Animal care and tumor implants

All animal work was reviewed and approved by the Institute of Animal Care and Use Committee at the University of Alabama at Birmingham. Tumor implantation used a 27.5 gauge needle and injected 2x10⁶ cells/100µl DMEM media (without FBS).
subcutaneously into the right flank of five-week-old female nude athymic mice (Jackson Laboratory, Bar Harbor, ME) \(N=24\). Tumor growth was measured biweekly until day 24 using calipers. Given basic tumor diameter measures along the transverse \(d_t\) and longitudinal \(d_l\) dimensions tumor size was calculated using the equation: \(\pi \times (d_t/2) \times (d_l/2)\). These caliper measurements reflect tumor size and do not account for necrotic or apoptotic regions.

**Surgical resection**

Mice were sorted into three groups \((N = 3\) for MB+drug groups, \(N = 3\) for drug alone groups, \(N = 2\) per control groups\) so that average tumor size for each group was approximately equal \((18.7 \pm 1.9 \text{ mm}^2\) at day 0\). Prior to surgery, mice underwent baseline imaging and tumor caliper measurements. Mice underwent surgical removal of the tumor at 0% resection (no tumor was removed; sham surgery was administered), 50% resection (50% of the tumor remained), and 100% resection (surgeon removed 100% of the tumor noted by visualization and palpation). Regardless of the groups, all mice underwent identical surgical procedures. Briefly, a surgical blade (Feather 2976 #15, Osaka, Japan) was used to open a flap of skin in an “L” shape, leaving an estimated one centimeter of space around the tumor. The skin flap was sharply dissected from the surface of the tumor and the tumor was then removed at 0%, 50%, or 100% with respect to its designated group. A licensed surgical resident performed each tumor resection. Suturing was completed with 6-0 fast absorbing plain gut suture using a PC-1 conventional cutting 3/8 circle needle (1916, Ethicon, San Angelo, TX). After the wound was closed, mice were subcutaneously injected near the incision site with a 100 µg cocktail of 1 mg/mL Carprofen and 20 µg/mL of Buprenorphine to help relieve any residual pain from
Figure 1. Representative images showing surgical removal of tumor and flap placement. (a) Mouse is placed under isoflurane with tumor located on the right flank, (b) incision is made an estimated 1 cm from tumor in an “L” shape, (c) flap is lifted, (d) tumor surgically detached, (e) tumor is removed from mouse (shown is 100% resection), (f) surgical bed with remaining tumor (0%), (g) flap is sutured closed, (h) animal is removed from isoflurane, (i) mouse received a subcutaneous injection of pain medicine near surgical site.

surgery. A surgical outline of events is detailed with images in Figure 1. Mice were allowed to heal for four days before follow-up experimental studies began.
Cetuximab-IRDye

Cetuximab was labeled with near-infrared IRDye at the UAB Vector Production Facility according to Current Good Manufacturing Practices. This conjugation resulted in 1.8 IRDye molecules per cetuximab molecule. Cetuximab is a monoclonal antibody targeted to the epidermal growth factor receptor (EGFR) and has been FDA approved for treating HNC as a single agent therapy. EGFR is overexpressed in many solid tumors, including SCC of the head and neck (Masui, Kawamoto 1984). High expression of EGFR is linked to poor prognosis after treatment; therefore it is an excellent target for treating this disease (Haddad and Shin 2008).

Ultrasound-stimulated drug delivery

US-stimulated drug delivery was applied for 5 min using a 1.0 MHz single element (0.75 inch diameter) unfocused immersion transducer (Olympus, Waltham, MA) and the following US exposure parameters: peak negative pressure of 0.9 MPa, pulse repetition period of 15 sec, and a 5% duty cycle. The transducer was in series with a signal generator (AFG3022B, Tektronix, Beaverton, OR) and power amplifier (A075, Electronics and Innovation, Rochester, NY) allowing the entire tumor to be exposed to US energy. For the group of mice to receive US-stimulated cetuximab delivery, a 30 μL dose of MB contrast agent (Definity, Lantheus Medical Imaging, North Billerica, MA) was injected systemically via the tail vein in combination with 200 μg (100 μL) of Cetuximab-IRDye. The drug alone group received 200 μg (100 μL) of Cetuximab-IRDye only while control group mice received 100 μL saline injections. Following a two minute period to allow for adequate systemic circulation of MBs, mice were submerged in a custom-built 37ºC water bath and remained under isoflurane gas anesthesia for the

Bioluminescence imaging

Bioluminescence imaging occurred on days 0 (both pre and post-surgery), 4, 7, 10, 14, and 17. Mice were injected intraperitoneal with firefly luciferin (2.5 mg). After a 15 min delay for systemic circulation, all mice were imaged for bioluminescence expression using a small animal imaging system (IVIS-100, Xenogen, BioSciences, Cranbury, NJ, USA). All mice were imaged in the prone position (dorsal side up). Five mice were imaged per time sequence using a 1 sec exposure, an f/stop of 1, binning of 8, and at 25 cm camera distance. A low exposure time was required to eliminate oversaturation of the optical signal. A standardized circular region-of-interest (ROI) was placed around each mouse tumor and the total photon counts were quantified within the region.

Fluorescence imaging

Fluorescence imaging was completed on days 0, 4, 7, 10, 14, 17. On days that imaging coincided with US-stimulated drug delivery, optical imaging took place at least one hour post therapy using a dedicated small animal optical imaging system (Pearl Impulse, LI-COR Biotechnology, Lincoln, NE). Imaging was performed using the near-infrared 800-nm channel, exciting at a wavelength of 778 nm and emitting at 794 nm. Animals were maintained under isoflurane anesthesia and were prone positioned. Using commercial software (Pearl Impulse Software Version 2.0, LI-COR Biotechnology), an ROI was manually drawn around each tumor in the fluorescence image given a co-registered digital photograph for guidance. This was done separately for each image, as
Figure 2. Timeline showing tumor implantation, surgery, adjuvant US-stimulated drug delivery, and imaging. US-stimulated drug delivery began 4 days post-surgery. Bioluminescence imaging allows tracking of viable cells within a tumor, while fluorescence imaging monitors drug delivery to the tumor.

there are slight differences in mouse positioning and tumor size. If there was a complete tumor resection and no tumor was present when imaged, an ROI was drawn approximately around the area of tumor removal. Total fluorescence signal intensity within that ROI was measured and normalized by total pixel counts to quantify mean tumor fluorescence.

Survival Analysis

Survival analysis was performed for 60 days after surgery. If a tumor exceeded IACUC standards for size, exhibited signs of ulceration, or if the animal became sick or distressed, the animal was humanely euthanized and that day was recorded as the terminal date. Tumors that were completely resected were also monitored for regrowth. A complete timeline of the experimental design is detailed in Figure 2.

Statistical analysis

All experimental data was summarized as mean ± SE. An analysis of variance (ANOVA) test was used to assess differences within the different group data. A paired t-
test was used to compare the initial baseline bioluminescence image measurements with their subsequent values at day 17. This was also completed between baseline and day 10 using the fluorescence imaging data. A $p$-value of less than 0.05 was considered
statistically significant. All analyses were completed using statistical software (SAS 9.2, SAS Institute Inc, Cary, NC, USA).

RESULTS

All control group mice exhibited an increase in tumor size compared to groups receiving therapy (US-stimulated drug delivery or drug alone, \( p < 0.04 \)). As detailed in Figure 3 for the 0% tumor resection control group, caliper measurements revealed a 473.8% increase in tumor growth over baseline (day 0) at day 24. Also, the 50% tumor resection control group had a 42.6% increase at day 24 compared to baseline measures (post-surgery) while the 100% resection group showed a 36.3% decrease. There was no statistically significant differences in tumor growth between the 50% and 100% tumor resection control groups at day 24 (\( p = 0.25 \)).

Bioluminescence imaging was employed to quantify viable cancer cells within a given tumor region. Comparing pre- and post-surgery tumor viability on day 0 revealed that the 0% tumor resection group demonstrated no change following sham surgery. Conversely, bioluminescence from the 50% tumor resection group was shown to be decreased by 77% and the 100% complete tumor resection revealed a 99% decrease in viable tumor burden. After an initial analysis of tumor reduction following surgery, residual tumors were tracked individually as percent change from their post-surgical state. Each of the control groups produced an overall increase in bioluminescence signals following tumor removal (day 0) to day 17; 0% resection, 50% resection, and 100% resection revealed a 2-fold, 3071-fold, and a 701-fold increase, respectively. Control data exhibited a pronounced exponential increase over the drug alone and US-stimulated drug delivery group bioluminescence signal measurements. Compared to mice that received
Figure 4. Bioluminescence monitoring allows quantitative measurements of tumor viability after surgical resection. Graphs detailing out the (a) 0% resection group (b) 50% resection group, and (c) 100% resection group comparing US+drug to drug alone. US-stimulated drug delivery shows improved drug effectiveness (decreased tumor viability post-surgery) in each group compared to its drug alone counterpart.

drug alone, at day 17, mice subjected to US-stimulated drug delivery resulted in a 2-fold decrease in bioluminescence signal measures (viable tumor) for the 0% tumor resection group, 66-fold decrease in the 50% tumor resection group, and a 4-fold decrease in the 100% tumor resection group, Figure 4. For the mice that received repeated sessions of US-stimulated drug delivery, the 0% and the 100% tumor resection mice groups exhibited an overall decrease by day 17 in tumor viability compared to the initial post-
surgery baseline imaging. Bioluminescence data collected from the 50% tumor resection group mice revealed an increase in viability on day 17.

Figure 5. Fluorescence imaging after ultrasound-stimulated therapy. Fluorescence imaging revealed increased delivery in US-stimulated drug delivery groups compared to those that received drug alone in the 0% and 50% tumor resection groups. Graphs show % change in mean fluorescence intensity for (a) 0%, (b) 50%, and (c) 100% surgical resection groups. Shown is a representative image of tumor fluorescence from each group in vivo at day 10.
Fluorescence imaging was used to monitor delivery of the IR-labeled drug and more specifically to determine if US-stimulated drug delivery enhances tumor uptake and retention of the therapeutic agent. Figure 5 suggests that the greatest enhancement of drug uptake occurred on day 10 according to peak fluorescence image measurements. All surgical group mice receiving US-stimulated drug delivery showed a significant increase in tumor fluorescence compared to control tumors that received sham US therapy ($p < 0.04$). Mice tumors that did not undergo surgical resection but received US-stimulated drug delivery exhibited a mean fluorescence signal increase of 42.3% on day 10 when compared to mice subjected to systemic drug treatment only ($p = 0.10$). Also at day 10 post-surgery, tumors that underwent a partial 50% resection and subjected to US-stimulated drug delivery demonstrated a 41.6% increase in fluorescence measurements compared to residual disease treated with drug only ($p = 0.03$). Lastly, a comparison of day 10 fluorescence imaging results from mice groups that received US-stimulated drug delivery versus drug only revealed a 1.5% increase in drug delivery to the wound bed following surgical resection of the entire tumor ($p = 0.48$). On day 17 of fluorescence imaging, these trends continued, however the signal from the tumors was markedly decreased.

All mice in the 50% and 100% tumor resection groups showed complete survival over the 60 day study. For the 0% tumor resection group mice, none of the control animals remained on day 36. However, all of the mice subjected to drug treatment alone or US-stimulated drug delivery survived. For tumors that were completely (100%) resected, both the control and drug only dosed mice had a 66% reoccurrence rate of
disease, while the US-stimulated drug delivery group improved treatment of any residual
disease and importantly no tumor reoccurrence was observed.

