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Cell-type Selective Phototoxicity Achieved with Chlorophyll-a Derived Photosensitizers in a Co-culture System of Primary Human Tumor and Normal Lung Cells

Erin C. Tracy¹, Mary J. Bowman¹, Ravindra K. Pandey², Barbara W. Henderson², and Heinz Baumann^{1,*}

¹Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263

²Department of Cell Stress Biology/PDT Center, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263

Abstract

The ATP-dependent transporter ABCG2 exports certain photosensitizers (PS) from cells, implying that the enhanced expression of ABCG2 by cancer cells may confer resistance to photodynamic therapy (PDT) mediated by those PS. In 35 patient-derived primary cultures of lung epithelial and stromal cells, PS with different subcellular localization and affinity for ABCG2 displayed cell-type specific retention both independent and dependent on ABCG2. In the majority of cases, the ABCG2 substrate 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) was lost from fibroblastic cells more rapidly than from their epithelial counterparts, even in the absence of detectable ABCG2 expression, facilitating selective eradication by PDT of epithelial over fibroblastic cells in tumor/stroma co-cultures. Pairwise comparison of normal and transformed epithelial cells also identified tumor cells with elevated or reduced retention of HPPH, depending on ABCG2. Enhanced ABCG2 expression led to the selective PDT survival of tumor cells in tumor/stroma co-cultures. This survival pattern was reversible through HPPH derivatives that are not ABCG2 substrates or the ABCG2 inhibitor imatinib mesylate. PS retention, not differences in subcellular distribution or cell signaling responses, was determining cell type selective death by PDT. These data suggest that up-front knowledge of tumor characteristics, specifically ABCG2 status, could be helpful in individualized PDT treatment design.

INTRODUCTION

Selective tumor destruction is the ultimate goal of all cancer therapies, including photodynamic therapy (PDT). A relatively narrow window of selectivity is achieved in PDT through at least two mechanisms (1,2): first, moderately increased accumulation of the photosensitizer (PS) in tumor tissue over surrounding normal tissue, observed in animal tumor models (2–5) and to some extent in patients (6); second, locally delivered light. The PS differential is believed to be at least in part due to tumor physiology, such as leaky vasculature (7). Proof of actual tumor cell selectivity in studies on established cell lines has been elusive for PSs other than aminolevulinic acid (ALA) and its derivatives (8). Most pertinent to this study, Perry et al. (9) found no overt preference for the PS hematoporphyrin derivative among six cell lines representing the major human lung cancer histologies and a normal lung fibroblast line. Attempts to target cancer cells by adding moieties to the PSs that bind to specific epitopes with elevated expression on malignant cells have been met

*Corresponding author: heinz.baumann@roswellpark.org.

with mixed success (10–12). The addition of galactose or lactose to the carbon-17 position of pyropheophorbide-a (HPPH-Gal and HPPH-Lac) to facilitate binding to galactoside-specific lectins known to be elevated in certain tumor cells (13–15) did not result in the hoped for internalization of the PS through high affinity galactoside binding activity (3). This modification, however, strikingly altered the uptake of HPPH derivatives from mitochondrial to lysosomal deposition and appeared to involve interaction with abundant plasma membrane components with low carbohydrate specificity. Furthermore, the structural modification and altered subcellular distribution fundamentally changed the pharmacodynamic behavior of the PS, especially PS release by the cells. For HPPH, drug egress is controlled in part by the ABCG2 transporter, while HPPH-Gal or HPPH-Lac cannot be exported via this mechanism (16,17). Moreover, Morgan et al.(18) showed in a murine system in vivo that a tumor cell sub-population with elevated expression of ABCG2 may be more resistant to PDT and responsible for tumor regrowth when PS are employed that are subject to elimination by ABCG2. The identification of PSs that serve as ABCG2 substrates has relied exclusively experimental assessment and indicated that, besides HPPH, protoporphyrin IX, phytylporphyrin (19), chlorin e6 (16), hypericin (20), and in part Photofrin (21) to be subject to ABCG2 export. Ambiguous are the findings with hematoporphyrin (16,22,23) and unaffected by ABCG2 activity were Foscan (mTHPC) (16), NPe6 (21), and rhodamin 123 (22). This realization as well as the availability of a novel cell culture model prompted us to revisit the issue of cell type specificity with regard to PDT. Employing primary cultures of tumor and stromal cells derived from lung cancer cases treated at RPCI, we addressed the questions of subcellular site of photoreaction and its consequence on cell protein modification and intracellular signaling by applying various forms of PSs and cell treatments that deliver the photoreaction to distinct subcellular sites. Notable cell-type specific differences were detected in the cellular retention of specific PSs by normal and transformed lung epithelial cells, as well as between epithelial and fibroblastic cells. These findings suggest that most beneficial PDT of individual tumors may demand the selection of the optimal PS type and light treatment conditions for the target cells.

MATERIALS AND METHODS

Photosensitizers

2-[1-Hexyloxyethyl]-2-devinylpyropheophorbide-a (HPPH), HPPH-galactose (HPPH-Gal) (3), and HPPH-related 2-(1'-m-iodobenzyloxyethyl)-2-devinylpyropheophorbide-a conjugated with PEG-5000 at position 17² (compound 531-PEG) (unpublished data) were generated in the laboratory of Dr. R. Pandey. Stock solutions were prepared in 5% dextrose in distilled water containing 2% ethanol and 0.1% Tween 80 yielding PS concentrations ranging from 235 to 500 μ M. Aliquots were serially diluted in serum-free or serum-containing culture medium immediately prior addition to the cells. ¹⁴C-labeled HPPH and HPPH-Gal were prepared by incorporating [¹⁴C]-hexanol into HPPH as described (3).

