Published in final edited form as:

Isr J Chem. 2012 September 1; 52(8-9): 681–690. doi:10.1002/ijch.201200011.

Photodynamic therapy-induced angiogenic signaling: consequences and solutions to improve therapeutic response

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Abstract

Photodynamic therapy (PDT) can be a highly effective treatment for diseases ranging from actinic keratosis to cancer. While use of this therapy shows great promise in preclinical and clinical studies, understanding the molecular consequences of PDT is critical to designing better treatment protocols. A number of publications have documented alteration in angiogenic factors and growth factor receptors following PDT, which could abrogate treatment effect by inducing angiogenesis and re-establishment of the tumor vasculature. In response to these findings, work over the past decade has examined the efficacy of combining PDT with molecular targeting drugs, such as anti-angiogenic compounds, in an effort to combat these PDT-induced molecular changes. These combinatorial approaches increase rates of apoptosis, impair pro-tumorigenic signaling, and enhance tumor response. This report will examine the current understanding of PDT-induced angiogenic signaling and address molecular-based approaches to abrogate this signaling or its consequences thereby enhancing PDT efficacy.

Keywords

Angiogenesis; growth factors; photodynamic therapy; VEGF; EGFR

1. INTRODUCTION

In photodynamic therapy (PDT) a photosensitizing drug and its light-initiated activation are employed to damage diseased or abnormal tissues associated with conditions such as age-related macular degeneration, Barrett's esophagus, actinic keratosis, and many forms of cancer.^[1] Once excited, the photosensitizer can react with a substrate in the cellular environment thereby producing radicals and reactive oxygen species, or react directly with molecular oxygen, resulting in the formation of singlet oxygen.^[1a, 2] Among the reactive molecules formed, singlet oxygen is thought to be the most prevalent during typical PDT photochemistry, making it the main effector of direct cytotoxicity.^[3] This damage subsequently contributes to the development of secondary effects including ischemia, tissue hypoxia, and inflammation, as well as the altered expression of molecules involved in these

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processes, such as growth factor receptors, matrix metalloproteinases, cytokines, and proteins involved in signaling along apoptosis and/or survival pathways.^[4]

Tissue hypoxia in response to PDT is created through oxygen depletion by the photochemical reaction and local vascular damage that can functionally shut off blood flow to the tumor.^[5] The extent of vascular damage is dependent on the clearance rate of the photosensitizer from the circulation, together with the time interval between drug administration and initiation of light delivery (drug-light interval). A short drug-light interval generally produces more acute damage to the vasculature, while more direct oxidative damage to tumor tissue may accompany longer drug-light intervals.^[6] Moreover, illumination parameters used for treatment (such as the fluence rate), and the composition of the tumor microenvironment, can affect the development of tumor hypoxia during and/or after PDT.^[7] Regardless of whether it develops acutely during PDT or as a secondary consequence of the treatment, PDT-induced hypoxia can result in increased stabilization and by extension increased expression of the hypoxia-inducible transcription factor-1 alpha (HIF-1 α) protein.^[8] Under normoxic conditions, the prolyl hydroxylase domain protein 2 (PHD2) catalyzes the rapid hydroxylation of HIF-1 α , leading to its binding to von Hippel Lindau (VHL) protein and its targeting for ubiquitination and proteosomal degradation.^[9] However, this reaction is blocked in the absence of oxygen through stabilization of HIF-1 α . Once stabilized, HIF-1 α can bind to its partner, HIF-1 β , which is constitutively expressed, forming the functional transcription factor. Upon binding of HIF-1 α to HIF-1 β , the transcription factor can translocate to the nucleus where it mediates cellular response to hypoxia by inducing the transcription of hundreds of genes, many of which are responsible for survival, proliferation, metabolic regulation, and angiogenesis.^[10] Among these genes are survivin (cell survival), transforming growth factor- α (survival, proliferation), insulin-like growth factor 2 (survival, proliferation), and a variety of proangiogenic molecules including vascular endothelial growth factor (VEGF) and platelet derived growth factor B (PDGF-B).^[4a, 9] PDT also elicits a host of other molecular alterations that have the potential to impact tumor progression such as changes in signaling along the PI3K/AKT and MAPK pathways^[4a, 11], and alterations in expression of the epidermal growth factor receptor (EGFR)^[12] and the enzyme cyclo-oxygenase 2 (COX-2).^[13] Like HIF-1 α , activation of these pathways or proteins can ultimately result in increased expression of VEGF, which can have detrimental consequences to therapeutic outcome.

