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A new nanoconstruct for epidermal growth factor receptor-targeted photodynamic therapy of ovarian cancer

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Abstract

Targeted photodynamic therapy (TPDT) involves the administration of a photosensitizer (PS) conjugated with a targeting moiety followed by light activation. The systemic toxicity associated with conventional therapy may thus be significantly reduced in TPDT due to the dual selectivity provided by the spatial localization of the illumination as well as the target-localizing ability of the conjugate. Herein, a Photo-Immuno-Conjugate-Associating-Liposome (PICAL) for TPDT has been developed in which the FDA approved benzoporphyrin derivative monoacid A (BPD) and the Cetuximab antibody for epidermal growth factor receptor (EGFR) were associated into a stable Preformed Plain Liposome (PPL) by passive physical adsorption. Results have shown that the BPD molecules adsorbed into PICAL have stable optical behavior and a higher fluorescence quantum yield than free-BPD. The Cetuximab adsorbed into PPL selectively binds to cells that overexpress EGFR. The inhibition of EGFR signaling by PICAL has enhanced PDT-mediated ovarian cancer cell death.

Keywords

Targeted photodynamic therapy; EGFR; Liposome; Ovarian cancer; Drug delivery

Introduction

Ovarian cancer causes more deaths in the United States than any other type of female reproductive tract cancer, with an estimated 21,990 new cases and 15,460 deaths in 2011.¹ Ovarian cancer is a highly metastatic disease that is rarely diagnosed when disease is confined to the ovaries (stage I), where the 5-year survival rate exceeds 90%.² The majority of ovarian cancer patients are initially diagnosed with disseminated intra-abdominal disease (stages III–IV) and have a 5-year survival of less than 20%.² Although conventional therapies have made significant improvements, heterogeneity at the cellular and molecular levels together with the late stage of diagnosis strangles efforts to cure this disease. New therapeutic approaches are therefore necessary for the management of advanced and recurrent epithelial ovarian cancer. However, because of the complex nature of this disease, it is becoming increasingly evident that combination therapies using nanomedicine are the most likely to succeed. Such an approach can reduce the toxicity from the constituent modalities of the combination treatment while also enhancing the efficiency of each modality.^{3,4}

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Nanomedicine represents an innovative field with immense potential for improving cancer treatment; it has evoked enormous interest among physical and biological scientists and has already attracted hundreds of millions of dollars of research funding.⁵ The great appeal of nanomedicine lies in its promise of using the unique properties of nanoscale materials to address some of the most challenging problems of medical diagnosis and therapy.⁵ The current state of the art involves the development of targeted nanomedicines, which combine antibody engineering and nanomedicine.⁶ This has given rise to a new class of drug delivery carriers, the so-called immunoliposomes, which appear to have great potential for improved cancer treatment and have shown promising results *in vitro* and *in vivo*.⁷ Over the last few years, nanomedicine based photodynamic therapy (PDT) has emerged as a promising vehicle to improve upon targeted PDT (TPDT) by targeting epithelial and vascular growth factors⁸ and simultaneously increasing the delivered “payload” into tumor cells. The dual selectivity provided by the target localizing ability of nanomedicine and the spatial control of illumination could significantly reduce the systemic toxicity associated with classical PDT. PDT has also shown efficacy in killing ovarian cancer cells that were resistant to conventional drugs.⁹ In this *in vitro* study, we examined the efficacy of combining PDT with an antibody-based biologic treatment that induces cell cycle arrest by blocking activation of the epidermal growth factor receptor (EGFR).^{10,11}

EGFR over-expression in ovarian cancer has been associated with poor prognosis¹² and has been shown to correlate with poor survival outcomes in women with advanced staged ovarian cancers who have been treated with surgery and combination chemo-immunotherapy.¹³ The competitive inhibition of EGFR activity results in inhibition of cellular growth and division, as well as of metastasis, invasiveness, and angiogenesis.^{14,15} Cetuximab (C225) has emerged as an effective agent for treating metastatic colorectal cancer and was granted the FDA approval for this application in 2004. In 2011 the approval was expanded for use in combination with chemotherapy for the treatment of metastatic head and neck cancer. This success suggests combinations of Cetuximab in conjunction with either cytotoxic chemotherapy or radiotherapy are a promising approach for improved outcomes in patients with ovarian cancer.

