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Sonodynamic therapy combined with novel anti-cancer agents, sanguinarine and ginger root extract: Synergistic increase in toxicity in the presence of PANC-1 cells in vitro.

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Abstract

The presence of ultrasound-induced cavitation in sonodynamic therapy (SDT) treatments has previously enhanced the activity and delivery of certain sonosensitisers in biological systems. The purpose of this work was to investigate the potential for two novel anti-cancer agents from natural derivatives, sanguinarine and ginger root extract (GRE), as sonosensitisers in an SDT treatment with in vitro PANC-1 cells. Both anti-cancer compounds had a dose-dependent cytotoxicity in the presence of PANC-1 cells. A range of six discreet ultrasound power-frequency configurations were tested and it was found that the cell death caused directly by ultrasound was likely due to the sonomechanical effects of cavitation. Combined treatment used dosages of 100 μM sanguinarine or 1mM of GRE with 15 s sonication at 500 kHz and 10 W. The sanguinarine-SDT and GRE-SDT treatments showed a 6% and 17% synergistic increase in observed cell death, respectively. Therefore both sanguinarine and GRE were found to be effective sonosensitisers and warrant further development for SDT, with a view to maximising the magnitude of synergistic increase in toxicity.

Keywords: Sonodynamic therapy, pancreatic cancer, sonosensitiser, sanguinarine, ginger root extract.

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1 Introduction

A particularly promising application of ultrasound technology in the field of human therapeutics is the developing cancer treatment modality ‘sonodynamic therapy’ (SDT), a technique that has seen success across numerous in vitro and in vivo studies [1, 2, 3]. SDT involves the combined use of ultrasound-induced cavitation in the presence of a chemical agent known as a ‘sonosensitiser’, with the resulting cellular damage and cytotoxicity being synergistically greater than the additive effects of each element alone. This may occur via chemical augmentation of the drug activity and/or via the enhanced accessibility of the cells via ultrasonically induced mechanical effects such as sonoporation or mechanical damage. By inducing cavitation in close proximity to the tumour mass, this approach offers a less invasive but more targeted treatment than is achievable with conventional cancer therapies, and could support much-needed progress in the battle against the most aggressive human cancers.

By 2012 pancreatic cancer was ranked the twelfth most common human cancer by incidence rate, representing 2.4% of new cases worldwide [4]. Specifically, pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of all pancreatic cancers and has an associated median survival rate of 4.6 months in Europe, making it the fourth leading cause of cancer-related deaths on the continent and in the United States [5, 6]. More recent estimates suggest that pancreatic cancer could become the United States’ second leading cause of cancer-related deaths by the year 2030 [7]. PDAC is therefore the subject of the National Cancer Institute’s (NCI) first scientific framework for progress against recalcitrant cancers, which focuses on advancing effective therapeutics for this disease [8].

The main difficulty in treating human PDAC is due to its considerable resistance to conventional cancer therapies [8]. A key contributor to this resistance is the central anatomical location of the pancreas, where surrounding vital organs restrict direct access to the tumour site for treatment via surgical resection. Further, PDAC tumours have a characteristic tendency to manipulate the tumour microenvironment, resulting in high interstitial fluid pressures, increased production of extra-cellular matrix (ECM) proteins, desmoplasia, and weak tumour vasculature [8, 9]. The combination of these factors renders the conventional intravenous delivery of chemotherapeutic drugs largely ineffective, and suggests that a more targeted modality such as SDT would be a good therapeutic fit for the treatment of pancreatic cancer.

Chemical compounds derived from natural sources are commonly tested for anti-cancer properties as part of the search for new chemotherapy treatments. Two such compounds, namely the plant alkaloid sanguinarine (found in the Bloodroot plant sanguinaria canadensis)
and ginger root extract (GRE) \((\textit{zingiber officinale roscae})\), have both been reported to exhibit significant anti-cancer effects against several human cancer cell lines \textit{in vitro} and \textit{in vivo} \[10, 11, 12, 13\]. These naturally-derived compounds have the general advantage of being relatively abundant and have potential to cause fewer unwanted side effects in patients, when compared to synthetic chemotherapeutic agents. Specifically, sanguinarine (Figure 1a) has been shown to induce cell death via apoptosis \[11\] which occurs along pathways that interact with both cell internal and membrane functions \[12\]. Though the major active components of GRE (widely accepted to be [6]-shogaol and [6]-gingerol; Figures 1b, 1c) have demonstrated an ability to induce apoptosis in various cell lines when used in isolation \[14, 15, 16\], the GRE mixture has been shown to induce cell death via autosis with PANC-1 cells \[13\]. Across these studies, an accumulation of cells in the G0/G1 phase cell cycle was observed regardless of the predominant cell death mechanism, indicating the two substances share the anti-proliferative properties that are desirable in proven chemotherapeutic agents.

