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Photoimmunotherapy and irradiance modulation reduce chemotherapy cycles and toxicity in a murine model for ovarian carcinomatosis: perspective and results

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Abstract

Significant toxicities from multiple cycles of chemotherapy often cause delays or early termination of treatment, leading to poor outcomes in ovarian cancer patients. Complementary modalities that potentiate the efficacy of traditional agents with fewer cycles and less toxicity are needed. Photodynamic therapy is a mechanistically-distinct modality that synergizes with chemo and biologic agents. A combination regimen with a clinically relevant chemotherapy cocktail (cisplatin + paclitaxel) and anti-EGFR targeted photoimmunotherapy (PIT) is evaluated in a murine model for ovarian carcinomatosis. Mice received either 1 or 2 chemotherapy cycles followed by PIT with a chlorin$_{e6}$-Erbitux photoimmunoconjugate and 25 J/cm^2 light. PIT + 1 cycle of chemotherapy significantly reduced tumor burden, comparable to multiple chemotherapy cycles. Relative to 1 cycle of chemotherapy, the addition of PIT did not cause significant mouse weight loss, whereas 2 cycles of chemotherapy led to a significant reduction in weight. Irradiance-dependence on PIT efficacy was a function of the conjugation chemistry, providing an additional variable for optimization of PIT outcome.

Introduction

Overview

This article presents our findings on epidermal growth factor receptor (EGFR)-targeted treatment of disseminated ovarian cancer using photoimmunotherapy (PIT) in combination with a conventional chemotherapeutic cocktail (Figure 1). In keeping with the spirit of the special issue, the article is a combination of a review and original data. To orient the reader, a relatively comprehensive discussion of key barriers to advanced ovarian cancer treatment and the role that photodynamic therapy (PDT) might play in the management of this disease is presented. To put PDT and PIT for ovarian cancer in context, we start with a broad overview of current management strategies, followed by a discussion of limitations of chemotherapy and drug resistance. We then transition to why PDT and PIT might be logical modalities to complement traditional management strategies.

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Background and perspective: towards improved management of ovarian cancer with targeted photodynamic therapy-based combinations

Approximately three quarters of the 140,200 new cases of epithelial ovarian cancer estimated worldwide in 2011[1] will be diagnosed after the disease has metastasized to pelvic sites or beyond the peritoneal cavity.[2-4] The dissemination pattern for advanced stage ovarian cancer is characterized by metastases to vital peritoneal organs, as well as the mesothelial lining of the peritoneum, omentum, mesentery, and diaphragm.[3, 5] The grueling toxicities and persistently poor outcomes associated with conventional treatments for this diffuse metastatic disease [6, 7] emphasize the need for targeted and rationally-designed combination regimens that improve the therapeutic index of conventional therapies. [2, 4, 8-14]

Following initial surgical staging and operative debulking, the standard of care for advanced stage ovarian cancer has typically involved radiotherapy or intravenous administration of a chemotherapeutic cocktail with a platinum agent (cisplatin or carboplatin) and a taxane (paclitaxel or docetaxel) for up to six treatment cycles.[15] This approach has modestly improved initial response rates, but the disease recurs in 80% of patients.[4, 16] In recent years, intraperitoneal chemotherapy has been shown to improve both overall survival and progression free survival by 20-30% in patients with optimally debulked advanced stage disease.[15, 17, 18] One of the key studies demonstrating this clinical benefit was GOG 172, conducted by Armstrong et al.[17] . Based on this study and others,[15, 18] the National Cancer Institute issued a clinical announcement in January 2006 encouraging the use of intraperitoneal cisplatin in patients with optimally cytoreduced ovarian cancer.[15] Regardless of the route of administration, treatment-related toxicities and complications limit the number of cycles and chemotherapeutic dose intensity that can be delivered to patients, both of which are emerging as potentially important determinants of treatment efficacy in ovarian cancer.[19, 20]

Chemotherapy cycles, dose intensity, and treatment failure

Recent articles by Fauci *et al.*[20] and Yen *et al.*[19] highlight the prognostic significance of evaluating survival in ovarian cancer patients within the context of relative dose intensity of chemotherapy and the number of delivered cycles. Relative dose intensity (defined in Eq 1), is a well established prognostic indicator in breast cancer and lymphoma, but has not been well studied in ovarian cancer.[20]

$$\frac{\text{Delivered dose intensity(total delivered dose/actual time to complete treatment)}}{\text{Standard dose intensity(standard dose/planned time to complete treatment)}} \quad \text{Eq 1}$$

A retrospective analysis of outcomes in chemotherapy naïve patients treated with platinum and taxane agents revealed that an incomplete or protracted treatment course predicts a poor prognosis and that an optimal therapeutic window is critical to providing meaningful improvements in survival. The essentials of this conclusion were confirmed in a similar study by Yen et al. The reasons for treatment delays or early termination were primarily related to the significant toxicities and poor performances scores associated with each additional cycle of chemotherapy.[17, 19, 20] These findings highlight the critical need to identify complementary therapeutic modalities that potentiate the efficacy of chemotherapeutic agents and improve outcomes with fewer cycles, lower dose intensities, and less treatment-related toxicity.

