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Selecting the Optimal Parameters for Sonoporation of Pancreatic Cancer in a Pre-Clinical Model

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***Abstract* — This study evaluated sonoporation in a xenograft model of pancreatic ductal adenocarcinoma (PDAC) using 4 different ultrasound contrast agents (UCAs) to select the optimal UCA for augmenting chemotherapy treatment. Athymic, nude, female mice (n = 120) were inocluated with MIA PaCa-2 cells in the right flank, and randomized into 2 control groups (vehicle or standard of care chemotherapy with paclitaxel and gemcitabine), and 8 treatment groups. These consisted of chemotherapy and one of 4 UCAs: Definity® (Lantheus Medical Imaging, N Billerica, MA, USA), Lumason® (Bracco, Milan, Italy), Optison™ (GE Healthcare, Princeton, NJ, USA) or Sonazoid™ (GE Healthcare, Oslo, Norway) scanned in a high or a low acoustic power cohort (I_{SPTA} of 200 or 60 mW/cm2, respectively). Groups of 10 animals were treated once a week for 3 weeks with chemotherapy followed by a 10 minute infusion of one of the UCAs through a tail vein. Hemoglobin and oxygenation measurements were obtained weekly (at baseline, during treatment and 1 week post treatment) in subgroups (3 mice from each group) using 3D photoacoustic imaging on a Vevo 2100 LAZR scanner (Fujifilm Visualsonics, Toronto, Canada). The remaining mice were followed for tumor volume growth and survival. Groups were compared with two-way, repeated measures ANOVAs. Tumor volumes from the 4 treatment groups in the high acoustic power cohort were smaller than those of the group receiving chemotherapy alone ($p<0.006$). In the low acoustic power cohort, only mice receiving Definity showed a significant tumor volume reduction ($p=0.003$). Hemoglobin and oxygenation values across tumors were greater in the high acoustic power cohort ($p<0.001$), while the impact of UCAs was statistically significant for oxygenation (Definity and Sonazoid; $p<0.05$) and for hemoglobin within areas of detected blood flow (Optison; $p=0.014$). Hence, chemotherapy treatment of PDAC xenografts can be augmented with high acoustic power sonoporation, and Sonazoid appears promising as a sonoporation agent as we move towards a clinical trial in PDAC patients.**

Keywords — pancreatic ductal adenocarcinoma; microbubbles; ultrasound contrast agents; sonoporation; murine xenograft model

I. Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) is the tenth most common cancer diagnosed in the USA with 55,440 new cases in 2018 along with nearly 44,330 deaths this year and a five year survival rate of just 9% [1, 2]. Moreover, PDAC is expected to become the second leading cause of cancer related death by 2030 [2]. The PDAC microenvironment is poorly vascularized and has poor drug delivery in part due to the particularly dense stroma which consists of up to 90% of the tumor mass [3]. This makes PDAC a promising target for treatment augmentation with sonoporation, which uses oscillating microbubbles to weaken endothelial cell junctions and membranes and improve therapy delivery [4, 5]. Gas-filled microbubbles (1 - 8 μm in diameter) are designed as ultrasound contrast agents (UCAs) to enhancer echo signals from blood by acting as vascular tracers [6]. At acoustic pressures typically above 200 kPa, the UCAs start to oscillate nonlinearly [6, 7], which is the regime that can be exploited for sonoporation. Previously, a Phase 1 trial demonstrated that sonoporation in 10 human patients with PDAC can increase the efficacy of standard chemotherapy, significantly extending survival from 8.9 months (in 63 historical controls) to 17.6 months ($p = 0.011$) [5, 8]. However, in that trial the UCA used was selected based on availability.

Consequently, this study evaluated the effects of sonoporation using different UCAs to select the best agent and acoustic setting for augmenting chemotherapy delivery/efficacy in a subcutaneous pancreatic cancer xenograft model.

