"PHOTODYNAMIC THERAPY OF
SQUAMOUS CELL CARCINOMA
AN EVALUATION OF A NEW PHOTOSENSITIZING AGENT
AND PHOTOIMMUNOCONJUGATE"

by

ALAN WILLIAM HEMMING

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Department of __Surgery__
__Faculty of Medicine__
The University of British Columbia
Vancouver, Canada

Date __March 26 1993__

ABSTRACT:

Photodynamic therapy for cancer depends on the relatively selective distribution of photosensitizing agents to malignant as compared to normal tissues, rendering the malignant cells more susceptable to light mediated damage. Photodynamic therapy has been used with only moderate success to date. The purpose of this study was to compare a new photosensitizing agent, Benzoporphyrin derivative (BPD), to the standard agent presently in use, Photofrin II, in a hamster cheek pouch model of squamous cell carcinoma. As well we have investigated the potential of using a tumor specific monoclonal antibody - BPD conjugate to improve the tumor localizing properties of BPD.

Treatment consisted of photodynamic therapy with either Photofrin II, BPD, or a tumor specific antiepidermal growth factor receptor -BPD conjugate. Control groups of light alone, antiEGFr, tumor nonspecific antiCEA, and tumor nonspecific antiCEA-BPD conjugate were included with the contralateral cheek pouch of each animal acting as a dark control. An assessment of differential delivery of BPD to tumor and to normal mucosa was undertaken using a spectrophotometric assay. Parametric statistical analysis included student t tests and linear regression while non-parametric analysis was undertaken using Fisher's exact test.

Animals receiving BPD alone demonstrated tumor to tissue levels of approximately 2:1 while animals receiving the tumor specific

antiEGFr-BPD conjugate had significantly better tumor:tissue ratios of 26:1.($p < 0.005$)

Animals treated with Photofrin II had a one month cancer free survival of 27% while animals treated with BPD had an improved survival of 67% ($p=0.03$) The group treated with the tumor specific antiEGFr-BPD conjugate at a twentieth the total dose of BPD had an 80% one month cancer free survival which was not statistically different from the group treated with BPD alone.

Benzoporphyrin appears to be a more effective photosensitizing agent than Photofrin II and its tumor selectivity can be improved using a tumor specific monoclonal antibody conjugate.

TABLE OF CONTENTS: page

LIST OF TABLES page

LIST OF FIGURES

ACKNOWLEDGEMENT

I. INTRODUCTION.

1 Photodynamic Therapy: History

1.1 *Early Development of Photodynamic Therapy*

The first known use of phototherapy occurred over 6000 years ago when the ancient Egyptians used the technique to treat depigmented areas of skin [1]. They applied crushed leaves from plants, containing what we now know to be psoralens, to areas of depigmented skin and on exposure to sunlight this resulted in sunburn and eventual pigmentation of the underlying skin [2]. In more recent times chemical sensitization of living tissues was first reported in 1900 by Raab using the aniline dye, acridine to render unicellular organisms sensitive to light [3].

Tappenier and Jesionek (1903) were the first to utilize photodynamic therapy for the treatment of malignant disease when they treated skin cancers using topical eosin as a photosensitizer along with white light [4]. Over the next 75 years several compounds including methylene blue, tetracycline, chlorophylls and other porphyrins were found to be cytotoxic in combination with light [5,6].

1.2 *Porphyrins as Photosensitizers*

Auler and Banzer described uptake of hematoporphyrin (HP) by neoplastic tissues in 1942 and this was later confirmed using fluorescence by Figge et al [7,8]. Hematoporphyrin derivative (HPD), the porphyrin

initially popularized for photodynamic therapy is a synthetic derivative of hemoglobin demonstrated by Lipson et al in 1960 to have tumor localizing properties [9]. HPD, marketed as Photofrin, in fact consists of a mixture of different porphyrins and has been further purified to the components that are thought to localize to tumor, ethers and esters of dihematoporphyrin (DHE) [10,11]. DHE also consists of a complex mixture of hematoporphyrin dimers, trimers, tetramers, pentamers, and their dehydration byproducts with both ether and ester linkages [12,13,14]. This tumor localizing fraction marketed as Photofrin II (Quadralogic Technologies, Vancouver, Canada) has enhanced photodynamic cytotoxic effects and is presently the standard photosensitizing agent in use now [2].

2. Mechanism of Action

2.1 Photochemistry

Porphyrins and photosensitizers in general have a common mechanism of action. After absorbing light of an appropriate wavelength the sensitizer is converted from its stable electronic ground state (S_0) to a short lived excited state known as the singlet (S_1) state that may undergo conversion to a longer-lived excited state known as the triplet state (T_1). The triplet state is responsible for forming cytotoxic species or may undergo several competing processes including fluorescence decay [15].

It is this fluorescence decay of photosensitizers that has led to their additional use in the detection of malignancy [16,17,18].

Table I
Photochemistry involved in PDT.

S_0 + light → S_1	Absorbence
S_1 → S_0 + light	Fluorescence
S_1 → S_0 + heat	Internal conversion
S_1 + M → S_0 + M	Quenching
S_1 → T_1	Intersystem crossover

Where	S_0 = Sensitizer ground state
	S_1 = Sensitizer singlet state
	T_1 = Sensitizer triplet state
	M = Substrate

Interaction of the triplet state sensitizer with tissues can proceed via either a type I or type II reaction. Type I reactions involve hydrogen abstraction from the sensitizer to produce free radicals and are oxygen independent. The type II reaction exclusively involves the interaction between molecular oxygen and the triplet state to form singlet oxygen

($^1O_2$) which is highly reactive in living tissue [19].

Type II: $T_1 + O_2 \rightarrow S_0 + {}^1O_2$

$^1O_2 + M \rightarrow$ photo-oxidation

It is this type II reaction that is thought to be responsible for the cytotoxic effects of PDT and in fact the absolute requirement for oxygen in photosensitization has been documented in solution, in culture and *in vivo* [20,21,22].

An important concept is that the excited sensitizer after creating singlet oxygen, returns to its ground state and can again be excited. This allows a single sensitizer molecule to produce many times its own concentration of singlet oxygen.

2.2 Cellular Effects

At the cellular level, singlet oxygen causes lipid peroxidation and damage to both mitochondrial and outer cell membranes resulting in cell death [23]. At the nuclear level single strand breaks in DNA can be produced and as well it has been shown that both RNA-dependant DNA polymerase and DNA dependant RNA polymerase can be inactivated by porphyrin photosensitization [24,25,26].

3 Properties of an Ideal Photosensitizer

3.1 *Absorption*

For photodynamic therapy a sensitizer with absorption in the red or infrared region of the spectrum is desirable [14]. Absorption and scattering of red light by human tissue are much less extensive than of blue light [27]. Living tissues are best penetrated by light of a wavelength above 600 nm and in fact it has been shown by Bown et al that significantly improved tissue penetration is achieved as the wavelength of activating light is increased from 630 nm to 675nm and beyond [28]. As well as having an absorption peak at this preferred band the sensitizer must also have an efficient quantum yield at this excitation wavelength , i.e., the predominant result of activating the sensitizer should be the production of the excited triplet state and therefore singlet oxygen rather than the production of fluorescence decay [29]. Photofrin II has intense absorption in the violet region (400nm) and several additional absorption bands between 500nm and 600nm. Unfortunately it absorbs relatively poorly at 630 nm the only absorption band above 600nm and yet it is this wavelength that is most often used clinically [23]. Photofrin II although the standard photosensitizer in present use is not an ideal photosensitizer in terms of its absorption characteristics.

3.2 *Tumor selectivity*

The ideal photosensitizer should distribute to malignant as opposed

to normal tissues, thus in theory, causing maximum tumor destruction while minimizing the destruction of normal surrounding tissues and systemic side effects. Pharmacokinetics of Photofrin II have been analyzed using radiolabelled tracers and it has been shown that the porphyrin is retained in tumor more than in normal tissues such as skin, muscle, brain and lungs. However much larger concentrations are found in organs such as liver, spleen and kidney [30]. While the mechanism for delivery and retention of porphyrins in tumor is not known, evidence suggests that there is a high affinity between porphyrins and lipoproteins [31,32]. Porphyrins bind strongly to both HDL and LDL. It is interesting to note that LDL receptors are elevated in neoplastic cells as well as in those organs where there are relatively high concentrations of porphyrin [33].

Barel et al have shown that the amount of porphyrin retained in tumor is higher when porphyrin is bound to isolated LDLs pre-injection as opposed to free drug or to drug bound to isolated HDLs [34]. Kessel has shown that tumor nonlocalizing porphyrins are bound to albumin and also found a correlation between the distribution of porphyrin and the number of LDL receptors in various tissues [35].

An alternative hypothesis has been suggested by Korbelik et al that suggests that macrophages have a high affinity for porphyrins and that relatively high levels of photosensitizers found in tumor as well as in

liver, spleen and kidney are due to the high number of functioning macrophages at each of these sites [36].

While the mechanism of tumor selectivity is not completely defined, it is certain that the lack of complete selectivity accounts for the main side effect reported with photodynamic therapy which is cutaneous photosensitivitity [37,38,39].

4 Light Delivery & Dosage

4.1 *Light Delivery*

Energy delivered to the target site is the product of the power of the light source and the time of exposure to this light source. Sufficient energy for photodynamic activity can be delivered over a prolonged time using an ordinary low-watt light bulb equipped with a red filter and in fact in early studies this method was used [40,41]. This method is quite time consuming and therefore the majority of preclinical studies have used high intensity arc lamps equipped with filters in order to adjust the wavelength to the appropriate band [14,42,43,]. While any source of light with the appropriate light band may be used, clinical applications generally involve the transmission of the light into an enclosed cavity and therefore a very coherent light source, such as laser light that can be transmitted via optical fibres, is required. Argon pumped dye lasers are presently the most common light source for clinical use and have the

advantage of being tunable to various desired wavelengths [44].

4.2 *Light Dosage*

To a large extent light dosage in photodynamic therapy remains quite empiric and tumor type dependant with doses of between 50 - 300 J/cm^2 used with success [45,46]. It has been demonstrated that for a given wavelength of light increasing the light dose increases the depth of tissue penetration and tumor necrosis. Mang et al demonstrated that skin lesions 3-4mm in depth were adequately treated with Photofrin II 2.0 mg/kg and 72 J/cm^2 while lesions 5-10mm in depth required light dosage in excess of 108 J/cm^2 [47].

Patterson et al have validated the concept of a threshold dose for tumor necrosis in photodynamic therapy that states that tissue necrosis due to photodynamic therapy will occur if the number of photons absorbed by the photosensitizer per unit volume of tumor exceeds a critical value [48]. Grossweiner has gone on to develop a light dosimetry model for Photofrin II where the concentration of DHE in the tumor is 2-4 $\mu g/g$ and the necrosis threshold is on the order of 0.4 - 0.7 J/cm^3 or $\approx$ 2 X 10^{18} photons/cm^3 at 630 nm. [49]. This model can be used as a general guideline for light dosage in most animal models using Photofrin II, however it should be noted that changes in photosensitizer concentration result in a proportionately inverse change in the required light dose needed to achieve the necrosis threshold.

5 Clinical Trials

5.1 *Overview of Clinical Applications*

A large number of different tumors have been treated with Photofrin or Photofrin II and photodynamic therapy since the first clinical studies were initiated in 1978 by Dougherty et al [38]. In early studies patients treated by photodynamic therapy had long histories of cancer, either metastatic or local recurrences, which had failed more conventional treatments. Debulking of large tumors has in general been unsatisfactory and while interstitial treatment of solid tumors has been accomplished it has had limited success due to lack of sufficient light penetration [50]. Overall, complete response rates using Photofrin and Photofrin II have been approximately 20-40%.

The major side effect of photodynamic therapy seen in all of the clinical trials has been skin photosensitivity resembling that seen in patients with porphyria [51]. Patients are advised to avoid both direct and indirect sun exposure for a period of 4-8 weeks after intravenous injection [52]. Other side effects reported include nausea, vomiting, metallic taste, eye photosensitivity and liver toxicity [53]. Toxicity does not appear to be additive in patients previously treated with either chemotherapy or radiation and can apparently be used in conjunction with other modalities [54]. Photodynamic therapy has been disadvantageous in situations where major complications can be predicted with treatment of the tumor, as in

treatment of a full thickness esophageal tumor resulting in an esophageal fistula [50].