Another reason for the high rate of treatment failure associated with the standard management of ovarian cancer is innate and acquired chemoresistance, which is driven by a variety of mechanisms. [2, 4, 11, 21-24] Chemotherapies, with their spectrum of cellular and

molecular targets, rely primarily on proliferating cells to be effective.[4] However, even in rapidly proliferating tumors, a significant number of cancer cells are quiescent, which confers resistance.[4, 24, 25] Other key determinants of treatment response include drug pharmacokinetics and pharmacodynamics, the influence of the tumor-microenvironment, and inherent or acquired somatic mutations and epigenetic changes that cause chemotherapeutics to fail (e.g. alterations in DNA repair machinery, increased activity in drug inactivation enzymes, as well as upregulation of anti-apoptotic proteins such as Bcl-2/Bcl-X_L and downregulation of pro-apoptotic proteins BAX/BAD).[2, 4, 11, 21-23] Overcoming these complex resistance mechanisms requires new therapeutic approaches, which target molecular pathways that are mechanistically distinct from traditional chemotherapies and cooperatively enhance the efficacy of these drugs. [4, 6, 11, 16, 21]

Targeted inhibitors and the epidermal growth factor receptor

Early indications from preclinical and clinical studies using targeted inhibitors in combination with chemotherapy suggest modest, but promising, improvements in outcome compared to standard clinical regimens. The targets for these ongoing or recently completed studies include inhibitors for vascular endothelial growth factor,[10, 11, 16, 26-32] folate receptor,[10, 11, 33-36] the Src oncogene,[11, 37] the mTOR/PTEN/PI3K/Akt pathway,[10, 11, 38-40] platelet-derived growth factor,[10, 32, 41-43] poly-ADP-Ribose Polymerase,[10, 11, 44] and the EGFR, an important prognostic indicator and therapeutic target in ovarian cancer.[10, 11, 30, 45, 46]

The EGFR is a member of the ErbB tyrosine kinase family and plays a key role in normal ovarian follicle development.[47] Dysregulation of this pathway increases the growth potential of normal ovarian surface epithelium and contributes to malignant transformation of this peritoneal lining.[48-50] Studies by Siemens et al.[48] and others[49, 51-55] support the concept that EGFR activation stimulates EMT-associated events in ovarian cancer cells, including disruption of E-cadherin junctions, increased production of matrix metallaoproteinases, and higher potential for migration and invasion. In ovarian tumors, overexpression of EGFR gene copies and protein levels is associated with high tumor grade and large residual tumor size, both of which prognosticate poor outcomes.[56] Indeed, Psyrri *et al.*[57] showed that high EGF receptor expression in clinical ovarian cancer samples was found to be the most significant prognostic indicator of poor overall and disease free survival. These findings, along with additional studies by our group[12, 58-60] and others,[10, 61-65] establishes the value of the EGFR as a therapeutic target. It now remains to demonstrate how best to exploit this high value target.

The vast majority of EGFR inhibitors under clinical evaluation fall into one of two categories: i.) Small molecule tyrosine kinase inhibitors (TKI's) that block receptor phosphorylation or ii.) Monoclonal antibodies (mAbs) that inhibit ligand binding and prevent receptor dimerization. Cetuximab (Erbitux) is a clinically approved chimeric mouse-human antibody directed against the EGFR. As described by Li *et al.*,[66] the epitope for Erbitux resides in domain III of the EGFR, which overlaps with the ligand binding region of the receptor. Interaction of the antibody with the EGFR, therefore, primarily blocks ligand binding and prevents the formation of a ligand-stabilized extended conformation that is critical to receptor dimerization.[66] This receptor blockade prevents the EGFR from being activated and leads to induction of p27, a tumor suppressor protein that arrests cellular growth in the Gap 1 phase.[67, 68]

Several anti-EGFR TKI's and mAbs, including Erbitux, by themselves have shown modest clinical benefit and are approved for the treatment of solid tumors.[10, 49, 69, 70] There are also significant toxicities associated with targeted inhibitors, including anti-EGFR agents.[10, 49, 69] None of these agents have performed well enough against ovarian cancer to

be clinically feasible as monotherapies.[2, 4, 10, 12, 49] Collectively, these findings suggest that, as with most anti-cancer therapies, targeted inhibitors are most likely to be successful as part of rationally-designed combination regimens.[71] Our hypothesis has been that the best combinations might be those that synergize by acting along non-overlapping pathways of tumor growth and proliferation where photodynamic therapy (PDT) might play a unique role. [12, 72-74]

Photodynamic therapy

PDT is a biophysically driven cytotoxic modality that is mechanistically-distinct from traditional therapies and has been shown by us and others to reverse chemoresistance and synergize with chemo and biologic agents for the treatment of ovarian cancer.[12, 14, 74-79] PDT is based on the activation of a photosensitizer by light of a specific wavelength to photochemically generate active molecular species that are locally cytotoxic and have the potential to elicit systemic anti-tumor effects.

We[12, 14, 74] and others[80, 81] have shown that PDT enhances the efficacy of chemotherapeutics and targeted biologics in ovarian cancer. Del Carmen et al.[12] reported a synergistic enhancement of Erbitux efficacy by Verteporfin-PDT in a clinically relevant mouse model for advanced stage ovarian cancer.[82] A greater than 90% reduction in acute tumor burden was seen in mice treated with combination PDT + Erbitux. This dramatic reduction in tumor burden was synergistic relative to the monotherapies, and had been a difficult result to achieve in this advanced stage model. [83] Similarly, a synergistic enhancement in survival was observed in the group that received combination PDT + Erbitux, relative to the controls. Four mice remained alive until the end of the survival study (day 180 post tumor cell implantation). Three of these four surviving mice received combination PDT + Erbitux and did not show evidence of gross residual disease. All other animals in the study had visible tumors present at the time of necropsy.[12] Because the PDT + Erbitux combination regimen potentiated the efficacy of the individual modalities, a dramatic reduction in tumor burden was achieved with fewer PDT treatments and lower toxicity than had been previously reported by Molpus et al.[83] PDT has also been shown to enhance the efficacy of chemotherapeutic agents. The optimal parameters for the interaction between these modalities are dependent on the photosensitizer and chemotherapeutic agent used. [80] We have shown that PDT decreases the size and disrupts the structure of ovarian micronodules, and synergizes with carboplatin in a 3D model for micrometastic ovarian cancer.[14, 84] These studies have collectively provided promising insights into the use of PDT to enhance the efficacy of conventional chemo and biologic therapies.