Cells

Primary proliferating cultures of human epithelial cells and fibroblasts were prepared from surgical specimens obtained from the Tissue Procurement Services at RPCI under IRB-approved protocol CIC-00–17. While a low number of viable endothelial cells could be recovered from the same surgical lung specimens, it was impossible to generate homogenous proliferating cultures from these. Hence, the characterization of PS uptake and action was restricted to the epithelial and stromal cell populations. Non-tumor-involved lung tissue served as source of the “normal” cell types (alveolar pneumocytes II and bronchial epithelial cells (collectively termed “N-EC”) and pulmonary fibroblasts (“N-Fb”). Portions of dissected lung squamous carcinoma or adenocarcinoma served as source for tumor epithelial cells (“T-EC”) and tumor-associated fibroblasts (“T-Fb”). In the following, the

cell cultures are listed with laboratory protocol number assigned to the lung specimens (in parenthesis; i.e., L227 to L293) from which the cells were derived. The isolation and culturing of epithelial cells were carried out as described previously (24,25). Every epithelial cell culture was selectively grown and maintained in collagen 1-coated culture dishes in serum-free keratinocyte medium supplemented with recombinant EGF, bovine pituitary extract and cholera toxin (KSFM; Invitrogen). While serum-free condition enabled the isolation and propagation of proliferating epithelial cells in culture, it represented a condition distinct from *in vivo*. Because addition of serum to cultured epithelial cells caused their growth arrest and differentiation, we evaluated the effect of serum only on PS uptake and retention (see Result section and Fig. 2D and Fig. 8B). The purity of epithelial cultures was determined by immunostaining with AE1/AE3 pan-cytokeratin antibodies. In selected cases, the karyotype of normal and tumor cells was identified by spectral karyotyping (SKY). Cultures of N-EC and T-EC could routinely be maintained for 3–7 passages before cessation of proliferation due to senescence. Long-term cultures (>10 passages) of primary T-EC from surgical adenocarcinoma specimens or from squamous carcinoma xenografts (listed as “T-EC(1)” to “T-EC(10)”) were adapted to growth in DMEM containing 10% fetal calf serum. These cultures permitted the reconstitution of co-cultures with fibroblasts, the assessment of PS uptake and post-PDT recovery in presence of serum.

To obtain primary cultures of pulmonary and tumor stromal fibroblasts, lung or tumor tissues were minced, macrophages removed by mechanical extraction, and a portion of epithelial cells released by trypsin digestion. The remaining tissue was attached to the scored surface of culture plates and incubated with DMEM containing 10% fetal calf serum. Cells that grew out of the tissue fragments were passaged twice prior to use. These cells proved to be exclusively fibroblasts based on morphological and immunological criteria.

A subclonal line of the established human alveolar adenocarcinoma A549 cells (ATCC) was grown in DMEM containing 10% fetal calf serum and used for comparison with primary cultures of T-EC.

PS uptake and photoreaction

Cells were incubated with medium containing defined amounts of serum and PS. Exclusive binding to cell surface was achieved by incubating cells for 30 min on ice under slow rotary movement (~25 RPM). For microscopic assessment of PS distribution, cells were washed with ice-cold serum-free medium and imaged at 100 to 640X magnification on an inverted fluorescence microscope (Zeiss Axiovert 200) with Axio camera. Lysosomal localization was visualized by incubating cells 30 min prior to imaging with 0.5 μ M LysoTracker Green (Invitrogen). Filters were: for HPPH, λ_{ex} 410/440 nm and λ_{em} 675/750 nm; and for LysoTracker Green λ_{ex} 390/422 nm and λ_{em} 460/550 nm. Internalization of PS was achieved by incubating PS-treated cells in serum-free medium for 4 to 24 h at 37°. Serum-free medium was used to obtain a value for optimal uptake by reducing PS binding competition by serum proteins and minimizing the activity of ABCG2. Externalization/retention of PS was determined by incubating PS-treated cells with serum-containing medium for 4 to 24 h. The contribution of ABCG2 transporter to PS retention was evaluated by addition of 10 μ M imatinib mesylate (Novartis, East Hanover, NJ) to the culture medium. PS amount in cells were quantified by extraction in Solvable™ (PerkinElmer Life and Analytical Sciences) and measurement of fluorescence (λ_{ex} 415 nm; λ_{em} 667 nm) in a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA). The data was expressed in fluorescence units (FU)/mg cell protein or in pmole per 10⁶ cells. Uptake of ¹⁴C-labelled HPPH or HPPH-Gal (specific activity 5 Ci/mole and 4.8 Ci/mole, respectively) was determined by incubating cells with serum-free medium containing 0.8 μ M ¹⁴C-labeled PS for 4 h at 37°C then washed with serum-free medium and either released by trypsin digestion and counted in a hematocytometer, or solubilized within the

culture well. The radioactivity in the solubilized samples was counted in Beckman scintillation counter, corrected for background counts, and expressed in pmole PS per 1×10^6 cells. Experiments were carried out in triplicate.

Photoreaction was performed by illuminating PS-treated cell cultures in serum-free medium at 37° (within a tissue culture incubator) with 665 nm light of an argon-pumped dye laser for 3 to 27 min at a dose rate ranging from 1 to 14 mW/cm² to a total fluence of 3 J/cm². Depending upon the experimental design, cells were either extracted immediately after light treatment, or incubated at 37° for time periods ranging from 15 min (evaluation of PDT-activated signaling reactions) to 24 h (evaluation of PDT-induced gene expression).

Cell analyses

Survival was defined as the percent viable cells recovered after 24 h PDT and incubation in full growth medium. Cells were released by trypsin treatment and trypan blue-excluding cells counted in a hemocytometer. Recovery of proliferation was evaluated by re-plating a defined number of viable cells in full growth medium. The plating efficiency, cell division and apoptotic process were determined by daily photographic recording of the defined culture areas. Overall recovery and net growth of the cultures was measured by counting viable cells after 6 days. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and subjected to protein analyses as described (25,26). Briefly, protein extracts (5–20 µg) were separated on 6% to 10% SDS-polyacrylamide gels. On all gels, reference protein markers for loading, molecular size detection and cross-comparison among gels were included. After separation, proteins were transferred to Optitrans BA-S 85 reinforced nitrocellulose (Whatman GmbH, Dassel, Germany) and uniformity of protein loading and transfer was verified by transient staining with Ponceau red dye. Nonspecific interactions with probing antibodies were blocked by incubating the membranes with PBS-0.1% Tween 20–5% skim milk. Membranes were then reacted overnight at 4°C with one of the primary antibodies: STAT3 or ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA); PY705-STAT3, STAT1, PY701-STAT1, P-ERK1/2, p38, PT180/PY182-p38, EGFR, Akt, PS473-Akt, caspase 3, or poly(ADP-ribose)polymerase (PARP) (Cell Signaling Technology, Inc., Danvers, MA); ABCG2 (BXP-53; Alexis Biochemicals, San Diego, CA); or actin (Sigma-Aldrich, St Louis, MO). The immune complexes were visualized by reacting with peroxidase-coupled secondary antibodies and enhanced chemiluminescence detection (ECL) (Pierce Chemicals, Rockford IL). ECL images were recorded on X-ray films by various lengths of exposure to ensure recovery of signals within the linear range of digital scanning. The net pixel values of each band were integrated by using the ImageQuant TL program (Amersham Biosciences). The cross comparison of separate analyses and immunoblots relied on co-separated reference markers. STAT3 crosslinking was expressed by the percent conversion of monomeric STAT3 into the dimer form I of the STAT3 crosslinked complexes (26,27). The immunodetectable signals for total ERK were generally used as sample loading reference.

