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Evaluation of oxygen dependence on the *in vitro* and *in vivo* cytotoxicity of photoimmunotherapy using IR-700-Antibody conjugates

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Abstract

Photoimmunotherapy (PIT) using the near infrared-absorbing photosensitizing phthalocyanine dye, IRDye 700DX (IR700) conjugated with a tumor-targeting antibody such as Panitumumab (Pan) has shown efficacy *in vitro* studies and several pre-clinical models in mice with promise for clinical translation. PIT results in rapid necrotic cell death *in vitro* and tumor shrinkage *in vivo*. Photochemical studies with the Pan-IR700 conjugate showed that this agent can support generation of singlet oxygen and also generate reactive oxygen species after exposure to near infra-red (NIR) light. Moreover, *in vitro* studies using A431 cells, singlet oxygen scavengers abrogated the efficacy of PIT with Pan-IR700, while oxygen depletion to undetectable levels in an exposure chamber almost completely inhibited the cellular cytotoxicity of PIT. Survival of tumor bearing mice was prolonged in PIT treated animals but mice whose tumors were made transiently hypoxic prior to PIT had no benefit from the treatment. The results from this study support a central role for molecular oxygen derived species in cell death caused by PIT.

Graphical Abstract

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INTRODUCTION

The phenomenon of photodynamic therapy (PDT) is based on a visible light-absorbing chromophore, also called a photosensitizer (PS), and exposure to visible light, which are individually harmless but together elicit significant toxicity [1–4]. Although the principle was established over a century ago [5], research in the 1980s, primarily by Dougherty, has led to the use of a partially purified hematoporphyrin derivative (HpD) as the first clinically-approved PS in USA [6–11]. Mechanistic studies revealed the following: i) PS levels are 2–5 fold higher in solid tumors than in normal tissue as a consequence of enhanced permeability and retention (*EPR*); ii) Double selectivity in treatment is achieved by increased uptake in the tumor and phototreatment restricted to the target volumes. iii) oxygen is a necessary component of the therapeutic effect of PDT [12,13]. The photochemical mechanisms involved in the phototherapeutic effects have been described in earlier reports [14,15]. It involves light absorption by the PS to excite the ground state molecule to the triplet state via an intermediate singlet state. The triplet state sensitizer can transfer electrons to substrates (type I) generating radicals or transfer energy to molecular oxygen (type II) generating highly oxidizing singlet oxygen. Both type I and type II pathways generate reactive oxygen species (ROS) which can damage cellular targets [2,16,17]. Experimental evidence showed that tumor vasculature as well as tumor cells are targets for PDT and that the most significant damage occurs in the membrane [3,18–21]. ROS generated by a PS localized in a membrane can generate ROS which can cause damage by perturbing the membrane lipids [22]. Through a type I mechanism, free radicals generated in the membrane can participate in a radical chain peroxidation reaction utilizing tissue oxygen to generate lipid peroxides causing significant damage to the cell membrane [23]. Singlet oxygen generated through a type II pathway can add across unsaturated bonds in membrane lipids generating lipid endoperoxides, making membranes more polar [24]. Both these processes can significantly modify cell membranes to weaken, swell and burst, eventually causing irreversible necrotic cell death. They are also known to target other membranous organelles such as lysosomes and mitochondria as well as plasma membrane, which have been suggested to cause apoptotic cell death via release of cytochrome C [2,4,16].

Conventional PDT relies on passive targeting of the tumor since the PS enters the cell based on its physico-chemical properties and the *EPR* effect [25]. However these passive targeting