DISCUSSION

Many cancer-fighting therapies have shown strong antitumor effects in preclinical
models of primary tumors but fail to translate clinically for prevention of disease
recurrence after surgery (Block 2011, Grinshtein 2009, Schreiber 2006, Tucker 2012). Surgery promotes inhibitory factors that allow lingering immunosuppressive cells to repopulate minute pockets of residual disease quickly (Predina 2013). This system of immunosuppression in the post-surgical tumor microenvironment explains the resistance of cancer to conventional therapies despite its small residual volume (Predina 2013). Surgery has also shown to have no impact on the alterations of remaining cells or their resistance to drugs (Predina 2013). HNC treatment consisting of surgical resection and adjuvant therapy is generally considered effective; however, many of these cases result in cancer reoccurrence due to residual viable cancer cells left behind after surgery. This is partially due to vital internal structures that cannot be damaged/removed or incomplete visualization of the tumor. Notwithstanding, the devascularized wound bed following surgical removal of a tumorous mass leaves a difficult environment for treatment. US-stimulated drug delivery has been explored in numerous preclinical animal studies for improving drug delivery to solid tumors in the neoadjuvant setting. We believe that exploration of combination treatment with surgical resection is a necessity to advance this research towards clinical translation. In this paper, we evaluated the benefit of applying US-stimulated drug delivery to HNC-bearing mice that underwent surgical resection of the primary tumor burden. The use of a multimodality imaging strategy allowed

EGFR is a transmembrane protein with intrinsic tyrosine kinase activity that regulates cell growth in response to binding of its ligands, such as epidermal growth factor (EGF) and transforming growth factor α (TGF-α) (Ang 2002, Rubin Grandis 1998). Ligand binding induces EGFR dimerization and activates several EGFR-mediated signaling pathways. In order for cetuximab to properly operate, it has to bind to the proper receptor to begin the cascade pathway. Due to the immense treatment potential, clinical studies have been ongoing for the last decades analyzing EGFR targeted-based therapies. While cetuximab is not currently approved for use in adjuvant therapy, it is currently being explored in clinical trials. A phase II trial demonstrated superior cancer control at one year when using cetuximab in combination with radiotherapy, than radiotherapy alone (Mesía 2013). Adjuvant cetuximab is also being explored with either cisplatin or docetaxel in Stage III and IV SCC patients (Harari 2011). Also, there are other targeted EGFR agents that have moved forward to be used as adjuvant therapies. In SCC of the HNC, there is recruitment for a phase III trial studying the effects of lapatinib (an EGFR-targeted antibody) in patients following surgery. Clinical studies analyzing the effects of trastuzumab (monoclonal antibody against the human epidermal growth factor receptor 2, HER2) in combination with traditional chemotherapies as an adjuvant therapy have now become standard-of-care in breast cancer. The addition of this targeted antibody revealed a significant increase in anticancer effects and survival (Perez 2011). With promising results in both preclinical and clinical trials using EGFR-targeted antibodies, there is an expanding amount of clinical research ongoing for these
monoclonal antibodies as single therapies, as well as in combination with traditional
chemotherapy (Moon 2010). Research has shown that targeted therapies can improve
antitumor effects post-surgery; therefore, further heightening tumor retention and
delivery through non-invasive methods would be a valuable addition to current methods
of treatment.

Studies have shown that intravascular MB contrast agents exposed to US energy
can increase extravasation by breaking down gap junctions between endothelial cells
allowing transport of molecules across barriers that previously would be unable to cross
(Maeda 2009, Miller 2002, Zderic 2002, Zhao 2012). This unique therapy has been
applied to many disease types and for modulating delivery of a host of molecular agents.
A previous study has shown that tumors subjected to US-stimulated drug delivery using
cetuximab exhibited significantly increased antitumor effects compared to drug alone
when analyzing tumor size (Heath 2012). These effects were confirmed by both
immunohistologic analysis and diffusion-weighted magnetic resonance imaging (DW-
MRI), demonstrating a 20% increase in apparent diffusion coefficient (ADC) values (i.e.,
increased apoptotic activity and cell death) when comparing tumors treated with US-
stimulated drug delivery compared to drug alone. When exploring other in vivo
applications of US-stimulated drug delivery, previously reported methods analyzed
secondary indicators of tumor response to treatment by measuring size and evaluating
histological results post-mortem (Zhao 2011). MRI-based measures have also shown to
be effective in confirming US-stimulated drug delivery through the blood-brain barrier
(Kinoshita 2006, McDannold 2012, O'Reilly 2012, Treat 2012). The ability to image at
near-infrared fluorescence wavelengths decreases error by minimizing the fluorescence
signal contributed from background tissue, allowing for more precise measurements (Frangioni 2003, Hebden 1997). Optical imaging of fluorescently-labeled drugs has shown potential for monitoring preclinical molecular delivery in neoadjuvant models (Jiang 2010), in surgical-resection models (Day 2013, Heath 2012), and US-stimulated therapeutic models (Sorace 2013).

Bioluminescence imaging allows visualization of viable (luciferase-positive) tumor tissue in comparison to the more routine tumor size measurements which cannot differentiate viable from necrotic tissue. In this paper, it was shown that both the 0% and 100% tumor resection mice groups receiving US-stimulated drug delivery exhibited enhanced antitumor activity compared to animals receiving drug alone. This response was highlighted by a progressive decrease in the amount of viable tumor tissue from baseline measures. However, mice that underwent surgery to remove only 50% of the tumor showed an increased antitumor effect compared to drug alone, yet did not regress when compared to baseline values. It is important to note that all three control groups (including the 100% tumor resection group) exhibited an increase in tumor viability and physical size. Surgery alone was not sufficient enough to impede tumor growth. Although caliper measurements are not known for their precision, they demonstrated a consistent trend in that the control group tumor grew exponentially compared to the groups receiving US-stimulated drug delivery or drug alone. Analysis of fluorescence imaging results from mice that underwent 100% tumor resection revealed that there were no significant differences in drug uptake for tumors treated with US-stimulated drug delivery or drug alone. This is expected as the ROIs during fluorescence monitoring of these data sets may not have had a visible tumor. After a single dose of cetuximab (day
4), no changes in drug uptake (tumor fluorescence) was observed for any of the groups at one hour post-delivery, which is consistent with previous research that looked at immediate uptake of fluorescently-labeled IgG molecules after US-stimulated drug delivery in a primary tumor model (Sorace 2013). It is believed that multiple doses of US-stimulated drug delivery further enhanced delivery and is essential for delivery of targeted antibodies, as cetuximab has been previously shown to be effective in a multi-dose longitudinal study (Heath 2012). However, fluorescence imaging of the 0% and 50% tumor resection model demonstrated heightened drug delivery when systemic injections of cetuximab were supplemented with US-stimulated drug delivery. Day 10 showed the greatest enhancement with a 42% increase in fluorescence signal measurements in the US-stimulated drug delivery group mice compared to those administered drug alone. Fluorescence signal decreases overtime, therefore it is logical that signal peaked on the final day of drug administration. On day 17, there was a significant correlation between percent change in fluorescence and bioluminescence ($R^2 = 0.57, p < 0.001$). This allows us to conclude that the drug delivery was correlated to overall tumor viability response. US-stimulated drug delivery enhances localized cetuximab delivery, thereby enhancing tumor response to therapy. High variance found within the groups was due to low group numbers. Although no differences were found with a 60 day survival analysis, disease reoccurrence was observed in the 100% tumor resection group mice that did not receive US-stimulated drug delivery to improve treatment of any residual cancer cells.

This study produced encouraging preclinical results exploring the use of US-stimulated drug delivery in the adjuvant setting for the treatment of residual disease
following incomplete resection of HNC. Given HNC patients are at a high risk for recurrence of the primary tumor even after surgical intervention and follow-up drug or radiation treatment, developing methods that will help reduce this disease recurrence is critically important for improving patient prognosis and long-term survival. In conclusion, US-stimulated drug delivery is a promising technology and more research is warranted.

ACKNOWLEDGMENTS

This research was supported by grant 1RO1CA142637 from the National Cancer Institute, grant 2T32CA091078-06 from the National Institutes of Health, and through a pilot award from the Department of Radiology at the University of Alabama at Birmingham.

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CHAPTER 7

MOLECULAR ULTRASOUND IMAGING USING A TARGETED CONTRAST AGENT FOR ASSESSING EARLY TUMOR RESPONSE TO ANTIANGIOGENIC THERAPY

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Journal of Ultrasound in Medicine

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ABSTRACT

Objective: Contrast-enhanced ultrasound (US) and targeted microbubbles (MBs) have shown to be advantageous for angiogenesis evaluation and disease staging in cancer. This study explores molecular US imaging of a multi-targeted MB for assessing early tumor response to antiangiogenic therapy. Methods: Target receptor expression of 2LMP breast cancer cells was quantified using flow cytometry analysis and characterization established with antibodies against mouse \( \alpha_v \beta_3 \), p-selectin, and VEGFR2. Tumor bearing mice (N=15 per group) underwent contrast-enhanced US (CEUS) imaging of multi-targeted MBs. MB accumulation was calculated using destruction-replenishment techniques and time-intensity curve analysis. On day 0, mice underwent baseline imaging. Next, therapy group mice were injected with a 0.2 mg dose of bevacizumab and controls received matched saline injections. Imaging was repeated on days 1 and 3. After imaging was completed on day 3, mice were euthanized and tumors excised. Histological analysis of microvessel density and intratumoral necrosis was completed on tumor sections. Results: On day 3 after bevacizumab dosing, a 71.8% change in tumor vasculature was shown between therapy and control groups (\( P = .01 \)). Therapy group revealed a 15.4% decrease in tumor vascularity, while control group increased 56.4%. Conclusion: Molecular US imaging of angiogenic markers can detect early tumor response to drug therapy.

Key words: Microbubbles, targeted contrast agent, angiogenesis, contrast-enhanced ultrasound, antiangiogenic therapy
INTRODUCTION

Cancer accounts for nearly one in every four deaths in the United States, and affects nearly 12 million people (1). Cancerous masses, or tumors, cannot survive in the body beyond 1-2 mm unless they are vascularized (2, 3). Angiogenesis, the development of new capillary blood vessels, has been shown to be directly associated with malignancy and is necessary for cancer to survive and progress.

Proper staging of cancer is critical in determining the appropriate method of therapy, as well as accurately assessing prognosis. Drug treatments for cancer encompass two main categories: antimitotic and targeted. Antimitotic drugs, or chemotherapeutics, act by killing cells that are rapidly dividing. Targeted therapy, most commonly associated with antibody therapy, is more specific and uses monoclonal antibodies to target malignant cells. Antiangiogenic drugs, such as bevacizumab, are targeted vascular disrupting agents that attempt to stop new blood vessel formation and may stop or retard the growth and spread of tumors. These antiangiogenic therapies have been used clinically in conjunction with chemotherapy to help treat cancer such as colon, breast, lung and brain cancer (4). Specifically, bevacizumab has been proven in preclinical animal models to be effective within seven days of treatment using both magnetic resonance (5) and ultrasound (US) (6, 7) imaging.

Ultrasound contrast agents are subcapillary-sized, gas-filled microbubbles (MBs) small enough to pass through microvasculature (8, 9). Contrast-enhanced US (CEUS) is the application of MBs to traditional US imaging, originally designed to improve visualization of echocardiograms (10). CEUS imaging has since been explored for numerous preclinical and clinical opportunities, such as detection and cancer staging. Other applications of MBs
currently being explored include increasing drug-delivery through MB-mediated US therapy and evaluation of tumor response to therapy (11-13). Different cancers and their endothelial cells overexpress certain receptor proteins, which allow the application of MBs to be expanded to include the use of targeting tumor vasculature markers to provide added visualization of angiogenesis and vessel assessment (14-17). Molecular US imaging includes actively targeting MBs to single or multiple receptors in order to provide additional enhanced visualization for cancer detection, disease staging or analyzing receptor profiles (15, 18-20). A triple-targeted MB allows increased visualization of angiogenesis through increased receptor binding (13). Targeting MBs to p-selectin (CD62P), vascular endothelial growth factor 2 (VEGFR2) and αvβ3 integrin angiogenic molecular markers has been shown to effectively increase visualization of tumor vasculature by 60% over single-targeted strategies, and 40% of dual-targeted strategies in preclinical breast cancer models (13). Other groups have also shown the advantages of using targeted MB strategies through enhanced visualization and assessments of tumor vascularity compared to traditional CEUS (21-23). Molecular US imaging has potential to be used to develop targeted personalized diagnostics and therapy (24). In the present study, we explore molecular US imaging of a triple-targeted MB for assessing early tumor response to antiangiogenic therapy.

Materials and Methods

Cell Culture

2LMP human breast cancer cells (MDA-MB-231, lung metastatic pooled) were maintained in Dulbecco’s Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum
(FBS), and 1% L-glutamine. All cells were cultured to 70 to 90% confluence before passaging. Cells were grown at 37° C and in 5% CO₂ and 90% relative humidity. Appropriate cell numbers for all experiments were determined using a hemocytometer and trypan blue dye exclusion.

**In vitro Characterization**

Mouse angiosarcoma (SVR) endothelial cells were aliquoted (25x10⁴ cells/tube) and stained with primary antibodies against mouse α₅β₃, p-selectin, or VEGFR2 per manufacturer’s recommendations. Phycoerythrin (PE) labeled anti-mouse IgG (405406, Biolegend, San Diego, CA) was used as a secondary stain. Tubes with cells and secondary alone were used as controls to establish background signal. Cells were analyzed for fluorescent counts (50x10³ events) using flow cytometry (Accuri C6, Accuri Cytometers Inc, Ann Arbor, MI). All experimental groups were analyzed in triplicate.

