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Modeling Epidermal Growth Factor Inhibitor-Mediated Enhancement of Photodynamic Therapy Efficacy Using 3D Mesothelioma Cell Culture

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Abstract

We have demonstrated that lung sparing surgery with intraoperative photodynamic therapy (PDT) achieves remarkably extended survival for patients with malignant pleural mesothelioma (MPM). Nevertheless, most patients treated using this approach experience local recurrence, so it is essential to identify ways to enhance tumor response. We previously reported that PDT transiently activates EGFR/STAT3 in lung and ovarian cancer cells and inhibiting EGFR via erlotinib can increase PDT sensitivity. Additionally, we have seen higher EGFR expression associating with worse outcomes after Photofrin-mediated PDT for MPM, and the extensive desmoplastic reaction associated with MPM influences tumor phenotype and therapeutic response. Since extracellular matrix (ECM) proteins accrued during stroma development can alter EGF signaling within tumors, we have characterized novel 3D models of MPM to determine their response to erlotinib combined with Photofrin-PDT. Our MPM cell lines formed a range of acinar phenotypes when grown on ECM gels, recapitulating the locally invasive phenotype of MPM in pleura and endothoracic fascia. Using these models, we confirmed that EGFR inhibition increases PDT cytotoxicity. Together with emerging evidence that EGFR inhibition may improve survival of lung cancer patients through immunologic and direct cell killing mechanisms, these results suggest erlotinib-enhanced PDT may significantly improve outcomes for MPM patients.

Graphical Abstract

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Here, we report the development of a novel 3-D malignant pleural mesothelioma cell culture model using a patient-derived cell line and its use to quantify PDT dose-response (figure). The translational potential of this system is further demonstrated in evaluating efficacy of molecularly targeted therapeutics in combination with PDT.

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a cancer linked to asbestos exposure that affects the pleural lining of the lung and thoracic cavity and is characterized by locally aggressive disease progression followed later by systemic manifestations of disease. As such, the majority of patients present with locally advanced disease, and the primary morbidity for these patients arises from local disease burden (1). Patients with MPM generally experience a poor prognosis with median overall survivals ranging from 6–14 months and 3-year survival of less than 15% depending on stage at diagnosis, patient age, and histological classification (2–4). Definitive management for patients with MPM typically consists of a multidisciplinary approach involving combinations of surgical cytoreduction such as extended pleurectomy-decortication or extrapleural pneumonectomy, systemic therapies such as cytotoxic chemotherapy or immunotherapy, and adjuvant local therapies such as radiotherapy or photodynamic therapy (PDT) with real-time dosimetry (1, 5–9).

The ability of PDT to mediate cancer cell cytotoxicity over large surface areas with a relatively shallow depth of penetration makes it highly attractive as an adjuvant therapy to target microscopic disease that remains even after macroscopically complete surgical cytoreduction (10, 11). PDT is a form of phototherapy utilizing a light-sensitive compound (photosensitizer) that generates cytotoxic reactive oxygen species when exposed to a specific wavelength of light. In clinical trials of intraoperative, Photofrin-mediated pleural PDT (pPDT) combined with lung-sparing surgical cytoreduction (extended pleurectomy/

decortication, eP/D), patients with locally advanced epithelioid MPM demonstrated a median overall survival of almost 36 months (10, 12–14).

Despite adjuvant therapy, local recurrence rates remain high after combination eP/D-pPDT, and patients who experience early local recurrence (<12 months after surgery) experience a particularly poor prognosis. PDT-mediated activation of survival signaling pathways through the receptor tyrosine kinase EGFR (epidermal growth factor receptor) is a key component of cancer cell resistance to PDT-mediated cytotoxicity (15, 16). We have previously shown that PDT transiently activates EGFR/STAT3 and induces EGFR nuclear translocation in vitro for ovarian and lung cancer cells in monolayer, and that inhibiting EGFR via erlotinib can increase sensitivity of these cells to PDT-mediated apoptosis (17). Additionally, EGFR overexpression in epithelioid MPM patients is associated with a negative prognosis (18), suggesting that EGFR-inhibitor sensitized PDT might improve local control particularly in patients who experience early local relapse/poorer prognosis.