While the development of sanguinarine and GRE as anti-cancer drugs remains immature, the aforementioned cytotoxic effects are understood to result from each compound’s ability to generate reactive oxygen species (ROS) which damage cell mitochondria \[11, 12, 13\]. The generation of ROS is also a common sonochemical effect of collapsing cavitation microbubbles, and is one of the primary mechanisms by which SDT treatments are made more potent. It therefore follows that both compounds are viable candidate sonosensitisers for a novel SDT treatment for pancreatic cancer.

The focus of the present work is hence to investigate the extent of any synergistic increase in the cytotoxic effects of these novel SDT treatments using sanguinarine and GRE, when administered to \textit{in vitro} PANC-1 cells (i.e. any reduction in cell viability that is greater than the sum of that caused by the ultrasound and either compound alone). Given the reported anti-cancer properties of these two candidate compounds, ultrasound could further augment the toxicity of the sonosensitisers, and/or benefit from enhanced accessibility via mechano-acoustic effects. In order to understand the potential mechanisms of interaction between the ultrasound and the therapeutic agents, initial ultrasonic calibrations and toxicity testing were performed. The combined approach presents a unique way of developing SDT, enabling a mechanistic elucidation of the treatment fundamentals, which allows for a more informed and targeted follow-on approach. This involved confirming the reported toxicity of both compounds with this particular cancerous cell line, before testing a range of ultrasound parameter combinations to ensure the presence of acoustic cavitation in the setup. PANC-1 cells were chosen as the cell line is widely regarded as a primary tumour for \textit{in vitro} PDAC model \[17\]. PANC-1 has been found to be resistant to conventional chemotherapy \[18\] and
more aggressive with a greater metastatic potential than two other commercially available pancreatic cancer cell lines [17]. Analysis of these results then informed the design and configuration of the main combined treatment study. Any indication of a synergistic increase in toxicity would be a significant benefit to the treatment modality as it would allow a relatively low dosage of sonosensitiser to be used with eventual in vivo subjects, thereby minimising any unwanted cell damage in surrounding healthy tissue.

2 Materials and Methods

2.1 Chemicals and Consumables

Sanguinarine chloride hydrate in solid powdered form (CAS No. 5578-73-4; ≥98% HPLC purity), ginger root extract (GRE) in liquid form (CAS No. 84696-15-1; Food grade), and dimethyl sulphoxide (DMSO; CAS No. 67-68-5; ≥99.7% purity) were obtained from Sigma Aldrich (Poole, UK). PANC-1 cells (PDAC cells derived from a 56-year old Caucasian male) were obtained from Sigma Aldrich (Poole, UK). Dulbecco’s Modified Eagle’s Medium (DMEM), Modified Dulbecco’s Phosphate Buffered Saline (DPBS), Trypsin, Ethylenediaminetetraacetic acid (EDTA), L-glutamine and penicillin-streptomycin were obtained from Sigma Aldrich (Poole, UK). Fetal Bovine Serum (FBS) was purchased from Gibco Life Technologies and the CellTiter 96® MTS assay kit was supplied by Promega (Southampton, UK). Potassium iodide in powdered form (CAS No. 7681-11-0; ≥99% purity) was supplied by Fisher Scientific (Loughborough, UK).

2.2 Cell Culturing and Treatment

The adherent PANC-1 cells were cultured in Nunc™ T-75 flasks using DMEM supplemented with 10% FBS; 1% L-glutamine; 1% penicillin-streptomycin. PANC-1 cultures were maintained under standard conditions (37°C; 5% CO₂) in a humidified incubator. Cultures were harvested via trypsinisation and centrifugation using DPBS and EDTA with 2.5% Trypsin, before being transferred to Nunc™ T-25 flasks / Nunc™ 96-well plates as required. Cell counting was conducted using a hemocytometer and desired cell densities were subsequently calculated.

