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Sonodynamic treatment as an innovative bimodal anticancer approach: shock wave-mediated tumor growth inhibition in a syngeneic breast cancer model

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ABSTRACT

Despite the great advances in fighting cancer, many therapies still have heavy side effects, thus urging the development of highly selective and safe treatments with a wide range of applicability. Sonodynamic therapy (SDT) is an innovative bimodal anticancer approach in which two, normally non-toxic components - one chemical, a sonosensitizer and one physical, ultrasound - selectively combine to cause oxidative damage and subsequent cancer cell death.

In this study, we investigate the anticancer effect of SDT using shock waves (SWs) to activate protoporphyrin IX (PpIX) cytotoxicity on a Mat B-III syngeneic rat breast cancer model.

The SDT-treated group saw a significant decrease ($p < 0.001$) in magnetic resonance imaging (MRI) tumor size measurements 72 h after treatment with PpIX precursor 5-aminolevulinic acid (ALA) and SWs. This occurred together with significant increase ($p < 0.01$) in apparent diffusion coefficients between pre- and post-treatment MR tumor maps and strong increase in necrotic and apoptotic histological features 72 h post-treatment. Moreover, significant *HIF1A* mRNA expression up-regulation was observed along with the prominent selective cleavage of poly (ADP-ribose) polymerase (PARP) and increased autophagy related protein LC3A/B expression in SDT-treated tumors, as compared to untreated tumors 72 h post-treatment. Then, the anticancer effect of SDT can be boosted by SWs making them a valid technology for furthering investigations into this innovative anticancer approach.

KEYWORDS

Therapeutic ultrasound; Sonodynamic therapy; Shock waves; Porphyrin; Cancer.

INTRODUCTION

Progress in anticancer therapy can be achieved by placing emphasis on less thoroughly investigated bimodal anticancer approaches which, preferably consist of non-invasive treatments and which make use of synergistic effects between chemical compounds and external stimuli, such as heat, light or ultrasound (Urban et al., 2013; Ahmad et al. 1998; Hayashi et al., 2009). Due to its relatively low tissue attenuation coefficient, ultrasound has the ability to deeply penetrate biological tissues differently than light (Leighton, 2007). The absorption of ultrasound energy can lead to the heating of tissues and this has been used to therapeutic intent in high intensity focused ultrasound (HIFU) therapy (Kennedy, 2005). Recent efforts have discovered that benefits can also be acquired from non-thermal ultrasound effects, especially acoustic cavitation (Frenkel, 2008). Indeed non-thermal ultrasound can modulate cell membrane properties and activate specific chemical agents (Frenkel, 2008; Wood and Sehgal, 2015). Acoustic cavitation can be divided into two types, i.e. non-inertial (stable) and inertial (collapse or transient), which can only take place if acoustic pressure amplitude is higher than the pressure threshold for cavitation in the tissue under investigation. Inertial cavitation then produces vapor- or gas filled cavities (microbubbles) in the insonated milieu, that first increase in volume and then implode violently, producing ‘hot spots’ characterized by extremely high temperatures and pressure in a very small space. This phenomenon allows such harsh conditions to be reached without affecting bulk temperature and pressure and therefore promotes mechanisms, such as sonoluminescence and sonochemical reactions (Suslick and Flannigan, 2008). Inertial cavitation can therefore cause energy transfer which triggers electronic excitation, from the ground state into an excited state, in sonosensitive molecules (Tachibana et al., 2008).

Sonodynamic therapy (SDT) typically refers to the selective uptake of a porphyrin-based sonosensitizer into cancer cells, followed by exposure to ultrasound which generates highly reactive cytotoxic products, such as reactive hydroxyl radicals, hydrogen atoms, alkoxyl and

peroxyl radicals and singlet molecular oxygen, which in turn cause damage to cancer cells (Tachibana et al., 2008). The peroxyl radicals are the key sonodynamic radical species as they possess longer lifetimes and the ability to diffuse longer distances (Wallace, 1997).

