Sonoporation-Enhanced Delivery of STING Agonist Induced Robust Immune Modulation and Tumor Regression

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Sonoporation, the temporal cell membrane openings that can arise after ultrasound exposure, has been explored across many preclinical applications but has lacked translational momentum. This can be attributed to a lack of quantitative characterization of the sonoporation process. Additionally, the impact of sonoporation on modulating the tumor microenvironment is not well understood. Using a cyclic dinucleotide-based STING (stimulator of interferon genes) agonist, MSA-1, that is analogous to MK-1454, and approved microbubbles and diagnostic ultrasound, the authors report a 5 min sonoporation procedure enhanced the tumor local concentration of systemically administered MSA-1 by 6.58-fold 15 min post sonoporation. The improved pharmacokinetic profile translates into greater STING-mediated cytokine production, including TNF-α, IFN-α, and IFN-β. Furthermore, through immunophenotyping, the authors report sonoporation could sustain STING-mediated immune activation while reversing its impact on immune suppression. In both unilaterally and bilaterally inoculated syngeneic tumor models, the authors demonstrate that the sonoporation treated group significantly outperformed other controls at equivalent dosing conditions. These findings may further the understanding of the sonoporation process and its impact on immune modulation, which will accelerate the translational momentum of sonoporation.

1. Introduction

Immune regulatory checkpoint inhibition with monoclonal antibodies against targets, such as cytotoxic T lymphocyte antigen-4 (CTLA-4), and programmed cell death protein 1 (PD-1) has demonstrated unprecedented efficacy in treating various human cancers, including melanoma, renal cell carcinoma (RCC), and nonsmall cell lung cancer (NSCLC).[13] However, in most cases, the response rate remains relatively poor.[4–6] Additionally, severe, autoimmune-like adverse effects limit the use of this new class of cancer treatments.[7] Therefore, there is still a pressing need to extend cancer immunotherapy further to benefit broader patient populations. Various efforts to study the “cancer immunogram” have highlighted complex biology that necessitates a multipronged approach to identify additional immune pathways that may be modulated to tackle cancer.[8] The cyclic GMP-AMP synthase (cGAS)–STING pathway has been recognized for its involvement in the innate response to tumor
immunity resulting from self, double-stranded (ds) DNA produced by dying tumor cells. Upon detection of tumor-derived dsDNA, cGAS generates cyclic dinucleotides (CDNs) that bind STING and activate multifaceted tank-binding kinase 1 (TBK1)/interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to the release of type I interferon (IFN) and proinflammatory cytokines, ultimately promoting T cell priming and recruitment. 

STING agonists are being investigated in clinical trials (NCT04220866, NCT02675439, and NCT03010176), but most are based on CDNs, which exhibit poor metabolic stability and permeability due to their negative charges and small molecular weight, and must be delivered intratumorally (IT) for as long as 2 years to be effective. 

Aside from poor patient adherence, multiple IT injections severely limit STING agonist-based CDNs to a few readily accessible tumor types and can introduce the risk of disrupting the tumor microenvironment (TME) and peritumoral vascular network, potentially inducing cancer cell extravasation and metastases. 

Therefore, there is an urgent need for a delivery technology that could safely and noninvasively enable the systemic delivery of CDNs to deep-seated and difficult-to-permeate tumors.

Sonoporation has been used widely in vitro to deliver many agents across cellular barriers in cell cultures. This aspect of “direct cell sonoporation,” however, does not recapitulate the action of stable and inert cavitation effects through an in vivo endothelial blood barrier which is the key translational challenge for sonoporation. 

Fortunately, there exists an extensive body of preclinical data on sonoporation, which is a method of increasing the permeability of microvasculature by application of intraluminal microbubbles (MB) and concomitant ultrasound (US) exposure. 

MBs are gas-filled microspheres stabilized by a lipid, protein, or polymer shell. Their gas-filled, and hence compressible core, are responsive to ultrasound pressure waves. This process of alternate growing and shrinking is called cavitation. 

Depending on the ultrasound intensities, cavitation can be further categorized as stable cavitation and inertial cavitation. Studies have shown that sonoporation-mediated cavitation can temporarily and safely permeabilize insonated areas to allow systemic drugs to enter targeted tissues in higher concentrations than their normal pharmacokinetic/pharmacodynamic (PK/PD) characteristics dictate. 

Under acoustic intensities approved by the United States Food and Drug Administration (FDA) for diagnostic imaging, these studies have shown safety as well as meaningfully improved efficacy that would be expected from the improvement of tissue penetration. In addition to preclinical data, there have also been positive clinical studies where sonoporation has augmented gemicitabine in pancreatic cancer, blood–brain barrier opening for patients 

To finally, in a bilateral syngeneic mouse model, we demonstrated that sonoporation treatment resulted in complete regression of both primary and distant tumors in 44% of enrolled subjects, which was highly superior to the IT dosed group. These results show that by using sonoporation, it is feasible to achieve significantly higher MSA-1 delivery to the treated tumor without any toxicity. The delivery strategy of MSA-1 in this study can be further combined with immune regulatory checkpoint blockades to fully realize the immunomodulatory effect of sonoporation. 

In the future, we aim to align and position this approach for clinical trials via an arm in an active treatment cohort or leveraging FDA’s guidance document on the expanded access to investigational drugs for treatment to tackle some of the huge unmet needs in circumstances where lack of treatment efficacy can be attributed to lack of tumor exposure, ultimately helping patients in need.