PDT is a promising new modality that offers many advantages over alternative strategies: diagnostic properties, specific targeting of abnormal cells and the possibility to be combined with other therapies.¹⁶ It is a successful and clinically approved therapeutic modality used for the treatment of neoplastic as well as nonmalignant diseases.¹⁷ A phase II intra-operative PDT trial on patients with ovarian cancer showed increase in median survival without leading to significant objective complete responses.¹⁸ The lack of efficacy of PDT treatment on ovarian cancer¹⁸ as well as other malignancies¹⁷ results due to the tumor heterogeneity, the lack of tumor specificity for photosensitizer (PS) uptake, and the heterogeneity in tissue optical properties. Although a lot of recent work has focused on developing several new strategies to improve the performance of PDT agents^{19,20}, the lack of specificity still remains a challenge.

We have previously reported that benzoporphyrin-derivative monoacid ring A (BPD)-PDT combined with Cetuximab synergistically reduces tumor burden and enhances survival in a xenograft mouse model of disseminated ovarian cancer.³ Combining BPD-PDT with Cetuximab improved acute and long-term therapeutic outcomes with fewer treatment cycles and minimal toxicity.³

Portnoy et al.²¹ have described recently a new approach where a liposome-based near-infrared probe that combines both imaging and targeting abilities was developed. They found that the probes adsorbed into liposomes were more fluorescent than free probes and

have a larger quantum yield. Using confocal microscopy they found the nano-probe could specifically recognize A431 tumor cell line.²¹

Based on previous findings by our group,³ the fact that inhibition of EGFR signaling might be expected to augment PDT-mediated cancer cell killing,²² and the work of Portnoy et al.,²¹ we introduce a new construct named photo-immuno-conjugate-associating liposome (PICAL). PICAL is a targeted nanoparticle where BPD and Cetuximab are associated in preformed plain liposomes (PPL) to effectively target epithelial ovarian cancer cells. We report the fabrication and the selective cellular uptake of PICAL and its synergistic and preferential phototoxicity in an ovarian cancer cell model *in vitro*.

Methods

Preformed liposome

PPL were obtained from FormuMax Scientific Inc., Palo Alto, CA. They are composed of 18:0 PC, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol (CHOL), and 18:0 PEG2000 DSPE:1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (50:45:5 mol/mol). PPL are received in bulk solution composed of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% Sucrose, pH = 6.5 Ethanol < 7.5%.

BPD adsorption into liposome

BPD was obtained from USP Reference Standards (Rockville, MD). BPD concentration was determined using optical density measurements together with the well-characterized photophysical properties of BPD.²³

The association of BPD to the PPL was performed by addition of various quantities of BPD from stock solution in acetone to 200 μ l (8.5 mg of lipid) of PPL dispersion. The final volume of 500 μ l was achieved with de-ionized water (dH₂O). After incubation at 5°C for 24 hours under gentle stirring (200 rpm), the dispersion was filtered using a centrifugal filter (Amicon® Ultra, Cork, Ireland, 0.5 ml, 30 kDa) for 15 min at 12550 rpm. The amount of associated BPD was quantified by optical density measurements.

C225-FITC attachment to PPL (BPD)

C225 was conjugated to FITC dye using a protocol adapted from Goding's method.²⁴ (see Supplementary Materials for more details). Varying concentrations of this labeled C225 (C225-FITC) were incubated with BPD loaded PPL at 5°C for 24 hours under mild agitation (200 rpm). The incubated final volume of 500 μ l was achieved with dH₂O and HEPES (8 mM). PICAL were then separated from unincorporated C225-FITC with gel filtration by loading the preparation onto a Sepharose® CL-4B (Sigma-Aldrich) column. Fractions of ~ 0.1 ml each were collected in a 96-well plate and FITC or BPD fluorescence was measured using a plate reader. The identification of the purified PICAL containing BPD and C225-FITC was performed by comparing PICAL and free C225-FITC fluorescence peaks. The association efficiency of C225-FITC into BPD-loaded PPL was determined by comparing the integrated intensity of fluorescence spectra between associated and free C225-FITC (see Figure S2 and S3).