Statistical evaluation

Mean and standard deviation of experiments carried out at least in triplicate were calculated and used for the graphic presentations. Significance between control and experimental groups was examined using Student's t-test. A value of $P < 0.05$ was regarded as significant.

RESULTS

The structure of the PSs determines type of cellular uptake and retention

Previous studies with established lines of murine epithelial and fibroblastic cells have indicated that HPPH enters cells by diffusion and accumulates in mitochondria, while

carbohydrate derivatives of HPPH are endocytosed and concentrated in the endosomal/lysosomal compartment (3). Moreover, HPPH, in contrast to HPPH-Gal, is accessible to export *via* ABCG2 transporter action (17). We have revisited these observations to determine whether and to what extent the mode of uptake and retention of the HPPH derivatives are applicable to human cells relevant for lung tumor tissue and whether any of the processes are subject to cell type-specific differences.

Cellular uptake of HPPH and HPPH-Gal was determined using [^{14}C]-labeled PSs in primary cultures of normal or tumor epithelial cells or fibroblasts derived from surgical specimens of lung cancer patients (Fig. 1A). Considering the fundamentally different culture conditions that were necessary to generate and maintain proliferating cell cultures, the assessment of uptake was carried out in serum-free medium allowing application of identical treatment conditions for both epithelial cells and fibroblasts. Moreover, serum-free medium reduced the activity of drug export mechanisms (28) thereby enabled assessment of maximal PS uptake that generally was reached after 4 h incubation (3). In all cultures, the amount of cell-associated HPPH exceeded that of HPPH-Gal, which reflects in part the difference in internalization by diffusion versus endocytosis. Comparison of separate cell preparations of normal and transformed epithelial cells indicated a consistent ~2-fold higher uptake of HPPH than HPPH-Gal. A noteworthy, different behavior was observed in tumor-derived fibroblasts which exhibited overall lower PS uptake and diminished differences in HPPH and HPPH-Gal levels. The relative amounts of ^{14}C -labeled PS taken up in Fig. 1A could be confirmed by quantifying the uptake based on fluorescence of the extracted PSs. Moreover, because the various cell types used for the comparative assessment have similar cell masses, the relative values for PS per cell were essentially the same as for PS per mg cell protein (data not shown; see also Fig. 2A below).

The uptake kinetics, subcellular distribution and retention in both epithelial and fibroblastic lung cells were visually followed by fluorescent microscopy (Fig. 1B). The ability of the PSs to bind to cell surface was determined by incubating the cells for 30 min at 0°, a condition that proved to be effective not only to suppress endocytosis and intracellular vesicular membrane trafficking, but also to prevent transmembrane movement of the diffusible PSs. Upon raising the culture temperature to 37°, surface-bound PSs assumed the known subcellular distribution by immediate translocation of HPPH to the mitochondria through diffusion and slow-rate accumulation of HPPH-Gal in the endosomal/lysosomal compartment through endocytosis (Fig. 1B and Fig. 2B). These redistribution patterns were universally observed in all cell types and cultures tested. In the case of normal epithelial cells, the uptake and retention of PSs were not appreciably different when comparing cells from the early passage 1 or 2 to cells from late passage 5–7 (data not shown). The only notable changes occurred in the increased cell size and reduced proliferation of the late passage cells, which were associated with senescence.

Mindful of the known role of the ATP-dependent transporters in PS export (16,29), in particular of HPPH (17), we explored the loss of PS over time and discovered an appreciable cell type-specific difference (Fig. 1B & C). While major portions of HPPH or HPPH-Gal taken up by epithelial and fibroblastic cells were retained when the cells were maintained in serum-free conditions, HPPH was preferentially lost from cells in the presence of serum as expected (Fig. 1C). The loss of HPPH was particularly high in fibroblasts, which retained <1% of initially bound HPPH after 24 h. Under the same condition, epithelial cells retained 5–20%. The preferential effect of serum on cellular uptake of HPPH over HPPH-Gal was similarly evident when the cells were incubated in medium containing the PSs and increasing serum concentrations (example of fibroblasts in Fig. 2A and epithelial cells in Fig. 2D).

To evaluate the contribution of the ABCG2 transporter to the overall process of PS retention, the various cell preparations and cell lines were probed for immune-detectable levels of ABCG2. Despite exhibiting significant time- and serum-dependent losses of HPPH, all preparations of N-Fb, T-Fb and N-EC, and most of the T-EC were negative for ABCG2 protein (representative examples in Fig. 1D). However, among 35 lung tumor cases, two adenocarcinoma cell preparations (e.g., T-EC(L237) in Fig. 1D) were identified that, as well as the established line (A549) (16), express a high level of ABCG2 protein (Fig. 1D, left panel). The impact of ABCG2 expression on cellular HPPH accumulation will be addressed below (Fig. 6 & Fig. 8). Of note is that every preparation of murine T-Fb recovered from human lung tumor xenografts proved to be ABCG2-positive and showed a level of expression similar to that determined for the murine fibrosarcoma cell line, RIF (Fig. 1D, right panel).

When characterizing new PS derivatives (30), a third mode of cellular uptake was identified with 531-PEG, a pyropheophorbide that has a 5,000-molecular weight PEG moiety attached to carbon 17 (Fig. 2A, right panel). This PS did not appreciably bind to the surface of either epithelial or fibroblastic cells (data not shown) and, only after 24 h incubation, could an endosomal/lysosomal accumulation be observed (Fig. 2C). In contrast to HPPH and HPPH-Gal, the uptake of 531-PEG was enhanced in presence of serum (Fig. 2A, right panel) and was consistently higher in fibroblasts than epithelial cells. The mode of 531-PEG accumulation exhibits features described for the uptake by fluid-phase pinocytosis such as determined by fluorescent, non-diffusible markers (31,32).

