DOI: 10.1002/cmdc.200((will be filled in by the editorial staff))

The effects of ultrasound and light on indocyanine green-treated tumour cells and tissues

Nikolitsa Nomikou,[b] Christine Sterrett[a], Ciara Arthur[a], Bridgeen McCaughan[a], John F. Callan[a], and Anthony P. McHale*[a]

Photodynamic therapy (PDT) is emerging as a treatment modality for the management of neoplastic disease. Despite considerable clinical success, its application for the treatment of deep-seated lesions is constrained by the inability of visible light to penetrate deeply into tissues. An emerging alternative approach is to exploit the observation that many photosensitisers respond to ultrasound, eliciting cytotoxic effects on target cells and tissues and this has become known as sonodynamic therapy (SDT). The objectives of this study were (i) to determine whether or not the IR-absorbing dye, indocyanine green (ICG) could be employed as a sonosensitiser and (ii) to determine whether or not ultrasound could be employed to enhance ICG-mediated PDT. Exposing ICG-treated mouse fibrosarcoma cells to ultrasound at an energy density of 30 Jcm^{-2}, reduced cell viability by 65%. Prior exposure of ICG-treated cells to light (830nm) and subsequent treatment with ultrasound led to a 90% decrease in cell viability. In combination treatments a synergistic effect was observed at lower doses of ultrasound. Microscopic examination of cell populations treated with light or ultrasound demonstrated the production of intracellular reactive oxygen species (ROS). Using a mouse tumour model, treatment with light, ultrasound or a combination of light and ultrasound led to reductions in tumour growth of 42, 67 and 98%, respectively at day 27 post treatment. These results could provide a means of circumventing light-penetration issues currently challenging widespread exploitation of PDT for the treatment of cancer.

Introduction

In 1975 Dougherty *et al*. described the use of hematoporphyrin derivative (HpD) in combination with red light (630 nm) to completely treat mouse mammary tumour growth.[1] Using this approach clinical trials subsequently demonstrated significant therapeutic benefit in the treatment of skin and bladder cancer.[2,3] Although PDT was the first drug-device combination to be approved for the treatment of cancer by the US Food and Drug Administration almost 20 years ago, it is generally accepted that PDT remains under-represented as a clinically-accepted therapeutic regime in cancer treatment.[4] Despite its relatively limited acceptance in oncology, its clinical exploitation thus far has demonstrated that; (i) it is effective and safe with limited and easily overcome side effects, (ii) it is a site-specific treatment modality, (iii) it is indicated in situations where radiotherapy is contraindicated and (iv) it is minimally invasive.

One feature that most limits the widespread clinical application of PDT, is the inability of visible light to penetrate deeply into tissues and thus most clinical applications of PDT in cancer therapy are restricted to the treatment of more accessible lesions. Indeed HpD was originally chosen as a PS because it absorbed light at 630nm, a visible wavelength that exhibited significant transmission through tissues.[2,3] Since then alternative photosensitisers (PSs) have been sought that have the ability to absorb light in the near IR region (NIR) of the spectrum since NIR may penetrate more deeply into tissues. Indocyanine green (ICG), a NIR-absorbing fluorogenic agent, is commonly used in ophthalmological applications as a vascular imaging agent and is clinically approved for this purpose.[5] More recently it has been shown that ICG can serve as a PS, eliciting cytotoxic effects both *in vitro* and *in vivo* when used in combination with light at wavelengths in the region of 800-830 nm.[6,7,8] Although it has been suggested that this approach can be employed to target more deeply seated lesions, the limit of penetration of NIR light into tissues is still somewhat restrictive.

Over 10 years ago, it was demonstrated that many PSs can respond to ultrasound in a manner that results in cytotoxic effects and it has been shown that these toxic effects are mediated by the generation of cytotoxic ROS. Because of the tissue penetrating capabilities of ultrasound, it has been suggested that

[a] *C.Sterrett, C.Arthur, Dr. B. McCaughan, Dr. J. Callan, Prof. A.P.McHale*
Dept. of Pharmacy and Pharmacuetical Sciences,
Univeristy of Ulster, Coleraine, Co. Derry, BT51 1SA,
UK
E-mail: ap.mchale@ulster.ac.uk
[b] *Dr. N. Nomikou*
Research and Development
Sonidel Ltd.,
Tonlegee Dr.
Dublin 5, Ireland
Email: n.nomikou@sonidel.com

this approach could provide an alternative to light activation of clinically-approved PSs.[9,10,11,12] In our own laboratories we have demonstrated that treatment of the PSs, methylene blue and rose bengal, with light or ultrasound resulted in the generation of ROS in cell free systems.[13] It was also found that production of ROS could be enhanced when these PSs were treated with a combination of light and ultrasound and this, in turn, resulted in enhanced cytotoxicity *in vitro*.[13]

In relatively recent clinical studies, it has also been shown that PDT may be combined with SDT to afford a better cytotoxic effect and this has become known as sonodynamic photodynamic therapy (SPDT).[14] These above listed reports suggest that one of the major challenges to more widespread exploitation of PDT (i.e. accessing more deeply-seated lesions) could be overcome by (i) choosing PSs that absorb in the NIR region of the spectrum, (ii) replacing light with ultrasound as an external stimulus and/or (iii) by combining ultrasound with light as a means of enhancing the photodynamic effect. Here we report that the recognised PS, ICG, responds to ultrasonic fields by inducing a toxic effect in a mouse fibrosarcoma cell line. At earlier stages post treatment with ultrasound, ROS were detected in target cell populations. When NIR radiation (830 nm) and ultrasound were used in combination to 'activate' ICG in target cells, enhanced cytotoxicity was observed. In translating this latter approach to treating lesions in a mouse tumour model *in vivo*, significant reductions in tumour growth were observed and the effects were greater than those obtained with either of the individual stimuli. Based on these findings we suggest that such an approach may provide significant therapeutic benefit in site-directed cancer therapy.