VEGF is one of the most important growth factors in modulating angiogenesis, which is critical to tumor progression. Once a tumor reaches a certain size, it requires its own vasculature in order to maintain a supply of oxygen and nutrients supportive of continued growth. This is achieved by the secretion of growth factors such as VEGF by the tumor or host cells, subsequent stimulation of endothelial cells, and remodeling of the extracellular matrix to allow for proliferation and migration of neovascular endothelial cells that will form the tumor vasculature.^[14] Due to the importance of this growth factor in tumor progression, anti-angiogenic compounds such as the human anti-VEGF antibody bevacizumab, were developed to more effectively target tumors by destroying their vasculature.^[15] Over the past two decades, combinations of conventional cancer therapies, such as radiation and chemotherapy, with these anti-angiogenic compounds have been well-documented with positive outcomes in pre-clinical and clinical studies.^[16]

The past decade has seen an increasing enthusiasm toward applying this treatment paradigm to PDT in an effort to improve efficacy. However, understanding the molecular consequences of PDT is paramount to optimizing treatment protocols. A number of studies have begun to dissect out the signaling events that are activated and inhibited by PDT, and from this work, new preclinical protocols have been developed to examine the efficacy of combining targeted therapy against growth factor receptors and cytokines with PDT. This report is intended to highlight these studies with a specific focus on how to harness molecular targeting therapy, such as with anti-angiogenic compounds, in conjunction with PDT to improve tumoricidal potential.

2. EXPERIMENTAL SECTION

Tumor model and photodynamic therapy

H460 human non-small cell lung carcinoma cells (ATCC, Manassas, VA) were maintained in RPMI-1640 (ATCC) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/ml penicillin, and 100µg/ml streptavidin (all from Gibco, Carlsbad, CA). Cells were cultured at 37°C in a 5% CO₂ atmosphere. H460 tumors were propagated by the intradermal injection of 1×10⁶ cells in saline over the shoulder of nude mice (NCI-Frederick, Frederick, MD). Seven to ten days later, animals received PDT (or control) treatment when their tumors were ~5-7 mm in largest diameter. PDT was performed with the photosensitizer Verteporfin (Visudyne®, QLT Ophthalmics, Vancouver, CA), delivered i.v. at 1 mg/kg, 3h before illumination. Microlens-tipped fibers (Pioneer, Bloomfield, CT) were used to deliver 690 ± 5 nm light from a diode laser (B & W Tek, Inc) over a 1.1 cm diameter field. Laser output was measured with a LabMaster power meter (Coherent, Auburn, CA) and adjusted to deliver an irradiance of 75 mW/cm² at the tumor surface. During PDT, mice were anesthetized by inhalation of isoflurane in medical air, delivered through a nosecone (VetEquip anesthesia machine, Pleasanton, CA). Animal studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and animal facilities are accredited by the American Association for the Accreditation of Laboratory Animal Care.

Anti-angiogenic treatment

Combinations of PDT with anti-angiogenics utilized bevacizumab (Avastin®, Genentech, South San Francisco, CA) to target the tumor-derived human VEGF and an anti-mouse VEGF antibody (R&D Systems, Minneapolis, MN) to target host VEGF. Bevacizumab (10mg/kg) was administered i.p. at 0, 2, and 4h post-PDT and then daily for 14d or until study endpoint. Anti-mouse VEGF antibody (10µg) was administered by intratumor injection at 0 and 4h post-PDT, and then daily for 14d or until study endpoint. Local injections of control antibody (Goat IgG, R&D Systems) followed the same schedule as the anti-mouse VEGF antibody.

ELISA

Tumor-localized concentrations of human and mouse VEGF were assayed by ELISA (R&D Systems). Tumors were excised from euthanized animals at 24h post-PDT and immediately frozen in a slurry of dry ice and 70% ethanol. Frozen tumors were homogenized on dry ice

and the resultant powder was resuspended in cold 1X PBS. Samples were vortexed to create a homogeneous solution and were then freeze-thawed three times to lyse the cells. Following lysis, protein concentrations of cleared lysates were detected using BCA reagent (Thermo Scientific, Rockford, IL). Samples were diluted to 0.25-1mg/ml and loaded onto 96-well plates coated with either human or mouse VEGF. Standard ELISA protocols provided by R&D systems were followed for the remainder of the experiment. Plates were analyzed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA) and Microsoft Excel.