Protoporphyrin IX (PpIX) is one of the most commonly used sonosensitizers and its accumulation in cancer cells can be produced via the administration of its precursor, 5-aminolevulinic acid (ALA) (Chen et al., 2014). PpIX is produced during the heme cycle, while the negative feedback system, that prevents its accumulation, is modified in cancerous cells due to enzymatic defects (Collaud et al., 2004). Indeed, PpIX from ALA has been shown to accumulate preferably in cancerous cells over healthy cells and it is currently in widespread use in malignancy detection and treatment (Millon et al., 2010; Wachowska et al., 2011).

Despite the effectiveness that SDT has demonstrated in experimental tumor models (Gao et al., 2013; Song et al., 2014; Tsuru et al., 2012; Costley et al., 2015), we only have a limited understanding of the mechanism of interaction between ultrasound and sonosensitizer in tumor tissues, even though inertial cavitation seems to play a crucial role. The most important parameters for inducing inertial cavitation are the ultrasound insonation technique used and peak ultrasound wave pressure (Leighton, 2007). Therapeutic ultrasound usually produces non-thermal effects that are difficult to isolate from the thermal ones. Therefore, our group has introduced the use of shock waves (SWs) to trigger sonosensitizer cytotoxicity, in order to minimize the thermal effect produced by ultrasound and enhance inertial cavitation.

SWs are sharp discontinuities involving a sudden change in pressure and density which can induce *in vivo* bioeffects (Millán-Chiu et al., 2014). A typical pressure waveform at the focus in water consists of a compressive wave with a peak positive pressure in the range of 30–150 MPa and a phase duration of 0.5–3 μ s, followed by a tensile wave with a peak negative pressure that drops to 20 MPa and a duration of 2–20 μ s, which is responsible for cavitation occurring. The low peak negative acoustic pressures of common therapeutic ultrasound are usually 0.2 MPa and produce stable cavitation (Lukes et al., 2015).

SWs have been used in extracorporeal shock wave lithotripsy for many years where it non-invasively treats patients with stone diseases, while more novel applications, such as drug delivery and gene therapy, are currently emerging (Millán-Chiu et al., 2014; Rassweiler et al., 2011; Steinhäuser and Schmidt, 2014). The *in vivo* treatment of tumors by SWs alone has been shown to be ineffective in inhibiting tumor growth (Lukes et al., 2015), whereas some evidence has been obtained to suggest that combining SWs and sonosensitizer results in sonodynamic tumor growth inhibition (Canaparo et al., 2013; Canaparo et al., 2006; Serpe et al., 2011). Since strongest evidences are needed to confirm SDT as a real anticancer treatment option, in the present study, we investigate the SWs' ability to induce *in vivo* PpIX cytotoxicity leading to tumor growth inhibition in a Mat B-III syngeneic rat breast cancer model.

METHODS

Sonodynamic treatment

The rat mammary adenocarcinoma cell line, Mat B III, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), was maintained in McCoy's 5A modified medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA), at +37 °C in a humidified atmosphere containing 5% CO₂. Cell pellets (1×10^6 cells), in 0.5 mL physiological saline, were orthotopically injected into the abdominal mammary fat pad of inbred 12 week-old female Fisher 344 rats (Charles River Laboratories, Milano, Italy) under isoflurane anesthesia. In three separate experiments, the animals were randomly assigned to treatment groups, with at least four animals per group.

SDT was carried out when the subcutaneous tumors reached nearly 500 mm³ in volume, typically within nine days. Experimental groups were treated on day 9 with a single iv injection, into the tail vein, of either physiological saline (0.5 mL), ALA (375 mg/kg bw) or

SWs alone (0.88 mJ/mm^2 , 500 impulses, 4 impulses/sec) or a combination of ALA and SWs (375 mg/kg bw ALA 4 h before SW exposure at 0.88 mJ/mm^2 , 500 impulses, 4 impulses/sec). Aminolevulinic acid powder (Sigma Aldrich) was dissolved in physiological saline at a dose of 375 mg/kg bw immediately before each administration. All animals were sacrificed at the end of the study (day 12) and tumor tissue samples were maintained in 10% buffered formalin for histology and in Allprotect Tissue Reagent (Qiagen, Milano, Italy) for mRNA and protein analyses.