5.2 *Cutaneous and Subcutaneous Malignancies*

Cutaneous and subcutaneous malignancies represent the most accessible of all malignancies. Various cutaneous malignancies have been treated including malignant melanoma, squamous cell carcinoma, basal cell carcinoma, Kaposi's sarcoma and dermal metastatic breast carcinoma [55]. Response rates have varied according to tumor morphology with small superficial lesions usually showing complete response in 4-6 weeks and larger more invasive lesions often requiring multiple treatments for complete response. Phototoxicity was the primary side effect in all trials. Toxicity was reduced apparently without compromising effect by reducing the dose of photosensitizer used [56].

5.3 *Head and Neck Cancer*

Most patients with head and neck malignancies treated with photodynamic therapy have had squamous cell carcinomas refractory to traditional treatment however in several recent trials patients with early carcinoma in situ and patients with "condemned mucosa" consisting of multiple areas of malignant and premalignant change have been included [2,57]. In cases where the purpose of therapy was only to provide palliation success was achieved in a high percentage of cases, however complete response was only obtained in 20-30% of cases [58,59].

Gluckman reported on 13 patients with early cancers of the aerodigestive tract treated with photodynamic therapy, 11 of which demonstrated a complete response. However, on follow-up 4 of these recurred between 8 and 12 months. More encouraging results were obtained in the same series in patients with condemned mucosa, with 8 of 8 patients having an excellent response to treatment with only 1 patient going on to have a positive biopsy with 12 month follow-up [2].

Complications in these series were again mainly due to skin photosensitization and, in fact, in some palliative cases symptoms of skin photosensitization were of such severity to worsen the quality of life. In one patient, the carotid artery, which was encased with tumor, ruptured three days post photodynamic therapy with resultant death [2].

5.4 Genitourinary Cancer

Photodynamic therapy has probably had its best result in treatment of transitional cell carcinoma of the bladder. The lesions that have benefitted most are diffuse carcinoma in situ or very superficial bladder cancers, especially if multicentric [60]. Tsuchiya et al and Hisazumi et al report 75% and 83% complete response rates in treating superficial bladder cancers with 6-18 month disease free follow-up [61,62]. Multiple other studies report essentially the same result with complete response rates ranging from 60 -85% however several of these studies have been complicated by troublesome bladder volume contraction that in at least

two cases required subsequent cystectomy [63,64,65]. This has led to attempt photodynamic therapy at 514 nm which is a less penetrating wavelength and at least in theory should result in less bladder fibrosis and contracture [66].

5.5 Endobronchial Tumors

Photodynamic therapy has been used for both curative and palliative treatment of endobronchial lesions. Balchum et al treated 100 patients that presented with 40 -90% obstruction due to squamous cell, large cell, and carcinoid tumors, with palliation achieved in the majority, however survival was not affected. All patients required bronchoscopy to remove necrotic debris 2 to 4 days after photodynamic therapy at which time treatment was repeated if any gross tumor was left behind [67]. This mode of therapy for obstructing endobronchial lesions should be considered in light of ablative laser therapy, the primary alternative for these lesions, which has immediate response, carries no complications of photosensitivity and generally does not require a second bronchoscopy.

Hayata et al have reported on photodynamic therapy in eight cases of early stage, central lung cancers that would otherwise have required extensive resection or been unresectable [68]. Six of the eight cases had complete response after photodynamic therapy and remained disease free by endoscopic, cytologic and histologic evaluation with follow-up between 11 and 36 months. The resected specimens of two patients with partial

response were found to have tumor in areas that were thought to have been inadequately irradiated.

Preoperative photodynamic therapy has been used by Kato et al to convert inoperable to operable lung cancer in four of five patients with one patient remaining tumor free four years after resection. As well seven of ten patients with planned pneumonectomy underwent less extensive resection after photodynamic therapy [69].

Complications of treatment encountered included excessive bronchial mucous secretions, mucosal sloughing, fever, pneumonia, and pneumothorax. In addition there are several reports of death from hemoptysis four to five weeks after treatment [67]. Many of these complications have apparently been eliminated by adding the routine use of bronchoscopy two to four days after photodynamic therapy.

5.6 Esophageal and Gastrointestinal Tumors

Photodynamic therapy has been used for both cure and palliation of esophageal carcinoma. McCaughan et al reported seven cases of complete or near complete esophageal obstruction who were treated with photodynamic therapy with all patients being adequately palliated [70]. More recently photodynamic therapy has been applied to earlier, superficial lesions with curative result. Okunaka et al demonstrated complete response in four of six patients with early superficial squamous cell carcinoma with 5 of the six patients alive at 2 years [71]. Calzavara

et al reported complete response in eight of twenty-one patients with superficial esophageal carcinoma with three of the eight patients alive and disease free at two years post treatment [53].

Early stage gastric cancer has been treated with photodynamic therapy with Hayata et al treating four of sixteen cases with photodynamic therapy alone. Complete response was obtained in all four patients as demonstrated by endoscopy, cytology and histology however three of the four had recurrences between five and 27 months after treatment. The other 12 patients underwent surgical resection after photodynamic therapy with the resection specimen showing complete response in five, however seven of the twelve showed residual tumor [72].

Photodynamic therapy has been used for the treatment of colorectal cancer predominantly in unresectable cases for palliation or on the treatment of pelvic recurrences. Relief of pain from unresectable pelvic recurrences seems to be obtained in 50% of patients [73]. In a pilot study by Barr et al 2 of ten patients with colorectal cancer unsuitable for operation were disease free two years after photodynamic therapy [74]. Kashtan et al have as well documented significant palliation with marked decrease in tumor bulk in five of six patients with inoperable rectal carcinoma [75].

Complications associated with treatment of gastrointestinal malignancies include fistulas, hemorrhage especially from the bulkier

tumors and of course skin photosensitivity.

5.7 *Miscellaneous Malignancies*

Photodynamic therapy has been attempted in several other clinical settings however reports for the most part are few. One area that seems of particular interest to surgeons is intraoperative use of photodynamic therapy to improve clearance of resection margins. Nambisan et al have reported on intraoperative photodynamic therapy for advanced retroperitoneal sarcomas in ten patients and have as well used the fluorescence of the photosensitizer to guide resection. Two of the ten patients treated were tumor free at two years with no reported complications [76]. This intraoperative approach has also been attempted for intracranial malignancies. McCulloch et al reported on primary resection of glioblastoma with treatment of the tumor bed with photodynamic therapy, however noted significant cerebral edema post treatment [77]. This intraoperative technique appears promising and may offer advantages in situations where wide resection margins are difficult to achieve.

6 Benzoporphyrin Derivative

6.1 *Synthesis*

While clinical results of photodynamic therapy have shown some successes the results overall have been somewhat disappointing. All clinical trials have used either Photofrin or Photofrin II, which as previously pointed out have less than ideal properties for photosensitization in the clinical setting. The search for an ideal photosensitizer has led to the synthesis of a customized molecule, benzoporphyrin derivative or BPD. (Quadralogic Technologies, Vancouver, Canada.) The synthesis of BPD has been described by Richter et al in 1987 and its structure is shown in Figure 1. BPD as originally synthesized consists of four isomeric forms of which BPD monoacid A has the most desirable tumor localizing effects [78].

6.2 *Photosensitizing Properties of BPD*

BPD absorbs extremely well at 692 nm and therefore is activated by light that penetrates living tissue better than the wavelength that activates Photofrin II (Figure 2). BPD has been utilized *in vitro* and has been shown to be a more potent photosensitizer than is Photofrin II [79]. The lethal effects of photodamage with BPD are associated with membrane damage and there is no evidence for "dark toxicity" [80].

Biodistribution of BPD appears to be similar to that of Photofrin II with marked binding to plasma lipoproteins, predominantly HDL,

however as well to LDL. Precomplexing BPD to LDL increases the deposition of BPD to tumor. Tumor to skin ratios of BPD given alone are between 2-3:1, which is less than ideal, however this is increased to an acceptable 5:1 when precomplexed to LDL [81].

BPD undergoes inactivation of its photoactivity in the tissues with up to 60% of its activity lost at 24 hours [82]. This may actually be of benefit in reducing the duration of skin photosensitivity. Richter et al have reported on a mouse model in which skin photosensitivity with BPD is higher than Photofrin II in the first 24 hours, however animals exposed to light after 24 hours showed only minimal effects of photosensitivity [83].

BPD also offers the advantage of being a single compound without the problems associated with Photofrin II, which consists of a mixture of porphyrins. BPD in theory appears to approach the ideal photosensitizer more closely than does Photofrin II.

7 Immunoconjugates

7.1 History and Principles

Kohler and Milstein first described a general procedure for the production of monoclonal antibodies (MoAb) in 1975 known as the hybridoma technique [84]. Briefly mice are immunized with a source of antigen and then splenocytes from the immunized animals that include

immune B lymphocytes are fused with an established mouse myeloma cell line. Hybrid cells grow out under selective conditions that kill nonhybridized mouse myeloma cells. Hybrid cells combine the ability to produce a specific antibody from the B lymphocytes with the property of immortality from the myeloma cell line. This allows the large scale production of specific monoclonal antibodies to desired antigens [85].

The development of monoclonal antibodies has led to efforts to identify tumor specific antigens to which MoAbs can be directed. Initially it was hoped that simply binding the MoAb to the malignant cells would be sufficient to achieve tumor destruction, or at least inhibition, via a complement mediated system or antibody dependant cell mediated cytotoxicity and in fact passive administration of unmodified antibodies has been shown to have some anti-tumor activity [86]. In general however, unmodified antibodies have not proven to have a major effect on tumor destruction and the search has largely turned elsewhere.

The identification of oncogenes and their products has led to another mechanism to interrupt tumor biology. A fundamental difference between cancer cells and normal cells is the ability of the transformed cancer cell to go on dividing forever and to have reduced requirements for exogenous growth factors [87]. It has been shown that the oncogene v-erb B codes for a protein product homologous to the receptor for epidermal growth factor [88]. It is thought that cancer cells can produce

growth factors that promote the growth of that same cell. For example a cancer cell that over expresses EGF receptor would initially be stimulated by normal amounts of EGF resulting in the same cells release of EGF as well as possible up regulation of the EGF receptor [89]. This so-called autocrine mechanism of stimulation is thought to provide a growth advantage and suggests a possible step at which to intervene with a monoclonal antibody directed to EGFr. In animal models growth of tumors is inhibited by the treatment of the animals with a MoAb to EGFr, however cure is not achieved [90].

The next step was to modify the tumor specific antibody in order to make it more toxic. In theory this produces the "magic bullet " so long sought after, concentrating the toxic agent in the malignant tissue while sparing normal tissues. Initial attempts with immunoconjugates were made by adding potent toxins or chemotherapeutic agents to tumor specific MoAbs. Ricin a potent cytotoxin has been linked to MoAbs to both carcinoembryonic antigen and EGFr with excellent resulting tumor cell destruction [91,92]. Radiolabelled antibodies to CEA have been used clinically in both localization and treatment of human colon cancer with some success, demonstrating the possibilities of using other immunoconjugates in the treatment of cancer [93].

7.2 Photoimmunoconjugates

In 1985 Mew et al demonstrated the feasibility of using specific monoclonal antibody - hematoporphyrin conjugates to photosensitize and destroy selected cancer cell lines in culture [94]. Later in 1986 Oseroff et al created a monoclonal antibody-chlorin conjugate that was effective in selective destruction of human T-cell leukemia *in vitro* [95]. Interestingly the chlorin used had no tumor selectivity of itself however was chosen as a photosensitizer due to its absorption in the 680-690 nm range demonstrating the potential for future applications of this technology.

A problem that continued to arise was the difficulty in loading an appropriate number of photoactive molecules onto the tumor specific antibody. One solution suggested by Jiang et al was to use a polyvinyl alcohol carrier on which to preload the photosensitizer(BPD) and then link the polyvinyl alcohol to the MoAb. This technique was found to retain antibody specificity with up to 50 molecules of BPD bound to each antibody [96]. Using this method a monoclonal antibody - BPD conjugate directed towards a human squamous cell carcinoma antigen 5E8 was produced which successfully localized and destroyed tumor cells in culture [97].