strategies may also permit the accumulation of PS in normal tissue [26]. While accumulation of PS in normal tissues may not, by itself, induce toxicity, normal tissue adjacent to tumor regions receiving light exposure may cause regional off target effects resulting in reductions in the light dose and/or the number of tolerated treatments [26]. To minimize normal tissue toxicity in PDT, several groups have developed strategies of actively targeting the PS to the tumor surface using antibodies conjugated to the PS, a modality known as photoimmunotherapy (PIT) [27–34]. With this approach, a much higher tumor/background ratio of the PS is achieved. In an *in vitro* study using leukemia cells, Oseroff et al observed a 100 fold enhanced therapeutic effect in PIT when using a PS-Ab conjugate compared to the free PS alone, a process shown to be $^1\text{O}_2$ dependent [28,29,35,36]. Hydrophilic dye-antibody conjugates were described with improved efficacy of PIT only when internalized in cells [37,38]. More recently, results from our group have shown robust response to PIT when a water-soluble phthalocyanine dye (IR-700) conjugated with trastuzumab targeting the human epidermal growth factor receptor-2 or panitumumab targeting the human epidermal growth factor receptor-1 were effective in eliciting cytotoxicity *in vitro* and *in vivo* compared with minimal effects using IR700 alone [39]. Thus in PIT, hydrophilic dyes covalently linked to antibodies were found to be effective by binding to membrane targets and without having to localize intracellularly making this strategy to use a broad range of dyes. In EGFR expressing cells, cell death occurred rapidly and *in vivo* studies demonstrated tumor shrinkage. The robust and rapid responses observed with PIT using Ab-IR700 + NIR treatment in cellular and *in vivo* studies and about 30% protection observed with sodium azide ROS scavengers suggested that mechanisms other than ROS generation, might be responsible for the observed cell necrosis [39,40]. However related phthalocyanine dyes, when evaluated in photochemical and cellular photobiology studies, have been shown to produce $^1\text{O}_2$ and ROS efficiently and cause cytotoxicity in an oxygen dependent manner [41–46]. A better understanding of the mechanisms underlying the Pan-IR700 mediated PIT will help in using this therapeutic modality efficaciously[47].

With the successful evaluation of PIT using Ab-IR700 in several pre-clinical models and with the potential for its translation into the clinic, this study was undertaken to evaluate the role of ROS in the observed therapeutic effects of PIT in a well controlled *in vitro* environment and *in vivo* tumor models.

Material and methods

Reagents

A water soluble, silicon-phthalocyanine derivative, IRDye 700DX NHS ester (IR700; $\text{C}_{74}\text{H}_{96}\text{N}_{12}\text{Na}_4\text{O}_{27}\text{S}_6\text{Si}_3$, molecular weight of 1954.22) was obtained from LI-COR Bioscience (Lincoln, NE). Panitumumab, a fully humanized IgG₂ mAb directed against the human epidermal growth factor receptor 1 (HER1), was purchased from Amgen (Thousand Oaks, CA). All other chemicals used were of reagent grade.

Synthesis of IR700-conjugated panitumumab

Panitumumab (1 mg, 6.8 nmol) was incubated with IR700 (66.8 μg , 34.2 nmol, 5 mmol l⁻¹ in DMSO) in 0.1 mol l⁻¹ Na₂HPO₄ (pH 8.5) at room temperature for 2 h. The mixture was

purified with a Sephadex G50 column (PD-10; GE Healthcare). The protein concentration was determined with Coomassie Plus protein assay kit (Thermo Fisher Scientific Inc, Rockford, IL) by measuring the absorption at 595 nm with spectroscopy (8453 Value System; Agilent Technologies, Santa Clara, CA). The concentration of IR700 was measured by absorption with spectroscopy to confirm the number of fluorophore molecules conjugated to each mAb molecule.

Cell culture

A EGFR-expressing epidermoid cancer cell line, A431, was obtained from Dr. H.Kobayashi, National Cancer Institute, and cultured with RPMI 1640 (GIBCO) containing 10% fetal calf serum (culture medium) at 37 °C in a humidified atmosphere adjusted to 5% CO₂.

Animals and tumor implantation

All animal experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (National Research Council, 1996) and approved by the National Cancer Institute (NCI) Animal Care and Use Committee. Female athymic nude mice were supplied by the Frederick Cancer Research Center, Animal Production. A431 solid tumors were formed by injecting one million cells s.c. into the right hind legs of mice. Tumor volumes based on MRI images were calculated by the following formula; tumor volume = 0.5 x length x width x height.

