Comparing the Effects of Light- or Sonic-Activated Drug Delivery: Photochemical/Sonochemical Internalization

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ABSTRACT: Photochemical internalization (PCI) is a technique that uses the photochemical properties of photodynamic therapy (PDT) for the enhanced delivery of endolysosomal-trapped macromolecules into the cell cytoplasm. The released agent can therefore exert its full biological activity, in contrast to being degraded by lysosomal hydrolases. Activation of photosensitizers via ultrasound (US), called sonodynamic therapy (SDT), has been proposed as an alternative to light-activated PDT for the treatment of cancerous tumors. The use of focused US (FUS) to activate photosensitizers allows treatment at tumor sites buried deep within tissues, overcoming one of the main limitations of PDT/PCI. We have examined ultrasonic activation of photosensitizers together with the anticancer agent bleomycin (BLM) using sonochemical internalization (SCI), as an alternative to light-activated PCI. Our results indicate that, compared to drug or US treatment alone, US activation of the photosensitizer $AlPcS_{2a}$ together with BLM significantly inhibits the ability of treated glioma cells to form clonogenic colonies.

KEY WORDS: sonodynamic therapy, sonochemical internalization, photodynamic therapy, photochemical internalization, bleomycin, aluminum phthalocyanine disulfonate, glioma

I. INTRODUCTION

PCI is a technique that uses the photochemical properties of PDT for the enhanced and site-specific delivery of drugs into the cell cytoplasm.[1–5] Drugs that are internalized into cells via endocytosis end up trapped in intracellular endosomes and lysosomes. The concept of PCI is based on using photosensitizers that localize in the cell membrane and are carried into the cell during the endocytotic event. The photosensitizer remains in the endosome membrane while the macromolecule is localized within the lumen. Specific amphiphilic photosensitizers (e.g., $AlPcS_{2a}$ and $TPPS_{2a}$) preferentially accumulate in the membranes of endosomes, and upon light exposure the photosensitizer interacts with ambient oxygen to produce singlet oxygen. Singlet oxygen has a very short range of action (<20 nm), so only the area of the vesicular membrane where the photosensitizer is localized will be damaged by singlet oxygen–mediated reactions with amino acids, unsaturated fatty acids, and cholesterol in the membrane bilayer. The released agent can therefore exert its full biological activity, in contrast to being degraded by lysosomal hydrolases. Originally, the PCI method was shown to be effective for the liberation of drugs that have already been endocytosed and trapped in endocytic vesicles. This was based on illumination after drug incubation, the so-called "light after" strategy, as explained above. In contrast, a number of studies have demonstrated efficacy when photochemical disruption of endocytic vesicles occurs before delivery of macromolecules or the "light before" strategy.[6]

Sonodynamic therapy (SDT) has been proposed as an alternative to photodynamic therapy (PDT) for the treatment of cancerous tumors.[7–12] Whereas PDT uses light to activate the sensitizing drug, SDT uses US. The advantage of US versus light activation is that US has a much lower tissue attenuation

compared to visible light. This allows focused US (FUS) to deliver energy to tumors buried deep within tissues. For example, the penetration depth of 670-nm light in human brain is ~6 mm suggesting an effective treatment depth of ~1 cm.[13]

The mechanism of PDT has been examined in great detail, but there is still some debate as to the exact mechanism of SDT.[12,14] SDT has shown effect using both ALA and phthalocyanine, two classes of photosensitizer that have also been used for PCI.[11,15] Because PCI consists of PDT + drug, we hypothesized that SDT + drug (sonochemical internalization [SCI]) would give similar results. The purpose of the study reported here was to determine the increased efficacy of the anticancer drug bleomycin (BLM) by SCI and compare it to PCI." In vitro models using rat glioma monolayer cultures and clonogenicity assays were used.

II. MATERIALS AND METHODS

A. Cell Lines

The F98 glioma cell line was obtained from American Type Culture Collection (ATCC; CRL-2397) and was used in all cell monolayer experiments. F98 mimics human gliomas and was originally derived from transformed fetal CD Fisher rat brain cells following exposure to ethylnitrosourea on the 20th day of gestation.[16] The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA) with high glucose and supplemented with 2 mM L-glutamine, gentamycin (100 mg/mL), and 2% heat-inactivated fetal bovine serum (Gibco). Cells were maintained at 37 °C in a 7.5% CO_2 incubator.