**Animal Preparation**

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Thirty 6-week-old nude female athymic mice (Jackson Laboratory, Bar Harbor, ME) were implanted subcutaneously with 2x10⁶ 2LMP breast cancer cells in the left flank. Implanted tumors were allowed to grow 16 days before experimental studies began (average tumor size of 130.9 ± 7.4 mm²). Mice were then sorted by tumor size into two groups, therefore each group created had approximately the same average size tumors to ensure no biasing between groups (P = 0.74). Tumor area was calculated using a standard ellipse equation with the transverse and longitudinal caliper measurements.
Antibody-Microbubble Conjugation

Streptavidin coated MBs (Targestar-SA, San Diego, CA) were conjugated to biotinylated rat IgG antibodies against αVβ3 (13-0512, eBioscience, San Diego, CA), p-selectin (16-0622, eBioscience, San Diego, CA) and VEGFR2 (13-6410, BioLegend, San Diego, CA). These MBs are lipid coated perfluorocarbon microspheres averaging at 2.5 µm. Their lipid shell is coated with streptavidin conjugated to the distal tip of the polymeric spacer. Multi-targeted MBs were prepared by incubating streptavidin-coated MBs with equal amounts (20 µg) of each respective antibody for 20 min (13). Antibody-labeled MBs were washed using a centrifuge (400x) for 3 min to wash any unbound antibody. MBs were diluted with phosphate buffered saline (PBS) to a total volume of 1 ml and the final concentration was characterized using a hemocytometer. A new vial of multi-targeted MBs was prepared each day to ensure consistency across MB population. Microbubbles were between 1-8 µm.

Treatment

Bevacizumab (Avastin, Genentech, South San Francisco, CA) treatment was given to the treatment group following baseline molecular US imaging on day 0. The therapy, bevacizumab, is a recombinant humanized monoclonal antibody to VEGF. This drug functions by blocking the VEGF protein thereby breaking down current permeability and vascularity, while inhibiting the formation of blood vessel growth (25-27). Other antiangiogenesis inhibiting drugs include sorafenib, sunitinib and pazopanib (4). Mice were administered 0.2 mg (25 mg/ml) of bevacizumab (Avastin, Genentech, South San
Figure 1: Ultrasound setup utilizes combination MB perfusion destruction technique to analyze amount of bound MBs within the tumor, allowing noninvasive detection of tumor angiogenesis and vascularity.

Francisco, CA) diluted with saline to 100 µl via intraperitoneal (i.p.) injection. Control mice received 100 µl matched i.p. dose of saline.

Imaging

Molecular US imaging was performed on days 0, 1 and 3. Mice were weighed before each imaging session. For the US imaging, each mouse was anesthetized with isoflurane gas. Grey-scale US imaging was performed using a SONIX RP research scanner (Ultrasonix Medical Crop, Richmond, BC) equipped with a 7 MHz linear-array transducer and a pulse-inversion harmonic imaging feature. Targeted MBs (60 µl, 14 x 10^6 MBs/ml) were diluted to 100 µl with saline and intravenously injected through the tail vein. Mice were submerged in a custom-built 37°C water bath and remained under isoflurane gas anesthesia for the entirety of the US imaging. A two min waiting period after injection ensured adequate systemic circulation and binding of MBs to their target angiogenic molecular markers. The largest cross-section of each tumor was identified and
Figure 2: Timeline shows experimental processes of tumor implantation, antiangiogenic drug dosing, and multi-targeted CEUS imaging.

used as the imaging plane between days for consistency throughout the study. Following the post MB injection delay, tumors were imaged at a low mechanical index (MI) value of 0.1 for 10 sec to capture both bound and systemically flowing MBs in the image plane. Subsequently, a high-intensity MB destruction pulse sequence (MI of 1.2, 4 sec) was applied to destroy all MBs within the imaging plane. Low-intensity US imaging was then performed again for 20 sec to capture tumor reperfusion of MB agents (see Figure 1). The imaging sequence was saved for offline processing. Figure 2 visually demonstrates the experimental timeline of tumor implantation, treatment and imaging.
Data Analysis

Molecular US images were processed using custom MATLAB programs (Mathworks Inc, Natick, MA) to evaluate time-intensity curve information. The first image which followed the two min post MB injection (before MB destruction pulse sequence) was utilized for each mouse in order to analyze bound MBs in tumor vasculature. The last image of the second US imaging sequence (post MB destruction pulse sequence) was used as a baseline image in order to calculate all circulating MBs and possible tissue reflection/artifacts. Images were saved in their 32 bit post-scan converted format and uploaded into MATLAB for analysis. Each image sequence was registered and the same region-of-interest (ROI) was used for both images to ensure proper subtraction of image sequences. Following placement of a circular ROI to encompass intratumoral vascularity, pixel intensity was measured from both images (one before and one post MB destruction pulse sequence as described previously). Pixel intensity was calculated for both images by analyzing brightness on a grey-scale. Image 2 (post MB destruction) was subtracted from image 1 (before MB destruction) to calculate intensity from the bound MBs. The difference between these two mean ROI values is a surrogate measure of successfully targeted and bound MB populations. This procedure was repeated for each mouse on each day of imaging.

Histology

After experimentation on day 3, all animals were humanely euthanized and tumors were excised. Samples were sliced at the largest cross-sectional diameter (to approximate the US imaging plane) and sections stained for H&E and CD31. H&E
Figure 3: In vitro results showed receptor characterization and expression through flow cytometry mean fluorescent counts. Microscopy representative picture of a multi-targeted MBs attached to SVR cells are also shown.

sections were examined for cellular necrosis and reported as percent of the entire tumor cross-section (original magnification x5). CD31 stained histology slides were examined (original magnification x40) to identify five separate areas containing the greatest microvessel density (MVD). Individual vessels from these five areas were counted (original magnification x200), averaged, and recorded as MVD.

Statistical Analysis

Data was summarized as mean +/- SE. Analyses were performed using SAS statistical software (Cary, NC). Assessment of inter-group comparison of in vitro data was
completed using the analysis of variance (ANOVA) test. Analysis of in vivo experimental data was performed using a two-sample independent \( t \)-test. Changes in tumor size and mouse weight were compared between groups using a two-sample independent \( t \)-test.

RESULTS

In vitro Characterization

The normalized (background subtracted) fluorescent counts from this study are displayed in figure 3. Receptor expression using mouse SRV cells was confirmed for each of the receptors targeted: \( \alpha \nu \beta_3 \), VEGFR2 and p-selectin. Receptor expression calculated in vitro by flow cytometry varied between the three receptors targeted, with VEGFR2 significantly decreased in comparison to its other constituents (\( P < 0.01 \)). Although significantly different, all receptors are expressed within the murine SRV cells (Figure 3).

In vivo Characterization

There were no differences between control and therapy group animal weights on day 0 (\( P = .63 \)) or day 3 (\( P = .97 \)) (Figure 4). Marked changes in weight loss or gain can be an indication of declining animal health. Analysis of tumor size data indicated no differences between the groups on day 0 (\( P = .66 \)) or day 3 (\( P = .89 \)). On day 0, baseline molecular US imaging results showed no differences in tumor perfusion between control and therapy group mice (\( P = 0.08 \)). However, imaging results collected on day 1 post therapeutic dosing revealed a 50.4% change in intratumoral perfusion between therapy and control (\( P = .09 \)) with control and therapy group tumors exhibiting a 64.9% and 14.5% increase, respectively, from baseline measurements. Day 3 imaging results revealed a
Figure 4: Intratumoral perfusion, calculated through MB perfusion destruction technique, was seen to decrease in treatment group receiving antiangiogenic drugs compared to control group. Shown are representative images from one mouse per group of multi-targeted CEUS images of control and therapy groups on Day 0, 1 and 3 (Before MB destruction and after MB destruction are shown for each). A graph showing percent change in intratumoral enhancement is also shown. Day 3 the therapy group exhibited a 72% decrease in intratumoral enhancement compared to controls (P = .01).
71.8% difference between therapy and control group data (P = .01), with control and therapy tumors demonstrating perfusion changes of 56.4% and -15.4%, respectively (Figure 5).

Histology

Histological analysis indicated there were no significant differences in percent necrosis between therapy and control group samples (P = .15). A comparison of CD31 section data revealed differences in terminal MVD for the control and therapy tumor samples, 26.6 and 17.3 counts, respectively (P < .01), figure 4.

DISCUSSION

In this experiment, multi-targeted MBs are used to evaluate and assess early response to antiangiogenic treatment in breast cancer bearing mice. Within one day of administering bevacizumab, decreased tumor vascularity was observed in the therapeutic animal group compared to controls. By day 3, molecular US imaging of these same angiogenic markers detected a further decrease in tumor vascularity as determined by a significant reduction in targeted MB accumulation (compared to control data). Kim et al (2010) showed a DCE-MRI approach to assess early response to antiangiogenic therapy by evaluating pharmokinetic parameters through calculating transfer of contrast agent from the vascular space, yielding a surrogate measure of tumor perfusion (5). This study showed a decrease in intratumoral perfusion on day 3 post therapy when treated with bevacizumab alone, demonstrated through calculation of $K^{\text{trans}}$ which represents vascular perfusion ($K^{\text{trans}}$ increase of about 18%)(5). Hoyt et al. (2010) showed CEUS had the capability to see trends in data between antiangiogenic therapy and control groups in a murine model within 3
Figure 5: Histological results showed significant decreases in microvessel density (MVD) in therapy mice receiving antiangiogenic drug compared to control mice receiving saline (P < .01). No significant differences were seen in tumor size from day 0 to day 3 in control and treatment group (P = .89), indicating US detection of early tumor response occurred before tumor size could indicate a response.

days, but they were not shown to be significant by day 6 (7). Streeter et al. (2011) showed analysis of tumor angiogenesis with three-dimensional CEUS molecular imaging targeting $\alpha_v\beta_3$, suggesting volumetric imaging can reduce error associated with two-dimensional single plane imaging (28). Other significant CEUS research that has surfaced includes
using volumetric CEUS molecular imaging to determine differences in whole tumor perfusion after bevacizumab treatment (6). These promising results with volumetric CEUS showed tumor perfusion decreases in response to therapy which also correlated to decreases in tumor size when compared to their controls. Although early response to drug was not shown in this volumetric CEUS study, this technique shows advancements in the field of CEUS and gives promise to future studies of combining targeted molecular US imaging and volumetric scanning.

Evaluating early tumor response to treatment gives information about the progressive nature of the disease, which is essential in determining patient prognosis. Angiogenesis is essential for tumor growth and metastasis, therefore antiangiogenic therapy has become a popular targeted therapy used solo or in combination with chemotherapy to reduce tumor perfusion and impede growth. It has been shown that not all tumors respond to antiangiogenic therapy; therefore it is necessary to make an early determination of treatment response (29-33). Early detection of response will be cost-effective, as well as beneficial in finding the most efficient antitumor drug for each patient.

As mentioned, molecular US imaging uses MBs targeted to overexpressed proteins in order to characterize physiologic and molecular events, clarifying why it has become increasingly popular in assessing antiangiogenic therapy in cancer (34-36). Standardizing techniques to measure initial and longitudinal response to treatment is a necessary step in advancing cancer therapy (35). Targeted contrast-enhanced high-frequency US has been shown to facilitate in vivo molecular imaging of VEGFR2 expression of tumor vascular endothelium to compare metastatic and nonmetastatic breast cancer (37). Subharmonic US imaging had also shown potential in quantifying and assessing changes in tumor
vasculature over two weeks of antiangiogenic therapy (38). Clinical trials using dynamic contrast-enhanced Doppler US to assess antiangiogenic therapy in renal cancer have shown significant differences in contrast uptake in tumors that have a positive response to therapy over six weeks, yet Doppler is known to be a poor measure when compared to MVD (39, 40). There have been clinical trials using dynamic CEUS as a prognostic tool for cancer showing correlations between dynamic CEUS and response to sunitinib after 15 days of therapy (41). Lassau et al. have shown promising results using dynamic CEUS to study effects of anti-tumor drugs in clinical trials. Sensitivity and reliability are essential in monitoring angiogenic response to therapy; therefore using multi-targeting CEUS could enhance the capabilities and advance procedures that are currently being used to assess early tumor responses to treatment. However, targeted contrast agents can accomplish site-directed imaging or therapy by a variety of active and passive mechanisms.

