**Engineering Immunogenic Focused Ultrasound (FUS) Paradigms for Breast Cancer through Incorporation of Adenosine Receptor Blockade**

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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# Engineering Immunogenic Focused Ultrasound (FUS) Paradigms for Breast Cancer through Incorporation of Adenosine Receptor Blockade

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## Abstract

Focused ultrasound (FUS) is a non-invasive, non-ionizing method that precisely targets tumors while preserving surrounding healthy tissues. Cell disruption due to FUS induces the release of endogenous danger signals, such as ATP and leads to immunosuppressive effect. This study aims to combine this innovative technology with adenosine receptor blockers to unleash the immunogenicity of FUS. In order to achieve that, effects of two different FUS paradigms, thermal (T-FUS) and mechanical (BH) ablation, on tumor outgrowth were examined. Additionally, ATP level in tumor microenvironment were measured following the FUS treatment. To determine the most effective adenosine receptor blocker among four options, the impact of the blockers alone on primary and distal lesions were examined. Results revealed that FUS treated groups showed lower tumor outgrowth compared to control. T-FUS group showed more efficiency in reducing tumor outgrowth compared to BH. Measuring ATP levels in tumor microenvironment confirmed elevated ATP levels following the FUS treatment. The monotherapy study demonstrated ADO-5030 (A2B) blocker showed lower tumor size and less lung metastasis compared to control group. Among the blockers, it also showed the highest effect on reducing tumor outgrowth compared to others. In reducing lung metastasis, another A2B blocker ADO-5047 showed the most effective result compared to both control and drug groups. It is concluded that both FUS and adenosine receptor blockers showed effective results in reducing tumor outgrowth and distal lesions. Further experiments will investigate the effects of combined therapy involving FUS and A2B adenosine blockers.

Keywords: Focused ultrasound, Breast Cancer, Thermally Ablative, Mechanically Ablative, Adenosine Receptor Blocker

## Introduction

Every year approximately 2.3 million women are diagnosed with breast cancer worldwide and 240,000 in the United States [1]. After skin cancer, it is the most common cancer, covering %30 of new cases, in female patients. Average diagnosed age is 62 in the U.S. which has slight changes in racial and ethnic groups. For instance, mean age for breast cancer diagnosis among Black women is 60 which is lower than White patients with a mean of 64. Black breast cancer patients have the highest mortality rate. Part of the reason of this is that Black women are in a higher risk group for Triple Negative Breast cancer (TNBC) [2].

One of the major clinical challenges of breast cancer is its tendency to distant metastasis. TNBC is considered to be the most aggressive subtype with the highest risks of metastasis, distant metastasis in about 46% of cases, and recurrence. Following metastasis, the median survival time is a mere 13.3 months, and the recurrence rate post-surgery is substantial at 25%. Metastasis often affects the brain and visceral organs, typically occurring in the third year after diagnosis. TNBC constitutes 15-20% of all breast cancer cases [3]. The mean annual incidence is 13.7 per 100,000 women, with notably elevated and variable rates in African American women (mean=20.5, range: 0.0–155.1) [4]. TNBC is associated with shorter survival compared to other subtypes, and 40% of patients face mortality within the initial 5 years post-diagnosis. Non-TNBC patients experience a longer time to relapse (35–67 months on average) compared to TNBC patients, whose average time to relapse is shorter (19–40 months). The mortality rate for TNBC patients within 3 months after recurrence is notably high at 75% [5]. Currently, there are no FDA-approved targeted therapies for TNBC patients. This emphasizes the pressing requirement for innovative combination treatments that can effectively target both primary and distant lesions

while also prioritizing patients' comfort and maintaining their quality of life.

Focused ultrasound (FUS) is the non-ionizing, non-invasive concentration of sound waves into a localized ellipsoid volume [6]. FUS can be precisely directed with millimeter accuracy using MRI or ultrasound assistance. This precision enables the precise application of heat to damage and eliminate tumor tissue while protecting the healthy surrounding tissues in between and on the periphery [6]. Previous study has shown that FUS-treated cancer cells release endogenous danger signals, such as ATP, due to mechanical and/or thermal disruption [7]. When cells undergo apoptosis, they release higher amounts of ATP, leading to increased levels of extracellular adenosine. Elevated levels of extracellular adenosine have been found to reduce anti-tumor activity and enhance the mechanism of immunosuppression within the microenvironment of solid tumors [8].