Photoimmunotherapy

Motivated by our findings with combination of PDT and anti-EGFR therapy,[12] we considered delivering the combination as a single agent by chemically conjugating the photosensitizer chlorine6 (C_{e6}) to Erbitux . The advantage of this approach would be the additional selectivity of delivery of the PS. This is important because, as with other treatment modalities, tumor selectivity remains a key issue in PDT, particularly in complex treatment sites such as the peritoneal cavity.[85, 86] Modest preferential accumulation of photosensitizers in neoplastic tissue has been demonstrated in several tumor models including murine models for ascites and diffuse ovarian carcinomatosis.[83, 87-92] To further enhance selectivity for disease sites, photosensitizers have been conjugated to a variety of targeted macromolecular carries including mAbs.[58, 72, 74, 89, 93-109] Photoimmunoconjugates (PICs) were first described over 25 years ago, [93-95] and subsequently a variety of conjugation strategies and targeting moieties, including those directed against the EGFR, have been investigated by our group and others.[58, 72, 74, 89, 93-109] Using a hamster cheek pouch model of squamous cell carcinoma,

Hemming *et al.* showed that the tumor selectivity of free benzoporphyrin derivative (BPD) increased from approximately 2:1 to 26:1 upon conjugation to an anti-EGFR mAb, and also reported a trend towards increased tumoricidal efficacy.[89] Eighty percent of the animals that received photoimmunotherapy (PIT) (*i.e.* PIC + light) in this study were cancer free after 1 month, as compared to 67% of those treated with free BPD-PDT, but this difference was not statistically significant.[89] It is worth noting that the animals treated with PIT were injected with one-twentieth the BPD equivalent dose relative to the free BPD group. The resulting lower photodynamic dose delivered to the PIT group is particularly important in the context of studies by us and others [59, 60, 89, 92, 103, 105, 106, 110-117] demonstrating that photosensitizer photophysical properties, cellular localization patterns and phototoxic efficacy are altered upon conjugation to macromolecular carriers. The resulting impact on therapeutic outcome is dependent on a variety of factors including the molecular characteristics of the target disease and the trade-off between specificity and cytotoxicity for a particular treatment site.

Studies by Savellano et al.[59, 60] demonstrated the increased selectivity of Cetuximab conjugated BPD, although factors affecting efficacy appear to be more complex. Vrouenraets et al. have developed meta-tetrahydroxyphenylchlorin (mTHPC) and aluminum phthalocyanine tetrasulphonate ($AlPcS_4$)-based PICs directed against a variety of targets, including the EGFR, using mAbs with differential internalizing properties and binding capacities. Conjugates of the non-internalizing mAb U36 demonstrated very poor phototoxicity, even in a cell line that expressed high levels of the U36 defined antigen. However, an AlPcS4 conjugate using the internalizing anti-EGFR mAb 425 was 7500 times more toxic than the free AlPcS4 in A431 cells a human epidermoid carcinoma line that overexpresses the EGFR), and 60 times more toxic than the mTHPC-mAb 425 PIC. A more comprehensive follow up study comparing mTHPC and $AlPcS_4$, three mAbs, in 5 cell lines revealed that the binding capacity of internalized and surface bound PICs was a critical determinant of treatment response, and that internalization capacity alone was not correlated with efficacy.[103] Since the in vitro data in the above studies was acquired in monolayer cell cultures, the significance of these findings in vivo needs to considered within the context of the experimental systems.

Uptake and phototoxic efficacy of C_{e6}, the photosensitizer used in the present study, has been shown to increase upon association with macromolecular carriers. The extent and nature of this increase depends on a variety of factors including the charge, conjugation strategy and sub-cellular localization pattern of the macromolecular conjugates.[92, 110, 118, 119]. Soukos et al. have demonstrated the diagnostic and therapeutic potential of C_{e6}-Erbitux PICs in oral premalignant lesions.[58] Among the challenges associated with leveraging the selectivity and therapeutic benefits of PICs for complex treatment sights has been limited uptake and limited mAb specificity due to photosensitizer conjugation near the antigen binding site. To address this issue, we [58, 74, 98-103, 110, 120] developed an alternative conjugation approach using a poly-L-lysine linker to attach photosensitizers in a site-specific manner to the Fc carbohydrate moiety of mAb, distal from the antigen binding sites. In vitro evaluation of differentially charged PICs synthesized using this method revealed that uptake and phototoxicity of C_{e6} in OVCAR5 cells increased upon conjugation to the $F(ab')_2$ region of an OC125 antibody fragment (directed against CA125, a glycoprotein that is both expressed on the surface of, and shed by, non-mucinous epithelial ovarian cancers).[110] The most significant increase in uptake and cytotoxicity was observed with the cationic PIC, which may have been due to improved internalization and lysosomal degradation, compared to the anionic PIC and free C_{e6}. These results were verified with biodistribution and treatment response studies in a murine model for ovarian carcinomatosis.[101, 102] The cationically charged PIC had the highest tumor selectivity and delivered the most C_{e6} per gram of tumor than all other constructs evaluated in the

biodistribution study.[101] Consistent with these findings, treatment efficacy in the same model, as evaluated by median survival, was highest with the cationic PIC.[102] It is important to note that these results favoring the cationic PIC were based on intraperitoneal administration of the species. Comparable studies in a different tumor model with intravenous administration led to more favorable results for the anionic conjugate, potentially due to more rapid clearance of the cationic PIC from the blood.[99, 100] These promising results informed a study by Duska et al.[74] to evaluate the effect of combining PIT with cisplatin (CDDP) to treat CDDP resistant and sensitive ovarian cancer cells and patient tissue samples. PIT in combination with CDDP was shown to reverse chemoresistance and synergistically reduce tumor viability.[74]