2. Results

2.1. Dose Escalation and PK/PD Study of MSA-1 in Syngeneic Mouse Model MC38 After IV Administration

MSA-1 is a phosphorothioate CDN analog to cGAMP that is generated to improve STING agonist activity (Figure S1, Supporting Information). Since MSA-1 was previously positioned as an IT dosing regimen, we first conducted a dose-escalation study of intravenously (IV) administered MSA-1 and assessed its dose-dependent antitumor efficacy and PK/PD characteristics on the MC38 syngeneic mouse model. We then combined a subefficacious dose of MSA-1 with FDA-approved intraluminal MB and 5 min concomitant US exposure from a commercially available diagnostic ultrasound machine (Scheme 1). Surprisingly, this 5 min sonoporation substantially elevated tumor local concentration of MSA-1 and resulted in the enhanced production of type I IFNs and proinflammatory cytokines TNF-α and IL-6. The percent complete regression (CR), tumor-specific growth rate, as well as percent survival were all significantly improved in the sonoporation-treated group. We then hypothesized that the improved efficacy in the sonoporation-treated group could be attributed to two distinct mechanisms: enhanced PK, and improved immunomodulation. The immunophenotyping experiment showed that sonoporation treatment sustained STING-mediated immune activation while reversing some of the STING-mediated immune suppression.

Finally, in a bilateral syngeneic mouse model, we demonstrated that sonoporation treatment resulted in complete regression of both primary and distant tumors in 44% of enrolled subjects, which was highly superior to the IT dosed group. These results show that by using sonoporation, it is feasible to achieve significantly higher MSA-1 delivery to the treated tumor without any toxicity. The delivery strategy of MSA-1 in this study can be further combined with immune regulatory checkpoint blockades to fully realize the immunomodulatory effect of sonoporation. 

In the future, we aim to align and position this approach for clinical trials via an arm in an active treatment cohort or leveraging FDA’s guidance document on the expanded access to investigational drugs for treatment to tackle some of the huge unmet needs in circumstances where lack of treatment efficacy can be attributed to lack of tumor exposure, ultimately helping patients in need.
1 or 0.3 mpk single dose could be combined with the sonoporation approach.

To correlate the antitumor efficacy with local tumor concentration of MSA-1, we measured the levels of MSA-1 in the tumor and plasma 10 min after single IV administration of 0.3, 1, 3, and 10 mpk dose (Figure 2A,B). Both the plasma and tumor showed dose-dependent MSA-1 concentrations. For tumor local concentrations specifically, at 3 mpk, the efficacious dose, the concentration was $0.328 \times 10^{-6} \text{ M}$, whereas at 1 mpk, the sub efficacious dose, the concentration was $0.177 \times 10^{-6} \text{ M}$. Therefore, we rationalize that if sonoporation could increase the MSA-1 delivery efficiency by 85%, then we could expect meaningful antitumor efficacy from a single dose of 1 mpk, a threefold dose reduction. Similarly, if sonoporation could increase the MSA-1 delivery efficiency by 181%, then we could expect meaningful antitumor efficacy from a single dose of 0.3 mpk, a tenfold dose reduction. As a direct result of the dose-dependent MSA-1 concentrations in the tumor and plasma, we also observed dose-dependent type 1 interferon and proinflammatory cytokine concentrations 4 h after IV administration, both in the tumor and plasma (Figure 2C–F). For 1 and 0.3 mpk specifically, the systemic and tumor local concentrations of the proinflammatory cytokines were undifferentiated, suggesting that the sub efficacious 1 or 0.3 mpk single dose could be safely combined with the sonoporation approach.
Figure 1. Dose escalation study of MSA-1 in syngeneic mouse model MC38 after IV administration. C57BL/6 female mice were inoculated on the right flank with $0.5 \times 10^6$ MC38 mouse colon adenocarcinoma cells and when tumor volumes reached 100 mm$^3$, treatments of PBS (vehicle), 0.3, 1, 3, and 10 mpk of MSA-1 were administered. (x1) indicates single injection on day 1; (x3) indicates injections on day 1, 4, and 7. A) Tumor volumes were measured at predefined timepoints until animals reached a tumor volume of 2000 mm$^3$. Volume measures are presented as mean tumor volume ± SEM. The number of mice that achieved CR is indicated. $n$ = 9–10 mice/arm. B) Bonferroni adjusted-Mantel–Cox test was used to compute statistical significance.* $p < 0.05$; ** $p < 0.01$. C) Bodyweight measures are presented as mean percent bodyweight change ± SEM.

2.2. Sonoporation Significantly Improved the Antitumor Efficacy of MSA-1 at 1 Mpk Single Dose

After establishing the baseline performance of MSA-1 in the MC38 tumor model, we then investigated its antitumor efficacy when combining MSA-1 at either 1 or 0.3 mpk with sonoporation. After dosing, the mouse was immediately transferred to a treatment platform where a layer of ultrasound induction gel was applied onto the surface of the shaved tumor to enable the coupling with a P4-2s transducer covered with a 2 cm gel standoff and controlled by a Mindray TE7 ultrasound system (Figure S3, Supporting Information). Throughout the ultrasound procedure, the mouse was under anesthesia.

Since sonoporation is composed of two functional components, namely the microbubbles and ultrasound, we further segregated the impact of each attribute and combined them individually with 1 mpk (Figure 3A; and Figure S4, Supporting Information). As expected, there was no difference between vehicle and vehicle plus sonoporation, suggesting that the sonoporation process itself does not directly contribute to tumor-killing. When comparing 0.3 with 0.3 mpk plus sonoporation, we did not observe a significant improvement in efficacy. However, the 1 mpk dose combined with sonoporation achieved 30% complete regression versus none in the 1 mpk group without sonoporation. Sonoporation treated group also yielded a significantly slower specific tumor growth rate compared to all other control groups (Figure S5 and Table S2, Supporting Information). For reference, three doses of IT administered MSA-1 at 1 mpk achieved 80% complete regression (Figure S6, Supporting Information). When combining the individual component of the sonoporation process with 1 mpk, no significant improvement in the percent complete regression was noted. However, 1 mpk plus ultrasound did offer significantly improved percent survival compared to 1 mpk alone (Figure 3B). Additionally, 1 mpk plus microbubble dose did not differ from 1 mpk alone, in either the complete regression or the percent survival, suggesting that in the absence of ultrasound, a microbubble dose by itself does not offer any favorable pharmacological intervention. Across all treatment groups, no abnormal body weight change was observed, highlighting that the sonoporation approach itself is safe and can be readily combined with systemically administered compounds (Figure 3C).
2.3. Sonoporation Drastically Enhanced Tumor Local Concentration of MSA-1 and Resulted in Substantially Elevated Cytokine Production