8 Epidermal Growth Factor Receptor

In order to use a photoimmunoconjugate an appropriate cell surface must be expressed by the malignant tissue. Epidermal growth factor receptor would appear to be a good marker to target with some possibility of future clinical use as it has been shown to be over-expressed in a variety of human neoplasms including squamous cell carcinomas of the head and neck, esophagus and lung [98,99,100]. A number of other non-squamous cell neoplasms such as colon, thyroid, breast and bladder have demonstrated an increase in expression of EGFr as well [101,102,103].

Epidermal growth factor receptor is a 170,000 MW protein that spans the cell membrane and mediates the cell's initial response to EGF and perhaps TGFα [104]. It has an extracellular region that binds EGF and an intracellular region that possesses tyrosine specific protein kinase activity [105]. The erb-b oncogene codes a product homologous for a portion of the EGF receptor [88]. When activated by EGF the EGF receptor promotes DNA synthesis and cell growth. The overexpression of EGFr appears to be a step in malignant transformation of cells and is thought to confer a growth advantage to those cells via an autocrine mechanism of growth factor stimulation [89].

EGF receptor, if not bound, is generally shed into the surrounding cell matrix during normal receptor turnover. When binding occurs

however, the receptor and substance bound to it are internalized via endocytosis [106].

Both the overexpression of EGFr by certain neoplasms, its role in malignant cell growth and its mechanism of action suggest that EGFr would be an ideal marker for a photoimmunoconjugate.

II. Experimental Rationale & Purpose.

1. Experimental Rationale

As outlined above photodynamic therapy has been used in both the preclinical and clinical stages with varying amounts of success. Photofrin and Photofrin II have been the primary photosensitizing agents used in photodynamic therapy of tumors and yet neither possesses photosensitizing properties that can be considered to approach in any way what might be considered ideal. Recent advances in biochemistry have allowed photosensitizing agents to be custom designed to provide photochemical properties that more closely approximate that of the ideal photosensitizer, specifically a modified chlorin structure, benzoporphyrin derivative or BPD.

The development of monoclonal antibodies to tumor specific cell surface markers suggests an elegant method of delivering a photosensitizing agent selectively to malignant tissue, in theory sparing the surrounding normal tissues illuminated during treatment as well as reducing if not eliminating the systemic toxicities associated with other photosensitizers during photodynamic therapy.

The theoretical benefits of using benzoporphyrin derivative may be seen to have basis in reality by comparing the response and cure rates of a specific tumor after photodynamic therapy with either Photofrin II, the standard photosensitizing agent in use, or benzoporphyrin derivative.

The ability *in vivo* of a monoclonal antibody - photosensitizer conjugate to distribute to malignant and not normal tissues can be assessed in two ways. One method is to treat a specific malignancy and infer from lack of side effects, particularly skin photosensitivity, that the agent is selective. The other and more objective method is to construct an assay for the specific photosensitizing agent in both normal and malignant tissues to assess relative levels of agent in the tissues.

Lastly the photodynamic ability of the monoclonal antibody - photosensitizer conjugate, specifically an anti-EGFr - BPD conjugate, to effect tumor destruction may be assessed in the same model as in comparing Photofrin II to the new agent, benzoporphyrin derivative. If response and cure rates of the monoclonal antibody - photosensitizer conjugate are equivalent to, or better than, the best of the other two agents and a strong tumor selective effect can be demonstrated this would be strong supportive evidence that photoimmunoconjugates offer advantages in photodynamic therapy and deserve consideration for the clinical setting.

2. Purpose

The purpose of this thesis is to:

1) evaluate a new photosensitizing agent, benzoporphyrin derivative against the standard photosensitizing agent Photofrin II.

2) assess the tumor localizing properties of a photoimmunoconjugate.

3) evaluate the effectiveness of a photoimmunoconjugate in photodynamic therapy.

III. MATERIALS & METHOD.

1. Animal Model:

The Syrian golden hamster cheek pouch model has been described previously [18]. In brief, Dimethylbenzanthracene (Sigma Chemical St. Louis) impregnated, silicone coated sutures were prepared and inserted submucosally into both cheek pouches of 95 male outbred Syrian golden hamsters (Charles River, Montreal, Canada) age 4-6 wks. A separate group of 15 male Syrian golden hamsters had a similarly placed suture in only one cheek pouch. This has previously been shown to induce squamous cell carcinomas in 85% of animals by 12 weeks(Figure 3) [18]. In our experience all animals developed tumor by 18 wks although 8 animals required reimplantation with suture at 4 weeks when it became evident that the original sutures had fallen out with initial inflammation caused by the DMBA. Animals were housed at room temperature with a 12-hr light /dark cycle. Water and laboratory chow were given ad libitum. Procedures were done under intraperitoneal barbiturate anesthetic (Sodium pentobarbital, 5 mg/kg). Euthanasia was performed using an overdose of the same barbiturate one month post photodynamic therapy. At the time of photodynamic therapy animals weighed between 175 and 200 grams. All procedures received approval of the University of British Columbia animal care committee prior to the institution of the study.

2. Monoclonal Antibody Selection

Both a tumor specific antibody as well as a nonspecific control antibody were required for our purposes. In order to identify the relative specificities of our chosen antibodies the following was undertaken (Figures 4+5).

Tumors along with samples of normal mucosa from 3 separate animals were harvested after euthanasia by sharp dissection away from underlying tissues. Specimens were embedded in OCT (polyvinyl alcohol/ethylene glycol/nonreactive ingredients) and snap frozen in liquid nitrogen. Standard hematoxylin and eosin frozen sections were done to confirm the presence of squamous cell carcinoma. 5 micron frozen sections were cut from both tumor and normal mucosa, air dried and fixed in acetone. The sections were then rehydrated with Tris buffer (pH 7.6) and incubated at room temperature for 5 minutes with non immune normal goat serum (Cedarlane Laboratories, Hornby, ONT, Canada) to block nonspecific binding. The primary antibodies used were a mouse monoclonal IgG1 to the extracellular domain of the EGFr (Sigma St. Louis Mo.) [107] and a mouse monoclonal IgG1 to Carcinoembryonic Antigen (Pierce Laboratories Rockford Illinois). Dilutions of 1:100 and 1:50 respectively were applied for one hour and then washed with Tris buffer. Endogenous peroxidase was blocked by applying 1.5% peroxide for 20 minutes. After a second wash with Tris buffer the secondary antibody-peroxidase

conjugate was applied for 90 minutes (goat anti-mouse/horseradish peroxidase, Biocan Scientific, Mississauga,ONT, Canada). The slides were then washed with Tris buffer, placed in an acetate buffer (0.1 M, pH 5.2) for 3 minutes and developed over 15 minutes with a substrate solution of 3-amino-9-ethyl carbazole(6 mg AEC in 1.5 ml N,N dimethylformamide, 28.5 ml 0.1 M acetate buffer and 0.3 ml 3% H_2O_2). Subsequently the sections were washed with distilled water, counter stained with Carrazzi's hematoxylin, mounted with an aqueous medium and dried. For each set of slides and for each primary antibody used, a negative control omitting the primary antibody and a positive control of a known EGFr expressing human oral squamous cell carcinoma or a known CEA expressing human colonic carcinoma were simultaneously stained. The staining was rated as positive if any portion of the slide showed staining.

3.   Photosensitizers & Preparation of Monoclonal Antibody Conjugates

3.1   *Photosensitizers*

Both Photofrin II and Benzoporphyrin derivative were kindly donated by Quadralogic Technologies (Vancouver, Canada). BPD and its synthesis have been well described elsewhere [79]. Crystallized BPD monoacid A was made into solution by dissolution in DMSO to make a

stock solution of 5mg/ml BPD/DMSO. This was kept frozen at -40 degrees Celsius until use at which time it was diluted with sterile phosphate buffered saline (PBS) for injection. The solution was kept in the dark until injected. The Photofrin II supplied as a crystalline powder was dissolved in 5% dextrose water immediately prior to use.

3.2 *Antibody Purification and Conjugation*

Both the tumor specific EGFr-BPD conjugate and the nonspecific CEA-BPD conjugate were prepared using a method that has been described in detail previously and is outlined below [14].

The antiEGFr IgG1 was supplied in ascites (Sigma Immunochemicals St. Louis. Mo.) purified over a recombinant protein G column(Pierce Laboratories, Rockford Illinois), lyophilized and stored in PBS. The concentration of antibody in solution was assayed using the Biuret method and found to be 3.8 mg/ml. A portion of this sample was used as the primary antibody in immunohistochemical staining of a known EGFr positive oral squamous cell carcinoma to confirm the maintenance of antibody specificity. The antiCEA IgG1 was supplied as a purified lyophilized powder.

Benzoporphyrin derivative is loaded onto modified polyvinyl alcohol carrier, which is then further substituted with 3-mercaptopropionic acid such that three free thiol(SH) groups are introduced per carrier molecule. The PVA-BPD-SH was supplied by Quadralogic

Technologies. (Vancouver, Canada).

Both specific antiEGFr conjugate and nonspecific antiCEA conjugate were prepared in the same manner. Antibody, initially in phosphate buffered saline, underwent volume reduction and buffer exchange to carbonate buffer(pH 8.5). Antibody was then transferred to an amber vial flushed with nitrogen. SMBS (sulfo-m-maleimido-benzoyl-N-hydroxysulfosuccinimide ester, 5mg/ml) was added to give a molar ratio of SMBS to antibody of 30:1, the vial again flushed with nonreactive nitrogen gas and the mixture stirred for two hours in the dark. The carbonate buffer was then exchanged to a 0.05M acetate buffer pH(5.4) and the BPD-PVA-SH added to the MoAb-SMBS with 5% PVA added to make a final concentration of 0.8% PVA. The mixture was stirred overnight at 4°C in the dark.

This results in a molar ratio of BPD: monoclonal antibody of 25:1 and has been shown *in vitro* to maintain both antibody specificity as well as the photosensitizing properties of BPD [97]. The BPD-monoclonal antibody conjugates were mixed with sterile PBS immediately prior to use.

4 Assay of BPD Delivery to Tissues.

The method of tissue porphyrin assay used has been adapted from that described by Straight [108]. The 15 animals with tumor in one

cheek pouch were divided into four groups for intravenous injection of either 2.5 mg/kg BPD, 1mg/kg BPD-anti EGFr conjugate(containing 120μg BPD/mg antibody), 1mg/kg BPD-antiCEA conjugate(120μg BPD/mg antibody) or 1.5 cc PBS(control). Three animals were in each group except the control group that consisted of six animals. Intravenous access was achieved via a left internal jugular cutdown. The animals were then kept in the dark for 6 hrs and sacrificed. Tumor and normal mucosa were harvested and divided by group into 1gm wet weight samples. Pooling of control group samples was undertaken prior to division in order to achieve control samples representing the average baseline characteristics of all tumors collected. The samples were then minced and 1.2 ml of distilled water was added to each 1 gm sample and the resulting mix homogenized mechanically (Brinkman Homogenizer Model PT 10/35). For the control (PBS) group a known amount of BPD was added to each sample prior to homogenization in order to construct a standard curve. Samples were then lyophilized overnight (Lab-Con Co., Freeze dry-3, Fisher Scientific Vancouver, Canada). The dry weight of the samples was determined after which each sample was reconstituted with 1.0 ml of reagent grade water to each 50mg dry weight of tumor and sonicated. (Bransonic 52, Branson Equipment Shelton CT). Each 1 gm wet weight sample would provide 100 - 150 mg dry weight of tumor. 100 microL aliquots of homogenized tissue were then added to

1.0 ml methylbenzonium hydroxide (1.0 M in methanol, Sigma Chemical, St. Louis Mo.) and hydrolysed at 60 °C for two hours. This step extracts the porphyrin from the tissue and monomerizes any porphyrin polymers that may be present. The samples were then cooled to room temperature and solubilized with 2.0 ml of DMSO added to each sample. The fluorescence of each sample was then assessed on a fluorometer(Perkin Elmer LS-5, Oakbrook Illinois) using an excitation wavelength of 418nm and an emission wavelength of 691nm (Figure 6). A standard curve (Figure 7, Table II) was constructed using the control samples with known amounts of BPD added while the concentrations of porphyrin in the BPD, BPD-antiEGFr and BPD-antiCEA treated animals were calculated by measuring the fluorescence at 691 nm and comparing this to the standard curve.

