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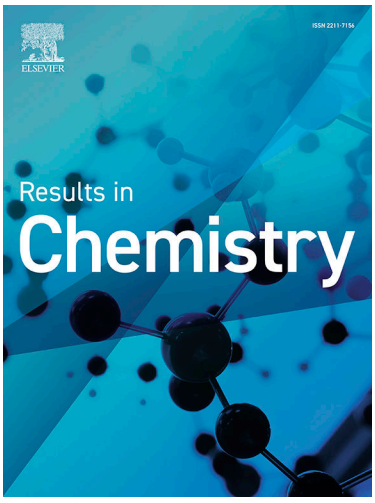

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Cell-Cell Death Communication by Signals Passing Through Non-Aqueous Environments: A Reply

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ABSTRACT

The effects of the emission of low intensity light from cells and organelles, known as biophotons, or ultraweak photon emission, are not well understood and subject to debate. Potapovich & Kostyuk recently proposed that the induction of oxidative stress generates non-chemical death signals which can induce cell death in neighbouring, chemically isolated cells (termed detector cells). Given the significance of these results, here we attempt to replicate their findings. We found treatment of "inductor cells" with duroquinone dissolved in ethanol does indeed induce significant cell death in neighbouring "detector" cells relative to distant control cells (64.53% ± 14.42 vs 99.72% ± 6.09 cell viability), closely reproducing their original results. However, this was no longer true if the induction drug was dissolved in a less volatile solvent, suggesting that their original findings may have been a result of volatile solvent-based transmission as opposed to light-based non-chemical signalling.

INTRODUCTION

Cells spontaneously and continuously emit low intensity light, known as "biophotons", in a phenomenon known as ultraweak photon emission (UPE) or BioAutoLuminesence (BAL). These photons, spanning the spectrum from ultraviolet (UV) to near infrared (NIR)[1], appear to be a by-product of reactive oxygen species (ROS) production, itself a by-product of cellular metabolism. The oxidation of biomolecules, including proteins, lipids and nucleic acids, by ROS[2] leads to a series of reactions forming energetically excited carbonyls. Energy transfer from such species to nearby chromophores or molecular oxygen then produces excited chromophores or singlet oxygen respectively. The decay of these excited species to their ground states in turn yields the emission of biophotons [3] [4]. As the mitochondria are the chief source of ROS within a cell, they are generally regarded as a major source of biophotons[5]. A precise role, if indeed if one exists, for biophotons remains unclear – yet a common consensus is that they may facilitate non-chemical signalling between cells or organelles. Numerous studies have demonstrated evidence for such pathways in an array of models, showing biophotons influencing the respiratory rate in isolated mitochondria[6], activating chemically isolated neutrophils[7], influencing levels of cellular protein and morphology[8], and perturbing calcium signalling[9]. Yet the role of biophotons as a means of non-chemical communication remains disputed, with arguments against including a failure to eliminate other means of communication such as volatile transmission, or that the intensity of biophotonic emission is far too low for cells or organelles to register over ambient noise[10]. However, as we have suggested, it may be no coincidence that many of the metabolic pathways that life depends on utilise chromophoric compounds – suggesting photonic dissipation of energy coupled to electron transfer is fundamental due to their quantum structure[11]; the implications are thus that internal generation, and absorption, of UPE is certainly a possibility.

Recently, Potapovich & Kostyuk reported that the stimulated production of signals from cells undergoing oxidative stress may induce apoptosis in neighbouring, chemically separate populations of

cells. They proposed that these signals may be biophotons, although there was no direct detection of biophotons in the article[12]. The authors seeded two cancer and two non-cancer cell lines into columns of clear polystyrene 96 well plates (See Supplementary Figure 1). Cells in the central column, referred to as "inductor" cells are surrounded each side by "detector" cells. The "inductor" cells were treated with drugs known to induce oxidative stress. To determine whether there was measurable biophoton communication between chemically isolated cells, cell viability in the "detector" cells, was compared to that of the control cells seeded at either end of the plate. The authors observed a significant decrease in "detector" cell viability compared to the control, suggesting that the biophoton emission was inducing cell death.