II. Methods

A. Tumor model and treatment groups

This study was conducted at Thomas Jefferson University (TJU; Philadelphia, PA, USA) following Institutional Animal Care and Use Committee (IACUC) approval and in accordance with the and Animal Care Policies of TJU. One hundred and twenty (120) athymic, nude, female mice were inoculated with MIA PaCa-2 cells (ATCC, Manassas, VA, USA) in the right flank. The 6 to 10 weeks old mice received a subcutaneous injection of $5x10^6$ cells in 100 μl phosphate buffered saline with 20% Matrigel. When tumors reached ~250 mm^3 (after ~4 weeks), the mice were randomized into 2 control and 8 treatment groups.

One control group was treated with vehicle only, while the other group received standard of care chemotherapy; specifically, paclitaxel, which was administrated at a dose of 30 mg/kg intraperitoneal (IP) once weekly and gemcitabine, which was treated with 100 mg/kg IP once weekly.

Supported by National Institutes of Health grant R01 CA199646. Lantheus Medical Imaging supplied Definity, while GE Healthcare supplied Sonazoid and Optison.

The 8 treatment groups received chemotherapy and one of 4 UCAs: Definity® (Lantheus Medical Imaging, N Billerica, MA, USA), Lumason® (or SonoVue; Bracco, Milan, Italy), Optison™ (GE Healthcare, Princeton, NJ, USA) or Sonazoid™ (GE Healthcare, Oslo, Norway). In turn the groups defined above were further stratified by being imaged with a Logiq E9 ultrasound scanner (GE Healthcare, Waukesha, WI, USA) in a high or a low acoustic power cohort, which corresponded to I_{SPTA} values of approximately 200 or 60 mW/cm^2, respectively.

Each of the treatment groups were allocated 10 mice (i.e., 80 mice in total), while the control groups consisted of 10 to 15 mice per group. The high and low acoustic power cohorts were treated over 2 separate time periods 2 months apart (for logistical reasons) and therefore, separate control groups were assigned to each cohort (i.e., a total of 40 control animals).

B. Experimental procedures

Groups were treated once a week for 3 weeks. Paclitaxel was dosed IP 2 hours prior to UCA infusion, and gemcitabine was dosed IP 15 minutes prior to infusion to allow for maximal proportion of blood in the plasma at the time of the UCA infusion. The dosages for all 4 UCAs were adjusted to approximately the same microbubble concentration (1.2 - 3.0 x 10^8 bubbles/mL) based on the maximum human dose allowed for Definity. The UCAs were diluted for a total volume of 200 µl, which was and infused through a tail vein over 10 minutes using an automatic infusion pump (model VS-20019 Vevo Infusion Pump; Fujifilm Visualsonics, Toronto, Canada) following chemotherapy administration as described above.

Imaging for sonoporation was performed on a Logiq E9 ultrasound scanner with a C6 curvi-linear transducer (bandwidth 1.0 – 6.0 MHz) modified to allow access to pulse parameters and acoustic output power values (I_{SPTA} values) within the color Doppler pathway. The highest line density was used with 12 pulses (20 µs pulse length) transmitted at a frequency of 2.1 MHz and the probe was positioned in a clamp to eliminate any operator motion during the infusion (Fig. 1).

Subharmonic imaging (SHI) was implemented on the system by transmitting 4 cycle pulses at 2.5 MHz (f_0) and receiving at 1.25 MHz ($f_0/2$) [9]. Above a certain acoustic pressure threshold (typically > 200 kPa) and close to twice their resonance frequency the UCA microbubbles are able to generate a marked subharmonic frequency component (at half the transmit frequency) [6, 9]. The subharmonic generation is specific to the UCA and does not occur in tissue. By selectively receiving at the subharmonic frequency it is possible to isolate vascular signals from tissue signals and we have previously demonstrated the utility of SHI for imaging of PDAC [9]. SHI was performed at the end of the infusion to verify vascular access.