MPM cells are likely to encounter a range of ECM proteins as they grow and invade into the parietal or visceral pleura and growth in multicellular MPM spheroids is associated with apoptosis resistance (19). Indeed, growth in 3D culture can be associated with changes in EGFR expression and altered sensitivity to EGFR and HER-2 inhibitors depending on the cell line when compared to the same cells in monolayer (20). Moreover, the integrin $\alpha 5 \beta 3$, one of the tumor-ECM anchoring complexes, contributes to erlotinib resistance and cancer stem cell properties in a range of epithelial tumors (21). Here, we chose to evaluate EGFR inhibitors in 3D cell culture to include these critically important cell-cell and cell-matrix interactions and potentially improve the translational relevance of our studies. These studies detail the novel development of a 3D MPM cell culture model and its use in evaluating EGFR inhibition, to compare the response of MPM cell lines to PDT after EGFR inhibition, and to present 3D culture models of MPM to more accurately model how these cells would respond to an erlotinib and PDT combination *in vivo*.

MATERIALS AND METHODS

Cell culture

AB12, M30, OK6, EMP, EMP-GFP, EMM (EMP + human mesothelin), and REN were a kind gift of Drs. Albelda and Moon at the University of Pennsylvania and MSTO-211H was purchased from ATCC. Cells were grown in RPMI-1640 supplemented with 10% FBS and 1% Penicillin/Streptomycin (EMP, EMP-GFP, and EMM), RPMI-1640 supplemented with 10% FBS, 1% L-glutamine, 1% 10 mM HEPES, and 1% Penicillin/Streptomycin (AB12, OK6, MSTO-211H, and REN), or DMEM supplemented with 10% FBS, 1% 200 mM L-glutamine, 1% 10 mM HEPES, and 1% penicillin/streptomycin (M30).

Western blotting

Antibodies purchased were PARP (Cell Signaling 9542), actin (Sigma A-5441), pY1069 EGFR (Millipore 07–715), peroxidase-conjugated AffiniPure donkey anti-mouse secondary (Jackson Labs 715–035-151), and peroxidase-conjugated AffiniPure goat anti-rabbit secondary (Jackson Labs 111–035-144). For acinar protein collection, medium was aspirated

carefully, and the dishes were rinsed 3 times with room temperature PBS. Each 35 mm dish received 2 ml Cell Recovery solution (Corning 354253) or collagenase, and the ECM pad containing the acinar bodies was gently scraped loose from the dish. The dishes were placed on a tray on ice and rocked for 1 hour. Once the ECM digestion was complete, the samples were transferred to a 2 ml microcentrifuge tube using a wide bore pipet tip and the acini were pulse centrifuged into a loose pellet. The ECM containing medium was aspirated, and the acini were rinsed 2 times in PBS. PBS was removed, and the samples were lysed in dye-free 2X LDS lysis buffer with 200 mM DTT, sonicated, heated at 90° for 5 minutes, and syringe sheared. Protein quantitation was performed by Amido Black Assay. Samples were lysed in dye-free 2X LDS lysis buffer with 200 mM DTT, boiled at 90° for 5 minutes, and syringe sheared. Samples were loaded into a Bis-tris NuPAGE precast gel and bands were transferred onto 0.2 µm PVDF membrane and blocked in either 3–5% milk or 5% BSA solution in PBS-tween. Membranes were probed overnight at 4° and washed with PBS-tween before secondary antibody incubation. Secondaries were probed for 1 hour at room temperature with gentle rocking, and membranes were rinsed with PBS-tween before 30 second incubation in Amersham ECL solution and film exposure.

Erlotinib/Photofrin-PDT treatments

A 4 mM stock solution of erlotinib-HCl (LC Laboratories) in DMSO was diluted in media to indicated concentrations. Cells were incubated with erlotinib and/or 10 µg/ml Photofrin overnight, washed in fresh media with or without erlotinib, and then irradiated with a 632 nm laser (Ceralas) at indicated fluence rates in media with or without erlotinib.

Photofrin uptake

Cells were incubated in 10 µg/ml Photofrin-containing growth medium overnight, washed for 30 min in fresh medium and then imaged with a Zeiss Observer.Z1 Live Cell microscope using a custom photosensitizer filter cube (excitation 405; emission LP600; Chroma Technology Corporation). A constant exposure time was used for each imaging session. Images were analyzed in ImageJ (integrated density function) to show Photofrin fluorescence intensity in total nodule area. Images were optimized for figure display using the ImageJ HiLo lookup table, with equivalent brightness and contrast settings for all conditions.

Agarose 3D culture formation

Ultra low melting point agar was mixed in equal volume with 2X serum. 200 µL of this agar mix were plated in each well of a plastic, 24-well plate and allowed to incubate at 37°C to promote gelling for 15–30 mins. Cells were then seeded on top of the base agar layer at concentrations of 5,000 cells or 10,000 cells in 200 µl volumes. These were then allowed to attach at 37°C for 30 mins. Finally, an additional layer of agar was added on top of the cells in the same concentrations and volumes as the base layer.