5. Photodynamic Therapy

The 90 animals with tumor in both cheek pouches were divided into groups receiving the following treatments; PBS(control n=15), Photofrin 10 mg/kg(n=15), BPD 2.5 mg/kg(n=15), BPD-antiEGFr conjugate, 1mg/kg,(120μg BPD/mg antibody n=15) BPD-antiCEA conjugate 1mg/kg(120μg/mg antibody n=10), antiCEA alone 1mg/kg(n=10), and antiEGFr alone 1mg/kg(n=10). Each animal received photodynamic treatment to only one cheek pouch with the other side acting as a dark control. All tumors were 6-8 mm in diameter at time of treatment.

Intravenous access was achieved via a left internal jugular cutdown under pentobarbital anesthetic. The animals were then kept in the dark for 6 hrs at which time they underwent photodynamic therapy. The exception to this was the group receiving Photofrin II which was left in the dark for 24 hrs as it has been shown that the maximum tumor to normal tissue levels for Photofrin II are achieved at around 24-48 hrs, while for BPD this is achieved between 3-6 hrs. [82,109] Animals were then treated by everting the tumor bearing cheek pouch and delivering 200 J/cm^2 of light to the tumor using a high intensity xenon arc lamp equipped with high and low band pass filters (Oriel Corp., Stratford CT, USA). The width of the wavelength band used for the Photofrin II group was between 600-630 nm while for all other groups it was 680-710 nm. Energy delivered at tumor level was measured using a surface radiometer(Gentec Model TPM-310, Gentec, Sainte-Foy, Quebec, Canada). The incident light density was 320 $milliwatt/cm^2$. The tissue being treated was kept moist using room temperature normal saline and the tissue temperature monitored with a temperature probe throughout the treatment. Temperature did not rise more than 0.5 degrees Celsius during treatment. The animals were then returned to their cages and had both cheek pouches examined at 24 and 48 hours after treatment with biopsies taken at one week and then repeat biopsies taken at sacrifice one month after treatment. Biopsies were snap frozen in OCT and underwent standard

hematoxylin and eosin staining . Slides were evaluated for the presence or absence of residual cancer.

6. STATISTICS:

Data was stored on a 386 PC using Dbase IV and statistical analysis performed using SPSS 4.0 statistical software. Non-parametric analysis of treatment differences was undertaken using Fisher's exact test, while student t tests were used for parametric analysis of difference between means. The equation of the standard curve for the porphyrin assay was generated using a "best fit" regression analysis. Significance was specified as $p < 0.05$. Values are reported as mean $\pm$ S.E.M. when appropriate.

IV. RESULTS:

1. Antibody Specificity

Multiple sections of the three squamous cell carcinomas revealed positive staining for Epidermal growth factor receptor in all sections. The pattern of staining(i.e., diffuse or focal) varied from tumor to tumor as well as between different sections of the same tumor, however was strongly positive in all cases. Sections cut from the same areas as for EGFr staining showed a complete absence of staining using the anti-CEA monoclonal as a primary antibody. Normal mucosa revealed no areas of positive staining for either EGFr or CEA.

2. BPD Assay

The standard curve generated for the tissue porphyrin assay is shown in figure 7. A linear regression model provided the "best fit" with a correlation coefficient of 0.97 with 1.0 being a perfect correspondence to the equation generated. The animals given 2.5 mg/kg of BPD showed a tumor tissue concentration of BPD of 7.8 $\pm$ 0.7 μg/g with normal mucosa of the same animals containing 5.0 $\pm$ 0.8 μg/g, a difference that was significant at p=0.046. The animals receiving the BPD-antiEGFr conjugate demonstrated a tumor tissue concentration of BPD of 6.8 $\pm$ 0.6 μg/g while normal mucosa taken from the same animals had a much lower level of BPD of 0.26 $\pm$ 0.09 μg/g (p =

0.0016)(Figure 8). No statistical difference was demonstrated between BPD and the BPD-antiEGFr conjugate in terms of the concentration of BPD delivered to the tumor although the amount of BPD given to the BPD-antiEGFr conjugate receiving animals was approximately 20 times less than the animals receiving BPD alone. BPD concentration in normal mucosa of animals receiving the BPD-antiEGFr conjugate was significantly lower than levels found in the normal mucosa of the animals receiving BPD alone ($0.26\mu g/g$ vs. 5.0 $\mu g/g$, p = 0.002). The levels of BPD in tumor and normal mucosa of animals receiving the BPD-antiCEA conjugate were below the limits of our assay and could not be distinguished from the zero $\mu g/g$ BPD controls of the standard curve.

Standard Curve Data

μg BPD Added	Fluorescence
0	5.2
0	4.5
1	22.5
1	21
2	29
2	37.6
2	28
3	49.1
3	47.7
3	36.5
4	67.5
4	69.9
4	68.7
6	77.8
6	71.7
8	94.2
8	96
10	101.7
10	107.4

TABLE II

Assay Data

Group	Fluorescence	μg BPD/g tumor
BPD/Tumor	116.8	9.88
BPD/Tumor	82.1	6.63
BPD/Tumor	89.8	7.36
BPD/Tumor	89.7	7.35
BPD/Mucosa	56	4.19
BPD/Mucosa	73	5.78
BPD/Mucosa	65.1	5.03
AntiEGFr/Tumor	81.8	6.61
AntiEGFr/Tumor	84.3	6.84
AntiEGFr/Tumor	70.6	5.56
AntiEGFr/Tumor	99.7	8.28
AntiEGFr/Mucosa	13	0.16
AntiEGFr/Mucosa	15	0.35
AntiCEA/Tumor	6	0
AntiCEA/Tumor	12	0
AntiCEA/Mucosa	8	0
AntiCEA/Mucosa	9	0

TABLE III

3. PHOTODYNAMIC THERAPY

Control animals receiving light alone had no response to treatment at 48 hours and all animals demonstrated squamous cell carcinoma at 1 week biopsy as well as at 1 month biopsy at time of sacrifice. 2 of the 15 animals required sacrifice at three weeks post treatment due to advanced disease. These 3 week results are included as 1 month results for those animals. Control animals receiving antiEGFr alone, antiCEA alone, and the nonspecific BPD-CEA conjugate uniformly had no response to treatment at 48 hrs, demonstrated squamous cell carcinoma at 1 week biopsy as well as at sacrifice 1 month post treatment. Again 2 antiEGFr animals and 1 BPD-CEA animal required sacrifice at approximately 3 weeks post treatment due to advanced disease and these results have been included as 1 month results. None of the dark controls on any animal showed any response at 48 hrs and all animals had tumor present at 1 week and 1 month.

The 15 animals undergoing PDT with Photofrin II uniformly exhibited a strong response at 48 hrs demonstrating tumor necrosis with mucosal sloughing and marked submucosal edema in the area exposed to light. At one week only necrotic tumor bed was visible, however, biopsy of this area revealed residual squamous cell carcinoma in 11 of the 15 animals. At one month 27% (4 of 15) of animals had no histologic evidence of persistent tumor.

The 15 animals undergoing PDT with BPD alone uniformly exhibited a strong response at 48 hours, demonstrating an effect similar to that of Photofrin II with tumor necrosis and marked submucosal edema in the area exposed to light. At one week only necrotic tumor bed was visible(Fig 9a,9b,9c) however biopsy of the tumor bed revealed residual squamous cell carcinoma in 5 of the 15 animals. One month biopsy demonstrated a 67% disease free rate(10 of 15).

The 15 animals undergoing PDT with the BPD-EGFr conjugate uniformly exhibited a strong response at 48 hrs with marked tumor necrosis. It appeared that there was less submucosal edema and mucosal sloughing in the area around the tumor than had been evident in either the BPD or Photofrin II treated animals. Biopsies taken at 1 week revealed no presence of squamous cell carcinoma in 12 of the 15 treated animals and these findings were confirmed at sacrifice one month after treatment.

Photodynamic therapy with BPD gave a 1 month disease free rate of 67% which was significantly higher than the 27% disease free rate achieved with Photofrin II (p= 0.03)Figure 10.   Both BPD and Photofrin II gave a better 1 month disease free rate than the light receiving control. (p < 0.0001 and p = 0.049 respectively)

Animals treated with the BPD-antiEGFr conjugate showed a 1 month disease free rate of 80% (12 of 15). This was significantly higher

than either the group treated with light alone, (p = 0.02) or the other control groups of nonspecific antibody-BPD conjugate, nonspecific antibody alone and tumor specific antiEGFr alone (p = 0.04). The BPD-EGFr conjugate treated group showed a significantly higher cure rate than did the Photofrin II group p=0.004. While on the surface the photoimmunoconjugate appeared to give a better cure rate than BPD alone (80% vs. 67%) this was not statistically significant (p=0.23).

GROUP	CANCER FREE AT 1 MONTH	CANCER AT 1 MONTH
LIGHT ALONE	0	15
PHOTOFRIN II	4	11
BPD	10	5
antiEGFR	0	10
antiCEA	0	10
antiCEA-BPD	0	10
antiEGFR-BPD	12	3

Table IV: Results of photodynamic therapy in treatment and control groups.

V. DISCUSSION:

The expression of EGFr in the hamster cheek pouch squamous cell carcinoma model has been investigated recently by Shin et al who demonstrated that normal cheek pouch mucosa did not express identifiable levels of EGFr while squamous cell carcinomas induced by DMBA expressed very high levels of EGFr [110]. This specificity for tumor and not normal mucosa makes an antiEGFr monoclonal antibody an ideal delivery vehicle for photosensitizing agents in the hamster cheek pouch model.

Immunohistochemical staining of the squamous cell carcinomas in our experiment confirmed overexpression of EGFr by SCC cells with no identifiable staining of normal mucosa.   In addition the staining confirmed the specificity of the particular antiEGFr monoclonal antibody, and nonspecificity of the antiCEA monoclonal antibody in use for the duration of the experiment.

BPD biodistribution has been investigated by Richter et al, who found that BPD, while accumulating in tumor shows no real specificity for tumor tissue, with significant concentrations found in other normal tissue [82]. The results of our tissue assay confirm this finding, showing BPD concentrations of 7.8 $\pm$ 0.7 $\mu g/g$ of tumor vs. 5.0 $\pm$ 0.8 $\mu g/g$ of BPD in normal mucosa; a tumor /tissue ratio of 1.6:1.   An ideal photosensitizing agent should localize to the tumor and not to the

surrounding tissue, a property that in theory should be conferred upon BPD by its conjugation to a tumor specific monoclonal antibody such as antiEGFr. In practice this has in fact turned out to be the case with tumor concentrations of BPD remaining relatively high (6.8 $\pm$ 0.6 μg/g) and concentrations in normal tissue being reduced, (0.26 $\pm$ 0.09 μg/g) producing a tumor/tissue ratio of BPD of approximately 26:1. This tumor specificity appears to be a function of the specificity of the antibody rather than of the BPD binding to IgG as the BPD-antiCEA non specific antibody conjugate showed no increased affinity for the tumor. This agrees with findings of Mew et al for a hematoporphyrin - monoclonal antibody conjugate used in M1 tumor bearing mice [111].

Photofrin II is the present standard for photosensitizing agents and has been used in photodynamic therapy for a variety of cancers including colorectal, bladder, esophagus and head & neck tumors, however it has had variable success [112,60,71,2]. Benzoporphyrin derivative offers theoretical advantages over Photofrin II in that its peak absorption and maximum activation is at a longer wavelength (691) nm which penetrates living tissue better than does the corresponding wavelength for Photofrin II (418nm) [79]. The wavelength (630nm) at which Photofrin II is activated in clinical use produces much less than maximal activation of the porphyrin [19]. In our experiment we have evaluated the relative effectiveness of both BPD and Photofrin II in photodynamic therapy of

squamous cell carcinoma. BPD appears a more effective agent for photodynamic therapy producing a 67% one month disease free rate versus a disease free rate of only 27% for Photofrin II. The rate of complete response obtained for Photofrin II is similar to that reported in the literature of between 10 and 40% [38,45]. There is to date little *in vivo* work with the recently developed BPD although in one series, treatment of M-1 tumors induced in DBA/2J mice produced an 83% cure rate although no histologic confirmation was obtained [78].

