Effects of combined photochemical internalization and hyperthermia are sensitively dependent on radiant exposure

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ABSTRACT

Combination therapies of photochemical internalization (PCI) and hyperthermia (HT) were investigated in an *in vitro* system consisting of human glioma spheroids. Spheroids (350-400 μm dia.) were irradiated with 670 nm laser light in an incubator at temperatures ranging from 37 to 50 ^{o}C. For each temperature investigated (45 min. heating time), spheroids were divided into 5 groups: control, dark control, bleomycin-only, photodynamic therapy (PDT), and PCI. PDT and PCI spheroids were exposed to radiant exposures ranging from 0.5-3.0 J cm^{-2} using an irradiance of 5 mW cm^{-2}. Toxicity was evaluated from spheroid growth kinetics. The combination of PCI and HT resulted in growth delays over a very narrow range of radiant exposures (1.5 – 2.5 J cm^{-2}) and temperatures (40 – 42 ^{o}C).

Keywords: photochemical internalization, hyperthermia, photodynamic therapy, human glioma spheroids

1. INTRODUCTION

PCI is a technique which improves the utilization of macromolecules in cancer therapy in a site-specific manner[1]. The concept is based on the use of specially designed photosensitizers, which localize preferentially in the membranes of endocytic vesicles. Upon exposure to light of appropriate wavelengths (usually in the far red of the visible spectrum), the photosensitizers induce the formation of reactive oxygen species, in particular, singlet molecular oxygen. Similar photochemical reactions are the basis for PDT which is used for the treatment of a wide variety of cancers and non-cancerous conditions[2]. The photooxidation of the endocytic membranes leads to the release of the contents of these vesicles into the cytosol. In this way, macromolecules encapsulated by the vesicles will reach the cytosol and exert their biological activity instead of being degraded by lysosomal hydrolases. The PCI-based relocation and activation of the macromolecules has the advantage of minimal side effects since the effect is localized to the area exposed to light. The endosomal escape of macromolecules including genes, oligonucleotides and proteins by means of PCI has been documented both *in vitro* and *in vivo* and has been shown to increase the therapeutic effect in a synergistic manner[3-6].

HT has been used in the treatment of a variety of cancers, either alone or as an adjuvant, for a number of decades[7]. HT-induced cytotoxicity depends on a number of factors including temperature and heating time. HT typically occurs in a temperature range of 40 to 44 ^{o}C over times ranging from 30 – 60 min[8]. Impaired blood flow, characteristic of most tumors, reduces their thermoregulatory abilities resulting in preferential heating during HT. Furthermore, reduced blood flow results in both oxygen and nutrient deprivation in tumors thus making them more heat sensitive[9]. At the cellular level, elevated temperatures have been shown to inhibit both protein and DNA synthesis. Inhibition of DNA repair enzymes has also been observed as has damage to the cell membrane[10].

HT is a strong sensitizer of radiotherapy and a number of cytotoxic drugs: combination therapies consisting of HT and radiotherapy or HT and chemotherapy have been shown to produce additive effects, as long as the treatments are

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administered without a significant time gap[8]. PDT has been used in combination with HT both *in vivo* and *in vitro*. For example, Dereski et al.[7] found that the effects were greater than additive when HT was given post-PDT. Hyperthermic temperatures were found to inhibit repair of sub-lethal damage thereby making hypoxic cells more sensitive to PDT. Using a murine mammary adenocarcinoma model, Chen et al.[11] showed that the combination of PDT and HT produced a synergetic tumor response. Hirschberg et al.[12] examined synergistic effects of 5-aminolevulinic acid-mediated PDT and HT concurrently on human and rat glioma spheroids. The results showed that, when administered separately, PDT and HT were not very effective, however, concurrent administration of the two treatment modalities resulted in significant toxicity.

The effects of combined PCI and HT were examined in an *in vitro* system consisting of human glioma spheroids. Radiant exposures and temperatures were varied in order to evaluate optimum light-temperature combinations as determined from spheroid growth kinetics.

2. MATERIALS AND METHODS

2.1 Cells

The human glioma cell line (ACBT; University of California, Irvine) was obtained from biopsy. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES buffer (pH 7.4), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37°C and 5% CO_2.