Detection of singlet oxygen

Light dependent bleaching of N,N dimethyl-4- nitrosoaniline (RNO) in the presence of IR700 alone or AI-PCS alone was used as an indicator of the efficiency of singlet oxygen generation. Experimental samples were mixed in cuvettes. The reaction mixture consisted of 3 ml volume with 0.05 M phosphate buffer, maintained at pH 7.5, 10 nM IR700 or AI-PCS, 50 µM RNO and 10 mM Imidazole compound. Sodium azide was added at a concentration of 100 µM. Reactions were carried out at room temperature (25 °C). Samples were kept in the dark or illuminated with a red light emitting diode (LED) which emits light at 670–710 nm wavelength (L690-66-60; Marubeni America Co., Santa Clara, CA, USA) at 40 mW/cm² or 20 mW/cm². Optical densities were measured at 440 nm in the spectrophotometer.

Detection of superoxide

Superoxide reactions were conducted at the same setting as the assay of singlet oxygen generation using 80 mW/cm² of LED light. The superoxide detection system utilized the reduction of ferricytochrome c, which was measured by an increase in absorbance at 550 nm. The reaction mixtures consistent of 3 ml samples with 0.05 M phosphate buffer, maintained at a pH 7.5, with 6.7 nM IR700, 20 µM cytochrome c and 3.3 µg/ml catalase. EDTA and superoxide dismutase were added at 3.3 mM and 67 µg/ml respectively.

Photoimmunotherapy

A431 cells incubated with Pan-IR700 for 1 hour were irradiated with the LED and 50 mW/cm² 50,100 sec (2.5 J/cm² and 5 J/cm² respectively) were applied to the cell culture

plate. Mice with implanted tumors in right hind limb were exposed to 60 J/cm² for 20 minutes.

Fluorescent staining

Fluorescent double staining was carried out using a detection kit for apoptotic/necrotic cells (Promokine; Heidelberg, Germany) which includes Ethidium homodimer III (EtD) and Annexin V labeled with FITC (AV) as relevant fluorescent stains. Fluorescent staining was performed immediately after PIT with the light dose of 5 J/cm² according to the manufacturer's instructions.

LDH cytotoxicity assay

Cytotoxicity induced by PIT in A431 cells in vitro was measured by monitoring LDH leakage into extracellular space. Cells were plated at twenty thousand cells/well in 96 well plates and treated with PIT in phenol red free RPMI medium containing 1% fetal calf serum. 24 hours after PIT, the medium was collected and LDH was measured using an LDH Cytotoxicity Detection Kit (Takara Bio Inc, Tokyo, Japan).

Hypoxia experiments

A431 Cells were incubated in 96 well glass bottom plates (In Vitro Scientific, Sunnyvale, CA) with Pan-IR700 at a concentration of 12.5 µg/ml in culture medium for 1 hour and were then washed and replaced with assay medium. The cells on a plate were transferred to a culture chamber on a shaker where oxygen concentrations could be altered. The cells were incubated for 90 minutes at each oxygen concentration using cylinders with gas mixtures containing different percent oxygen with the remainder composed of N₂ and 5% CO₂. The outlet was constantly monitored for oxygen content to ensure accurate conditions. The cells were exposed to NIR light while in the chamber.

ROS Scavenger experiments

Cells were incubated with Pan-IR700 in the same manner as described for the hypoxia experiment and the medium was replaced with assay medium containing each scavenger.

In vivo PIT

The experiment was initiated 3 days after tumor implantation when the tumors were approximately 40–50 mm³. A431 tumor-bearing mice were randomized into 4 groups of at least 6 animals per group for the following treatments; (1) 300 µg of Pan-IR700 i.v. followed by PIT at 60 J/cm² on day 1 after injection(PIT); (2) 300 µg of Pan-IR700 i.v. followed by PIT at 60 J/cm² on day 1 after injectionwith ipsilateral inguinal ligation of the femoral artery using an external rubber band 10 min prior to and during PIT (PIT + ligation); (3) no treatment (control); (4) 300 µg of Pan-IR700 i.v. was administered. On day 1 after injection, 30 min of ipsilateral inguinal ligation was performed on day 1 after injection without light therapy. Mice were monitored daily and tumor volumes were measured every other day until the tumor volume reached 1000 mm³ or a mouse presented with signs of severe organ system dysfunction, whereupon mice were euthanized with carbon dioxide.