C. Photoacoustic Imaging

3D photoacoustic imaging was performed on a Vevo 2100 LAZR scanner with an LZ250 probe (Fujifilm Visualsonics, Toronto, Canada) to determine hemoglobin signal (HbT), oxygenation levels in detected blood (SO2 Avg), and oxygenation levels over the entire tumor volume (SO2 Tot) [10]. These measurements were obtained weekly for four weeks (at baseline, during treatment and 1 week post treatment) in subgroups consisting of 3 random mice from each group.

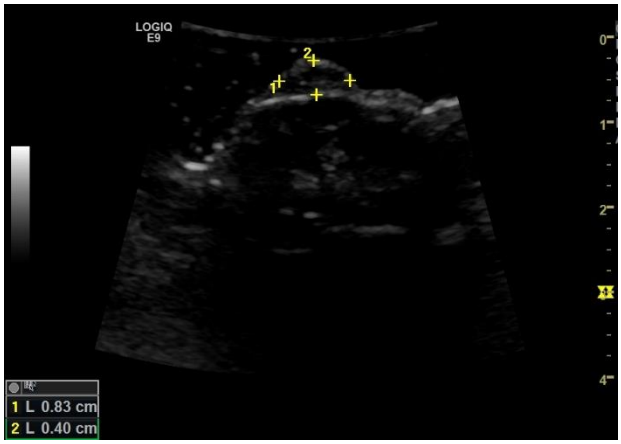

Fig. 1. An example of a subcutaneous murine PDAC tumor being measured for size (8 x 4 mm in this plane). The flank of the mouse can be seen below the tumor.

D. Outcomes

Three representative mice per group (i.e., the mice from the photoacoustic subgroups) were sacrificed after four weeks and excluded from overall survival. These representative mice prior to sacrifice were infused with tomato lectin 30 minutes prior to sacrifice to allow perfused vascular structures to be visualized on microscopy [11].

Organs (kidneys, liver) and tumor were flash frozen in liquid nitrogen immediately after collection, and then sectioned using a cryostat. Frozen sections were then stained using CD31 (Dianova, Hamburg, Germany). These slides were imaged utilizing a DM4 B microscope (Leica Microsystems, Wetzlar, Germany) and perfusion was assessed.

The remaining mice were followed for tumor volume growth and survival, until they reached the euthanasia criteria mandated by the IACUC. In this murine xenograft model of PDAC this criteria was a tumor volume exceeding 1500 mm^3.

E. Statistical Analysis

Statistical analyses were performed using Stata 15.1 (StataCorp, College Station, TX, USA). Comparisons between groups were performed using two-way, repeated measures ANOVAs with a *p*-value of 0.05 or lower being considered statistically significant.

III. Results

As expected, all tumor volumes in the 8 treatment groups and in the chemotherapy alone group were significantly smaller than those from the vehicle only group ($p = 0.015$). This appeared to correlate with an increase in drug delivery/response, since when comparing tumor volumes from the treatment groups in the high acoustic power cohort to the group receiving chemotherapy alone, all 4 UCA treated groups had significantly smaller tumors ($p < 0.006$) with Optison having the greatest reduction ($p = 0.001$; Fig. 2). In the low acoustic power cohort, only mice receiving Definity showed a significant tumor volume reduction ($p = 0.003$; Fig. 3), while all other comparison were not significant ($p > 0.05$). There were no differences in overall survival for either cohort ($p > 0.4$).

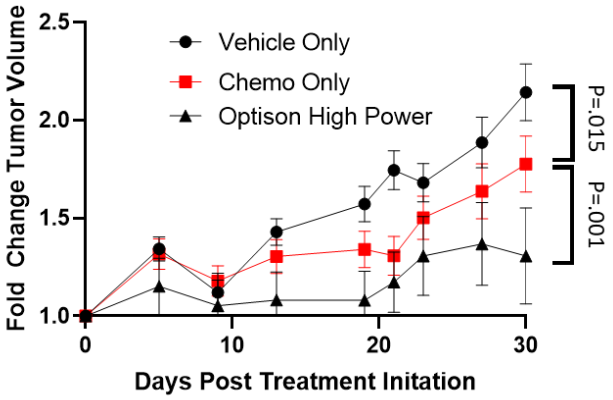

Fig. 2. Comparison of vehicle vs chemotherapy alone (i.e., the control groups) vs. mice treated with chemotherapy augmented by sonoporation with Optison as the UCA in the high acoustic power cohort.

