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Photodynamic Therapy in Combination with Antiangiogenic Approaches Improve Tumor Inhibition

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1. Introduction

Photodynamic therapy is a non-surgical and minimally invasive procedure that is rapidly developing as a cancer treatment modality. It involves the administration of a photosensitizer that selectively accumulates in the tumor tissue, which is subsequently activated with light of specific wavelength that interacts with molecular oxygen to form toxic, short-lived species known as singlet oxygen, which causes tumor cell death (Macdonald & Dougherty, 2001). The evident advantage of PDT over other conventional cancer treatments such as chemotherapy and radiotherapy is its selective targeting and reduced toxicity (Dolmans et al., 2003). The treatment is relatively non-invasive as it usually only requires targeted illumination of the tumor site. PDT can also be repeated without detrimental consequences to the patients. Currently, PDT is being successfully used for the treatment of early lung cancers (Moghissi et al., 2007; Usuda et al., 2006) and in dermatology for the treatment of non-melanoma skin cancers and precancerous diseases (Klein et al., 2008). PDT has also been successfully employed to treat early carcinomas of the oral cavity and larynx to preserve normal tissue and improve cure rates (Biel, 2007). In the past 20 years, PDT has been successfully used for the treatment of dermatological diseases, ophthalmic diseases, head and neck cancers, brain tumors, pulmonary and pleural mesothelial cancer, cardiovascular disease, gastroenterological cancer, urological disease and gynaecological cancer (Z. Huang, 2005).

However, PDT is an oxygen consuming modality, and an inherent consequence of PDT is local hypoxia. This condition arises either due to direct oxygen consumption during treatment or indirectly due to the destruction of tumor vasculature. As a result, cells under hypoxic stress may switch to an adaptive response by inducing hypoxia inducible factor like

HIF-1α thus triggering angiogenesis. Angiogenesis is the formation of new blood vessels from pre-existing vessels. It is a vital process in the progression of cancer from small, localized neoplasms to larger, growing, and potentially metastatic tumors (Folkman, 2002). Therefore, the process of tumor angiogenesis is triggered by the tumor's release of pro-angiogenic signals such as vascular endothelial growth factor (VEGF), which bind to receptors on nearby vessel endothelial cells. VEGF is a potent regulator of tumor angiogenesis that plays a critical role by increasing blood vessel permeability, endothelial cell growth, proliferation, migration and differentiation (Ferrara, 2004). It is upregulated in response to hypoxic conditions in tumor via the transcription of hypoxia-inducible factor (HIF-1) (Pugh & Ratcliffe, 2003). Cellular and circulating levels of VEGF have been elevated in haematological malignancies and are adversely associated with prognosis (Giles, 2001).

Reports on tumors treated with PDT showed an upregulation of various angiogenic factors like VEGF, HIF-1α, cyclooxygenase-2 (COX-2), basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMPs) (Solban et al., 2006; Yee et al., 2005). Studies have shown the upregulation of HIF-1α, VEGF, COX-2 and bFGF after hypericin-mediated PDT treated tumors, suggesting that PDT-induced damage to tumor microvasculature and the resultant hypoxia upregulated the expression of certain proangiogenic factors (Zhou et al., 2005). They also reported that the inclusion of various angiogenic inhibitors along with PDT treatment enhanced the PDT effectiveness. Currently, anti-angiogenesis agents are being developed to target different growth factors and molecular pathways that play a major role in tumor angiogenesis.

This chapter evaluates expression of VEGF after PDT and also the efficacy of PDT by combining monoclonal antibodies (angiogenesis inhibitors) against VEGF and epidermal growth factor receptor (EGFR) to improve the overall bladder tumor responsiveness. The following approaches were adapted in this study: (i) evaluating the expression of VEGF after PDT, (ii) targeting the VEGF pathway using monoclonal antibody, Avastin, to inhibit tumor angiogenesis and also to study the effect of Avastin on other angiogenic growth factors; (ii) targeting the EGFR pathway, using the monoclonal antibody Erbitux to inhibit tumor angiogenesis and to assess its effect on the EGFR pathway and finally (iii) combining both Avastin and Erbitux with PDT to assess the importance of blocking the two major angiogenic pathways, VEGF and EGFR, to improve treatment outcome.

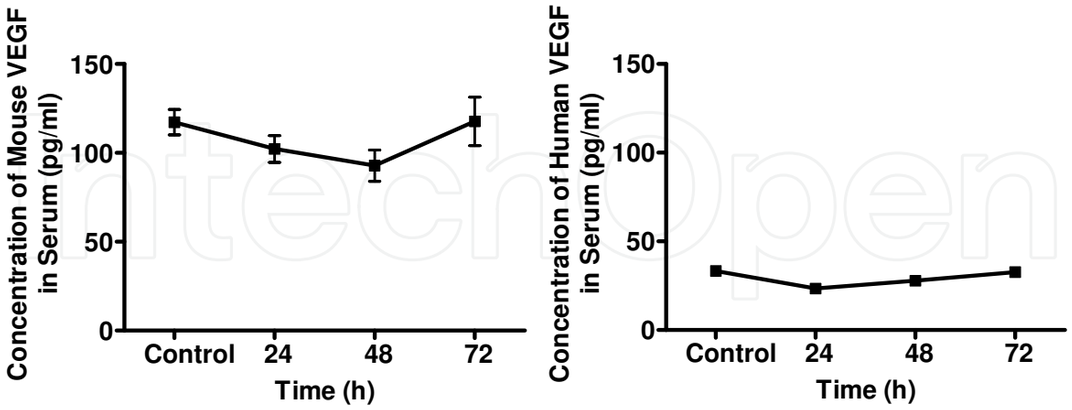
PDT followed by Avastin inhibited VEGF expression and other important growth factors to improve tumor response in bladder carcinoma xenografts. In a similar way, PDT and Erbitux suppressed growth factors related to the EGFR pathway to produce better treatment outcome. It was noticed that PDT induced tumor destruction can be maintained and significantly enhanced by the administration of Erbitux. VEGF and EGFR pathways play a major role in angiogenesis of bladder tumors. Combining angiogenic inhibitors with PDT protocol to block VEGF and EGFR pathways has proven to be effective in controlling tumor regrowth. Therefore, antiangiogenesis agents may augment the activity of PDT by inhibiting its counterproductive upregulation of VEGF and EGFR. The success achieved by combining angiogenic inhibitors with PDT can provide information for potential target mechanisms, which can be translated into clinical studies with better response rate, less local and systemic toxicity and improved overall survival in patients.

2. Photodynamic therapy induced VEGF

Vascular endothelial growth factor is one of the most important regulators of angiogenesis that acts as a switch to trigger tumor recurrence by promoting proliferation, migration and

tube formation of endothelial cells. Moreover, VEGF binds to the tyrosine kinase receptors, VEGFR-1 and VEGFR-2 thus initiating a downstream signaling cascade that promotes angiogenesis (Kowanetz & Ferrara, 2006). In vitro studies have clearly demonstrated that VEGF is a potent mediator of angiogenesis as it helps in the proliferation and migration of the endothelial cells to form tube like capillaries (Bernatchez et al., 1999). Studies have reported that hypoxia plays a major role in the expression of VEGF in tumor tissue (Robbins et al., 1997). It has also been reported that PDT produced significant increases in VEGF within treated lesion (Ferrario & Gomer, 2006). The expression of VEGF in areas surrounding tumor necrosis has also suggested that hypoxia within tumors played a major role in angiogenesis (Senger et al., 1986; Shweiki et al., 1992).

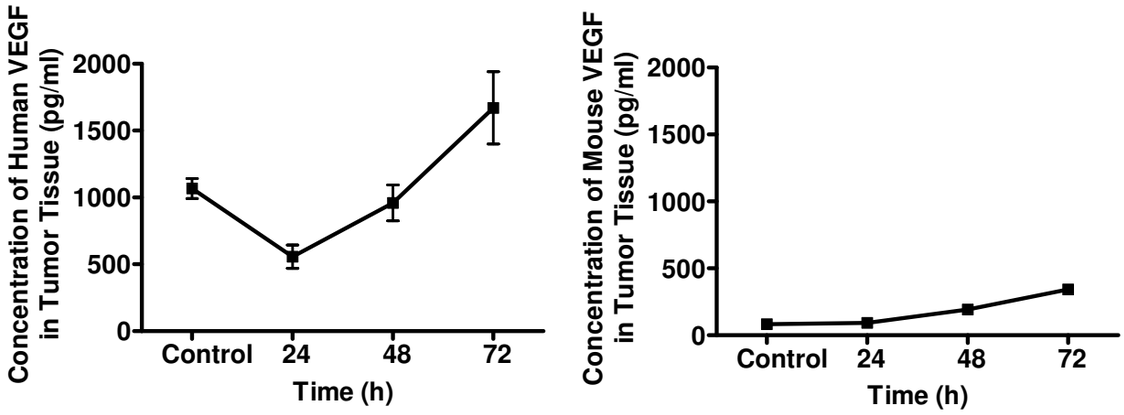
Photodynamic therapy can produce a significant effect on the expression profile of VEGF in serum and tumors. Experiments were conducted in a xenograft model to evaluate VEGF expression at 24 h, 48 h and 72 h after treatment to understand the initiation of regrowth post hypericin PDT (Bhuvaneswari, Gan et al., 2007). Controls in the experiments were animals with untreated tumors. As human nasopharyngeal carcinoma cells was used as xenografts in a mouse model, both human and mouse VEGF were estimated in serum and tumor tissue. The decrease in mouse VEGF in serum immediately post treatment was not significant, but it reached control levels within 72 h. Greater amount of mouse VEGF compared to human VEGF in serum could indicate the involvement of host environment in modulating the PDT response of the tumor (Figure 1). At 24 h post PDT, both the mouse and human VEGF levels in tumor tissue decreased compared to the control group but elevated by 72 h (Figure 2). The decrease of VEGF observed at 24 h post PDT could be explained through the postulation that the residual tumor cells from the initial PDT treatment could be reoxygenated after 24 h following PDT (Uehara et al., 2001) or may be due to reversal of temporary vascular occlusion (Tsutsui et al., 2002). Downregulation of VEGF immediately after PDT and its subsequent upregulation at 72 h could indicate that regrowth in tumors after PDT begins as early as 72 h. It can be argued that both tumor angiogenesis and recurrence may therefore be mediated by PDT via the enhancement of VEGF expression within the treated tumor mass (Tsutsui et al., 2002).