Single and dual targeted molecular US imaging strategies from Willmann et al. showed a positive enhancement of tumor vasculature compared to traditional CEUS (23, 42). Following this methodology, a triple-targeted MB was developed by Warram et al. showing the synergistic effects of targeting three different molecular agents simultaneously. This approach has been proven to enhance visualization of tumor angiogenesis for cancer staging and prognosis (13), which allows increased detailed information about tumor angiogenesis. Using a multi-targeted MB, we are able to see significant differences in tumor vasculature after dosing with an antiangiogenic drug. This molecular imaging approach was also supported with histology evidence of significant differences in microvessel density from antiangiogenic therapy on day 3. This imaging approach gives information regarding tumor response to therapy before changes in tumor
size can be detected (current standard of care). This gives an early assessment that could be added to Response Evaluation Criteria in Solid Tumors, which designates the current standard for measuring response to therapeutics in cancer is through tumor measurements (43). Successful evaluation of early tumor response to therapy could increase efficiency of anticancer treatment, thereby improving patient prognosis and results.

Molecular US imaging of multiple tumor-specific protein markers shows potential to evaluate early tumor response to antiangiogenic therapy. In conclusion, multi-targeted MBs allow a noninvasive approach to determine early tumor response to antiangiogenic therapy through molecular US imaging.

ACKNOWLEDGEMENTS

This research was supported in part by NIH grant UL1RR025777, NCI grant CA13148-38 UAB Comprehensive Cancer Center and NIH grant T32EB004312 from the National Institute of Biomedical Imaging and Bioengineering.

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CHAPTER 8

BIODISTRIBUTION OF P-SELECTIN TARGETED MICROBUBBLES

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Submitted to *Journal of Drug Targeting*

Format adapted for dissertation
ABSTRACT

Purpose: To evaluate binding of P-selectin targeted microbubbles (MB) in tumor vasculature; a whole-body imaging and biodistribution study was performed in a tumor bearing mouse model. Procedures: Antibodies were radiolabeled with Tc-99m using the HYNIC method. Tc-99m labeled anti-P-selectin antibodies were avidin-bound to lipid-shelled, perfluorocarbon gas-filled MB and intravenously injected into mice bearing MDA-MB-231 breast tumors. Whole-body biodistribution was performed at 5 min \( (n=12) \) and 60 min \( (n=4) \) using a gamma counter. Tc-99m-labeled IgG-control-MB group \( (n=12 \text{ at } 5 \text{ min}; \ n=4 \text{ at } 60 \text{ min}) \), Tc-99m-labeled IgG-control-Ab group \( (n=5 \text{ at } 5 \text{ min}; \ n=3 \text{ at } 60 \text{ min}) \), and Tc-99m-labeled anti P-selectin-Ab group \( (n=5 \text{ at } 5 \text{ min}; \ n=3 \text{ at } 60 \text{ min}) \) were also evaluated. Planar gamma camera imaging was also performed at each time point. Results: Targeted-MB retention in tumor \( (60 \text{ min}: 1.8 \pm 0.3 \% \text{ID/g}) \) was significantly greater \( (P=0.01) \) than targeted-MB levels in adjacent skeletal muscle at both time points \( (5 \text{ min}: 0.7 \pm 0.2 \% \text{ID/g}; \ 60 \text{ min}: 0.2 \pm 0.1 \% \text{ID/g}) \) while there was no significant difference \( (P=0.17) \) between muscle and tumor retention for the IgG-control-MB group at 5 min. Conclusions: P-selectin targeted MBs were significantly higher in tumor tissue, as compared with adjacent skeletal tissue or tumor retention of IgG-control-MB.

Key words: P-selectin, Microbubbles, Biodistribution, Cancer, Targeted delivery
INTRODUCTION

Improving targeted delivery of anti-cancer drugs to a solid primary tumor can improve overall effectiveness of current systemic and targeted therapies, while reducing total dose and systemic toxicity. Ultrasound contrast agents are perfluorocarbon, gas-filled, lipid microbubbles (MBs) with a diameter of 1-3 µm. The stability of MBs within microvasculature, combined with their non-toxic and non-immunogenic properties has led to pre-clinical investigations of MBs to improve tumor delivery of therapeutic compounds [1], plasmids [2], and viral vectors [3]. Various drug delivery strategies have been investigated using MBs to improve cancer therapy. Some pre-clinical research utilizing MB-assisted delivery involves a physical association between the MB and therapeutic compound [2, 4]. One such approach includes labeling hydrophilic pDNA to the exterior of protein-shelled MBs using non-covalent interactions [5]. Other studies have taken advantage of the unique lipid shell component in conjunction with lipophilic compounds, such as Paclitaxel, to physically join the compound to the MB core [1, 6]. Additional approaches involve double-emulsified MBs that physically encapsulate hydrophilic macromolecules such as pDNA [7], Doxorubicin [8], and adenovirus [9]. In the latter studies, complete encapsulation of the agent was proven advantageous for systemic or localized delivery because the payload was shielded from immune response and sequestering mechanisms. In all of these strategies, the performance of the MB to transport and deliver a molecule to the targeted region is dependent upon the ability of the MB to specifically accumulate within that tissue.

Targeting MBs to commonly over-expressed receptors in a specified region-of-interest have been shown to improve overall MB accumulation at target sites [10]. The
active targeting of MBs is achieved by conjugating receptor-specific ligands to the outer shell via biotin-avidin chemistry or covalent linkage [11]. Ligand-modified MBs bind specifically to molecular receptors within the vasculature of the targeted tissue, while unbound MBs are filtered from the circulation [12]. Improved MB accumulation using targeted strategies has been demonstrated in the molecular imaging of tumor angiogenesis [13-15], inflammation [16-18], and intravascular thrombi [19, 20]. One cellular target currently under investigation is the cell adhesion molecule, P-selectin (CD-62P), which is commonly over-expressed in tumor endothelial cells [21]. P-selectin is expressed on stimulated endothelial cells and activated platelets; it contributes to the recruitment of leukocytes in areas of inflammation common in tumor vasculature [22, 23]. In addition, the presence of P-selectin permits the adhesion of platelets and cancer cells to the tumor endothelium. Strategies for improving MB accumulation have utilized the expression of P-selectin in echocardiography, atherosclerotic plaque detection, and tumor detection [24-26]. The overexpression of P-selectin in the tumor vasculature by stimulated endothelial cells makes it a viable target for improving intravascular MB retention.

The challenges associated with systemically delivered therapeutic agents include both non-specific sequestration and immunogenicity from toxic chemical compounds and viral therapy. The well characterized safety of MBs [27], combined with the ability to target specific molecules within the tumor makes this approach a viable tool for the safe and specific delivery of these agents to improve overall patient treatment and survival. The current study propels this drug delivery technique forward by elucidating the whole-body biodistribution of P-selectin targeted MBs.
MATERIALS AND METHODS

Culture Methods and Tumor Model

MDA-MB-231 breast cancer cell line was purchased from the American Tissue Type Collection (Manassas, VA) and maintained in DMEM, 10% FBS, and 1% L-glutamine. The cell line was cultured at 37°C and 5% CO₂ while maintained to 70-90% confluence before passaging. To generate the tumor model, 2x10⁶ cells were subcutaneously implanted in the flank of 6-week-old athymic female nude mice (Frederick Cancer Research, Hartford, CT). Cell numbers were determined with hemocytometer and trypan blue dye exclusion. Tumors were allowed to grow to a mean diameter range of 8-10 mm. Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham approved all animal protocols.

Preparation of Radiolabeled Antibodies

Radiolabeling of biotinylated rat IgG anti mouse CD-62P (PSGL-1) (553743, BD Pharmingen, San Diego, CA) and biotinylated rat IgG antibody (SouthernBiotech, Birmingham, AL) was performed using the HYNIC method as previously described [28]. Briefly, a fresh 1.8 mmol/L solution of succinimidyl 6-hydrazinonicotinate (HYNIC) in dimethylformamide was prepared. Forty picomoles were transferred to glass vials, followed by freezing at −90°C, and then solutions were vacuum dried using an Advantage Benchtop Freeze Dryer (Virtis Co., Inc.) with the shelf temperature at −75°C and trap at −90°C. The vials were sealed under vacuum and kept frozen at −80°C until use. Each vial was reconstituted with 1.0 mL of sodium phosphate buffer (0.15 mol/L, pH 7.8) containing 0.3 mg of IgG antibody (HYNIC/antibody molar ratio of 18; [29]). After
3 hr incubation at room temperature, the mixture was transferred to a Slide-A-Lyzer dialysis cassette having 10,000 molecular weight cutoff (Pierce) and immersed in 1.0 L phosphate buffered saline (PBS, pH 7.4) overnight at 4°C. The HYNIC-modified antibody was labeled with Tc-99m using SnCl₂/tricine as the transfer ligand [30], and unbound Tc-99m was removed by G-25 Sephadex size-exclusion chromatography. Protein concentrations of the collected fractions were measured by Lowry assay [31]. The level of free Tc-99m was measured by thin layer chromatography (TLC) using separate strips eluted with saturated saline and methyl ethyl ketone. Experiments were separated into two days.

**Targeted Microbubbles**

Streptavidin coated MBs (Targestar-SA) were obtained from Targeson (San Diego, CA). MBs were conjugated to the antibodies by means of biotin-streptavidin chemistry as previously described [26]. Briefly, streptavidin-bound MBs (1 vial per group 2.8x10⁹ MB/vial) were incubated with the respective antibodies (100 µg per group, 3.5 µg per 1x10⁸ MB) for 20 min followed by 2x centrifuge washing (400x for 3 min) to wash out unbound particles. The amount of antibody used during conjugation served to saturate the available streptavidin on the MB. MB concentration was determined via hemocytometer to ensure equal amounts of MBs were injected between groups.

The amount of antibody within the MB dose administered per injection was calculated by dividing activity injected by the decay-corrected specific activity (µCi/µg). To determine the µg/MB, the amount of antibody injected was divided by the number of MB administered per injection. This value was converted to micromoles, and then
multiplied by $6.023 \times 10^{17}$ (molecules per micromole) to yield number of molecules per MB.

Whole-body Biodistribution

Prior to experiments, mice were sorted based on tumor sizes to achieve equal distribution of tumor size in all groups. Experiments were performed over a 2-day period with day one biodistribution performed 5 min post intravenous (tail vein) injection of P-selectin-MB ($n=12$) (0.274 µg, $2.88 \times 10^5$ MB), IgG-control-MB ($n=12$) (0.363 µg, $2.88 \times 10^5$ MB), P-selectin-Ab ($n=5$) (0.274 µg), and IgG-control-Ab ($n=5$) (0.363 µg). Day two biodistribution was performed 60 min post intravenous (tail vein) injection of P-selectin-MB ($n=4$) (0.239 µg, $2.49 \times 10^5$ MB), IgG-control-MB ($n=4$) (0.378 µg, $2.88 \times 10^5$ MB), P-selectin-Ab ($n=3$) (0.239 µg), and IgG-control-Ab ($n=3$) (0.378 µg). All injections for MBs and antibody alone were diluted to a total volume of 60 µl with saline. Planar gamma camera imaging was performed on day two. The biodistribution procedure was performed as previously described [32]. Briefly, syringes containing dose were counted before and after injection using an Atomlab 100-dose calibrator (Biodex Medical Systems, Shirley, NY) to determine the exact dose. At 5 min and 60 min post dose, animals were sacrificed and all tissues collected in previously weighed scintillation vials. All tissue samples were then weighed and the Tc-99m activity was measured using a calibrated gamma ray counter (MINAXIg Auto-gamma 5000 series Gamma Counter; Packard Instrument Company), decay corrected to dosing time, and converted to absolute radioactivity. The percentage of injected dose per gram of tissue (%ID/g) was determined and used for comparison.
Gamma Camera Imaging

Imaging studies were conducted using X-SPECT, a SPECT/CT dual-modality imaging instrument manufactured by Gamma Medica, Inc, (Northridge, CA). Acquisitions (60 sec) were performed in planar mode using high-resolution low-energy parallel-hole collimators. Imaging was performed during day two at 4 min and 59 min post injection. Injection of radioactivity and subsequent imaging was staggered between the groups to account to radionuclide and MB decay. Quantitative analysis was performed using exported images and ImageJ software. ROI masks were generated and applied to each tumor to uniformly measure tumor pixel intensity normalized to total mouse intensity. Data shown as %ID ± SD.