Several reports of monoclonal antibody-photosensitizing agent conjugates have been made in the past [113-115]. A BPD-monoclonal antibody conjugate has recently been demonstrated to be effective *in vitro* in killing human squamous cell carcinomas [97]. Our preliminary results in treating squamous cell carcinoma in a hamster cheek pouch model suggest that the BPD-monoclonal antibody conjugates are effective in cancer cell destruction *in vivo* as well.

Patrice et al have suggested a clonal selection mechanism for failure in photodynamic therapy [44]. Clonal selection would appear to be even more likely when using a monoclonal antibody-BPD conjugate and may well explain the treatment failures seen in the photoimmunoconjugate treated group.

VI. SUMMARY.

Photodynamic therapy using Photofrin for malignant disease has been used with disappointing success to date. Benzoporphyrin derivative is modification of the chlorin structure designed specifically to enhance its photoactive properties and improve the results of photodynamic therapy. We have shown that BPD is more effective at eradicating squamous cell cancer than is Photofrin in a hamster cheek pouch model.

Unfortunately BPD itself has little tumor localizing properties, as shown by our assay, where tumor levels of BPD were only 1.6 times as high as in normal tissues. We have dramatically improved the tumor localizing ability of BPD by creating a monoclonal antibody-BPD conjugate directed at a tumor specific antigen, Epidermal Growth Factor receptor. When twenty times less BPD was given as a tumor specific conjugate, tumor levels of BPD remained high while drastically reducing levels of BPD found in normal tissues. The tumor localizing properties of the antiEGFr-BPD conjugate allowed us to achieve a tumor: tissue ratio of 26:1.

This tumor specific antiEGFr-BPD conjugate when used in photodynamic therapy in the hamster cheek pouch squamous cell cancer model gave us a cure rate of 80%. This was better than the 27% cure rate achieved with Photofrin. As well, although given at one twentieth the dose of BPD, the antiEGFr-BPD conjugate gave a cure rate

statistically similar to that of BPD when given alone (67%).

VII. CONCLUSIONS.

Benzoporphyrin derivative is a more effective photosensitizing agent for photodynamic therapy than is the current standard agent Photofrin II. Tumor to normal tissue ratios of BPD can be increased by using a tumor specific monoclonal antibody-BPD conjugate which, in theory, will decrease the side effects clinically encountered with photodynamic therapy. Monoclonal antibody-BPD conjugates were shown to be at least as effective as BPD alone in eradicating squamous cell carcinoma in a hamster cheek pouch model.

VIII. BIBLIOGRAPHY.

1.	Edelson RL. Scientific American 1988; August: 68-75.

2.	Gluckman JL. Laryngoscope 1991; 101 : 36-42.

3.	Raab O. Biol 1900; 19: 524.

4.	Tappenier H, Jesionek A. Muench Med Woschsder 1903; 1: 2042.

5.	Bellin JS, Mohos SC, Oster G. Cancer Res 1961; 21: 1365-1371.

6.	Fowlks WL. J Invest Derm 1959; 32: 233-247.

7.	Auler H, Banzer G. Z Krebsforsch 1942; 53: 65-68.

8.	Figge FHJ, Welland GS, Manganielle LOJ. Proc Soc Exp Biol Med 1948; 68: 640-641.

9.	Lipson RL, Baldes EJ, Olsen EM. JNCI 1961; 26: 1-12.

10.	Kessel D, Cheng M. Photochem Photobiol 1985; 41: 277-282.

11.	Pottier R, Truscott TG. Int J Radiat Biol 1986; 50: 421-452.

12.	Bonnet R, Berenbaum MC. Adv Exp Biol Med 1983; 160: 241-250.

13.	Kessel D. Photochem Photobiol 1986; 44: 193-196.

14.	Pandey RK, Bellnier DA, Smith KM, Dougherty TJ. Photochem Photobiol 1991; 53: 65-72.

15.	MacRobert AJ, Bown SG, Phillips D. CIBA Foundation Symp 1989; 146: 4-16.

16.	Van Leengord E, Versteeg J, Vanderveen N. J Photochem Photobiol 1990; 6: 111-119.

17. Benson RC, Farrow GM, Kinsey JH, et al. Mayo Clin Proc 1982; 548-555.

18. Kluftinger AM, Davis NL, Quenville NF, Lam S, Hung J, Palcic B. Surg Oncol 1992;1:183-188.

19. van Lier JE, Spikes JD. CIBA Foundation Symp 1989; 146: 17-25.

20. Moan J, Sommer S. Cancer Res 1985; 45: 1608-1610.

21. Mitchell JB, McPhearson S, DeGraff W, et al. Cancer Res 1985; 45: 2008-2011.

22. Gomer CJ, Razum MJ. Photochem Photobiol 1984; 40: 435-39.

23. Gomer CJ, Ferrario A, Hayashi N, et al. Lasers Surg Med 1988; 8: 450-463.

24. Gomer CJ. Cancer Lett 1980; 11: 161-67.

25. Munson BR, Fiel RJ. Res Commun Chem Pathol Pharmacol 1977; 16: 175-78.

26. Munson BR. Int J Biochem 1970; 10: 957-60.

27. Wan S, Parrish JA, Anderson RR, Madden M. Photochem Photobiol 1981; 34: 679-681.

28. Bown SG, Tralau CJ, Coleridge-Smith PD, Akdemir DTJ, Wieman TJ. Br J Cancer 1986; 54: 43-52.

29. Bonnet R, Berenbaum M. CIBA Foundation Symp 1989; 146: 40-59.

30. Gomer CJ, Dougherty TJ. Cancer Res. 1979; 39: 146-151.

31. Reyftmann JP, Morliere P, Goldstein S, Santus R, Dubertren L, LaGrange D. Photochem Photobiol 1984; 40: 721-729.

32. Jori G, Reddi E, Salvato B, Pagnan A, Ziron L. Cancer Lett 1984; 24: 291-294.

33. Maziere JC, Santus R, Morliere P, et al. J Photochem Photobiol 1990; 6: 61-68.

34. Barel A, Jori G, Perin A, et al. Cancer Lett 1986; 32: 145-150.

35. Kessel D. Cancer Lett 1986; 33: 183-188.

36. Korbelik M, Krosl G, Chaplin DJ. Cancer Res 1991; 51: 2251-55.

37. Li J, Guo Z, Jin M, et al. J Photochem Photobiol 1990; 6: 149-155.

38. Dougherty TJ, Kaufman JE, Goldfarb A, et al. Cancer Res 1978; 38: 2628-2635.

39. McLear PW, Hayden RE. Am J Otolaryngol 1989; 10: 92-98.

40. Dougherty TJ, Grindley GB, Fiel et al. JNCI 1975; 55: 115-129.

41. Wilson BC, Patterson MS. Phys Med Biol 1986; 31: 327-360.

42. Elmets CA, Bowen KD. Cancer Res 1986; 46: 1608-1611.

43. Berenbaum MC, Hall GW, Hoyes AD, et al. Br J Cancer 1986; 53: 81.

44. Patrice T, Foultier T, Yaltayo S, et al. J Photochem Photobiol 1990; 6: 157-165.

45. Jin ML, Yang BQ, Zhang W, Ren P. J Photochem Photobiol 1990; 7: 87-92.

46. Nseyo UO, Dougherty TJ, Boyle D, et al. J Urol 1985; 133: 311- 315.

47. Mang TS, Dougherty TJ, Potter WR, Boyle DJ, Somer S, Moan J. Photochem Photobiol 1987; 45: 501-506.

48. Patterson MS, Wilson BC, Graff R. Photochem Photobiol 1990; 3: 343-349.

49. Grossweiner LI. Lasers Surg Med 1991; 11: 165-173.

50. Dougherty TJ, Kaufman JE, Goldfarb A, Weishaupt KR, Boyle DG, Mittleman A. Cancer Res 1978; 38: 2628-2635.

51. Manyak MJ, Russo A, Smith PD, Glatstein E. J Clin Oncol 1988; 6: 380-391.

52. Thomsen K, Schmidt H, Fisher A. Dermatologica 1979; 159: 82-86.

53. Calzavara F, Tomio L, Corti L, et al. J Photochem Photobiol 1990; 6: 167-74.

54. Dougherty TJ, Weishaupt KR, Boyle DG. In "Principles and Practice of Oncology" (VJ DeVita, S Hellman, SA Rosenberg ED) pp 2272-2279. Lippincott, Philadelphia, 1985.

55. Kessel D. Biochem Pharm 1984; 33: 1389-1393.

56. Tomio L, Calzavara F, Zorat PL, et al. In "Porphyrin Localization of Tumors" (DR Doiron, CJ Gomer ED) pp 829-841. LISS, New York, 1984.

57. Davis RK. Otolaryngol Clin North Am 1990; 23: 107-119.

58. Wile AG, Novotny J, Mason GR. Am J Clin Oncol 1984; 6: 39-63.

59. Gluckman JL, Weissler MC. Laser Med Science 1986; 1: 217-219.

60. Rosenberg SJ, Williams RD. Urol Clin North Am 1986; 13: 435-444.

61. Tsuchiya A, Obara N, Miwa M, et al. J Urol 1983; 130: 79-82.

62. Hisazumi H, Misaki T, Miyoshi N. J Urol 1983; 130: 685-687.

63. Benson RC, Kinsey JH, Cortese DA, et al. J Urol 1983; 130: 1090-1085.

64. Benson RC. Mayo Clin Proc 1986; 61: 859-61.

65. Hisazumi H, Miyoshi N, Naito K, Misaki T. J Urol 1984; 131: 884-887.

66. Bellnier DA, Prout GR, Lin CW. JNCI 1985; 74: 617-21.

67. Balchum OJ, Doiron DR, Huth OC. Lasers Surg Med 1984; 4: 13-30.

68. Hayata Y, Kato H, Amemiya R, et al. In "Porphyrin Localization and Treatment of Tumors" (DR Doiron, CJ Gomer ED) pp 747-759. LISS, New York, 1984.

69. Kato H, Konaka C, Ono J, et al. J Thorc Cardiovasc Surg 1985; 90: 420-429.

70. McCaughan JS, Williams TE, Bethel BH. Ann Thorac Surg 1985; 40: 113-120.

71. Okunaka T, Kato H, Conaka C, et al. Surg Endosc 1990; 4: 150-153.

72. Hayata Y, Kato H, Okitsu H et al. Semin Surg Oncol 1985; 1: 1-11.

73. Herrera-Ornelas L, Petrelli NS, Mittelman A, Dougherty TJ, Boyle DG. Cancer 1986; 57: 677.

74. Barr H, Brown SG, Krasner N, Bounos PB. Int J Colorect Dis 1989; 4: 15-19.

75. Kashtan H, Papa MZ, Wilson BC, Deutch AA, Stern HS. Dis Col Rectum 1991; 34: 600-605.

76. Nambisan RN, Karakousis P, Holyoke ED, Dougherty TJ. Cancer 1988; 61: 1248-52.

77. McCulloch GAJ, Forbes IJ, Lee See K, et al. In "Porphyrin Localization and Treatment of Tumors" (DR Doiron, CJ Gomer ED) pp 709-719. LISS, New York, 1984.