(a) (b)

Fig. 1. Concentration of (a) mouse VEGF and (b) human VEGF in serum in the control and at 24 h, 48 h and 72 h post PDT. Error bars represent the standard error of the mean concentration of mouse VEGF in serum at 24 h, 48 h and 72 h, n = 8. The mouse VEGF in serum decreased at 24 h and 48 h post treatment compared to the control group. However at 72 h post PDT the mouse VEGF levels increased and were comparable to the control group.

Mouse VEGF levels were found to be significantly lower than human VEGF in the tumor tissue and this could be attributed to the number of host cells versus the number of tumor cells present within the treated region. Similar observations were reported by Gomer et al. (Gomer et al., 2006). Detection of VEGF has long been known as a potential serum diagnostic marker for malignant diseases. Increased serum VEGF concentrations have been measured in various types of cancer, including brain, lung, renal and ovarian cancer (Kondo et al., 1994). High serum VEGF has been strongly associated with poor clinical outcome in lymphoma patients (Salven et al., 2000). Overexpression of VEGF is known to be common in NPC, which is related to hypoxia up-regulated expression involving a HIF-dependent pathway, and is associated with poor prognosis. Targeting the hypoxia pathway may be useful in the treatment of NPC (Hui et al., 2002). Patients with nasopharyngeal carcinoma having high VEGF levels in serum have been associated with a worse progression-free survival. A recent study has also shown increased microvessel density in oral cancer tissues in VEGF-positive tumors and indicated that upregulation of VEGF was correlated with tumor angiogenesis and disease progression (Shang et al., 2007).



(a) (b)

Fig. 2. Concentration of (a) mouse VEGF and (b) human VEGF in tumor tissue in control and at 24 h, 48 h and 72 h points post PDT. Error bars represent the standard error of the mean concentration of human VEGF in tumor tissue at 24 h, 48 h and 72 h, n = 8. Mouse and human VEGF were significantly higher in the tumor tissue (320-1700 pg/ml) compared to serum (33-110 pg/ml). Mouse VEGF in tumor tissue increased at 48 h and 72 h post PDT. The increase in VEGF levels from 24 h to 72 h was found to be statistically significant ($p<0.05$) (Figure 2b). Controls were animals with untreated tumors.

Immunofluorescence results also confirmed the increased expression of VEGF post PDT in the tumor tissue (Figure 3). Several groups have reported the upregulation of VEGF following PDT (Bhuvaneswari, Gan et al., 2007; Ferrario et al., 2006; Uehara et al., 2001; Yee et al., 2005). Ferrario et al. revealed that PDT-mediated hypoxia and oxidative stress could be involved in photofrin-mediated PDT induced expression of HIF-1α and also increased protein levels of the HIF-1 target gene VEGF, in treated mouse mammary carcinoma xenografts (Ferrario et al., 2000). In a similar study, the same group also reported significant overexpression of HIF-1α and VEGF after photofrin-mediated PDT in a xenograft model of Kaposi's sarcoma (Ferrario et al., 2006). Increased expression of VEGF was noticed from 0 h to 6 h in tumors treated with haematoporphyrin mediated PDT compared to control tumor

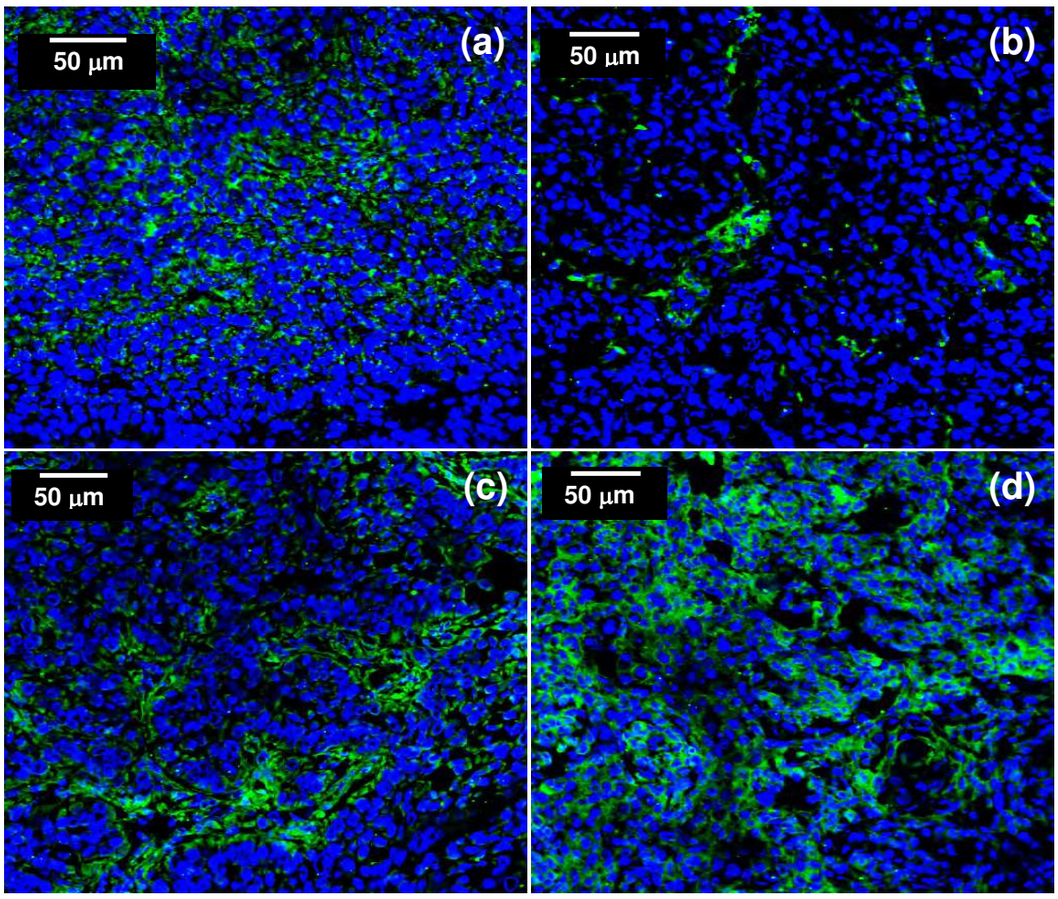


Fig. 3. Immunofluorescence was performed to confirm the expression of VEGF in tumor tissue at different time points post PDT. In the confocal images, the green FITC fluorescence staining indicated the expression of VEGF. (a) control, (b) 24 h post PDT, (c) 48 h post PDT and (d) 72 h post PDT. Magnification: 200X, scale bar = 50 mm. Around 13% and 15% (IF score 2) of scattered staining in certain regions of the cytoplasm was observed in the control (Figure 3a) and at 48 h post PDT (Figure 3c). Minimum VEGF expression of 5% (IF score 1) was observed at 24 h post PDT (Figure 3b). Maximum VEGF staining of 26% (IF score 3) was noticed at 72 h time point (Figure 3d).

in a mouse squamous cell carcinoma model (Uehara et al., 2001). Similar observations were noted by Jiang et al. whereby VEGF levels significantly increased after photofrin-PDT in intracranial glioblastoma xenografts (Jiang et al., 2008). In earlier studies, the same research group had reported increased VEGF levels in normal rat brain that induced the formation of aberrant new vessels following treatment with high dose PDT. In another study it was demonstrated that low dose PDT increases endothelial cell proliferation and VEGF expression in nude mice brain (Zhang et al., 2005). In addition, the upregulation of VEGF in photofrin mediated PDT was also observed in the brain tissue adjacent to tumor in a dose dependent manner (Jiang et al., 2004). Solban et al. investigated the effect of subcurative PDT using photosensitizer benzoporphyrin derivative (BPD) in an *in vivo* orthotopic model of human prostate cancer that demonstrated increased VEGF secretion 24 h following PDT and suggested vascular damage and/or a direct effect of BPD to be responsible for this

increase (Solban et al., 2006). Kosharskyy et al. observed increases in not only VEGF secretion but also incidences of lymph node metastases after subcurative PDT in an orthotopic model of prostate cancer (LNCaP), that created conditions favorable for enhanced tumor growth and metastasis (Kosharskyy et al., 2006). The same group also investigated the use of an optical molecular imaging strategy to monitor VEGF expression *in vivo* and effectively labeled and imaged bound VEGF released from the extracellular matrix in response to photodynamic therapy (Chang et al., 2008). Increased secretion of HIF-1α and its target gene VEGF has been observed in hypericin-mediated PDT in both nasopharyngeal and bladder carcinoma (Bhuvaneswari, Gan et al., 2007; Bhuvaneswari, Yuen et al., 2007). Moreover, cellular mediated long drug light interval (DLI) hypericin-PDT induced greater expression of pro-angiogenic growth factors compared to vascular mediated short drug light interval PDT in bladder carcinoma (Bhuvaneswari et al., 2008). Zhou et al. (Zhou et al., 2005) demonstrated that the expression of HIF-1α and VEGF increased in PDT-treated tumor samples collected 24 h post-PDT in a mouse model of human nasopharyngeal carcinoma. Mono-L-aspartyl chlorin e6 (NPe6) PDT of cytokine-overexpressing Lewis lung carcinoma (LLC/IL-2) tumors revealed that the expression of GADD-5alpha and VEGF are induced after PDT and in particular the expression levels were much higher as compared with those in LLC tumors, 12 h after PDT (Ohtani et al., 2008). However, the application of ALA-PDT resulted in a lowered rate of metastatic spreading and decreased VEGF level in blood serum of 3LL-bearing mice that has been attributed to vascularization disturbances in tumor tissue (Lisnjak et al., 2005). Hypocrellin mediated PDT in human brain tumor cells induced expression of proangiogenic VEGF and of antiangiogenic SFH-1, angiostatin, p43, allograft inflammatory factor-1 and connective tissue growth factor suggesting favorable and deleterious effects of hypocrellin-PDT on tumor outgrowth (Deininger et al., 2002). Based on the above studies, it can be inferred that PDT using photosensitizers i.e., photofrins, hypericin, hypocrellins and chlorin e6 increases VEGF concentrations within the tumor tissue and acts as a key regulator of angiogenesis and tumor recurrence post treatment.