2.2 Spheroid formation

Spheroids were formed using a modified centrifugation method first described by Ivascu and Kubbies[13]. Briefly, 5 000 ACBT cells were detached from the growth flask; the exact number of cells was determined from coulter counting. The cells were pipetted into one well of a 96-well plate. Each round bottomed well was coated with matrigel and the plate was centrifuged at 1000g for 10 min. Following centrifugation, the well plate was placed in a CO_2 incubator for 48 h to allow the spheroids to take on their usual 3-dimensional shape.

2.3 PDT and PCI treatments

After 48 hours, 350-400 μm diameter spheroids were transferred from the 96-well plate to 35 mm irradiation dishes where they were incubated in 1 μg/mL photosensitizer ($AlPcS_{2a}$; Frontier Scientific Inc., Logan, UT) for 18 h at 37 °C and 5% CO_2. Following incubation, spheroids were washed and placed into new medium. Spheroids in the PCI group were then subjected to an additional incubation in 0.25 μg/mL of bleomycin for 4 h. Following incubation, spheroids in both PDT and PCI groups were irradiated with 670 nm light from a photodiode laser (Intense, New Brunswick, NJ) coupled to a 200 μm diameter optical fiber fitted with a micro-lens. In all cases, spheroids were subjected to an irradiance of 5 mW cm^{-2} for a range of times required to deliver radiant exposures ranging from 0.5 to 3.0 J cm^{-2}. All irradiations were performed at 37 °C in a temperature controlled incubator. This was accomplished by inserting the laser-coupled optical fiber through a small aperture located at the top of the incubator.

Following light exposure, 16 spheroids from each dish were placed into separate wells of an agarose coated 48-well plate containing 750 μL of DMEM. Spheroid growth kinetics were monitored by recording two orthogonal diameters of each spheroid using a microscope with a calibrated eyepiece micrometer. Spheroid growth was measured 24 h after treatment and then twice a week over a 29-day period. After each measurement, 400 μL of DMEM was removed and replaced with 400 μl of fresh DMEM to provide the spheroids with adequate nutrients.

2.4 Hyperthermia

The 35 mm dishes containing medium and spheroids were placed in a temperature controlled incubator for 40-50 min. at temperatures ranging from 40 to 50° C. In the case of the combined treatments (PDT + HT; PCI + HT), spheroids were placed in the incubator 40 min. prior to light irradiation. Irradiances and radiant exposures were identical to those

described previously. Following treatment, spheroids were placed in a 48-well plate and monitored as previously described. In all cases, the temperature of the incubator was monitored using a standard laboratory thermometer.

2.5 Statistical analysis

All data were analyzed and graphed using Microsoft Excel. The two orthogonal diameter averages were used to calculate the mean diameter of the spheroids. Each experiment included approximately 16 spheroids per group and was repeated 2-3 times and the average volume was calculated ($V = 4/3\ \pi r^3$). Standard errors were used throughout to calculate the error bars.

3. RESULTS

3.1 PCI at a radiant exposure of 0.5 J cm^{-2}

As illustrated in Figure 1, complete spheroid growth inhibition was observed in all groups (including controls) at 45 ^{o}C while significant growth was observed for all spheroids at 40 ^{o}C. The data indicate that the threshold for complete heat-induced cell death occurs somewhere between 40 and 45 ^{o}C. No significant difference in growth kinetics was observed between the two treatment groups (PDT and PCI) at the lower temperature. This suggests that the radiant exposure was inadequate.

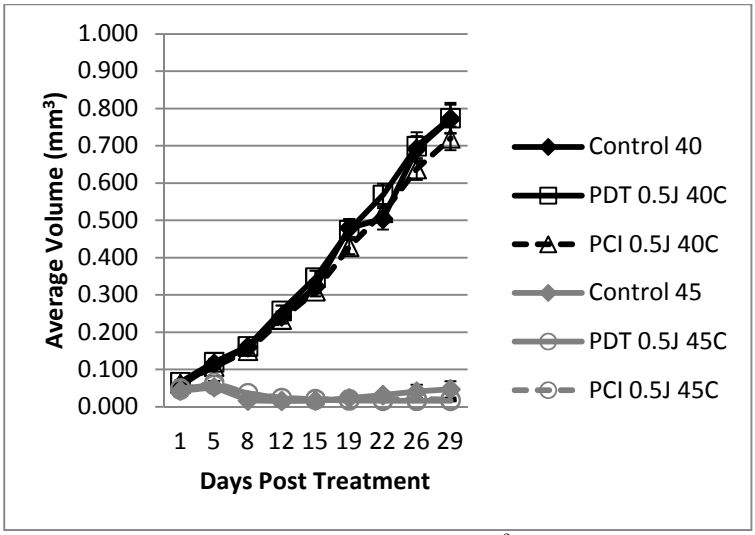

Figure 1. Average spheroid volume over a 29-day period at 40 and 45 ^{o}C for a radiant exposure of 0.5 J cm^{-2}.