Present study

Motivated by the need to reduce chemotherapy cycles and mitigate toxicity, we evaluate the efficacy of EGFR-targeted PIT in combination with a clinically relevant chemotherapy cocktail (Figure 1B). An Erbitux- C_{e6} conjugate is used for a dual purpose: (i) to selectively deliver C_{e6} to EGFR overexpressing target tissue, and (ii) to simultaneously engage the receptor blocking function of the mAb thus inhibiting EGFR mediated cell proliferation and growth. An initial comparison of PIT efficacy between C_{e6} directly conjugated to Erbitux (Direct PIC) versus indirectly conjugated via a poly-L-lysine linker (Indirect PIC) is conducted. The effect if irradiance, which has been show to be important in PDT (with free photosensitizer),[121-124] but has never been evaluated in PIT(where the PS is bound to macromolecules with consequent altered photophysical properties), is assessed. Lastly, the ability of EGFR-targeted PIT to potentiate the efficacy of cisplatin and paclitaxel in fewer treatment cycles is determined. All studies are conducted in a xenograft murine model for ovarian carcinomatosis (Figure 1A) using the NIH:OVCAR-5 human ovarian cancer cell line.[12, 82, 83, 102]

II. Experimental Section

Cell culture

NIH:OVCAR-5 human ovarian cancer cells were kindly provided by Dr. Thomas Hamilton at the Fox Chase Cancer Institute (Philadelphia, PA). Cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) and maintained at 37°C in an atmosphere of 5% carbon dioxide (CO_2). For harvesting, cells were grown to 80% to 90% confluence disaggregated with trypsin-EDTA (Life Technologies, Inc [GIBCO-BRL], Gaithersburg, Md). Cells were centrifuged at 200 g for 10 minutes, resuspended in phosphate buffered saline without Ca^{2+} or Mg^{2+}, and counted on a hemacytometer.

Ovarian cancer mouse model

All animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. An orthotopic xenograft mouse model previously developed in our laboratory was used.[82] Six to eight-week-old female Swiss athymic Nu/Nu mice weighing 20 to 25 g (Cox Breeding Laboratories, Cambridge, Mass) were injected intraperitoneally with 31.5×10^6 NIH:OVCAR-5 cells in 2 mL of sterile phosphate buffered saline. This model reproducibly produced intraabdominal carcinomatosis adherent to all peritoneal surfaces within 10 to 14 days post-inoculation (Figure 1A).[82] Mice received proper care and maintenance in accordance with institutional guidelines. The mice had continuous access to food and water and were housed in laminar flow racks in pathogen-free condition. We monitored the mice daily for general health status; mice were sacrificed at the end of the study period or if they appear moribund or had an excessive tumor burden.

Photoimmunoconjugates

The procedure for preparing PICs has been described previously.[58, 74, 98-103, 110, 120] Briefly, poly-L-lysine (average molecular weight 25,000) was treated in dimethyl sulphoxide (DMSO) with the N-succinimidyl ester of chlorin e6 to give pl-chlorin e6. The resulting mixture was then reacted with N-succinimidyl 3-(2-pyridyldithio) propionate to form the functionalized derivative pl-chlorin e6-N-succinimidyl 3-(2-pyridyldithio) propionate. MAb Erbitux was partially reduced for 1 hour with 5 mM mercaptoethylamine hydrochloride, dialyzed, and then reacted with pl-chlorin e6-N-succinimidyl 3-(2-pyridyldithio) propionate for 24 hours to form the photoimmunoconjugate, pl-chlorin e6-Erbitux; this photoimmunoconjugate was dialyzed and purified on Sephadex G-200 columns and characterized by absorption and fluorescence spectrophotometry, and polyacrylamide gel electrophoresis.

Chemotherapy

Paclitaxel (Bristol-Myers Squibb Oncology, Princeton, NJ) and cisplatin (Baxter Healthcare Corporation, New Providence, NJ) were obtained from the Massachusetts General Hospital Pharmacy. Paclitaxel was dosed at 15 mg/kg, and cisplatin was dosed at 5 mg/kg. Each dose was diluted in phosphate buffered saline for a total volume of 0.4 to 0.6 mL/dose/mouse. Each drug was administered intraperitoneally. After the drugs were administered, each mouse was observed for acute toxicity and then allowed to return to its cage. Chemotherapy was administered 14 and 19 days after inoculation for mice receiving either one or two cycles of chemotherapy, respectively.