EPR imaging

Technical details of the EPR scanner operating at 300 MHz, data acquisition based on a single-point imaging modality, image reconstruction, and the oxygen mapping procedure were described in an earlier report [48]. After the animal was placed in the resonator, the oxygen-sensitive tracer TAM (OX63, GE Healthcare) was injected i.v. at a dose of 1.125 mmol/kg bolus. The EPRI measurement was carried out before and after 10 min of inguinal ligation. Mice were anesthetized by isoflurane inhalation (4% for induction and 1.5% for maintaining anesthesia) during MRI and EPR imaging. During EPRI and MRI measurements, the breathing rate of the mouse was monitored with a pressure transducer (SA, Instruments Inc. Stony Brook, NY) and maintained at 60 ± 10 breaths per minute. Core body temperature was maintained at 37°C with a flow of warm air.

Statistical analysis

Data are expressed as mean $\pm$ S.D. ($n = 3-6$). Statistical analyses were carried out using a statistics software R. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni test or log rank test.

RESULTS

Our prior studies using chemical detection methods for singlet oxygen with sulfonated phthalocyanines substituted with Al, Zn etc, have shown that these PS generate $^1\text{O}_2$ efficiently with quantum yields ranging between 0.35 – 0.45 [46]. The ability of IR700, a related phthalocyanine dye containing Si, to generate $^1\text{O}_2$ either alone or as an immunoconjugate was tested in aqueous solution. A dye (RNO) bleaching assay with imidazole as a scavenger for singlet oxygen was used [49] in this photochemical reaction and the relevant reactions are given below:

When solutions containing IR700 (10 nM), imidazole (10 mM) and RNO (50 mM) were exposed to 685 nm light, the absorbance decreased steadily at 440 nm as a function of light exposure time (Figure 1A, filled squares). When azide (100 μM), an efficient scavenger of singlet oxygen was included in the reaction mixture (Figure 1A, filled triangles), a significant inhibition of the dye bleaching was noticed. The IR700 dye conjugated with the antibody panitumumab was also tested for $^1\text{O}_2$ generating ability (Figure 1A, filled diamonds). The results show that IR700 retained its $^1\text{O}_2$ generating ability even when

conjugated with an antibody. To obtain quantum yields of $^1\text{O}_2$ generation by IR700, we used Al-PCs as a standard whose quantum yields have been determined previously[46]. The similar absorption maxima of these two dyes made it convenient for comparison without having to make corrections for light output from the exposure system. The results show that the quantum yield for $^1\text{O}_2$ generation for IR700 and the Pan-IR700 were ~ 0.30 which is sufficient to elicit phototoxicity in cellular studies.

Type I photochemistry mediated by PS in triplet states (^3PS) are mediated by reduction or oxidation reactions. On a thermodynamic basis, the oxidation (or H-abstraction) by triplet IR700 is favored based on its mid-point reduction potential (0.77 Volt vs normal hydrogen electrode) and can induce many H-abstractions (0.3–0.6 Volt vs normal hydrogen electrode) from membrane lipids with allylic hydrogen atoms compete with type II processes which generate singlet oxygen. To test this possibility, ferri cytochrome c reduction assay was used to detect superoxide generated by type I reactions [50] as given by the following equations:

When solutions containing an IR700 (6.7 μM) and a H-atom donating oxidizable substrate EDTA (3.3mM) were exposed to 685 nm in the presence of ferricytochrome c (20 μM) and its reduction was monitored at 550 nm, a light dose dependent decrease in absorbance was noticed suggesting a conversion of ferricytochrome c to ferrocytochrome c (figure 2, filled circles). To examine whether superoxide mediated this reduction, this reaction was conducted in the presence of the enzyme superoxide dismutase (67 $\mu\text{g/ml}$). Under these conditions, the reduction was completely inhibited. Absence of IR700 or absence of EDTA did not support the reduction of ferricytochrome c (data not shown). These results suggest that IR700 is capable of participating in Type I photochemistry to generate ROS. The results shown in Figures 1 and 2 suggest that IR700 dye can mediate cellular damage through singlet oxygen and ROS generation.

The studies by Mitsunaga et al [39] have shown that the cancer cell line A431 expresses HER-2 significantly and PIT with the IR700-Ab using trastuzumab (antiHER2) or panatumumab (antiEGFR1) elicited significant necrotic cell death. Figure 3A shows results from PIT-induced cytotoxicity in A431 cells treated with Pan-IR700. The assay reports on both apoptotic and necrotic cell death (Figure 3A). The results show that while PIT induces

cell death both by apoptosis and necrosis, the latter appeared to contribute most (Figure 3B) as observed previously [39].