Adenosine, a purine nucleoside, regulates numerous physiological and pathological signaling functions through the activation of four G protein-coupled receptors (GPCRs): A1, A2A, A2B, and A3. Notably, two of these receptors, A2AR and A2BR, exhibit immunosuppressive effects. Elevated adenosine levels in the tumor microenvironment pose a substantial obstacle to anti-tumor immunity, making blockers of these receptors therapeutic targets [8]. A2BR blockers exert their anti-tumor effects by inhibiting receptors on cancer cells that are activated in response to hypoxia. Additionally, A2BR on tumor-associated antigen-presenting cells (APCs) hampers anti-tumor immunity by suppressing antigen cross-presentation from dendritic cells (DCs) to tumor-infiltrating lymphocytes (TILs), including CD8+ T cells [9]. TILs stand as key players in promoting favorable responses to chemotherapy and elevating overall clinical outcomes. Large adjuvant studies involving patients with HER2-positive breast cancer and TNBC consistently show that heightened levels of TILs in primary biopsies are associated with prolonged overall survival (OS) and a decreased risk of recurrence, regardless of the specific therapy chosen. Similar positive correlations are evident in patient groups undergoing neoadjuvant therapy. A strategy in cancer immunotherapy involves inhibiting immunosuppressive signaling pathways like PD-1, CTLA-4, adenosine A2A receptors (A2AR), and adenosine A2B receptors (A2BR). This aims to counteract immune evasion and boost the anti-tumor activity in the tumor microenvironment. While blocking these targets can be successful in curbing tumor growth, the outcomes can greatly differ across different types of tumors.

A study conducted by Zhenlin Hu, PhD, assessed the impact of high-intensity focused ultrasound (HIFU) on the liberation of endogenous danger signals from tumor cells and the subsequent activation of APCs in an in vitro setting. Dr. Hu and his team treated MC-38 tumor cells by mechanical and thermal HIFU. They stated that the findings that HIFU treatment induces the release of endogenous danger signals (ATP and hsp60). Exposure of DCs and macrophages to the supernatants of HIFU-treated tumor cells results in heightened expression of co-stimulatory molecules (CD80 and CD86). Additionally, this leads to increased secretion of IL-12 by DCs and elevated secretion of TNF-a by macrophages The results indicate that the signals released from mechanically damaged tumor cells were significantly more effective in activating APCs compared to those from thermally damaged tumor cells. This suggests a potential approach to boost anti-tumor immunity in the context of HIFU therapy [10]

In another study, Joel Linden, PhD, and collaborators discovered that immune suppression in 4T1 and various other tumors is, in part, a result of extracellular adenosine (ecto-adenosine) produced from ATP abundantly released by necrotic or apoptotic cells in the inflamed tumor microenvironment. Ectoenzymes, primarily CD39 and CD73, swiftly convert ATP to adenosine. The elevated release of ATP is expected to be particularly during FUS-induced tumor injury. In human TNBC, heightened tumor expression of CD73, responsible for converting extracellular AMP to adenosine, is associated with a poor prognosis [7]. While previous studies on adenosine receptor antagonists in cancer mainly targeted A2AR receptors abundant on T cells, prolonged A2AR blockade can induce T cell anergy and have highly variable effects on tumor growth. Although A2BR expression is low on T cells, it is abundant on macrophages and DCs [11]. Ablating A2BR in mice with tumors leads to increased infiltration of macrophages and CD103+ dendritic cells, facilitating cross-priming of adoptively transferred tumor antigen–specific CD8+ T cells [11]. Consequently, A2BR signaling plays a crucial role in shifting myeloid APCs from tumor-protecting Myeloid-Derived Suppressor Cells (MDSCs) to tumor-killing phenotypes, such as M1 macrophages.