B. Photosensitizer/Sonosensitizer

The term photosensitizer, as opposed to sonosensitizer, is used throughout this report regardless of its activation (US or light energy). Aluminum phthalocyanine disulfonate ($AlPcS_{2a}$; Frontier Scientific, Inc., Logan, UT), a membrane-localizing amphiphilic photosensitizer, was used in all experiments.

C. Bleomycin Toxicity

Viability of treated cells was assayed using a clonogenic assay. Cells from the F98 cell line were plated out in 35-mm tissue-culture dishes at 100 cells/dish and incubated overnight to allow them to adhere. Varying concentrations (0.1–10 μg/mL) of BLM (Sigma, St. Louis, MO) in culture medium were added to the cultures. The cells were allowed to grow for an additional 4 days, whereupon they were stained with 0.5% crystal violet in 95% ethanol. Colonies containing >50 cells were scored as survivors. The colonies were photographed and their numbers were counted visually. All experiments were performed in duplicate and results are a combination of at least four separate experiments.

D. SDT/PDT Treatment

Cells from the F98 cell line were plated out in 35-mm tissue-culture dishes as described above. The cells were then incubated in 1 μg/mL $AlPcS_{2a}$ in DMEM for 18 hours. Following incubation, cells were washed three times with phosphate buffered saline (PBS), and 1 mL of fresh medium was added. Continuous wave (CW) ultrasound radiation at a frequency of 1 MHz was performed with a portable US generator (SonoCare Plus, Roscoe Medical, Inc., Middleburg Heights, OH). The cultures received a range of US exposures (0–15 J/cm^2) delivered during an interval of 3 minutes (0–83 mW/cm^2). PDT cultures were irradiated with 670-nm light from a fiber-coupled diode laser (Intense, North Brunswick, NJ). Cells were exposed to a range of radiant exposures (0.1–2.0 J/cm^2) delivered at a light irradiance of 5 mW/cm^2. The clonogenicity of the treated cells was assayed as described previously.

E. SCI/PCI Treatment

The protocol for US activation (SCI) is shown in Figure 1a,b. F98 cells were plated out in 35-mm tissue-culture dishes at 100 cells/dish and incubated overnight, after which 1 μg/mL $AlPcS_{2a}$ was added to the cultures and the incubation continued

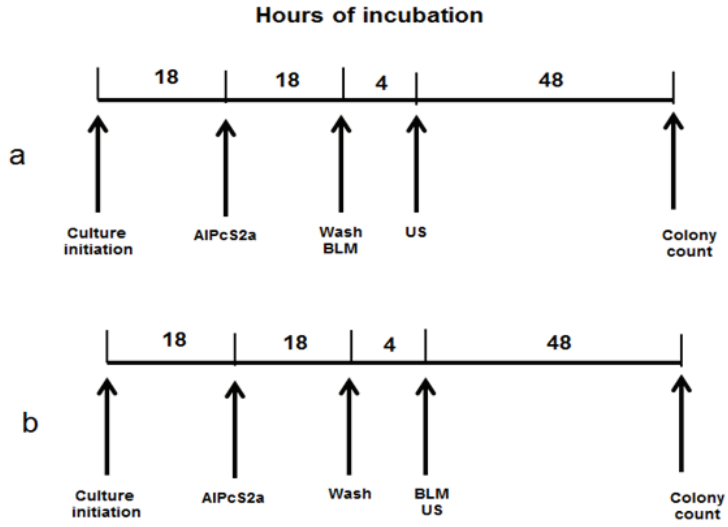

FIG. 1: SCI protocols. (a) US after drug. BLM incubated with cells for 4 hours followed by US exposure. (b) US before drug. A 4-hour wash was followed by the addition of BLM and US exposure.

for an additional 18 hours. Following a triple wash and 4 hours of incubation in fresh medium, BLM was added either immediately after the wash (Figure 1a) or after the 4-hour incubation period (Figure 1b). US-irradiated control cultures were done either with photosensitizers only or BLM only and followed the above protocol. PCI light treatment was performed in a similar manner, with the following exceptions: BLM was added after the 4-hour incubation period just before light illumination (light before protocol). Cells were irradiated with 670-nm light at an irradiance of 5 mW/cm^2 (radiant exposures of 0.1 or 0.2 J/cm^2).