3. PDT in combination with Avastin

Combination of anti-angiogenic agents with the PDT regime has been shown to be effective in inhibiting tumor regrowth and improving tumor response (Bhuvaneswari et al., 2009). Studies have reported that transplantable BA mouse mammary carcinoma treated with PDT and non-specific antiangiogenic peptides, IM862, a dipeptide and EMAP-II, a single chain polypeptide, increased tumor regression by inducing apoptosis and inhibiting VEGF production. However, the anti-angiogenic agents by themselves did not produce the desired outcome (Ferrario et al., 2000). Use of novel antiangiogenic monoclonal antibodies, MF1 and DC101 along with PDT against vascular endothelial growth factor receptors VEGFR-1 and VEGFR-2, respectively, reduced the tumor volume significantly and prolonged the survival time of glioma-implanted animals (Jiang et al., 2008). PDT followed by administration of an antiangiogenic agent, TNP-470, abolished the increase in VEGF levels caused by subcurative PDT and reduced local tumor growth in an orthotopic model of prostate cancer (LNCaP) (Kosharskyy et al., 2006). Synthetic RTK inhibitors SU5416 and SU6668 when combined with hypericin PDT significantly extended survival of tumor-bearing host mice (Zhou et al., 2005). Combining PDT with humanized monoclonal antibody Avastin (bevacizumab)

resulted in significant increase in long-term responsiveness of treated Kaposi's sarcoma tumors when compared to monotherapies (Ferrario et al., 2006). Chang et al. (Chang et al., 2008) used an *in vivo* optical imaging technique that produces wavelength-resolved fluorescence hyperspectral images to study changes in tumoral VEGF concentration following PDT and Avastin treatment. The *in vivo* antigen blocking experiment showed that Avastin pretreatment before imaging blocked the tumoral VEGF, and also that VEGF-specific contrast agent labeling decreased in tandem with the pretreated Avastin dose, demonstrating that VEGF-specific contrast agent specifically binds to the VEGF protein.

Since VEGF and its receptors represent central molecular targets for antiangiogenic intervention, addition of Avastin (bevacizumab) along with PDT can increase the treatment efficacy. Avastin is a recombinant, partially humanized, monoclonal IgG1 antibody that binds to and inhibits the biological activity of human VEGF thus preventing interaction with its receptors. Avastin along with chemotherapy has been approved in the United States of America (USA) for the treatment of colorectal cancer and NSCLC and in other countries for the treatment of breast cancer, prostate cancer and renal cell carcinoma (Shih & Lindley, 2006).

In this study, the potential of combining anti-angiogenic agent Avastin that is specific to VEGF, with photodynamic therapy to enhance treatment efficacy by improving the tumor responsiveness was investigated. As Balb/c nude mice are immunocompromised, human bladder carcinoma cells were injected to establish subcutaneous tumor grafts. Subcutaneous models were used for our experiments because of the simple inoculation procedure, reproducibility of tumor growth and easy accessibility of the tumor for measurement and treatment. MGH bladder tumors form vascularized solid tumors.

The tumor regression experiments conducted in the xenograft model clearly indicated that combining Avastin with PDT can impede the angiogenesis process and improve the response of treated tumors. This has been demonstrated by the significant decrease in the tumor volume of the combination therapy group of PDT + Avastin compared to the control and high dose PDT groups (Figure 4). This demonstrates that by targeting the VEGF pathway, post-PDT angiogenesis can be reduced. It should be noted that Avastin is a monoclonal antibody that targets human VEGF and not mouse VEGF. The tumor volume of high dose PDT treated group was significantly greater than the low dose PDT group and this could be due to the difference in fluence rates administered. High fluence rate can deplete tumor oxygen to a greater extent, thereby reducing the primary cytotoxic processes of PDT and affecting tumor control. On the other hand, low fluence rate treatments can be more effective in decreasing vascular lesions even if the same overall fluence is maintained. Other studies have concluded that lower fluence rate treatments can preserve the status of oxygen for a more effective PDT (Chen et al., 2002; Sitnik et al., 1998; Tromberg et al., 1990) and it is well recognized that light fluence rates play a major role in the tumor oxygenation status during PDT exposure. But we should also understand that oxygen-conserving low fluence rate PDT cannot always be effective if it is unable to produce the desired tumor damage and it is essential to estimate the lower limits of fluence rate that would be required for effective treatment thus potentially allowing the tailoring of treatment to specific situations (Henderson et al., 2006; H. W. Wang et al., 2004). The group of animals that were administered only Avastin showed greater tumor response compared to the high dose PDT and control groups, this could be due to the fact that Avastin by itself can target and bind to human VEGF. Though not statistically significant, better tumor response was observed in the combination therapy group compared to Avastin only and low dose PDT groups, however a greater sample size would be required to establish these findings. Complete cure was not achieved in these groups by end of 30-day post treatment, as Avastin does not

target mouse VEGF produced by the host environment. Tumors were excised to analyze the expression of VEGF and other angiogenic proteins at the end of the 30-day tumor growth experiments. This time point was chosen to study the long-term effect of Avastin on the expression profile of angiogenic proteins.

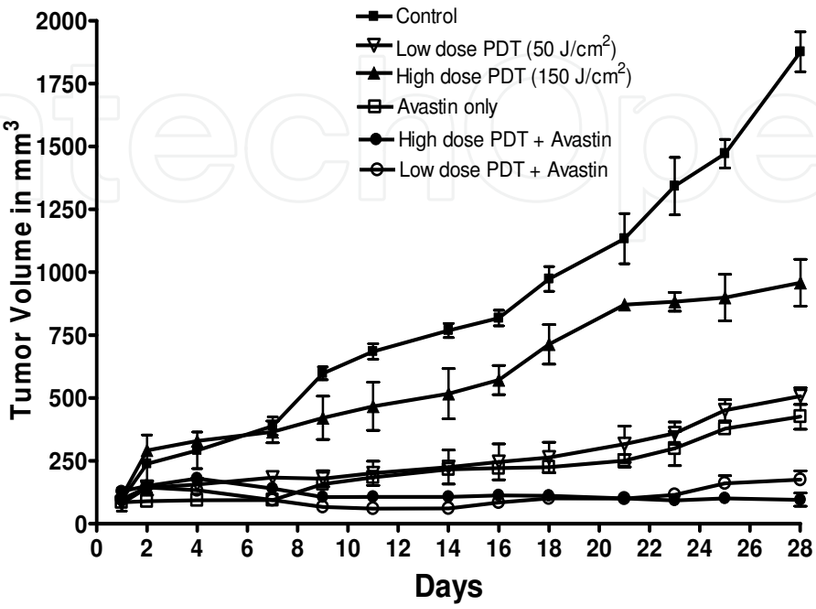


Fig. 4. Tumor volume charted against days, to assess the tumor response in various treatment groups. The combination therapy group of PDT and Avastin exhibited greater tumor response in comparison with other groups. Each group represents the mean (bars, SE) of 10 animals.

Next, circulating human VEGF concentrations in mice was investigated to analyze tumor-derived VEGF. A low but detectable amount of human VEGF was observed in most tumors (Figure 5). It has been shown that high fluence rates during PDT can influence the inflammatory responses associated with PDT (Henderson et al., 2004). In the same way, the results also suggest that high dose PDT could have triggered inflammatory responses within the treated tumors that may enhance VEGF secretion. Though it was expected that Avastin would specifically bind to the circulating human VEGF, measurable amount of VEGF was documented in the animals that received Avastin alone. One of the reasons for detecting circulating VEGF in all the treatment groups could be the extended period of VEGF transcriptional activation, (Liang et al., 2006) and it is likely that low level of VEGF can be generated, continuously or in pulses, during the angiogenesis process that could vary significantly from tumor to tumor. Also, the data did not seem to exhibit any correlation between VEGF secretion and tumor volume. The smaller tumors in the Avastin only group expressed relatively greater amount of VEGF compared to the tumors in the control and high dose PDT treated groups, this observation can be attributed to multiple factors such as tumor vascularization, tumor invasiveness, tumor infiltrating macrophages and the production of cytokine IL-1α that has been shown to influence the secretion of VEGF (Borg et al., 2005).

Immunohistochemistry was performed to detect VEGF and this method has been used in earlier studies for quantifying VEGF (Harper et al., 1996; Saito et al., 1999). As VEGF is a

secreted protein, it was observed mainly in the cytoplasm and the extracellular matrix (Figure 6). These data demonstrate that tumors under oxidative stress express greater amounts of VEGF. Also, significantly lower occurrence of VEGF was observed in the combination therapy of PDT and Avastin. These results are consistent with an earlier report by Solban et al. (Solban et al., 2006) on subcurative PDT performed on an orthotopic model of prostate cancer that showed increased VEGF secretion and also demonstrated that VEGF induction can be abolished by administering p38 MAPK inhibitor along with PDT.

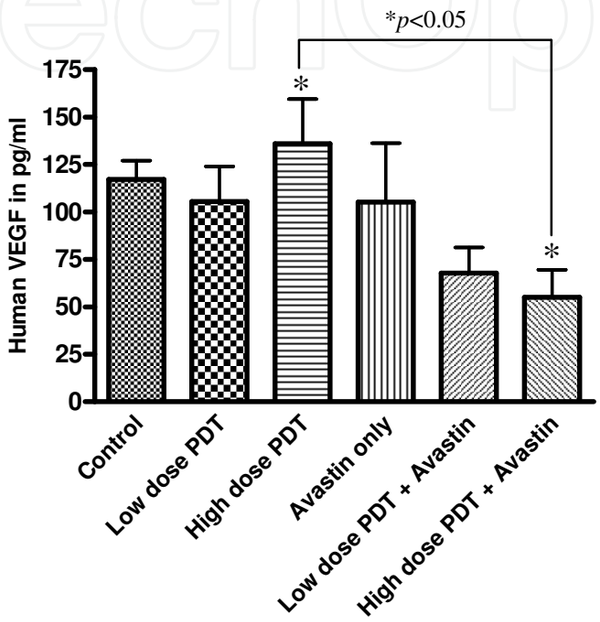


Fig. 5. Relative concentration of human VEGF measured in pg/ml in serum for various treatment groups. Greater expression of VEGF was observed in the high dose PDT group compared to the combination therapy group of high dose PDT + Avastin. Each group represents the mean (bars, SE) of 10 animals.