Collagen I 3D culture formation

Rat tail collagen I (Corning) was mixed with 10X medium and reconstitution buffer (2.2 g NaHCO₃ and 4.77 g of HEPES in 100 mL of 0.05 N NaOH) to form 2.75 mg/mL collagen I.

The pH was adjusted to between 7.1 and 7.4 with NaOH and HCl and the collagen was incubated on ice for 3–5 minutes then centrifuged at 4°C to remove bubbles. 100 µL of the collagen was spread around glass-bottom dishes (MatTek), then incubated at 37°C for 25 min. Cells were seeded at 15,000 cell/mL with a total volume of 200 µL on top of this basement membrane. Cells were allowed to incubate at 37°C for 30 min to promote adherence. The wells were then quenched with additional media. Growth and acini formation were monitored daily via microscopy.

Agarose/collagen I combination

2% agarose and 2 mg/mL collagen were mixed in equal portions giving 1% agarose and ~1.00 mg/mL collagen. Similar materials and procedure were followed as in the collagen basement membrane with a plastic, 24-well plate in which glass coverslips were placed. Growth and acini formation were monitored daily via microscopy.

Hanging drop

Cell suspensions were placed on the underside of a petri dish lid in small volume drops and inverted. Here, cells were used at four different concentrations—2,000, 6,000, 10,000, and 30,000 cells per droplet. Growth and acini formation were monitored via microscopy.

Matrigel basement membrane

Growth factor reduced (GFR) Matrigel (Corning) was thawed on ice at 4°C overnight. Using pre-chilled pipet tips and MatTek glass bottom dishes, the coverslips were coated with various volumes of Matrigel based on area. In experiments with 50% Matrigel (Figure 1C), dilutions were made in cold PBS. The dishes were then allowed to incubate at 37°C for 30 min to promote gelation. After gelation, two different concentrations of cells were plated on top of the Matrigel basement membrane – 500 µL at 15,000 cell/mL or 200 µL at the same concentration. These were then allowed to incubate at 37°C to allow cell attachment. Media volume was brought up to 2 ml using 2% supplemented GFR Matrigel and replaced with fresh media twice/week. Growth and acini formation were monitored via microscopy or IncuCyte System (Essen BioScience) imaging.

Live-dead acinar staining

Acinar dishes were incubated with 2 ml serum-free medium containing 2 µM calcein AM (Life Technologies C3100MP) and 4 µM ethidium homodimer (Fisher E1169) for 40 minutes in the dark at 37°. At the end of the incubation, staining medium was removed and fresh serum-free medium was added. Dishes were imaged on a Zeiss Observer.Z1 Live Cell microscope using the same exposure settings for all conditions, and images were optimized for figure display using the ImageJ HiLo lookup table, with equivalent brightness and contrast settings for all conditions.

Flow cytometry apoptosis analysis

EMP cells were grown on Matrigel for 1 week with 2% Matrigel RPMI. Cells were incubated overnight in media containing 8 µM erlotinib and/or 10 µg/ml Photofrin, washed for 30 min in fresh media containing erlotinib, then exposed to light via a Ceralas 632 nm

laser. 24 hours after PDT, cells were incubated with Dispase for 30 min to dissolve the Matrigel, and Versene and trypsin were used to create a single-cell suspension. Cells were fixed in cold 70% ethanol, and a buffer containing PBS, 0.1% triton, 0.1% sodium citrate, 100 µg/ml RNase A, and 50 µg/ml propidium iodide was added 30 min before flow cytometry. Data was analyzed in FlowJo.

Statistical analysis

The Shapiro-Wilk normality test was used to determine distribution of data. Two-tailed Student's t test was used to analyze normally distributed data, and Wilcoxon-Mann-Whitney Rank Sum tests were used to analyze non-normal data. Results were considered significant if $p < 0.05$ (*), and $p < 0.01$ (**), < 0.005 (***) or < 0.001 (****) and are additionally labeled in figures. Error bars indicate standard deviation in all figures.