2.3 Natural Compound Screening Study

Two consecutive screening studies were conducted to confirm the reported cytotoxic effects of sanguinarine and GRE when administered to PANC-1 cells [10, 11, 12, 13], and
subsequently determine appropriate dosages for use in the combined treatment study. Quantitative assessment of cell viability took place using MTS proliferation assay in conjunction with a BioTek Synergy HT plate reader and Gen5 computer software.

10 mM stock solutions of sanguinarine and GRE in DMSO were prepared and further diluted with supplemented DMEM solution to the desired dosages. Across both screening studies the tested dosages ranged from 0.5-20.0 µM for sanguinarine and 1.0-100 µM for GRE in accordance with other published works [10, 13], with the second study testing a narrower range of dosages as prompted by the results of the first. PANC-1 cells were plated in a 96-well plate at a seed density of 4,000 cells/well with 200 µL/well supplemented DMEM solution. Cells were incubated for 24 hours prior to drug dosing to allow cells to detach. Each well was dosed with 200 µL of either sanguinarine- or GRE-supplemented DMEM solution at the required concentration. Cell viability measurements were recorded 24, 48 and 72 hours after dosing using 120 µL/well MTS in supplemented DMEM solution according to the manufacturer’s instructions (dilution factor of 6), with plates incubated for 3 hours. All reported results represent the mean and standard deviation of four experiments.

2.4 Ultrasound Calibration Study

A number of preliminary tests were carried out to confirm the presence of acoustic cavitation within the experimental set up used in this investigation. For sonication experiments a T&C Power Conversion Inc. AG 1006 Amplifier/Generator was used in conjunction with one of two electrically-driven piezoceramic plate transducers, with resonance frequencies of 500 kHz and 1.0 MHz. Each of these tests were conducted using six different power-frequency configurations: 500 kHz, 10/20/30 W and 1.0 MHz, 20/30/40 W. A schematic of the equipment configuration used is shown at Figure 2.

The first of these tests determined the discrepancy between the incident ultrasound power as set using the amplifier, and the power received by the liquid in the submerged T-25 flask as measured calorimetrically. This was calculated in accordance with an established method [19] with temperature readings taken at 15 s intervals. The second test also followed an established dosimetric method [19] to determine the extent of radical species formation during sonication.

The concentration of tri-iodide ions in the T-25 flask’s contents after sonication exposure at 15 s intervals was measured via UV spectroscopy at 355 nm (ε = 26,303 L/mol·cm), and used as a proxy for the extent of the observed sonochemical activity. All reported results represent the mean of three experiments. Evidence of sonoluminescence occurring within the T-25 flask contents was observed during sonication and photographed at 30 s exposure.
time using an Andor iXon X3 Low Light Camera (Figure 3), further indicating the presence of acoustic cavitation [20, 21].

2.5 Cell Sonication Study

A series of experiments involving the sonication of un-dosed PANC-1 cells were conducted to determine an appropriate configuration of ultrasound parameters, namely frequency, power and T-25 flask exposure time, for use in the combined treatment study.

PANC-1 cells were split into T-25 flasks at a seed density of $2 \times 10^6$ cells/flask, before adding 5 mL/flask supplemented DMEM solution and incubating for 24 hours prior to sonication. Each T-25 flask was submerged in 500 mL distilled water (Figure 2) at a starting temperature of 35 °C and sonicated at one of the six tested frequency-power combinations (Section 2.4) for an exposure time of 15 s. Following sonication all flasks were incubated for a further 24 hours prior to the measurement of cell death via hemocytometry. Both the anchorage independent cells (detached) in suspension with supplemented DMEM solution that were deemed dead, and the anchorage dependent cells (adhered) that were deemed alive and harvested via trypsinisation, were counted based on a 20 µL sample. This method of cell viability measurement was pre-validated by comparison with a trypan blue exclusion assay, and an MTS assay (data not shown) and is generally used in similar areas of in vitro research [22, 23, 24]. Hence, the pre-validation demonstrated that the detached cells would invariably take up the dye (indicating that they are not viable), while the adhered cells would invariably reject the dye (indicating that they are viable). It was therefore deemed that comparing the adhered and detached cell counts was appropriate for the investigation. This had the added advantage of reducing the amount of cell handling steps, which may have altered the observed viability for only the adhered cells. All reported results represent the mean and standard deviation of two experiments.