F. Statistical Analysis

All data were analyzed and graphed using Microsoft Excel. The arithmetic mean and standard error were used throughout to calculate averages and errors. Statistical significance was calculated using Student's *t*-test as well as Welch's *t*-test. Two values were considered distinct when their *p* values were below 0.05.

Synergism was calculated when analyzing SCI treatments. The following equation was used to determine whether the SCI effect was synergistic, antagonistic, or additive:[17]

$$\alpha = \frac{SF^{a} \times SF^{b}}{SF^{ab}}$$

In this scheme, SF^a and SF^b represent the survival fraction for a specific treatment. If two treatments are to be compared, the survival fraction of each separate treatment are multiplied and divided by the survival fraction when both treatments are applied together (SF^{ab}). The resulting number (α) describes the cumulative effect. If $\alpha > 1$, the result is synergistic. If $\alpha < 1$, the result is antagonistic, and if $\alpha = 1$ the result is simply additive.

III. RESULTS AND DISCUSSION

A. Endosomal Escape of $AlPcS_{2a}$ after US or Light Irradiation

Fluorescence microscopy was used to verify the uptake and intracellular localization of the photosensitizer $AlPcS_{2a}$ in the absence or presence of US or light (Figure 2). The F98 cells were incubated with 1 μg/mL $AlPcS_{2a}$ for 18 hours, followed by 4 hours of incubation in drug-free medium before microscopy. The photosensitizer (red in Figure 2) is taken up in the F98 cells and localized in granular organelles representing endosomes and lysosomes

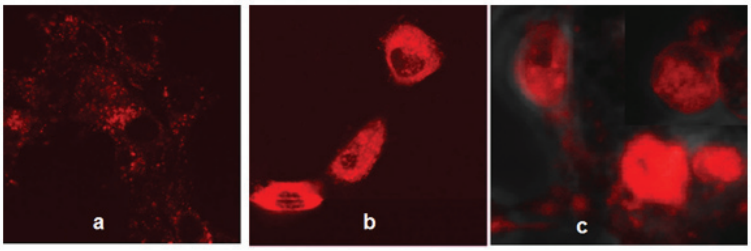

FIG. 2: Light- or US-induced endosomal escape of $AlPcS_{2a}$. (a) Photosensitizer (red) was taken up in the F98 cells and localized in granular organelles (endosomes/lysosomes); (b) 1 hour after light exposure; (c) 1 hour after US exposure. The diffuse $AlPcS_{2a}$ fluorescence throughout the cytosol indicates an induced endosomal escape of the photosensitizer by both light (b) and US (c) irradiation.

(Figure 2a), as previously observed for other cell types. One hour after light (b) or US (c) exposure, a diffuse $AlPcS_{2a}$ fluorescence throughout the cytosol was observed, indicating US- or light-induced endosomal escape of the photosensitizer.

B. Effects of PDT/SDT and BLM on F98 Colony Formation

To determine the optimal parameters for evaluating the effects of SCI on the F98 monolayers, titrations of drug concentration, light, and US energy doses were performed. The results are shown in Figure 3. As seen in Figure 3a, F98 cells are sensitive to BLM exposure. The LD_{50} for BLM was ~1.0 μg/mL; nearly all cells were killed at a concentration of 10 μg/mL (data not shown). The effects of PDT/SDT treatment on the ability of F98 cells to form colonies are shown in Figure 3b,c. As expected, the data show an increase in PDT or SDT toxicity with increasing light and US exposure. The cells appear to be more sensitive to $AlPcS_{2a}$-PDT compared to $AlPcS_{2a}$-SDT, as indicated by the 50% survival dose (LD_{50}) of 1 J/cm^2 compared to 7.5 J/cm^2 for PDT and SDT, respectively. Although the exact mechanism for SDT is unknown, it has been postulated that US inertial cavitation, a process characterized by the formation, oscillation, and collapse of gas-filled bubbles, is central to the production of singlet oxygen. When the cavitation phenomenon becomes dominated by inertial forces, the bubbles collapse violently, leading to very high local temperature elevations and the emission of light (sonoluminescence).[18,19] It has been suggested that in SDT, singlet oxygen generation results from indirect photoactivation of the sensitizing drug via sonoluminescence. Once excited, the sensitizer generates singlet oxygen in exactly the same way as in PDT. Results of the present work clearly demonstrate the efficacy of SDT in the F98 glioma cell line using $AlPcS_{2a}$ (Figure 3c). Not surprisingly, at sufficiently high exposures (15 J/cm^2), US alone is capable of causing significant cytotoxicity. Although numerous *in vitro* and *in vivo* SDT studies have been conducted with a wide variety of photosensitizers (mostly porphyrins), the present study is, to our knowledge, the first to use $AlPcS_{2a}$. This is important because photosensitizers with this type of amphiphilic structure are the most efficient for PCI. The hydrophilic domain prevents penetration through cellular and, hence, endosome membranes. The photosensitizer must maintain its position within the endocytic vesicle while the macromolecular drugs are trapped within the vesicle in order to reduce photochemical destruction of the macromolecules. A closely related photosensitizer (chloroaluminum phthalocyanine tetrasulfonate) was investigated for its SDT effect in a murine carcinoma model.[20]