The conditions used for measuring PS uptake were specifically selected for gaining maximal values that define the cell-type specific binding, internalization, subcellular distribution and plasma protein-dependent release. Since, however, serum-free culture condition and the pulse-chase technique do not adequately reflect the *in vivo* conditions expected for PS-treated patients or mice, we determined whether the relative uptake of HPPH and HPPH-Gal by epithelial cells changes as a function of serum/plasma protein concentration. T-EC(9-1) cultures were incubated in medium with identical concentrations of the two PSs in the presences of 0 to 10% fetal calf serum or 100% human plasma. The subcellular distribution and relative amount of PS was visualized by fluorescence microscopy after 4 and 24 h incubation (Fig. 2D). Despite prominent concentration-dependent competition of plasma protein for PS uptake, relative amounts of HPPH and HPPH-Gal detected in the cells were essentially the same at all condition with HPPH exceeding HPPH-Gal by a factor of around two.

Cell type-specific PS level, but not subcellular site of PS accumulation, correlates with the magnitude of photoreaction, cellular signaling and cell death

The quantitative differences in PS uptake and retention directed by the culture conditions were proportionally reflected in the PS-mediated photoreactions (Fig. 3). In the example of T-EC cultured in serum-free medium and exposed to therapeutic light at 37°, mitochondrial HPPH and surface-bound HPPH-Gal produced dose-dependent photoreactions that led to a similar profile of immediate cell responses including the oxidative crosslinking of STAT3, loss of EGFR, and activation of p38MAPK (Fig. 3A, left panel). These responses were followed by a proportional, PDT dose-dependent cell death (Fig. 3B). When the cells, after loading with PS for 30 min, were subjected to a 24 h chase period with PS-free medium, HPPH remained associated with mitochondria, but its cellular concentration was reduced by ~50% (Fig. 1C). Under the same condition, the amount of cell-associated HPPH-Gal remained constant although essentially all was transferred to the endosomal/lysosomal compartment (Fig. 2B). Despite the change in subcellular locations of the PSs, light treatment generated again comparable cellular response patterns that were proportional to the PS concentrations (Fig. 3A&B, right panel). The consequence of cell-type specific

uptake and retention on cellular signaling and survival was highlighted by the results with fibroblasts in which the preferential loss of HPPH over HPPH-Gal generated a shift toward an HPPH-Gal-dominated PDT (Fig. 3C & D).

Since the photoreaction was effective in activating stress signaling pathways in both epithelial and fibroblastic cells (Fig. 3), the involved kinase activities could conceivably contribute to the PDT outcome. This notion was tested by exposing T-ECs to the same PDT dose, but delivered at three different fluence rates (Fig. 4), thus adjusting the severity of damage at any given time and length of treatment while delivering the same cumulative PDT dose. The equal extent of crosslinked STAT3 confirmed that the same overall PDT dose was delivered to the cell culture. The longer duration of a milder photoreaction resulted in a more effective activation of stress signaling (Fig. 4A) which translated into an approximately two-fold increased cell killing (Fig. 4B). A similar two-fold enhancement of cellular killing by PDT delivered at lower fluence rate was observed for A549 cells and fibroblasts (data not shown).

A prominent cell type-specific consequence of HPPH and HPPH-Gal PDT was detected in the rate of post-PDT recovery of the primary cell cultures (Fig. 5). When determining the ability of PDT-surviving epithelial cells and fibroblasts to regain normal proliferation rates, we observed that epithelial cells (N-EC, T-EC and A549) showed a progressive decline in resumption of proliferation as a function of initial PDT damage. While viable cells from cultures with 24 h post-PDT survival rates below 20% showed close to normal plating efficiencies, they were unable to regain normal proliferation rates (Fig 5A). Adherent cells underwent cell divisions and displayed an essentially normal cell cycle stage distribution throughout 14 days of post-PDT culturing (data not shown). However, the rate of apoptotic cells was elevated and the persistent presence of cleaved PARP in such cultures indicated ongoing apoptosis (Fig. 5B) suggesting that these cells harbored non-repairable damage caused by PDT. A distinct course of recovery was determined for fibroblast cultures. Viable cells from cultures with PDT survival rates even below 1% were able to regain normal proliferation rates (Fig. 5A).

Epithelial cells show transformation-dependent changes in PS uptake and retention

The cell analyses (Fig. 1–Fig. 3) showed differences in the uptake of HPPH, HPPH-Gal and 531-PEG between epithelial and fibroblastic cells and suggested that these potentially could be exploited to achieve cell type-targeted PDT. To assess whether the oncogenic transformation of the epithelial cells had any additional impact on PS uptake and PDT reactions, we carried out comparative analyses of primary cultures of epithelial cells derived from tumor and non-tumor lung tissues of 35 individual cancer patients. The study included 14 squamous carcinomas and 21 adenocarcinomas representing pathological stages of pT1a to pT4 with a histology grade from 1 to 3. Eight additional squamous carcinomas, which had been maintained for 4 to 7 passages as xenografts in Scid mice, were included in the characterization to evaluate the stability of the PS-uptake phenotype of this cancer type.

All non-transformed lung epithelial cell cultures, including those derived from bronchial brushing, and approximately ~50% of the tumor epithelial cell cultures displayed essentially the same PS uptake and retention phenotype as shown for the representative examples of T-EC(L234) (Fig. 1B) and T-EC(L297) (Fig. 6A, top row). This epithelial cell phenotype showed consistent quantitative differences to that defined for fibroblasts derived from the same tissues (Fig. 3). The pair-wise analysis of epithelial cells derived from normal and tumor tissue of the same patient involved the determination of HPPH and HPPH-Gal uptake during a 30-min incubation and retention during a subsequent incubation for 4 and 24 h in medium containing 10% fetal calf serum. The results indicated that the initial uptake rates of the PSs were comparable among all EC cultures (Fig. 6A). The retention of HPPH, however,

deviated in some of the tumor-derived epithelial cell cultures from the “normal” phenotype. One type, found in 8 squamous carcinoma and 3 adenocarcinoma (example T-EC(L278) in Fig. 6A center row) displayed a several-fold lower release of HPPH. The other type, found in 2 squamous carcinoma and 2 adenocarcinoma cultures (example T-EC(L237) in Fig. 6A, lower row) had a significantly increased release of HPPH. These cells lost internalized HPPH at a rate of that determined for fibroblasts. The overall lower retention by these T-EC cultures relative to the corresponding N-EC cultures was proportionally reflected in a lower HPPH PDT signaling (Fig. 6B) and, consequently, more resistance to PDT killing (Fig. 6C). The phenotype of enhanced HPPH loss could be correlated with an expression of the ABCG2 transporter only in the two adenocarcinoma (including T-EC(L237)), which had an expression exceeding several-fold that of A549 cells (Fig. 1D).