2.6 Combined Treatment Study

The combined treatment study was designed to investigate the extent of any observed synergistic increases in cytotoxic effects of a SDT treatment using sanguinarine and GRE as measured relative to the additive cytotoxic effects observed when using either compound and ultrasound exposure independently. The study therefore included six experimental conditions: un-dosed, not-sonicated (CELLS); un-dosed, sonicated (CELLS+US); dosed, not-sonicated (CELLS+SANG, CELLS+GRE); dosed, sonicated (CELLS+SANG+US, CELLS+GRE+US). Cell death was measured 24 and 48 hours post-sonication.
T-25 flasks of PANC-1 cells were prepared as per the cell sonication study (Section 2.5). The un-dosed experiment T-25 flasks (CELLS, CELLS+US) were re-filled with 5 mL/flask fresh supplemented DMEM solution. The dosed experiment T-25 flasks (CELLS+SANG, CELLS+GRE, CELLS+SANG+US, CELLS+GRE+US) were dosed with sanguinarine or GRE diluted in 5 mL/flask supplemented DMEM solution to the appropriate concentrations - 100 µM sanguinarine and 1 mM GRE. This is equivalent on a mol compound per cell basis to the 200 µL dosages administered to 4,000 cells/well in the drug screening study. All T-25 flasks were incubated for 1 hour prior to sonication.

The sonicated experiment T-25 flasks (CELLS+US, CELLS+SANG+US, CELLS+GRE+US) were sonicated at 500 kHz, 10 W, 15 s, as per the cell sonication study (Section 2.5). The non-sonicated experiment T-25 flasks (CELLS, CELLS+SANG) were removed from the incubator for the entirety of the sonication experiments. Post-sonication, all T-25 flasks were incubated for 24 and 48 hours prior to measurement of cell death in each flask via hemocytometry, as per the cell sonication study (Section 2.5). All reported results represent the mean and standard deviation of three experiments.

3 Results and Discussion

3.1 Natural Compound Screening

The cell viability measurements as taken 24, 48 and 72 hours after dosing with sanguinarine and GRE across the narrower range of dosages tested in the second screening are presented in Figure 4. Cell viability values were measured relative to an un-dosed control cell culture. For both compounds across all time periods the cell viability was observed to decrease with increasing dosage, indicating a dose-dependent toxicity for both sanguinarine and GRE in the presence of PANC-1 cells. This largely confirms the findings of other published works using these compounds at similar dosage levels with other cancer cell lines [10, 13], with any minor discrepancies in observed viability measurements likely due to differences in the population size of the dosed cell cultures. At low dosages of both compounds, cell viability was reduced after 24 hours but increased over the 48- and 72-hour observation periods. This trend is reversed at greater dosages of both compounds, where the reduction in cell viability was greater after 48 and 72 hours than was observed 24 hours after dosing. This is pertinent to the scheduling of the sonication procedure of a combined treatment, which should complement the time at which the sonosensitiser is most active. Though not specifically tested in this investigation, it is believed this toxicity was caused by the same ROS-mediated mechanisms referred to in the existing work relating to these two compounds [10, 11, 12, 13].
Based on these findings the dosages of each compound for the combined SDT treatment tested in the combined treatment study (Section 3.3) were chosen to be 5 μM sanguinarine and 50 μM GRE. These dosages were chosen to correspond to an approximate reduction in cell viability of 50% relative to the un-dosed control culture, as determined via linear interpolation of the 24-hour data presented in Figure 4. Note these dosages were subsequently scaled up in the combined treatment study (100 μM sanguinarine, 1mM GRE) to account for both the larger cell culture populations in the T-25 flasks (4,000 cells/well to 2,000,000 cells/flask), and the increased solvent DMEM volume (200 μL/well to 5 mL/flask).

3.2 Sonication Studies

3.2.1 Ultrasound Calibration

The measurements of both the calorimetric power received by the T-25 flask, and tri-iodide concentration after 15 s of sonication resulting from the dosimetric test are presented for each of the six tested frequency-power configurations in Table 1. For the same incident power as set using the amplifier, a greater calorimetric power was observed when sonicating at 500 kHz than when sonicating at 1.0 MHz. This suggests that sonication at the lower frequency increases the likelihood of more cavitational activity (i.e. higher bubble number and stronger collapse intensity) in the flask’s fluid contents, which would augment any microbubble-cell interaction in a sonicated cell culture. The observed yield of tri-iodide ions in the flasks sonicated at 500 kHz are an order of magnitude greater than those observed after sonication at 1.0 MHz, indicating that a greater amount of cavitation-mediated ROS production occurs at this lower frequency. This is in agreement with established ultrasound theory that relates lower sonication frequencies to a longer rarefaction cycle, and increased time in which a cavitation microbubble can collapse- as is necessary for ROS production [20].