Statistical Analysis

Statistical analysis was completed using the SAS 9.2 software (Cary, NC). Analysis of variance (ANOVA) was used to analyze the differences between the group means and variation among and within the groups. Comparisons were performed using a Tukey-Kramer method (Tukey’s HSD) multiple comparisons test. When comparing %ID/g per tissue within the same animal (e.g., muscle vs. tumor), a paired $t$-test was utilized. All data is given as mean ± standard deviation (SD). A difference of $P < 0.05$ was considered statistically significant.
RESULTS

Radiolabeling

The antibodies and MBs were successfully labeled with Tc-99m, with 4% or less free Tc-99m in the preparations. For day one radiolabeling, specific activity was determined to be 37.25 μCi/μg for the anti P-selectin radiolabeled antibody and 33.61 μCi/μg for the rat IgG radiolabeled antibody. During day two radiolabeling, specific activity was determined to be 22.04 μCi/μg for the anti P-selectin radiolabeled antibody and 29.54 μCi/μg for the rat IgG radiolabeled antibody. Values for μg/MB and antibody molecules/MB are presented in Table 1.

P-selectin-MB Retention in Liver, Spleen, and Lungs

Liver retention was significantly higher ($P<0.05$) for the IgG-control-MB group (48.5 ± 7.3 %ID/g at 5 min, 42.7 ± 2.1 %ID/g at 60 min) compared to all other groups (Fig.1a). Liver retention in all groups remained stable from 5 min to 60 min with the exception of the P-selectin-Ab which decreased significantly ($P<0.05$) from 15.7 ± 1.8 %ID/g at 5 min to 9.2 ± 3.1 %ID/g at 60 min. Notably, liver retention was 82% and 84% higher at both 5 and 60 min in the IgG-control-MB group compared to the P-selectin-MB group. For the spleen, retention levels were greater for the MB groups compared with Ab.

<table>
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<th>P-selectin-MB (day 1)</th>
<th>P-selectin-MB (day 2)</th>
<th>IgG-control-MB (day 1)</th>
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<tr>
<td>µg/MB</td>
<td>9.55x10^{-7}</td>
<td>8.33x10^{-7}</td>
<td>1.26x10^{-6}</td>
<td>1.32x10^{-6}</td>
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<td>Antibody molecules/MB</td>
<td>3.83x10^{6}</td>
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Fig. 1. Comparison of percent injected dose per gram (% ID/g) values at 5 min and 60 min for P-selectin targeted microbubbles (MB), IgG targeted control MB, P-selectin antibody, and IgG control in (a) liver, (b) spleen, and (c) lungs. Data are means ± SD. Asterisk denotes statistically significant difference, $P<0.05$.

groups, with no significant difference ($P>0.05$) between P-selectin-MB (16.9 ± 6.4 %ID/g at 5 min, 23.4 ± 6.5 %ID/g at 60 min) and IgG-control-MB (20.6 ± 7.4 %ID/g at 5 min, 18.0 ± 6.6 %ID/g at 60 min) at the 5 min and 60 min time points (Fig. 1b). Lung retention remained highest in the MB groups at both time points, however IgG-control-MB was significantly higher ($P<0.05$) than P-selectin-MB at both 5 min (100.2 ± 19.3 %ID/g for IgG-control-MB, 56.3 ± 10.1 %ID/g for P-selectin-MB) and 60 min (61.7 ± 4.1 %ID/g for IgG-control-MB, 42.4 ± 4.1 %ID/g for P-selectin-MB) (Fig. 1c).
Fig. 2. Comparison of percent injected dose per gram (% ID/g) values at 5 min and 60 min for P-selectin targeted microbubbles (MB), IgG targeted control MB, P-selectin antibody, and IgG control in (a) blood and (b) kidney. Data are means ± SD. Asterisk denotes statistically significant difference, $P<0.05$.

**P-selectin-MB Clearance in Blood and Kidney**

Blood clearance from 5 min (26.4 ± 4.4 %ID/g) to 60 min (3.6 ± 0.5 %ID/g) was greatest ($P<0.01$) for the P-selectin-MB group (87%), while the IgG-control-MB group (9.9 ± 0.9 %ID/g at 5 min, 9.4 ± 1.2 %ID/g at 60 min) was not significantly different ($P>0.05$) (Fig. 2a). Likewise, the blood clearance was significantly greater ($P<0.05$) for the P-selectin-Ab (79%) over the control antibody (42%) from 5 min to 60 min. For the kidneys, there was significantly greater ($P<0.05$) retention of the P-selectin-MB (12.9 ±
Fig. 3. Comparison of percent injected dose per gram (% ID/g) values at 5 min and 60 min for P-selectin targeted microbubbles in heart, stomach, large intestine, small intestine, cecum, reproductive organs, brain, and femur. Data are means ± SD. Asterisk denotes statistically significant difference, $P<0.05$.

1.4 %ID/g) over IgG-control-MB (8.8 ± 2.3 %ID/g) at the 5 min time point. However, P-selectin-MB were significantly cleared ($P<0.05$) at the 60 min time point (9.1 ± 0.6 %ID/g), while the IgG-control-MB retention was not significantly different at the 60 min time point (10.2 ± 2.3 %ID/g) (Fig. 2b).

P-selectin-MB Retention in Other Tissues

Fig. 3 shows the retention of P-selectin-MB at the 5 min and 60 min time points in heart, stomach, large intestine, small intestine, cecum, reproductive organs, brain, and femur. The greatest P-selectin-MB retention was observed in the stomach (12.2 ± 0.9 %ID/g) at 60 min. The stomach also demonstrated the greatest increase in retention from 5 min to 60 min (3.2 ± 1.4 %ID/g at 5 min). The cecum also demonstrated an increase in P-selectin-MB retention from 5 min (0.9 ± 0.2 %ID/g) to 60 min (1.9 ± 0.1 %ID/g). The heart (6.8 ± 1.6 %ID/g at 5 min, 1.77 ± 0.1 %ID/g at 60 min) and brain (0.7 ± 0.2 %ID/g at 5 min, 0.2 ± 0.03 %ID/g at 60 min) showed the only significant decrease
Fig. 4. Comparison of percent injected dose per gram (% ID/g) values in tumor and muscle tissue at (a) 5 min and (b) 60 min P-selectin targeted microbubbles (MB), IgG targeted control MB, P-selectin antibody, and IgG control groups. Data are means ± SD. Asterisk denotes statistically significant difference, *P*<0.05.

(P<0.05) in retention over time. IgG-control-MB exhibited similar trends in these tissues compared to P-selectin-MB (*P*>0.05).

P-selectin-MB Retention in Tumor and Muscle

For the tumor, there was significantly higher (*P*<0.05) retention of P-selectin-MB (1.3 ± 0.4 %ID/g) over IgG-control-MB (0.4 ± 0.1 %ID/g) at 5 min (Fig. 4a). Tumor retention for all groups increased from 5 min to 60 min (Fig. 4b), which was unique to tumor. P-selectin-MB retention in tumor (1.3 ± 0.4 %ID/g at 5 min, 1.8 ± 0.4 %ID/g at 60 min) was significantly greater (*P*<0.01) than P-selectin-MB levels in adjacent skeletal muscle at both time points (0.6 ± 0.2 %ID/g at 5 min, 0.2 ± 0.1 %ID/g at 60 min). There was no significant difference (*P* =0.17) between muscle (0.3 ± 0.1 %ID/g) and tumor (0.4 ± 0.1 %ID/g) retention for the IgG-control-MB group at 5 min. The greatest tumor retention occurred at the 60 min time point with the P-selectin-Ab group (3.5 ± 0.3 %ID/g).
**Fig. 5.** (a) Comparison of % ID calculated by mean intensity normalized by the total mouse intensity at 5 min and 60 min during planar gamma camera imaging between P-selectin targeted microbubbles (MB) and IgG targeted control MB in tumor tissue. Data are means ± SD. (b) Representative image of P-selectin targeted MB during planar gamma camera imaging at 60 min. Asterisk denotes statistically significant difference, $P<0.05$.

Planar Gamma Camera Imaging

Planar gamma camera imaging was performed to compare with whole-body biodistribution. Fig. 5a shows analysis of pixel intensity in the tumor normalized by total intensity found in the mouse at 5 min and 60 min time points for P-selectin-MB and IgG-control-MB groups. The trend of increased tumor retention for the MB groups was observed in the P-selectin-MB exhibiting a 98% increase ($P=0.03$) over time (6.0 ± 2.23% at 5 min, 11.9 ± 3.93 at 60 min). The IgG-control-MB group showed a 57% increase ($P=0.07$) from 5 to 60 min (4.4 ± 0.4 % at 5 min, 6.9 ± 1.8 % at 60 min). In Fig. 5b, a representative image of planar gamma camera imaging shows Tc-99m tumor retention for the P-selectin-MB group at 60 min.
DISCUSSION

Accurate measurement of targeted MBs and appropriate controls, in all tissues will aid in the development of these novel-imaging agents. These steps establish specific targeting, as well as non-target tissues involved in elimination. Reported here is the whole body biodistribution of tumor bearing mice after intravenous injection of P-selectin-MBs. This full body biodistribution comparing P-selectin-MBs and control MBs can be more generally applied for other mechanisms to target MBs.

Tumor retention of P-selectin-MBs was greater than IgG-control-MBs with a 3.2-fold enhancement of MB accumulation. In addition, there was greater retention of P-selectin-MBs in the tumor compared with adjacent skeletal muscle at both time points, 2-fold at 5 min and 9.3-fold at 60 min. This trend of enhanced tumor accumulation by P-selectin targeting was also observed in the antibody alone groups with greater tumor retention of P-selectin-Ab compared to the IgG-control-Ab. These results confirm the hypothesis that P-selectin targeting is beneficial for MB delivery to tumor. An observed trend that was unique to the tumor tissue was the increase in retention from 5 min to 60 min for all groups. This could be attributed to the Enhanced Permeability and Retention (EPR) effect common to tumor tissue [33]. In a study by Willmann et al. in 2008, F-18 labeled anti-VEGFR2 MBs were evaluated in angiosarcoma tumor bearing mice using dynamic micro-PET imaging [34]. In that study, tumor retention was reported to be 1.14 %ID/g at 4 min post iv injection and 1.35 %ID/g at 60 min post while skeletal muscle retention was reported unchanged at 0.84 %ID/g at 4 min and 60 min. These results were relatively similar to the current study findings, however the deviation between the tumor
and muscle tissue was greater for the P-selectin-MBs, which could also be attributed to the different modality, tumor type, and receptor density used within the studies.

For the filtration organs, Tc-99m liver accumulation remained stable from 5 min to 60 min for all groups except the P-selectin-Ab group, which was significantly reduced by 41%. This observation of static retention in the liver was also observed in the previously mentioned study performed by Willmann et al. These findings would imply that the capacity of the liver to sequester and process the injected doses requires more than 60 min. Delayed liver clearance was also shown to occur in a similar study of un-targeted MB biodistribution where MB clearance in the liver was first observed at the 6 hr time point [35]. This retention by the liver is most likely due to MB uptake by Kupffer cells, whose normal function is to phagocytose foreign particles, and have been shown to play a crucial role in MB uptake in the liver [3]. At each time point, the lung and liver retention was significantly higher for the IgG-control-MB group compared to the P-selectin-MB group. However, P-selectin-MB retention was greater than IgG-control-MB group in the blood and tumor at each time point, which would account for the lack of retention in liver and lung. Unique to the lung, Tc-99m retention was significantly greater in the MB groups at both time points compared to antibody alone. Considering the minor amount of pulmonary macrophage contribution to lung clearance in mice [36], the increased MB retention could be explained by the non-specific pulmonary entrapment that often occurs using MBs with a diameter greater than 5 μm [37]. In the spleen, there was a slightly higher retention of MB groups compared with antibody alone; this was most likely caused by previously reported involvement of splenic macrophages and mononuclear phagocyte system in the clearance of MBs [2]. There was no significant
difference in the spleen for the MB groups at 5 min and 60 min however there was a
significant decrease in targeted and control antibody from 5 min to 60 min, a trend that
further supported the role of splenic macrophages to engulf MBs in the spleen. In the
kidneys, there was greater retention of the radiolabeled antibody groups compared to the
MB groups at 5 min, a trend that was also observed in the Willmann et al. study [34].
However, there was a significant clearance of both P-selectin-MB and P-selectin-Ab in
the kidneys that was not observed in the IgG control groups suggesting an additional
benefit of targeting to improve P-selectin-MB circulation and bioavailability while
limiting kidney accumulation over time. Expediting kidney clearance could prove useful
in decreasing systemic circulation of unbound particles. As expected, organs filtered
MBs and antibodies differently, illustrating the importance of the control groups used
during the whole-body biodistribution. This analysis also demonstrates the differences
between targeted and control MBs, with decreased filtration of targeted MBs compared to
controls.