BPD and C225 release study

The *in vitro* release of BPD and C225 from PICAL was measured by dialysis using a Mini GeBAflex-tube (10–250 μ l, MWCO 6–8 kDa, Gene Bio-Application) and a Float-A-Lyzer G2 (1 ml, MWCO 300 kD, Spectrum Laboratories), respectively. For BPD release, an aliquot of PICAL solution (250 μ l) was placed in a tightly sealed dialysis tube. Then, the

tube was immersed in 1 ml of release medium, i.e., PBS at 4° C or culture media (RPMI-1640, 10% of FCS and 1% of penicillin/streptomycin) at 37° C under stirring (200 rpm). The release medium (200 µl) was taken at specified times and refilled with the same volume of fresh medium. The dialyzed PICAL were examined by DLS, and the results showed that the dialysis technique doesn't affect PICAL size. The cumulative release ratio of BPD was determined by fluorescence spectroscopy.

For C225 release, 500 µl of PICAL solution was placed in a tightly sealed dialysis tube. Then, the tube was immersed in 50 ml of release medium, i.e., PBS at 4° C or free phenol culture media at 37° C. PICAL solution samples (200 µl) were taken at specified times, and concentrations were quantified by fluorescence spectroscopy.

Optical measurements

All photophysical measurements were performed with freshly-prepared solutions at room temperature. Fluorescence quantum yields were measured according to literature procedures using fluorescein in 0.1 N NaOH as a standard (quantum yield $\Phi = 0.90$).²⁵ The optical instruments used are described in the Supplementary Materials.

Cell culture and incubation conditions

Human epithelial ovarian carcinoma cells (Ovar-5) were purchased from Fox Chase Cancer Center, Philadelphia, PA. Human breast carcinoma cells (CAMA-1) and human epidermoid carcinoma cells (A431) were purchased from ATCC (Rockville, MD). Cells were grown according to ATCC descriptions (see Supplementary Materials for more details).

Immunoblot and FACS Analysis

Negative and positive EGFR cell lines were first identified using western blots and flow cytometry. These were used to investigate the affinity of PICAL to recognize and target EGFR. A431 and CAMA-1 were incubated with either PPL, PICAL or drugs free media and their PEGFR expressions were quantified. Descriptions of the western blot procedure and the flow cytometry experiments are detailed in the Supplementary Materials.

Cell uptake experiments

Cellular uptake experiments were done using a method adapted from a previously published procedure.²⁶ Briefly, cells were plated in 0.5 ml of media in 24 well-plates at densities such that the cells reached 90% confluence at the end of the incubation period. After an initial period of at least 14–20 hours, which allowed for cells to attach and begin dividing, cells were incubated with PICAL by replacing the original 0.5 ml of media with the same volume of media containing PICAL. From this point onward, cells were handled under low-light conditions to avoid photosensitizing the cells or photobleaching the PS. At the end of the specific incubation time, the media containing PICAL was removed, and cells were washed twice with 1 ml of PBS. Thereafter, cells were solubilized by adding 300 µl of aqueous-based tissue solubilizer (Solvable, PerkinElmer, Waltham, MA) and incubated at 37° C for 2 hours under subdued light conditions. To quantify the BPD content, aliquots (200 µl) of the solubilized cell samples were removed, and BPD fluorescence was measured. The remaining lysed cells (25 µl) were used to perform cellular protein quantification using a BCA protein assay. BPD and protein concentrations were determined using free-BPD fluorescence and bovine serum albumin absorbance standards curves. The results were expressed as nanomole of BPD per mg of cellular protein. Fluorescence ($\lambda_{exc} = 405$ nm, $\lambda_{em} = 695$ nm) and absorbance ($\lambda_{abs} = 562$ nm) measurements were performed using a 96-well plate on a plate reader (Spectramax M5). The protein content of the cells was measured to be 173.06 ± 1.65 and 116.14 ± 2.74 µg protein/ 10^6 cells for Ovar-5 and CAMA-1, respectively.