<table>
<thead>
<tr>
<th>Incident Frequency (MHz)</th>
<th>Incident Power (W)</th>
<th>Calorimetric Power (W)</th>
<th>Tri-iodide (I₃⁻) Concentration; 15 s exposure (mol dm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>10.0</td>
<td>0.42</td>
<td>1.90 x 10⁻⁶</td>
</tr>
<tr>
<td>0.5</td>
<td>20.0</td>
<td>0.86</td>
<td>2.56 x 10⁻⁶</td>
</tr>
<tr>
<td>0.5</td>
<td>30.0</td>
<td>1.37</td>
<td>4.12 x 10⁻⁶</td>
</tr>
<tr>
<td>1.0</td>
<td>20.0</td>
<td>0.76</td>
<td>1.27 x 10⁻⁷</td>
</tr>
<tr>
<td>1.0</td>
<td>30.0</td>
<td>1.19</td>
<td>4.56 x 10⁻⁷</td>
</tr>
</tbody>
</table>

Table 1-Ultrasound calibration results, values represent the mean of three measurements.
The observation of sonoluminescence in the T-25 flask contents shown in Figure 3 is a further indication of the presence of sonochemical cavitation activity in this system, and can be considered as a qualitative validation of the dosimetric results shown in Table 1. More specifically, this observation confirms that the sonication transmission efficiency through the bulk fluid and T-25 flask is sufficient to generate cavitation activity. Sonoluminescence also indicates the presence of high-energy bubble collapse, as the power threshold for sonoluminescence is higher than sonochemistry [25]. In other words, the sonoluminescence occurs with a much higher energy than sonochemistry.

3.2.2 Cell Sonication

The cell death measurements resulting from the final un-dosed cell sonication study (exposure time of 15 s) as taken 24 hours after sonication for the six tested frequency-power configurations are presented in Figure 5. Cell death measurements were taken as the ratio of the hemocytometry count of unadhered cells in suspension in each T-25 flask to the sum of the counts of unadhered and adhered cells in each T-25 flask. Significant cell death was observed across each of the six experiments relative to the non-sonicated control experiment. For the same incident power as set using the amplifier, a greater amount of cell death was observed when sonicating using the 500 kHz transducer than was observed when using the 1.0 MHz transducer. This is in agreement with the finding of the calorimetric test in Section 3.2.1.

It is likely that this cell death is caused by the same combination of sonomechanical and sonochemical mechanisms outlined in other published works. The hydrodynamic shearing forces and acoustic streaming that occurs around stable cavitation microbubbles, and the jetting phenomena caused by collapsing microbubbles, are well-known features of a cavitating fluid [20]. These characteristics have been shown to cause significant damage in the form of membrane rupture and cell lysis, should the fluid forces be sufficient in magnitude [26, 27, 28]. Alternatively, the extreme physical conditions generated during the collapse of microbubbles are known to facilitate the production of radical oxygen species (ROS), such as peroxyl and hydroxyl radicals and singlet oxygen [20]. Ultrasound-induced ROS have been shown to cause cellular damage and cytotoxicity across a number of studies, mediating autosis and necrosis via interaction with cell internals [21] and membrane damage via peroxidation of the phospholipid bi-layer [2, 29].

Plots of these cell death measurements against the calorimetric power and tri-iodide results from the ultrasound calibration experiments respectively (Section 3.2.1) are presented in
Figure 6. Figure 6a shows a positive correlation between the incident calorimetric power and cell death for both frequencies, indicating that the dominant cavitational mechanism involved here is power-dependent. Figure 6b shows that a similar extent of cell death was achieved by sonication at 1 MHz and 500 kHz despite the much greater yield of ROS (by proxy of the tri-iodide measurement) observed at the lower frequency. Considered together, these results suggest that the reduction in cell viability resulting from sonication in the absence of a sonosensitiser for this experimental setup is predominantly due to sonomechanical effects.

An appropriate ultrasound frequency-power configuration for the combined SDT treatment in the combined treatment study was deemed to be one that minimised direct cell damage (so that any synergistic increase can be easily identified), but increased the potential for interaction with the sanguinarine and GRE sonosensitisers. Given the reported involvement of ROS in the cytotoxic action of both compounds (Section 3.1) and the analysis presented here, this configuration was chosen to be 500 kHz; 10 W.