These results have significant implications not only in terms of biophotonic and non-chemical signalling, but for the countless studies that investigate cell death, viability, or oxidative stress in 96 well polystyrene plates. We therefore set out to reproduce the results of this study by replicating the cell viability experiments.

MATERIALS AND METHODS

Cell Culture

MCF7 Cells were grown in Minimum Essential Media (MEM) (Merck, UK) supplemented with 10% Foetal Bovine Serum (FBS), 1% l-glutamine and 1% penicillin/streptomycin. Cells were maintained in a humified 5% CO_2 environment.

Experimental Set up:

MCF7 cells were seeded in columns 2, 5, 6, 7 and 10 of 96-well polystyrene plates (See Supplementary Figure 1). at an initial seeding density of $3.0x10^4$ cells per well to match the set-up described by Potapovich & Kostyuk[12]. Cells in columns (8 wells per column) 2 and 10 (Controls) and 5 and 7 (Detectors) were treated with 2% volume/volume (v/v) ethanol or 2% v/v DMF in serum-free MEM for 24 hours. Cells in column 6 (Inductor) were treated with 200 µM duroquinone prepared in ethanol or dimethylformamide (DMF), or 100 µM cisplatin in serum-free media for 24 hours prepared in ethanol or DMF.

Viability Assay:

Following treatment, cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Treatments were aspirated from cells, washed with PBS, and replaced with serum-free MEM. Wells were spiked with 5 mg/mL MTT solution and incubated for 3 hours at 37°C 5% CO_2. Solution was removed from wells, and the resultant formazan crystals dissolved in DMSO. Absorbance was measured at 570 nm minus background at 690 nm using the BMG Optima.

Statistics:

Differences in mean cell viability were analysed with One-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism 8.2.0, USA).

RESULTS

The initial experiment was designed to replicate Potapovich & Kostyuk's original experiment as closely as possible: inductor MCF7 cells were treated with 200 µM duroquinone, whilst control and detector cells were treated 2% v/v ethanol. Cell viability was measured 24 hours after treatment. The results, shown in **Figure 1**, indicate that direct addition of duroquinone, dissolved in ethanol, induced a significant decrease in inductor cell viability compared to control cells (Mean decrease of 101.30% ± 4.17, p = <0.0001). Moreover, it also brought about a significant reduction in viability albeit to a lesser extent in the detector cells compared to control cells (Mean decrease of 30.67% ± 4.17, p = <0.0001). These results clearly reproduced those reported by Potapovich & Kostyuk, suggesting some form of non-aqueous communication between the inductor and detector cells.

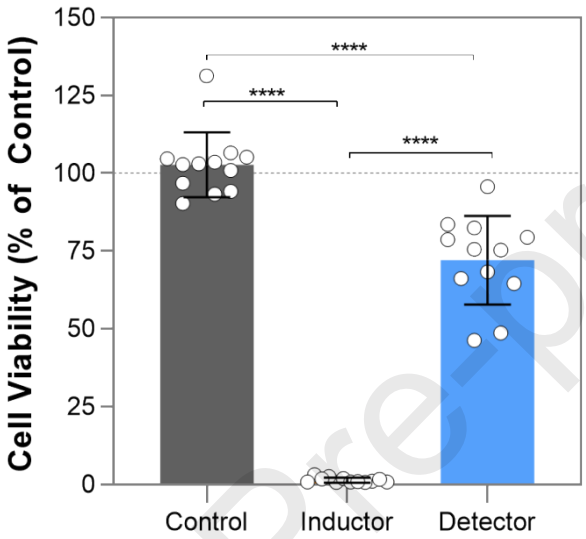

Figure 1: The effect of duroquinone inductor cell treatment on detector cell viability*. MCF7 "inductor" cells were treated with 200 µM duroquinone prepared in ethanol for 24 hours. Cell viability assessed via MTT Assay. Data presented as mean cell viability expressed as a percentage of the control ± Standard Deviation (SD). Differences between means analysed via one-way ANOVA followed by Tukey's multiple comparison test. Differences considered significant when $p < 0.05$. ****: $p < 0.0001$. $n = 12$.*

To explore this phenomenon further, the experiment was repeated with an alternative cytotoxic drug: the chemotherapeutic agent cisplatin. The results (**Figure 2**) show that whilst cisplatin induced a substantial and significant decrease in inductor cell viability compared to the control (Mean decrease of 88.71% ± 2.57, p = < 0.0001), there was no significant difference between detector and control cell viability (Mean decrease of 1.04% ± 2.57, p = 0.914). Thus, the apparent non-aqueous signalling pathway seen using duroquinone was absent using cisplatin, despite both drugs exhibiting substantial cytotoxic effects on the inductor cells.