RESULTS

Development of 3D MPM models

Generally, cancer cells grown in 3D with the corresponding increases in cell-cell and cell-extracellular matrix contacts develop resistance to drug-induced apoptosis. As discussed above, EGFR pathway expression, activation, and response to inhibitors differ when cancer cells are grown in contact with ECM proteins compared to monolayer cultures. Additionally, since MPM *in vivo* develops into a 3D mass known to generate aggregates in pleural fluid, unlike the normal layer of pleural mesothelium, a 3D model of this malignancy is particularly relevant (22). Therefore, we first tested and characterized a number of methods for growing MPM cell lines in 3D culture conditions. Among potential 3D models of MPM tumors, we tested agarose-embedded, agarose-collagen I combinations, and agarose overlay preceded by hanging-drop. The results found that cells either proliferated poorly (agarose) or were too mechanically unstable and inconsistent for further experimental manipulation (hanging-drop). Consequently, the method of collagen basement membrane was attempted. Rat tail type I collagen was diluted to a desired concentration in glass-bottom dishes, and MPM cells were then seeded on top of the collagen basement membrane. These cells did spontaneously form acini in REN and EMP with sufficient acinar growth around day 7, but many of the cell lines tested did not proliferate well in these conditions (data not shown).

Based on previously published methods for culturing breast, ovarian, and pancreatic cancer cell lines in 3D conditions (23–26), we next evaluated GFR-Matrigel, finding it to create an effective and consistent pattern of cell growth for most of the MPM cell lines tested. Respective to area of coverslip/glass-bottom dish, specific volumes of Matrigel were plated onto the bottom of the dish while maintaining cold temperatures to prevent its premature gelling. This technique proved to be the most effective, with consistent acinar proliferation displayed in REN and EMP epithelioid MPM cell lines around day 7, and other cell lines exhibiting a range of phenotypes (Figure 1A and B). The mouse cell line AB12, for example, grew with an invasive and branching pattern consistent with its biphasic MPM subtype encompassing mostly sarcomatoid cells mixed with a smaller proportion of epithelial cells (27, 28). Promisingly, the human biphasic MPM cell line MSTO-211H

maintained its histological phenotype in these in vitro conditions as well (29). Neither OK6 nor M30 appeared to proliferate well in these conditions.

In order to determine effects on EGFR activation after Photofrin-PDT in these 3D MPM models, we grew EMP cells on various combinations of Matrigel and collagen I ECM, treated them with 4 J/cm² PDT, and evaluated their EGFR phosphorylation status 24 hours after treatment (Figure 1C). In all of the observed ECM conditions, PDT increased pY1069-EGFR (right panel). This suggests that matrix composition does not affect PDT-mediated EGFR activation.

After plating EMP cells on Matrigel, we used IncuCyte imaging to track their proliferation potential and any phenotypic changes over time. As shown in Figure 2A, these cells reliably form round nodules, approaching their maximum size after approximately two weeks of growth. Additionally, we were able to use IncuCyte imaging to track and analyze changes in GFP-transfected EMP cells in response to therapeutic interventions. Here, we see that as the cells begin to die and nodules deteriorate a few hours after PDT, we can also measure the GFP loss (Figure 2B). Loss of GFP has previously been shown to correlate with standard apoptotic markers like annexin/propidium iodide (30, 31), and GFP has been used as a metric for viable cell number in other 3D culture contexts (32), so this process has many potential uses for real-time monitoring of treatment response in 3D nodules grown on a variety of basement membrane products. From these results, we established that 24–48h after PDT would be the most appropriate time interval to observe the effects of EGFR inhibition on PDT-mediated cytotoxicity.

EGFR inhibition and Photofrin-PDT combination in 3D MPM cell culture

Using this 3D MPM model, we next evaluated whether EGFR inhibition improves PDT-mediated cytotoxicity. EMP cells were grown on Matrigel for 10 days, incubated overnight in erlotinib and Photofrin, and then illuminated the next day. 24 hours after PDT, we imaged the nodules for calcein fluorescence and ethidium homodimer-stained nuclei. Live-dead staining of EMP acini show a clear increase in the amount of intercalated ethidium homodimer after erlotinib and PDT compared to PDT alone (Figure 3A). This is quantified in Figure 3C by counts of ethidium homodimer foci in each EMP nodule. Here, we also see a slight (though significant) increase in ethidium homodimer staining in erlotinib-treated nodules compared to untreated. These results are also corroborated via western blotting for PARP cleavage, where we see cleavage at 4 J/cm² Photofrin-PDT with erlotinib, but no signs of apoptosis at the same dose without erlotinib (Figure 3B). With these methods of visualizing cell death, we do not see any toxicity in 8 μ m erlotinib-alone conditions.