Tumor response

To assess long-term therapeutic outcome, mice were followed daily after light delivery until their tumor volume exceeded that measured pre-treatment. Tumor volume was measured in two orthogonal directions and calculated using the formula: volume = diameter $\times$ width² $\times$ $\pi/6$. A cure was defined as an absence of tumor regrowth at 90 days after PDT.

Statistics

Means and standard errors are reported for summary statistics. Student's t-tests (JMP, SAS Institute, Inc; Cary, NC) were used to assess the effect of PDT or anti-angiogenics on human and mouse VEGF levels. Data were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

PDT increases both tumor- and host-derived VEGF

As a potent angiogenic factor, VEGF is a key molecule in the initial development and post-treatment recurrence of solid tumors. VEGF-stimulated proliferation and migration of endothelial cells, increases in vascular permeability, and downregulation of the adaptive immune response all contribute to a microenvironment primed for tumor growth.^[16b] A widespread role for VEGF in PDT response is evident; reports in murine models of human glioblastoma, mouse mammary carcinoma, mouse squamous cell carcinoma, and human prostate cancer have all documented increased expression of VEGF following PDT.^[8a, 14a, 17] Moreover, Zhang, et al^[18] reported increases in VEGF, as well as increased endothelial cell proliferation, in the normal brain of animals that received low dose PDT. The latter study emphasizes the importance of understanding the effects of PDT on the microenvironment because increased angiogenesis in tumor-adjacent normal tissue could aid tumor progression and diminish therapeutic efficacy.

In agreement with the above reports, we have also found PDT to induce increases in VEGF production, in this case after Verteporfin-mediated PDT of human tumor xenografts of non small-cell lung cancer (H460). At 24h after PDT, a significant increase in human VEGF was detected, characteristic of the known effects of PDT on tumor microenvironment (Figure 1A). This change was modest, however, from 189.7 ± 28.4 pg/mg protein in untreated controls to 347.2 ± 47.2 pg/mg protein in PDT-treated tissues. It should be noted that while levels of human VEGF varied somewhat between control conditions, neither light-alone nor Verteporfin-alone led to VEGF levels that were significantly higher than those in untreated controls ($p > 0.23$ for both light- and drug-only compared to untreated). This speaks to inter-

tumor heterogeneity in baseline VEGF levels and suggests that PDT-induced increases may be even more modest than indicated by comparison to the untreated tumor controls.

Our results demonstrating a modest increase in human VEGF levels at 24h after PDT are in agreement with those of other groups using various endpoints and treatment conditions. For example, Solban, et al^[17a] reported a ~2-fold increase in human VEGF levels 24h after Verteporfin-PDT of an orthotopic model of human prostate cancer (LNCaP). Likewise, Bhuvanewari, et al^[19] showed a modest increase in serum VEGF levels 30d after hypericin-PDT in a murine model of human bladder carcinoma. This group also demonstrated modest, but statistically significant, increases in VEGF 48-72h after hypericin-PDT in a murine model of human nasopharyngeal carcinoma.^[20]

In contrast to our findings with human VEGF, we identified very large increases of ~10-fold in tumor-localized murine VEGF after PDT; levels increased to 627 ± 110.5 pg/mg protein at 24h after PDT, compared to 53.7 ± 16.3 pg/mg protein in untreated tumors (Figure 1B). This large increase in mouse VEGF levels can potentially be attributed to a variety of cell types, since numerous host cells (i.e. - endothelial cells, macrophages, neutrophils, T lymphocytes) are potential sources of VEGF.^[16b, 21] Interestingly, others have also observed increases in host VEGF after both Photofrin- and hypericin-PDT in murine models of Kaposi's sarcoma and nasopharyngeal cancer, respectively, but these changes were more modest.^[8c, 20] The stronger induction of host-derived VEGF in our present results could be reflective of photosensitizer-dependent differences in the response of host tissues to PDT and are worthy of further investigation. These results emphasize the importance of considering the contribution of the host tissue to the tumor microenvironment, especially with respect to the secretion of growth factors and other cytokines that are typically associated with inflammatory and angiogenic responses as a result of PDT.