SDT was carried out using an “energy-focused” piezoelectric device (Piezoson 100; Wolf, Knittlingen, Germany) and the energy at the focal point, i.e., energy flux density (EFD) per impulse, was recorded as mJ/mm^2 . Focal area is defined as the area in which 50% of the maximum energy is reached (10 mm length in the SW axis direction and 2.5 mm diameter perpendicular to this axis). Tumor bearing rats were anesthetized with 1-2% isoflurane in air and O_2 , fixed to a board in a supine position with the tumor facing upwards and ultrasound gel applied to the naked skin. A secured, acoustically adapted gel pad allowed a 5 mm SW penetration depth to be achieved by the transducer. The experimental protocol was approved by the Ethics Committee of the University of Torino, Italy.

Magnetic resonance imaging

Animals were imaged using a dedicated whole body rat coil in a high field (7T) magnetic resonance imaging (MRI) scanner (Bruker, PharmScan, Germany). Rats were anesthetized with isoflurane that had been vaporized with O_2 . Isoflurane was used at 3.0% for induction and at 1.0% -2.0% for maintenance. Spin echo (RARE) imaging ($\text{TR/TE/NEX} = 3000/8.5/2$, slice thickness 1.5 mm, FOV 50 mm, matrix 256×256 ; 15 slices, imaging time = 2.5 min) was used to calculate tumor volume and analyze tumor morphology. Typically, 15–20 coronal slices were acquired to cover the whole tumor. Each rat was scanned on day 8 (24 h pre-treatment) and 12 (72 h post-treatment) in order to characterize SDT tumor response. Diffusion-weighted images were acquired at a repetition time of 3100 ms and an echo time of

32 ms. Seven images were obtained with different gradient scalings, resulting in b-values of 0, 100, 300, 400, 600, 800, 1000 mm^2 . Signal intensities measured from the images that had been acquired at different b-values $S(b)$ and were numerically fitted against the model, $S(b) = S(b=0) e^{-b\text{ADC}_z}$, on a pixelwise basis. The resulting longitudinal component of the apparent diffusion coefficient (ADC) values were then displayed and analyzed as a parametric map. The tumor ADC values at the two time points (24 h pre- and 72 h post-treatment) were then analyzed.

Histopathological analysis

Tumor samples were fixed in 10% formalin 72 h post-treatment, blocked in a paraffin resin, cut to 4 μm , deparaffinized in xylene and rehydrated with alcohol. Sections were then stained with hematoxylin-eosin for histological examination by light microscopy (Leica DM600, Wetzlar, Germany).

Real Time RT-PCR

Tumor samples were collected in Allprotect Tissue Reagent (Qiagen) 72 h post-treatment and stored at -80°C . Total RNA was obtained using the AllPrep[®] DNA/RNA/protein Kit (Qiagen) and concentration ($\mu\text{g}/\text{mL}$) was determined using the Quant-iT[™] RNA Assay Kit (Invitrogen, Milano, Italy) on a fluorometer Qubit (Invitrogen). RNA sample integrity was determined using the total RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Milano, Italy). 500 ng of total RNA was reverse transcribed in a 20 μL cDNA reaction volume using the QuantiTect[®] Reverse Transcription Kit, while real time PCR analysis was carried out using SsoFast[™] EvaGreen on the MiniOpticon[™] Real Time PCR system (Bio-Rad, Milano, Italy). A QuantiTect Primer Assay was used as the gene-specific primer pair for *APAF-1* (QT01611225), *BAD* (QT00190407), *BCL2* (QT00184863), *HIF1A* (QT00182532), *MMP9* (QT00178290), *NFE2L2* (QT00183617), *NQO1* (QT00186802), *RNR1* (QT00199374), and *TP53* (QT00193522). The transcript of the reference gene ribosomal 18s and 28s RNA (*RNR1*) was used to normalize mRNA data. The PCR protocol conditions used have

previously been reported (Canaparo et al., 2013) and data analysis quantification was performed using Bio-Rad CFX Manager Software version 1.6 (Bio-Rad).