Cell type-specific retention of PSs permits selective killing of cells

The studies of isolated cell types provided information about potential cell type-specific differences in PS biology, such as the preference of epithelial cells for HPPH and of fibroblasts for HPPH-Gal. To assess whether these differences might be exploited for tumor treatment required, a confirmation that the differences deduced from single cell type cultures persisted in appropriate co-culture systems was determined. To generate such cultures, primary lung tumor epithelial cells that exhibited enhanced HPPH retention were adapted to proliferate in the presence of serum and, thus, be culture-compatible with fibroblasts. To test the reproducibility of the model, cells from 3 adenocarcinoma (surgical specimens) and 4 squamous carcinoma (xenografts) were reconstituted in co-cultures with normal pulmonary fibroblasts or fibroblasts derived from tumor tissue. Detection of PS uptake and monitoring post-PDT recovery were facilitated by the fact that epithelial and fibroblastic cells proliferate in segregated clusters and have characteristic morphologies. Essentially the same results were obtained in each case.

Treatment of the co-cultures for 30 min with HPPH resulted in a comparable HPPH uptake by both cell types (Fig. 7A, left panel). A subsequent incubation in serum-containing, but PS-free medium led within 4 h to a drastic reduction of HPPH in fibroblasts, but only to a minimal loss of internalized HPPH from tumor cells (Fig. 7A, right panel). The preferential retention of HPPH made the tumor cells more vulnerable to PDT treatment. The reciprocal feature of cell-type-specific PS accumulation could also be demonstrated by taking advantage of the higher level of endocytosis of 531-PEG or HPPH-Gal over HPPH by fibroblasts (Fig. 7B & D). Following 24 h continuous incubation of a co-culture with 531-PEG in regular culture medium, fibroblasts exhibited a higher level of lysosomal PS than epithelial cells (Fig. 7B). In contrast, the co-culture treated for 30 min with low dose HPPH followed by 4 h incubation with serum-containing medium resulted in a predominant tagging of tumor cells. Light treatment resulting in an initial ~90% cell death in the culture was sufficient to eliminate either fibroblast or tumor cells from the recovering culture (Fig. 7C). A similar design of reciprocal cell killing was possible by application of high dose of HPPH-Gal or low dose of HPPH in co-culture. While the two PSs accumulated in both cell types (Fig. 1&Fig. 3), the difference in concentration generated during the chase period of 4 h was sufficient to produce a PDT reaction that killed ~95% of the cells in the culture and led to a preferential survival and proliferation of either tumor cells or fibroblasts (Fig. 7D). By applying a stringent HPPH-Gal PDT treatment to ensure killing of all fibroblasts, the surviving tumor cell population demonstrated a strongly delayed recovery of proliferation (Fig. 7D). Even after 16 days, the surviving tumor cells showed continued release of apoptotic, cleaved PARP-positive cells (determined by daily photographic recording, data not shown). The impaired recovery of T-EC was comparable to that described in Fig. 5. While fibroblasts were subject to substantial killing in the HPPH-exposed co-culture in

which T-ECs were preferential removed, the surviving population recovered more readily to normal proliferation rates in line with the finding shown in Fig. 5A.

Cell-type specific retention of HPPH involves passive and active components

Our analyses of primary cell types have indicated that epithelial cells have a higher propensity to retain HPPH than fibroblast (Fig. 1&Fig. 3). Moreover, the oncogenic transformation appears to modify this property of the epithelial cells (Fig. 6A) resulting in ~1/3 of the cases having a further enhanced retention. A reduction of HPPH retention was found in ~1/10 of the cases. These data suggested that at least two separate mechanisms are involved, one that determines the organelle-restricted retention of HPPH and the other the removal of HPPH. The biochemical basis for the HPPH retention remains to be identified. However, the observation that remnants of cells, which had been fixed to the culture dish and permeabilized by high-dose PDT (several-fold lethal dose), were still able to bind HPPH in a subcellular localization pattern equivalent to that seen for intact cells and this suggested the presence of membrane components that serve as binding targets for HPPH. To assess whether this passive HPPH binding property was in part responsible for the difference in HPPH-retention observed between epithelial and fibroblastic cells, we subjected a co-culture of T-Fb and T-EC, the latter with confirmed high HPPH-retention capability, to high dose PDT. Bacteriopheophorbide-carboxylic acid (λ_{ex} 782 nm, λ_{em} 810 nm) was used as PS because its spectral properties do not overlap with HPPH (Pandey et al. unpublished). After exhaustive washing with serum-containing medium, the cellular remnants of the co-culture were then exposed to HPPH in presence of serum. A preferential binding of HPPH to T-EC structures were observed that showed a spatial specificity as seen with intact cells (Fig. 8A). This interaction was stably maintained during 24 h washing with serum-containing medium.

The enhanced loss of HPPH from some of the T-EC preparation was tentatively attributed to active enzymatic export mechanisms, such as members of the ATP-binding cassette gene family. In the example of T-EC(L237) the abundant expression of ABCG2 was identified and conceivably could account for the efficient efflux of HPPH. The cell-type specific process of the ATP-dependent transport process was confirmed by using a co-culture system composed of T-EC(L237) and ABCG2-negative T-Fb. The uptake and retention of HPPH was determined in the presence or absence of imatinib mesylate, an efficient inhibitor of ABCG2 (Fig. 8B). While imatinib did not appreciably alter the initial uptake of HPPH by either cell type (Fig. 8B, left panels), it specifically prevented the loss of HPPH from the T-ECs during a subsequent chase period in the presence of serum (Fig. 8B, center panels). A comparable, enhanced retention of HPPH by T-EC(L237) cells over T-Fb was evident when the uptake process involved a 4-hour incubation of the co-culture with HPPH in the presence of 10% serum (Fig. 8B, right panels).