Results and Discussion

The effects of light and ultrasound on ICG-treated RIF-1 cells

As mentioned above, overcoming the challenge presented by limited light penetration through living tissues remains one of the major obstacles to broad ranging clinical acceptance of PDT as a cancer treatment modality. Increasing the wavelength of irradiating light is one way of addressing this and indeed it has been shown that an increase in wavelength from 630nm to 693nm, increased tissue penetration by about 40% for bladder transitional cell carcinoma and by about 50% for normal bladder tissues.[15] However, even when optimal transmission with NIR radiation is achieved, light penetration is still only measured in mm and this would preclude non-invasive treatment of lesions in many internal organs. ICG is a dye that has routinely been used clinically for imaging purposes, absorbs maximally in the 800nm region of the electromagnetic spectrum and as mentioned above, has been found to exhibit the properties of a PS.[6,7,8] Since it has been shown that many conventional PSs can respond to ultrasound, it was of interest to determine whether or not this might be the case with ICG. Indeed it was also an objective of this part of the study to determine whether or not ultrasound could be employed to enhance the cytotoxic effects delivered by ICG when used in combination with light.

To the above ends, it was decided to treat the RIF-1 mouse tumour cell line[16] with ICG and subsequently expose the treated cells to NIR radiation, ultrasound and a combination of both stimuli. This cell line is a mouse radiation-induced fibrosarcoma and provides an ideal model target because it can also be employed to establish tumours in syngeneic mice.[16] The cytotoxic effects delivered by these treatments were then determined by measuring target cell viability using an MTT assay as described previously.[13] Where cells were treated with ICG and a combination of light and ultrasound, they were initially treated with a dose of light (37.8 Jcm^{-2}) that was pre-determined (data not shown) to deliver a 20% decrease in cell viability and immediately treated with the indicated dose of ultrasound. This light dose compared favourably with that used in a previously-reported study describing the use of between 20 – 99 Jcm^{-2} in treating HeLa cell populations that had been exposed to various concentrations of ICG. [17]

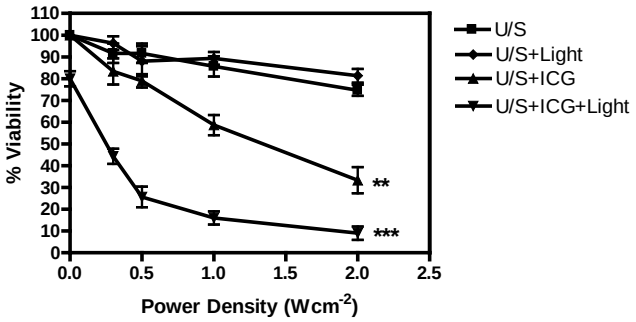


Figure 1 The effect of light and ultrasound on RIF-1 cells in the presence and absence of ICG. Cells were treated with light at an energy density of 37.8 Jcm^{-2} and with ultrasound at the indicated power densities. Cells were treated with ultrasound (■) and ultrasound plus light (♦) in the absence of ICG and ultrasound (▲) and ultrasound plus light (▼) in the presence of ICG. Cell viability of surviving fractions following treatment was determined using the MTT assay. Data points represent the mean ± the standard error where n = 3. For group comparisons of data with samples treated with ultrasound in the absence of ICG at 2 Wcm^{-2} and following ANOVA analysis and use of the MCT test, ** represents $p < 0.10$ and *** represents $p < 0.001$ (where $F = 63$).

The data obtained are shown in Fig.1 and the cell viability data are normalised with respect to cells incubated with ICG but receiving no other treatment. Dark toxicity of this dose of ICG was determined to be 2 % (± 0.2 %) and exposure of cells to light in the absence of ICG had no effect on cell viability. It should be noted that when the temperature of the medium in wells was recorded during treatment with light, the temperature increased by 1°C during treatment with light in the absence of ICG and by 2°C when treated with light in its presence. When treated with ultrasound in the absence of ICG, cell viability decreased steadily to 75 % at the highest ultrasound dose and similar results were obtained when both stimuli were employed (Fig.1). However, when target cells were sensitised with ICG, a statistically significant decrease in cell viability to 35 % was observed at the highest ultrasound dose (2 Wcm^2; E = 30 Jcm^{-2}). In a previously reported study using rose bengal as a sonosensitiser, it was found that at an ultrasound frequency of 1.9 MHz, reducing the cell viability to 30% required an ultrasound energy dose of 120 Jcm^{-2}. [10] In another study using ultrasound at a frequency of 1.2 MHz, rhodamine as the sensitiser and a human lymphoma cell line as a target, cell viability was reduced to 20 % using an ultrasound energy dose of 69 Jcm^{-2}.[12] Although direct comparisons are difficult because of variability in ultrasound frequencies and because it is difficult to determine the precise energy densities delivered to targets, this level of comparison does suggest that ICG may have a role to play as a sensitiser in SDT-based approaches. In our study it was interesting to note that a decrease in cell viability of 20 % in the presence of ICG