In both tumors and host tissues, hypoxia-initiated activation of HIF-1 α represents one major pathway through which VEGF production may be induced.^[8a, 10b] This has presented a unique problem in the treatment of cancers with PDT because while the therapy can be highly efficacious in killing tumor cells through vascular shutdown and hypoxia, the likelihood of recurrence may be greater if the tumor is able to re-establish its blood supply through an increase in VEGF-mediated angiogenesis. While much literature exists to demonstrate a link between HIF-1 α and VEGF induction following PDT, some reports have implicated signaling events along other pathways to be important. For example, Solban, et al^[17a] suggested that PDT-induced increases in VEGF secretion in prostate cancer cells (LNCaP) could be attributed to the p38/MAPK signaling pathway. In these studies, the silencing of Hif-1 α through RNA interference, or treatment of cells with the COX-2 inhibitor NS-398 or the PI3K/AKT inhibitor LY294002, did not impact PDT-induced increases in VEGF. However, treatment of cells with the p38/MAPK inhibitor SB202190 attenuated PDT-induced increases in VEGF, indicating that this pathway was critical for this specific cell line. An important note made in this study is that cellular response to PDT is "system specific", suggesting that PDT treatment planning requires careful consideration of the specific tumor to be treated as well as its molecular characteristics.^[17a]

Although a PDT-induced increase in tumor VEGF is well-established in a variety of tumor models, there are reports that this does not always hold true for VEGF in the serum. For example, Osiecka, et al^[22] found that treatment of murine fibrosarcoma tumors in vivo resulted in decreased levels of serum VEGF 24h after PDT. A similar observation was made by Thong, et al^[23] in a murine model of human nasopharyngeal carcinoma. Osiecka, et al suggested that the PDT-induced decrease in VEGF could result from the damage and transient decrease in cells that secrete VEGF in the blood as a result of the light treatment. Thong, et al attributed the decreased VEGF levels observed in their study to a number of factors, including the use of a low fluence rate, longer drug-light interval, and a multi-fractionated approach to light delivery in which animals received several short bursts of PDT in an effort to preserve tumor oxygenation. In a more recent study by this group, the discrepancies observed between decreased serum VEGF and increased tumor VEGF appear to have a temporal explanation. In this study, both mouse and human VEGF levels were decreased in the serum 24h post-PDT, but rebounded to control levels by 72h post-PDT. In the tumor tissue, the authors also noted a small decrease in human and mouse VEGF 24h after PDT, but by 72h post-PDT, levels were increased compared to controls, which they suggested was an indication of tumor regrowth.^[20] Taken together, these results stress the importance of evaluating not only study-dependent differences in treatment conditions, but also the temporal effects of PDT in multiple tissues to obtain a clearer picture of the biological consequences of PDT.

Combinations of PDT with anti-angiogenics

Given the expected limitations that treatment-induced increases in VEGF expression could place on therapeutic response, a number of groups have examined the effect of combining anti-angiogenic agents with PDT to improve outcome. This hypothesis was not without precedence – in the 1990s, researchers began combining anti-angiogenic compounds with either chemotherapy^[24] or radiation therapy.^[25] Dimitroff, et al^[26] and Ferrario, et al^[8a] were among the first to combine anti-angiogenic compounds with PDT. Dimitroff, et al demonstrated the anti-angiogenic properties of a broad spectrum tyrosine kinase inhibitor (PD166285) and a fibroblast growth factor receptor (FGFR)-specific tyrosine kinase inhibitor (PD173074), as evidenced by their ability to inhibit microcapillary formation and angiogenesis in vitro. Combinations of these agents with HPPH-PDT served to prolong the tumor-free interval in murine mammary 16c tumors.^[26] Ferrario, et al reported that combining Photofrin-PDT with anti-angiogenic compounds EMAPII or IM862 resulted in decreased levels of VEGF post-PDT and increased curative outcome for tumors of mouse mammary carcinoma. Similarly, in bladder carcinoma xenografts, a reduction in tumor volume was observed in animals treated with PDT and bevacizumab.^[19, 27] Using in vivo confocal endomicroscopy, it was observed that PDT + bevacizumab caused greater vascular destruction than PDT alone.^[27] Likewise, the PDT-induced increase in VEGF observed in vivo was significantly reduced when PDT was combined with bevacizumab.^[19] Interestingly, this study also observed that the addition of bevacizumab to PDT decreased expression of HIF-1 α protein levels, although PDT as a single entity, under the conditions that they employed, did not increase HIF-1 α levels compared to untreated controls.^[19] The authors infer that HIF-1 α protein expression may have been high in control tumors due to aberrant signaling, and indeed, increased expression and activation of HIF-1 α can be

achieved in an oxygen-independent manner involving activation of molecular signaling pathways induced by cell surface growth factor receptors.^[28] Nevertheless, their findings that combination therapy with bevacizumab and PDT served to decrease both HIF-1 α and VEGF expression is consistent with the known effect of HIF-1 α signaling on VEGF levels, while also pointing to a need for further investigation on the role of HIF-1 α in mediating the molecular consequences of combining anti-angiogenics with PDT.