78. Richter AM, Waterfield E, Jain AK, et al. Br J Cancer 1991; 63: 87-93.

79. Richter AM, Kelly B, Chow DJ, Towers GHN, Dolphin D, Levy JG. JNCI 1987; 79: 1327-1332.

80. Kessel D. Photochem Photobiol 1989; 49: 579-82.

81. Allison BA, Pritchard PH, Richter AM, Levy JG. Photochem Photobiol 1990; 52: 501-507.

82. Richter AM, Cerruti Sola S, Sternberg ED, Dolphin D, Levy JG. J Photochem Photobiol 1990; 5: 231-44.

83. Richter AM, Yip S, Waterfield E, Logan PM, Slonecker CE, Levy JG. Photochem Photobiol 1991; 2: 281-286.

84. Kohler G, Milstein C. Nature 1975; 256: 495-496.

85. Houghton AN, Scheinberg DA. Semin Oncol 1986; 13: 165-79.

86. Bernstein ID, Tam MR, Nowinski RC. Science 1980; 207: 68-71.

87. Sherr CJ. Mol Biol Med 1987; 4: 1-10.

88. Downward J, Yarden Y, Mayes, et al. Nature 1984; 307: 521-527.

89. Earp HS, Austin KS, Blaisdell J. J Biol Chem 1986; 261: 4777-4780.

90. Masui H, Kawamoto T, Sato JD, et al. Cancer Res 1984; 44: 1002-1007.

91. Levin LV, Griffen TW, Childs LR, Davis S, Haagensen DE. Cancer Immunol Immunother 1987; 24: 202-206.

92. Taetle R, Honeysett JM, Houston LL. JNCI 1988; 13: 1053-1059.

93. Doerr RJ, Abdel-Nabi H, Krag D, Mitchell E. Ann Surg 1991; 214: 118-124.

94. Mew D, Wat C, Towers GHN, et al. Cancer Res 1985; 45: 4380-4386.

95. Oseroff AR, Ohuoha D, Hasan T, Bommer JC, Yarmush ML.
Proc Natl Acad Sci USA 1986; 83: 8744-48.

96. Jiang FN, Jiang S, Liu D, et al. J Immunol Methods 1990; 134:
139-149.

97. Jiang FN, Liu DJ, Neyndorff H, Chester M, Jiang S, Levy JG.
JNCI 1991; 83: 218-225.

98. Partridge M, Gullick WJ, Langdon JD, Sherriff M.
Br J Maxillofacial Surg 1988; 26: 381-389.

99. Eisbruch A, Blick M, Lee JS, Sacks PG, Gutterman J.
Cancer Res 1987; 47: 3603-3605.

100. Berger MS, Gullick WJ, Greenfield C, et al. J Path 1987; 152:
297-307.

101. Kluftinger AM, Robinson BW, Quenville NF, Finley RJ,
Davis NL. Surg Oncol 1992; 1: 97-105.

102. Toi M, Nakamura N, Mukaida H. Cancer 1990; 65: 1980-
1984.

103. Masuda H, Sugenoya A, Kobayashi S. World J Surg 1988; 12:
616-622.

104. Haley JD, Hsuan J, Waterfield MD. Oncology 1989; 4: 273-
283.

105. Carpenter G. Moll Cell Endocrin 1983; 31: 1-19.

106. Schreiber AB, Liberman TA, Lax Y, Yarden Y,
Schlessinger J. J Biol Chem 1983; 258: 846-853.

107. Gooi HC, Schlessinger J, Lax Y, et al. Bioscience Reports
1983; 3: 1045-52.

108. Straight RC, Spikes JD. Adv Exp Med Biol 1985; 193: 77-89.

109. Bugelski PJ, Porter CW, Doughterty TJ. Cancer Res 1981; 41:
4606-4612.

110. Shin DM, Gimenez IB, Lee JS, et al. Cancer Res 1990; 50: 2505-2510.

111. Mew D, Wat C, Towers GHN, Levy JG. J Immunol 1983; 130: 1473-1477.

112. Barr H, Krasner N, Boulos PB, Chatlani P, Brown SG. Br J Surg 1990; 77: 93-96.

113. Hasan T, Lin CW, Lin A. Prog Clin Biol Res 1989; 288: 471-477.

114. Hasan T. Proc SPIE 1988; 997: 42-47.

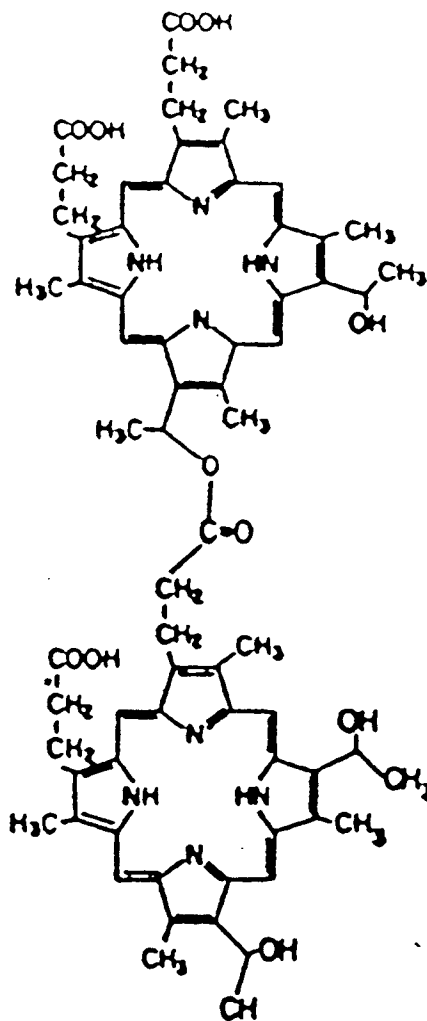115. Oseroff AR, Ara G, Ohuoha D, et al. Photochem Photobiol 1987;46: 83-96.
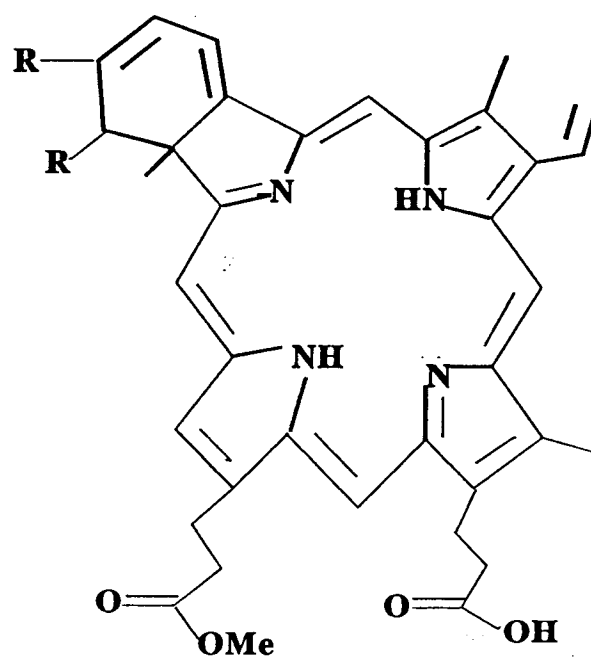
Figure 1a: Photofrin II or DHE