DISCUSSION

By using PSs with known subcellular preference in primary human lung cell cultures, we discovered a cell type-specific mitochondrial retention of HPPH in epithelial cells and an elevated accumulation of HPPH-Gal and pegylated pheophorbide in the endosomal/lysosomal compartment of fibroblastic cells. These features allowed the design of the preferential destruction of either fibroblastic or epithelial cells in co-culture systems that reflect the cellular combinations found in human lung tumor tissue. The finding of transformation-dependent changes in porphyrin transport mechanism in epithelial cells also indicated that a pretreatment evaluation of tumor tissue for ABCG2 expression and the ability of isolated tumor cells to retain PS may be helpful for customizing PDT of individual cancer cases.

Here we address the question whether a tumor cell type-specific PDT is measurable and, if so, what is its cellular manifestation. Basically, one can envision two non-mutually exclusive properties of the target cells: (1) the uptake and retention of PSs is subject to cell type specific mechanisms and, thus, the amount and subcellular location of the PSs determine the level of PDT response; or (2) independent of the amount of PS in the cells, the response to the PS-mediated photoreaction is dictated by cell-type specific stress reactions, expression of protective pathways and the ability to mobilize survival and recovery mechanisms (33). Aside of confirming the known properties of the PSs that determine cellular and tissue distribution, such as the competitive interaction with plasma proteins, binding to cell surface, mode of cellular uptake and intracellular distribution, the data indicated that subcellular retention and active export by drug transporting reactions are the most notable determinants for the PS accumulation that distinguish epithelial and fibroblastic cells. The pair-wise comparison of normal and tumor epithelial cell cultures has also indicated measurable differences in PDT in that some tumor epithelial cells showed enhanced PDT resistance. However, this transformation-associated property appears to correlate with a reduced PS accumulation rather than increased expression of survival functions. While the analyses of individual cell types did reveal quantitative differences in the egress process to account for the lower retention of HPPH by fibroblasts versus epithelial cells, the relevance of the difference for PDT could only be confirmed in the context of tissue-relevant co-cultures of cell types.

The application of HPPH and HPPH-Gal confirmed in the primary human lung cells the alternative uptake mechanisms: diffusion and predominant mitochondrial accumulation of HPPH and endocytosis of surface-bound HPPH-Gal followed by deposition of the PS in the endosomal/lysosomal compartment. The precise biochemical partners that determine the intracellular retention of either PS is unknown, but is generally believed to represent major membrane components constituting the respective subcellular compartments (34). The comparative analysis of the cell types indicated that not the uptake process but the capacity of the cells to bind PSs at intracellular sites is the basis for the cell type specific properties. The intracellular membrane composition of lung epithelial cells appears to be particularly effective in HPPH binding. The fact that this binding activity was also demonstrable in cellular residues post PDT suggests that cell-type specific differences in membrane composition contribute to the elevated HPPH-retention in epithelial cells. In part, this binding activity is further elevated in a subset of transformed cells.

The contrasting higher accumulation of lysosomal/endosomal PSs in fibroblasts has tentatively been ascribed to the higher endocytotic rate of these cells compared to epithelial cells. To what extent the retention of the PSs in the intracellular vesicles are dependent on interaction of the PSs with membrane or luminal constituents remains to be determined. The stability of endosomal/lysosomal PS deposition (Fig. 1B and Fig. 2B & C) and retention during continued cell division cycles suggests low turnover rates of the captured PSs. Whether there is a cell-type specific difference in this process is not yet known.

The study also revealed that the level of HPPH retention in certain transformed epithelial cells is strongly influenced by active export processes, correlating with the elevated expression of ABCG2 (Fig. 8B). Our evaluation of the cells for ABCG2 expression was restricted to the western blot-detectable protein in the primary cell cultures (Fig. 1D). This analysis indicated that ABCG2 expression appears to be enhanced in a subset of transformed epithelial cells derived from adenocarcinoma and that this expression is stable in culture. The absence of detectable ABCG2 in non-tumor epithelial cells or fibroblasts can not rule out that ABCG2 may be expressed in subpopulations of the cell culture (18,35) or that the gene is expressed in the same cells within the lung tissue, but silenced in the cultured cells. Knowing that lung tumors are generally associated with inflammation and inflammatory

cytokines along with hypoxia have been noted to enhance ABCG2 expression (36–38), a contribution of in situ inflammatory milieu may assist in setting the level of ABCG2 activity in lung tumor tissue (39). This stimulatory effect is lost upon isolation of the cells. However, treatment of cultured ABCG2-negative epithelial cells with various combinations of inflammatory cytokines, including oncostatin M, IL-1b, TNFa, TGFb, or IFNg, or irritants such as the TLR2 and TLR 4 ligands lipooligosaccharides and lipopolysaccharides, respectively, failed to induce ABCG2 expression (data not presented). The fact that stable ABCG2 expression was detected in certain tumor cell lines, but not in others, suggested that activation of ABCG2 gene was not a generic consequence of the cellular adaptation to tissue culture conditions and that the chosen protein analysis provided a useful descriptor for the particular cell culture. By extension, the identification of ABCG2 expression in given tumor tissue may assist in designing the application of optimal PDT regimen (16,17). Moreover, the observation that murine fibroblasts from a lung tumor xenograft, but not human fibroblasts from the original primary tumor, express ABCG2 may imply a distinct role of stroma in defining the HPPH level and PDT response in xenograft versus primary tumor. The identification of the stromal compartment in the uptake process of PSs by xenografts containing human epithelial cancer cells with defined PS retention phenotypes is currently under study.

The subcellular site and amount of the PSs can be controlled to a large extent by the mode of PS treatment and incubations. A wealth of earlier reports had indicated that the photoreaction mediated by PS localized to plasma membrane, mitochondria or lysosomes will inevitably have site-specific immediate targets and, thus, produce characteristic profiles of reactions that eventually converge on the activation of lethal pathways (33,40–42). Our approach in defining the PDT response utilized only two readouts, the activation of cellular signal transducing pathways within a 15 min photoreaction and cell survival at 24 h and later time points. This permitted us to probe the magnitude of PS as a function of PS amount and localization, but did not indicate the mode by which the cells were killed. Regardless of the type of photosensitizer and its subcellular localization, the oxidative crosslinking of cytoplasmic STAT3 and the activation of the stress MAPK pathway were strikingly similar for both fibroblasts and epithelial cells (Fig. 3 & Fig. 4). This may relate to the fact that the necessary reactive intermediates generated at the distinct subcellular sites need to reach a common cytoplasmic position where STAT3 and initiating MAPK members reside. Still elusive is an explanation of the mechanisms by which EGFR at the plasma membrane is removed within the timespan of the photoreaction (Fig. 3). While an immediate activation of a membrane-localized protease, such as a member of the ADAMS family member (43), may explain the process of receptor loss, we have not been able to detect the presence of soluble EGFR products in the post PDT culture medium (data not presented). Hence, the alternative possibility of an immediate endocytosis and intracellular proteolysis is not excluded (44).