We tested the ability of singlet oxygen scavengers sodium azide (hydrophilic) and biliverdin (lipophilic) to inhibit Pan-IR700 mediated phototoxicity in A431 cells. The results show that while sodium azide at 10 mM as high concentration as it can even quench species other than singlet oxygen provided complete protection to Pan-IR700 mediated phototoxicity, lipophilic biliverdin offered the same level of inhibition at a 20 fold lower concentration. (Figure 4A,4B) The lipophilic singlet oxygen scavenger offered more efficient protection against PIT than the hydrophilic sodium azide because the latter was excluded from the cell membrane while the former had ready access to the cell membrane. These results suggest that similar to PDT, oxygen may be a required component for inducing cell death with PIT [13,51]. When the ROS scavenger, butylated hydroxyl toluene (BHT)[22] was tested for protective effects in IR-700-Pan mediated PIT, no changes in cytotoxicity in A431 cells was noticed (Figure 4C). The results from these studies suggest that the IR-700-Pan mediated cytotoxicity operates predominantly through a singlet oxygen mediated (type II) process with minimal contributions through type I pathway involving free radicals.

To examine the role of molecular oxygen in photodamage caused by IR700-Ab mediated PIT, we treated cells under identical conditions but at different levels of oxygen ranging from ~ 0% to 21% and monitored the cytotoxicity measured by LDH leakage. The results (Figure 5) show that while there was minimal toxicity induced by PIT when the oxygen content was maintained at ~0% O₂, cells incubated with Pan-IR700 (12.5 µg/ml) and exposed to NIR underwent minimal toxicity. This pattern was found to be similar at the two light doses (2.5 and 5 J/cm²) tested. As the oxygen concentration was gradually increased to 1% the toxicity induced by PIT rapidly increased, reaching a maximum at an oxygen content of 4% with no further increases in toxicity above 4% oxygen content. These results suggest that oxygen is an essential component of PIT in agreement with previous reports [13,44,46,51].

Many therapies are oxygen dependent. For instance, radiation therapy depends on oxygen for inducing cytotoxicity but reaches a maximum at oxygen levels >10%. In contrast, maximal toxicity with PIT is observed at < 5% oxygen concentration. This difference may be related to the actual targets of each of these two modalities. While DNA is the main target of radiation therapy, membrane damage is generally accepted as the main mode of damage [17,23,24]. Oxygen solubility differences in aqueous (0.2 mM maximum) and lipid compartments such as the cell membrane (5 mM maximum) may explain the rapid increase in toxicity mediated by PIT at much lower levels of oxygen in the exposure chamber.

The oxygen content within the tumor influences the response to PIT. When the blood supply to the tumor was occluded for 90 minutes, quantitative changes in tumor pO₂ in response to restricting blood supply to the tumor, were monitored by Electron Paramagnetic Resonance (EPR) imaging. EPR imaging is a low field magnetic resonance imaging technique which uses a paramagnetic tracer. The effect of molecular oxygen, which is paramagnetic, on the relaxation properties of the injected tracer, is used to determine pO₂ in the regions of interest [48]. Figure 6A shows pO₂ maps obtained by EPRI in the leg of a mouse with an implanted

A431 tumor injected with the paramagnetic tracer, Oxo 63. The pO_2 maps prior to ligation (left) show that the tumor is well oxygenated but with some regions of hypoxia. When the tumor pO_2 was obtained – mins after ligation, a global reduction of oxygen levels was recorded. The histogram in Figure 6B shows quantitative changes in the tumor after hypoxia induction by ligation. The histogram shows that a significant fraction of the tumor has $pO_2 < 10$ mm Hg after ligation. Figure 6C shows median changes in pO_2 after ligation where a >10 mmHg drop in pO_2 was noticed after ligation.

