



## Molecular imaging of tumor photoimmunotherapy: Evidence of photosensitized tumor necrosis and hemodynamic changes

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### ABSTRACT

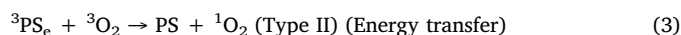
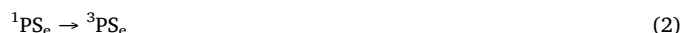
Near-infrared photoimmunotherapy (NIR PIT) employs the photoabsorbing dye IR700 conjugated to antibodies specific for cell surface epidermal growth factor receptor (EGFR). NIR PIT has shown highly selective cytotoxicity *in vitro* and *in vivo*. Cell necrosis is thought to be the main mode of cytotoxicity based mainly on *in vitro* studies. To better understand the acute effects of NIR PIT, molecular imaging studies were performed to assess its cellular and vascular effects.

In addition to *in vitro* studies for cytotoxicity of NIR PIT, the *in vivo* tumoricidal effects and hemodynamic changes induced by NIR PIT were evaluated by <sup>13</sup>C MRI using hyperpolarized [1,4-<sup>13</sup>C<sub>2</sub>] fumarate, R<sub>2</sub>\* mapping from T<sub>2</sub>\*-weighted MRI, and photoacoustic imaging. *In vitro* studies confirmed that NIR PIT resulted in rapid cell death *via* membrane damage, with evidence for rapid cell expansion followed by membrane rupture. Following NIR PIT, metabolic MRI using hyperpolarized fumarate showed the production of malate in EGFR-expressing A431 tumor xenografts, providing direct evidence for photosensitized tumor necrosis induced by NIR PIT. R<sub>2</sub>\* mapping studies showed temporal changes in oxygenation, with an accompanying increase of deoxyhemoglobin at the start of light exposure followed by a sustained decrease after cessation of light exposure. This result suggests a rapid decrease of blood flow in EGFR-expressing A431 tumor xenografts, which is supported by the results of the photoacoustic imaging experiments. Our findings suggest NIR PIT mediates necrosis and hemodynamic changes in tumors by photosensitized oxidation pathways and that these imaging modalities, once translated, may be useful in monitoring clinical treatment response.

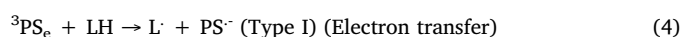
### 1. Introduction

Photodynamic therapy (PDT) is a well-known cancer treatment modality in which a visible light-absorbing chromophore or photosensitizer (PS) is administered intravenously followed by selective irradiation of the tumor with visible light [1]. The PS accumulates preferentially in the tumor *via* the enhanced permeability and retention (EPR) effect.

The clinical application of PDT is limited for several reasons. Selective illumination of the tumor not only results in tumor killing but can also cause damage to adjacent normal tissue [1–3]. In addition, the patient remains light-sensitive after exposure to the PS. Finally, the photochemical mechanisms underlying PDT are thought to be mediated by free radicals and singlet oxygen.



or



where PS<sub>g</sub> and PS<sub>e</sub> are ground state and excited state of photosensitizer, respectively.

<sup>1</sup>PS<sub>g</sub> is excited to <sup>1</sup>PS<sub>e</sub> by irradiating light (1), which then transits to <sup>3</sup>PS<sub>e</sub> *via* intersystem crossing (2). Energy transfer results in generation of singlet oxygen in type II reaction (3), whereas electron transfer results in generation of free radical in type I reaction (4). Free radicals

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generated through Type I photochemical mechanisms by the PS can lead to membrane lipid peroxidation, while singlet oxygen generated by a Type II mechanism adds across double bonds of membrane lipids [5–7]. Oxidative stress supported by Type I and Type II mechanisms cause membrane damage [6]. Thus, while PDT is used in selected cases, it is still not widely applicable in cancer treatment [1,4].