required an ultrasound energy dose of 6-7 Jcm^{-2} whereas a light dose of 37.8 Jcm^{-2} was required to deliver the same cytotoxic effect. Furthermore, when cells were treated with light and subsequently with ultrasound, cell viability decreased dramatically to 10 % at an energy dose of 30 Jcm^{-2} delivered at an ultrasound power density of 2 Wcm^{-2}. Indeed from Fig.1 it can be seen that the major effect on cell viability in the combined treatments occurred when cells were treated at an ultrasound power density of 0.3 and 0.5 Wcm^{-2}. These results suggested a degree of synergy between treatments with light and ultrasound because the additive effect of light treatment alone (20% reduction in cell viability) and ultrasound alone (28 % reduction in viability at 0.5 Wcm^{-2}) would be expected to deliver a 48 % decrease in cell viability whereas the data in Fig.1 demonstrate a 75 % decrease in cell viability for the combined treatment. It is also worth emphasising at this stage that when the temperature was measured during the above treatments, increases of no greater than 2-3^{o}C were observed and since treatments were performed at room temperature the temperature did not rise above 37^{o}C at any time. The latter observation would tend to preclude photothermal involvement resulting from treatment with NIR.[18] To the best of our knowledge this is the first demonstration that ICG may be employed to sensitise tumour cells to ultrasound. The data obtained with combined light and ultrasound activation also suggest that this clinically-approved imaging agent may find a role in cancer therapies that seek to derive the best attributes of PDT and SDT. [14, 19]

Production of intracellular reactive oxygen species

It has been shown that exposure of some PSs to ultrasound can lead to the generation of ROS in extracellular environments and our own studies, at relatively low ultrasound intensities, have demonstrated the generation of ROS in cell free systems when the PS methylene blue and rose bengal are stimulated with ultrasound.[13] Interestingly, we have further demonstrated enhanced ROS generation when a combination of light and ultrasound are used in the above-mentioned cell free systems. These results confirm earlier studies that suggest the involvement of ROS in cytotoxic events resulting from ultrasound-induced activation of some PSs.[9, 10] In a number of other reports, it was found that exposure of cells to ultrasound following treatment with PSs led to an increase in intracellular ROS and it was suggested that these species were mediating ultrasound-induced apoptosis.[17, 20, 21] In order to determine whether or not ultrasound treatment could result in the generation of intracellular ROS species in ICG-treated cells, target cells were treated with ICG and subsequently exposed to ultrasound as described in the experimental section. The total ultrasound dose was 36 Jcm^{-2}. As a positive control ICG-treated cells were also treated with light, since it had previously been demonstrated that exposure of ICG-treated cells to NIR resulted in the production of ROS.[22] In cases where ICG-sensitised cells were exposed to light, the laser module (830nm) was employed and exposures equated to a total energy dose of 37.8 Jcm^{-2}. Other controls consisted of cells treated with ICG in the absence of light or ultrasound and cells treated with light and ultrasound in the absence of ICG. Following treatment, cell populations were exposed to the ROS probe, 2',7'-dichlorodihydrofluorescein diacetate (DHFA) which generates cell-associated fluorescence in the presence of ROS and has been used as an indicator of intracellular ROS generation in other SDT studies.[23] This analysis was performed 3 h after treatment and the data obtained are shown in Fig.2. The photomicrographs clearly demonstrate that in the presence of ICG alone or when cell populations are treated with light or ultrasound in the absence of ICG, no significant fluorogenic signal was obtained (Fig.2; Panels right E, A and C, respectively).

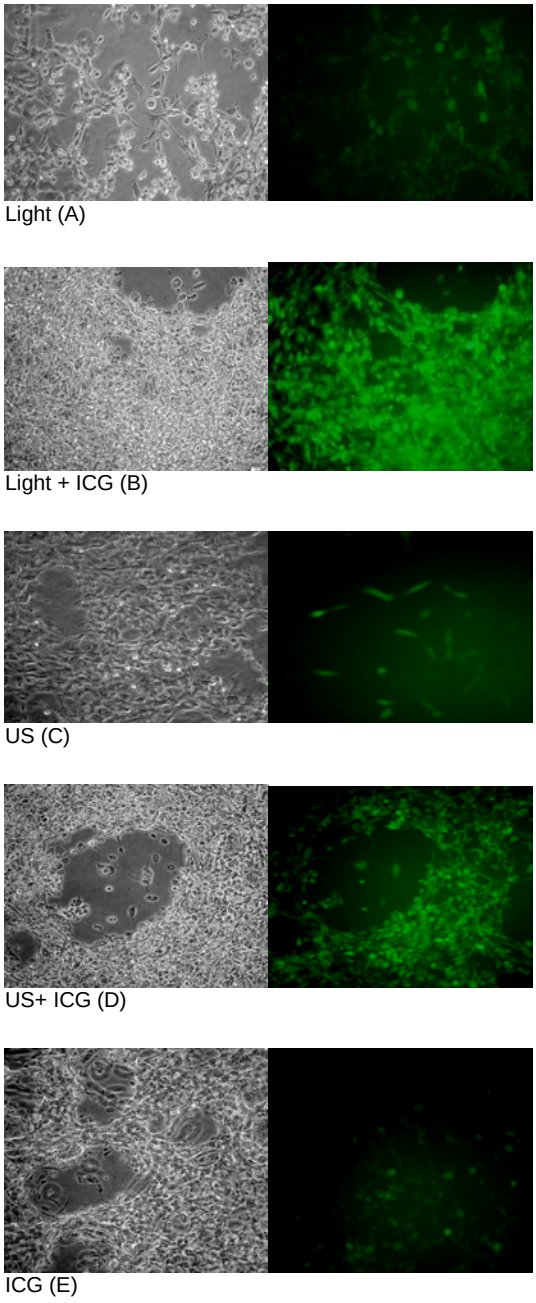


Figure 2. Photomicrographic analysis of ROS production following treatment of RIF-1 cells with light alone (panel A); light plus ICG (panel B); ultrasound alone (panel C); ultrasound plus ICG (panel D) and ICG alone (panel E). Black and white panels on the left represent images captured under white light and those on the right represent images captured using a filter with an excitation wavelength of 492-495nm and an emission wavelength of 517-527nm.