C. SCI-/PCI-Mediated BLM Efficacy on F98 Colony Formation

Results of the two treatments (SCI and PCI) are shown in Figure 4a,b, respectively. The inhibition of colony formation using the strategy of US irradiation before exposure to BLM was clearly much more pronounced than that seen for the strategy of US exposure after drug incorporation (Figure 4a).

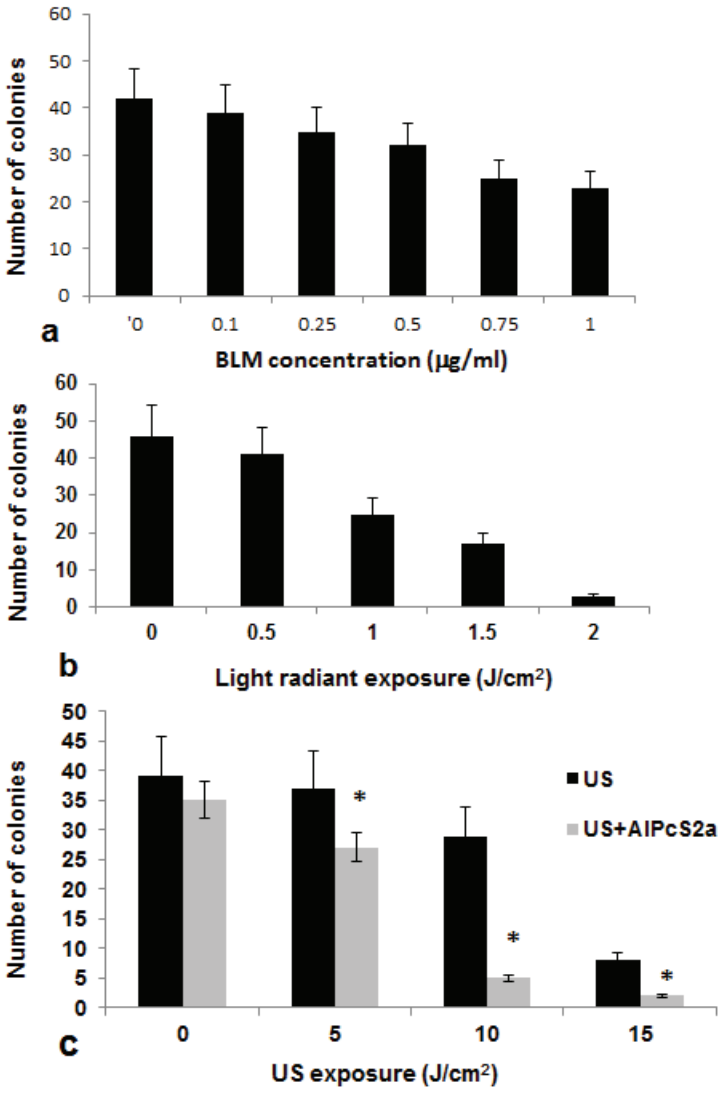

FIG. 3: Effects of BLM, PDT, and SDT on colony formation. A total of 100 F98 cells were seeded per 35-mm tissue-culture dish. (a) Cells were incubated with BLM concentrations of 0–1 μg/mL. (b) PDT-treated cells (1 μg/mL $AlPcS_{2a}$ for 18 h; λ = 670 nm; radiant exposure of 0–2 J/cm²; irradiance of 5 mW/cm²). (c) SDT-treated cells (1 μg/mL $AlPcS_{2a}$ for 18 h; US exposure of 0–15 J/cm²; exposure time of 3 min). Asterisks (*) denote significant differences ($p < 0.05$) between US-only controls and SDT-treated cells. Each data point represents the mean of four experiments. Error bars denote standard errors.