Binding of PICAL to the EGFR was investigated using a competition assay with high concentrations of either C225 or IgG (AP112, Gt x human IgG, Chemicon, Temecula, CA) antibody. Competition experiments were conducted on Ovar-5 by pre-incubating cells with high concentrations (10 times higher than associated C225 in PICAL) of either C225 or IgG for 1 hour at 37° C under 5% CO₂. Positive control cells were incubated with antibody free-media as well. Thereafter PICAL was added and cells were incubated 90 min, washed twice with PBS and BPD, and cellular uptake quantified.

Photocytotoxicity study

Ovar-5 cells were plated in 24 multiwell plates and incubated overnight at 37° C under 5% CO₂. A few wells did not receive cells to serve as blank controls. Attached cells were incubated with 250 nmol/l of free or associated BPD (PICAL) in culture media for 90 min. The C225 concentration was set to 3 µg/ml. The cells were then rinsed twice with PBS, re-fed with 500 µl of fresh culture media, and exposed to laser light with a 690-nm fiber-coupled diode laser (Model 7401; High Power Devices, Inc.) at a fluence rate of 100 mW/cm². Dark controls were prepared in the same manner. Plates were then incubated 24 hours at 37° C under 5% CO₂. Cell survival was measured by a colorimetric method, the tetrazolium salt MTT (see the Supplementary Materials for details). Six-fold replicates were run, and experiments were repeated at least three times.

Results

Stability of PPL

The high stability of PPL in the presence of culture media at 37° C under 5% CO₂ over weeks can be thought as a first approximation to gain insight into the behavior of PPL in culture cells *in vitro*. Moreover, the PPL stability in PBS at different temperatures allows their long-term storage (Figure S1). The stability of PPL in serum is mostly yielded by polyethylene glycol (PEG) that may inhibit the adsorption of human serum proteins into the liposome. Modification of the liposome surface by grafting PEG provided steric protection.²⁷ Moreover, PPL lipid composition could also provide steric stabilization that prevents protein binding and aggregation in the presence of plasma *in vitro* as well as *in vivo*.²⁸

BPD adsorption into PPL

The PPL passively adsorbs BPD with a maximum efficiency of $86 \pm 18\%$ (Table 1). The measured hydrodynamic size of PPL (BPD) was 95 ± 2 nm. The BPD associated with the PPL increased from 2.35 to 14.2 nanomoles when the BPD in solution was increased from 2.62 to 32.45 nanomoles, however, the efficiency of associated BPD decreases from 86 to 44% respectively (Table 1).

C225-FITC association into PPL (BPD)

The PPL loaded with BPD passively adsorbs C225 with a maximum efficiency of $72 \pm 11\%$ (Table 2). Similarly, others have reported that liposomes of equimolar phospholipid and cholesterol composition adsorb 34–89% of a mouse monoclonal IgG₁ into the liposomal surface after 24 hours of incubation.²⁹

The measured hydrodynamic size of PICAL was 98 ± 2 nm, and the zeta potential was -10 mV. The associated amount of C225 into PICAL increases from 0.03 to 0.06 nanomoles when the amount of added C225 in solution increased from 0.04 to 0.36 mg. However, the efficiency of C225 association decreased from 72 to 17% respectively (Table 2).

The adsorption of BPD and C225 together into PPL has a different behavior than described above. While the C225 association efficiency decreased from 72 to 17%, the BPD association efficiency increased from 46 to 77%. These results suggest that both C225 and BPD are incorporated into the same volume of the construct where the increase in one leads to the displacement and decrease of the other: a competitive association between both compounds within the liposome.

The adsorption of C225 into liposome containing BPD increases with incubation time. While only less than 10% of the total amount of C225 is associated after 2 hours incubation, increasing the latter to 24 hours results in up to 83% of C225 association.