### 3.3 Combined Treatment Study

The cell death measurements from the combined treatment study, taken at 24 and 48 hours after sonication for the six experiment configurations are presented in Figure 7. Across both 24- and 48-hour observations, the extent of cell death observed in all dosed and/or sonicated experiments exceeded that observed in the un-dosed and non-sonicated control experiment (CELLS). Normalising the 24-hour results to account for the baseline cell death in the control experiment, the observed cell death in both combined SDT experiments (CELLS+SANG+US, CELLS+GRE+US) exceeded the sum of the observed cell deaths for the respective isolated experiments (CELLS+US plus CELLS+SANG, and CELLS +US plus CELLS+GRE, respectively). This indicates that a synergistic increase in cytotoxic effect was observed in the combined SDT experiment for both compounds, with an increase of 6% in the sanguinarine treatment and 17% in the GRE treatment, as measured 24 hours after sonication. The measurements recorded 48 hours after sonication indicate no increase in the combined sanguinarine experiment (CELLS+SANG+US), and an increase (non-synergistic) of just 2% in the combined GRE experiment (CELLS+GRE+US). Statistical analysis of the 24-hour data for both combined treatments relative to the sum of the isolated experiments was performed using Student’s t-test. The combined GRE treatment was found to be statistically significant for p<0.01, while the sanguinarine SDT treatment was found only to be significant for p<0.2.

The findings of this combined treatment study therefore validate both sanguinarine and GRE as effective sonosensitisers for a novel pancreatic cancer SDT treatment. Discrepancies
between the trial studies and the isolated experiments of this study with regard to the observed cytotoxicity measurements may be noted. The CELLS+SANG; CELLS+GRE experiments were more toxic than in the screening study; and the CELLS+US experiment less toxic than the sonication study. However, the combined treatment experiments: CELLS+SANG+US, CELLS+GRE+US gave toxicity measurements of less than 100% which validates the reported findings. Although the relatively modest synergistic increase of the sanguinarine-SDT treatment’s cytotoxicity was shown not to be statistically significant here, the GRE-SDT treatment demonstrated a substantial increase in toxicity of 24 hours post-sonication, indicating its strong affinity for a novel anti-cancer treatment with ultrasound-induced cavitation. It is however worth noting that these effects were observed for a single dose of compound with sonication at a single frequency-power configuration- the effect of varying these parameters on the magnitude of this synergy has not been investigated as part of this work.

A less severe sonomechanical effect that might explain the synergistic increase in cell death of the combined SDT treatment is the phenomenon of ‘sonoporation’. Here the cell membrane undergoes a transient permeation due to liquid jets from collapsing microbubbles at its surface, allowing an increased uptake of macromolecules from the surrounding fluid [2, 30]. The increased uptake of the molecules from the surrounding liquid relies on the presence of microbubbles produced by ultrasound and can lead to enhanced drug delivery [31]. The degree permeability of the membrane is dependent on the applied ultrasonic parameters [32] and pore sizes have been reported in the 100s of nanometer range [33]. The pore size is likely dependent on the conditions applied and for this mechanism to be responsible for the synergistic increases observed here, the chosen sonication frequency-power configuration would need to generate microbubbles of a particular size relative to the PANC-1 cells to allow the necessary ‘controlled’ cell damage. Published work has investigated the effect of these ultrasound parameters on bubble size distribution [34], and could inform any changes to the present configuration that would enhance this sonoporation effect. Though the evidence in Section 3.2.2 suggests that the direct toxicity of the ultrasound cavitation alone is due to sonomechanical effects, the synergistic increase in toxicity of the combined SDT treatments observed here cannot definitively be attributed to these same effects. Though an increased toxicity by this mechanism would be independent of the anti-neoplastic method of the compound involved, the ability to increase the effective drug uptake would be particularly desirable in the treatment of dense, resistant PDAC tumours in later in vivo models.