We hypothesized that one of the 2 main mechanisms resulting in sonoporation-mediated MSA-1 antitumor efficacy enhancement is increased drug delivery into the tumors. To test this, we conducted a PK study where mice received 1 mpk MSA-1 or 1 mpk MSA-1 plus sonoporation were sacrificed at designated time points and both the tumor and plasma concentrations of MSA-1 were quantified. As soon as 20 min postdosing, or 15 min postsonoporation, the median tumor local concentration of MSA-1 increased from $0.126 \times 10^{-6}$ m in the MSA-1 alone group to $0.834 \times 10^{-6}$ m in the MSA-1 plus sonoporation group, a 6.58-fold increase (Figure 4A,B). 1 h postdosing or 55 min postsonoporation, while all samples in the MSA-1 group fell below the limit of quantification (LOQ), 3 out of the 5 samples in the MSA-1 plus sonoporation group yielded a positive value above LOQ, further supporting our hypothesis that the 5 min sonoporation process significantly enhanced the tumor local concentration of MSA-1 (Figure 4C,D). The improved PK profile of MSA-1, as a result of sonoporation treatment, further resulted in substantially improved PD cytokine levels. 1 h postdosing, both TNF-α, and IFN-β levels were significantly increased from MSA-1 control in the tumor (Figure 4E–H). TNF-α, IFN-α, and IFN-β levels were substantially increased as compared to MSA-1 control in the tumor 4 h postdosing (Figure 4I–L). Due to large sample variability, even though IL-6 showed an upward trend in the sonoporation treated group, the increase in that cytokine was not statistically significant. Interestingly, 1 h postdosing, IL-6, and TNF-α from the plasma as well as 4 h postdosing, IL-6 from the plasma showed an increase in the sonoporation treated group (Figures S7 and S8, Supporting Information). These results collectively suggest that the 5 min sonoporation treatment significantly increased the delivery of systemically administered MSA-1 into the tumor and elicited greater STING-mediated cytokine production as compared to MSA-1 control.

2.4. Sonoporation Sustained MSA-1’s Immune Activation Mechanisms While Reversed Its Immune Suppression Mechanisms

In addition to the sonoporation enhanced delivery of MSA-1 into the tumor, we also hypothesized that the 5 min sonoporation could trigger immunomodulation to synergize with MSA-1’s mechanism of action in regressing tumors. To investigate sonoporation’s potential immunomodulatory effect on the tumor microenvironment, we analyzed the immune cell population within the tumor 5 days after treatments. First, we noticed that 1 mpk plus sonoporation treatment resulted in significantly smaller tumors, which further validates our previous findings (Figure S9, Supporting Information). From the lymphoid lineage, we observed that both ultrasound and sonoporation increased the infiltration of CD8+ and GranzB+ CD8+ T cells (Figure 5A,B). Furthermore, sonoporation enhanced the infiltration of effector CD8+ T cells (CD44+CD62L−) that are responsible for tumor cell killing. (Figure 5C). Additionally, ultrasound and sonoporation sustained MSA-1 mediated NK cell recruitment (Figure 5D–F). No noticeable trend was observed...
Sonoporation significantly improved the anti-tumor efficacy of MSA-1 at a 1 mpk single dose. C57BL/6 female mice were inoculated on the right flank with $0.5 \times 10^6$ MC38 mouse colon adenocarcinoma cells and when tumor volumes reached 100 mm$^3$, treatments were administered. A) Tumor volumes were measured at predefined timepoints until animals reached a tumor volume of 2000 mm$^3$. Volume measures are presented as mean tumor volume ± SEM. The number of mice that achieved CR is indicated. $n$ = 8–10 mice/arm. B) Bonferroni adjusted-Mantel–Cox test was used to compute statistical significance. **$p < 0.01$. C) Bodyweight measures are presented as mean percent body weight change ± SEM.

2.5. MSA-1 in Combination with Sonoporation Eradicated Distantly Located Nonultrasound-Treated Tumors

Encouraged by the results, we further hypothesized that in combination with sonoporation, 1 mpk MSA-1 could regress not only primary tumors that received direct ultrasound treatment but also distantly located tumors that did not. To test this hypothesis, we inoculated mice on both the left and right flanks with MC38 cells and only treated the right tumor with ultrasound for 5 min after IV administration of MSA-1 and microbubbles. We also introduced a control group where 1 mpk MSA-1 was IT administered to the right tumor. We then monitored the growth profile of both the right (primary) and left (distant) tumors.