Furthermore, we have observed that the zwitterionic organic buffer ion HEPES increases the C225 adsorption efficiency into preformed liposomes. HEPES concentrations above 5 mM (up to 60 mM) induce an association efficiency of $73 \pm 8\%$ while between 1 and 3 mM the efficiency drops to $34 \pm 12\%$.

BPD and C225 release study

To evaluate PICAL stability during storage and to mimic *in vivo* conditions, time-dependent changes in BPD/C225 concentration were measured in different physiological media. After being incubated 20 days in PBS at 4° C, aliquots of PICAL were placed in dialysis tubing which was immersed either at 4° C in PBS or at 37° C in culture media. After 5 hours of incubation in culture media, only 5% of the loaded BPD has been released from the PICAL. This rose to 15% release after 21 hours of incubation (Figure 1A). Hence PICAL may retain a substantial proportion of the adsorbed BPD when in the blood stream under *in vivo* conditions. In PBS, a negligible amount of BPD (1.3%) was released after 21 hours of incubation (Figure 1A).

For the C225 release study, a small amount (12%) of C225 was released from PICAL after 24 hours incubation in PBS. Whereas in culture media, C225 was rapidly release from PICAL. After 2, 7 and 24 hours incubation periods, the measured amounts of C225 release from PICAL were 15, 36 and 41%, respectively (Figure 1B). This high degree of release of the C225 at 24 hours maybe advantageous in making the C225 available for biological action for a longer period after the PDT (see discussion).

BPD and C225 release profiles from PICAL (Figure 1) showed high adsorption stability in PBS at 4° C. This stability enables the storage of PICAL in PBS at 4° C over four weeks. In culture media, C225 and BPD are released more quickly than in PBS, However only 2% of the BPD and 20% of the C225 are released from PICAL after 5 hours of incubation in culture media. These results suggest that the liposome adsorption strategy could be a good approach to deliver hydrophobic and hydrophilic drugs in the blood stream without significant loss.

Optical Properties of PICAL

In culture media, the optical properties of PICAL associated BPD are markedly more stable than that of free-BPD (Figure S4). This may be beneficial for *in vitro* as well as *in vivo* applications.

The results illustrated in Figure 2 show that the same amount of BPD fluoresces 35-fold higher at 695 nm when it's associated in PICAL than when it is free in PBS. This behavior may be partially explained by favorable hydrophobic interactions between the aromatic moieties of the porphyrin systems in aqueous media.^{23,30}

The fluorescence quantum yields (Φ_F) of free and associated BPD were also determined using the comparative method. The results showed that associated BPD in PPL has a 30-fold higher quantum yield ($\Phi_F = 0.02$) than free-BPD ($\Phi_F = 0.0006$). This higher fluorescence quantum yield can be explained by the fact that the liposome has the potential to entrap a large number of BPD that is predominantly present in monomeric form.^{31,32} These results showed that the adsorption strategy into liposomes allows the BPD to be a fluorescent labels for fluorescence imaging applications and a PDT agent.

Immunoblot and FACS Analysis

Results showed that binding strength correlated with expression of EGFR with C225 monoclonal antibody. A431 cell line make the strongest binding with EGFR and CAMA-1 cells showed no affinity to EGFR which make them the best candidate in our study as a positive and negative EGFR model (Figure S5, S6).

Results confirm that CAMA-1 expresses no P-EGFR. A431 that was incubated with either PICAL, PPL or control as well as non-treated cells demonstrated an EGFR expression (Figure 3). P-EGFR expressions were observed by single bands of apparent molecular weights of 200 kDa. This finding confirmed that PICAL recognized and bound strongly to EGFR.