When analyzing P-selectin-MBs alone, retention was highest at the 5 min time
point in the lung (56.3 %ID/g) followed by the blood (26.4 %ID/g) and then the liver
(26.1 %ID/g). The relatively high signal in the stomach and cecum is attributed to the
normal gut localization of free Tc-99m pertechnetate that ultimately is an artifact of the
radiolabeling strategy used in this study [38]. At the 60 min time point, P-selectin-MB
retention remained relatively constant in the lung, liver, and spleen however there was an
86% clearance in the blood. This represented the greatest amount of blood clearance
observed in all groups from 5 min to 60 min. The constant levels in lung, liver and
spleen suggest the Tc-99m-labeled P-selectin was not released from the MBs in those
tissues. Similarly, the blood clearance suggests the Tc-99m-labeled P-selectin remained with the MBs and was also not released into the circulation where a longer half-life would be expected.

While P-selectin is overexpressed in tumor vasculature, it is also expressed in other regions of the body, including inflammation processes. This could be a limitation to the study; however it was shown that targeting does enhance the retention of the targeted groups in the tumor when compared to controls. Other targeted agents explored in cancer therapies including VEGFR2, ICAM-1, and integrin αVβ3 are also expressed in other tissues, and not specific to the tumor. Further studies would be beneficial to compare the performance of P-selectin targeted MBs to alternate targeted MBs. Also, there was significant amount of blood clearance after injection of the P-selectin-MB group in comparison to the other groups, exhibiting the necessary traits for filtration. Differences in the blood flow and shear stress can directly affect targeting and retention of molecules within a region-of-interest. There has recently been studies examining P-selectin targeting in ischemic tissues where blood flow is altered [39, 40], however it has also been shown that targeting and shear stress is not linearly related [41]. The difference in blood flow is a limitation when comparing skeletal muscle to tumor. This study relies on targeting a receptor which is overexpressed within the tumor, and limitations included, still shows increased retention of targeted MBs over control MBs. Differences in blood flow also directly affect Abs and MBs differently, as MBs have a much shorter half-life than Ab. Ab have an estimated 11 day half-life [42], while MBs have an estimated 2 min half-life [2]. These differences can directly affect tumor uptake, as well as organ filtration and sequestration.
Avidin-biotin chemistry was utilized in this study to conjugate Ab to MB. Clinically, this strategy is not relevant. Instead, targeting peptides are being explored that are covalently linked to MB. Consequently, the Ab ratios used here would not translate to clinical use. The difference in the number of Ab per MB found during the study relative to previously published studies may be the result of alternative labeling and quantification strategies. Most of the previously reported methods utilized a three-step conjugation where a biotin-streptavidin-biotin bridge is used. In a study assessing renal tissue injury and inflammation using P-selectin targeted MBs by Lindner et al [43], 75 μg of biotinylated Ab was used to conjugate 1x10^8 MBs. In their study, the yield was found to be 3x10^5 molecules of Ab per MB, as determined by fluorescent techniques. In a similar study investigating P-selectin targeted MBs under shear flow by Takalkar et al [41], 75 μg of biotinylated Ab was also used to conjugate 1x10^8 MBs. The yield was found to be 1x10^5 molecules of Ab per MB, as determined by fluorescent techniques. In the current study, 100 μg of biotinylated Ab was used to conjugate 1 vial of streptavidin coated MBs (Targeson-SA, 2.8x10^9 MB/vial), which equates to 3.5 μg per 1x10^8 MB. To determine the molecules of Ab per MB, radiological techniques were used due to the unique radiation component of the study design. This is another study parameter that differs from previously reported studies that could also account for the variances in labeling.

One limitation of this study was the use of avidin chemistry to adhere the antibody to the MB. While this strategy ensures a stable bioconjugate, the avidin utility reduces the translational potential of the targeted MB construct due to the immunogenicity of the avidin molecule. Alternative strategies of MB targeting being
developed include the use of targeted peptides that are covalently bound to MBs. Initial studies using this motif have been performed and results suggest the strategy to be safe and specific [44, 45]. One study recently concluded a phase 0 clinical trial using VEGFR2 as the targeting moiety during prostate cancer evaluation [46]. This component would allow for a more feasible clinical translation of targeted MBs for both imaging and drug delivery. Although there are limitations, this study provides initial evidence of a promising tool for targeted drug delivery using P-selectin and ultrasound contrast agents.

CONCLUSIONS

If MBs are continued to be investigated as drug delivery carriers, this study demonstrates that targeting improves tumor retention compared to non-targeting. The study also shows that targeting MBs to P-selectin reduces liver and lung retention compared to non-targeted MBs. While there is more MB retention in all tissues compared to tumor tissue, there is less retention of targeted MBs in tissues and greater tumor retention relative to non-targeted MBs. With the potential of targeted MBs to improve CEUS and drug delivery in patients, the current work is invaluable to elucidate the systemic biodistribution of targeted MBs in general. The innate role of P-selectin to sequester platelets and rolling monocytes is well suited to reproduce the desired mechanism for vascular MB adhesion. The P-selectin molecule is a promising candidate to improve MB accumulation in target tissues, however other cell adhesion molecules and receptors should be continually investigated and may prove to be more efficacious. This study provides validation of P-selectin targeted MBs as a more specific approach for systemic drug delivery in a solid tumor.
ACKNOWLEDGMENTS

This work was supported the UAB Small Animal Imaging Shared Facility NIH Research Core Grant (P30CA013148) and the Susan G. Komen Breast Cancer Foundation (KG090969). The authors would like to thank Reshu Saini and Soojin Kim for their contributions to this project.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES


These chapters have presented novel advancements utilizing the field of US to improve methods in cancer imaging, monitoring, and drug delivery. Cancer is currently the second leading cause of death in the United States, affecting 1 in every 2 males, and 1 in every 3 women. This alarming statistic encourages the continuing research effort to find an effective approach to improve cancer patient outcome. Enhancing localized delivery to the tumor burden improves overall therapeutic response, and thereby decreases systemically circulating drugs. For an effective therapy, the amount of drug delivered is directly related to the response obtained. Localized or targeted augmentation not only improves anticancer response, but can decrease toxic side effects currently associated with many cancer treatments. Individualized cancer treatment is essential in improving overall patient outcome; individuals with the same type of cancer will have a distinct response to treatment. Another important aspect involved in patient prognosis is assessment of therapeutic response. Knowledge involving vascular activity and the ability to monitor changes within the tumor can give important validation of whether or not a particular drug or treatment is effective. Although current RECIST criteria do not incorporate this component, it has been shown essential in both preclinical and clinical studies for predicting cancer response. The earlier predictive response is determined, the more accurate patient prognosis and survival outcome is obtained. Early determination can directly impact patient care and the choice of treatment, therefore imaging can be directly related to patient response and survival outcome. Cancer researchers are continuing to develop novel ideas for improving both monitoring and therapy. However, we are still in dire need for improvements to further improve patient prognosis.
Improving both monitoring and therapy using MBs allow for a noninvasive and nontoxic approach that can improve overall patient outcome.

US-stimulated therapy has demonstrated the ability to increase molecular delivery both in vitro and in vivo in order to improve cell membrane permeability and drug delivery. This novel drug delivery approach has been developed for utilization in cancer therapy with intentions to be universally applied to many cancer types and drugs. In vitro optimization was evaluated and quantified through fluorescent uptake within cells. Cells in suspension and monolayers were both used within this study; therefore further analysis to compare the different methodologies was completed for comparison (as seen in Appendix A). Evaluation of US-stimulated therapy in vivo was essential to assess whole tumor and vascular response for optimization within a primary tumor animal model. Optimization of US-stimulated therapy in combination with traditional, anti-mitotic chemotherapeutic drugs significantly enhanced therapeutic response in an aggressive breast cancer animal model (Chapter 1). This was confirmed with both retardation of tumor size and histological analysis of anti-cancer effects. Further investigation expanded US-stimulated therapy in vivo to a variety of cancer cell types and in combination with additional anti-cancer drugs. Using the previously established optimized parameters, US-stimulated drug delivery showed improved effectiveness compared to drug alone constituents to improve drug efficiency in head and neck cancer (HNC). Enhancement of drug delivery and improvement utilizing this therapy was demonstrated with a platinum based drug, cisplatin, and a targeted antibody therapy, EGFR-targeted cetuximab. Along with tumor size regression, diffusion weighted- MRI (DW-MRI) and histological analysis gave additional significant validation of cancer response to treatment in the US-
stimulated therapy groups (Chapter 2). These experiments establish a generalized protocol and validation for using US and MBs as a potential nontoxic and noninvasive approach to enhance universal cancer drug delivery.

Optical imaging was hypothesized to give a noninvasive in vivo imaging approach to monitor molecular delivery with US-stimulated therapy. To the best of our knowledge, this was the first manuscript of its kind evaluating tumor uptake of various molecules noninvasively in a longitudinal study following US-stimulated therapy. Monitoring in vivo delivery directly following therapy gives insight into US-stimulated therapeutic timing and length of effectiveness. Optical imaging offers an inexpensive, preclinical method for evaluation of small and large sized molecules during drug delivery. This technique shows potential for improving overall drug delivery with US-stimulated therapy as seen in Chapter 3. Fluorescent optical imaging gave a direct validation of enhanced delivery to the tumor burden after a single dose of US-stimulated therapy, with initial validation of duration of effectiveness. This approach was further evaluated and confirmed using immunohistochemistry to confirm uptake of cetuximab-IRDye after US-stimulated therapy in a primary tumor model (seen in Appendix B). US-stimulated therapy was shown to enhance cetuximab delivery and tumor retention, thereby increasing fluorescent expression analyzed through in vivo monitoring and histology.

After initial exploration of delivering anti-cancer agents or molecules to a primary tumor for optimization and exploration of the potential of US-stimulated drug delivery, further translational opportunities were investigated. US-stimulated therapy was hypothesized to further improve tumor retention of Ad. This hypothesis was validated when US-stimulated therapy demonstrated improvement in tumor uptake and retention of
a single dose of intratumorally injected adenovirus (Chapter 4). One major limitation preventing the translation of Ad therapy in the clinical setting is the inadequate percent of delivery to the intended region. US-stimulated techniques show potential for improving gene delivery and therapy, which shows significant potential in cancer research.

Success of US-stimulated therapy was confirmed and identified in many clinically translatable opportunities and currently demonstrates potential to be universally applied to many cancer types with a variety of anti-cancer drugs and molecules. Due to the vast applicability of US-stimulated therapy, there is significant potential as a role in cancer research. Investigating further into the mechanisms of US-stimulated therapy required studying the safety of this unique treatment. It was hypothesized that the assessment of biological effects from US-stimulated therapy can improve knowledge and potential for clinical translation. In vitro analysis of cell death gave initial validation of a safe modality for low pressures when utilizing a 1.0 MHz pressure, as seen in Appendix C. In order to evaluate a wider range of US-stimulated therapy pressures, a 2.25 MHz frequency was used in vivo to explore the biological effects generated internally. Utilizing clinically translatable monitoring devices: MRI, CEUS, and histology, we evaluated the effective changes in vascular perfusion and permeability within a tumor following US treatment at various pressures. It was shown that utilizing currently established low pressure optimized US parameters, there were no negative bioeffects noted during molecular delivery in vivo (Chapter 5). However, it was also shown that at high US pressure parameters US-stimulated therapy creates vascular disruption or collapse, thereby decreasing vascular permeability and perfusion. As clinical translation becomes closer to reality, it is a necessity that we stay within the safe regions of US exposure. This
noninvasive imaging analysis of US-stimulated therapy furthers our knowledge and shows potential to improve drug dosing and monitoring of US-stimulated therapy in combination with a variety of anti-cancer fighting agents.