Currently HIFU therapy is predominantly centered on thermal ablation of tumor tissue. Through comparing mechanical and thermal ablation, this project seeks to offer an alternative and more efficient FUS treatment for further cancer research. This project diverges from prior studies in three key aspects: 1) the use of novel cell line (4T1-LUC and E0771), 2) developing an in-vivo model, and 3) the

development of a combined therapy integrating FUS and adenosine blockers.

In this project, we postulate that adenosine receptor blockers will increase the immunogenic potential of FUS compared to FUS monotherapy by inhibiting the immunosuppressive effect of adenosine receptors. To achieve this, we plan to create a customized in vivo system to address murine breast cancer through focused ultrasound thermal and mechanical ablation. Subsequently, we will systematically adjust particular parameters (such as ablation fraction, pulse length, and sonication duration) associated with the extent or intensity of the ablation. Through this, our goal is to identify the optimal FUS parameters for the release of ATP. In the second part of the project, we assess adenosine receptor blockers alone as therapeutic solution to reduce tumor outgrowth and distal lung metastasis.

## Results

### *In-vitro ATP assessment*

First we conducted an experiment to assess ATP release from 4T1 cells following the FUS treatment. In order to achieve that we designed in-vitro study that includes T-FUS (n=3), BH (n=3), Sham (n=3) and control (n=3) groups. After FUS treatment supernatants were collected in various amounts to prevent exposure and transferred to 96-well white plate. Samples were mixed with the required amount CellTiter-Glo. Results gathered from luminescence imaging showed higher ATP concentration in T-FUS group compared to Sham and Control groups among 1:5 groups (Figure 1). Among the 3:10 and 4:10 ratio groups, overexposures were observed in T-FUS wells therefore for final analysis only 1:5 groups were used. BH group showed lower ATP release compared to the Sham (Figure S1) therefore it is excluded from final result. The possible explanation for this unexpected outcome is missing targeting for BH group. Refer Figure S2 for experimental design.

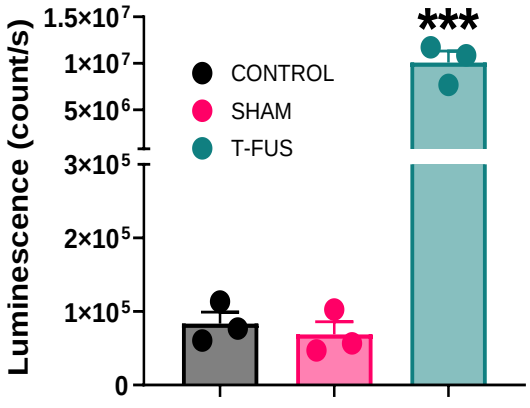


**Figure 1. In-vitro ATP assessment.** T-FUS group showed significantly higher ATP level compared to Sham group.

### *In-vivo ATP assessment*

To assess extracellular ATP in tumor microenvironment we designed an in-vivo study where we compared thermal and mechanical ablation of FUS to non-treatment (Sham) group. FUS groups involved 4 mice per group whereas Sham had 8 to match each experimental group during the imaging. At the beginning of the treatment 3 BH mice were lost due to low tolerance to sedative. After given obstacles, study conducted only on one mouse per FUS group and two Sham mice. To have bioluminescence images from tumor area, D-luciferin and Firefly Luciferase were administered a minute before and at the end of the FUS treatment, respectively. Due to transportation between operation and imaging room, bioluminescence reading started 10 minutes after the FUS treatment. Results revealed that BH group showed higher ATP release in tumor microenvironment compared to Sham group (Figure 2). Bioluminescence imaging from T-FUS mouse showed more signals from the tail compared to tumor area due to accumulation of administered reagents. Therefore, max radiance graph showed inconsistent data (Figure 2). Refer to Figure S3 for experimental design.