However, in the presence of ICG and following treatment with light or ultrasound, a clear fluorogenic signal was observed from cell monolayers indicating the generation of intracellular ROS (Fig.2; Panels right B and D, respectively). These results clearly demonstrate that exposure of ICG-treated cells to ultrasound resulted in the production of intracellular ROS. From the images captured using white light it is clear that ICG-sensitised cell populations treated with light and ultrasound exhibit stress as

indicated by their rounded-up morphology (Fig.2; Panels left B and D, respectively).

It has previously been demonstrated that excitation of ICG with light at 805nm resulted in a Type II photodynamic reaction resulting in the generation of $^1O_2$ and the subsequent generation of lipid peroxides.[22] In terms of serving as a sonosensitiser the data obtained in our study are similar to those previously reported with other sonosensitisers using the same approach to detect intracellular ROS. The results also suggest that the intracellular accumulation of ROS mediate cell death following exposure of ICG-sensitised cell populations to ultrasound.[23] Whether or not ultrasound-induced generation of intracellular ROS derive directly from sonodynamic effects or is simply a consequence of mitochondrial dysfunction however remains to be seen and this is the subject of ongoing studies. It should be noted that in studies designed to detect ROS production in cell free systems, we were unable to detect ROS production during treatment of ICG even at ultrasound power densities of 2.6 Wcm^{-2} (1 MHz, 50% duty cycle at a pulse frequency of 100 Hz) (39 Jcm^{-2}) (unpublished results). Although this may appear to preclude ultrasound-induced generation of ROS by ICG via a sonochemical/sonodynamic mechanism, particularly at the ultrasound power densities employed in our experiments, we believe that further studies may be required to resolve this issue. In this context it is perhaps important to note that all of our cell treatment studies were performed in the wells of a 96 well plate and the ultrasound probe was fixed to the bottom of that well. In all cases the ultrasound treatment dose was based on the transducer output parameters delivered by the sonoporator. However, this does not take into account the potential aberrant behaviour of ultrasound during transmission into the wells of the 96 well plate and potential ultrasound dosing issues resulting from echogenic effects caused by the medium/air interface as suggested in a recent study.[24] One consequence of these effects might be the exposure of cells to higher energy densities or acoustic pressures than those calculated from the emission outputs indicated by the ultrasound generator. As a result of these recent observations, we are currently examining whether or not higher ultrasound power densities will provide evidence of ROS production from ICG in cell free systems. Since it has been shown previously that ICG can serve as a photothermal sensitiser to near IR laser radiation the temperature was monitored during our treatments. [18] In our studies an ICG-dependant increase in temperature of 1^oC was observed following treatment with the laser whereas no significant ICG-dependant increase in temperature was observed during treatment with ultrasound. This could suggest that photothermal effects are not involved in the mechanism by which ICG is exerting its effects in the current study although highly localised thermal effects at a microscopic level cannot be ruled out at this stage.

The effects of light, ultrasound and a combination of both on growth of ICG-treated tumours.

Since it was demonstrated above that ICG could serve as a sonosensitiser and that ultrasound could be used to enhance ICG-dependant photodynamic cytotoxic effects *in vitro,* it was decided to determine the effects of light, ultrasound and a combination of light and ultrasound on growth of tumours *in vivo*. To this end, RIF-1 tumours were established in syngeneic mice as described previously [16] and injected intratumourally with ICG. Intratumoural injection was chosen as the route of administration in order to preclude any tissue delivery issues resulting from administration via an intravenous route. Control animals receiving treatments in the absence of ICG were injected intratumourally with diluent. All animals were allowed to rest for a period of 30 min prior to treatment to ensure diffusion of the intratumourally-injected ICG or diluent through the tumour tissues. For treatment with light, tumours were irradiated for a period of 3 min instead of the 2 min employed during the *in vitro* studies in order to compensate for tissue attenuation effects. In addition, the laser beam was rotated slowly around the tumour area during treatment and the beam was visualised using a digital camera. In cases where tumours were treated with ultrasound, a 3 min treatment using an ultrasound power density of 3.5 Wcm^{-2} with a duty cycle of 40 % (pulse frequency = 100 Hz) was employed in order to compensate for tissue attenuation and because these parameters were similar to conditions previously shown to have no effect on tumour growth with this and other tumour models.[25,26] Once again, the ultrasound transducer was rotated around the tumour area in order to ensure complete treatment. In addition, when combination treatments with light and ultrasound were carried out, ultrasound treatment was performed immediately after treatment with light. Following treatment, tumour volumes were determined and tumour growth was expressed as the % increase in size above the average tumour volume immediately prior to treatment.