For the primary tumors that received 1 mpk MSA-1 IV plus sonoporation, three out of the nine tumors were fully eradicated (Figure 6A; and Figure S12A, Supporting Information). In comparison, seven out of the nine tumors that received 1 mpk MSA-1 IT were fully regressed. No regression was observed in the other groups. Surprisingly, for the distantly located tumors that did not receive ultrasound treatment, three out of the nine tumors were fully eradicated in the 1 mpk MSA-1 IV plus sonoporation group versus none in the 1 mpk MSA-1 IT treated group (Figure 6B; and Figure S12B, Supporting Information). No other tumor regression was noted in other treatment groups. When looking at the overall survival, 1 mpk MSA-1 plus sonoporation was
Sonoporation drastically enhanced tumor local concentration of MSA-1 and resulted in substantially elevated cytokine production. C57BL/6 female mice were inoculated on the right flank with $0.5 \times 10^6$ MC38 mouse colon adenocarcinoma cells and when tumor volumes reached 100 mm$^3$, treatments of 1 mpk MSA-1, color-coded orange, and 1 mpk MSA-1 plus sonoporation, color-coded blue, were administered. A,B) Violin plot of tumor and plasma concentrations of MSA-1 20 min postadministration are shown. C,D) Violin plot of tumor and plasma concentrations of MSA-1 20 min postadministration are shown. E-H) Violin plot of tumor IL-6, TNF-$\alpha$, IFN-$\alpha$, and IFN-$\beta$ cytokine levels 1 h postdosing are shown. I-L) Violin plot of tumor IL-6, TNF-$\alpha$, IFN-$\alpha$, and IFN-$\beta$ cytokine levels 4 h postdosing are shown. Statistics were calculated using a two-tailed t-test. *$p < 0.05$. $n = 5$ mice/arm/timepoint.

3. Discussion

First-generation STING agonists, the CDN analogs of cGAMP, when administered systemically in animals, induce equipotent inflammatory cytokine productions both in tumor and normal tissues, owing to ubiquitous STING expression.[41] Thus, CDN-based STING agonists currently undergoing clinical trials, MK-1454 and ADU-S100 for example, are dosed intratumorally which limits their application to a narrow set of tumors. To address a broad spectrum of cancers, approaches that could enable systemic administration and promote the targeted delivery to tumors are urgently needed. Various particulate-based delivery strategies have been proposed, and, despite their excellent preclinical proof-of-concept, the clinical translation foresees to be challenging due to the lack of a line of sight or perceived high chemistry, manufacturing, and control (CMC) complexity.[42–46] Platforms requiring complex and multistep synthesis can be very difficult to manufacture on a large scale, specifically, each step creates challenges in understanding the control of impurity removal, raw material quality, and the impact on the final critical quality attributes of the nano- or microconstruct drug product, which complicates the widespread adoption or exploration of these novel delivery systems.

In this report, we show that sonoporation, using microbubbles and noninvasive ultrasound commonly used in clinical practice, can enable the systemic administration of the STING agonist, MSA-1, and allow MSA-1 to enter tumors in higher concentrations than their normal PK/PD characteristics provide. To the best of our knowledge, this is the first comprehensive study that employs an already approved microbubble and diagnostic ultrasound, in combination with a STING agonist to quantitatively and mechanistically investigate the impact of sonoporation in a comparable to 1 mpk MSA-1 IT. Additionally, 1 mpk MSA-1 plus sonoporation outperformed all other three groups and resulted in 33% complete regression (Figure 6C).
Figure 5. Sonoporation sustained MSA-1’s immune activation mechanisms while reversed its immune suppression mechanisms. C57BL/6 female mice were inoculated on the right flank with 0.5 × 10^6 MC38 mouse colon adenocarcinoma cells and when tumor volumes reached 100 mm^3, treatments of PBS (vehicle), PBS plus sonoporation, 1 mpk MSA-1, 1 mpk MSA-1 plus ultrasound, and 1 mpk MSA-1 plus sonoporation were administered. Five days post-treatment, mice were euthanized and tumors were collected for immunophenotyping. A–F) Selective immune cells from the lymphoid lineage are presented as a percentage of CD45^+ cells. G–L) Selective immune cells from the myeloid lineage are presented as a percentage of CD45^+ cells. Statistics were calculated using a one-way ANOVA followed by Dunnett’s test referencing the vehicle. *p < 0.05. **p < 0.01; ***p < 0.001; ****p < 0.0001. n = 8 mice/arm.

Since MSA-1 was previously pursued as an IT dosing regimen, we first conducted a dose-escalation study to identify the minimally efficacious IV dose for the MC38 syngeneic female mouse model. The primary reason we chose female mice was due to the origin of the MC-38 murine CRC cell line, which is derived from a Grade-III adenocarcinoma that was chemically induced in a female C57BL/6 mouse and used since then as a transplantable mouse tumor model.[47] A clear separation on the tumor growth curve was noted where doses at or below 1 mpk failed to regress tumor growth, whereas doses at or above 3 mpk did (Figure 1A; Figure S2, Supporting Information). Therefore, 3 mpk is the minimal efficacious IV dose of MSA-1 for the MC38 model. Through PK examination, we hypothesized that if the tumor delivery efficiency for 1 mpk can be enhanced by 85%, improved antitumor efficacy can be expected from this current subefficacious dose (Figure 2B). Additionally, PD cytokine profiling confirmed that doses higher than 1 mpk resulted in equivalent or higher inflammatory cytokine production, which could jeopardize the safety profile of IV administration. Doses at or below 1 mpk, however, resulted in equal or lower inflammatory cytokine production, which suggests these dosing regimens can be safe upon IV administration (Figure 2C–F).