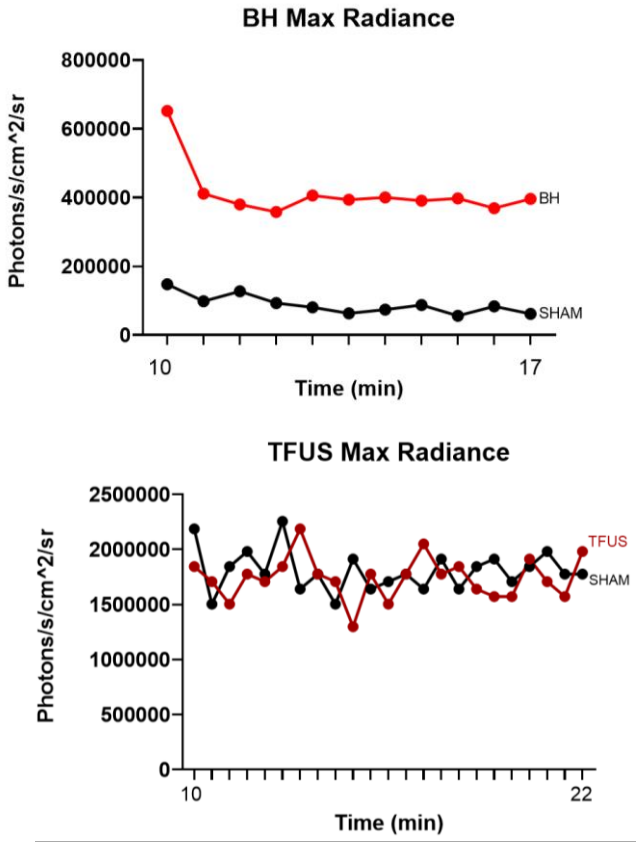


**Figure 2. In-vivo ATP assessment.** At top max radiance graph for BH, mechanically ablative FUS showed increased ATP level compared to Sham. At bottom graph shows max radiance for T-FUS group.

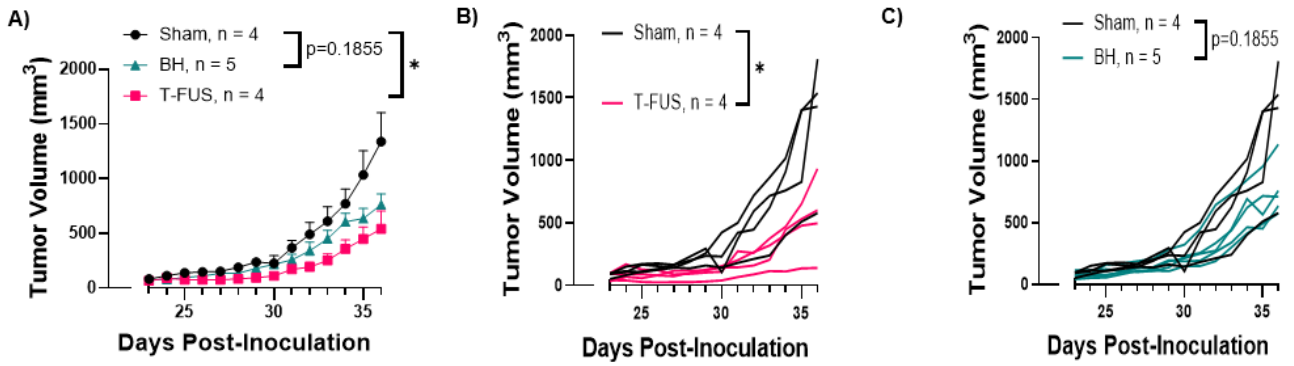


**Figure 3. E0771 outgrowth study.** On the right (A), graph shows average tumor volume for each experimental group. FUS treated groups showed significantly (p=0.0274) reduced tumor outgrowth compared to Sham. Individual outgrowth data showed that T-FUS (B) treatment showed higher effect compared to BH (C) treatment. compared to Sham.