Photoimmunotherapy

In vivo PIT was conducted 20 days after tumor inoculation; mice were given an intraperitoneal injection containing 1 mg/kg chlorin e6 equivalent. Twenty-four hours later, mice were anesthetized by intraperitoneal administration of a 0.04 mL cocktail; the anesthetic cocktail contained 30 mg/mL ketamine and 5 mg/mL xylazine. The method of light delivery has been described previously.[102] In brief, 2 mL of 0.1% intralipid solution (soybean oil emulsion for intravenous use) (Kabi Pharmacia, Inc; Clayton, NC) was injected intraperitoneally immediately prior to treatment to enhance light scatter. The mouse was placed in the supine position. Light was delivered intraperitoneally via an 8.0 mm × 0.4 mm cylindrically diffusing tip using a solid state diode laser (BWF 665-1, B&W TEK, Newark, DE) at a fluence rate of either 30 or 180 mW/cm^2. A total of 25 J/cm^2 of light was delivered at a wavelength of 665 nm. The power output was measured by an integrating sphere and an oscilloscope. The diffusing fiber was introduced into the peritoneal cavity via a 22-gauge catheter that traversed the abdominal wall. One fourth of the total light energy was delivered to each quadrant. At the conclusion of treatment, the mice recovered in an animal warmer until they awoke and resumed normal activity.

Fourteen days after tumor inoculation, mice were given a small identification tattoo on their abdomen using a minute (<0.1 mL) amount of a diluted solution of India ink. The mice were randomly divided into six groups: (*i*) control (N = 19), (*ii*) one cycle of chemotherapy (N =5), (*iii*) two cycles of chemotherapy (N = 6), (*iv*) PIT only (N = 5), (*v*) one cycle of chemotherapy followed by PIT (N = 5), and (*vi*) two cycles of chemotherapy followed by PIT (N = 5). Batches of 7 to 10 tumored mice were randomly assigned to each treatment group. For every batch, 3 mice were assigned to the no treatment group to ensure consistency of tumor growth.

Treatment evaluation

All mice were weighed at the start of the study and at each treatment interval. All mice were sacrificed for necropsy by CO_2 inhalation on Day 24 of the experiment. Prior to sacrifice, all mice were grouped together and randomly picked for order of sacrifice. Two investigators (TAD and IR) performed all necropsies together. The first investigator did the necropsy and the second investigator confirmed completeness of the dissection. Wet tissue weights of excised tumor were obtained (Mettler AE 163, Mettler Instrument Corp, Hightown, NJ). Treatment response was assessed by comparing the extent of gross residual disease in treated animals with the extent of disease in untreated controls. For toxicity studies, mouse weight at the time of sacrifice was used as an indicator of the mouse tolerance for treatment.

After weighing, all resected tissue was fixed in 10% phosphate buffered formalin (Mallinckrodt Inc, Paris, Ky) and embedded in paraffin. Sections, cut 5 μm thick, were stained with hematoxylin and eosin for microscopic evaluation to confirm the presence of carcinoma. Immunohistochemistry was performed on formalin-fixed, paraffin-imbedded specimen slides using a monoclonal mouse anti-proliferating cell nuclear antigen-clone PC 10 (DAKO Corporation, Carpinteria, Calif). Initial blocking serum and peroxidase-conjugated secondary antibody was used from the Vectastain ABC Kit-Peroxidase Mouse IgG and Vectastain Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). Control sections were run concurrently, following the same method, except using phosphate buffered saline in place of the primary antibody. All sections were lightly stained with Gill's 3 hematoxylin (Richard Allen Scientific, Kalamazoo, MI) and coverslipped for evaluation.

Statistical analysis

All values are expressed as mean ± standard deviation. A two-tailed student's t-test was used to analyze the effect of irradiance and conjugation strategy on tumor reduction (Figure 2). For both tumor burden and weight loss (Figure 3 and Table 1), we first examined the synergistic effect of the PIT and one cycle chemo therapy by testing the interaction of the two treatments using analysis of variance. In addition to comparing between each individual group and the control group, we were also interested in the following comparisons: one cycle of chemotherapy plus PIT vs. one cycle of chemotherapy alone, two cycles of chemotherapy plus PIT vs. two cycles of chemotherapy alone, one cycle of chemotherapy alone vs. two cycles of chemotherapy alone, one cycle of chemotherapy plus PIT vs. two cycles of chemotherapy plus PIT, and one cycle of chemotherapy plus PIT vs. two cycles of chemotherapy alone (a total of ten pair-wise comparisons). The unadjusted p values and Sidak adjusted p values (to account for the inflation of type I error from multiple comparisons) were reported for these pre-specified comparisons. P values < .05 were considered statistically significant.

III. Results and Discussion

Results

To establish the role of irradiance and conjugation strategy on PIT efficacy (Figure 2), mice received 1 mg/kg chlorin e6 equivalent of either the direct or indirect PIC. Twenty-four hours later, a total fluence of 25 J/cm^2 was delivered equally among four quadrants in the peritoneal cavity at either a "high" (180 mW/cm^2) or "low" (30 mw/cm^2) irradiance. A trend towards irradiance-dependent reduction in residual tumor weight was observed with the both the indirect and direct PIC. In the indirect PIC group, residual tumor weight was significantly lower with low irradiance PIT (327.1 ± 74.9 mg, n=8) than high irradiance PIT (543.2 ± 67.0 mg, n=9)(P<0.05). No statistically significant difference in residual tumor weight was observed following PIT with the direct PIC in the high irradiance (543.3 ± 111.5 mg, n=11) versus low irradiance (471.0 ± 81.1 mg, n=12) groups (P>0.05). Compared to the

direct PIC, PIT with the indirect PIC did not produce a statistically significant reduction in residual tumor weight at either the low or high irradiance ($P>0.05$). Based on these findings, the high irradiance PIT with the direct PIC was used for subsequent combination studies to minimize the irradiation times and reduce stress on the animals.