The survival of mice implanted with A431 tumors was measured following treatment with Pan-IR700 with and without focal ischemia prior to phototreatment. Control groups (untreated and not ligated) were also included. The protocol of Pan-IR700 administration, ligation, and phototreatment is shown in Figure 7 A and the Kaplan- Meier survival curves for the individual groups are shown in Figure 7B. The results show that while PIT was effective in extending survival, ligation to induce hypoxia prior to PIT compromised the effectiveness of PIT and the survival of these mice was closer to untreated controls. Data from these studies support the critical role of oxygen in mediating PIT-induced cytotoxicity.

DISCUSSION

PDT is an approved treatment modality for several malignant and non malignant diseases [2,7–9,20,21,26,52,53]. The treatment involves destruction of the targeted region by the simultaneous presence of a photosensitizer and the application of light of appropriate wavelength in an oxygen dependent process [54]. The relatively higher levels (2–5 fold) of accumulation of the PS through *EPR* and the ability to restrict the delivery of the excitation light confers double selectivity for PDT, allowing more treatment fractions than conventional radiotherapy or chemotherapy which are dose-limiting [55]. Further, the lack of cross-resistance with chemotherapy makes PDT a useful addition to chemotherapy [26]. Passive targeting of PS in tumors by *EPR*, results in a modest selectivity in accumulation in tumors compared to normal tissue but their long clearance times can cause non-target toxicities, especially in the skin, that last for weeks [21,26]. Actively targeting the PS to the tumor site by conjugation with a tumor specific antibody makes it possible to have high levels of the PS accumulate at the tumor cell surface with minimal binding to non-target sites [20]. Such strategies were developed and tested successfully in several *in vitro* and *in vivo* models [33,36,47]. Recently we developed a dye-immunoconjugate covalently linking the near IR absorbing water-soluble phthalocyanine derivative, IR700, to trastuzumab (antiHER2) or panatumumab (antiEGFR1) and showed that in tumor cells expressing the receptor selective cell death could be achieved with PIT, however, unconjugated IR700 had no effect [39]. The response to PIT was immediate and cell death proceeded predominantly through necrosis followed by tumor shrinkage and accompanied by increased survival. Such tumor specific PIT strategies will be, in general, more effective, especially in anatomic sites such as in the peritoneal cavity where delivering light doses specifically to the tumor will be difficult and using non targeted PS will result in unacceptable normal tissue damage.

Tumor oxygenation status has a significant influence in the outcome of radiotherapy [56]. Unrepaired DNA double strand breaks are responsible for radiation-induced cell death. Oxygen is required to create additional free radicals and increase the initial radiation-

induced DNA damage induced by ionizing radiation and there is a 3-fold increase in resistance to radiation under hypoxic conditions; hypoxic tumors respond poorly to radiotherapy compared to well oxygenated tumors. It is also important to understand the dependence of molecular oxygen on the efficacy of *in vitro* and *in vivo* PIT using IR700-Ab conjugates. The target for radiation-induced cytotoxicity is the chromosomal DNA surrounded by aqueous phase where oxygen solubility is at a maximum of 0.2 mM. In the case of PIT where the PS is actively targeted to the tumor cell membrane compartment, oxygen status in the membrane may play a role. Since lipidic regions' oxygen solubility is in the mM range, a radiobiologically hypoxic tumor which is resistant may still respond to PIT since membrane reservoir of oxygen may still be sufficient to support photochemical oxidation which results in damage to the membrane.

The photochemical studies with free and antibody conjugated IR700 were shown to support both type I and type II processes to generate ROS and $^1\text{O}_2$ (Figure 8). Cellular studies show that oxygen is an essential component in inducing apoptotic and necrotic cell death and singlet oxygen scavengers are able to reduce such damage. The singlet oxygen generating reactions are schematically given in the following equations:

Singlet oxygen generated by the IR700-Ab can add across double bonds of unsaturated lipid components in the membrane and alter the membrane structure and function and compromise the cellular homeostasis.

Similarly ROS generated by the IR700-Ab through a type I process (Figure 2) can also catalyze lipid peroxidation chain reactions as shown below:

Reactions 9–10 continue as a chain till oxygen in the membrane is consumed. Thus, a single photochemically generated radical L $^\cdot$ (eq. 8) can proceed in a chain reaction till oxygen is consumed in the lipid even while inflicting significant damage to the membrane. Thermodynamically, the excited triplet state with an oxidation potential of 0.7 V (vs. normal hydrogen electrode) has the ability to oxidize membrane lipids, and with abundant unsaturated lipids with allylic hydrogen atoms in the membrane, this process may also be

equally favored. However, after the initial radical generation, the propagation steps of lipid peroxidation (eq. 9–10) are oxygen dependent. Our photochemical experiments and cellular and *in vivo* data provide evidence for oxygen dependence of PIT damage using IR700-Ab.