***E0771 outgrowth study***

In order to assess the FUS effect on tumor outgrowth, mice were randomized into three experimental groups, two of which underwent thermally (18 watts, seconds duration) and mechanically (5 millisecond burst, 2 Hz, 10 seconds duration) ablative FUS treated with a custom-built FUS system. Starting from the treatment day tumor sizes were measured daily by caliper. Results from average tumor volume demonstrated statistically significant (p=0.0274) between Sham and T-FUS group (Figure 3A). Sham mice showed higher tumor outgrowth compared to FUS groups. Comparing FUS mice individual outgrowth (Figure 3B&C) revealed that thermally ablative FUS has a higher effect on reducing tumor outgrowth rate compared to BH.

***Adenosine Receptor Blockers Monotherapy study***

In-vivo monotherapy experiment with five groups was designed to assess the effect of adenosine receptor blockers on tumor outgrowth and spread of lung metastasis. Mice inoculated with luciferase-expressing 4T1 cells received adenosine receptor blockers and vehicle intratumorally three times per week starting from day 12 post-inoculation. Starting from 6$^{th}$ dose, injection volume reduced 20 uL from 100 uL. Tumors measured daily by caliper until day 36. On day 36, tumors and lungs were harvested and imaged by using LAGO imaging system. Results gathered from caliper measurements showed that A2B:ADO-5030 group had lower tumor outgrowth compared to other drugs and sham groups. However, when bioluminescence tumor images analyzed A2B:ADO-5030 and Theophylline groups showed closer total emission data where they are lower than other groups. A2A: CPI-444 and A2B: ADO-5047 showed higher tumor volume compared to Sham. Despite average tumor results, the Sham group showed higher lung metastasis expression compared to adenosine receptor blocker groups. Among drug groups there were inverse proportions between tumor volumes and lung metastasis expressions where A2B:ADO-5030 and A2B: ADO-5047 showed highest and lowest metastasis expressions, respectively (Figure 4).

**Discussion**

In this study we reported that FUS is an effective technology in reducing tumor outgrowth in breast cancer murine model. Our results revealed that FUS treated mice showed reduced tumor outgrowth compared to non-treatment group. It is also observed that thermally ablative FUS showed greater effect on tumor outgrowth compared to mechanically ablative FUS treatment.

Our findings from the adenosine receptor antagonist monotherapy study demonstrated the therapeutic effect of adenosine receptor antagonist on tumor outgrowth and the spread of metastasis. Results revealed that A2B receptor blocker ADO-5030 and non-selective blocker Theophylline showed better results in order to reduce tumor outgrowth compared to A2A receptor blockers and Sham group. It should be noted that this study ended on day 29 post inoculation after 9$^{th}$ dosing due to reaching humane end point. It is expected that ADO-5030 would show more effective results in a longer treatment timeline. In order to reduce lung metastasis another A2B receptor ADO-5047 showed the least lung metastases expression from bioluminescence reading. All adenosine receptor blockers showed reduced lung metastasis compared to sham group.

While our studies have important strengths, there are some limitations with respect to assessing ATP release right after FUS treatment. In in-vitro ATP assessment experiment we observed an increased ATP level in T-FUS group compared to Sham and control. We were not able to gather reliable data from BH group since it showed a similar trend to control group. This unexpected result might be due to