The effect of different treatment regimes on the expression profiles of angiogenic proteins was also investigated. The study established differential expression of proteins in the angiogenesis pathway as different PDT combinations were administered (Figure 7). The protein angiogenin initiates cell migration, proliferation and induces neovascularization *in vivo* (Hartmann et al., 1999). In our experiments it was upregulated in high-dose PDT treated tumors compared to all other groups and this may be due to hypoxia induced production of angiogenin. Similarly studies have established positive correlation between hypoxia and angiogenin expression in human malignant melanoma, (Hartmann et al., 1999) and human primary breast carcinoma (Campo et al., 2005). A study on gastric carcinoma cancer has shown angiogenin expression in cancer tissues to be positively correlated with VEGF (Chen et al., 2002) and our results show that blocking the VEGF pathway using PDT with Avastin does downregulate the expression of angiogenin. Both bFGF and VEGF seem to differentially activate the Raf pathway in the angiogenesis process (Alavi et al., 2003) and as bFGF has shown to promote angiogenesis indirectly by the upregulation of VEGF in endothelial cells, (Pepper et al., 1992) the reduced expression of bFGF in the combination

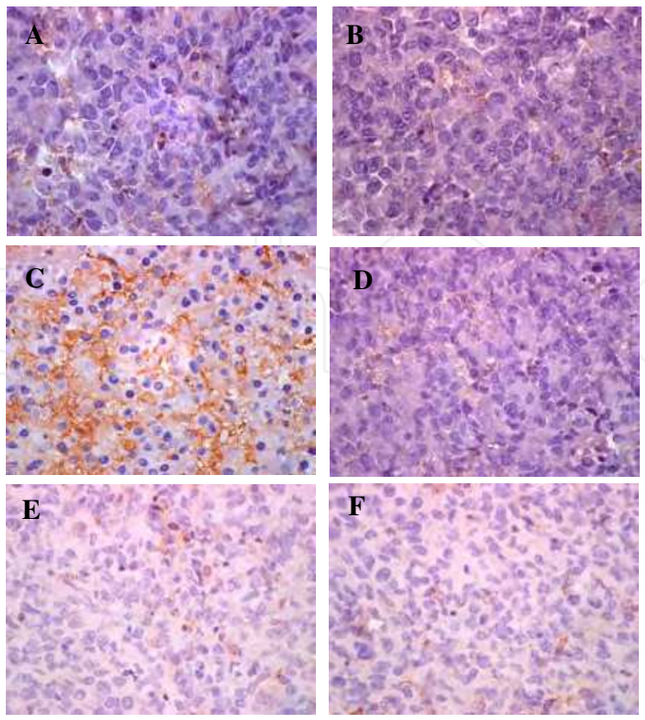


Fig. 6. VEGF expression was assessed in tumors treated with various treatment regimens using immunohistochemistry A - Control (untreated tumor), B - Low dose PDT, C - High dose PDT, D - Avastin only, E -Low dose PDT + Avastin and F - High dose PDT + Avastin. VEGF, a secreted protein was observed in the cytoplasm and extracellular matrix. 30% Immunostaining for VEGF was observed in high dose PDT treated tumors. Control, low dose PDT and Avastin only groups exhibited <5% of staining. Minimal staining of less that 2% was observed in the combination therapy groups of PDT and Avastin. All sections are shown at a magnification of × 630.

therapy groups could mean that inhibiting VEGF may possibly attenuate bFGF expression as well. Amplication of bFGF by a HIF-1α-dependent pathway, (Calvani et al., 2006) may be one of the reasons for the upregulation of bFGF in PDT treated tumors. Similary EGF, a key EGFR ligand that promotes angiogenesis (Ciardiello, 2005) and was upregulated in high dose PDT treated tumors, is known to be HIF-1α regulated (Vaupel, 2004). We observed downregulation of EGF in the combination therapy groups suggesting that VEGF and EGFR pathways are closely related, sharing common downstream signaling pathways. (Tabernero, 2007) PlGF-1 is expressed in placental tissues, colon and mammary carcinomas and it belongs to the VEGF family (Cao et al., 1996). No PIGF expression was noted in the low dose PDT treated group as the oxidative stress in these tumors was expected to be minimal due to low fluence rate administered during PDT. However, the Avastin only and combination therapy treated tumors expressed minimal PIGF, which may suggest that Avastin, which binds to VEGF, has negligible effect on PIGF. Furthermore as PIGF binds only to VEGF receptors, it has been documented that PIGF can be downregulated by blocking the VEGFR-1/FLT1 receptor pathway (Ahmed et al., 2000). As both EGF and PIGF was not observed in the control tumors and induced only post PDT treatment, we conjecture that these proteins may not play a major role in angiogenesis of MGH bladder tumors, based on our experimental data that show increased tumor volume in control groups. After PDT

treatment, the tumors are in hypoxic condition which is one of the factors causing cytokine expression (Gomer et al., 2006). The role of IL-6 in angiogenesis is mediated through the induction of VEGF (T. Cohen et al., 1996). The increased expression of interleukins in the control and PDT treated groups may have resulted due to greater tumor volume and PDT induced inflammation, respectively. Compared to high dose PDT group, the tumors in combination therapy group produced lower levels of cytokines and this we theorize to be the role of Avastin in reducing angiogenesis by binding to VEGF, thus reducing the expression of post PDT inflammatory proteins. Expression of IL-6 was elevated in most of the treatment groups compared to IL-8 suggesting its importance in PDT-induced inflammation.

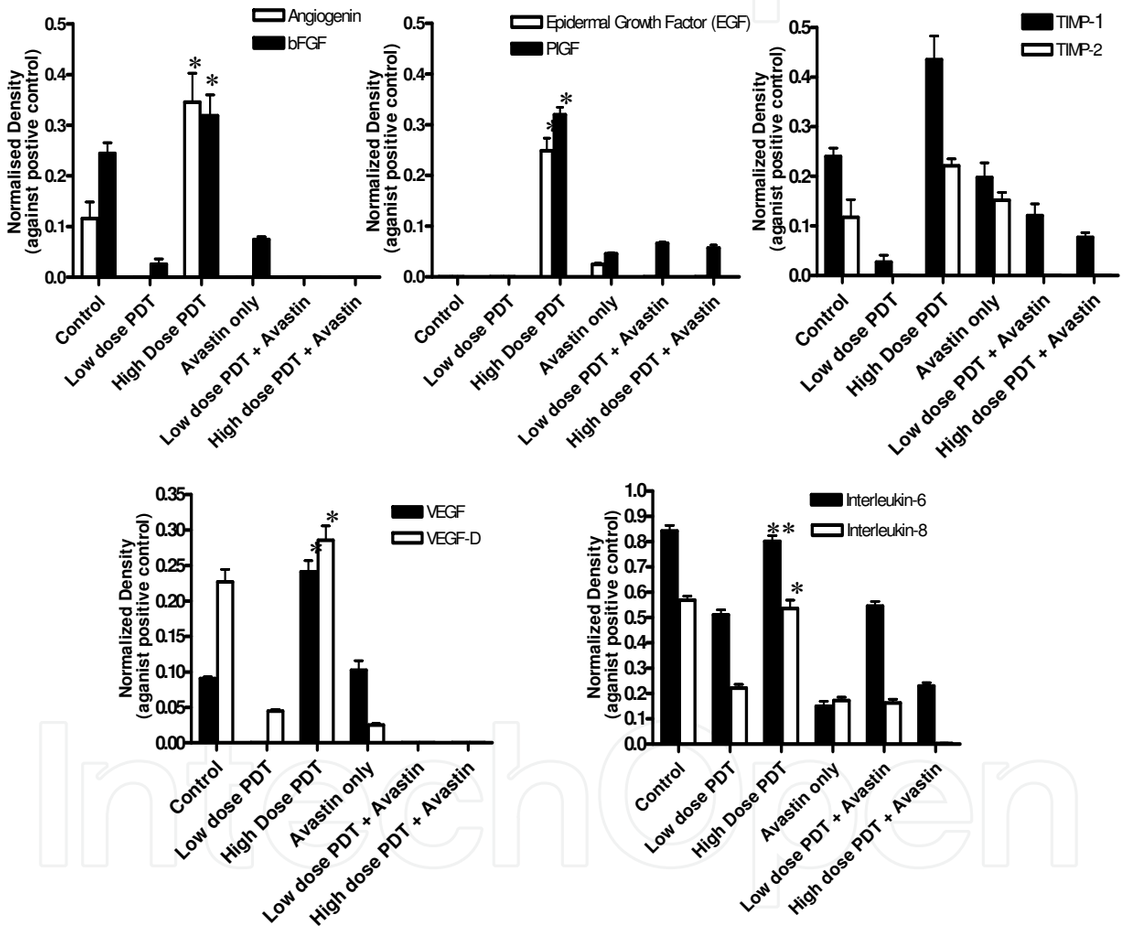


Fig. 7. Antibody arrays were used to analyze the expression of angiogenic proteins in the treated tumors. Density of proteins was plotted and normalized against the positive control Actin, (a) Angiogenin and bFGF, (b) EGF and PIGF, (c) TIMP-1 and TIMP-2, (d) VEGF and VEGF-D and (e) IL-6 and IL-8. Each group represents the mean (bars, SE) of 5 tumors (i.e. one membrane was used per tumor). Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple comparison tests. * = $p<0.001$, ** =$p<0.01$ when high dose PDT group was compared with the combination therapy groups of low dose PDT + Avastin and high dose PDT + Avastin.

On the other hand, TIMPs are natural inhibitors of MMPs. A stimulatory role of TIMP-1 in angiogenesis has been proposed in an earlier study (Wurtz et al., 2005) which reports that by inhibiting MMPs, TIMP-1 may prevent angiostatin and endostatin production, thus playing a positive role in tumor angiogenesis. In contradiction to an earlier report (Ferrario et al., 2004) that PDT suppresses TIMP-1 expression in mouse mammary carcinoma, we noticed upregulation of TIMP-1 in the high dose PDT treated tumors. Nevertheless, this dissimilarity can be attributed to the different tumor systems and the PDT protocol administered. VEGF which is also known as VEGF-A is involved in angiogenesis and lymphangiogenesis and VEGF-D, a secreted protein stimulates lymphangiogenesis and metastasis in tumors (Hoeben et al., 2004). Reports have shown that subcurative PDT in an orthotopic model of prostate cancer increases VEGF secretion and also cause lymph node metastasis. It was also demonstrated that the administration of anti-angiogenic agent TNP-40 abolished this increase and reduced tumor growth (Kosharskyy et al., 2006). In our study VEGF was not detected in low dose PDT group and that could be due to lower oxidative insult to the tumor tissue compared to the high dose PDT group. Furthermore, downregulation of VEGF levels was observed in the PDT + Avastin treated tumor as the combination treatment effectively suppressed the VEGF signalling cascade. However, we noticed minimal expression of VEGF using IHC and ELISA and this we attribute to the different tumor microenvironment as tumors were collected from different animals though they were treated with the same treatment protocol. In conclusion, the results demonstrate that by targeting the VEGF pathway, post-PDT angiogenesis can be inhibited. Furthermore, suppressing the VEGF pathway can also downregulate other angiogenic mediators.