3.2 PCI at a radiant exposure of 1.5 J cm^{-2}

Figure 2 shows that PDT+HT and PCI+HT at 1.5 J cm^{-2} and 40 ^{o}C were more effective than treatments at the lower radiant exposure (0.5 J cm^{-2}; Figure 1). For both temperatures investigated, PCI induced greater growth inhibition than PDT. Both PDT and PCI were more effective at the higher temperature thus illustrating the therapeutic advantage conferred by hyperthermia. The effectiveness of combined PCI and hyperthermia at 1.5 J cm^{-2} can be appreciated by comparing the final spheroid volume (ca. 0.5 mm^3) to the final volume obtained for the combined treatments at 0.5 J cm^{-2} (ca. 0.7 mm^3: Figure 1).

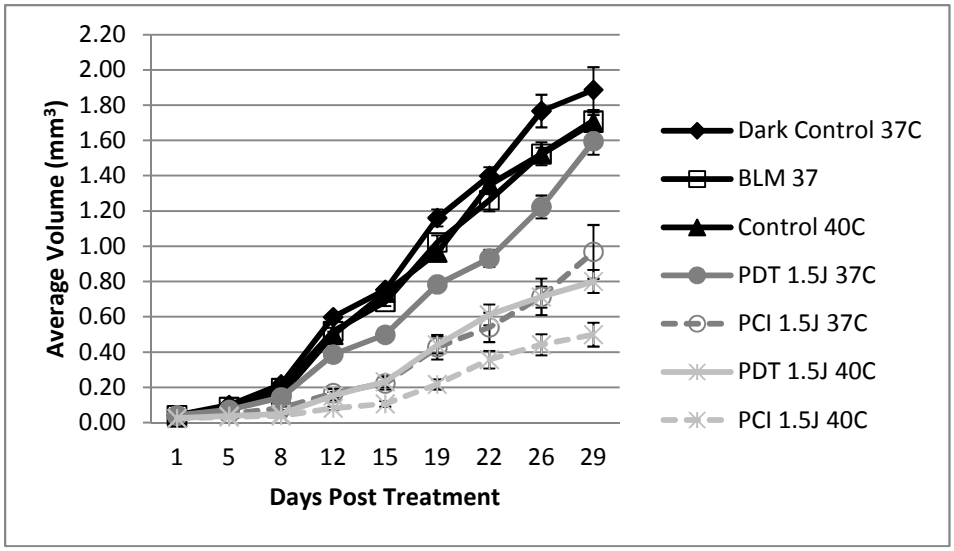

Figure 2. Average spheroid volume over a 29-day period at 37 and 40 ^{o}C for a radiant exposure of 1.5 J cm^{-2}. Dark controls correspond to spheroids incubated with photosensitizer. BLM denotes spheroids incubated with bleomycin only.

3.3 PCI at a radiant exposure of 2.5 J cm^{-2}

The enhanced efficacy at 2.5 J cm^{-2} is evident from the growth kinetics data in Figures 3 and 4. Of particular relevance is the combined PCI – hyperthermia data in Figure 4 showing almost complete spheroid growth inhibition as evidenced from the mean spheroid volume at the end of the observation period (0.2 mm^3).