As shown in Figure 3, all treated mice showed a reduction in tumor weight relative to the no treatment group (601.2 ± 202.0 mg, n=19). Tumor weights following treatment with PIT alone (472.2 ± 111.0 mg, n=5) or 1 cycle of chemotherapy alone (Chemo 1 cycle) (580.8 ± 189.0 mg, n=5) were not significantly lower than no treatment (none of the unadjusted or adjusted P was < 0.05). PIT in combination with 1 cycle of chemotherapy (Chemo 1 Cycle + PIT) resulted in a residual tumor weight (267.2 ± 252.5 mg, n=5) that was significantly lower than Chemo 1 cycle alone (unadjusted P=0.001, adjusted P=0.011). However, there was no significant evidence of synergistic effect from Chemo 1 Cycle and PIT (ANOVA, P=0.24 for treatment interaction). Chemo 1 Cycle + PIT had lower residual tumor weight when compared to Chemo 2 cycles (314.0 ± 193.8 mg, n=6) but the difference did not reach statistical significance (unadjusted P=0.68, adjusted P=0.99). The addition of PIT to 2 cycles of chemotherapy (Chemo 2 cycles + PIT) was not statistically different from Chemo 2 cycles (unadjusted P=0.34, adjusted P=0.98). Relative to no treatment, Chemo 2 cycles + PIT resulted in the lowest residual disease among all treated groups (203.6 ± 71.1, n=5) (unadjusted P<0.001, adjusted P=0.002).

Mouse weights (in grams) at the time of sacrifice, as a metric for treatment-related toxicity, are detailed in Table 1. Relative to no treatment (23.0 ± 2.6), both the Chemo 2 cycles alone group (10.1 ± 3.6) and the Chemo 2 cycles + PIT group (19.0 ± 1.4) showed a significant reduction in mouse weight (unadjusted P=0.039 and 0.008, respectively). However, the statistical significance diminished after adjustment for multiple comparisons (adjusted P = 0.33 and 0.078, respectively). Relative to Chemo 1 cycle (23.5 ± 1.3), Chemo 2 cycles showed a significant reduction in mouse weight (20.1 ± 3.6) (unadjusted P=0.045, adjusted P=0.37). The addition of PIT did not significantly change the mouse weight at sacrifice when used alone or in combination with one or two cycles of chemotherapy (none of the unadjusted or adjusted P was < 0.05).

Expression of proliferating cell nuclear antigen, a nuclear protein frequently seen in cells undergoing division, is used as a marker for cellular proliferation. In our study, tissue from the control mouse showed uniform, strong staining with a mAb against proliferating cell nuclear antigen (PCNA). In contrast, tissue from mice treated with combination chemotherapy (1 cycle) + PIT showed a weaker and irregular staining pattern. A representative section of diaphragmatic tissue is shown in Figure 4.

Discussion

EGFR targeted treatments with Erbitux or small molecule inhibitors are currently in use for many cancers with variable results.[49, 63, 66, 125, 126] For ovarian cancer, the effects are modest and temporary and chemotherapy following surgery still remains a mainstay for the management of this disease. The main problems associated with chemotherapy or Erbitux monotherapy are high toxicity and acquired resistance resulting from multiple cycles of treatments.[49, 63, 66, 125, 126] The current study, along with previous studies from our group[12, 58, 72, 74, 101, 102, 108] and those of others,[49, 71, 125, 127-139] appears to address some of these problems. The present study shows that chemotherapy and PDT doses may be decreased and yet obtain tumor reductions that are comparable to, or greater than, those achieved with higher doses of either monotherapy alone. We suggest that EGFR-targeted PIT be considered a serious contender as a therapeutic strategy to potentiate the efficacy of standard chemotherapy for disseminated ovarian cancer, with the goal of minimizing treatment cycles and mitigating treatment-related toxicities.

Paclitaxel and platinum-based agents are widely used as first-line therapies for ovarian cancer.[2, 3, 6, 15] Although the majority of patients experience clinical remission of their disease, the tumor often recurs.[4, 140] The effectiveness of cytotoxic therapies is limited by treatment-related toxicities and the development of acquired drug resistance.[4, 140] Moreover, the development of resistance to an individual drug is often associated with broad cross-resistance to structurally similar drugs, which leads to low response rates for salvage chemotherapy. Goldie and Coldman have suggested that most malignant cells have initial intrinsic sensitivity to chemotherapy, but develop spontaneous chemoresistance at variable rates. Clinically, this explains the ability to achieve clinical remission with standard chemotherapy even if the resistant cell lines are present.[141]

PDT is mechanistically distinct from chemotherapy and has been shown to reverse chemoresistance and to synergize with chemo and biologic agents.[12, 14, 72-74, 80] Our results show that the combination of PIT and chemotherapy significantly reduces tumor burden compared with 1 cycle of chemotherapy alone or PIT alone. The amount of tumor burden reduction achieved with 1 cycle of chemotherapy + PIT was comparable to (and showed signs of trending lower than) 2 cycles of chemotherapy. Additionally tumor reduction by PIT appears to be greater after one cycle of chemotherapy as compared with enhancement after two cycles of chemotherapy. This observation may be due to the experimental design. Since PIT was done 7 days after one cycle of chemotherapy, the effect may have been more pronounced as compared with PIT done after the second cycle (2 days post-chemotherapy).

Our observation with necropsies of mice 24 hours after PIT using an Erbitux-chlorin e6 conjugate did not reveal any significant ascites or hydrothoraces. Previous clinical trials using intraperitoneal PDT have been done in ovarian cancer patients under suboptimal conditions using a nonselective photosensitizer (Photofrin; Aptalis Pharma, Birmingham, AL). Delaney et al reported that 59% of patients developed pleural effusions, and 15% of patients required thoracentesis or prolonged intubation.[142] This postoperative edema may be related to a mechanism of tumor destruction via vascular damage from Photofrin-PDT, which, depending on the treatment parameters, may cause vessel constriction and macromolecular vessel leakage as well as prostaglandin release.[143] C_{e6}, used in the present study, causes blood flow stasis due to platelet aggregation without vessel leakage.[144] Our study takes advantage of this important difference in the mechanism of action between the two photosensitizers to overcome the limitations seen in the human studies using Photofrin.