In Chapters 1-4, US-stimulated therapy was shown effective to enhance localized delivery of a variety of molecules (chemotherapeutics, antibodies, and adenovirus) in primary neoadjuvant tumor models. For clinical translation, it is important to show this therapy’s potential as both a neoadjuvant and adjuvant therapy. US-stimulated drug delivery was hypothesized to improve response in residual disease and reduce reoccurrence in HNC following surgical resection. All previous research explored within this field has been completed on primary tumor models in a neoadjuvant setting. US-stimulated adjuvant therapy significantly improves the ability to extravasate drugs as surgery exposes a difficult to penetrate devascularized wound bed. US-stimulated drug adjuvant therapy was shown to decrease tumor viability when used in combination with a targeted antibody therapy, cetuximab, in comparison to systemic injections of cetuximab alone in a longitudinal study with HNC (Chapter 6). Increased drug delivery to the tumor burden was evaluated using previously established optical imaging techniques. Clinically, HNC treatment consists of combination chemotherapy, radiation, or surgery because of its high rate of recurrence in the primary location. This transition of combining US-stimulated drug delivery with previously established clinical protocols is important for future translation and translation to universal applications. This experiment gives validation of US-stimulated therapy as an effective tool for increasing drug delivery to residual disease, thereby reduced recurrence in HNC.
Functionalizing MBs has been made possible by binding antibodies to the outside shell of the MBs and targeting to overexpressed receptors. This was completed with a streptavidin-biotin interaction and has been proven to improve MB-cell interaction. This interaction creates an improved molecular imaging or drug delivery strategy through enhanced accumulation of MBs within the desired ROI, such as the tumor. Using multi-targeted MBs to p-selectin, VEGFR2 and αVβ3, it was hypothesized that molecular US imaging will improve the overall information received regarding tumor vascular and response to antiangiogenic drugs. It was shown that US can determine significant response to therapy on day 3 after a single dose of antiangiogenic drug in an *in vivo* breast cancer tumor-bearing mouse model (Chapter 7). Histological analysis confirmed this through MVD staining. The most important aspect of this technique for utilization in cancer imaging was that the addition of targeted MBs allowed detection of tumor response to therapy before current standard of care methods. Both molecular US imaging and histology detected a change within the cancerous region; however tumor size showed no difference between therapy and control groups. The ability to predict responders versus non-responders at an early stage in cancer treatment is directly related to survival and a good patient outcome. Another aspect of targeted MBs which has potential in cancer research is the ability to enhance drug delivery. In order to further explore this area of interest, a full body biodistribution of radiolabeled P-selectin targeted MBs was completed and analyzed in comparison to IgG (non-specific) targeted MBs. P-selectin was shown to be an effective targeted for MBs in breast cancer, and this exploration demonstrated increased binding and tumor retention with targeted MBs at both 5 and 60 min post injection (Chapter 8). This study further investigated filtration
mechanisms between targeted and non-targeted MBs, as well as MBs versus Ab alone. Improving tumor retention, as well as reducing filtration of the radioactivity was completed through this targeted approach, thus giving evidence for effective drug delivery with targeted MBs. Because targeting MBs improves MB-cell interactions, it also shows potential for further increasing US-stimulated therapy. Preliminary in vitro investigations show that targeted MBs further increase chemotherapeutic delivery and overall anticancer response when combined with US-stimulated drug delivery compared to non-targeted MBs (as shown in Appendix D). This unique therapy, termed molecular US therapy, promotes additional MB-cell interaction during US exposure, thereby increasing the apparent effectiveness of cell membrane permeabilization.

Because MBs have shown to be effective in both monitoring and drug delivery, there is potential for future investigations within cancer research to combine these techniques. Utilizing targeted MBs in US-stimulated drug delivery in combination with monitoring response to treatment gives potential for an all-in-one approach to improve patient outcome in a nontoxic, noninvasive manner. CEUS and US-stimulated drug delivery in vivo were briefly explored in combination to prove feasibility of combination monitoring and US-stimulated therapy (Appendix E), however many more studies will need to be completed to prove effectiveness. These experiments demonstrate that there is potential to monitor tumor response with molecular US imaging while simultaneously utilize the targeted MBs in molecular US therapy, thereby creating a theranostic approach. More research will need to be completed in both molecular US therapy and in the field of combination CEUS and US-stimulated therapy. Molecular US therapy and
imaging is a novel strategy that shows potential to be applied universally across many cancer types and in combination with a variety of anti-cancer therapeutics.

This dissertation research presents effective strategies using traditional US contrast agents to enhance systemic drug delivery, while utilizing alternative US imaging methods to analyze cancer therapy. These techniques have led to a safer and more translatable cancer therapy. US-stimulated therapy has demonstrated applications in diverse anti-cancer fields, including drug delivery, gene delivery, neoadjuvant, and adjuvant therapy. Targeting MBs has shown potential to enhance MB-cell interaction for improved imaging and drug delivery strategies in preclinical animal models. The tremendous potential of this field is validated in this dissertation and demonstrates the ability to impact numerous cancer types through noninvasive advancements in both therapy and monitoring. In summary, this dissertation investigates US research utilizes MBs as effective tools in the treatment of cancer. Essentially, use of these methodologies has revealed a nontoxic, noninvasive approach for improvements in cancer monitoring and therapy, leading to greater success in cancer treatment.

Future directions in US research utilizing MBs rely on the theranostic potential to combine diagnostic imaging and monitoring with therapy. I believe this dissertation research encompassing US-stimulated therapy and molecular US imaging and drug delivery lays the groundwork for future directions in cancer treatment, however more research is necessary before clinical translation. Because MBs are nontoxic and nonimmunogenic, they create an ideal imaging contrast agent, while their impact in the drug delivery field also improves efficiency of current treatments without creating additional toxicity. The next stages of MB research in cancer treatment need to begin
combining molecular targeting strategies for monitoring disease states in conjunction with US-stimulated therapy. This combination strategy has the potential to create the greatest impact in the clinical setting. Molecular US imaging, molecular US drug delivery, and US-stimulated therapy have potential to improve personalized medicine, better tailoring cancer treatment to each individual patient. As preclinical research further develops to progress US strategies utilizing MBs towards clinical translation, it is essential that we develop strategies that will exploit the unique characteristics of MBs in order to create the greatest benefit for all aspects of cancer treatment: detection, monitoring, and therapy.
GENERAL LIST OF REFERENCES


APPENDIX

A CELL SUSPENSION VERSUS MONOLAYER

US-stimulated therapy relies on the mechanic oscillations of contrast agents to transiently induce intracellular molecular delivery. When analyzing effects of US-stimulated therapy, researchers have interchangeably used both cell suspension and monolayer techniques to validate molecular uptake. Given these two different states, the goal of this study was to evaluate the differential influences of MB-mediated US therapy on membrane permeabilization and molecular cell uptake when applied to cell suspensions or monolayers. 2LMP breast cancer cells (1x10^⁶) were suspended in polystyrene tubes or fixed on acoustically transparent plates (OptiCell). Calcein, a non-permeable fluorescent molecule, and MBs (Definity) were added to both cell suspension and monolayer. Cells underwent US-stimulated therapy in a 37ºC waterbath using a single element immersion transducer in series with a power amplifier and signal generator. The following parameters were held constant: 5.0 min total duration, 0.01 sec PRP, 1.0 MHz transmit frequency. Duty cycle (0.01%, 0.1%, 1.0%) and MI (0.1, 0.5) were varied. Control cells were analyzed under the same conditions, except sham US was used. Following therapy or sham treatment, cells were washed, trypsinized, and analyzed using flow cytometry. In vitro fluorescent uptake was counted. Data was summarized as mean ± SE.

Intensity measurements are independent of peak to peak voltage and show same \( I_{SPTA} \) % change over each frequency (monolayer % increase over suspension). Monolayers received a 15 to 55% increase in intensity when compared to cells in suspension, depending on frequency, under identical US conditions as seen in Figure 1.
Figure 1. **US-stimulated delivery in cancer cells in vitro: comparison of suspension vs monolayer.** Intensity values show similar trends in both monolayer and suspension as the voltage increases (increase in pressure & MI) across various frequencies (top). Percent change of intensity received by the monolayer compared to suspension in shown below. At various frequencies, it is shown that the changes between the two methodologies are different; therefore it is difficult to make a direct comparison between the two.

Monolayer cells showed increased uptake with increased frequency. Suspension cells showed peak uptake at 1.0 MHz frequency.

At an MI of 0.1, over the various duty cycles, studied cell suspensions exhibited an increased fluorescent uptake affect compared to monolayer cells ($P = 0.002$) indicating increased levels of membrane permeabilization as seen in Figure 2. US-
Figure 2. US-stimulated uptake of fluorescent molecules in vitro: comparison of suspension versus monolayer. As the frequency is altered, the uptake also varies. In the monolayer groups, the uptake increases as the frequency increases (shown above), however in the suspension groups, uptake peaks at 1.0 MHz. (shown below).

stimulated therapy at an MI of 0.1 exhibited considerable higher levels of membrane permeability compared to results collected using an MI of 0.5 ($P = 0.27$). As the duty cycle and effective US exposure increased, fluorescent uptake also exhibited an
increasing trend in both cell suspension and monolayer. Molecular uptake, resulting from increased membrane permeabilization from US-stimulated therapy, is affected by the direct MB-cell interact. If cells are freely flowing in comparison to stationary monolayers, MB-cell interactions may react differently, thereby creating differences in frequency or size of pores. It is important to use caution when using these different techniques of cell suspension or monolayer interchangeably. The mechanisms of US therapy differ between cell monolayers and cell suspension. Improved models for this drug delivery mechanism need to be developed. *In vivo* and *in vitro* experiments using US therapy cannot be directly compared due to this difference, although similar trends have been shown throughout.
B CETUXIMAB-IRDYE DELIVERY WITH ULTRASOUND-STIMULATED THERAPY

Chapter 3 evaluated optical imaging as a tool to further evaluate differences in tumor retention and uptake following US-stimulated therapy. A limitation of this study was that histological analysis was not completed due to the tumors being homogenized for evaluation of fluorescence release \textit{ex vivo}. This study investigates the uptake of IRDye-labeled cetuximab after US-stimulated therapy both \textit{in vivo} and through histological analysis of fluorescence.

Using a dual flank tumor animal model \((N = 8)\), uptake of IRDye-labeled cetuximab was evaluated post US-stimulated drug delivery. Histological analysis confirmed uptake of fluorescent molecule (with targeted drug). Dual-flank tumors create a built-in control for each animal. Fluorescent labeled-drug and MBs were injected intravenously and allowed to circulate for 2 min. Following circulation, one tumor per mouse received US therapy. Animals received localized US therapy for 5 min to one tumor in a 37°C waterbath. The other tumor provided a built-in control, receiving the same dose of MBs and drug, without the US therapy. Mice were imaged with the 800nm channel of a small animal imaging system, the Pearl Impulse, at 1, 5, 10, 20, and 30 min post therapy \textit{in vivo}. Tumors were extracted for H&E histological evidence to compare fluorescent dye in the fixed tumor samples using a qualitative fluorescent imaging system (Odyssey, LI-COR Biotechnology). Paired, \(t\)-test statistical analysis was used for the comparison of US-stimulated drug delivery and drug alone in the dual-flank tumor model.
Figure 1. Optical imaging of IRDye-cetuximab and US therapy using a mouse model. Molecular delivery was analyzed for one 30 min post therapy with a small animal fluorescent imaging chamber. In vivo imaging post therapy shows therapy group with an increased amount of fluorescent expression compared to control group as shown in representative images (P < 0.01) shown top. Shown bottom left is the histogram analysis of IRDye-cetuximab extravasation in representative tumor of therapy (top) and control (bottom). Shown bottom right is histological analysis of therapy vs control tumors of IRDye-cetuximab extravasation showing a 19% increase in therapy group (P = 0.17).
US-stimulated therapy plus IRDye-labeled cetuximab drug showed an increased delivery to therapy tumors compared to control. ROI analysis of IRDye in tumor allowed quantification of molecular uptake in each tumor. Tumors were extracted and histological analysis was completed for verification and quantification of IRDye in each tumor. Verification was completed with the Pearl Odyssey fluorescent imaging system and quantification was completed with custom Matlab software. In vivo fluorescent analysis showed enhanced delivery of IRDye-labeled cetuximab to the tumors which received MB-mediated US therapy throughout the longitudinal study compared to tumors receiving drug alone ($P < 0.01$), as seen in Figure 1. Histological results supported in vivo analysis, showing increased IRDye in tumors receiving US therapy ($P = 0.17$).

US-stimulated therapy shows potential to improve drug delivery for enhanced drug localization. This experiment shows translation of US therapy to antibody drugs used in cancer therapy and gives potential to further explore this technique for other molecules compared to traditional chemotherapeutics.
C ULTRASOUND-STIMULATED THERAPY: CELL VIABILITY

Suspended 2LMP breast cancer cells underwent US-stimulated therapy using various US parameters in the presence of calcein, a membrane non-permeable fluorescent tracer. The US setup involved an unfocused, single element (0.75”) immersion transducer (Olympus) in series with a signal generator (AFG3022B, Tektronix) and power amplifier (A075, Electronics and Innovation) as similarly described in the optimization of US parameters in vitro section (Chapter 1). Cell viability curves will be used to identify a range of conditions where US therapy is efficacious in vitro. The influences of US parameters, i.e. transmit frequency, mechanical index (MI) (0.1 - 2.0), duration of exposure (0.1 - 1000 sec), pulse repetition period (PRP) (0.01 - 5 sec), using Definity microbubbles at a standard dose (10 μl) were investigated and quantified using flow cytometry. Cell viability was determined through calcein AM uptake. Cell death was determined through propidium iodide uptake in cells thirty minutes after US-stimulated.