Western Blot analysis

Tumor samples were collected in Allprotect Tissue Reagent (Qiagen) 72 h post-treatment and stored at - 80 °C. Total protein was obtained using the AllPrep[®] DNA/ RNA/ protein Kit (Qiagen) and concentration (µg/mL) was determined using the Quant-iT[™] Protein Assay Kit on the fluorometer Qubit (Invitrogen). Equal amounts of protein (30 µg) were heat denaturated in sample-loading buffer (50 mmol/L Tris-HCl, pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 0.10% bromophenol blue, 10% glycerol), resolved by SDS-PAGE (Any kD[™] Mini-PROTEAN[®] TGX[™] Gel, Bio-Rad) and transferred to nitrocellulose membranes using Trans-Blot[®] Turbo[™] Transfer System (Bio-Rad). Membranes were stained with Ponceau Red (Sigma Aldrich) after the transfer procedure to check for the complete protein transfer and equal lane-to-lane protein loading. Filters were then blocked with Tris-buffered saline containing 0.05% Tween (Sigma Aldrich) and 5% non-fat dry milk and incubated overnight with the following primary antibodies (Abcam Company, Burlingame, California): beta actin (β-actin, Abcam 8226), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam 9484), LC3A/B (Abcam 128025), poly-ADP-ribose polymerase (PARP, Abcam 32138) and Ras homolog gene family member A (RhoA, Abcam 86297). Peroxidase-conjugated IgG (Abcam) was used as secondary antibodies. The membrane-bound immune complexes were detected using an enhance chemiluminescence system (ECL, GE Healthcare, Milano, Italy). Quantification of the bands was performed by densitometric analysis using TotalLab Software, version 2006 (Nonlinear Dynamics, Newcastle on Tyne, UK).

Statistical analysis

Results are expressed as the average value ± standard deviation (SD) throughout. Statistical analyses were performed on Graph-Pad Prism 5.0 software (La Jolla, CA, USA). The two-

tailed Mann-Whitney U test and the Kruskal-Wallis test were used to calculate the threshold of significance. Statistical significance was set at $p < 0.05$.

RESULTS

Sonodynamic treatment effect on tumor growth

MRI tumor size measurements clearly show that the groups demonstrated no significant difference in tumor size before treatment, while the SDT-treated group saw a tumor volume decrease, of about 60% ($0.79 \pm 0.39 \text{ cm}^3$), 72 h after the treatment.

This is considerable when compared to the untreated group ($2.08 \pm 0.20 \text{ cm}^3$), the ALA- ($1.56 \pm 0.74 \text{ cm}^3$) and SW- ($1.64 \pm 0.28 \text{ cm}^3$) treated groups (Figure 1A). SWs then induced *in vivo* PpIX cytotoxicity causing cancer cell death which was confirmed by histological analyses. Indeed, histological sections of control tumors, i.e. untreated (Figure 1B), ALA- (Figure 1C) or SW-treated (Figure 1D) tumors, showed that the integrated tumor cells were densely distributed, whereas tumor sections of SDT-treated group highlighted a strong increase in pyknosis, cell debris, cell shrinkage, increased intercellular space and necrotic subregions (Figure 1E). Moreover, no evidence of blood vessel injury and/or blood cell extravasation was found in the tumor sections of SW-treated groups, whether they had received ALA or not, as special attention was paid to detecting morphological alteration that may have been caused by SW mechanical effects.

MRI can provide *in vivo* information about tissue physiology and morphology, whereby it furnishes a non-invasive assessment of the tumor response by monitoring treatment-induced changes in tissue properties. Indeed, water molecule diffusion indirectly characterizes the tissue microenvironment as it is associated with cellular integrity and pathological conditions (Charles-Edwards and deSouza, 2006). Diffusion weighted (DW)-MRI detects water molecule displacement and its potential for monitoring a tumor's response to PDT has been demonstrated in several studies. Wang et al. (2010) have stated that morphologic changes,

such as broken cells, lead to changes in water diffusion within the tumor and therefore provides the mechanism by which DW-MRI detects early tumor response. We therefore monitored the tumor response to SDT using DW-MR images 24 h pre- and 72 h post-treatment. DW-MRI and ADC values were analyzed for all treatment groups: sonosensitizer alone, SWs alone and the combination of sonosensitizer and SWs.