Therefore, we chose MSA-1 at both 1 and 0.3 mpk to combine with the sonoporation procedure, which is comprised of two main components: an IV administration of 0.025 mL Imagent perflexane lipid microspheres and a 5 min ultrasound insonation administered via a Mindray TE7 commercial ultrasound system with a P4-2s transducer. The system settings for insonation were in Contrast Mode, 2.2 MHz center frequency at a mechanical index (MI) of 0.531, and thermal index (TI) of 0.1. Total insonation time was 5 min with periodic 1100 ms ultrasound bursts of 1.304 MI administered every 10 s. In contrast to most other preclinical studies where only one MI insonation regimen was employed, we, on the other hand,
Figure 6. MSA-1 in combination with sonoporation eradicated distantly located nonultrasound-treated tumors. C57BL/6 female mice were inoculated on both the left and right flank with $0.5 \times 10^6$ MC38 mouse colon adenocarcinoma cells each and when both tumors reached an average of 100 mm$^3$, indicated treatments were administered. Ultrasound was only applied to the primary tumor. 1 mpk MSA-1 IT was injected into the primary tumor. A) Primary tumor volumes were measured at predefined timepoints until animals reached a combined tumor volume of 2000 mm$^3$. Volume measures are presented as mean tumor volume $\pm$ SEM. B) Distant tumor volumes were measured at predefined timepoints until animals reached a combined tumor volume of 2000 mm$^3$. Volume measures are presented as mean tumor volume $\pm$ SEM. $n = 9$ mice/arm. C) Bonferroni adjusted-Mantel–Cox test was used to compute statistical significance. * $p < 0.05$; *** $p < 0.001$.

Incorporated two different MI insonation regimens in our 5 min treatment. At a MI of 0.531, we expected stable cavitation whereas, at a MI of 1.304, we expected inertial cavitation. Collectively, these alternating cavitation phenomenonst greatly enhanced the antitumor efficacy of MSA-1 at 1 mpk (Figure 3A; and Figure S4, Supporting Information). Compared to all other control groups, MSA-1 at 1 mpk plus sonoporation significantly increased the number of complete regressed tumors. Furthermore, it also improved the percent survival profile without compromising safety (Figure 3B,C).

Intrigued by the results, we hypothesized that the improved antitumor efficacy was a result of two dominant mechanisms, namely, the improved PK/PD profile, as well as favorable immune modulation. To test the hypothesis, through the PK/PD study, we showed that the sonoporation procedure resulted in a significant elevation of the tumor local concentration of MSA-1 upon IV administration. Specifically, 15 min postsonoporation, we reported a 6.58-fold increase of tumor MSA-1 concentrations in the sonoporation-treated group (Figure 4A). Additionally, 55 min postsonoporation, we also reported a meaningful increase of tumor MSA-1 concentrations in the sonoporation-treated group as the control group failed to yield any value above the LOQ (Figure 4C). This improved PK profile translated into better tumor local cytokine levels 1 and 4 h postdrug administration (Figure 4E–L; and Figures S7 and S8, Supporting Information). Therefore, the data from this PK/PD study supported our hypothesis that the improved PK/PD profile in the sonoporation treated group resulted in improved antitumor efficacy.

STING activation can be considered a double-edged sword; on one hand, STING facilitates antitumor immune response through promoting the infiltration of effector T cells and natural killer (NK) cells and eradication of tumor cells. On the other hand, STING activation may hamper immune response by inducing the infiltration of immune suppressive cells, such as MDSC, and enhance the activity of indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the transformation of tryptophan into kynurenine, which hampers the antigen-presenting ability of DCs.[48] To this end, the impact of sonoporation on immune modulation was analyzed separately.

We and others have shown that STING activation in the TME can facilitate the recruitment of effector CD8$^+$ T cells and NK cells (Figure 5A–F).[9,16,49–51] The impact of sonoporation on this lineage was additive as the mean percentage of some of these effector lymphocytes was trending upward in the sonoporation treated group versus drug alone. Surprisingly, we noted that in the absence of microbubbles, the mean percentage of all these effector populations trended upward in the ultrasound-treated group. Based on the lack of antitumor efficacy improvement from the ultrasound-treated group, we did not anticipate such dramatic and favorable immune modulation on the lymphocyte lineage.

Through literature research, we concluded that in the absence of microbubbles, the cavitation nuclei—the degree of permeabilization enhancement across the peritumoral endothelial cells...
as a result of ultrasound insonation—is barely marginal. Consequently, the PK/PD profile from the ultrasound-treated group should be different from the drug-alone group. However, ultrasound can, for example, result in increased reactive oxygen species (ROS) generation, oxygen ions, or oxygen-containing radicals and altered redox status in the TME. \[52,53\]

The role of oxidants in inducing local inflammation has been extensively investigated in all manner of experimental models and ultimately, through bystander activation or stimulation with the antigens released from inflamed tumor tissue, additional T cells, and NK cells recruited to the tumor. \[54,55\] We also confirmed that STING activation could result in the increased recruitment of immunosuppressive MDSCs. \[56\] Surprisingly, sonoporation treatment dampens the recruitment of both G-MDSC and M-MDSC (Figure 5G, H), as well as immunosuppressive mononuclear cells (Figure 5I). The exact mechanism remains to be elucidated but we suspect this might be attributed to IFN’s skewed impact on the monocytes and granulocytes process and interferon-independent activities of STING. \[57\] It is generally recognized that MDSCs are capable of inducing strong immunosuppressive effects through the expression of various cytokines and immune regulatory molecules, stimulation of other immunosuppressive cells, and depletion of metabolites critical for T cell functions. \[58\] Thus a dampened effect of sonoporation treatment on MDSCs could contribute favorably to the overall tumor cell killing. It is reported that STING activation can be associated with the enhanced activity of IDO and the resulting elevated kynurenine could hamper the antigen-presenting ability of APC. \[48\] Interestingly, we observed that sonoporation treatment relieved the negative impact on the recruitment of APC from STING activation (Figure 5J–K). Collectively, the immunophenotyping result suggests that both ultrasound and sonoporation treatment sustained STING activation’s positive impact on immune activation while only the sonoporation treatment reversed STING activation’s negative impact on immune suppression.