4. PDT in combination with Erbitux

Erbitux was approved by the US Food and Drug Administration (FDA) for use in combination with irinotecan for the treatment of metastatic colorectal cancer and it is also being used for the treatment of metastatic squamous cell carcinoma of the head and neck (SCCHN) (Wong, 2005). Results of a large phase II study on irinotecan-refractory, colorectal cancer patients have shown a significant response of 22.9% when Erbitiux was combined with chemotherapy agent, irinotecan (Cunningham et al., 2004). In another study, the response rate was significantly improved when Erbitux was combined with cisplatin in the first-line treatment of recurrent or metastatic SCCHN (Burtness, 2005). A randomized trial that compared radiotherapy plus Erbitux with radiotherapy alone in patients with stage III or IV non-metastatic SCCHN, demonstrated significantly longer locoregional control with radiotherapy plus Erbitux than with radiotherapy alone; moreover, progression-free survival were significantly longer and the overall response rate was significantly better with the combination therapy (Griffin et al., 2009). Erbitux given concurrently with radiotherapy yields a significant clinical benefit over radiotherapy alone without any increase in radiotherapy-associated toxicity, this was demonstrated in the results of a recent phase III randomized study (Bernier & Schneider, 2007).

In the *in vivo* tumor regression study, we demonstrate that the combination therapy of Erbitux with PDT can improve the tumor response by attenuating the angiogenic process (Figure 8). A similar study conducted on a mouse model of human ovarian cancer in which C225 (Erbitux) was combined with PDT regimen produced synergistic reductions in mean tumor burden and significantly greater median survival (del Carmen et al., 2005). In this study, PDT treated tumors did not exhibit significant tumor regression compared to combination therapy groups

and this could be attributed to the high fluence rate that was administered during PDT. High fluence rate can deplete tumor oxygen to a large extent, thereby stimulating the production of stress induced survival molecules that reduce the effectiveness of PDT and affect tumor control (Henderson et al., 2004). More importantly, the use of high light dose for this experiment was to test our hypothesis that combining PDT with Erbitux can improve tumor control and also to evaluate the effectiveness of Erbitux in reducing EGFR concentrations. The investigations have indicated that Erbitux alone as monotherapy was not effective in controlling tumor growth. One of the possible reasons for this observation could be the fact that tumors overexpressing EGFR might not be sensitive to Erbitux. Although we would assume that tumors overexpressing EGFR would respond well to anti-EGFR therapy, studies have demonstrated that the level of EGFR expression does not have any impact on tumor response rates as a significant number of EGFR-positive tumors could be resistant to Erbitux (Ellis & Hoff, 2004; Vallbohmer et al., 2005). The group that received the combination therapy of PDT and Erbitux exhibited accelerated growth a week after PDT which could be due an increase in the expression of angiogenic growth factors either due to hypoxia induced by oxygen depletion during PDT light irradiation or incomplete treatment. Our earlier results have shown increased expression of angiogenic growth factor VEGF at 72 h post PDT (Vallbohmer et al., 2005). In this study, the regular administration of Erbitux after PDT treatment could have blocked the EGFR pathway and reduced angiogenesis. Therefore, our data supports the hypothesis that combination therapy of PDT and Erbitux would be more effective in preventing angiogenesis compared to monotherapy alone.

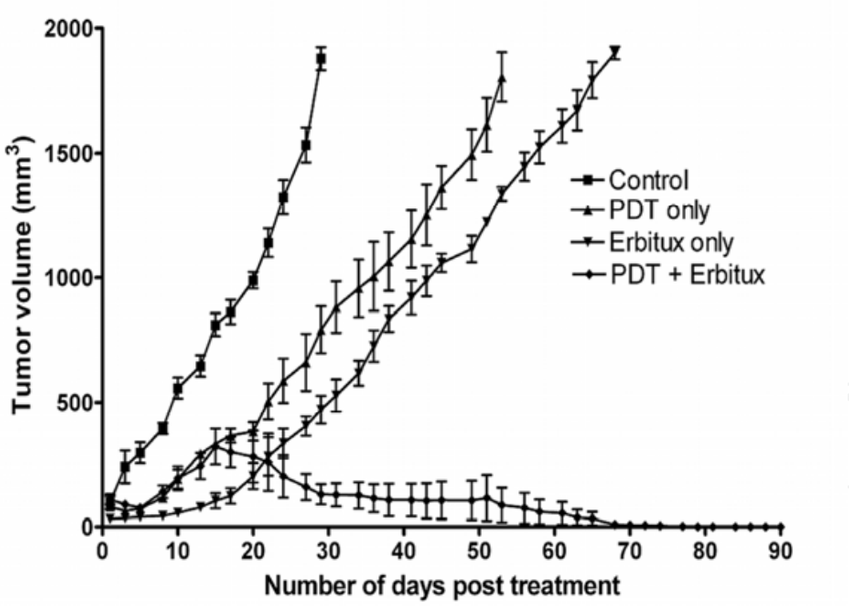


Fig. 8. Mean tumor volume charted against number of days post treatment, to assess the tumor response in various treatment groups. The combination therapy group of PDT and Erbitux exhibited greatest tumor response in comparison with all other groups. Each group represents the mean response (bars, SE) of 10 animals.

To further substantiate our results we performed western blotting and immunohistochemistry to determine the EGFR levels in all the treatment groups. EGFR

immunoreactivity was localized mainly in the cell membranes and to a lower extent in the cytoplasm as well (Figure 9). It has been well established that the core of solid tumors is hypoxic, and that hypoxic tumor environment is sufficient to trigger EGFR expression in tumors (Franovic et al., 2007). Previous studies have reported the downregulation of EGFR after PDT (Ahmad et al., 2001; Tsai et al., 2009); in marked contrast our results demonstrated an increase in EGFR expression post hypericin-mediated PDT. This observation could be attributed to numerous reasons such as the light/drug dosage, the complexity of tumor microenvironment and the properties of the photosensitizer (Henderson et al., 2004). Combined antitumor activity of Erbitux with standard chemotherapy and radiotherapy is well documented in the treatment of different types of tumors and is reported to be more efficacious than individual monotherapies (E. E. Vokes & Chu, 2006). In this study, combination modality of PDT and Erbitux was effective in reducing the expression of EGFR and that could have lead to the regression of tumors in this group.

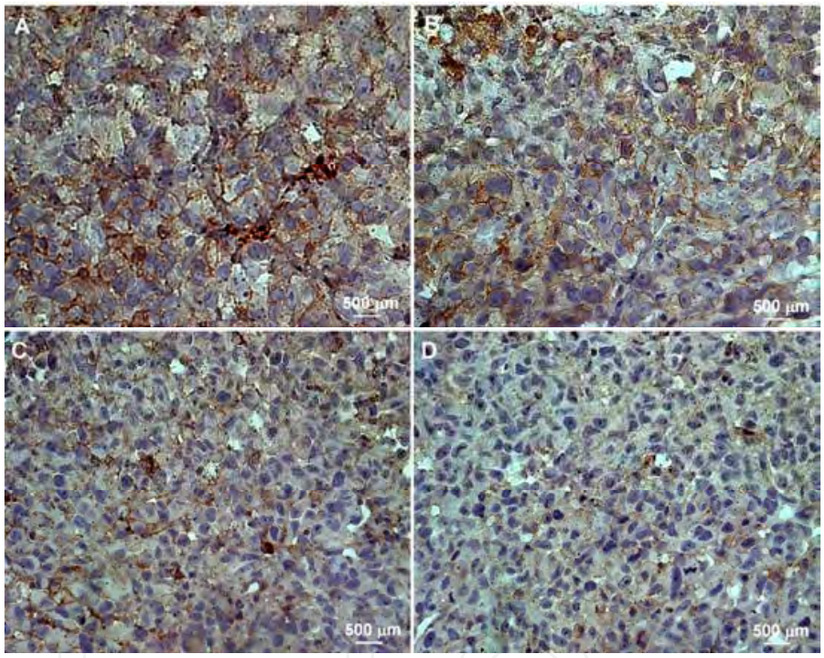


Fig. 9. EGFR expression was assessed in tumor sections using immunohistochemistry. The brown colored membrane staining indicates EGFR positive immunoreactivity. (A: Control, B: PDT, C: Erbitux and D: PDT +Erbitux). PDT and Erbitux (D) resulted in significant reduction of EGFR expression of 4-6% (EGFR score 1) compared to monotherapy (B: PDT and C: Erbitux) and control groups (A). Maximum EGFR tumor cell membrane staining of 21-24% (EGFR score 3) noticed in the untreated tumors. The monotherapy groups of PDT only and Erbitux only, exhibited 15-17% (EGFR score 2) and 11-13% (EGFR score 2) staining respectively. Magnification: 630X.

In the current study, we have also shown that PDT plus Erbitux increased apoptosis in the treated tumors compared to PDT only and inhibitor only monotherapies (Figure 10). Erbitux has been known to increase apoptosis in various tumor models by different mechanisms, including upregulation of pro-apoptotic Bax protein (Mahtani & Macdonald, 2008), decrease in the expression of anti-apoptotic molecule Bcl-2 (S. M. Huang et al., 1999) and the activation of

pro-apoptotic caspases (Iwase et al., 2008). Hypericin-PDT is also known to induce apoptosis in a dose-dependent manner with higher doses leading to necrosis. Based on the lack of tumor inhibition in the monotherapy groups, it can be noted that tumors treated with PDT alone and Erbitux alone induced limited apoptosis in bladder carcinoma tumors. Therefore in this investigation, we observe that the combination therapy has significantly increased tumor cell apoptosis and inhibited tumor progression. Preclinically, many studies have shown that treatment with Erbitux in combination with radiotherapy or chemotherapy enhances apoptotic cell death than individual therapies. In a similar manner, PDT induced apoptosis, could have been enhanced by the combination of Erbitux to the treatment regime.