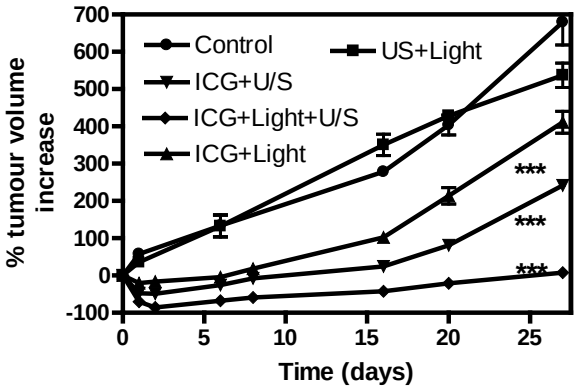


Figure 3. The effect of light and ultrasound on RIF-1 tumours in the presence and absence of ICG. Tumours were grown in C3H/HeN mice and were either untreated (♦), treated with ultrasound and light in the absence of ICG (■), treated with ICG and ultrasound (▼), (ICG and light (▲) or ICG and a combination of light and ultrasound (◄). Data points represent the mean ± the standard error where n = 3 with 9 measurements per data point. For treatment group comparisons relative to the untreated control at day 27 using one way ANOVA and the MCT test, *** represents p< 0.001 (where F = 105).

The data obtained from these studies are shown in Fig.3 and it should be stated that in cases where animals were treated solely with light, ultrasound or with ICG, no significant effect on tumour growth was observed. In addition, when tumours were treated with the combined stimuli in the absence of ICG, no significant effects on tumour growth were detected (Fig.3). However when tumours were treated with ICG and subsequently with either light or ultrasound alone, significant reductions (P<0.001) in tumour growth were observed when compared with growth of the untreated tumours. In these studies ultrasound treatment appeared to have a greater effect on growth than treatment with light, although this must be placed in the context of using a total energy of 252 Jcm^{-2} per tumour during ultrasound

treatments and 57.7 Jcm^{-2} per tumour using light. In a recent report similar results were obtained using ultrasound treatment alone, protoporphyrin IX as a sonosensitiser and tumours derived from the mouse hepatoma cell line, H 22.[27] In that study tumours were treated for 3 min with ultrasound using a focused transducer emitting at a frequency of 1.43 MHz and an ultrasound power density of 3 Wcm^{-2}. This yielded a total ultrasound energy dose of 540 Jcm^{-2} (based on a 3 min exposure) at an acoustic pressure of 0.27 MPa. Following such treatments, an approximate 45 % decrease in tumour growth was observed at 15 days. In our studies using ICG as the sensitiser, it was found that at day 25 a 50 % reduction in tumour growth was observed (Fig. 3) following treatment with a total ultrasound energy dose of 252 Jcm^{-2}. The acoustic pressure at the ultrasound intensity employed in this part of our study was previously shown to be in the region of 0.227 MPa.[28] In addition, at earlier stages after treatment (1 – 5 days), we were able to demonstrate tumour size regression and the tumour size only returned to the starting volume at day 16 following treatment with ICG and ultrasound (Fig.3). In a study using a chemically-induced mouse mammary tumour model and the sonosensitiser ATX-70, Yumita *et al.* demonstrated complete inhibition of tumour growth following treatment with ultrasound although in that case the ultrasound frequency was 1.92 MHz and the total ultrasound dose per tumour treatment ranged from 900 – 2,700 J. [29] At these doses and particularly at this frequency, cavitational effects could not be ruled out although the authors did pay particular attention to controlling tumour temperature in order to avoid hyperthermic effects. The results presented here clearly demonstrated that ICG could be employed to sensitise tumour tissues to ultrasound and could potentially play a role in SDT-mediated therapeutic approaches for cancer.

In addition to demonstrating that ICG may serve as a sonosensitiser the data in Fig.3 also clearly demonstrate that the combined treatment with light and ultrasound provided very significant inhibitory effects ($p < 0.001$ relative to the control) on tumour growth. Although studies with ultrasound *in vitro* are notoriously difficult to extrapolate to *in vivo* studies, the data clearly demonstrate therapeutic benefit associated with the combined treatment. Indeed, at earlier stages following treatment with the combined stimuli (days 1 – 5), tumour volumes regressed to almost immeasurable sizes and even at day 25 the tumour sizes had not fully recovered to their initial pre-treatment volume. Although the pre-treatment tumour volumes in these studies were relatively small (0.3 cm^{-3}), preliminary studies in our laboratories with larger initial lesions (> 1 cm^{-3}) demonstrated similar regression from the initial pre-treatment volume, although in some of those studies, severe necrosis was noted at the centre of these larger tumours, suggesting catastrophic compromise to the tumour vasculature. Although there is clear evidence of a cooperative effect in these studies with the combined treatments using light and ultrasound, a synergistic effect is not evident from the data in Fig.3. Demonstrating synergy would require significant manipulation of dosing regimes in terms of light, ultrasound and ICG concentrations and these aspects will be examined in future studies.

In considering the data shown in Figs.1 and 3 above, it is clear that a combination of light and ultrasound provides significant therapeutic benefit. It is also clear that both stimuli, when used separately, result in the generation of intracellular ROS. It could therefore be suggested that ultrasound is augmenting photo-induced ROS production, resulting from Type II-photodynamic events,[22] thereby enhancing the overall cytotoxic effect. An alternative hypothesis could involve initial ROS-induced destabilisation of cell membrane structure/function by lipid peroxidation resulting from photodynamic activation of the ICG and subsequent catastrophic physical/mechanical perturbation following exposure to ultrasound. This hypothesis might be supported by earlier work carried out by our group demonstrating that pre-treatment of tumours with intense, short-during electric pulses resulted in significant sensitisation of those tumours to ultrasound at doses similar to those employed here. [25,26]