Figure 1b: Benzoporphyrin derivative monoacid A

Absorbence
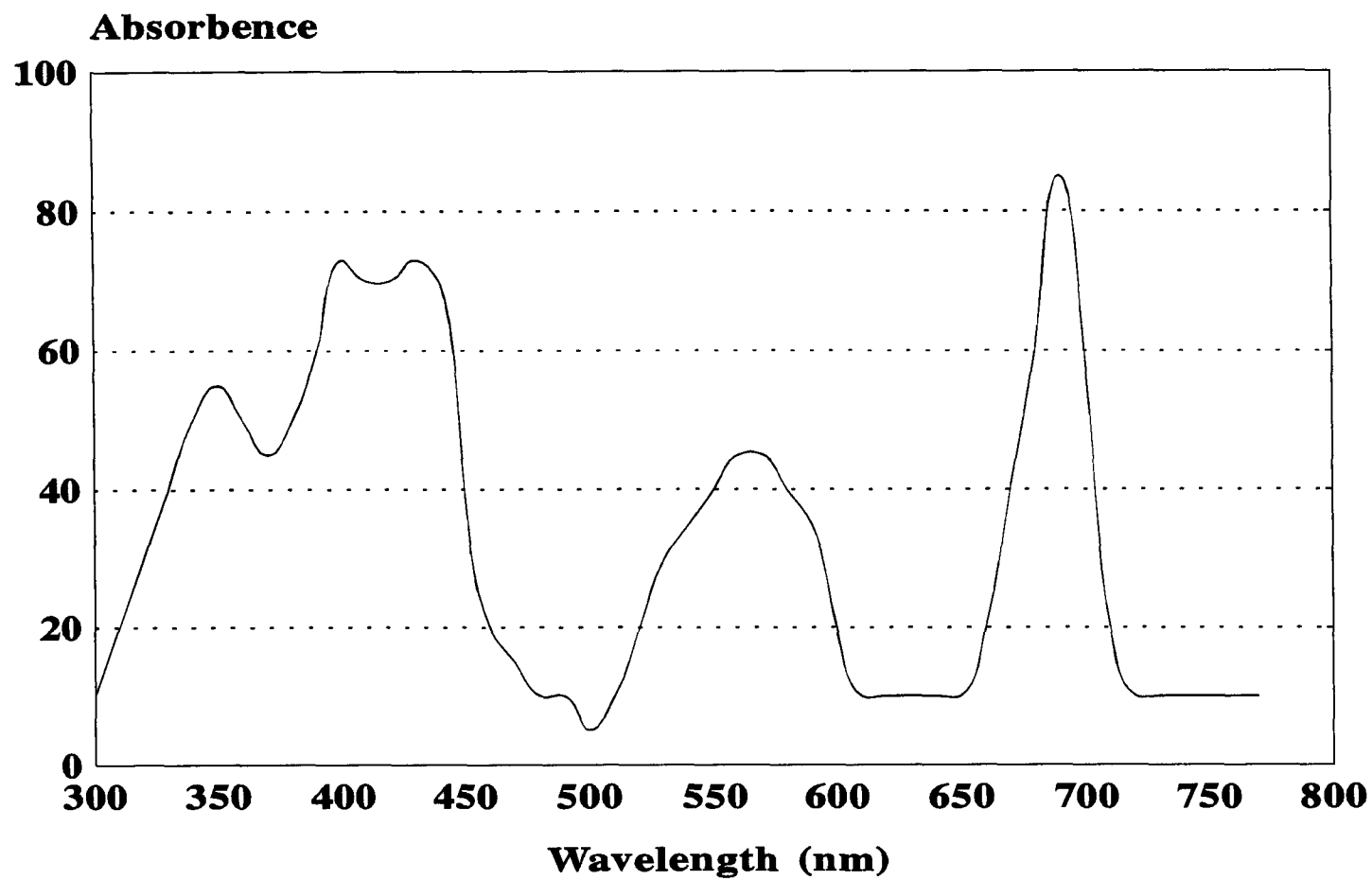

Figure 2: Absorbence spectrum of BPD
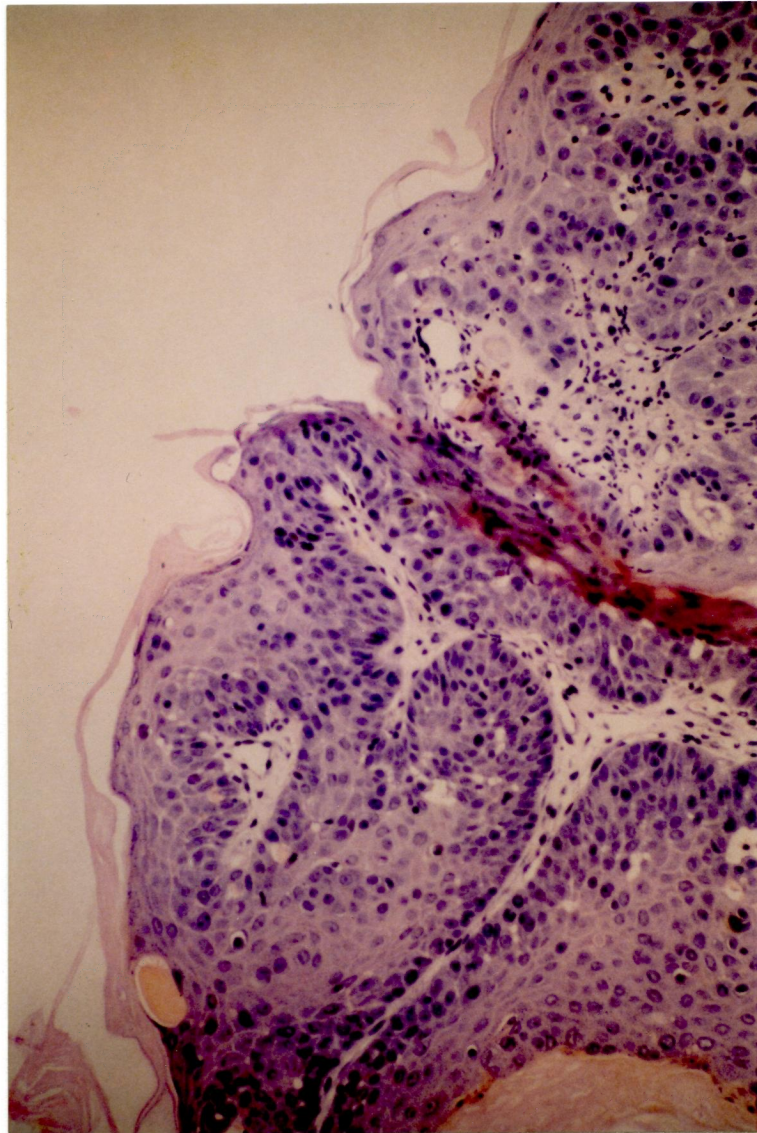
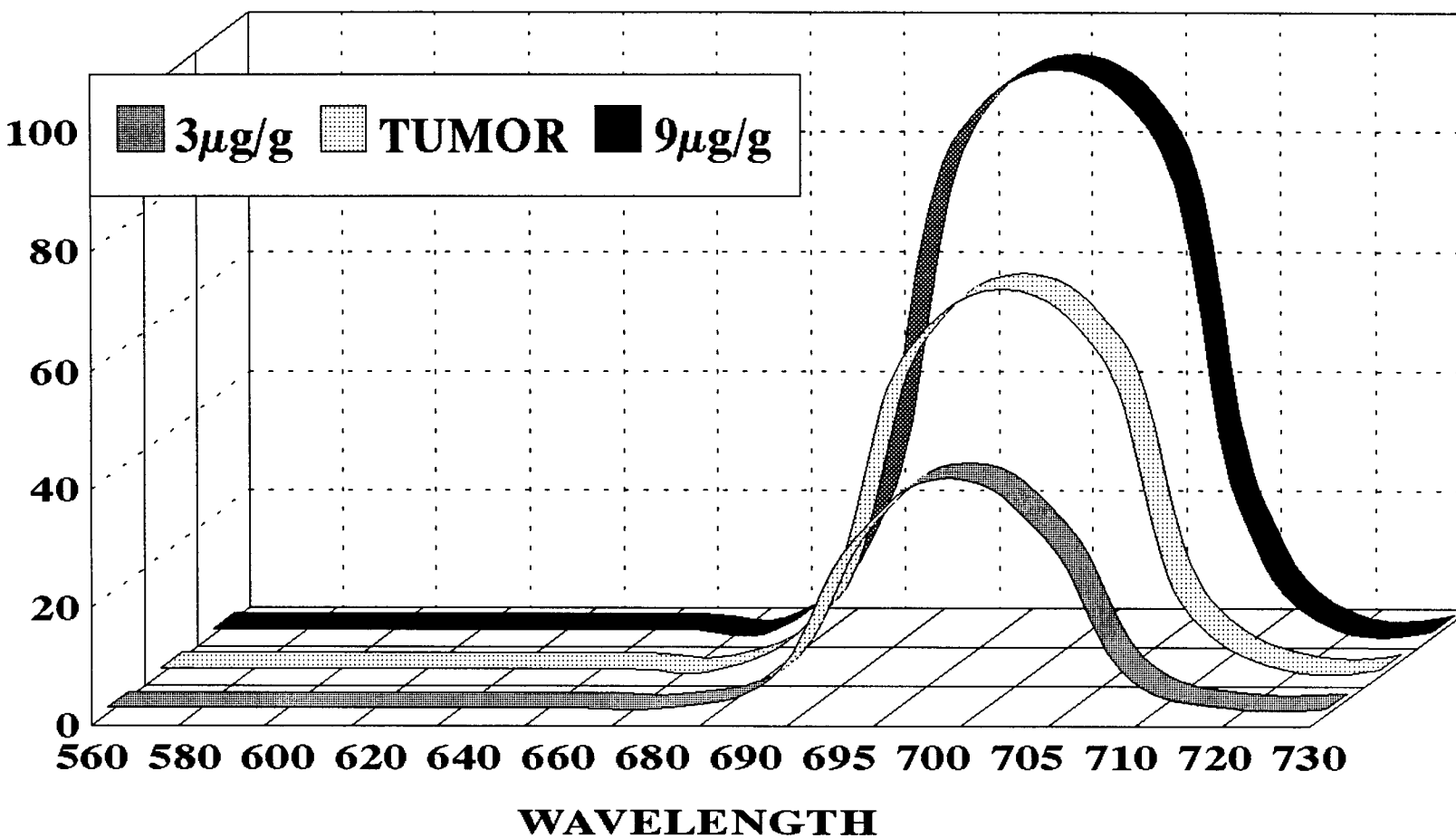**Figure 3:**
Squamous cell carcinoma arising in the cheek pouch of a hamster after being exposed to DMBA. [Hematoxylin & Eosin X 100]

FITC

PEROXIDASE

☐ = Mouse monoclonal
IgG1 to marker
(EGFr or CEA)

⬭ = antimouse IgG
labelled with
FITC or
peroxidase
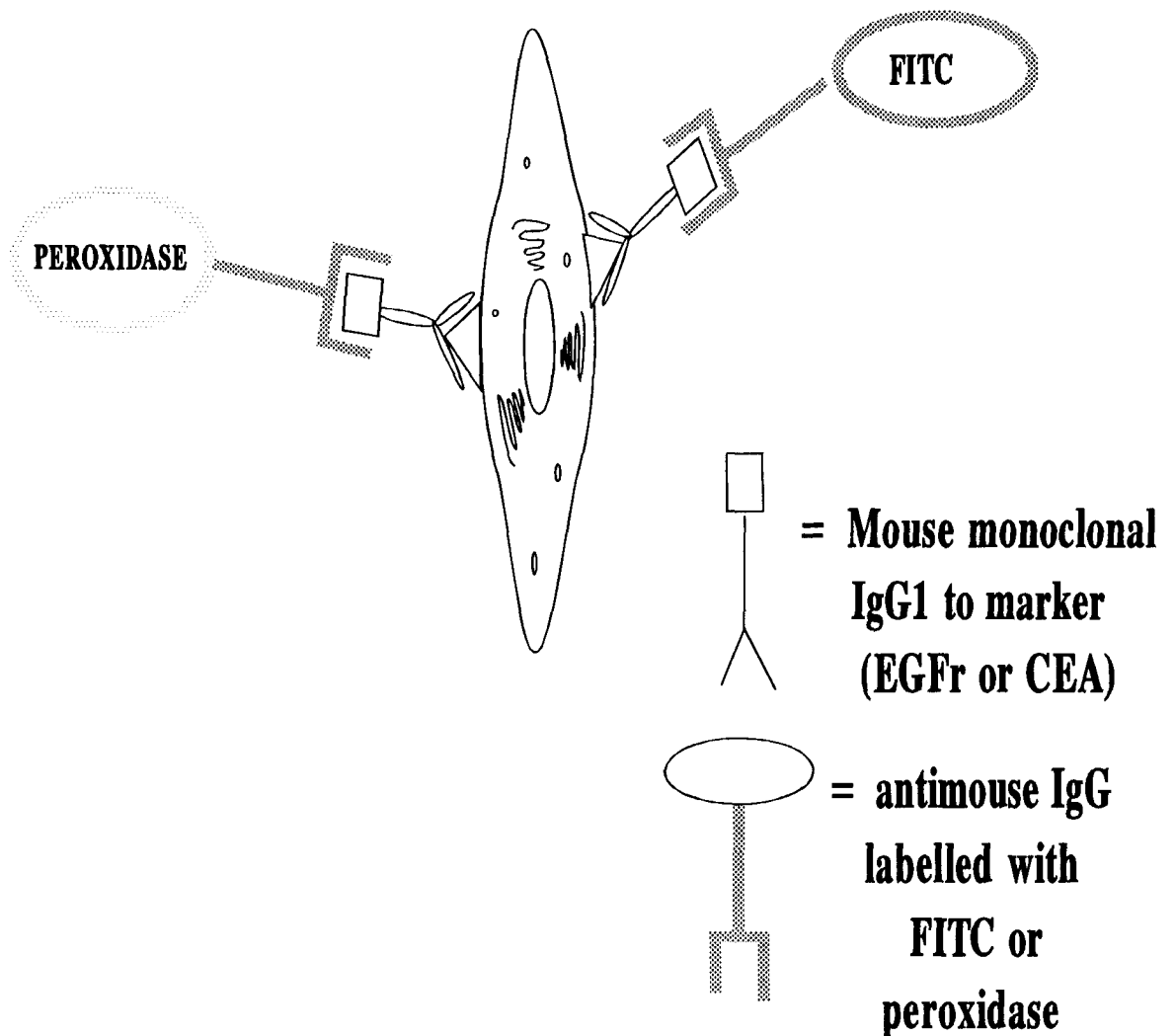
Figure 4:
Immunohistochemical method for
detecting recptor expression. A primary
mouse anti-receptor antibody is applied
after which a secondary labelled antibody
directed against mouse IgG is added.
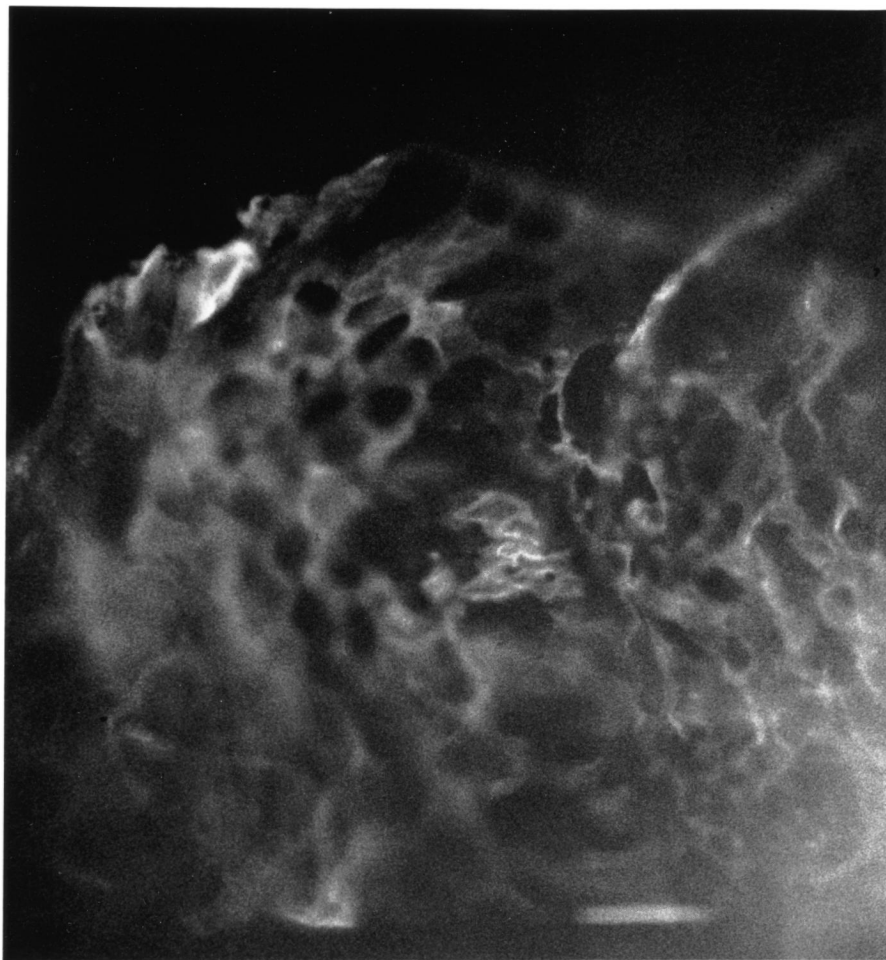
Figure 5:
EGFr expression by Hamster SCC.
Lighted areas represent binding of
flurescent antimouse IgG to primary
mouse anti-EGFr as shown in Figure 4.

FLUORESCENCE

WAVELENGTH

Figure 6:

Fluorescence peaks obtained using tumor samples containing known amounts of BPD were compared to tumor samples of animals given BPD at therapeutic dosage.