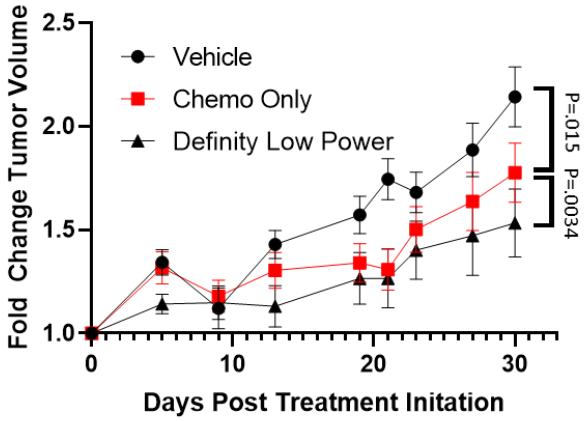

Fig. 3. Comparison of vehicle vs chemotherapy alone (i.e., the control groups) vs. mice treated with chemotherapy augmented by sonoporation with Definity as the UCA in the low acoustic power cohort.

An example of the photoacoustic imaging performed is shown in Figure 4. Total hemoglobin and oxygenation values across tumors were significantly greater in the high than in the low acoustic power cohort ($p < 0.001$). The UCA treatment groups only differed for oxygenation values across the entire tumor volume where Definity ($p = 0.048$) and Sonazoid ($p = 0.003$) showed statistically significant increases and for hemoglobin within areas of detected blood flow in the Optison group ($p=0.014$).

When the PDAC tumor specimens were evaluated microscopically for perfusion proximal to blood supply (as measured by tomato lectin proximal to CD31) there was a qualitative increase in perfusion with three out of the four treatment groups as demonstrated in Figure 5. The only UCA that did not show this was Lumason (cf., Fig. 5). This result was consistent irrespective of the acoustic power cohort.

A summary of the results for the 8 treatment groups is presented in Table 1.

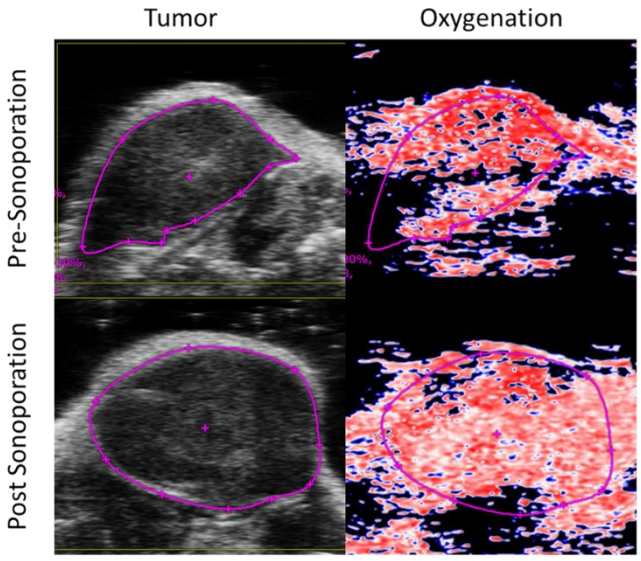

Fig. 4. Comparison of tumor oxygenation at baseline prior to sonoporation with Sonazoid in the high acoustic power cohort and after sonoporation treatment has been completed. This image depicts an overall increase in oxygenation froom 49% to 53% within the imaging plane (and from 41% to 54% within the entire tumor volume of this animal).