Work from our lab also shows a therapeutic benefit to combining PDT with anti-angiogenic compounds. In H460 xenografts, we found that administration of bevacizumab post-PDT decreased human VEGF levels ~6-fold at 24h post-PDT, while, as expected, administration of mouse anti-VEGF antibody after PDT had no effect on human VEGF secretion (Figure 2A). A similar 6-fold decrease in mouse VEGF was observed in animals treated with Verteporfin-PDT followed by anti-mouse VEGF, but bevacizumab had no effect on mouse VEGF levels (Figure 2B). It should be noted that a non-specific ~1.5-fold decrease in mouse VEGF was observed in tumors treated with the goat-IgG antibody control ("PDT + Ab control") or bevacizumab ("PDT + bevacizumab") (see both in Figure 2B) compared to PDT treatment alone ("Verteporfin-PDT", Figure 1B). Importantly, however, the VEGF-targeted mouse antibody produced an additional 6-fold decrease in mouse VEGF that is far greater than this non-specific effect. Overall, these data suggest a role for both host and tumor in the post-PDT induction of angiogenic factors important for tumor progression. Tumor response studies validate this point in that we observed no benefit to therapeutic response when Verteporfin-PDT was combined with only bevacizumab. In contrast, the combination of PDT with both mouse anti-VEGF antibody and bevacizumab produced a 33.3% cure rate, compared to 8.3% among animals that received only PDT (Figure 3).

Clearly, there is a benefit to be had in the post-PDT administration of anti-angiogenics in many treatment protocols, which leads to the secondary question as to the importance of the timing of anti-angiogenic delivery relative to PDT. This question was explored in an orthotopic model of prostate cancer by Kosharsky, et al^[29] who showed a benefit to combining TNP-470 with Verteporfin-PDT when the anti-angiogenic compound was added after, but not before PDT. These results were corroborated in a study by Ju, et al^[30] who reported the simultaneous delivery of PDT and an anti-VEGF antibody to abrogate regrowth of ocular neovasculture; in contrast, treatment was ineffective if antibody administration preceded PDT. These authors speculated that pre-PDT delivery of the anti-angiogenic may have diminished photosensitizer delivery or altered immunological components of the PDT response. Interestingly, however, studies of radiation and chemotherapy have employed pre-treatment administration of anti-angiogenics to prune and "normalize" abnormalities in tumor vasculature. Under these conditions, anti-angiogenic treatment served to enhance tumor accumulation of chemotherapeutics or decrease tumor hypoxia and, as a result, improved therapy response.^[31] These results speak to the value of continuing investigations on the molecular and biological consequences of anti-angiogenics in different temporal combinations with PDT.

Inhibition of other molecular pathways in a combined modality approach

While the increased expression of VEGF post-PDT is well-documented, it is important to note that this is not the only molecular alteration observed. For example, PDT induces changes in expression/activation of EGFR, PI3K/AKT and MAPK signaling, COX-2, survivin, and HIF-1 α .^[4a, 12-13] Therefore, it would stand to reason that combining PDT with inhibitors against these pathways would also provide enhanced therapeutic effect. Indeed, such combinations have been reported with positive results. For example, PDT efficacy can be improved by adding inhibitors against COX-2 or heat shock protein 90 (Hsp90) to the treatment protocol.^[4a, 8d, 13, 32] Ferrario, et al^[13, 32a] showed a large increase in the percentage of tumor-free animals following PDT combined with COX-2 inhibitors (celecoxib and NS-398), as well as a decrease in expression of inflammatory cytokines and VEGF. In a more recent study, treatment with the Hsp90 inhibitor, 17-AAG, was reported to decrease the expression of several proteins required for tumorigenesis including survivin, Hif-1 α , and VEGF, in a mouse mammary carcinoma cell line. In an in vivo model of this disease, an increase in curative response was observed following combination PDT and 17-AAG treatment. It was suggested that the enhanced efficacy of the combination treatment resulted from the inhibition of multiple pathways that would otherwise be activated by Hsp90, such as AKT, HIF-1 α , and survivin.^[8d] Therefore, combining PDT with these types of inhibitors could serve as a multi-pronged attack in killing the tumor.