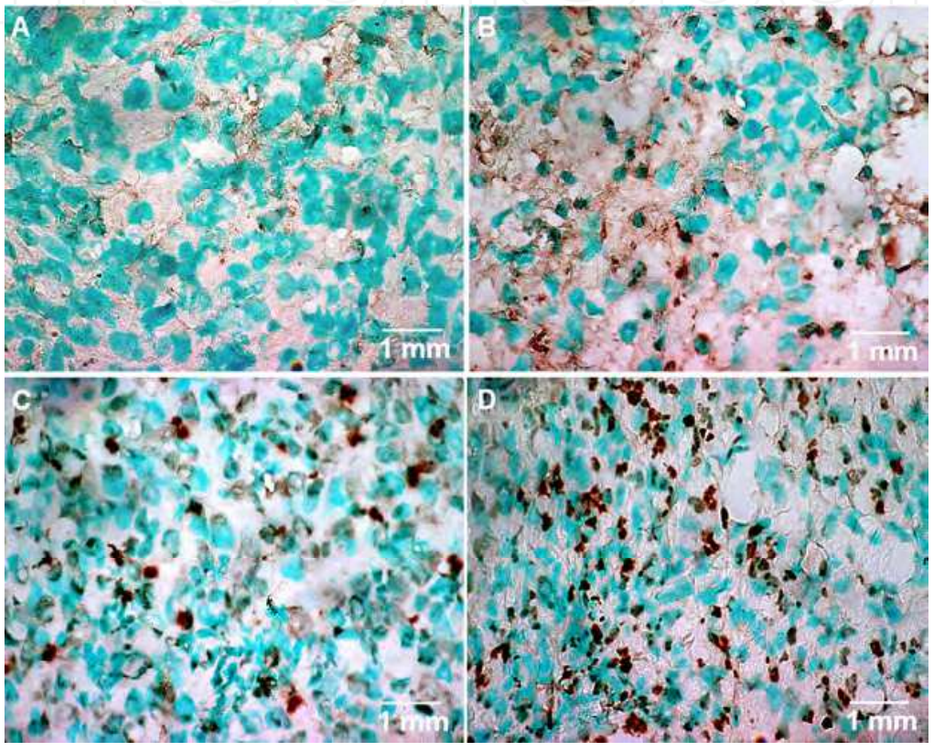
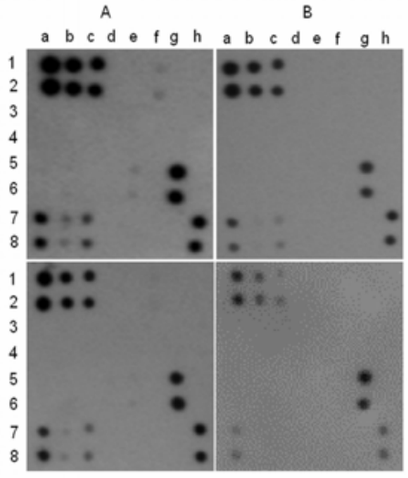


Fig. 10. The tunnel assay was performed on the tumors that were harvested from the animals at the end of the treatment. Few isolated positive nuclei were noticed in (A) untreated tumors, (Apoptotic index (AI) – 6%). Both (B) PDT only (AI - 14%) and (C) Erbitux only (AI - 16%) treated tumors showed increased apoptosis compared to control. High levels of apoptotic nuclei were clearly exhibited by tumors treated with the (D) PDT plus Erbitux combination therapy (AI - 32%, $p < 0.001$). Magnification: 630X.

By using EGF phosphorylation antibody array membranes, we examined the relative level of phosphorylation of specific sites for human EGFR receptors. Interestingly, we noted the phosphorylation of Threonine 686 site of ErbB2 in all the groups. Studies have suggested that the dysregulation of cellular protein kinase C (Ouyang et al., 1996) and protein kinase A (Monje et al., 2008) activity could phosphorylate ErbB2 on Thr-686 for the activation and proliferation of tumor cells (Figure 11). However, our findings suggest that ErB2 on Thr-686 may not be essential for regulation of tumor proliferation, as tumor control was observed in the PDT + Erbitux treated group. Phosphorylation of EGFR tyrosine 845, only noticed in control tumors, is implicated in the stabilization of the activation loop, providing a binding

surface for substrate proteins and is capable of regulating receptor function and tumor progression (Cooper & Howell, 1993). c-Src is known to be involved in the phosphorylation of EGFR at Tyr845 (Biscardi et al., 1999). The major autophosphorylation sites of ErbB2 are Tyr1248 and Tyr1221/1222 that lead to Ras-Raf-MAP kinase signal transduction pathway (Kwon et al., 1997). In control tumors, ErbB2 was phosphorylated at tyrosine 1221/1222 and is associated with high tumor grade and with shorter disease-free survival and overall survival (Frogne et al., 2009). Similarly, ErbB4 is able to induce phosphorylation of phosphatidylinositol 3-kinase regulatory subunit which is a pro-survival protein that prevents apoptosis (B. D. Cohen et al., 1996; Gallo et al., 2006). Our data suggests that dephosphorylation of ErbB4 tyrosine 1284 is critical for tumor regression in the dual treatment group.



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Pos1	Pos2	Pos3	Blank	Neg	EGFR (Tyr845)	EGFR (Tyr992)	EGFR (Tyr1045)
Pos1	Pos2	Pos3	Blank	Neg	EGFR (Tyr845)	EGFR (Tyr992)	EGFR (Tyr1045)
Blank	Blank	Blank	Blank	EGFR (Tyr1068)	EGFR (Tyr1086)	EGFR (Tyr1148)	EGFR (Tyr1173)
Blank	Blank	Blank	Blank	EGFR (Tyr1068)	EGFR (Tyr1086)	EGFR (Tyr1148)	EGFR (Tyr1173)
EGFR (Ser1046/ 1047)	EGFR (Ser1070)	ErbB2 (Tyr877)	ErbB2 (Tyr1112)	ErbB2 (Tyr1221/1222)	ErbB2 (Tyr1248)	ErbB2 (Thr686)	Blank
EGFR (Ser1046/ 1047)	EGFR (Ser1070)	ErbB2 (Tyr877)	ErbB2 (Tyr1112)	ErbB2 (Tyr1221/1222)	ErbB2 (Tyr1248)	ErbB2 (Thr686)	Blank
ErbB2 (Ser1113)	ErbB3 (Tyr1289)	ErbB4 (Tyr1284)	Blank	Blank	Neg	Blank	Pos4
ErbB2 (Ser1113)	ErbB3 (Tyr1289)	ErbB4 (Tyr1284)	Blank	Blank	Neg	Blank	Pos4

Fig. 11. Phosphorylation statuses of EGFR sites were determined using antibody arrays. Increased phosphorylation of ErbB2(Thr686), ErbB2(Ser1113) and limited phosphorylation of EGFR(Thy845), ErbB2(Tyr1221/1222), ErbB3(Tyr1289) and ErbB4(Tyr1284) sites was seen in the control group. In the monotherapy groups, ErbB2(Thr686), (Ser113) and ErbB4(Tyr1284) sites were phosphorylated. Inhibition of most of the EGFR phosphorylation sites was observed in combination therapy groups except for ErbB2(Thr686) and (ser1113).

EGFR-mediated Ras-Raf-MEK-ERK and PI3K-PTEN-AKT pathways play an important role in transmission of signals from membrane receptors to downstream targets that regulate apoptosis, cell growth and angiogenesis. Components of these pathways include genes such as Ras, B-Raf, PI3K, PTEN and Akt that can be mutated or aberrantly expressed in human cancer. Though we did not investigate these genes, it should be noted that they could cause resistance to anti-EGFR therapy. Numerous studies have reported Kras mutations as a predictor of resistance to Erbitux therapy and are associated with poor prognosis in colorectal cancer (Lievre et al., 2006) and non-small cell lung carcinoma (Riely et al., 2009). In a similar way, Braf mutation is also known to cause resistance to anti-EGFR therapy in colorectal cancers (Li et al., 2006) and primary lung adenocarcinomas (Schmid et al., 2009). Mutation of PTEN tumor suppressor gene in human cancer cells leads to activated EGFR downstream signaling including PI3-kinase/AKT and have been linked to resistance to anti-EGFR targeted therapies (M. Y. Wang et al., 2006). However, in this study we investigated the role of EGFR target genes cyclin D1 and c-myc that are involved in cell proliferation. Our RT-PCR results showed downregulation of cyclin D1 and c-myc in the tumors treated with the combination therapy (Figure 12). Amplification of cyclin D1, a key cell cycle regulatory protein, appears to be an important event in bladder cancer and is often associated with cell proliferation and poor prognosis in human tumors (Le Marchand et al., 2003). In our study, downregulation of EGFR also resulted in reduction of cyclin D1. This observation could be due to the administration of Erbitux, that is known to cause cell cycle arrest in the G(1)/G(0)-phase, also increases the expression of cyclin-dependent kinase inhibitors (Huether et al., 2005). c-myc, another EGFR target gene that can obstruct the induction of apoptosis in tumor cells and lead to uncontrolled cell growth was reduced in the PDT + Erbitux treated tumors. Over-expression and amplification of c-myc can play an important role in metastatic progression that indicates poor prognosis in different cancers (Peng et al., 1997). These results suggest that EGFR target genes could play a role in tumor inhibition in bladder cancer by arresting cell cycle growth and inducing apoptosis.

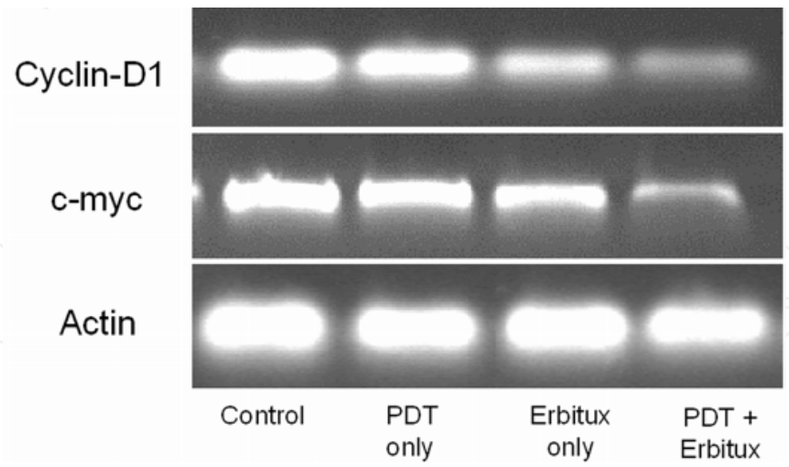

Fig. 12. The effect of EGFR inhibition on target genes cyclin-D1 and c-myc was evaluated at the RNA level. Cyclin D1 is an important regulator of G1 to S-phase transition and overexpression of cyclin D1 has been linked to the development and progression of cancer. c-Myc is activated in a variety of tumor cells and plays an important role in cellular proliferation, differentiation, apoptosis and cell cycle progression. Downregulation of cyclin-D1 and c-myc was observed in the tumors treated with PDT and Erbitux ($p<0.05$) when compared with the other groups.