Figure 2 shows representative ADC maps, at days 8 and 12 after tumor cell inoculation, of an untreated rat (Figure 2A, B) and a SDT-treated rat (Figure 2C, D) together with the corresponding T2-weighted images (Figure 2E, F and G, H). An increase in ADC, within the tumor and surrounding edema, is clearly visible by the bright region in the ADC map of the SDT-treated rat at day 12, 72 h post-SDT (Figure 2D) compared with the ADC map at day 8, 24 h pre-SDT (Figure 2C). No significant differences were observed in ADC maps of untreated rat between day 8 and 12 (Figure 2A, B). The average ADC value of the SDT-treated tumors increased at day 12, 72 h after SDT ($p < 0.01$) compared to the average ADC value of the SDT-treated tumors at day 8, 24 h pre-SDT (Figure 2I). The average 24 h pre-SDT ADC value was $1.02 \pm 0.15 \times 10^{-3} \text{ mm}^2/\text{s}$ and the average 72 h post-SDT ADC value was $1.37 \pm 0.06 \times 10^{-3} \text{ mm}^2/\text{s}$. Increased ADC values in the SDT-treated tumors might then indicate higher water diffusivity in 72 h post-SDT tumors compared with 24-h pre-SDT tumors. In the untreated rat no significant differences were observed between ADC maps at day 8 and 12 (Figure 2A, B, H). No significant differences in the average ADC value of untreated, sonosensitizer alone or SW alone-treated groups were observed (Figure 2I).

Sonodynamic treatment effect on gene and protein expression

As PpIX sonosensitization can induce cell death via oxidative damage and apoptosis, we analyzed a panel of genes involved in these pathways according to our previous *in vitro* work (Canaparo et al., 2013). mRNA gene expression was evaluated in samples from all experimental groups 72 h after treatment. Figure 3 shows increased mRNA expression of oxidative stress related genes, such as the transcription factor Hypoxia Inducible Factor 1

Alpha Subunit (*HIF1A*) ($p < 0.05$), in the SDT-treated tumors as compared to the untreated tumors. Moreover, an increase in the Apoptotic Peptidase Activating Factor 1 (*APAF1*) mRNA expression, although it was not statistically significant, and a decrease in extracellular matrix endopeptidases Matrix Metalloproteinase 9 (*MMP9*) mRNA expression were observed, whereas no significant differences in mRNA gene expression was observed for the apoptosis related genes, *APAF1*, *BAD* and *BCL-2*, or the oxidative stress related genes, *NQO1* and *NFE2L2*. No statistically significant differences in the mRNA expression of the panel of genes was observed in ALA alone or SWs alone-treated tumors as compared to untreated tumors (data not shown).

Since the selective cleavage of poly (ADP-ribose) polymerase (PARP) by several caspases, especially caspase-3, is a prominent apoptosis event, we performed a western blot analysis to investigate whether caspase-3 cleaves 113 kDa PARP to generate 85 and 29 kDa polypeptides in the SDT-treated group as compared to other experimental groups. The results in Figure 4A show representative images of significant cleavage in the SDT-treated group as compared to untreated, ALA alone or SWs alone-treated groups, at 72 h post-treatment. The PARP analysis results prompted us to focus our attention on SDT-treated tumors and investigate the protein expression of other cell death effectors. An increased expression of LC3A/B protein expression was observed in tumor tissues 72 h after SDT (Figure 4B), this protein is an indicator of autophagy, the basic catabolic mechanism for the degradation of unnecessary or dysfunctional cellular components via the action of lysosomes. A slight increase in RhoA protein expression, a small GTPase protein known to regulate actin cytoskeleton in the formation of stress fibers, was also detected in the SDT-treated tumors with respect to untreated tumors (Figure 4B).