In accordance with the general principle of PDT, the lethal progression of the PDT reaction in our cell models is proportional to the PS amount associated with the cells and light-dose delivered. It is accepted that the cellular reactions activated during and post light treatment contribute to the overall PDT response (33,41). Since these mostly involve enzymatic reactions, the level and duration of their activation should likely be manifested in the overall outcome. Indeed, the application of different fluence rates revealed that light delivered at a lower rate has an enhancing effect on the lethal outcome (Fig. 4B), suggesting that prolonged stimulation of the stress pathways is beneficial for optimized PDT design. The prediction is also in agreement with the previously made but unexplained observation that light delivery to subcutaneous tumors at a lower fluence rate improves tumor control by a mechanism distinct and in addition to the well described maintenance of tumor oxygenation (45).

High dose PDT results in a markedly delayed recovery of epithelial cell proliferation. A manifestation of this delay is the persistently elevated apoptotic rate in the post-PDT cell culture. Considering that the cell cycle stage distribution did not indicate any gross alteration relative to untreated cells (data not shown), the failure of the cells to regain normal proliferative activity was attributed to the inability of the cells to repair cellular damage caused by the photoreaction and continuous apoptotic loss of cellular progenies. Whether PDT has generated in epithelial cells irreparable chromosomal aberrations (46) or not (47,48) remains to be determined. Since fibroblasts were able to more efficiently overcome high dose PDT, an improved survival of stromal cells within treatment fields is predicted.

The study of the lung tumor cells has indicated potential markers that need to be established if optimal PDT conditions for individual tumors is designed. To determine whether the same markers also apply to other epithelial tumors for which PDT is considered, we have initiated equivalent analyses of primary cell cultures derived from head/neck cancer tissue (squamous carcinomas). Preliminary data (not presented here) show that those tumor epithelial cells have similarly increased retention of HPPH over fibroblasts. Expression of ABCG2 in primary head/neck tumor cases remains to be determined, but has been reported for established tumor cell lines (17,49,50). A contrasting, low HPPH retention phenotype was expected for a fibroblastic tumor. This was confirmed in the example of a murine fibrosarcoma, RIF, as already demonstrated previously (3). RIF cells also have an enhanced retention of HPPH-Gal as predicted for fibroblastic cells and this retention correlates with lysosomal deposition.

The fact that we were able to manipulate (through use of different PSs, PDT regimen, inhibition of pump mechanisms) PS accumulation/retention in primary epithelial and fibroblast co-cultures in ways that allowed preferential elimination of one or the other cell type suggests that up-front determination of ABCG2 expression may be important in individualized PDT treatment design.

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Figure 1.

Uptake and retention of PSs by primary human lung cells. A, Monolayers of primary cultures of normal (N-EC), tumor epithelial cells (T-EC from squamous carcinoma xenograft 9-1) and tumor fibroblasts (T-Fb), and A549 established adenocarcinoma cell line in 24-well culture plates were incubated for 4 h with [14 C]-labeled HPPH or HPPH-Gal in serum-free culture medium. Cell-associated radioactivity was normalized to cell number (mean $\pm$ SD, N=3). B, Subcellular localization of PSs in primary T-EC(L234) and T-Fb(L298) was monitored over 24–48 h. Cells in 6-well culture plates were incubated for 30 min on ice in serum-free medium containing the indicated PSs. After washing and recording the surface-bound fluorescence, the cells were incubated at 37° in the PS-free media as listed

(chase). After the indicated culture periods, the cell-associated PSs were visualized by fluorescence. All panels represent fluorescent images captured by 1 sec exposure at 100X magnification. C, Uptake and retention of PSs as a function of serum in the chase medium. Replicate cultures of T-EC, T-Fb and A549 cells were incubated for 4 h in serum-free medium at 37° (=Uptake) and followed by 4 or 24 h chase in either serum-free medium or medium containing 10% fetal calf serum. The cultures were extracted and amounts of cell-associated PS determined by fluorometry. The arbitrary fluorescent units (FU) were normalized to the amount of cell protein in each extract and expressed as percent of the fluorescence taken up by each culture during the initial 4-h incubation (mean $\pm$ SD, N= 3–5). D, Immunoblot analysis of representative cell cultures for the expression of ABCG2. The same blots were probed for ERK1/2 to demonstrate protein loading.

Figure 2.

Effect of serum protein on PS uptake and confirmation of lysosomal localization of PS derivatives. A, T-Fb cultures in 24-well plates were incubated for 4 h in serum-free DMEM containing 400 nM HPPH or HPPH-Gal and the indicated concentration of fetal calf serum. The uptake of 531-PEG was carried out by incubating T-Fb with 800 nM PS for 24 h in medium containing the indicated concentration of fetal calf serum. The amounts of cell-associated PS were quantified by fluorometry and expressed relative to the amount of cell protein in each extract (mean $\pm$ SD, N=4-5). B, Cultures of T-EC (derived from squamous carcinoma xenograft 9-1) were incubated for 30 min on ice in KSFM containing 800 nM HPPH or 1600 nM HPPH-Gal followed by a 24 h chase period at 37° in PS-free KSFM.

Localization of acidic endosomal/lysosomal organelles was visualized with lysotracker green. Phase microscopic and fluorescent images (merged) are shown. C, Uptake and co-localization of 531-PEG with acidic endosomal/lysosomal organelles are shown for T-Fb that were incubated with 800 nM 531-PEG for 24 h. D, T-EC(9-1) cultures were incubated in the media containing serum or plasma as indicated at the bottom in the presence of either 400 nM HPPH or 400 nM HPPH-Gal. After 4 h and 24 h, the cell-associated fluorescences was recorded by imaging using timed exposures as listed at the top. The images of the cells maintained in plasma were subjected to identical contrast enhancement for visualization of the fluorescent signals.

Figure 3.