5. PDT in combination with Avastin and Erbitux

In this study, the potential of combining anti-angiogenic agent Avastin that is specific to VEGF and Erbitux that targets EGFR along with PDT to improve bladder tumor response was investigated. To achieve this, the inhibitory effect of the anti-angiogenic compounds was tested using angiogenic assays. Cell migration is a highly integrated multistep process that is essential for invasion and metastasis of tumors. Directed cell migration is normally initiated in response to extracellular cues such as chemoattractants, growth factors and the extracellular matrix (Ridley et al., 2003). In this study, stimulation with VEGF, a proangiogenic protein, increased the migration of tumor cells. On the other hand, antiangiogenic agents, Avastin and Erbitux reduced the migratory potential of the tumor cells. Avastin is known to significantly reduce proliferation and migration capacity, and increase apoptotic rates in endothelial cells (Carneiro et al., 2009). A similar study has reported 40-60% inhibition of cell migration of SCC cells when incubated with Erbitux in a dose dependent manner (S. M. Huang et al., 2002). One of the most important and crucial events in cancer metastasis is the invasion of basement membrane. The invasion assay results in this study have clearly demonstrated the stimulatory effect of VEGF on endothelial cell migration (Figure 13). VEGF plays an important role in tumor invasion and metastasis through its specific action on endothelial cells in tumor tissue (Khosravi Shahi & Fernandez Pineda, 2008). *In vitro* studies have clearly demonstrated that VEGF is a potent mediator of angiogenesis as it helps in the proliferation and migration of the endothelial cells to form tube-like capillaries (Bernatchez et al., 1999). Tumor cells exposed to hypoxia induced by PDT triggers the expression of VEGF mRNA that in turn releases the VEGF protein (Dvorak et al., 1995; Shweiki et al., 1992).

Endothelial barrier disruption by VEGF-mediated Src activity has been shown to potentiate tumor cell extravazation and metastasis (Weis et al., 2004). Another study has reported that interference with VEGF function could be sufficient to abrogate tumor invasion (Skobe et al., 1997). Reduced invasion of endothelial cells through the basement membrane was observed in the Avastin and Erbitux treated cells, suggesting their role in preventing the disruption of the basement membrane. Another important step in angiogenesis is the formation of a functional vascular system for tumor growth and metastasis. Endothelial cell tube formation is a consequence of various biological activities, including cell migration, vacuolization, cell-cell junction formation and cell elongation. It is well established that VEGF plays an important role in the process of angiogenesis by facilitating endothelial cell migration and tube formation similar to the observations in this study (Ferrara, 2004). Another report has also identified Hedgehog signaling as an important component of the molecular pathway leading to vascular tube formation (S. A. Vokes et al., 2004). Avastin and Erbitux were used successfully to reduce and inhibit tube formation, thus signifying their role in blocking major angiogenic processes. Similarly, another study demonstrated that bevacizumab could significantly impair tube formation capabilities in tumor derived endothelial cells and also noted a continuing effect after 14 days of treatment even after omitting the antibody (Grau et al., 2011). Treatment with Cetuximab has also shown to reduce cell-to-cell interaction of human umbilical vascular endothelial cells (HUVEC), resulting in disruption of tube formation (S. M. Huang et al., 2002). Further substantiating these *in vitro* findings, Avastin and Erbitux also inhibited angiogenesis in a mouse plug matrigel assay, as evaluated by haemoglobin content levels.

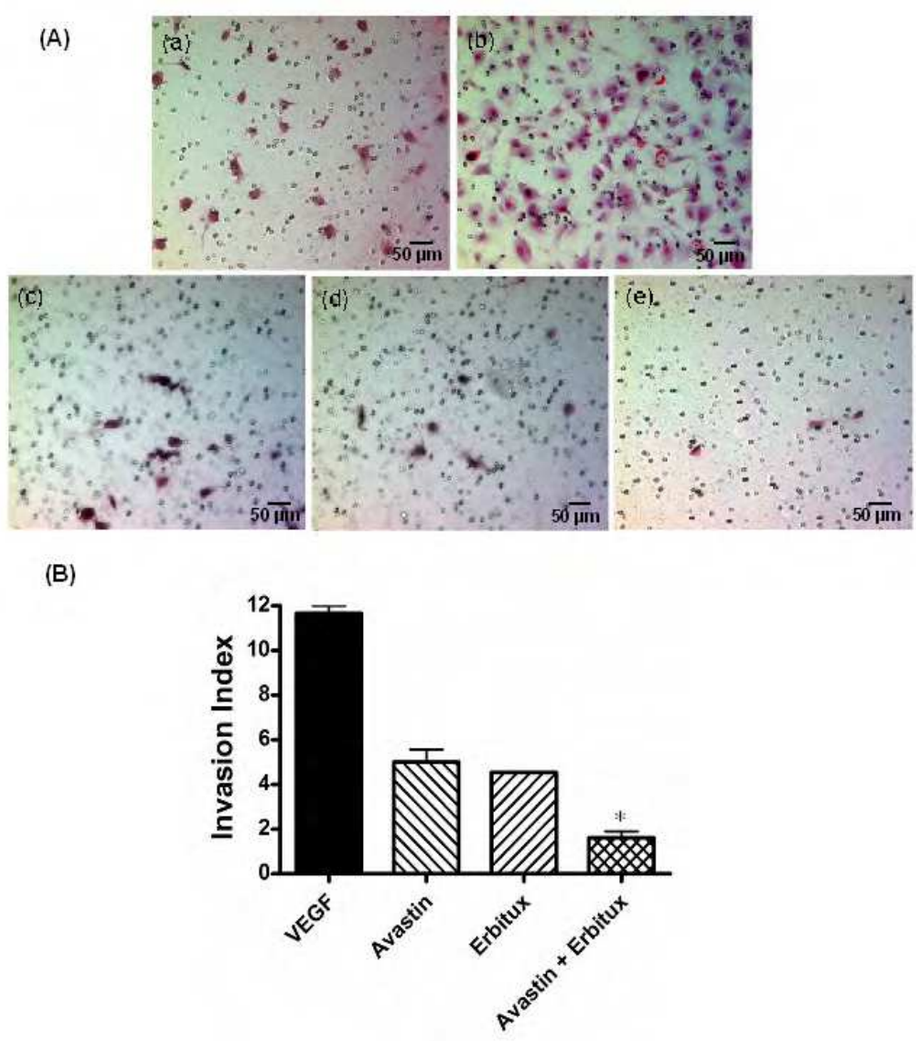

Fig. 13. Effect of angiogenesis stimulator and inhibitors on HUVECs *in vitro*. (a) control, (b) VEGF, (c) Avastin (d) Erbitux and is (e) Avastin + Erbitux. (A) High invasion of endothelial cells through basement membrane were noticed in VEGF treated wells Avastin and Erbitux inhibited the migration of endothelial cells. The figures are representative of the results from three separate experiments. (B) The invasion index was calculated based on the number of cells in the test samples compared to the control samples. Calculations for each group were performed in triplicate. Invasion index for VEGF was high and was lowest for the combination of angiogenesis inhibitors (*=$p<0.001$). Error bars represent the standard error of the mean invasion index in comparison to control in all the groups, n = 6.

Avastin and Erbitux monotherapies and also Avastin + Erbitux remarkably suppressed the sprouting of endothelial cells and induction of new blood formation in the matrigel plugs (Figure 14). It has been demonstrated that the effects of blocking angiogenesis can be observed on tumor transplanted onto animals (O'Reilly et al., 1994). The antiangiogenic activities of Avastin and Erbitux may be explained by their inhibitory action on the proliferation, migration and differentiation of the tumor cells, by inhibiting VEGF and EGFR respectively.

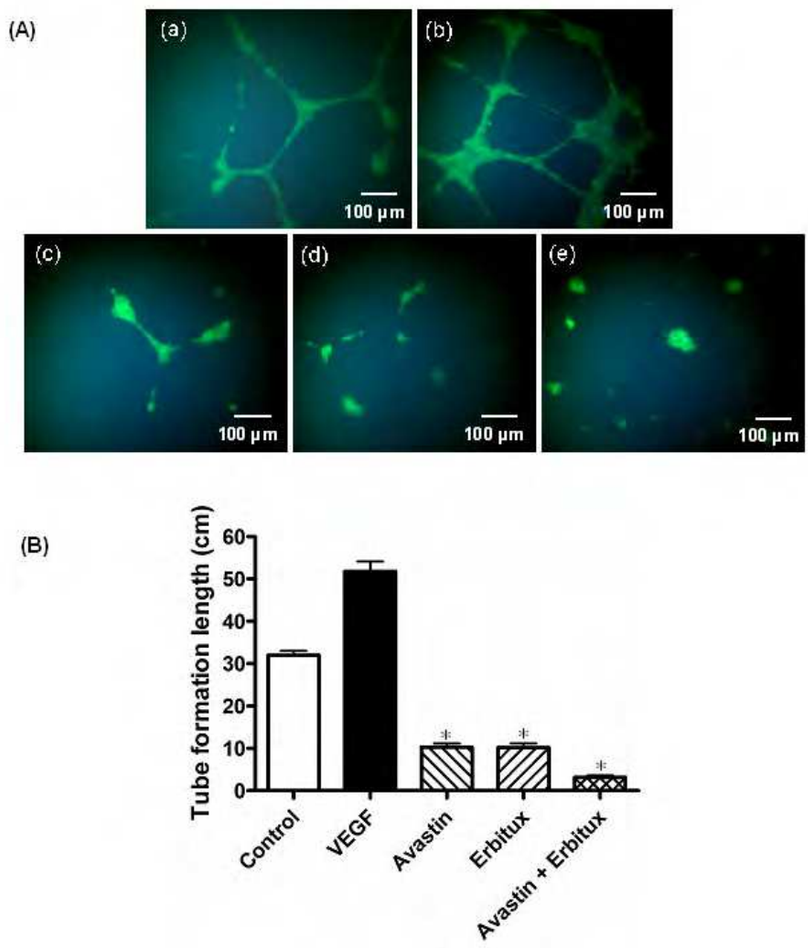

Fig. 14. (A) Endothelial cell tube formation was assessed using VEGF, Avastin and Erbitux. (a) control, (b) VEGF, (c) Avastin (d) Erbitux and (e) Avastin + Erbitux. VEGF induced tube formation and Avastin and Erbitux decreased growth inhibited tube formation of endothelial cells. Combining Avastin and Erbitux, almost completely abrogated tube formation (B) The total tube length of each treatment group was quantified by the software, Datinf Measure (Tubingen, GmbH, Germany). The figures are representative of the results from three separate experiments. Error bars represent the standard error of the mean invasion index in comparison to control in all the groups, n = 8.