Another major molecular target currently being evaluated in the context of combination therapy is the epidermal growth factor receptor (EGFR). EGFR is one of four receptors in the ErbB family of receptor tyrosine kinases. The active receptor exists as a dimer of the ErbB-1/HER1 proteins and is activated upon binding of ligand (e.g. – EGF, TGF- α) to the extracellular binding domain.^[33] Downstream targets of EGFR activation include the PI3K/AKT and MAPK signaling pathways that regulate cell survival, motility, proliferation, and angiogenesis, all of which contribute to a favorable environment for tumor growth.^[33-34]

Overexpression of EGFR is linked to poor prognosis in a variety of cancers, including non-small cell lung carcinoma, breast, ovarian and prostate cancers, head and neck squamous cell carcinoma, and colorectal cancer.^[14c] Several agents are currently in use to inhibit EGFR in tumors where the receptor is overexpressed, including an anti-EGFR antibody (cetuximab) and two small molecule inhibitors of EGFR (gefitinib, erlotinib).^[14c, 35] While the efficacy of these drugs as stand-alone treatments is modest, improved benefit has been observed when these agents are used in combination with other standard therapeutics such as chemotherapy and radiation. In a murine model of human non-small cell lung carcinoma, it was reported that combination treatment with cisplatin/erlotinib resulted in a 98% inhibition in growth of A549 tumor xenografts, compared to 30% when cisplatin was employed as stand-alone therapy.^[36] Likewise, in BxPC-3 pancreatic tumor xenografts, addition of cetuximab or erlotinib increased the therapeutic benefit of gemcitabine/radiation compared to gemcitabine/radiation alone.^[37]

More recent studies have evidenced a similar benefit when combining PDT with EGFR inhibition. Among the first to use this combination therapy regimen were del Carmen, et al^[38], where they reported that treatment of intraperitoneal ovarian cancer xenografts with

PDT and cetuximab reduced tumor burden compared to treatment with either PDT or cetuximab alone. Importantly, overall survival of mice receiving combination therapy was also improved compared to mice receiving either therapy individually.^[38] Bhuvaneswari, et al^[39] showed a similar effect in bladder carcinoma xenografts whereby combination PDT and cetuximab treatment resulted in better tumor response than PDT or cetuximab alone. Increased apoptosis, as well as decreased expression of downstream targets of EGFR, cyclin-D1 and c-myc, were also reported.^[39]

Many studies of combination therapy have found success in the combination of one specific molecular targeting agent with a standard therapy such as chemotherapy or radiation. However, a common problem observed when treating patients with EGFR inhibitors is the development of resistance to the drugs, ultimately contributing to disease recurrence. Resistance to EGFR inhibitors is suggested to occur in a number of ways: 1) presence of mutations in K-RAS, 2) EGFR-independent activation of PI3K/AKT signaling, and/or 3) secondary mutations in EGFR.^[40] The first two mechanisms are considered “primary” resistance, since these are factors intrinsic to the tumor. The third mechanism is one of “acquired” resistance, because it typically occurs in response to EGFR inhibition therapy. “Acquired” resistance to EGFR inhibition therapy may be associated with increases in VEGF levels, arising from selective pressure imparted on the tumor cells by EGFR inhibition.^[41] Similarly, in cells exhibiting “primary” resistance, large increases in tumor (human) and host (mouse) VEGF levels can be found in resistant cells compared to cells that are sensitive to EGFR-inhibition. Interestingly, combining EGFR inhibition with bevacizumab to block VEGF was shown to inhibit tumor growth in models of both “primary” and “acquired” resistance more than either treatment alone.^[40]

By extension, an even greater therapeutic benefit can be achieved when multiple molecular targeting drugs are combined with PDT. Bhuvaneswari, et al^[42] showed that combining PDT with both bevacizumab and cetuximab resulted in greater efficacy than single combinations of the drugs with PDT. This study also notes that this unique treatment protocol inhibits endothelial cell invasion, tube formation, and new vessel growth. Thus, blocking both VEGF and EGFR signaling was strongly anti-angiogenic and dramatically impacted the ability of the tumor to form new vasculature in response to PDT insult.^[42]