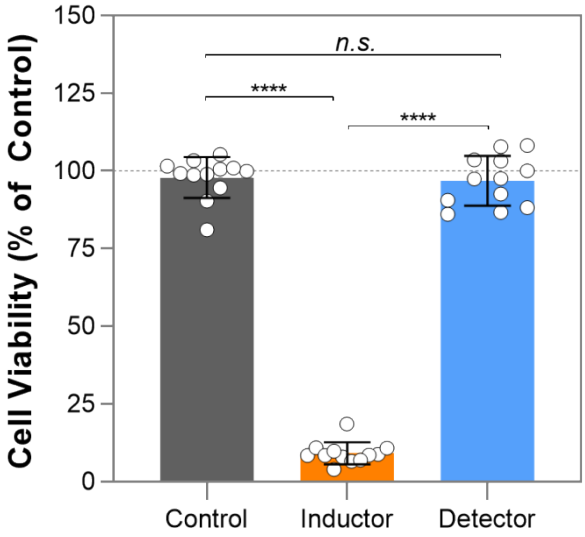

Figure 2: The effect of cisplatin inductor cell treatment on detector cell viability*. MCF7 "inductor" cells were treated with 100 µM cisplatin prepared in DMF for 24 hours. Cell viability assessed via MTT Assay. Data presented as mean cell viability expressed as a percentage of the control ± Standard Deviation (SD). Differences between means analysed via one-way ANOVA followed by Tukey's multiple comparison test. Differences considered significant when $p < 0.05$. ****: $p < 0.0001$. n.s.: not significant. $n = 12$*

The drugs, both capable of inducing oxidative stress in cells, were initially prepared in different solvents as per standard practice: duroquinone in ethanol, and cisplatin in DMF. It was therefore hypothesised that the choice of solvent may be influencing the results. The experiment was therefore repeated, but this time duroquinone was prepared in DMF, while cisplatin in ethanol. Results from these experiments are shown in **Figure 3**. When duroquinone was prepared in DMF (**Figure 3A**), there was no significant difference in detector cell viability compared to the control (Mean decrease of 0.82 ± 3.80%, $p = 0.975$), despite duroquinone itself causing a substantial and significant decrease in inductor cell viability (Mean decrease of 95.80 ± 3.80, $p = <0.0001$). Conversely, when cisplatin was prepared in ethanol (**Figure 3B**), there was a significant decrease in detector cell viability compared to the control (Mean decrease of 25.10% ± 6.30, $p = 0.0172$), as well as the expected decrease in inductor cell viability compared to the control (Mean decrease of 67.99% ± 6.02, $p = <0.0001$). These results appear to suggest that results reported by Potapovich & Kostyuk may relate to a side-effect of unwarranted chemical communication rather than biophoton communication.