Encouraged by results from the antitumor efficacy study and the immunophenotyping, we then hypothesized that the improved PK/PD profile and favorable immune modulation after sonoporation treatment could potentially regress distantly located tumors. We conducted a study in a bilateral syngeneic mouse tumor model where we only treated the primary tumor with ultrasound immediately after IV administration of MSA-1 and microbubbles. We observed a similar tumor growth pattern on the primary tumor to the unilateral antitumor efficacy study where the sonoporation treated group offered better efficacy (Figure 6A; and Figure S12A, Supporting Information). Interestingly, the tumor growth pattern on the distant tumors suggests that sonoporation treatment could generate a systemic antitumor immune response and result in regressed distant tumors (Figure 6B; and Figure S12B, Supporting Information). In this study, we introduced an IT dosing group at an equivalent MSA-1 dose; despite its excellent efficacy against the injected primary tumors, it failed to regress the distantly located untreated tumors.

The survival analysis data (Figure 6C) as well as tumor volume analysis on both the primary and distant tumors (Tables S3 and S4, Supporting Information), collectively suggests that a) both the 1 mpk (x1) IV + US + MB and the 1 mpk (x1) IT group has significantly controlled the tumor growth on the primary tumors and improved the overall survival when compared to the 1 mpk (x1) IV group; b) for distant tumors, however, only the 1 mpk (x1) IV + US + MB group, but not the 1 mpk (x1) IT group, has significantly controlled the tumor growth; c) there is no difference on the overall survival between the 1 mpk (x1) IV + US + MB and the 1 mpk (x1) IT group, but 1 mpk (x1) IV + US + MB group better controlled the tumor growth on the distant tumors.

The interpretation of the results is that a) sonoporation significantly improved the overall survival of STING-treated subjects at an equivalent IV dose; and b) the combination between sonoporation and IV injection of STING agonist, despite not being able to yield better overall survival when compares to the current standard route of administration for STING agonist, intratumoral injection, at an equivalent dose, it did offer better tumor growth control on the distant tumors. IT injection of selected CDNs (S100, DMXAA, and MSA-1) successfully eliminated the injected primary tumors in a dose-dependent manner, yet their response on noninjected distant tumors varies. \[59,60\] Sivick et al. reported that a relatively high dose of S100, while effective in clearing the injected tumor, can diminish tumor-specific T cell responses and negatively affecting durable immunity. \[60\] The absence of T cell expansion with a comparatively high dose of S100 was due to a lack of T cell activation in the tumor-draining lymph nodes, presumably due to overt cell death and compromised infrastructure. Furthermore, Lemos et al. reported that hyperactivation of STING could result in tissue damage that could further prevent T cell trafficking and activation. \[61\] We suspect that those could collectively contribute to 1 mpk (x1) IT group’s inadequate control over the noninjected distant tumors and further highlight our combination strategy’s potential in managing distant tumors.

Sonoporation has been established in many different preclinical tumor models, including MDA-MB-435; HepG2; PaCa-2; PC3; LNCaPb1; C32; and orthotopic C6 rats. \[62\] Yet it has lacked translational momentum. \[63\] Key to this next step into the clinic will be the characteristics of the microbubble employed as well as the ultrasound parameters chosen. These choices will significantly impact the adoption potential of this approach by minimizing the time of the treatment and facilitating its use in an outpatient environment. Microbubbles can be prepared in any in vitro lab with readily available ingredients. However convenient and inexpensive lab-scale microbubbles are to prepare, in order to be safely and routinely injected into patients, a wide range of risks including the potential to embolize capillaries must be considered. Only four microbubbles have been approved by the FDA, all for diagnostic purposes.

This work was conducted with Imageon perfluorane lipid microspheres (approved by the FDA in 2002). Imageon has several characteristics that allow it to be used as an important contributor to the sonoporation process. It has a flexible monolayer lipid wall with a neutral charge and does not require polyethylene glycol to prevent protein binding and reduce the risk of complement activation. Compared to other commercial microbubbles, it has a 0% propensity to be taken up by Kupffer cells and sequestered prematurely into the liver. \[64\] These surface characteristics of Imageon and the use of the vapor of a liquid perfluorocarbon to stabilize the microbubbles enable the use of relatively modest mechanical indices to collapse Imageon microbubbles to selectively induce inertial cavitation in the desired tissue location. The flexible shell is efficient at absorbing ultrasound energy such that the expansion and contraction characteristics of
Imagent’s stable cavitation can translate ultrasound energy into biomechanical effects that temporarily permeabilize endothelial walls. Imagent microbubble size is also effective for sonoporation using commercially available broadband frequency transducers. Commercial microbubbles are all considered “polydisperse” in that there is a range of microbubble sizes centered around a median diameter. Reconstituted Imagent demonstrates a biophasic size distribution centered around both 1 and 4.5 μm, both sizes would be acoustically active in stable cavitation upon exposure to typical broadband transmit pulses from modern diagnostic ultrasound systems. This also provides a larger median gas volume which has been shown to be a key dose parameter in the blood–brain barrier opening with sonoporation. Inside the microbubble, the choice of gases is a critical design feature. Imagent was engineered with the lowest solubility gas of FDA-approved microbubbles—perfluorohexane. Perfluorocarbon solubility decreases significantly for every additional carbon atom. Perfluorohexane has a water solubility 1000 times lower than sulfur hexafluoride and 704 times lower than octafluoropropane, the gases used in other FDA-approved microbubbles. This factor increases the circulation time by ≈50% in vivo. Additionally, the Imagent vial headspace also includes nitrogen gas that, when reconstituted, osmotically equilibrates the pressures inside the microbubble with the dissolved gases in the surrounding aqueous suspending medium (e.g., blood), thereby also contributing to longer circulation time.