Molecular-Targeted PDT

An alternative approach to conventional combination therapy is the conjugation of photosensitizers to antibodies against molecular targets with potential roles in angiogenic signaling. Photoimmunotherapy (PIT) has shown great promise in the laboratory as a way of increasing the specificity of photosensitizers to tumor cells as well as enhancing the efficacy of molecular targeting antibodies.^[43] In a mechanistic study of this approach, Savellano and Hasan^[43b] designed a photosensitizer immunoconjugate (PIC) between Verteporfin and cetuximab. Treatment of various cell lines with this PIC showed increased PDT-induced cytotoxicity only in cells which overexpressed EGFR. This approach can trade potency for specificity since the extent of PDT-induced cytotoxicity was greater in cells treated with free Verteporfin; nonetheless, it is preferential to find therapies that can spare as much normal tissue as possible when performing PDT. Since drug specificity to tumor cells is strongly

desired, improving PIC-based PDT may only require careful dosimetric consideration; for example, efficacy might be improved by increasing drug-light interval, light dose, or both.^[43b] Similarly, Abu-Yousif, et al^[44] showed that treatment of OVCAR5 cells with Verteporfin-Cetuximab PDT resulted in decreased cell viability compared to non-treated controls, and that treatment was specific to cells overexpressing EGFR, further validating the ability of PICs to spare normal tissue from PDT-induced damage. Importantly, these authors also showed PIC-mediated PDT decreased EGF-induced activation of pEGFR, pAKT (Ser473), and pMAPK/pERK, all of which can be involved in the induction of angiogenesis.

In a very recent study by Mitsunaga, et al^[45], a new PIC was developed by conjugating trastuzumab (anti-HER2 antibody) or panitumumab (anti-HER1 antibody) to a near-infrared dye. In tumor xenografts of A431 or 3T3-HER2 cells, PIT with these agents resulted in reduced tumor volume and significantly prolonged survival following treatment. How this combination therapy compares to stand-alone PDT was not reported. An interesting note from this study and a previous study by Soukos, et al^[43c] is that use of PICs can aid in distinguishing tumor from its surrounding normal tissue because these conjugates are designed to specifically target tumor cells. Such photodiagnostic applications could prove useful at several tumor sites, including in the oral cavity where pre-cancerous lesions can be difficult to visualize.^[43c]

Following the same principle as PIT, another method to target cancer cells with PDT was reported by Hu, et al.^[46] In this instance, the authors took advantage of the overexpression of the cell surface receptor, tissue factor (TF), on tumor cells. TF is a single-pass transmembrane protein that is expressed on the surface of endothelial cells following vascular injury or during angiogenesis. However, oncogenic transformation can result in overexpression of TF in the tumor cells, which aids tumor progression by inducing angiogenesis^[47] and activating MAPK and PI3K/AKT signaling pathways.^[48] In order to selectively target TF, Hu, et al conjugated Verteporfin (VP) to the serine protease factor VII (FVII) which binds with high affinity to the extracellular domain of TF. This would therefore specifically target the photosensitizer to tumor cells and the neovascular endothelial cells of the developing tumor vasculature where TF is overexpressed, while sparing the surrounding normal tissue and the quiescent endothelial cells of the normal vasculature. Levels of apoptosis and necrosis were greater in the FVII-VP treated cells compared to PDT alone or control cells. In vivo, the therapy was shown to be effective in decreasing tumor growth in subcutaneous mouse breast cancer tumors.

Molecular targeting of PDT toward an anti-angiogenic effect can also be achieved through conjugation of photosensitizing agents to specific “carrier molecules” that can target cell surface receptors involved in angiogenic signaling.^[49] These carrier molecules come in a variety of forms from short peptides to synthetic nanoparticles. For example, Tirand, et al^[50] conjugated a chlorin-based photosensitizer (TPC) to a VEGF-receptor heptapeptide in an attempt to target endothelial cells, specifically. The authors looked at the effect of TPC-heptapeptide PDT and found a dramatic decrease in human umbilical vein endothelial cell (HUVEC) survival in vitro compared to conventional TPC-PDT. However, this study did not evaluate the effect of TPC-heptapeptide PDT on tumor cell kill in vivo. Kameyama, et