DISCUSSION

Umemura et al. (1990) and Tachibana et al. (1993) were the first to introduce the sonodynamic treatment in the 1990s using SDT as a new bimodal anticancer approach to trigger sensitizer cytotoxicity and overcome the main drawback of PDT, i.e., light's relatively limited capacity to penetrate human tissues. Reactive oxygen species (ROS) and the resulting oxidative stress both play a pivotal role in sonodynamic induced cancer cell death (Misík and Riesz, 2000). The subcellular localization pattern of the sonosensitizer is extremely important for sonodynamic treatment efficiency due to the very short lifetimes and very short diffusion distances of some of the radical products derived from the sonosensitization produced during SDT. Thus, the SDT effect is determined by parameters that include both the sonosensitizer and ultrasound delivery patterns.

The sonosensitizer PpIX accumulated mainly in the mitochondria of cancer cells, where it is produced from ALA. Interestingly, it was found that the relative change in PpIX fluorescence intensity may be able to discriminate breast cancer from normal mammary epithelial cells (Millon et al., 2010). Several studies have indicated that apoptosis may be responsible for SDT induced cancer cell death and that mitochondrial dysfunction may play a central role.

As *in vivo* pressure measurements of SWs have shown that *in vivo* waveforms are roughly 30% lower in peak amplitude than those measured in water, we carried out *in vivo* treatments using higher energy levels than those used in previous *in vitro* works (Canaparo et al., 2013; Canaparo et al., 2006).

Our results show that tumor growth was significantly reduced by ALA and SWs-based SDT (Figure 1A) and provide a glimpse of possible future developments in the use of SWs, which is very much an emerging research area with numerous exciting prospects. Moreover, DW-MRI was used to monitor SDT tumor response, as this technique might be able to predict treatment efficacy at an early time. Indeed, MRI provides the non-invasive *in vivo* monitoring of tumor tissue and may allow an assessment of tumor response to be carried out by monitoring changes in treatment-induced tissue properties. An increase in water molecule

mobility in necrotic tumors over the restricted diffusion in viable tumors may result in an increase in MRI ADC values (Charles-Edwards and deSouza, 2006). Wang et al. (2010) have reported that changes in ADC values may provide a useful tool with which to monitor early tumor response and determine the effectiveness of anticancer treatments, such as PDT. Induced tumor necrosis is characterized by massive cell damage, reduced cell density and increased intercellular space and thus also by the liberation of water molecules from cellular membrane restriction. Our results show that significant differences in ADC values 72 h after SDT (Figure 2I) are consistent with the tumor necrosis and nonvascular cell injury observed in the respective histological sections (Figure 1E). Moreover, no differences in histological sections and ADC values were found between untreated tumor tissues and those that had only been treated with ALA alone or SWs alone. Lukes et al. (2015) reported that mechanical stress induces cell tumor damage only with high overpressure SWs with positive pressure peak amplitudes of 372 MPa, which are significantly higher than those used in this work (90 MPa).

By analyzing the mechanism of action that underlines SDT, Tabuchi et al. (2008) have proven *in vitro* that there is a significant down-regulation of the genetic network associated with cellular proliferation, gene expression and cellular development and a significant up-regulation of the genetic network associated with cellular movement, cell morphology and cell death after SDT. Our *in vivo* data also indicate that SDT affects gene and protein expression and provide novel insight into its bio-molecular mechanisms. Interestingly, the oxidative stress-related gene, *HIF1A*, was significantly up-regulated and the cellular movement related gene, *MMP9*, was significantly down-regulated, 72 h after SDT (Figure 3). Moreover, we observed the *in vivo* cleavage of PARP, a cellular substrate for caspase 3 and 7, indicating that apoptosis was induced in the SDT-treated tumors (Figure 4A). Since autophagy appears to promote or inhibit apoptosis according to specific stimuli and differing cell type (Cui et al., 2007; Pan et al., 2013) it was decided that the LC3A/B expression profile

should be investigated and, in fact, an increased level of expression was found in the SDT-treated tumors (Figure 4B). Referring to autophagy and apoptosis in SDT, Su et al. (2015) demonstrated that apoptosis and autophagy occurred with dependency on ROS production, whereas Wang et al. (2013) revealed that autophagy inhibition enhanced SDT-induced apoptosis in murine leukemia L1210 cells. Finally, a slight increase in RhoA expression (Figure 4B) was also observed. Our attention was focused on RhoA as it has been reported that PpIX from ALA may sensitize inside-out signaling proteins, such as Rho, that influence integrin binding to the substratum ligands and control the formation of stress fibers and general cellular response. Interestingly, the constitutive activation of RhoA has been demonstrated to negatively regulate cell migration due to the formation of excess stress fibers (Uzdensky et al., 2004)