However, for cells incubated with PICAL, a second band appears at 300 kDa. We have confirmed this observation by repeating the same western blot experiment three times. The observed heavy band at 300 kDa may be explained by C225 antibody fragments that could make strong bonds with EGFR. Antibody fragments of $F(ab')_2$ have a molecular weight of 100 kDa, and it has been suggested that the presence of those fragments could be due to a partial cleavage of disulfide bridges in the heavy and light chains due to reducing agent exposure.³³ Moreover, the authors reported other bands having molecular weight of 70–80 kDa. This may be caused by an intermolecular disulfide bridge between Fab' fragments and light or heavy chains.³³

Cellular uptake

To evaluate the affinity of PICAL towards EGFR in live cells *in vitro*, Ovar-5 and CAMA-1 were incubated with PICAL having different associated C225 concentrations. The incubation was performed at different times at 37° C under 5% CO₂. For both cell lines (Ovar-5 and CAMA-1) the amount of BPD uptake didn't change significantly with increasing incubation time (Figure 4). The results showed that BPD uptake by Ovar-5 increased with increasing amount of associated C225 (Figure 4A). At 0.5 µg/ml of associated C225, we measured an uptake of ~ 0.05 nmol/mg of BPD. Increasing the associated C225 concentration from 2.1 to 3.5 µg/ml induced an increase of BPD uptake from 0.10–0.15 nmol/mg to ~ 0.3 nmol/mg of protein (Figure 4A). For the CAMA-1 cell line, increasing the associated C225 from 0.9 to 3.7 µg/ml, we measured similar amounts of BPD cellular uptake (~ 0.05 nmol/mg of protein) (Figure 4B).

The fact that BPD uptake increased selectively in cells that over expressed EGFR with increasing amount of associated C225 proved that PICAL recognized EGFR and that BPD is delivered into cancer cells.

To confirm these findings, competitive inhibition of C225 with PICAL on Ovar-5 was performed. Figure 5A illustrates the results. Pre-incubating cells for 1 hour with C225 (30 µg/ml = 10 times higher the associated C225 in PICAL) followed by incubation with PICAL induced a BPD cellular uptake of 0.10 nanomoles/mg of protein. However, incubating Ovar-5 with PICAL alone increased BPD cellular uptake by almost two-fold (0.2 nmoles/

mg). As a control, the same cellular uptake experiment was performed using IgG, a non-specific EGFR antibody, as a competitive inhibitor. The results showed, as expected, no competitive inhibition between IgG and PICAL (Figure 5B).

Photodynamic activity

To evaluate the PDT efficiency of PICAL on ovarian cancer cells, Ovar-5 were incubated 90 min with either C225, liposome C225 (L-(C225)), free C225, free-BPD or PICAL. These cells were then washed and irradiated with 1, 5 and 10 J/cm² of 690 nm laser light at 100 mW/cm². No dark toxicity was observed on cells incubated with either BPD or PICAL. Similarly, C225 and L-(C225) induced no cytotoxic effects (Figure 6). It seems that the inhibition of EGFR with a monoclonal antibody C225 had failed to induce any cell death which may be a resistance of Ovar-5 cells to C225. This behavior was previously reported in literature by our group using *in vitro* and *in vivo* models as well as other clinical studies.

Both BPD and PICAL-based PDT induced cell death increased in efficacy as the total light dose increased from 1 to 10 J/cm². However, PICAL demonstrated a stronger PDT effect than BPD. The recorded cell viability of BPD and PICAL-based PDT and related student's t-test at 1 J/cm² is (BPD 87 ± 21% and PICAL 56 ± 13%, P-value = 0.02), at 5 J/cm² is (BPD 76 ± 20% and PICAL 39 ± 16%, P-value = 0.008) and at 10 J/cm² is (BPD 56 ± 15% and PICAL 7 ± 4%, P-value = 0.00006) (Figure 6). When the total light dose is increased from 1 to 5 J/cm², the cell death increased by 1.5-fold. Further increasing the light dose from 5 to 10 J/cm² led to an additional 5.6-fold increase in cell death.