In contrast to PDT, which uses a non-targeted PS, in near-infrared photoimmunotherapy (NIR PIT), PSs are covalently linked to an antibody to provide tumor selectivity [8,9]. The highly hydrophilic PS, IR700, when conjugated to an antibody targeting cell membrane receptors expressed in tumors, can accumulate selectively at doses able to induce phototoxic effects [8–10]. For example, using an anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) conjugated to IR700, NIR PIT demonstrated potent and selective cytotoxic effects immediately after light exposure in tumor xenografts and in spontaneous EGFR-expressing cancer, resulting in dramatic reductions in tumor size [11–13]. *In vitro*, cells exhibited rapid swelling and membrane rupture, indicating a necrotic mechanism. Systematic studies showed that: a) tumor killing was dependent on IR700-Ab dose and light exposure; b) in targeting the EGFR receptor, panitumumab was superior to cetuximab; c) multiple NIR PIT cycles proved superior to a single treatment; and d) antibody fragments were as efficacious as whole antibodies [14–17].

NIR PIT studies also showed a nearly immediate increase in vascular permeability that facilitated the delivery of intravenous therapeutics, such as liposomal encapsulated doxorubicin, resulting in a synergy between NIR PIT and chemotherapy [18–20]. More recent studies have shown that NIR PIT can be used to target the immunosuppressive Treg cells which help cancer cells evade the normal immune response. This not only causes regression in treated tumors but also leads to responses in untreated tumors, opening avenues for incorporating this modality in cancer immunotherapy [21,22].

Several imaging techniques have been used to monitor the effects of NIR PIT. Bioluminescence imaging and fluorescence lifetime imaging have been used to follow treatment responses preclinically [23,24]. Clinically translatable imaging techniques include positron emission tomography (PET) with  $^{18}\text{F}$ -fluorodeoxyglucose ( $^{18}\text{F}$ ]FDG) uptake, magnetic resonance imaging (MRI) measuring changes in T1, measurement of the apparent diffusion coefficient (ADC), and detection of macromolecular contrast agent uptake, all of which showed early profound changes [19,25]. For instance,  $^{18}\text{F}$ ]FDG uptake was found to be significantly reduced immediately following NIR PIT, suggesting that this treatment modality causes rapid cytotoxicity [25]. However, *in vivo* imaging of cell necrosis and related hemodynamic changes have been lacking, but would potentially be useful for treatment monitoring.

Cell necrosis and EPR are considered hallmarks of NIR PIT. Biomarkers that can detect these endpoints would be useful in a clinical setting to monitor the response to NIR PIT.  $^{13}\text{C}$ -labeled fumarate, a substrate for fumarate hydratase (FH), can be hyperpolarized for MRI and used to non-invasively image necrotic death [26,27]. Fumarate is converted to malate by FH, which is an intracellular enzyme. Fumarate is cell-impermeable and remains unmodified in intact tissue. Upon the rapid necrotic cell death seen following NIR PIT, we hypothesized that FH would leak out and convert fumarate to malate in the extracellular milieu. Blood flow and blood oxygenation can also be monitored by  $\text{T}_2^*$ -weighted MRI and photoacoustic imaging (PAI), as these methods are influenced by deoxyhemoglobin and oxyhemoglobin, respectively [28–30]. The current study evaluates these imaging techniques to monitor therapeutic responses to NIR PIT *in vivo*.