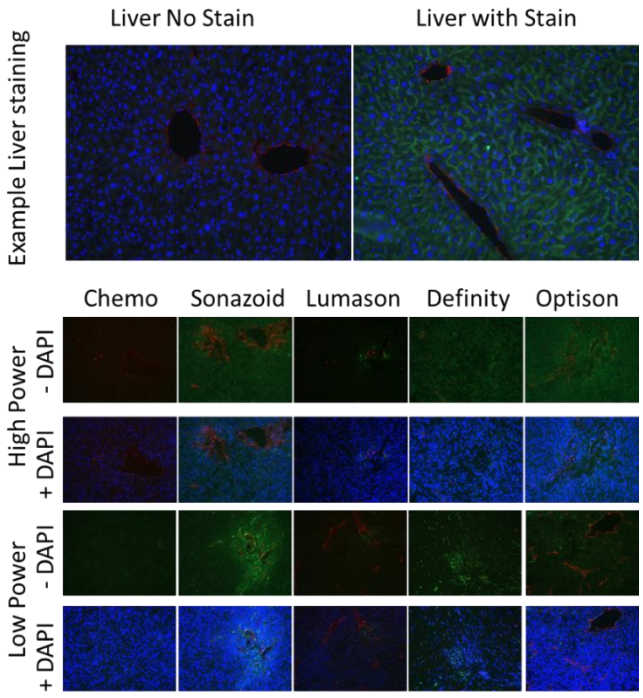

Fig. 5. Representative images of mouse livers that have been stained with CD31 (red) showing vasculature, DAPI showing nuclei (blue), and +/- tomato lectin demonstrating perfusion (green). Representative mice from the *in vivo* experiments were perfused with tomato lectin and then sacrificed. Tumors were flash frozen and then stained for CD31. Images are representative from each treatment group demonstrating varying degrees of perfusion.

TABLE 1. SUMMARY STATISTICS OF THE EFFECTS VS CHEMO FOR EACH UCA ACROSS HIGH AND LOW ACOUSTIC POWER TREATMENTS.

Treatment type	Acoustic power	Significant tumor volume reduction vs chemo	Increase in tomato lectin staining	Increase in oxygenation levels
Definity	low	✓	✓	×
Lumason	low	×	×	×
Optison	low	×	✓	×
Sonazoid	low	×	✓	×
Definity	high	✓	✓	✓
Lumason	high	✓	×	×
Optison	high	✓	✓	×
Sonazoid	high	✓	✓	✓

IV. DISCUSSION

This pre-clinical study evaluated the effects of sonoporation using four different UCAs to select the best agent and acoustic setting for augmenting chemotherapy delivery/efficacy in a subcutaneous PDAC xenograft model. Results supported the notion that chemotherapy treatment of pancreatic xenografts can be augmented with high acoustic power sonoporation, and that optimal acoustic parameters are UCA-dependent.

When focusing on the high acoustic power cohort, both Definity and Sonazoid showed efficacy across several parameters (cf., Table 1). However, for the most quantitative evaluations (i.e., based on photoacoustic imaging) Sonazoid showed greater effect and this UCA was therefore selected for our future human trial.

Nonetheless, it should be mentioned that the lack of statistically significant improvements in overall survival when augmenting chemotherapy with sonoporation (irrespective of the UCA tested) was a disappointment. Moreover, this is at odds with prior work using Lumason (or SonoVue) for sonoporation in a xenograft model of PDAC [12]. Resolving this issue will require more experiments.

V. CONCLUSION

In this study, preliminary results suggest that chemotherapy treatment of pancreatic xenografts can be augmented with several different agents with an increased efficacy (i.e., tumor volume reduction) observed with high vs low acoustic power. Across UCAs Sonazoid with high acoustic power sonoporation demonstrated the most consistent increase in parameters related to increased perfusion, which likely contributed to the increase in efficacy demonstrated in the Sonazoid treatment group. Hence, Sonazoid with a higher acoustic power may be most effective as a sonoporation agent in further work as we move towards a clinical trial in PDAC patients.

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