Discussion

The mechanism behind the adsorption of BPD molecules into PPL was studied by many investigators, and it's believed that the hydrophobic and ionic interaction could be the reason and that the modulation of the liposome structure helps to obtain the proper porphyrin localization in the phospholipid bilayer.¹⁹ In 1988, Ricchelli et al.,³⁴ published a work on the porphyrinliposome interaction where they examined the influence of the physical-chemical properties of the phospholipid. The authors showed that below the critical temperature for the phase transition of the lipids, hematoporphyrin is preferentially distributed in the outer lipid monolayer of liposomes of dipalmitoyl-phosphatidylcholine while most hematoporphyrin molecules are located in the inner monolayer in liposomes of dimyristoyl-phosphatidylcholine (DMPC).³⁴ Lavi et al., have also reported that the addition of cholesterol or a saturated phospholipid such as DMPC to the bilayer moves the porphyrin deeper into the bilayer.³⁵

The adsorption of C225 into the liposome could be explained by the interaction of hydrophobic regions of C225 and the lipid domain of the liposome,³⁶ thus leading to hydrophobic bonding between the antibody and liposome. More generally, it is assumed that hydrophobic and ionic interactions are the two principal driving forces for protein adsorption, combined with an entropy gain caused by conformational changes of the protein during adsorption.³⁷ The data on the release kinetics (Figure 1B) of the construct present interesting possibilities. The presence of 41% free C225 over 24 hours could be helpful. The initial construct with high C225 will bind to the target, deliver the BPD and PDT (with BPD) is typically conducted within the first 3 hours. The C225 released either naturally (Figure 1B) or due to the illumination will continue to act via the inhibition of the EGFR pathway thus potentially augmenting the treatment outcome.

The influence of HEPES in the hydrophobic interaction is due to the piperazine ring. In fact, the importance of this ring is recognized. Its role in achieving a good phospholipid coating and as a linking agent between a coated surface and anionic phospholipid membranes has

been studied. Indeed, in the presence of HEPES the amino groups are protonated, and the polar head groups of the phospholipids enhanced the attachment of phospholipids.³⁸ The same investigators have demonstrated in other work that the presence of buffer ions having the piperazine ring leads to efficient phospholipid coating, followed by improved protein immobilization.³⁹

Regarding the C225 effect, our results corroborate with our previous *in vivo* study of the synergistic response observed using a combination treatment³ as well as with another *in vitro* study using a conjugated system.⁴ Furthermore, our C225 results agree with other clinical studies suggesting EGFR inhibition alone in ovarian tumors that over-express EGFR is unlikely to result in clinical response.⁴⁰ The reason for the lack of the efficiency of C225 is still not clear, but some investigators have shown that nuclear EGFR contributes to acquire resistance to C225. In addition, increased nuclear EGFR localization correlates with poor clinical outcome in patients with ovarian cancer.²⁶

Our PDT results suggest that a nanotechnology based approach is a useful dual strategy for the selective destruction of cancer cells that over expressed EGFR while also exerting the receptor-blocking biological function of C225. Moreover, the PDT results are in agreement with our previous published data⁴ where BPD conjugated to PEGylated Cetuximab sensitizes EGFR-expressing Ovar-5 cells to EGF-induced phosphorylation of EGFR and downstream signaling 24 hours following PDT while C225 alone inhibited these phosphorylation events.⁴

The mechanism behind the enhanced efficiency of BPD-based PDT when combined with C225 is still unclear. Indeed the interactions between EGFR signaling and PDT are complex and may to some extent be dependent on the cell line or the PDT method used.²² Some investigators^{41,42} have suggested that EGFR activation is important for survival of carcinoma cells after PDT, and that PDT increases EGFR signaling. Others^{43,44} have found that PDT causes a temporary degradation or inactivation of cell surface receptors, including EGFRs. More recently, Edmonds et al. found that inhibition of EGFR signaling led to increased PDT cytotoxicity through an apoptosis mechanism and demonstrated that PDT stimulates the nuclear accumulation of both EGFR and STAT3.⁴⁵ The authors suggested that targeting these survival pathways is a potentially promising strategy that could be adapted for clinical trials of PDT for patients with serosal spread of malignancy. Interestingly, all of these cases converge to the fact that the inhibition of EGFR signaling might be expected to augment PDT-mediated cancer cell killing.²²