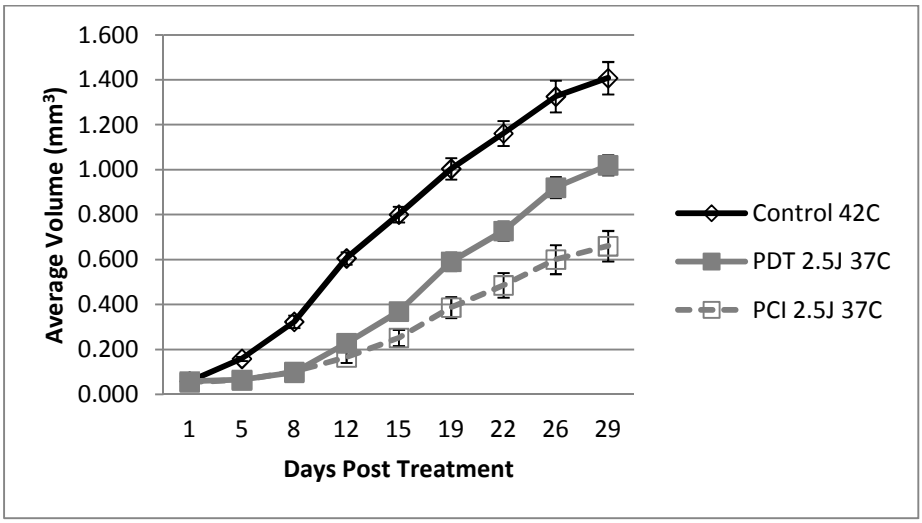

Figure 3. Average spheroid volume over a 29-day period at 37 ^{o}C for a radiant exposure of 2.5 J cm^{-2}.

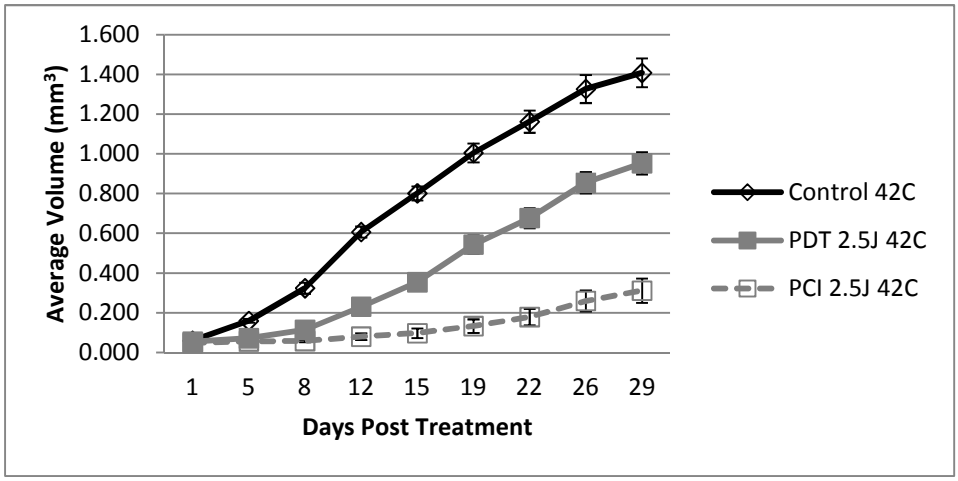

Figure 4. Average spheroid volume over a 29-day period at 42 °C for a radiant exposure of 2.5 J cm^{-2}.

3.4 PCI at a radiant exposure of 3.0 J cm^{-2}

Complete growth inhibition was observed in all treatment groups, even at physiological temperatures (37 °C; Figure 5). The data suggest that this light level was too high.