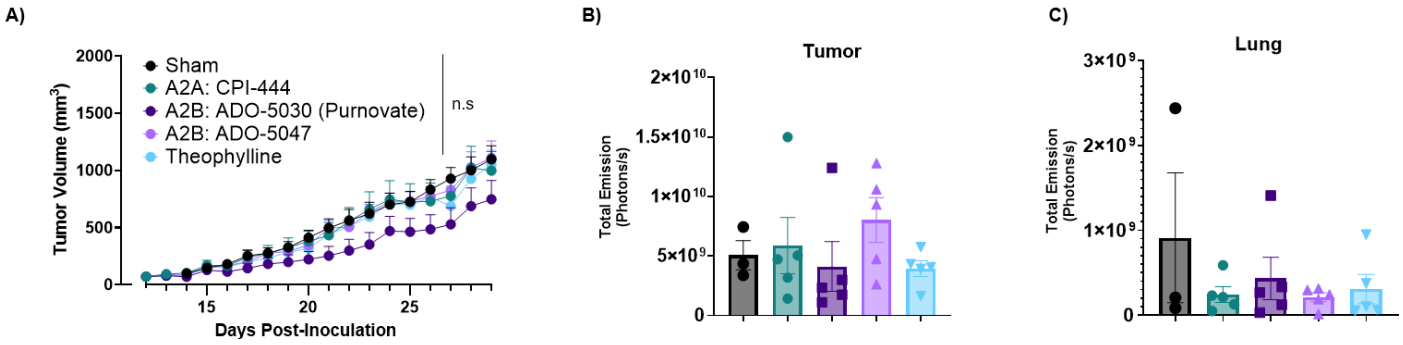


**Figure 4. Adenosine receptor blockers monotherapy study.** On the right (A), graph shows average tumor volume for each experimental group. Figure B and C shows total emission data gathered from tumor and lungs bioluminescence images, respectively.

missing targeting. We were working with 1.5 mL PCR tubes and due to the small size of the sample and used FUS system we were not able to confirm targeting. The in-vivo study had different limitations in order to assess ATP release in tumor microenvironment. We confirmed the elevated ATP level in BH group. However, due to transportation between imaging system and operation rooms, we may not be able to observe peak expression during bioluminescence reading.

In further studies we will explore combined therapeutic solutions by using FUS and adenosine receptor blockers, building upon the insights gained in this study.

Findings from our study also suggest further exploration on additional pathways to prevent immunosuppressive effect of elevated adenosine level in tumor microenvironment.

## Materials and Methods

### *Cell Culture*

*4T1-LUC.* Luciferase-expressing 4T1-LUC cells were cultured in complete growth medium containing Roswell Park Memorial Institute (RPMI) supplemented with 10% Fetal Bovine Serum (FBS) during cell passing in T175 flasks. Cells were maintained at 37°C and 5% CO2.

*E0771.* E0771 medullary breast adenocarcinoma cells were cultured in complete growth medium containing high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS during cell passing in T175 flasks. Cells were maintained at 37°C and 5% CO2.

### *In-vitro ATP assessment*

Before FUS treatment started, 4T1 cells were washed three times with phosphate-buffered saline (PBS) and the medium was replaced with fresh RPMI without FBS (20 uL per $1x10^6$ cells). $3x10^6$ cells were transferred to 1.5 mL PCR tube (3 tubes per group). FUS groups received the following treatments; T-FUS group 18 watts for 15 seconds, BH group 5 millisecond burst, 2 Hz for 10 seconds. A custom-built FUS system with four therapeutic 2.5 MHz transducers was used. The sham group waited in hot water (37°C) for 15 seconds whereas the control group only waited in ice. After FUS treatment cells were centrifuged at 279 g for 5 minutes. From each tube 10,15 and 20 uL supernatant were collected and transferred into 96-well white plate. To each well, required amount RPMI, to reach 50 uL in total volume, and 50 uL CellTiter-Glo® reagent were added and mixed for 2 minutes. The plate was covered and incubated in room temperature for 10 minutes before starting luminescence reading (TECAN Spark Multimode Microplate Reader).

### *In-vivo ATP assessment*

The study included 3 groups: T-FUS (n=4), BH (n=4), and sham (n=8) to assess extracellular ATP in the tumor microenvironment. C57/Black 6 mice were injected orthotopically with 1E6 E0771 cells on the mammary fat pad. Mice were treated with PG4 FUS system with following parameters; 1mm spacing between sonication points on five planes that 1mm apart from each other. Mice received the same FUS treatment as described above, according to their assigned groups. For bioluminescence imaging each mouse intravenously received 200 uL D-luciferin 1 minute before FUS treatment starts and 100 uL Firefly Luciferase + PBS, 1:99 ratio, right after treatment ends. To minimize the residue in catheters 200 uL saline were also injected after each administration. For injections 18 inches catheters were used. Mice were imaged with the LAGO bioluminescence imaging system.