Fluorescence
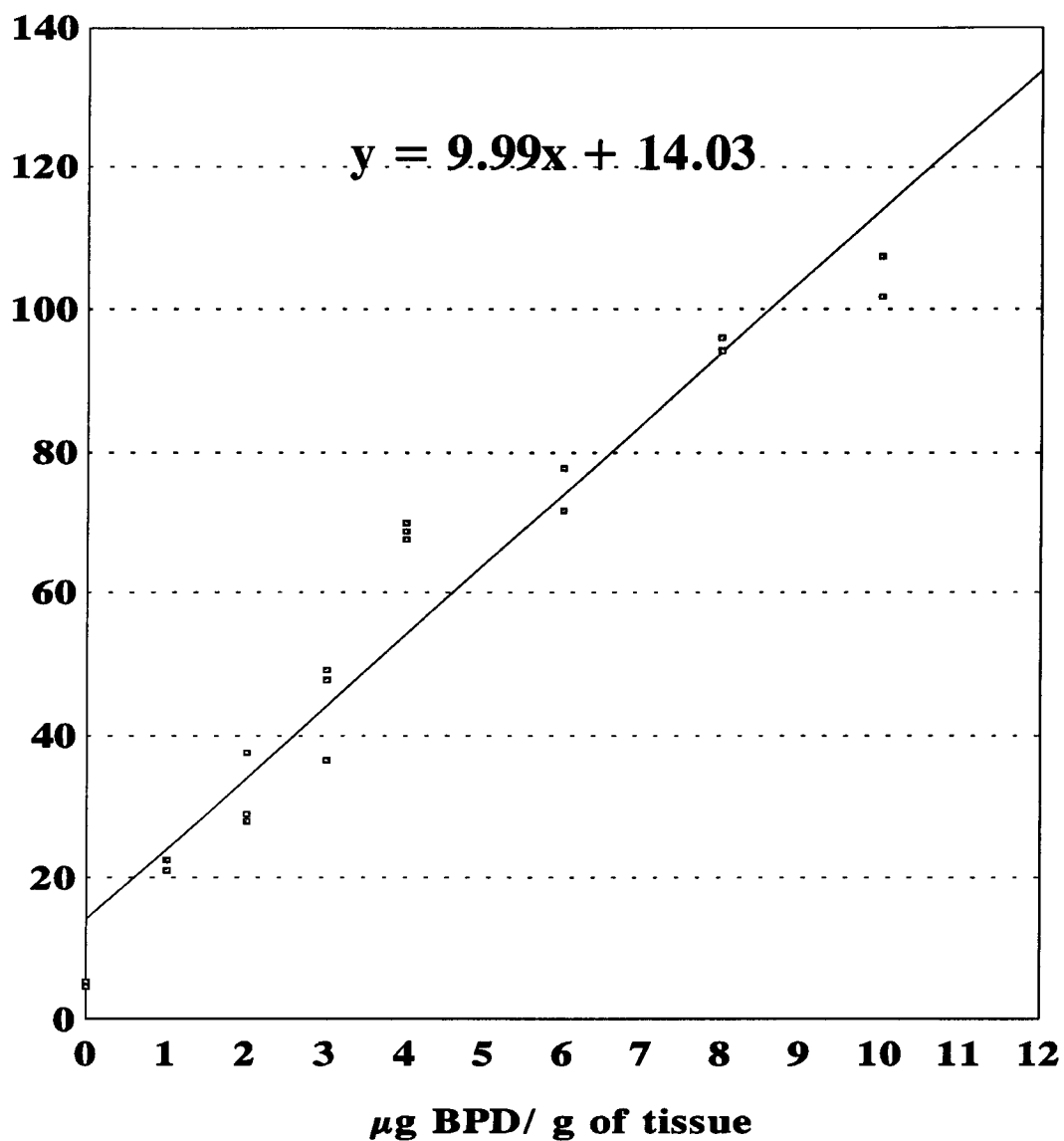
Figure 7:
Standard curve generated for the BPD assay. A linear regression model gave the "best fit" with a correlation coefficient of 0.97 for this regression line.

BPD μg/g

Legend: Tumor, Mucosa

Y-axis: 0, 2, 4, 6, 8, 10

X-axis: BPD, MoAb-BPD
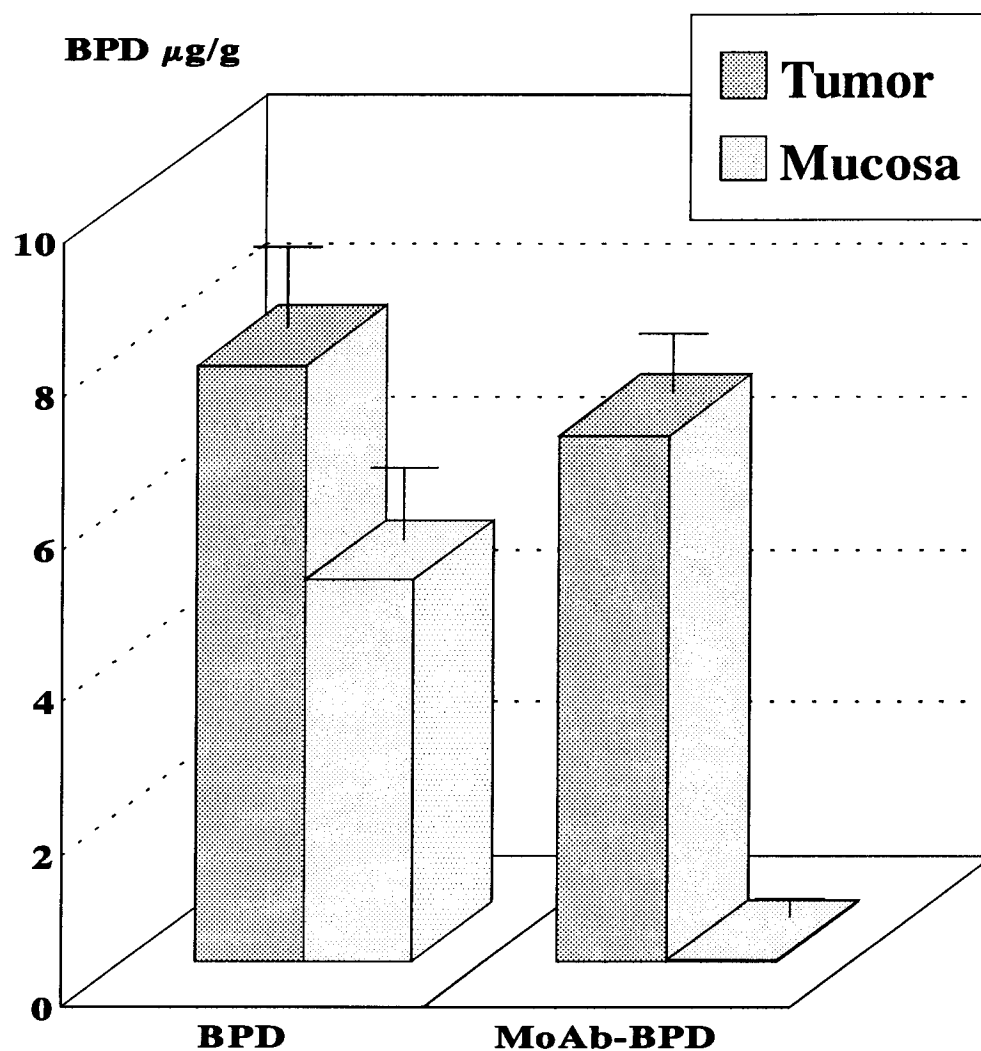
Figure 8:
BPD levels are higher in tumor than in normal mucosa when given alone. (p=0.046) When the tumor specific BPD-MoAb conjugate is given tumor levels of BPD remain high however levels of BPD in normal tissues are markedly reduced. (p=0.0016)

Figure 9a:
Squamous cell carcinoma prior to treatment
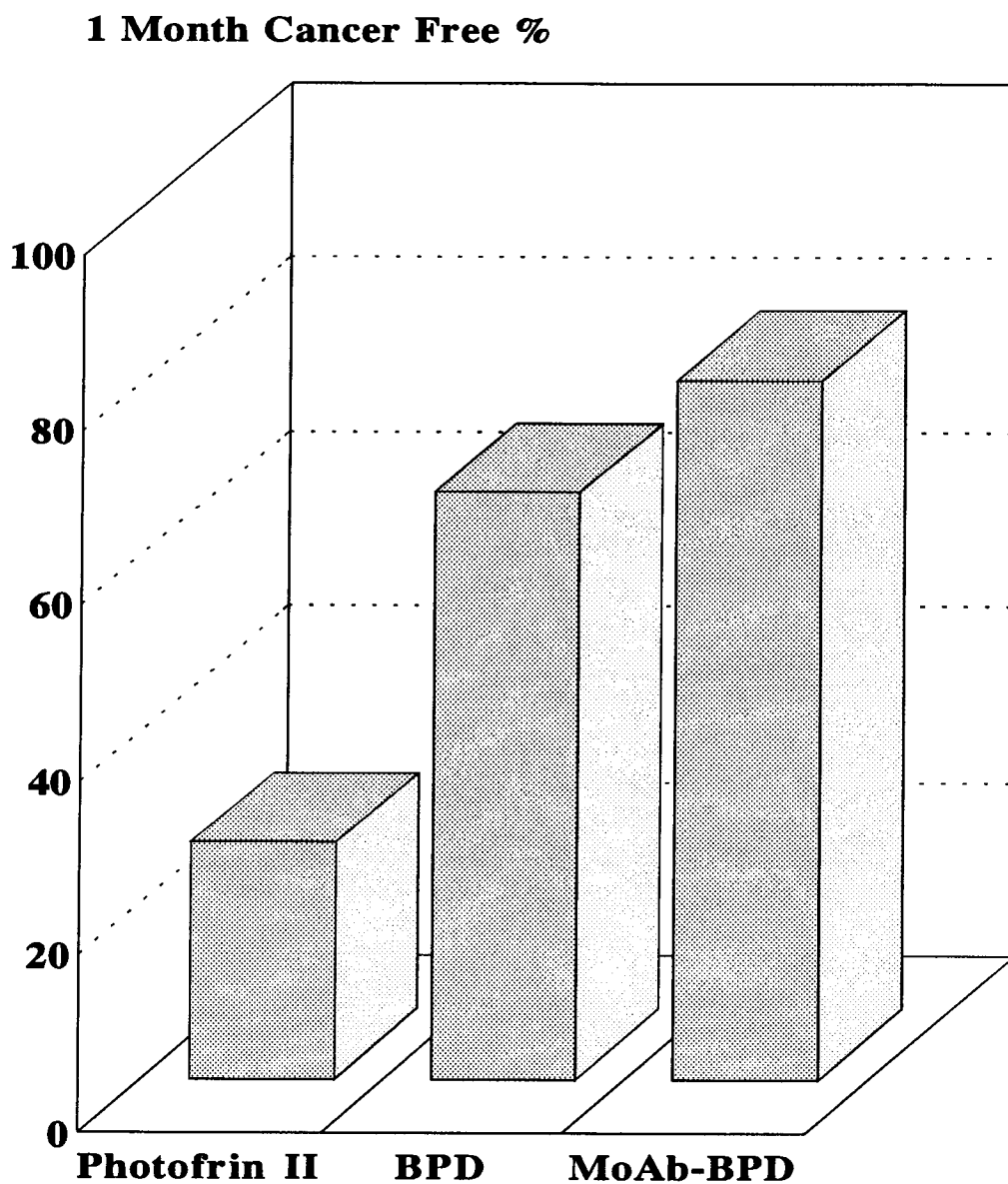**with photodynamic therapy.**

Figure 9b:
The same tumor as in Fig.9a one week post photodynamic therapy. Notice the marked tumor necrosis.

Figure 9c :
The same tumor as in Fig. 9a and 9b one
month after photodynamic therapy.
Histologic examination of the area shows
no evidence of residual tumor.

1 Month Cancer Free %

Figure 10
Animals treated with BPD or the tumor
specific BPD-EGFr conjugate showed
significantly better cancer free survival than
did those treated with Photofrin II.