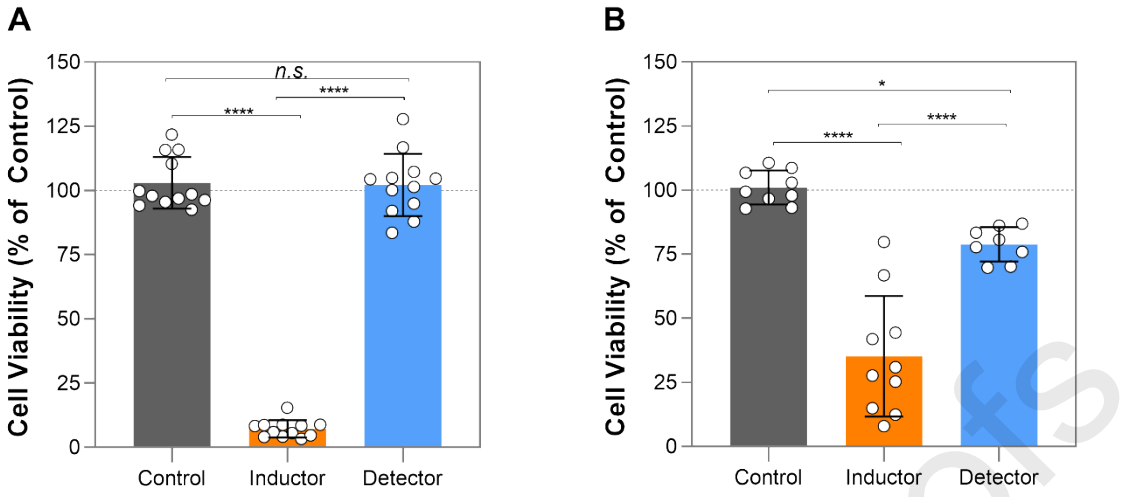

Figure 3: The effect of solvent choice on inductor cell treatment on Detector cell viability*. MCF7 cells were treated with 200 µM duroquinone dissolved in DMF **(A)** or 100 µM cisplatin dissolved in ethanol **(B)** for 24 hours. Cell viability assessed via MTT Assay. Data presented as mean cell viability expressed as a percentage of the control. Differences between means analysed via one-way ANOVA followed by Tukey's multiple comparison test. Differences considered significant when $p < 0.05$. *: $p < 0.05$, ****: $p < 0.0001$. n.s.: not significant. $n = 12$.*

DISCUSSION

Interaction between light and mammalian cellular components is well established and is remerging in the field of photobiomodulation (PBM, formally known as low-level laser therapy (LLLT)), in which specific wavelengths of light are used in a variety of medicinal purposes[13]. Cells contain a large range of chromophores, such as cytochromes, NADH and FAD, Fe-S centres, interactions with which exert a spectrum of physiological effects. Broadly, higher intensity photons ranged towards the blue and UV regions of the electromagnetic spectrum appear to be associated with DNA damage, increased ROS production, and decreased adenosine triphosphate (ATP) synthesis[14], whilst red to near-infrared (NIR) light causes increased ATP production, and an increase in ROS production that is associated with a downstream epigenetic influence that confers increased cellular proliferation[15]. However, lower dose UV can be viewed as hormetic, as it induces multiple adaptive changes; the fact that NADH absorbs in the UV, is fluorescent, and can eject an electron, and plays a key role in both calcium and sirtuin modulation is perhaps suggestive[11]. Whether biophotons play a similar role remains debated, particularly as their intensity is orders of magnitude below what is used in PBM applications. Studies have demonstrated that biophotons can enable non-chemical signalling between cells, influencing proliferation[8], calcium signalling[16], cellular morphology[17], and mitochondrial function[6,7]. As the production of biophotons is linked to the production of ROS, it has been suggested that they may play a role in the propagation of stress signals. Potapovich & Kostyuk were the first to report that this effect may even lead to the induction of apoptosis in a distant, non-chemical fashion.

Our initial result reproduced the findings of Potapovich & Kostyuk, producing what appears to be evidence of a form of non-aqueous communication, possibly facilitated by biophotons, between cells undergoing oxidative stress and their adjacent, untreated neighbours (although it should be noted that there was no direct detection of biophotons in the article). However, when probed further we found that the effect could only be replicated when each cytotoxin was prepared in ethanol, but not when DMF was used as the solvent. This raises the question of why the apparent biophotonic, non-chemical signalling effect is only present when each drug is prepared in ethanol. Each drug induces oxidative

stress, albeit through different mechanisms: duroquinone increases ROS production by stimulating NADPH oxidases such as Nox4[18], and cisplatin inhibits mitochondrial DNA transcription, ultimately increasing ROS through subsequent mitochondrial dysfunction[19]. Furthermore, each drug was observed in this study to induce a substantial decrease in viability in the directly treated detector cells, independent of the solvent used. Both DMF and ethanol, whilst toxic at higher doses, had no significant effect on the viability of the control cells. Where the solvents do diverge is in their volatility: ethanol, with a boiling point of 78°C is substantially more volatile than DMF, which has a boiling point of 153°C. Thus, as the "signalling effect" reported by Potapovich & Kostyuk and reproduced in the current study only takes place in ethanol but not DMF, we are led to conclude that it is the volatility of the solvent (what chemical it may be carrying) that is inducing the effect, rather than the emission and absorption of biophotons. Volatile communication, perhaps most predominantly associated with plant cells[20], has been observed in physically disconnected cultures of cancer cells[21]. Indeed, chemical communication of this manner has been proposed as an alternative mechanism to explain the observed effect in similar "non-chemical communication" experiments[10].