Conclusions

It is clear from our data that ICG can serve to sensitise tumour cells and tissues to ultrasound and could potentially be employed as a sonosensitiser in SDT-based treatments for cancer. Since ICG is already a clinically-approved imaging agent its use for such purposes would be attractive from a regulatory perspective. It is also clear from our studies that ultrasound can be employed to enhance the cytotoxic effects of ICG-mediated PDT. Although we have shown that ultrasound can induce the generation of intracellular ROS in the presence of ICG, the mechanism by which it serves as a sonosensitiser or has the ability to enhance the cytoxicity of ICG-mediated PDT remains unclear. Regardless of the precise mechanism by which ultrasound enhances ICG-induced PDT effects on tumour growth, it is clear that the approach provides significant therapeutic benefit. Indeed the data presented here are supportive of emerging clinical observations that demonstrate significant benefit associated with the combined use of light and ultrasound in SPDT-based therapeutic regimes. In one notable clinical study reporting the treatment of advanced breast carcinoma using an experimental sonosensitiser (SFI®), patients were initially treated with light at 630nm (20 $mWcm^{-2}$) for a period of 30 min (total energy dose = 36 J) and subsequently with ultrasound at a frequency of 1 MHz, a power intensity of 2 Wcm^{-2} for a period of 20 min. (total energy dose = 2,400 J).[14] Although the number of patients was very limited in this trial (n = 3) and all had complications resulting from presentation with advanced disease, the SPDT was well tolerated and the limited discomfort experienced during treatment with ultrasound was alleviated by rotation of the transducer over the area to be treated. Hypothetically, using a similar approach incorporating the observations from our study above, the experimental sensitising drug, SFI® would be replaced with the already clinically-approved ICG and the light source at 630 nm would be replaced with a source emitting at 830 nm. These modifications would be expected to result in more efficient light transmission through tissues as a result of less tissue attenuation at this wavelength and this would in turn, result in more efficient photodynamic activation of the sensitiser. Ultrasound could then be applied in a manner similar to that described above in the clinical procedure and our data suggest that a more effective therapeutic effect would result. Although further characterisation of the phenomena presented in this paper is required, we believe the data support the suggestion that ICG may have a role to play either as a sonosensitiser in SDT-based regimes or in SPDT-based cancer treatment modalities.

Experimental Section

Cell culture and establishing tumours: RIF-1 (radiation induced fibrosarcoma) cells were employed in this study because they may be cultured *in vitro*, form tumours in syngeneic mice and have been used as treatment targets in our laboratory in related studies.[16] Essentially the cell line was routinely maintained in RPMI 1640 tissue culture medium supplemented with glutamine (GlutaMAX^{TM}, Invitrogen, UK) and 10 % (w/v) foetal bovine serum in a humidified 5 % CO_2 atmosphere at 37^{o}C. Single cell suspensions were prepared from monolayers by treating with 0.05% (w/v) trypsin containing 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS). Cells were subsequently harvested by centrifugation and washed in PBS prior to use. In order to establish tumours, 0.1ml aliquots of cells containing 1 x 10^6 cells in PBS were injected intradermally into the flanks of 6-8 week old recipient C3H/HeN mice. All animals were treated humanely and in accordance with licensed procedures under the UK Animals (Scientific Procedures) Act, 1986.

Determining cell viability: Cell viability was determined by direct counting using a haemocytometer and trypan blue-based dye exclusion to determine cell concentrations in stock cell suspensions. In addition, to determine the cytotoxic effects imparted by various treatments, cell viability was determined using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide)-based assay as described previously.[13,16] In all cases data derived from the MTT assay were confirmed using the trypan blue-based dye exclusion assay.

Treatment of cells *in vitro:* Single cell suspensions of RIF-1 cells, suspended in serum-containing medium, were dispensed into the wells of 96-well tissue culture plates to yield a concentration of 1 x 10^4 cells per well. Plates were incubated in a humidified 5 % CO_2 atmosphere at 37^{o}C overnight after which the complete medium was replaced with 195 µL serum free medium. ICG (Sigma Aldrich, UK) was prepared as a stock solution of 5 mgmL^{-1} in distilled water and 5 µL aliquots of this solution were dispensed into 195 µL of serum free medium in each well, yielding a final concentration of 125 µgmL^{-1}. Plates were incubated for a period of 1 h and the contents of each well were replaced with 200 µL of serum-containing medium. For treatment with light, a Photon laser diode module (PM-605P; Photonic Products, USA), emitting at 830 nm with a power density of 315 mWcm^{-2}, was placed underneath the tissue culture well to be treated. Care was taken to ensure that the beam was centred on the well using a CCD camera to visualise the beam. Each well was treated for 2 min. and cell viability was determined using the MTT assay referred to above after overnight incubation at 37^{o}C in a humidified 5 % CO_2 atmosphere. The treatment energy dose was derived from W (power) = J (energy) s^{-1} and expressed as Jcm^{-2}.

In cases where cells were treated with ultrasound, a SP100 Sonoporator (Sonidel Ltd., Ireland) was employed. This device emits ultrasound at a frequency of 1 MHz and has a pulse repetition rate of 100 Hz. Cells were prepared as indicated above in the wells of 96-well tissue culture plates, treated with ICG as described above and the bottom of each tissue culture well to be treated was placed in contact with the ultrasound-emitting probe of the SP100. Adequate contact between the bottom of the tissue culture well and the surface of the ultrasound transducer was facilitated using an ultrasound gel. In addition, care was taken to ensure that wells, surrounding the well to be treated, were not employed for tissue culture in order to prevent overlapping treatment with ultrasound as the area of the ultrasound transducer was slightly greater than the bottom of each tissue culture well. Cells were treated with ultrasound for 30 s at a duty cycle of 50 % (pulse frequency = 100 Hz) at various ultrasound power densities (intensities) and these are expressed in Wcm^{-2} (I_{SATP}: intensity; spatial average temporal peak). Following treatment, cells were incubated at 37^{o}C in a humidified 5 % CO_2 atmosphere overnight. Cell viability was determined using the MTT-based assay referred to above. The ultrasound energy dose expressed as Jcm^{-2} was calculated from the ultrasound power density emitted by the transducer and derived from W (power) = J (energy) s^{-1}.