The cellular studies show that even under moderate hypoxia, PIT using IR700-Ab may be effective, unlike radiotherapy where resistance can occur at $pO_2 < 10$ mmHg. One of the reasons IR700-Ab-induced damage becomes resistant only below a pO_2 of < 5 mmHg is that the membrane is a known reservoir of oxygen. Even when the tumor pO_2 is around 10 mm Hg, the membrane pO_2 may be high enough to support reactions (eq. 9–10) to cause sufficient damage to the membrane.

In conclusion, our studies show that PIT is oxygen dependent and that the photochemical pathways utilized likely proceed through both non-radical (singlet oxygen) and radical pathways.

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Highlights

We demonstrate that the photoimmunoconjugate Pan-IR700 can generate singlet oxygen and reactive oxygen species.

We show that in vitro cell killing by Pan-IR700 is predominantly mediated by singlet oxygen in an oxygen dependent process.

In vivo photodynamic therapy clearly improved the survival time that was canceled by making tumor hypoxic by ligature.

Singlet oxygen plays a central role in PIT mediated by Pan-IR700.

Figure 1.

A The chemical assay showed that IR700 retained its 1O_2 generating ability even when conjugated with an antibody. Filled squares: reaction mixture of IR700 (10 nM), imidazole (10 mM) and RNO (50 mM), filled triangles: reaction mixture of IR700 (10 nM), imidazole (10 mM), RNO (50 mM) and sodium azide (100 μ M) : filled diamonds: reaction mixture of Pan-IR700 (10 nM), imidazole (10 mM) and RNO (50 mM). B comparison of quantum yields between Al-PCs and IR700. IR700 exhibited 1O_2 generating ability comparable to Al-PCs.

Figure 2.

Superoxide, detection via type I photochemistry from IR-700. Reduction of ferricytochrome c by superoxide was cancelled by adding superoxide dismutase. Solid line: reaction mixture of IR700 (6.7 μ M), EDTA (3.3mM) and ferricytochrome c (20 μ M), dashed line: reaction mixture of IR700 (6.7 μ M), ferricytochrome c (20 μ M) and SOD (67 μ g/ml), dotted line: reaction mixture of IR700 (6.7 μ M), EDTA (3.3mM), ferricytochrome c (20 μ M) and SOD (67 μ g/ml).

Figure 3.

PIT induced necrotic and apoptotic cell death. (A) necrotic cells were stained in red by ethidium homodimer III and apoptotic cells were stained in green by annexin V. (B) the ratio of necrotic cells and apoptotic cells (n=4). Results are mean $\pm$ SD.

Figure 4.

Singlet oxygen scavengers showed complete inhibition of cytotoxicity. 500 μ M biliverdin exhibited equivalent inhibition to 10 mM NaN_3 . (A) by lipophilic singlet oxygen scavenger biliverdin (n=4), (B) by hydrophilic scavenger NaN_3 (n=3) and (C) by ROS scavenger BHT (n=4).. Results are mean $\pm$ SD. *P < 0.05.

Figure 5. Cytotoxicity induced by PIT was inhibited under hypoxic condition. Results are mean $\pm$ SD. Filled circles: PIT 5 J, filled squares: PIT 2.5 J.

Figure 6.

Inguinal ligation with rubber band induced focal hypoxia. (A) representative EPR images of tumor pre and post ligation. (B) histogram of pixel values of pO_2 in the tumor. C: average intratumor pO_2 difference between pre and post ligation ($n=3$). Results are mean $\pm$ SD.

Figure 7. Kaplan-Meier plot showed inhibition of PIT effect on survival. (A) schematic of treatments for each group. (B) Kaplan-Meier plot. Statistically significant difference $P < 0.05$ was observed in survival curve between PIT group and PIT+Ligation group by log rank test. (control group; $n=7$, other groups; $n=6$)

Figure 8.
Schematic of mechanism of PIT mediated by IR-700