CONCLUSIONS

To conclude, our results indicate that the observed "non-chemical" signalling effect identified by Potapovich & Kostyuk is unlikely to be caused through biophotons, and instead may be driven by volatile communication between the physically disconnected populations of cells. It is also possible that other factors, such as the presence of photon-absorbing compounds in the media, such antibiotics, could also inhibit a form of UPE inter-cellular communication. However, despite this, biophotonic communication or signalling remains an understudied, yet fascinating field which warrants closer investigation and could well still yield a phase shift in our understanding of biology with the advent of newer and more robust techniques Certainly, theoretically, it is possible and could support that life is perhaps using non-trivial quantum effects.

FUNDING

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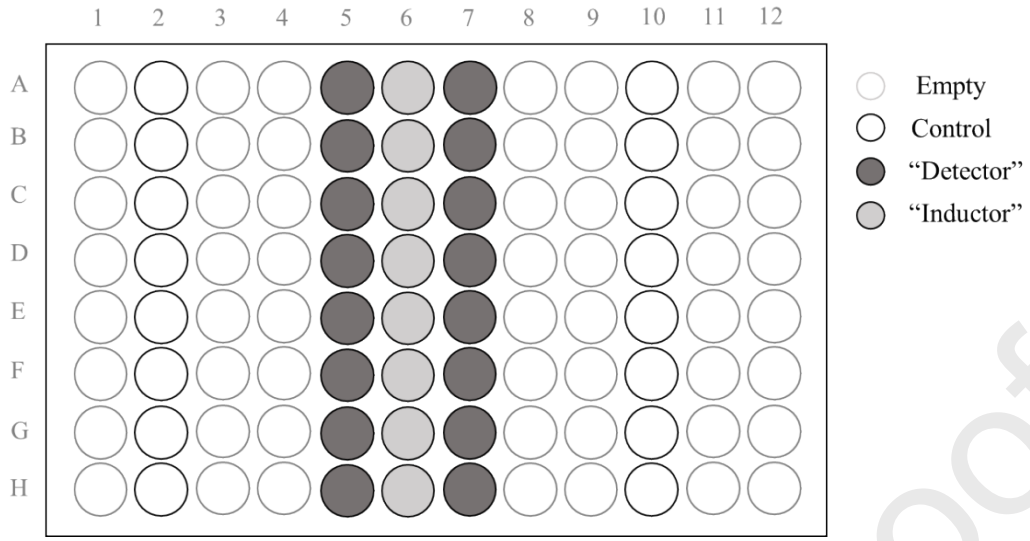

***Supplementary Figure 1: Plate layout as per Potapovich & Kostyuk**. Cells were seeded in columns 2,5,6,7 and 10 of a 96-well clear polystyrene plate. Column 6, the "Detector" wells, were treated with oxidative stress-inducing drugs. The effect of this treatment was measured in cells seeded in columns 5 and 6, the "Detector" wells, compared to cells seeded in columns 2 and 10, the control wells.*

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Rhys Mould: Conceptualization, Methodology, Investigation, Formal Analysis, Visualisation, Writing – Original Draft

Louise Thomas: Writing – Review and Editing

Geoffrey Guy: Conceptualization, Funding acquisition, Writing – Review and Editing

Alistair Nunn: Conceptualization, Funding acquisition, Writing – Review and Editing

Jimmy Bell: Supervision, Conceptualization, Funding acquisition, Writing – Review and Editing

- Cells emit light (biophotons), by the process of ultraweak photonic emission (UPE)
- UPE may have a role in cellular signalling
- Previous publication suggested UPE induces cell death in neighbouring cells
- Our results show that volatile communication is however responsible for this effect