To confirm an increase in the fraction of apoptotic cells after erlotinib-PDT compared to PDT alone, we processed the treated EMP nodules into ethanol-fixed/permeabilized single cells for flow cytometric analysis of nuclear DNA fragmentation via propidium iodide staining. Apoptotic cells have fragmented DNA and therefore less propidium iodide fluorescence than cells in the G1 peak (Figure 4) (33). The percentage of sub-G1 cells shown in Figure 4A (normalized to the PI fluorescence mode) and Figure 4B (total cell counts) one day after PDT is highest for EMP treated with erlotinib and PDT compared to any other condition, although effects of this combination treatment (27% sub-G1) are only

slightly more than additive compared to the effects of erlotinib (7.0%) and PDT (15.8%) as single agents based on this metric of viability evaluation.

Erlotinib potentiates PDT efficacy without altering Photofrin uptake

We have previously demonstrated that erlotinib sensitizes lung and ovarian cancer cells to BPD-mediated PDT without altering intracellular levels of BPD (17). Nevertheless, small molecule kinase inhibitors like erlotinib have a theoretical potential to increase photosensitizer levels (34). To establish whether erlotinib affects Photofrin uptake in our 3D MPM model system, we compared Photofrin uptake in EMP acini in Matrigel overlays with or without simultaneous erlotinib incubation. Based on analysis of Photofrin fluorescence intensity in imaged acini, erlotinib doses used in this study do not alter photosensitizer levels in a 3D model of MPM (Figure 5), similar to previous data in lung and ovarian cancer cells grown in monolayer (17).

DISCUSSION

Taken together, these results demonstrate that EGFR inhibition sensitizes MPM cells to PDT-mediated apoptosis even with the addition of cell-cell and cell-matrix interactions, suggesting that this may be a potentially effective way to improve outcomes in patients undergoing eP/D-pPDT. After attempting multiple potential methods of 3D culture for these MPM cell lines, we determined that Matrigel overlay was most effective and relevant to the defined experimental questions, and found that MPM cells grow similarly to breast, ovarian, and pancreatic tumor cell lines in Matrigel (23–26). If larger 3D cultures are desired, Broadus et al. (22) describe an effective method for the formation of non-adherent MPM spheroids that is likely more effective than the hanging-drop procedure used here. Compared to monolayer cultures, when MPM cells are grown as non-adherent spheroids in the absence of artificial ECM, they upregulate genes associated with the immune response and lymphocyte/cytokine stimulation while downregulating apoptosis-related genes (35). Survival signaling (including EGFR) and invasive behavior of cancer cells in the presence of ECM basement membrane also differs significantly from monolayer (36, 37) and is often altered depending on the biochemical and biophysical properties of the ECM in contact with cells (38, 25). Here we found that, regardless of the biophysical composition of the ECM, EMP mesothelioma cells had increased phospho-EGFR after PDT (Figure 1C), at a later timeframe than we had previously observed in monolayer (17). Thus, our findings in 3D MPM culture validate and extend our previous observation that EGFR inhibition using erlotinib sensitizes cells to PDT-mediated toxicity in lung and ovarian cancer monolayer culture, supporting the translational relevance of our findings.

Despite the high expression of EGFR in MPM (18), EGFR-TKIs like erlotinib have not been effective single-agent therapies in clinical trials (39, 40). This resistance may be due to the lack of tyrosine kinase domain *EGFR* mutations, since these mutations are predictive of response in lung cancer patients (41–43). Regardless of single-agent response to small molecule inhibitors or receptor-targeting antibodies against EGFR, they have been shown to increase PDT efficacy in vitro and in vivo (15, 17, 44, 45). One potential mechanism for this increase is due to the ability of EGFR inhibitors like erlotinib to decrease the ability of ATP-

binding cassettes to efflux drugs (46), since porphyrin photosensitizers are substrates for these transporters (34, 47, 48). Although our previous in vitro monolayer work showed no difference in photosensitizer concentration after erlotinib treatment (17), we have also seen slight increases in photosensitizer uptake after a three-day course of erlotinib in a murine model (44). In the 3D model presented here, without the influence of vasculature, we found no difference in Photofrin uptake, suggesting that decreases in viability after erlotinib/PDT are due to erlotinib's influence on survival signaling. EGFR can translocate to the nucleus after PDT along with STAT3, protecting the cells from apoptosis, and pretreatment with erlotinib is likely to prevent the occurrence of some of this post-PDT survival signaling (17).