In order to identify thermal effects elicited by treatments, the temperature in each well during treatment was recorded. This was achieved by introducing a hypodermic needle thermocouple (Hypo-33-1-T-G-SMP-M, Omega, UK) to each well. This was coupled to an analogue to digital recorder (ADC) (TC-80, Pico Technologies, UK) interfaced with a PC. Temperatures were recorded at 1s intervals during treatments and no greater than a 2-3^{o}C increase in temperature was observed during treatments.

Detection of intracellular ROS production: As shown previously, intracellular generation of ROS can be detected using the fluorescence indicator 2',7'-dichlorodihydrofluorescein diacetate (DHFA) and this is based on the oxidation of the hydrolysed non fluorogenic DHFA by ROS to a fluorogenic product.[13, 23] Essentially cell suspensions were dispensed into 96-well plates and grown until cells were 90 - 95 % confluent. Cell monolayers were treated with ICG at a final concentration of 125 µgmL^{-1} in PBS. Following a 1 h incubation period, the cells were washed lightly with PBS and 200 µL aliquot of serum free medium was placed in each well. Cells were either treated with light at a wavelength of 830 nm for 2 min. as described above or with ultrasound for 1 min at an ultrasound frequency of 1 MHz and a power density of 1.2 Wcm^{-2} using a 50 % duty cycle (pulse frequency = 100 Hz) as described above. Controls consisted of cells treated with light or ultrasound in the absence of ICG but with addition of an equivalent volume of ICG diluent (distilled water) or treated with ICG in the absence of light or ultrasound. Following treatment, the medium was removed from all wells, replaced with serum-containing medium and incubated at 37^{o}C in a humidified 5 % CO_2 atmosphere for 3 h. Treated cells were then washed with PBS and DHFA was added to each well at a concentration of 8.2 µM. Cells were incubated at 37^{o}C for 30 – 40 min., washed with PBS and following addition of complete medium, incubated at 37^{o}C for a further 30 min. Samples were then examined using a Nikon TDM inverted fluorescence microscope fitted with a x10 objective lens (mag. X100). Fluorescence was induced using an excitation wavelength of 492-495nm and an emission wavelength of 517-527 nm and digital images of fluorescent and white light fields were captured using a Nikon Coolpix 5000 camera.

Treatment of tumours in vivo: RIF-1 tumours where established in host animals as described above and permitted to grow until they reached an average volume of 0.3 cm^3. Tumours were then injected with 50 µl of a 2mgmL^{-1} solution of ICG in PBS. This was prepared by pre-dissolving ICG at a concentration of 8 mgmL^{-1}in distilled water and subsequently diluting in PBS. Animals were permitted to rest for a period of 30 min and were then treated with

light at 830 nm for 3 min using the source described above. During treatments, the beam was visualised using a digital camera to ensure coverage of the tumour area and the emitting surface of the source was placed at a distance of 1 cm from the surface of treated tumours. In cases where tumours were treated with ultrasound, contact between the emitting surface of the ultrasound transducer and the tumour surface was facilitated using an ultrasound gel. In all cases tumours were treated with ultrasound at a frequency of 1 MHz using a power density (intensity) of 3.5 Wcm^{-2} (I_{SATP}) for a period of 3 min and using a duty cycle of 40 % (pulse frequency = 100 Hz). Where animals were treated with a combination of light and ultrasound, the ultrasound treatment was carried out immediately after light treatment. In addition, where control animals were treated with stimuli in the absence of ICG, animals were injected with the diluent. During all treatments, animals were anaesthetised by inhalation using a 2 % (v/v) isofluorane stream in a 100 % oxygen carrier supplied at a flow rate of 2 $Lmin^{-1}$. After treatment, animals were allowed to recover from anaesthesia and the tumour volume was determined using the formula $4/3(\pi R^3)$, where R was determined from the average of 3-leg measurements taken at the relevant times.

Statistical analysis: Statistical analysis of significance was performed using ANOVA and data groups were compared using the Tukey Multiple Comparison Test (MCT) in GraphPad Prism version 4.

Acknowledgements

During this work Dr. N. Nomikou was partially funded under a EuroStars Program project administered by Enterprise Ireland.

Keywords: indocyanine green · cytotoxicity· sonodynamic therapy · cancer · ultrasound

[1] T.J. Dougherty, G.B. Grindley, R. Friel, K.R. Weishaupt, D.G. Boyle. *Natl. Cancer Inst.* **1975**, *55*, 115-121.

[2] T.J. Dougherty, J.E> Kaufman, A. Goldfarb, K.R. Weishaupt, D. Boyle, A. Mittleman. *Cancer Res.* **1978**, *38*, 2628-2635.

[3] J.F. Kelly, M.E. Snell. *J.Urol.* **1976**,*115*, 150-151.

[4] P. Agostinis, K. Berg, K.A. Cengel, T.H.Foster, A.W. Girotti, S.O.Gollnik, S.M.Hahn, M.R. Hamblin, A.Juzeniene, D.Kessel, M. Korbelik, J.Moan, P.Mroz, D. Nowis, J.Peitte, B.C.Wilson, J. Golab. *CA-Cancer J. Clin.* **2011**, *61*, 250-281.