al^[51] constructed a synthetic nanoparticle that contained Verteporfin and was conjugated to an anti-EGFR antibody to achieve tumor cell specificity. When mice bearing A431 tumors received this nanoparticle and light, the authors noted prolonged survival compared to controls. In a different context, Renno, et al^[52] conjugated Verteporfin to a “homing” peptide for VEGFR-2 in order to target neovascular endothelium in a rat model of choroidal neovascularization (CNV). The authors chose this peptide because of its specificity for VEGFR-2 and its ability to compete for binding with VEGF, ultimately inhibiting VEGF-mediated angiogenesis. In this study, it was found that while treatment with standard Verteporfin-PDT and VEGFR-2 targeted Verteporfin-PDT similarly closed CNV areas 24h post-illumination, the damage to the normal retinal tissue (i.e. – photoreceptors, retinal pigment epithelium, choroid) was greater with conventional PDT compared to targeted PDT. Importantly, the authors suggest that a benefit to using this specific type of targeted PDT is that it is highly specific for angiogenic vessels, thereby limiting damage to the surrounding normal tissue.

4. CONCLUSIONS

It is evident that PDT has great therapeutic benefit in treating a wide variety of cancers. However, to broaden the use of this therapy in the clinic, it is important to evaluate how PDT impacts the tumor microenvironment, and how this can vary depending on the molecular characteristics of individual cancers. Great strides have been made in understanding the molecular effects of PDT, especially with respect to expression of angiogenic factors such as VEGF. The finding that PDT could induce increased expression of VEGF was an important observation that has led to the development of new treatment methods that combine PDT with anti-angiogenic compounds. Indeed, our lab and others have found that addition of anti-angiogenic compounds to typical PDT protocols attenuates the PDT-induced increase in VEGF and enhances therapeutic responses. These studies, coupled with the increasing body of evidence for the addition of other molecular targeting agents to improve PDT outcomes, will serve as a valuable foundation on which to base future investigation of combining PDT with drugs that exploit the tumor microenvironment.

ACKNOWLEDGEMENTS

Support was provided by National Institutes of Health through R01-CA-085831, R01-CA-129554, PPG-CA-087971, PPG-CA-087971-S1, and T32-CA-009677.

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Figure 1.

Verteporfin-PDT induces VEGF secretion. A) Human VEGF (hVEGF) ELISA was performed on tumors removed 24h after Verteporfin-PDT. Verteporfin-PDT treated tumors (“Verteporfin-PDT”) had significantly more VEGF than untreated tumor controls (“Tumor”). VEGF levels in Light only (“Light”) and Verteporfin only (“Verteporfin”) controls were not significantly different from either Verteporfin-PDT or tumor controls. B) Mouse VEGF (mVEGF) ELISA showed a statistically significant increase in VEGF 24h after Verteporfin-PDT compared to tumor, light, and Verteporfin controls. $n = 6-9$ animals per group in PDT-treated and untreated controls; $n = 3-6$ animals per group for light and Verteporfin only controls. VEGF levels are expressed as pg VEGF/mg of total protein loaded. Data are expressed as mean $\pm$ SE. * $p < 0.05$, Students’ t-test.

Figure 2.

Combining anti-angiogenic therapy with Verteporfin-PDT attenuated PDT-induced increases in VEGF. A) 24h post-PDT, hVEGF levels were significantly decreased in tumors treated with bevacizumab and Verteporfin-PDT (“PDT + Bevacizumab”) compared to PDT + antibody controls (“PDT + Ab control”) or PDT + anti-mouse VEGF antibody (“PDT + anti-mVEGF”) as determined by ELISA. B) ELISA showed a significant decrease in mVEGF 24h after PDT in tumors receiving Verteporfin-PDT + anti-mVEGF treatment compared to PDT + Ab control or PDT + bevacizumab groups. $n = 3-6$ animals per group. VEGF levels are expressed as pg VEGF/mg of total protein loaded. Data are expressed as mean $\pm$ SE. * $p < 0.05$, Students’ t-test.

Figure 3.

Verteporfin-PDT tumor responses are benefited by adding human and mouse specific anti-angiogenics. Treatment of animals with Verteporfin-PDT (“PDT alone”) shows an 8.3% cure rate, which is improved to 33.3% with the addition of anti-mouse VEGF and bevacizumab (“PDT + anti-mVEGF + Bevacizumab”). Note that treatment with PDT and bevacizumab alone (“PDT + Bevacizumab”) or PDT with goat-IgG antibody controls (“PDT + Ab Control”) did not confer any therapeutic benefit compared to PDT alone. Controls for untreated tumor, light alone, and Verteporfin alone did not experience any regrowth delay (data not shown). $n = 6-12$ animals per group.