CONCLUSION

In this work, we have shown as SWs can be considered a promising technology for *in vivo* sonodynamic applications since they can be focused by extracorporeal device to reach different depths in the body and activate sonosensitizer leading to cancer cell death. Indeed, the strength of this therapeutic approach relies on the possibility to selectively activate the drug in situ with a consequent improvement in drug specificity and efficacy.

We would also like to stress that significant progress in this field can only be achieved through multidisciplinary research demonstrating the validity of this approach at preclinical level either for primary or metastatic cancer sites. Moreover, it is worth noting that the methodology has a great clinical translatability either in terms of the necessary devices for the focused shock waves application or in terms of the required bioactive anticancer agents (e.g. porphyrin derivatives) each one already approved for clinical use.

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DISCLOSURE STATEMENT

All Authors declare that there are no conflicts of interest associated with this publication.

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FIGURE LEGENDS

Figure 1. Effect of SDT on Mat B III/ Fisher 344 tumor growth. Rats with growing tumors were treated with ALA and SWs (375 mg/Kg bw iv and 0.88 mJ/cm² for 500 impulses, 4 impulses/sec, respectively) 9 days after tumor cell inoculation and tumor volumes were determined by MRI on days 8 (24 h pre-treatment) and 12 (72 h post-treatment). A: the tumor volumes of each experimental group (n = 12) are reported as mean \pm SD. Representative haematoxylin-eosin section images of an untreated tumor (B), a ALA-treated tumor (C), a SW-treated tumor (D), a ALA and SWs (SDT)-treated tumor (E), 72 h after treatment (10x magnification). Statistical significance versus untreated rats, *** p < 0.001.

Figure 2. Diffusion weighted (DW)-MR images and ADC values at day 8 (24 h pre-treatment) and day 12 (72 h post-treatment) after tumor cell inoculation. Representative ADC maps and corresponding T₂-weighted images of an untreated tumor at day 8 (A, E) and 12 (B, F) and of a SDT-treated tumor at days 8, 24 h pre-treatment, (C, G) and 12, 72 h post-treatment (D, H). An ADC increase, within the tumor and surrounding edema, is visible in the SDT-treated tumor (D) but not in the untreated tumor (B). ADC values for each experimental group (n = 12) are reported as mean \pm SD. (I). Statistical significance versus pre-treatment ADC values, ** p < 0.01.

Figure 3. SDT effect on mRNA expression 72 h post-SDT. *RNR1* (ribosomal RNA 18S and 28S) was used as a reference gene to normalize the data. The combined treatment between ALA and SWs (375 mg/Kg bw iv and 0.88 mJ/cm² for 500 impulses, 4 impulses/sec) induced alterations in mRNA levels that are compared with those of the control, i.e. untreated rats, stated as 1 and shown by the dotted line. Data are reported as mean \pm SD for three separate experiments, each experiment with at least four animals per group (n=12). Statistical significance versus untreated tumor: * p < 0.05.

Figure 4. SDT effect on protein expression 72 h post-treatment. For each experimental group, two different representative samples are showed. A: sonodynamic treatment with ALA and

SWs (375 mg/Kg bw iv and 0.88 mJ/cm² for 500 impulses, 4 impulses/sec) induced significant cleavage of poly (ADP-ribose) polymerase (PARP) 113 kDa nuclear protein, into two fragments of 29 kDa (C-terminal catalytic domain) and 85 kDa (N-terminal DNA-binding domain) in the SDT-treated tumors, as compared to untreated tumors, SWs alone or ALA alone-treated tumors. B: detection of the 22 kDa RhoA protein and of the 15 kDa LC3A/B protein. GAPDH was used as loading control.