PIC-mediated PIT provides multiple therapeutic benefits including enhanced selectivity for target tissue, increased payload delivery and inherent cytostatic/cytotoxic potential.[58, 83, 92, 97, 101, 103, 110] We used an Erbitux-based PIC to deliver C_{e6} to selectively target the EGFR-expressing ovarian cancer tissue. EGFR expression is associated with aggressive ovarian cancer and poor prognosis and therefore serves as a viable PIT target.[12, 69, 125] Treating cancer cell lines that express functional EGFR with Erbitux potentiates the cytotoxic action of conventional treatment modalities. Previous experience has shown that treatment with Erbitux alone in the same murine xenograft model decreased tumor burden, and the combination Erbitux and PDT not only further decreases tumor burden synergistically but also increased survival times including a 30% cure rate.[12]

Chemotherapy leads to permeability of the gut during treatment as well as loss of protein and decreased absorption of nutrients. Our study uses the weight of mice as an indirect measure of the toxicity of treatment. Our murine model used the NIH-OVCAR-5 cells, and ascites were not a main finding at 24 days after tumor inoculation; thus, ascites did not give significant input to the weight of the mouse at the time of necropsy. Our operative finding at the time of necropsy in mice treated with chemotherapy was significant for the decreased

tumor burden. However, this reduction in tumor burden was offset by increased weight loss. Monotherapy with two cycles of chemotherapy produced mice that appeared more cachectic than those treated with only one cycle of chemotherapy, PIT alone, or PIT in combination with one cycle of chemotherapy. PIT, as monotherapy or in combination with chemotherapy, did not cause weight loss. It is probable that the mice would have regained the weight had the study continued past 24 days.

Samuels et al. evaluated cancer cachexia in a murine model of colon carcinoma and noted that treatment with chemotherapy in nontumored control mice caused a 17% reduction in intestinal protein mass. [145] This decrease of intestinal protein mass was also seen in untreated tumor-bearing mice. However, treatment of tumors with chemotherapy did not increase the intestinal protein loss. Complete and rapid recovery of intestinal protein mass was seen after successful treatment with chemotherapy.

IV. Conclusions

Our study supports the current trend towards the development of rationally designed combination therapies by showing that EGFR-targeted PIT combined with cisplatin-paclitaxel chemotherapy significantly reduces tumor burden with fewer treatment cycles and lower toxicity in a complex model of ovarian intraperitoneal carcinomatosis. Based on previous findings that synergistic interaction between PDT and conventional therapies is dependent on the treatment sequence, the nature of the photosensitizer, and the chemo and biologic agents,[12, 14, 74, 80] the current results merit further investigation to optimize the order, schedule and doses of the combination regimen. Additionally, leveraging minimally invasive imaging systems for treatment planning, and online monitoring of tumor reduction will expedite protocol optimization.

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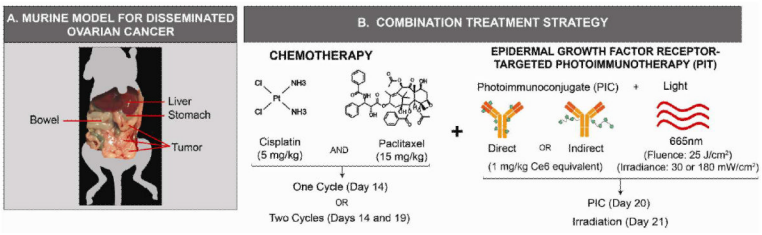

Figure 1. Orthotopic mouse model for disseminated ovarian cancer and experimental schema

A mouse model for intraperitoneal ovarian carcinomatosis (A), which mimics the complex dissemination pattern of advanced stage disease, was used to evaluate the efficacy of EGFR-targeted PIT in combination with chemotherapy (B). The impact of irradiance (30 or 180 mW/ cm^2) and conjugation chemistry (Direct or Indirect PIC) on PIT tumoricidal efficiency was determined. For the combination treatment, mice received either one or two cycles of a clinically relevant chemotherapy cocktail (cisplatin and paclitaxel) followed by a single PIT treatment, and acute tumor burden was evaluated.