In summary, we showed in this study that the passive adsorption process is an effective technique to associate drugs and molecular moieties into a preformed plain liposome. The association of BPD into the liposome confers to BPD a high optical stability in culture media as well as a higher fluorescence quantum yield in comparison with free-BPD. Overall, our data show that the rational application of nanomedicine formulations for a combination of biologic and photochemical therapies may be efficient for the treatment of advanced and recurrent epithelial ovarian cancer and deserves further investigation *in vivo* in appropriate animal models to establish its potential utility for the treatment of ovarian cancer. Although it is difficult to project exactly what a final product might be, we speculate that it could be EGFR conjugated liposomes stored at 4°C in PBS over four weeks or more. The shelf-life could be increased by adding appropriate stabilizers to avoid desorption of BPD and C225. EGFR overexpression is associated with advanced and resistant disease, whereas PDT in experimental models is effective against chemo- and radioresistant ovarian cancer cells.³ Combination therapy could minimize or solve the major limitations of both PDT and Cetuximab therapy: the nonspecificity of the PS toward targeted tumors and C225-associated toxicities. The results and strategies presented here need to be tested in

appropriate *in vivo* models of cancer and could be envisaged to treat a variety of EGFR-overexpressing cancers,⁴⁶ such as lung⁴⁵, colorectal and prostate cancer⁴⁷ using PICAL constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

BPD (A) and C225 (B) release from PICAL in PBS at 4°C and culture media (RPMI-1640, 10% FCS and 1% of penicillin/streptomycin) at 37°C.

Figure 2.
Absorbance and fluorescence curves of BPD and PICAL in PBS.

Figure 3.

Western blot analysis of P-EGFR expression in Ovcar-5 and C225 cells. β-actin was used as the loading control. NT (non-treated cells), Ctrl (cells incubated with EGF), Lip (cells incubated with liposome and thereafter EGF) and PICAL (cells incubated with PICAL and thereafter with EGF)

Figure 4. Cellular uptake of BPD by EGFR expressing Ovar-5 cells (A) and non-EGFR expressing CAMA-1 cells (B) at different incubation times and at different associated C225-FITC concentration. The BPD concentration was set to 250 nM.

Figure 5. Competitive cellular uptake of PICAL. BPD concentration was set to 250 nM (A) C225-FITC (2.8 $\mu\text{g/ml}$) and C225 (30 $\mu\text{g/ml}$) (B) C225-FITC (1.5 $\mu\text{g/ml}$) and IgG (10 $\mu\text{g/ml}$).

Figure 6.

Viability of Ovar-5 cells after 90 min incubation with C225, liposome C225 (L-(C225)), free C225, free-BPD or PICAL. BPD and C225 concentration was set to 250 nM, and 3 $\mu\text{g}/\text{ml}$, respectively. Cells incubated with BPD or PICAL were irradiated thereafter with 1, 5 and 10 J/cm^2 of 690 nm laser light at a fluence of 100 mW/cm^2 . To evaluate the effect of light alone on cells, non-treated cells (Ctrl irr) were irradiated as well with 10 J/cm^2 .

Table 1

The amount of BPD added, associated and its association efficiency in PICAL.

BPD		
Added (nmol)	Associated (nmol)	Associated (%)
2.62 (2)	2.35 ± 1.53	85.8 ± 18
4.44 (8)	3.27 ± 0.37	73 ± 8
5.02 (6)	2.71 ± 0.39	54 ± 7
13.89 (5)	7.43 ± 1.73	54 ± 10
21.11 (3)	9.05 ± 2.19	43 ± 3
32.45 (2)	14.2 ± 0.13	44 ± 0.1

Each value is an average of (number of experiments) ± standard deviation.

Table 2

The amount of C225 added and associated in PICAL and the association efficiency of BPD and C225.

C225			BPD
Added (mg)	Associated (nmol)	Associated (%)	Associated (%)
0.04 (6)	0.029 ± 0.005	72 ± 11	46 ± 4
0.08 (4)	0.053 ± 0.011	64 ± 13	52 ± 5
0.14 (13)	0.049 ± 0.017	35 ± 12	53 ± 11
0.36 (5)	0.056 ± 0.012	17 ± 5	77 ± 5

Each value is an average of (number of experiments) ± standard deviation.