## 2. Materials and methods

### 2.1. Animals, tumor model, and PIT

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animal Resources (National

Research Council, 2011) and were approved by the NCI Animal Care and Use Committee. Female athymic nude mice were supplied by the Frederick National Laboratory for Cancer Research (Frederick, MD). A431 and H520 cell lines were purchased from the American Type Culture Collection (Manassas, VA). A431 and H520 solid tumors were formed by injecting  $2 \times 10^6$  cells and  $5 \times 10^6$  cells, respectively, subcutaneously into the right hind legs of mice. The tumor volume was calculated by using the formula for a prolate ellipsoid: length  $\times$  width  $\times$  depth  $\times$   $\frac{1}{2}$ . The growth of A431 tumors ( $n = 5$  for both control groups and PIT-treated groups) was monitored from when the tumor volume was approximately  $200 \text{ mm}^3$  to a volume of  $1500 \text{ mm}^3$ . Mice were treated with PIT once at day 4 after tumor cells injection. During MRI and PAI measurements, the mouse breathing rate was monitored with a pressure transducer (SA Instruments Inc.) and was maintained at  $80 \pm 10$  breaths per minute. Core body temperature was also monitored with a nonmagnetic rectal temperature probe (FISO, Québec, Canada) and was maintained at  $36 \pm 1^\circ\text{C}$ .

Panitumumab-IR700 (Pan-IR700) was prepared using previously described methods [11]. For the *in vitro* study, cells were incubated with  $12.5 \mu\text{g}/\text{mL}$  Pan-IR700 for 30 min, washed with PBS three times, and then exposed to NIR light illumination with infrared NIR LED laser at a wavelength of  $690 \text{ nm}$  ( $5 \text{ J}/\text{cm}^2$ ). The cells were then washed with PBS three times and evaluated by flow cytometry. For *in vivo* studies, 24 h prior to PIT treatment, tumor-bearing mice were pretreated with  $200 \mu\text{g}$  of Pan-IR700 (i.p.). For  $\text{T}_2^*$ -weighted MRI and PAI experiments, the NIR light was delivered using a fiberoptic cable coupled with an LED laser ( $36 \text{ J}/\text{cm}^2$ ). For  $^{13}\text{C}$  MRI experiments and tumor growth experiments, the same LED laser was used ( $200 \text{ J}/\text{cm}^2$ ).

### 2.2. *In vivo* fluorescence imaging studies

Both A431 and H520 tumors were studied after they reached volumes of approximately  $300 \text{ mm}^3$ . Fluorescence images of Pan-IR700 were obtained with a fluorescence camera (Carestream, Carestream Health, Inc., NY) using a  $690 \text{ nm}$  excitation and  $750 \text{ nm}$  emission filters 24 h after i.p. injection of  $200 \mu\text{g}$  of Pan-IR700. The fluorescence images were then overlaid on X-ray images of each mouse. The scale of signal detection is indicated in Fig. 1B.

### 2.3. Flow cytometry analysis

The affinity of tumor cells for Pan-IR700 was assessed by incubating cells ( $5 \times 10^6$ ) with or without Pan-IR700 on ice for 1 h followed by three washings in Dulbecco's PBS (DPBS) + 1% fetal bovine serum (FBS). PIT-induced lipid peroxidation was assessed by incubating PIT-treated cells with rabbit anti-4-hydroxy-2-nonenal (4-HNE) Michael Adducts polyclonal antibody for 30 min on ice (1:250; EMD Millipore, Billerica, MA), followed by three washings in DPBS + 1% FBS and incubation with Alexa Fluor-conjugated goat anti-rabbit secondary antibody (1:250; Thermo Fisher Scientific Inc., Waltham, MA) for 1 h on ice. Following staining, the cell fluorescence was measured by flow cytometry (FACSCALIBUR, BD Biosciences, San Jose, CA).

### 2.4. Histostaining

Tumor-bearing mice were euthanized, and tumor tissues were removed from mice. Tumor tissues were frozen in OTC compound (SAKURA) using ultracold ethanol. Frozen tumors were sectioned to  $10 \text{ mm}$  thick using a cryostat, and the sections were thaw-mounted on glass slides. After fixed with 4% paraformaldehyde, sections were stained with hematoxylin and eosin and mounted on permount for histological observation.

### 2.5. FH enzyme activity assay

A serum sample was obtained from untreated mice and from NIR

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