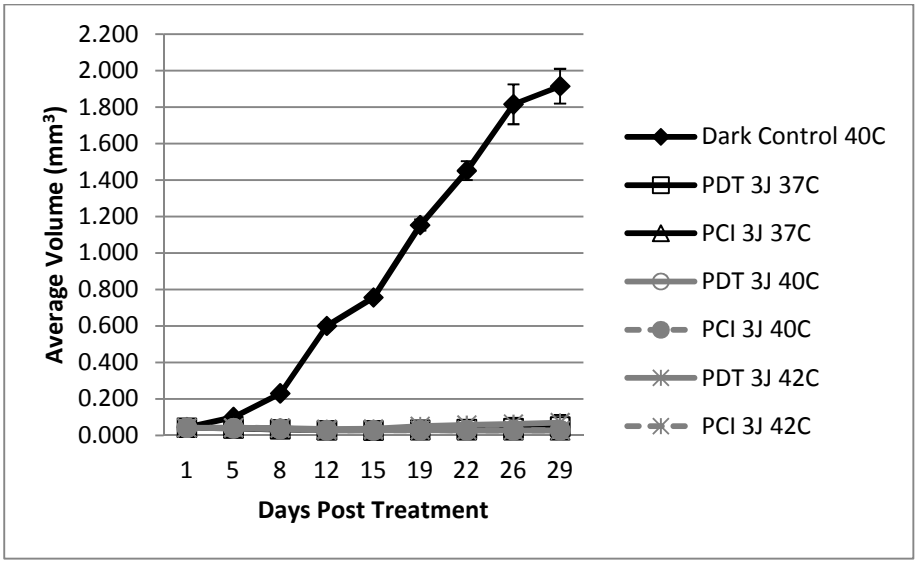

Figure 5. Average spheroid volume over a 29-day period at 37, 40 and 42 °C for a radiant exposure of 3.0 J cm^{-2}.

4. DISCUSSION

PCI is a specialized application of PDT that may be used to enhance the delivery of macromolecules including chemotherapeutic agents such as bleomycin. PCI is appealing for a number of reasons including: (1) the lack of size restrictions on the therapeutic molecules to be delivered, (2) high site specificity which confines the biological effect to light-illuminated volumes thus minimizing side effects of the drug, (3) increased therapeutic efficacy of a wide range of macromolecules allowing for the possibility of lower drug doses resulting in decreased morbidity, (4) high suitability for

combination with other strategies for targeted drug delivery, thereby increasing the potential for further therapeutic improvements[1,3, 14-16]

BLM is a 1.5 kDa glycopeptic antibiotic that has been used in a number of chemotherapeutic regimens for the treatment of a variety of cancers including, squamous cell carcinomas of the head and neck, esophagus, bronchus, and skin, as well as Hodgkin's and non-Hodgkin's lymphoma[3]. BLM has also been used in brain tumor treatments since the early 1970's and is delivered directly to the tumor since it does not traverse the intact blood-brain barrier[17]. The molecule exerts its toxic effects by causing single and double-strand DNA breaks[18,19]. BLM induces single- and double-strand DNA breaks with a 10:1 ratio resulting in cell arrest in the G2-M phase. To induce these effects, BLM must pass through the cellular membrane and diffuse to the nucleus[20]. BLM has a high intrinsic cytotoxicity, but since it cannot diffuse through the cell membrane, its capabilities are limited[21]. Due to the limited penetration of BLM through the plasma membrane, tumor cells have a relatively low sensitivity to this anti-cancer agent. BLM enters cells via receptor-mediated endocytosis resulting in its encapsulation in endosomes. Once in the cytosol, most endosomes end up in the lysosomes along with their contents. Therefore, the vast majority of BLM never reaches the DNA[22]. Due to its poor efficacy, BLM is typically used in combination with other agents[21]. Alternatively, BLM efficacy can be enhanced via the PCI technique.

PCI-mediated delivery of bleomycin has been investigated previously in glioma spheroids[23] and the results are in qualitative agreement with the findings of the present work, i.e., at sufficient radiant exposures (1.5 J cm^{-2}), PCI was found to enhance the efficacy of BLM. In the previous study, PCI was examined at relatively low light levels ($\leq$ 1.5 J cm^{-2}) and all studies were performed at physiological temperatures (37^{o}C). The studies described herein extend the previous work by examining the effects of higher light doses and, most importantly, the combination of PCI and HT. HT has previously been shown to enhance the efficacy of chemotherapy and radiation. Additionally, the cellular effects of HT were thought to be ideal for possible synergism with PCI. For example, BLM binds to DNA causing damage which often results in cell death, unless the damage is repaired. Since HT inhibits DNA repair, it's postulated that the addition of this therapeutic modality will enhance the cytotoxicity of PCI-mediated delivery of BLM.

5. CONCLUSIONS

The results of the present study show that HT and PCI interact in a synergistic manner over a very narrow range of light and temperature exposures. A temperature of 45 ^{o}C resulted in total spheroid death, while all spheroids subjected to 42 ^{o}C survived. Therefore, the hyperthermic threshold was estimated to be between 42 and 45 ^{o}C. This provided the rationale for the temperature range investigated (40 – 42 ^{o}C). No significant differences in growth kinetics and survival were observed between PCI- and PDT -exposed spheroids at radiant exposures < 1.5 J cm^{-2}. In contrast, all PDT and PCI spheroids irradiated with 3.0 J cm^{-2} died, suggesting that the useful light range is between 1.5 and 3.0 J cm^{-2}.

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