This was the case at both BLM concentrations investigated (0.125 and 0.25 μg/mL) and at the two lower US exposures used (2.5 and 5 J/cm²). At 10 J/cm², the direct toxic effects of US in the presence of photosensitizer (SDT effect) were overriding, with a more or less complete inhibition of colony formation. As shown in Figure 4b, significant PCI effects were observed at the highest light exposure (0.2 J/cm²) at both BLM concentrations investigated. Based on the data in Figure 4b, PCI demonstrated a synergistic effect according to the calculated α values at the highest light exposure (2.1 and 2.8 for BLM concentrations of 0.1 and 0.25 μg/mL, respectively).

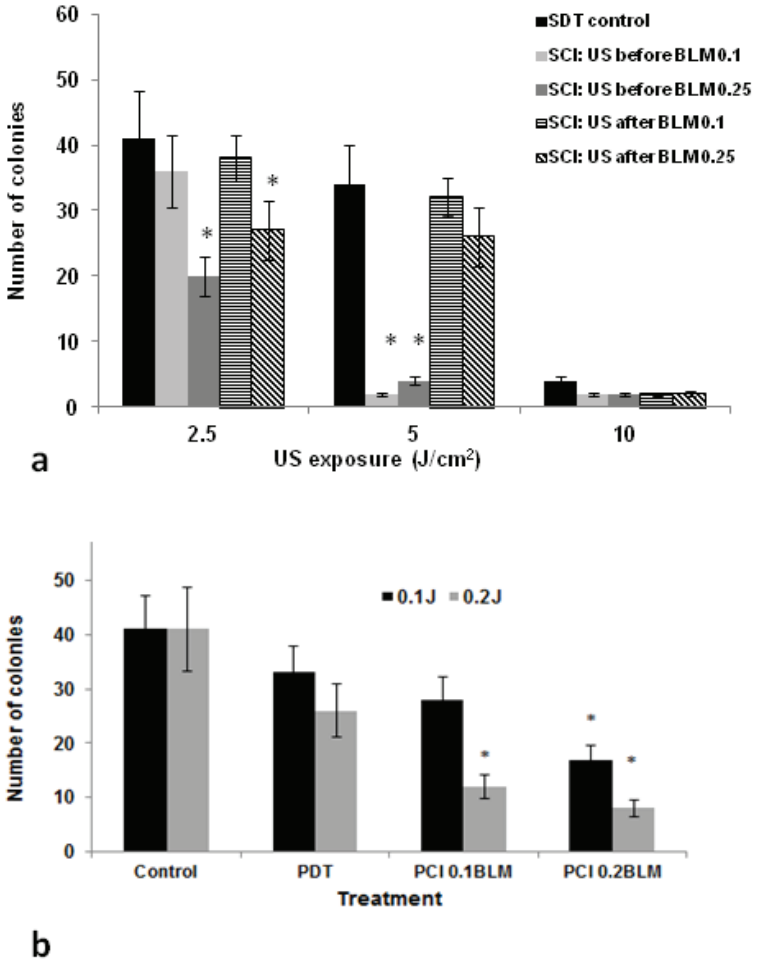

FIG. 4: Effects of BLM, SCI, and PCI on colony formation. (a) A total of 100 F98 cells were seeded per 35-mm tissue-culture dish and incubated in 1 μg/mL $AlPcS_{2a}$ for 18 hours. BLM concentrations of 0 (SDT control), 0.1, or 0.25 μg/mL were added either 4 hours before US or immediately after US (1 MHz frequency; exposure of 2.5–10 J/cm^2; exposure time of 3 min). (b) PCI light before drug protocol (0.1 and 0.2 J/cm^2 radiant exposure and 5 mW/cm^2 irradiance). Each data point represents the mean of three experiments. Error bars denote standard errors. Asterisks (*) denote significant differences ($p < 0.05$) compared to SDT (a) or PDT (b) control values.