The tumor regression data demonstrated that combining Avastin + Erbitux with PDT can impede the angiogenic process and improve the response of treated tumors (Figure 15). The tumor volume of the PDT treated group was significantly greater than the Avastin treated and Erbitux treated groups as high fluence administered during treatment can deplete tumor oxygen to a large extent, releasing stress induced survival molecules that reduce the effectiveness of PDT and affect tumor control (Gomer et al., 2006). Although tumor regression was also observed in Avastin + Erbitux only treated group, complete cure was not observed. Thus targeting EGFR and VEGF without PDT treatment might not be sufficient to cause regression of most bulky tumors. One of the possible explanations for this observation may be related to pericytes that respond to angiogenic stimuli and promote

endothelial stability through matrix deposition, and have macrophage-like function (Lu et al., 2007). Also, the tumors overexpressing EGFR might not be sensitive to Erbitux. Although it is normally assumed that tumors overexpressing EGFR would respond well to anti-EGFR therapy, studies have demonstrated that the level of EGFR expression does not have enough impact on tumor response rates as a significant number of EGFR-positive tumors could be resistant to Erbitux (Ellis et al., 2004; Vallbohmer et al., 2005). Complete cure was noted in tumors treated with PDT and continued Avastin + Erbitux therapy. Thus the data from the present study supported the hypothesis that Avastin and Erbitux are capable of binding and neutralizing secreted VEGF and EGFR respectively, thus causing regression of tumor vessels, and preventing tumor recurrence.

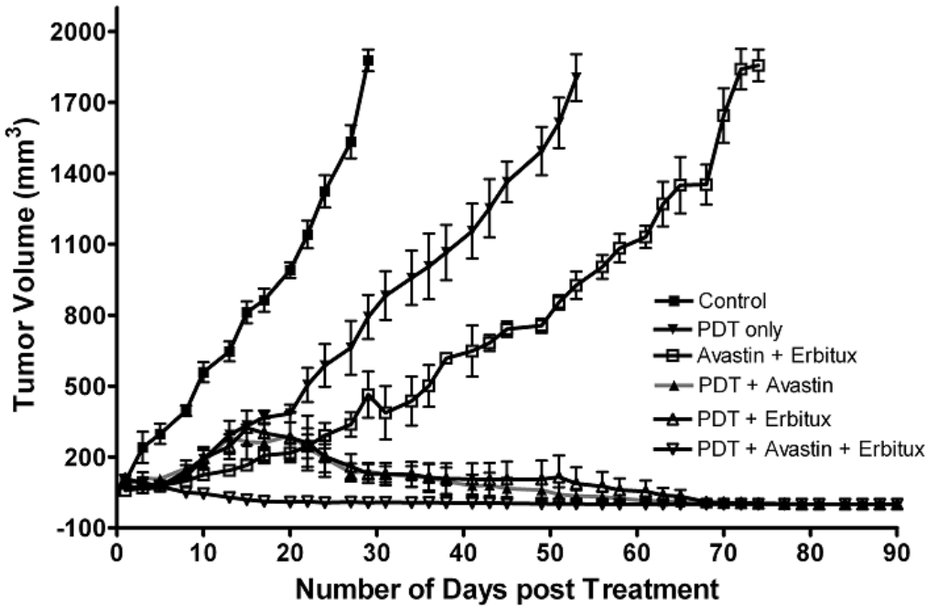

Fig. 15. Tumor volumes were charted against days to assess the tumor response in various treatment groups. The combination therapy groups of PDT + Avastin, PDT + Erbitux and PDT + Avastin + Erbitux exhibited greater tumor response in comparison with other groups. Each group represents the mean (error bars, SE) of 10 animals.

VEGF and EGFR expression was suppressed in the tumors treated with PDT and inhibitors (Figure 16). The data in this study demonstrate that tumors treated with PDT expressed greater amounts of VEGF, which is consistent with an earlier report by Solban et al. (Tortora et al., 2008) on subcurative PDT performed on an orthotopic model of prostate cancer that showed increased VEGF secretion. Also, significantly lower occurrence of VEGF was observed in the combination therapy of PDT and Avastin. On the other hand, previous studies have reported the downregulation of EGFR after PDT (Ciardiello et al., 2006), in marked contrast the results of this study demonstrated an increase in EGFR expression post hypericin-mediated PDT. This observation could be attributed to numerous reasons such as the light/drug dosage, the complexity of tumor microenvironment and the properties of the photosensitizer (Henderson et al., 2004). Combined antitumor activity of Avastin and Erbitux with standard chemotherapy and radiotherapy is well documented in the treatment

of different types of tumors and is reported to be more efficacious than individual monotherapies (Press & Lenz, 2007).

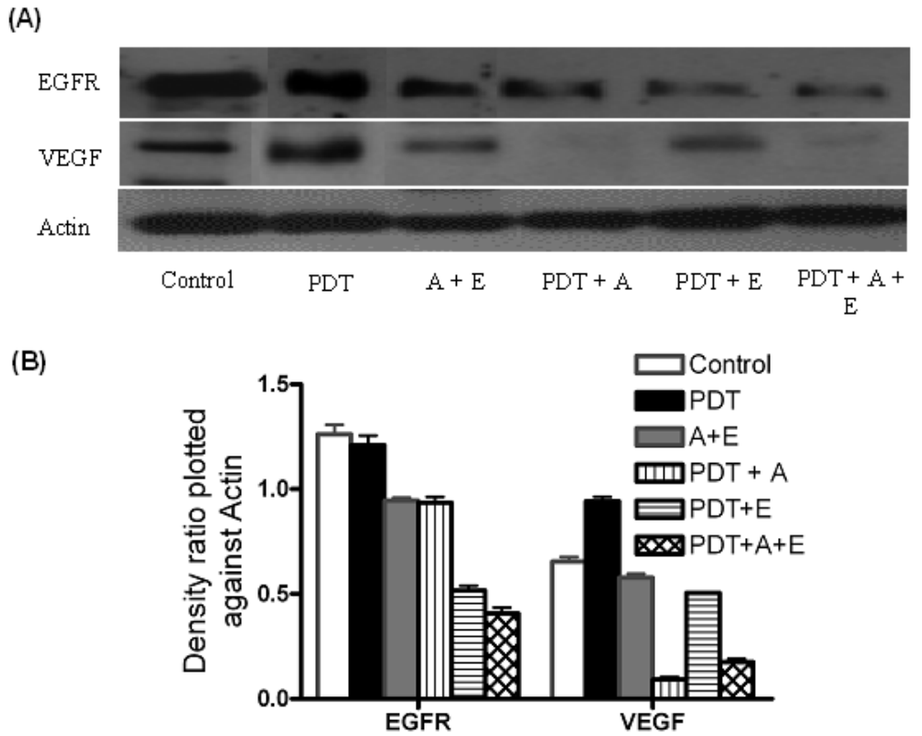

Fig. 16. (A) Expression of EGFR and VEGF was detected in the treatment groups using western immunoblot analysis. Expression of actin was used to monitor protein loading. (B) Ratio of EGFR and VEGF density was plotted against actin.

In this study, combination modality of PDT + Avastin + Erbitux was effective in reducing the expression of VEGF and EGFR which could have led to the greater tumor regression in this group. Combination of Avastin and Erbitux with PDT also improved treatment efficacy by suppressing angiogenic proteins. Though immediate tumor inhibition was noticed in the groups treated with both the inhibitors and PDT, the overall outcome post 90-day treatment for both single agent and double-agent inhibition remained the same. Although tumor regression was also observed in Avastin + Erbitux only treated groups, complete cure was not observed. Thus targeting EGFR and VEGF without PDT treatment might not be sufficient to cause regression of most bulky tumors. One of the possible explanations for this observation may be related to pericytes that respond to angiogenic stimuli and promote endothelial stability through matrix deposition, and have macrophage-like function. Also, the tumors overexpressing EGFR might not be sensitive to Erbitux. Although it is normally assumed that tumors overexpressing EGFR would respond well to anti-EGFR therapy, studies have demonstrated that the level of EGFR expression does not have enough impact on tumor response rates as a significant number of EGFR-positive tumors could be resistant to Erbitux. Complete cure was noted in tumors treated with PDT and continued Avastin + Erbitux therapy.

6. Conclusion

In conclusion, it has been demonstrated that VEGF is upregulated due to hypoxic conditions induced by hypericin-mediated PDT. Also, VEGF acts as a potent angiogenesis-stimulating factor that has potential as a tumor biomarker to determine the outcome of photodynamic therapy. Combination treatment of PDT with Avastin that binds to VEGF and blocks receptor binding improved the tumor response of bladder carcinoma xenografts and suppressed the VEGF pathway by causing the downregulation of important angiogenic mediators. In the similar way, the regular administration of Erbitux, an EGFR inhibitor after PDT treatment can block the EGFR pathway and reduce angiogenesis. Therefore, the combination therapy of PDT and Erbitux was more effective in preventing angiogenesis compared to monotherapy alone. In another study the combination of both Avastin and Erbitux with PDT was capable of binding and neutralizing secreted VEGF and EGFR respectively, thus causing regression of tumor vessels, normalizing surviving mature vasculature and preventing tumor recurrence.

To summarize, combining angiogenesis inhibitors with PDT increased therapeutic efficacy and this method is a promising approach to cancer therapy. The challenge is to choose the appropriate anti-angiogenic agent in combination with optimal light dosimetry PDT for potential clinical application. The success seen with the combination of inhibitors with conventional treatments can provide information for potential target mechanisms, which may translate into better response rate with less local and systemic toxicity and improved overall survival rates.

7. Acknowledgements

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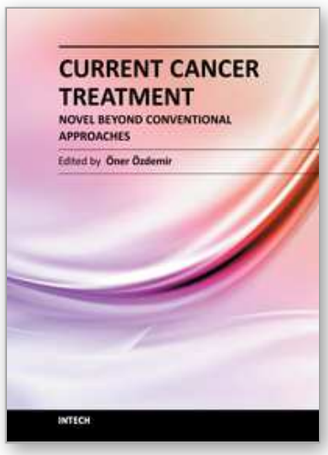

Current Cancer Treatment - Novel Beyond Conventional Approaches

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Currently there have been many armamentaria to be used in cancer treatment. This indeed indicates that the final treatment has not yet been found. It seems this will take a long period of time to achieve. Thus, cancer treatment in general still seems to need new and more effective approaches. The book "Current Cancer Treatment - Novel Beyond Conventional Approaches", consisting of 33 chapters, will help get us physicians as well as patients enlightened with new research and developments in this area. This book is a valuable contribution to this area mentioning various modalities in cancer treatment such as some rare classic treatment approaches: treatment of metastatic liver disease of colorectal origin, radiation treatment of skull and spine chordoma, changing the face of adjuvant therapy for early breast cancer; new therapeutic approaches of old techniques: laser-driven radiation therapy, laser photo-chemotherapy, new approaches targeting androgen receptor and many more emerging techniques.

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