In Dimcevski’s pioneering clinical paper, low mechanical indexes were chosen with a long insonation time of 31.5 min with multiple IV injections of microbubbles. With a net transmit duty cycle of 0.3%, cumulative ultrasound exposure was less than 6 s for the entire treatment. The design goal of our study was to reduce the insonation time to under 10 min with a single IV injection so as to reduce the time a technologist needs to hold the transducer in place over the tissue of interest to make it practical for clinical adoption. By adding intermittent higher mechanical index pulses every 10 s, our goal was to enable both stable and inertial cavitation to enhance the permeability effect of sonoporation using Imagent. Other diagnostic clinical studies researching microbubble contrast have used MI’s of greater than 1.6 without significant safety issues in multiple tissues. For a therapeutic context, especially in oncology, the 1.304 MI pulses used intermittently in this study are expected to also yield a clinically acceptable safety profile.

During the preparation of this manuscript, we also noted newer generations of STING agonists that have been developed, including an orally available non-nucleotide STING agonist MSA-2 and a non-nucleotide cGAMP mimetic SR-717. Cross-examination of various STING agonists is warranted on attributes, such as efficacy, toxicity, route of administration, and patient compliance.

In summary, by combining 1 mpk MSA-1 with a 5 min sonoporation treatment from FDA-approved microbubbles and a commercially available diagnostic ultrasound system, we report a clinically translational approach to enable the systemic administration of a nucleotide STING agonist. Through rigorous animal testing, we show that the sonoporation treatment enhanced the antitumor efficacy of IV administered MSA-1 at 1 mpk, both in unilaterally inoculated syngeneic mouse tumor model as well as in bilaterally inoculated model. The underlying mechanisms that attributed to the efficacy improvement were twofold: the improved PK/PD profile as a result of cavitation-mediated peritumoral endothelial permeabilization; and the positively modulated TME. We further recognize that certain models, such as MC-38 and LL-2, are relatively sensitive to immunotherapy such as STING agonists, whereas others are resistant to monotherapy treatment. Therefore, we hypothesized that combining the subefficacious dose of STING agonist in a sensitive model (including MC-38, CT26, B16F10, and LL-2), with sonoporation would allow the benefit of increased permeation due to sonoporation to be better highlighted. Sonoporation, as a tumor-targeting strategy, can be generally applicable to many.

Our approach does not require any modification of the therapeutics and uses an optimally designed FDA-approved microbubble with diagnostic ultrasound equipment that is available in many cancer centers, making the clinical translation much easier than particulate-based approaches. Additionally, the modularity of our approach, as a result of the ultrasound beam focusing, opens opportunities for many tumors that are anatomically deeply seated and difficult to reach with intratumoral techniques. Our next step is to align and position this approach for clinical trial via an arm in an active cancer treatment cohort.

4. Experimental Section

All animal studies were performed in accordance with guidelines of the Institutional Animal Care and Use Committee at Merck & Co., Inc., Kenilworth, NJ, and mice were kept in a pathogen-free facility.

**Treatment of MC38 Tumors:** For the therapeutic study, female Balb/c mice (Jackson Laboratory) of age 6–8 weeks were subcutaneously inoculated with 0.5 × 10^6 MC38 cells per mouse on the right flank on day 0. On day 8, animals were dosed with PBS or MSA-1 formulated in PBS at 10, 3, 1, and 0.3 mg kg⁻¹ IV or 20 μg intratumorally. In some experiments, Imagent microbubbles (Vesselon) and/or Mindray TE7 commercial ultrasound system (Mindray North American) were applied for indicated groups of animals. The tumor volume was measured 2 times per week and the volume was estimated with the following equation: volume = 0.5 × length × width^2. Animals were euthanized when the tumor volume reached 2000 mm^3 or had active ulceration.

**Sonoporation:** Insonation of mice was performed using a Mindray TE7 commercial ultrasound system (Mindray North America) with a P42S transducer fixed by clamps in a vertical position oriented in approximately a transverse plane. A latex probe cover with the tip filled with acoustic gel (Parker Laboratories) provided an approximate 2 cm soft standoff from the tumor. The fixture holding the transducer was able to be lowered using a rotary indexing mechanism to avoid putting too much pressure on the mice. Air bubbles in the probe cover gel were displaced so that there was a clear gel pathway for the ultrasound beam from the transducer face to the tumor. A 2 cm thick by 9 cm diameter ultrasound gel pad was placed underneat the mice to reduce reflections from the procedural surface. Mice were shaved over the tumor and an additional gel couplant was applied. The system settings for insonation were in Contrast Mode, 2.2 MHz center frequency at a mechanical index (MI) of 0.531, and thermal index (TI) of 0.1. Total insonation time was 5 min with periodic 1100 ms ultrasound bursts of 1.304 MI administered every 10 s.

**Pharmacodynamic Cytokine Analysis:** For the pharmacodynamic study, mice were dosed IV with MSA-1 formulated in PBS at 0.3, 1, and 3 mg kg⁻¹. In some experiments, Imagent microbubbles (Vesselon) and/or ultrasonic insonation were applied for indicated groups of animals. At predetermined time points postinjection, 400 μL blood was collected in Microtainer Blood Collection Tubes with Lithium Heparin/ PST Gel (Becton, Dickinson, and Company) and kept on ice. The samples were centrifuged at 10 000 g for 5 min at room temperature, and 100 μL of the plasma was
analyzed by TNF-α, IL-6, and type I IFN (IFN-α and IFN-β) ELISA (Meso Scale Diagnostics) following the manufacturer’s instructions.