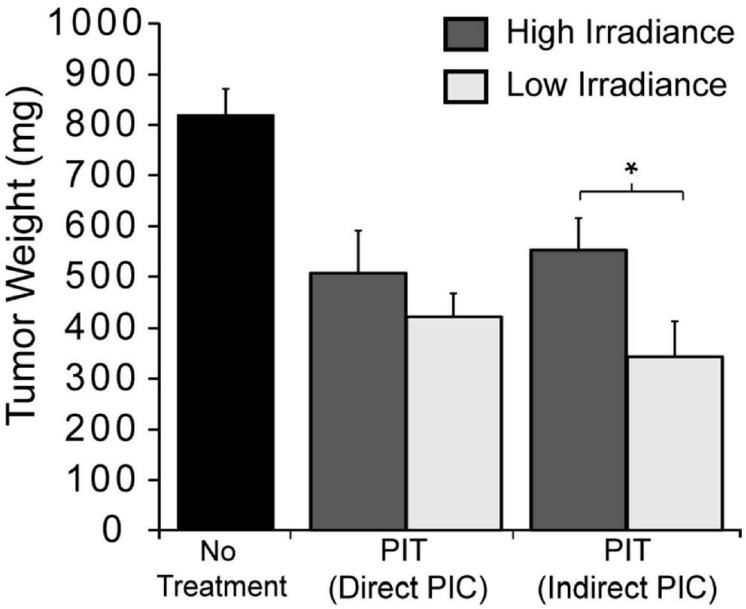

Figure 2. Role of irradiance and conjugation strategy on PIT efficacy

An Erbitux-based photoimmunoconjugate (PIC) with $Chlorin_{e6}$ (C_{e6}) conjugated either directly to the mAb (Direct PIC) or via a poly-L-lysine linker (Indirect PIC) was injected into tumored mice (1mg/kg C_{e6} equivalent). After 24 hours, a total fluence of 25 J/cm^2 was equally distributed in the peritoneal cavity at either a high irradiance (180 mw/cm^2) or low irradiance (30 mw/cm^2). In the Indirect PIC group, low irradiance PIT resulted in a residual tumor weight (327.1 ± 74.9 mg, n=8) that was significantly lower than high irradiance PIT(543.2 ± 67.0 mg, n=9)(student's t-test, $P<0.05$). In the Direct PIC group, there was a trend towards lower residual tumor weight with low irradiance PIT (471.0 ± 81.1 mg, n=12) compared to high irradiance PIT (543.3 ± 111.5 mg, n=11), but the difference was not significant. No statistically significant reduction in residual tumor weight was observed between the Direct and Indirect PIC at either the low or high irradiances.

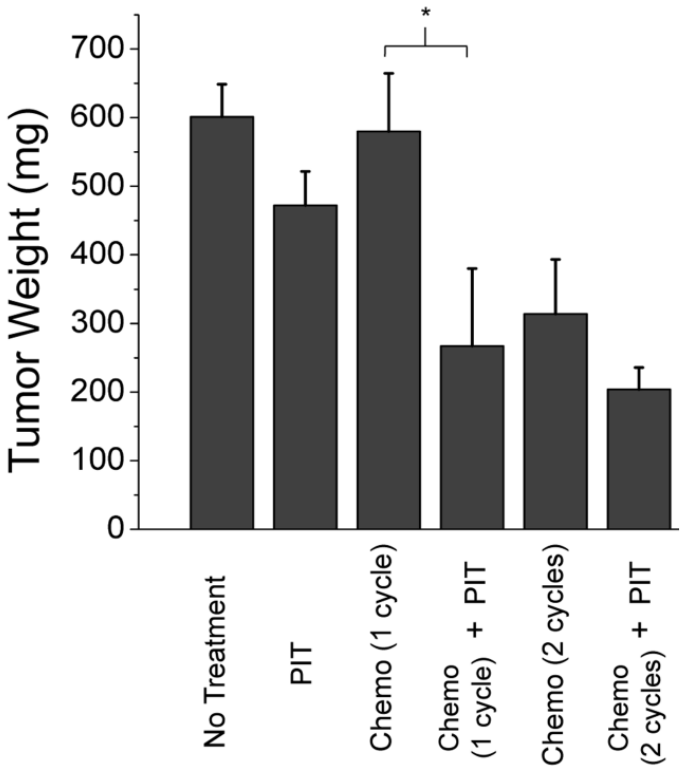

Figure 3. PIT in combination with chemotherapy significantly reduces tumor burden with fewer treatment cycles

PIT in combination with 1 cycle of chemotherapy (Chemo 1 Cycle + PIT) resulted in a residual tumor weight (267.2 ± 252.5 mg, n=5) that was significantly lower than 1 cycle of chemotherapy alone (ANOVA, unadjusted P=0.001, adjusted P=0.011). Chemo 1 Cycle + PIT showed a trend towards a residual tumor weight that was lower than 2 cycles of chemotherapy (314.0 ± 193.8 mg, n=6). The addition of PIT to 2 cycles of chemotherapy resulted in the lowest residual tumor weight (203.6 ± 71.1, n=5), relative to no treatment (unadjusted P<0.001, adjusted P=0.002).

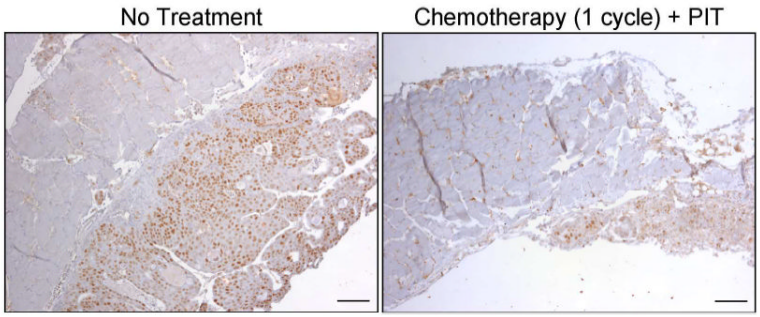

Figure 4. Treatment-based expression of proliferating cell nuclear antigen (PCNA)
Weak and irregular PCNA expression was observed following treatment with combination Chemotherapy 1 cycle of + PIT (right panel), relative to no treatment (left panel).

Table 1

Mouse Weight (in Grams) at Time of Sacrifice by Treatment Group

Treatment Type	
No Treatment	23.0 ± 2.6
PIT	23.5 ± 2.1
Chemotherapy (1 cycle)	23.5 ± 1.3
Chemotherapy (1 cycle) + PIT	22.0 ± 3.7
Chemotherapy (2 cycles)	20.1 ± 3.6
Chemotherapy (2 cycles) + PIT	19.0 ± 1.4