In addition to direct effects of EGFR inhibition on PDT induced cytotoxicity, EGFR-TKIs may influence the anti-tumor immune response. For example, the EGFR inhibitors erlotinib and gefitinib amplify lung cancer susceptibility to natural killer cells via increasing expression of the NKG2D ligand ULBP1 on the tumor cells (49). PDT is also able to up-regulate NKG2D ligands on gastric and lung tumor cell lines (50) and in murine colon cancer in vivo (51), so the combination of these two treatments may further stimulate the innate immune response. The utility of an EGFR-TKI in this case would not be dependent on the *EGFR* mutational status, so would be applicable to *EGFR* wild-type MPM. Since we were able to successfully extract MPM cells from the Matrigel matrix for flow cytometry (Figure 4), we also plan to confirm similar changes in immunogenicity in this model.

Overall, we have developed and described a novel method of 3D cell culture for MPM that retains some ancestral phenotypic characteristics less obvious in monolayer; i.e., histological MPM subtype. Using this model, we found that erlotinib increases direct cytotoxicity of Photofrin-mediated PDT, without altering Photofrin uptake. These results lend support to the potential of receptor tyrosine kinase inhibitors like erlotinib to improve PDT response clinically.

Additionally, as part of this process, we were able to show that this model is appropriate for a wide range of further analysis, including flow cytometry. We plan, for example, to observe changes in protein expression (such as mesothelin and markers of immunogenicity) when MPM cells are grown in contact with different substrates (Matrigel or collagen I), as this may mimic the potential changes in ECM microenvironment that MPM cells experience as they invade through the pleura.

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Figure 1.

Growth phenotypes of MPM cell lines in ECM overlay. **(A)** Various mesothelioma cell lines were grown for up to 10 days on Matrigel beds with regular phase-contrast imaging. Some grew as round acini, others with a branching morphology, and M30 and OK6 did not proliferate in these conditions **(B)** Images of each MPM cell line at approximately 7 days. Scale bars are 100 μ m. **(C)** EMP cells were grown on combinations of Matrigel and collagen I for 10 days then treated with Photofrin-PDT (right panel). After 24 hours, Western blotting was performed for pY1069-EGFR and actin.

Figure 2.

(A) IncuCyte timelapse images for 3D EMP Matrigel acini, from 5 days after plating (left) to 20 days after plating (right). Scale bars are 300 μm . (B) IncuCyte timelapse images of EMP-GFP grown for 10 days before beginning timelapse imaging. These were then exposed to Photofrin (designated Day 1 after beginning timelapse; left images), illuminated for PDT (designated Day 2) and regularly imaged until Day 4 (right images). Loss of GFP in EMP acini correlates with loss of viability, as quantified on the bottom panel.

Figure 3. Erlotinib sensitizes EMP 3D cultures to Photofrin-PDT.

(A) Live-dead staining of EMP acini 24 hours (top images) and 48 hours (bottom images) after 4 J/cm² Photofrin-PDT, with calcein fluorescence indicating viable cells and ethidium homodimer intercalating into DNA of dead cells. These are 2×2 stitched 10x images with 200 μm scale bars. (B) Western blot for PARP in EMP acini after PDT. (C) Quantification of dead cells (mean ethidium homodimer foci per nodule) at 24 and 48 hours after PDT (+/- 8 μM erlotinib) from sets of above images (50 acini per condition). Error bars show standard deviation and indicated p-values are * p=0.011, *** p=0.002, and **** p<0.001.

Figure 4.

Erlotinib increases the sub-G₁ population of PDT-treated nodules. EMP cells grown on Matrigel were processed for flow cytometry 24 hours after PDT. After gating out doublets and cellular debris, DNA fluorescence is shown in a logarithmic scale. **(A)** Propidium iodide-stained nuclei peak in fluorescence at G₁ and are normalized to the mode. The apoptotic cell population containing fragmented DNA is designated as sub-G₁. **(B)** For the sub-G₁ population only, the number of cells in each condition is shown. Here, untreated cells are 1.19% sub-G₁, erlotinib are 6.99%, PDT are 15.8%, and erlotinib+PDT are 27.0%.

Figure 5. Erlotinib does not alter photosensitizer levels. **(A)** Photofrin levels in EMP acini grown on Matrigel beds with or without erlotinib after overnight incubation with 10 ng/ml Photofrin and 8 μM erlotinib. These are 2 $\times$ 2 stitched 10x images with 200 μm scale bars. **(B)** ImageJ quantification of total fluorescence intensity (integrated density) of Photofrin averaged over 12 stitched images for each condition. Error bars show standard deviation.