Figure 1. Cell viability after US-stimulated therapy in vitro. No significant differences were seen in cell death after altering the duration of exposure (shown left) or altering the MI (shown right) at 1.0 MHz frequency in breast cancer cells.
therapy. Flow cytometry analysis allowed us to graph cell viability vs. cell death (FL-1 vs. FL-3 with fluorescent signal) and determine percent death compared to sham US-stimulated therapy tubes (cells that underwent identical conditions without receiving US intensity). At 1.0 MHz frequency, the frequency in which was deemed optimal for US therapy, cell death resulting from US-stimulated therapy was not significant over various mechanical indexes and durations of exposure ($P > 0.05$) as seen in Figure 1.
As cancer research expands into new treatments and we individualize therapies for each patient, it has become important to create novel therapeutic techniques in preclinical models. Traditionally, chemotherapy treatment results in systemic toxicity and insufficient dosage to the tumor. The goal of successful drug delivery is to reduce toxicity to healthy tissue, while enhancing delivery to the agent to the desired site.

Molecular US therapy utilizes targeted US contrast agents to increase US-stimulated therapy through specifically targeting cells of interest. MB-mediated US therapy utilizes contrast agent oscillations to create temporary pores in the cellular membrane. The use of properly targeted MBs increases the amount of agents that accumulate in a specified region (e.g., tumor), thereby increasing the physical interaction of MBs with the endothelial cells (Willmann et al. 2008, Warram et al. 2011). Using targeted MBs in combination with therapeutic strategies shows potential to both localize and target drug treatment, creating further positive enhancements in local drug delivery.

In this study, US therapy was used in conjunction with Ab labeled MBs to determine if localization of targeted MBs would further improve drug uptake compared to that observed using non-targeted (conventional) MBs. This strategy would create a novel molecular US therapeutic technique.

2LMP breast cancer cells were harvested in DMEM media with 10% FBS and 1% L-glutamine. Cells were grown to 70 to 90% confluency, trypsinized and then counted with a hemocytometer. Cells were cultured in 37°C and 5% CO₂. Cancer cells (1x10⁶) were fixed on acoustically transparent tissue culture plates (Opticell, Thermo Scientific, NY). US parameters were selected by analyzing a intracellular fluorescent reporter signal.
using flow cytometry after molecular US therapy was applied to cells incubated with extracellular calcein (membrane impermeable molecule), as seen in Chapter 1. The following parameters were held constant: 5.0 min duration of US therapy exposure, 0.01 sec PRP, 1.0 MHz US transmit frequency, and 20% duty cycle.

Cell receptors were induced using an Ad vector that encodes for an extracellular hemagglutinin (HA) tag and green fluorescent protein (GFP) reporter. Cells were infected with this Ad-HA-GFP vector to induce cell surface expression. Cells were incubated for 24 hr with Ad infectivity of 100 multiplicity of infection (MOI) (3.2x10^{11} plaque forming units per mL). Previous studies have verified successful infection by using flow cytometry for quantification of GFP expression. Successful antibody binding to the HA tag was further confirmed by flow cytometry after incubating infected cell populations with Cy5.5-labeled anti-HA antibodies.

Targeted MBs were created by conjugating either biotinylated anti-HA antibody (Ab) or isotype control Ab to the surface of biotin coated MBs through a streptavidin bridge. Streptavidin labeled MB (Targestar-SA, Targeson, San Diego, CA) were incubated with either 100 µg of biotinylated anti-HA antibody (Sigma Aldrich St. Louis, MO) or isotype control antibody (Southern Biotech, Birmingham, AL) for 20 min at room temperature. A wash step removed any residual unbound antibody. The final concentration of targeted MBs was characterized by a hemocytometer.

MBs (control or therapy) and chemotherapeutic drug (paclitaxel) was administered to the cells prior to US therapy. Paclitaxel was chosen due to previous experience with this drug in combination with US therapy, as well as being a common chemotherapeutic used in breast cancer. Cells underwent molecular US therapy in a 37°C
waterbath using a single element (0.75 inch) unfocused immersion transducer (Olympus, Waltham, MA) in series with a AFG3022B signal generator (Tektronix, Beaverton, OR) and a A075 power amplifier (Electronics and Innovation, Rochester, NY). After US exposure, cell culture plates were washed with phosphate-buffered saline (PBS) and trypsinized to recover a solution of treated cells. Cells were analyzed for expression of cell death at 24 and 48 hours post therapy through flow cytometry (Accuri C6, Accuri Cytometers Inc, Ann Arbor, MI) and ATP lite assays.

Data is presented as mean ± SE. All statistical data was analyzed using SAS software (SAS, Cary, NC). An unpaired two-sample t-test was used to assess differences between control MB-mediated US therapy and molecular US therapy groups. A P-value of less than 0.05 was considered statistically significant.

Using the system introduced by Saini et al., variable receptor densities can be induced in cell cultures using Ad vector techniques (Saini et al. 2011a, Saini et al. 2011b). Ab-labeled MBs exhibiting a high affinity to induced cell surface molecular targets were investigated for their potential to further impact cell membrane permeability when exposed to properly timed US fields. Preliminary results in our group depicted that at an MI of 0.1, targeted US therapy improves cell membrane permeability and extracellular fluorescent tracer uptake by an additional 43.3% when compared to MB-mediated US therapy using non-targeted control MBs.

On day 1 (24 hours post therapy) targeted US therapy plus drug produced a 25.2 ± 4.9% decrease in viable cells compared to non-targeted, control MB-mediated US therapy with drug (P < 0.001). Within two days, molecular US therapy consistently exhibited a significant increase of 25.1 ± 9.6% in anticancer effects compared to non-
targeted US therapy when evaluated through ATPlite assays ($P < 0.001$), as seen in Fig. 1. The ATPlite assays revealed a 23.8% decrease in cell viability on day 2 compared to day 1 in the chemotherapy plus molecular US therapy group ($P = 0.01$). Flow cytometry analysis indicated an 8.4% increase in propidium iodide expression (corresponds to non-viable cells) on day 1 and 16.2% increase on day 2 when comparing molecular US therapy to control US therapy ($P = 0.09$ and $P = 0.10$, respectively). This trend suggests that targeted MBs increase effectiveness of US therapy. Molecular US therapy promotes additional MB-cell interaction during US exposure, increasing the apparent effectiveness of cell membrane permeabilization.

This study utilizes a model system which allows induction of extracellular receptor proteins and highly specific MB targeting to analyze the effects of molecular US
therapy. This ensures that the amount of receptors is consistent and controlled between each experiment. The application of using targeted MBs has been explored for drug delivery release of encapsulated drugs, proteins, adenovirus and other various applications. Once MBs are attached to the ROI, high intensity US is induced to burst the MBs, releasing the payload to the localized region (Tsutsui et al. 2004, Warram et al. 2012). Targeting drug coated MBs has been explored to enhance drug delivery to the blood-brain barrier (Majumdar et al. 2010), as well as increase chemotherapeutic delivery to a localized region to decrease systemic toxicity (Cochran et al. 2011). Our study demonstrates a novel approach of using targeted MBs in conjunction with US therapeutic techniques to improve delivery and cellular uptake of an anticancer drug.

A previous study by Kooiman et al. (2011) exploring targeted MBs and US therapy quantified fluorescent uptake of propidium iodide. That study was completed in vitro by targeting CD31 receptors and using a 1.0 MHz US transmission frequency. Results demonstrated that 30% of cells internalized propidium iodide and was the first evidence that targeted MBs can be used to increase cell permeability. A 1.0 MHz frequency was also used in our experiments and was previously deemed efficacious for performing US therapy compared to other US frequencies. Analyzing molecular US therapy versus traditional US therapy alone in this study allows us to directly compare the increased effect of incorporating targeting MB contrast agents. Molecular US therapy exhibited a 25% increase in anticancer effects compared to US therapy alone using non-targeted MB contrast agents.

This study demonstrates that MBs targeted to overexpressed cellular receptors are a useful adjunct to US therapy. Molecular US therapy is a technique that may introduce
promising opportunities in cancer treatment but more research must be performed to unravel the underlying biological mechanisms leading to these encouraging anticancer effects. This trend suggests that targeted MBs increase effectiveness of US therapy. Molecular US therapy promotes additional MB-cell interaction during US exposure, increasing the apparent effectiveness of cell membrane permeabilization.
E CEUS EVALUATION OF ULTRASOUND-STIMULATED THERAPY

Analysis of US therapy in vivo has shown to be a more difficult task as the mechanisms are more complicated and molecules are difficult to track. The ability to monitor drug therapy has potential to reveal tumor uptake mechanisms and pending response to treatment for individual patients and essential in gaining further understanding in this technology. It was hypothesized that we will be able to track tumor growth and changes within the vasculature of the tumor over the three weeks of therapy to determine which mice are responding to treatment and with CEUS imaging; we will be able to further analyze US-stimulated therapy. Simultaneous therapy and imaging allows for dual purpose for the MBs, and could also reveal alterations in tumor perfusion.

Tumor-bearing mice (N = 12) were injected with MBs via tail-vein injection and received US-stimulated therapy for 5 minutes. Therapy parameters were optimized from Chapter 1 in vivo analysis. CEUS imaging was completed throughout the therapy process using both volumetric and planar US imaging. Results showed an estimated 25% reduction in MB circulation to the tumor when analyzed by volumetric US imaging, with an estimated 10% decrease from planar imaging as seen in Figure 1. MBs in circulation can be eliminated through liver filtration and can also become trapped within the lungs and small capillaries. Volumetric CEUS imaging exhibited increased destruction in MBs, creating some MB bursting, or inertial cavitation. This data supports our hypothesis of majority stable cavitation (5% decrease using US therapy in comparison to contrast-enhanced US imaging alone) with our US therapy parameters. This preliminary experiment allows us to have initial groundwork in dual therapy and imaging, and supports planar US imaging over volumetric for this application. Further investigation of US imaging of a temporal study
Figure 1. CEUS imaging of US-stimulated Therapy. Shown is US therapy monitored in N = 12 tumors to analyze MB intensity changes over five minutes of therapy. This technique compares volumetric and planar US imaging. No drug was given to these animals.

analyzing effects of drug plus US-stimulated therapy is needed. This experiment gave initial validation that the combination of CEUS and US-stimulated therapy is possible, which would allow combination monitoring and treatment.
F IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: October 19, 2012

TO: KENNETH L. HOYT, PH.D.
YH - G082 0019
FAX (205) 975-8522

FROM: Judith A. Kopp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Development of a Novel Targeted Contrast Agent for Ultrasound Imaging and Local Chemotherapeutic Drug Delivery
Sponsor: Internal
Animal Project Number: 121000773

As of October 2, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

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Animal use must be renewed by October 1, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 121000773 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7682.
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE:          October 8, 2012
TO:            KURT R ZINN, Ph.D.
               YH -G082 0019
               FAX: (205) 975-8522

FROM:          Judith A. Kapp, Ph.D., Chair
               Institutional Animal Care and Use Committee (IACUC)

SUBJECT:       Title: (Core) Laboratory for Multimodality Imaging Assessment
               Sponsor: Internal
               Animal Project Number: 121003656

As of October 8, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

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Animal use must be renewed by October 7, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 1210036561 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7882.
NOTICE OF APPROVAL

DATE: February 14, 2012
TO: KURT R ZINN, D.V.M., Ph.D.
    VH G082 0019
    FAX: (205) 975-6522

FROM: [Signature]
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: CENTER Comprehensive Cancer Center Core Support Grant (Dr. Partridge), Small Animal Imaging Core
Sponsor: NIH
Animal Project Number: 120105554

As of January 3, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

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<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>B</td>
<td>90</td>
</tr>
<tr>
<td>Mice</td>
<td>C</td>
<td>75</td>
</tr>
<tr>
<td>Rats</td>
<td>B</td>
<td>40</td>
</tr>
<tr>
<td>Rats</td>
<td>C</td>
<td>10</td>
</tr>
</tbody>
</table>

Animal use must be renewed by January 2, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120105554 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7652.