Alternatively, the observed synergistic increases could be attributed to a constructive interaction of the compounds with the sonochemical effects of acoustic cavitation. Any
additional ROS generated by collapsing microbubbles can augment the radically-driven pathways from toxic sonosensitisers like sanguinarine and GRE [35, 36]. The radicals produced by ultrasound can initiate radically-driven pathways via initial radical attacks on the therapeutic agents. The advantages of ultrasonic If this is a prominent mechanism in these treatments, the difference in the extent of the synergistic increases is likely due to the differing stoichiometry of both treatments as a result of using two different compound dosages. It is also worth noting that while sanguinarine is a single chemical compound, the GRE used in this investigation is a mixture of several chemical species [13] that may contain more than one sonochemically-sensitive component, which might also explain the differing extent of the two treatments’ increases in cytotoxicity.

Where other work has discussed the cytotoxic effects of sonicated systems caused by thermal effects [1], the ultrasound setting used in the combined treatment study for a sonication exposure time of 15 s was observed to cause a bulk fluid temperature increase of just 0.4°C during the calorimetric testing. As such, it is unlikely that the synergistic increase in cytotoxicity observed here is the result of bulk thermal effects. The significant findings of this investigation nonetheless warrant further development of these novel SDT treatments, with a view to optimising the two components to maximise the extent of the synergistic cell death effects. Progressing the treatment towards in vivo and clinical trials should attempt to achieve this optimum within the constraints of using compound dosages and ultrasound exposure levels that are deemed safe for human therapeutic use.

4 Conclusions

The present work aimed to evaluate the suitability of two novel naturally-derived anti-cancer agents, sanguinarine and ginger root extract (GRE), to be used as sonosensitisers in a sonodynamic therapy treatment for human pancreatic cancer. The dose-dependent cytotoxicity of both compounds reported in other works was confirmed here in the presence of in vitro PANC-1 cells. A range of six ultrasound power-frequency configurations were tested for the ability induce acoustic cavitation in this experimental setup, and suitability for a SDT treatment when combined with the two compounds. It was found that the cell damage and death resulting directly from the ultrasound-induced cavitation was likely due to sonomechanical effects, rather than sonochemical effects.

The main study involving the two combined SDT treatments found both sanguinarine and GRE to act as sonosensitisers, and cause a synergistic increase in the observed cell death relative to the additive effects of the compound and ultrasound alone. The GRE SDT experiment was found to cause a statistically significant synergistic increase in cell death of
17%, indicating its strong suitability for use in this developing treatment. Further work should aim to determine the primary mechanism by which the enhanced toxicity of both compounds is achieved, with a view to maximising the extent of the increase in toxicity by manipulation of the ultrasound parameters.

5 Acknowledgements

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6 References


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Figure 1. Novel anti-cancer agent schematics (sigmaaldrich.com). 1a shows sanguinarine; 1b shows [6]-shogaol; 1c shows [6]-gingerol.

Figure 2. Sonication equipment configuration schematic, drawn as used in the combined SDT study. A distance of 25 mm between the T-25 flask and the transducer plate was maintained for all studies involving sonication.

Figure 3. Photographic evidence of sonoluminescence observed during sonication (amplifier frequency-power configuration: 500 kHz; 10.0 W). 3a shows the T-25 flask prior to sonication; 3b shows the T-25 flask during sonication, with sonoluminescence present in the surrounding distilled water and within the flask itself.

Figure 4. Results of second screening study. 4a and 4b show the cell viability measurements from the MTS assay kit across all three days of observation, for sanguinarine and GRE respectively. All values represent the mean and standard deviation of four measurements.

Figure 5. Results of cell sonication study. Cell death as measured via hemocytometry 24 hours after 15 s sonication is presented for each of the six amplifier frequency-power configurations. All values represent the mean and standard deviation of two measurements.

Figure 6. Comparison of the cell sonication results with ultrasound calibration results. Figure 6a indicates that cell death is a function of incident power. Figure 6b indicates that across the two frequencies cell death is not a strong function of present ROS concentration.

Figure 7. Results of the synergistic effect study. 100 µM sanguinarine, 1 mM GRE. Figure 7a shows the cell death measurements as recorded 24 h after sonication at 500 kHz, 10 W. 7b shows the cell death measurements as recorded 48 h after sonication at 500 kHz, 10 W. All values represent the mean and standard deviation of three measurements.
Highlights

- Anti-cancer properties of sanguinarine and ginger root extract (GRE) confirmed.

- Both compounds used successfully as sonosensitisers with *in vitro* PANC-1 cells.

- GRE-Sonodynamic therapy treatment achieved 17% synergistic toxicity increase.

- Sanguinarine-Sonodynamic therapy treatment reached 6% synergistic toxicity increase.