**Pharmacokinetic Study:** For the pharmacokinetic study, mice were dosed IV with MSA-1 formulated in PBS at 0.3, 1, and 3 mg kg⁻¹. In some experiments, Imagent microbubbles and/or ultrasound insonation were applied for indicated groups of animals. At predetermined time points postinjection, in addition to blood, tumors were collected and kept on dry ice. Tumor homogenates were mixed with blank plasma to matrix match the tumor to a plasma calibration curve. Plasma and mixed tumor homogenates were protein precipitated with acetonitrile containing an internal standard analog. Supernatants from the protein precipitation were diluted into the water with 0.1% formic acid. Calibrators and unknowns were measured by ion-exchange liquid chromatography–mass spectrometry. Chromatographic separation was performed on a Shimadzu Nexera UPLC with an Imtakt Unison UK-Amino (50 x 2 mm, 3 mm) column maintained at 45 °C with a flow rate of 0.6 mL min⁻¹. Mobile phase A consisted of 70:30:1 acetonitrile, water, 1 M ammonium acetate. Mobile phase B consisted of 70:30:2:10.5 acetonitrile, water, 1 M ammonium acetate, isopropanol, ammonium hydroxide. Mass spectrometry quantitation was performed on an AB Sciex 6500.

**Immunophenotyping:** For analysis of immune cell population in filtration, fresh mouse tumors were enzymatically digested using the GentleMACS Mouse Tumor Dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The mixture was then filtered through 70 μm cell strainers to generate single-cell suspensions. The single-cell suspension from tumors was then washed and counted. Whole blood or two to three million cells from the tumor were incubated with a fixable viability dye FVS780 (BD Biosciences, San Diego, CA) and then blocked with antibodies to CD16/CD32 (BD Biosciences, San Diego, CA) for 10 min on ice. After Fc-blocking, samples were stained with fluorescence-conjugated antibodies in flow cytometry staining buffer with Brilliant Stain Buffer (BD Biosciences, San Diego, CA). The surface antibody cocktail included CD45 (Clone 30-F11), CD11b (Clone M1/70), CD8 (Clone S3–6.7), CD24 (M1/69), Ly6G (Clone 1A8), F4/80 (Clone BM8), Ly6C (Clone AL-21), F4/80 (Clone BM8), Ly6C (Clone AL-21), MHCII (Clone M5/114.15.2), CD11c (Clone N418), CD3e (Clone 145-2C11), CD4 (Clone GK1.5), CD49b (Clone DX5), CD335 (Clone 29A14), CD25 (Clone PC61), PD-1 (Clone RMP1-30), and PD-L1 (Clone MHI5). After surface staining, blood samples were lysed by BD FACs Lysing Solution, and then intracellular staining of Ki-67 (Clone 16A8), Granzyme B (Q16A602), and FOXP3 (Clone MF-14) on both blood and tumor samples was performed using eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. The stained samples were acquired on a BD Fortessa cytometer with DIVA software (BD Biosciences, San Diego, CA). Counting Beads (Thermo Fisher Scientific) were added to the blood samples before acquisition to enable absolute cell counts per blood volume. All the raw data were analyzed using FCS Express (De Novo Software, Pasadena, CA) according to the following gating strategies. Antibodies against CD45, CD3, TCRb, CD4, CD8, CD49b, CD335, CD25, FOXP3, Granzyme B, PD-1, Ki67 were used to define lymphocyte while antibodies against CD45, CD11b, Ly6G, CD4, F4/80, Ly6C, I-A/E, CD11c, PD-L1 were used to define myeloid populations. Gates were drawn on CD45⁺ leucocytes by doublet exclusion (forward scatter height versus forward scatter area; side scatter height versus side scatter area) and dead cell exclusion using fixable viability dyes. Gates were then drawn on the CD3⁺ T cell population, followed by CD4⁺ and CD8⁺ populations. Among CD4⁺ T cells, FOXP3⁺ CD4⁺ were Tregs. CD3⁻ CD335⁺ CD49b⁺ gated on CD45⁺ population defined NK cells. Granzyme B, Ki-67, and PD-1 were further gated on T or NK cells. Myeloid populations including gMDSC/neutrophils (CD45⁺ CD11b⁺ Ly6G⁺), M-MDSC (CD45⁺ CD11b⁺ MHCII⁺ F4/80⁻ Ly6C⁺), MACrophage (CD45⁺ CD11b⁺ MHCII⁺ F4/80⁻ Ly6C⁺), DC (CD45⁺ CD11c⁺ MHCII⁺ CD24⁻) or mixed monocytic cells (CD45⁺ CD11b⁺ MHCII⁺ F4/80⁻ Ly6C⁺) and total antigen-presenting cells (APC, CD45⁺ Ly6G⁻ CD11b⁺ MHCII⁺) were drawn. Fluorescence minus one, unstained, and isotype controls were included for assessment of surface and intracellular proteins. The percentage of each cell subset within viable CD45⁺ cells in the tumor and absolute cell number per μL of blood was calculated.

**Statistical Analysis:** All statistical analyses were performed using the GraphPad Prism software package (PRISM 8.1.1; GraphPad Software) unless otherwise specified. Biological replicates were used in all experiments unless otherwise stated. The survival benefit was determined using a log-rank test. All experimental results were indicated as the means ± SEM unless otherwise specified. One-way analysis of variance (ANOVA) was used when there were multiple comparisons. The student’s t-test was used for single comparisons. The specific statistical methods are provided in the figure legends and supporting documents.

**Supporting Information**
Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**
All authors (except authors affiliated with Vesselon) are employees or former employees of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA, and may hold stock or stock options in Merck & Co., Inc., Kenilworth, NJ, USA.

**Author Contributions**
J.X., N.S., R.S., B.J.L., A.T.P., and S.M. conceived, designed, or planned the studies. J.X., Y.W., H.F., K.L., and P.M. performed the experiments. J.X., N.S., Y.W., H.F., K.L., and P.M. analyzed the data. S.P., R.S., B.J.L., S.R., A.T.P., and S.M. aided in the interpretation of data. J.X. wrote the paper. All authors critically reviewed or revised the manuscript for intellectual content and approved the final version.

**Data Availability Statement**
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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clinical translation, drug delivery, STING agonist, ultrasound
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