[5] G.*Porini*, A. Giovannini, C.Amato, A.Ioni, M.Pantanetti. *Ophthalmol.* **2003**,*110*, 674-680.

[6] O. Bozkulak, R.F. Yamaci, O.Tabakoglu, M. Gulsoy. *Photodiagnosis Photodyn. Ther.* **2009***, 6*, 117-121.

[7] A.M. Mamoon, A.M. Gamal-Eldeen, M.E> Ruppel, R.J. Smith, T.Tsang, L.M. Miller. *Photodiagnosis Photodyn. Ther.* **2009**, *6*, 105-116.

[8] T.Funayawa, M.Sakane, T.Abe, N.Ochiai. *Photomed. Laser Surg.* **2012**, *30*, 47-53.

[9] S. Umemura, N.Yumita, K.Umemura, N.Nishigaki. *Cancer Chem. Pharmacol.* **1999**,*43,* 389-393.

[10] N.Miyoshi, T.Igarashi, P. Riesz. *Ultrason. Sonochem.* **2000**,*7,* 121-124.

[11] I.Rosenthal, J.Z.Sostaric, P.Riesz. *Ultrason. Sonochem.* **2004**,*11,* 349-363.

[12] W.Hiraoka, H.Honda, L.B.Feril, K.Kudo, T.Kondo. *Ultrason. Sonochem.* **2006***, 13,* 535-542.

[13] B.McCaughan, C.Rouanet, C.Fowley, N.Nomikou, A.P.McHale, P.A.McCarron, J.F.Callan. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5750-5752.

[14] X.Wang, W.Zhang, Z.Xu, Y.Luo, D.Mitchell, R.W.Moss. *Intg. Cancer Ther.* **2009**, *8,* 283-287.

[15] D.C.Shackley, C.Whitehurst, J.V.Moore, N.J.R. Georg, C.D.Betts, N.W. Clarke. *BJU Intl.* **2000**, *86,* 638-643.

[16] N.Nomikou, P.Tiwari, T.Trehan, K.Gulati, A.P.McHale. *Acta Biomat.* **2012***, 8,* 1273-1280.

[17] K. Skrivanova, J.Skorpikova, J.Svihalek, V.Morstein, R.Janisch. *Photochem. Photobiol. B: Biology,* **2000**,*85,* 150-154.

[18] X.Zheng, F.Zhou, B.Wu, W.R.Chen, D.Xing. *Mol. Pharm.* **2012**, *5,* 514-522.

[19] J.N.Kenyon, R.J.Fuller. *Curr. Drug. Ther.* **2011**, *6,* 12-16.

[20] N.Yumita, K.Okuraira, Y.Momose, S.Umemura. *Cancer Chemother. Pharmacol.* **2010**, *66,* 1071-1078.

[21] H. Honda, T.Kondo, Q.L.Zhao, L.B.Feril, H.Hitagawa. *Ultrasound Med. Biol.* **2004**, *30*, 683-692.

[22] W.Baumler, C.Ables, S.Karrer, T.Weib, H.Messmann, M. Landthaler, R.M. Szeimies. *B.J. Cancer.* **1999**, *80,* 360-363.

[23] Y.He, X.Xia, X.Xu, H.Yu, D.Bai, J.Xiang, A.W. Leung. *Ultrasonics*, **2010**, *66*, 1071-1078.

[24] K.Hensel, M.P. Mienkina, G.Schmitz. *Ultrasound Med. Biol.* **2011**, *37,* 2105-2115.

[25] A.M.Rollan-Haro, A.Smyth, P.Hughes, C.N.Reid, A.P.McHale. *Cancer Lett.* **2005**,*222,* 49-55.

[26] J.L.Larkin, D.Soden, C.Collins, M.Tangney, J.M.Preston, L.J. Russell, A.P.McHale, C.Dunne, G.C.O'Sullivan. *Eur. J. Cancer,* **2005**, *41*, 1339-1348.

[27] X.Wang, Y.Wang, P.Wang, X.Cheng, Q. Liu. *Ultrasonics,* **2011**, *51,* 539-546.

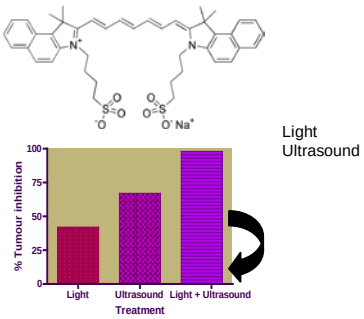
[28] Y.S.Li, E.Davidson, C.N.Reid, A.P.McHale. *Cancer Lett.* **2009**, *273,* 62-69.

[29] N.Yumita, N. Ikuyama, K.Sasaki, S.I. Umemura. *Cancer Chemother.Pharmacol.* **2007**, *60*, 891-897.

Received: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

FULL PAPERS

An ultrasound-responsive cancer therapeutic: We show that the near IR-absorbing dye, indocyanine green may be used to sensitiser cancer cells and tissues to ultrasound. This phenomenon may be used to directly treat solid tumours or may be combined with photodynamic therapy-based approaches to provide enhanced therapeutic outcomes.



*Dr.N.Nomikou, C.Sterrett, C.Arthur, Dr.B.McCaughan,Dr.J.F.Callan, Prof.A.P.McHale**

Page No. – Page No.

The effects of ultrasound and light on indocyanine green-treated tumour cells and tissues