### *E0771 outgrowth study*

The study was designed with 3 groups: T-FUS (n=4), BH (n=5) and sham (n=4). C57/Black 6 mice were injected orthotopically with one million (1E6) E0771 cells on the

mammary fat pad. Mice were treated with FUS on day 23 (post-inoculation) when they reached approximately 80 $mm^3$ volume. Mice were treated with PG4 FUS system with following parameters; 1mm spacing between sonication points on five planes that 1mm apart from each other. Mice received the same FUS treatment as described above, according to their assigned groups. Until day 36, when mice reached humane end point, tumors' size were measured by caliper daily and volume were calculated by equation below.

$$0.5 \times length \times width^2$$

### *Adenosine Receptor Blockers Monotherapy study*

The experiment included 5 groups: CPI-444 (A2A) (n=5), Theophylline (non-selective) (n=5), ADO-5047 (A2B) (n=5), ADO-5030 (A2B) (n=5) and Sham (n=3). C57/Black 6 mice were inoculated ectopically on the right flank with 400,000 luciferase-expressing 4T1 cells . Dosing started on day 12 when tumors volume reached approximately 60-80 $mm^3$. Drugs were injected intratumorally (IT) 3 times per week. For the first 5 dosing, mice received 100 µL per injection. In 6th-9th doses, mice received 20 µL per injection. In each dose, the given amount and possible losses were noted. When mice reached a humane endpoint, tumors and lungs were harvested and imaged by using LAGO bioluminescence imaging system.

### *Statical Analysis*

All statistical analysis, 2-way ANOVA, t-test and Mann-Whitney test, were performed using GraphPad Prism software.

### *Ethical Approval*

The experimental procedures conducted in this study were in accordance with the University of Virginia Animal Care and Use Committee guidelines for use of laboratory animals.

## End Matter

### *Author Contributions and Notes*

The authors declare no conflict of interest.

### *Acknowledgments*

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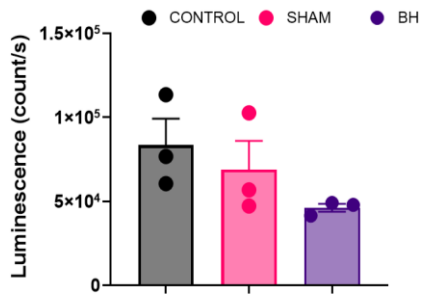
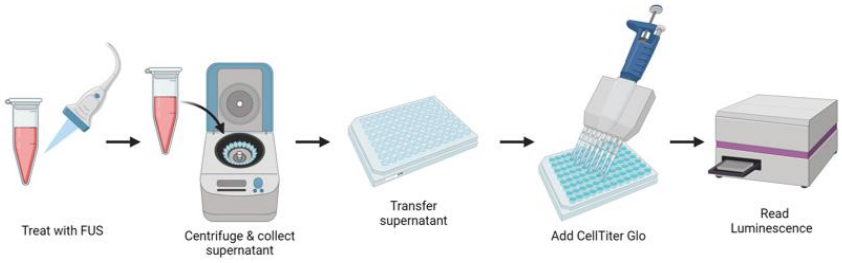
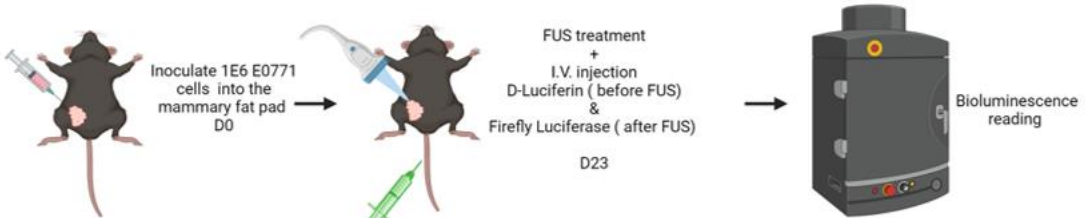



**Supplementary Figure 1. In-vitro ATP assessment.** Mechanically ablative treatment luminescence reading.




**Supplementary Figure 2. In-vitro ATP assessment.** Experimental design.




**Supplementary Figure 3. In-vivo ATP assessment.** Experimental design.