Effects of HPPH and HPPH-Gal PDT on signaling and survival of T-EC and T-Fb. A & B, Primary cultures of T-EC(L231) were incubated in replicate 24-well plates for 30 min at 37° in KSFM containing the indicated concentrations of HPPH or HPPH-Gal. After washing the cells with PS-free medium, the incubation was continued for half of the replicate cultures for 24 h in KSFM. Cells after initial PS uptake and after 24 h chase were treated for 9 min with 665 nm light (3 J/cm²) at 37°. One series of cell cultures were immediately extracted for immunoblot analysis of the indicated proteins (A) and 4 replicates of PDT-treated cultures were incubated for an additional 24 h to determine the percentage of surviving cells (B). C & D, T-Fb(L231) in replicate cultures in 24-well plates were incubated in DMEM

containing 0.5% fetal calf serum and the indicated concentrations of HPPH and HPPH-Gal. After washing the cultures, the cells were incubated in DMEM containing 10% fetal calf serum (=chase). Cultures after PS uptake and chase period were analyzed for PDT-mediated signaling (C) and cell killing (D) as done for T-EC.

Figure 4. Fluence rate affects cell signaling and survival. A, Replicate cultures of primary T-EC (squamous carcinoma xenograft 1–3) were incubated in KSFM with 50 nM HPPH and HPPH-Gal for 30 min at 37° followed by a 24 h chase in KSFM. Cultures were exposed to 665 nm light under the conditions indicated. The cells were extracted to determine the level of photoreaction (STAT3 crosslinking) and activation of the p38 stress MAPK pathway. B, T-EC cultures of 6 separate lung cases were incubated with 12.5 nM HPPH in KSFM followed by a 24 h chase period in KSFM. These cultures were subjected to PDT at different fluence rates as described in “A”. The percent of surviving cells were determined after an additional 24 h post-PDT incubation (mean $\pm$ SD).

Figure 5.

The severity of PDT action determines the post-PDT recovery of the proliferative activity of cells. A, Primary cultures of N-EC, T-EC, A549 and T-Fb were subjected to HPPH PDT yielding 24 h post-PDT survival rates from <1% to 100%. Viable cells (3,000–10,000 cells per well in 24-well plates) were reseeded in full culture medium. Plating efficiency and recovery of proliferating cultures was monitored over a 6-day period. The number of viable cells relative to the control cultures (no PDT) in each series was determined. The data are compiled from 5–11 separate experiments. B, Cultures of T-EC(L289) with initial 10% PDT survival, along with the control culture, were plated in KSFM into separate wells. After the times indicated adherent cell material was lysed and equivalent aliquots of the cultures were analyzed for PARP cleavage by immunoblotting. Debris collected from the 24-h post-PDT cultures was included to indicate the relative level of apoptotic material at 0 h.

Figure 6.

Iterated retention of HPPH by T-EC. A, Three phenotypes of HPPH retention detected in T-EC. The representative examples of primary T-EC demonstrate the uptake of HPPH (800 nM) in serum-free DMEM and the release in presence of DMEM containing 10% fetal calf serum during 4 and 24 h culture period. The cell-associated HPPH fluorescence was recorded by microcopy at 100X magnification. B&C, Manifestation of reduced HPPH retention resulted in lower PDT reaction. Duplicated cultures of N-EC and T-EC derived from lung case 237 were incubated for 30 min in KSFM containing the indicated concentrations of HPPH. After a 24-h chase period in PS-free KSFM, the cells were treated

at 37° with 665 nm light (3 J/cm²) and the response determined at the level of signaling (B) and survival (C). Is there a figure of HPPH uptake in L237 N-EC vs T-EC?

Figure 7.

Cell-type specific retention and action of PS in co-cultures of T-EC and T-Fb. A, A 5-day old co-culture of T-EC(L278) and T-Fb was incubated for 30 min at 37° in serum-free DMEM containing 800 nM HPPH followed by 4 h chase in DMEM with 10% fetal calf serum. The culture-associated HPPH-fluorescence was recorded by fluorescence microscopy using identical setting. B&C, Selective cell killing by 531-PEG and HPPH. Duplicate primary cell cultures generated from lung tumor case 237, containing approximately equal proportion of T-EC and T-Fb, were incubated in DMEM+10% fetal calf serum and either 200 nM 531-PEG for 24 h or 12 nM HPPH for 4 h. The cellular pattern of preferential 531-PEG retention by T-Fb and HPPH by T-EC was assessed immediately before light treatment

by fluorescence microscopy (B). The level of cell killing and recovery of T-EC or T-Fb was recorded by phase microscopy (C). D, Duplicate 3-day old co-cultures of T-EC (isolated from squamous carcinoma xenograft 1–3) combined with human T-Fb were treated for 30 min with serum-free DMEM containing 100 nM HPPH-Gal or HPPH. After a 4 h chase in DMEM with 10% fetal calf serum, the co culture was exposed to 665 nm light (3 J/cm²). The cellular change in the post-PDT cultures was monitored over a 16 day period by phase microscopy.

Figure 8.

Mechanisms that contribute to cell-type specific retention of HPPH. A, Preferential binding of HPPH to intracellular membrane structures of T-EC. T-EC (squamous carcinoma xenograft 1–3) in co-culture with human T-Fb were exposed to lethal PDT reaction using bacteriopheophorbtyin-mesic acid (1600 nM) and 782 nm light at 15 J/cm². The cells remnants that are crosslinked to the collagen matrix on the culture dish were incubated for 24 h with DMEM containing 10% fetal calf serum. The cellular material was then incubated for 10 min at 37° with medium containing 800 nM HPPH followed by washing for 24 h with DMEM with 10% fetal calf serum. HPPH fluorescence retained by the T-EC structures was visualized by fluorescence microscopy. B, ABCG2 activity contributes to the preferential

loss of HPPH from T-EC(L237). Replicate 2-day co-cultures of T-EC(L237) and T-Fb were incubated for 30 min at 37° with serum-free DMEM containing 800 nM HPPH. The cultures were then chased for 4 h with DMEM containing 10% fetal calf serum. One set of culture included 10 μ M imatinib mesylate. A separate incubation was performed with the uptake of HPPH carried out for 4 h in DMEM with 10 % fetal calf serum and 800 nM HPPH. One set of culture included 10 μ M Imatinib mesylate. The relative cellular distribution of HPPH was visualized by fluorescence microscopy using an identical setting.