There was a statistically significant ($p < 0.05$) difference in colony formation between cells exposed to single modality treatment (BLM or SDT) and cells subjected to SCI (Figure 5). Greater SCI effects were observed at higher BLM concentrations. Because SCI is a technique that relies on the combination of drug, photosensitizer, and US exposure, the resultant toxicities should show more than an additive effect of the single modalities. The degree of synergism was calculated by comparing the survival fractions of BLM or SDT alone with that of SCI treatment using the data from Figure 5. As evidenced from the calculated α values, SCI demonstrated a synergistic effect, especially at the higher BLM concentration: $\alpha = 7.5$ and 8.2 for 0.25 and 1 μg/mL BLM, respectively (the higher the α value, the greater the degree of synergism). Even in the absence of photosensitizer, US exposure increased the efficacy of BLM (Figure 5: BLM vs. BLM + US group). Calculated α values in this case

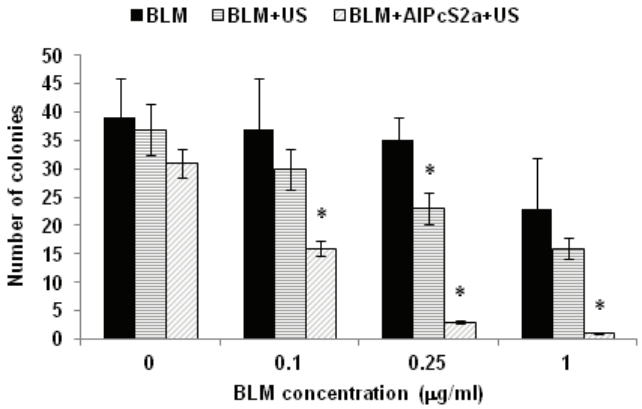

FIG. 5: Effects of US and US + $AlPcS_{2a}$ on BLM colony inhibition. 100 F98 cells were seeded per 35-mm tissue-culture dish and incubated in 1 μg/mL $AlPcS_{2a}$ for 18 hours. BLM concentrations of 0–1 μg/mL were added immediately after US (1 MHz frequency; radiant exposure of 5.0 J/cm^2; exposure time of 3 min). Each data point represents the mean of three experiments. Error bars denote standard errors. Asterisks (*) denote significant differences ($p < 0.05$) compared to BLM controls.

were 1.7 and 1.4 for 0.25 and 1 μg/mL BLM, respectively. A particularly interesting finding is that SCI efficacy is significantly improved if US is given before (US before) rather than after (US after) BLM administration (Figure 4a). This is in good agreement with studies showing improved PCI efficacy in light before versus light after protocols.[6]

The primary objective of this study was to determine whether SDT could be used to potentiate the effects of BLM (SCI) in a manner similar to that in which PDT has been used to enhance BLM efficacy (PCI).[5] As evidenced from calculated α values, SCI demonstrated a much higher degree of synergism compared to either BLM + US (in the absence of photosensitizer; Figure 5) or PCI at the US and light energies investigated.

Recent studies have shown that the optimum light sequence (before vs. after) is sensitively dependent on the type of compound to be delivered. For example, with nontargeted drugs such as BLM, equal or better effect has been observed using the light before approach, similar to what we have found with the US before protocol. For targeted drugs (e.g., immunotoxins), the light after approach does not work at all, most likely due to the photosensitizer relocating to the plasma membrane during light exposure.[21] The suboptimal results obtained with the light after approach are likely due to a combination of effects including enzymatic degradation of the compounds in endocytic vesicles before their release into the cytosol and induction of photochemical damage to the macromolecules in close proximity to the photosensitizer at the time of illumination.[6] In the case of SCI, similar explanations for the increased efficacy of US before compared to US after can be invoked, that is, it is assumed that SDT may damage BLM via singlet oxygen generation and that BLM may undergo enzymatic degradation in endocytic vehicles in US after protocols.

Although the mechanisms of SCI (and SDT) are somewhat speculative, the results presented here show that SCI is a highly effective technique for the delivery of chemotherapeutic agents such as BLM. Future studies will focus on delineating mechanisms and determining the utility of this new technology for the delivery of other compounds.

IV. CONCLUSIONS

SCI was found to potentiate the effects of a commonly used chemotherapeutic agent in *in vitro* systems of F98 rat glioma monolayers and spheroids. Specifically, the results show that, for the US and light irradiation parameters investigated, SCI demonstrated better therapeutic efficacy compared to PCI. SCI is a promising new technology that, like

PCI, may potentiate the efficacy of a wide variety of therapeutic compounds. However, unlike PCI, SCI is not limited by the poor tissue penetration inherent to light-based approaches and, as such, this US-based technology is ideally suited for the treatment of large and deep-seated lesions.

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