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Sensitivity of Different Human Lung Cancer Histologies to Photodynamic Therapy

Roger R. Perry, Wilbert Matthews, James B. Mitchell, Angelo Russo, Steven Evans, and Harvey I. Pass[1]

Thoracic Oncology Section, Surgery Branch [R. R. P., W. M., S. E., H. I. P.], and the Radiation Oncology Branch [J. B. M., A. R.], National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT

The relative sensitivities of different cancer histologies in a single site to photodynamic therapy (PDT) are unknown and methods to predict PDT sensitivity have not been described. The *in vitro* response to PDT of six established human lung cancer cell lines and one normal lung fibroblast cell line was studied using the clonogenic assay. Dose-response curves were determined for cells incubated in 25 μg/ml of Photofrin II for 2 h, followed by exposure to 630-nm light to total energies of 0–3150 J/m². None of the cell lines were sensitive to sensitizer alone or light alone. Differences in inherent PDT sensitivities as evaluated by survival curve parameters *n*, D_o, and light dose to yield 1% survival were observed among the cell lines. No clear correlation was found when inherent PDT sensitivity was compared with sensitizer uptake; however, a general association was noted between PDT sensitivity and the plating efficiency of the cell line. These data illustrate that differences in inherent PDT exist for *in vitro* systems. Such differences may explain some of the failures seen in clinical PDT.

INTRODUCTION

Thoracic malignancies generally have a poor prognosis. The overall 5-year survival rate for patients with carcinoma of the lung is approximately 10%, with or without multimodality therapy (1). The standard therapies for mesothelioma including pleuropneumonectomy, pleurectomy with radiation therapy, and radiation therapy combined with chemotherapy are generally ineffective (2). An innovative therapy which shows promise for the treatment of certain malignancies is PDT.[2] PDT has been used to treat both early and advanced endobronchial disease (3), for the palliation of advanced carcinoma of the esophagus (4), and for the treatment of malignancies at other sites, such as the bladder (5), skin (6), and brain (7). The major side effect of this treatment is skin photosensitivity.

In spite of increasing clinical use, little is known about the relative PDT sensitivities of different malignancies or about the relative PDT sensitivities of different malignant histologies in the same site. Different transitional cell carcinoma of the bladder cell lines take up different amounts of sensitizer, and the PDT sensitivity of one of the cell lines correlates with cell sensitizer levels (8). Conditions which effect sensitizer uptake also impact on PDT sensitivity (9). Different concentrations of sensitizer are required to achieve 90% cytotoxicity of explanted brain cancer cells from different tumors (10). These studies suggest that the degree of PDT sensitivity may be related to cell sensitizer levels. However, the precise cell parameter which correlates with PDT sensitivity remains poorly defined.

In order to clarify the issue of the relative PDT sensitivities of different cancer histologies, the major human lung cancer histologies (adenocarcinoma, small cell carcinoma, large cell carcinoma, squamous cell carcinoma, and mesothelioma) were studied *in vitro* under identical conditions and compared to the PDT sensitivity of normal human lung fibroblasts. Different cell parameters, such as cellular sensitizer uptake, total cell protein, and cell size were examined to determine whether any of these parameters correlated with the degree of PDT sensitivity.

MATERIALS AND METHODS

Drugs. Photofrin II was obtained from Photofrin Medical Inc. (Quadra Logic, Vancouver, British Columbia, Canada) as a sterile solution of 2.5 mg/ml dissolved in 0.9% NaCl solution.

Light Source. Exposure to light was accomplished by placing the Petri dishes on top of an X-ray viewbox with daylight bulbs (General Electric, Milwaukee, WI) filtered with a thin sheet of ruby red acetate (Visual Systems Inc., Rockville, MD). The emission spectrum of the light box ranged from 595–700 nM (10% peak intensity) with the peak at 630 nm. This was measured using a set of calibrated interference filters (Oriel Corporation, Stratford, CT; typically 50% transmission, 10 nm band width) and a radiometer (Model 550; EG&G, Salem, MA). The fluence rate as measured by a photometer (Model 1140; International Light, Newbury, MA) was 3.5 W/m². Cells were exposed to light for periods of time ranging from 1–15 min, resulting in delivered energies of 210–3150 J/m².

Cell Lines. The characteristics of the six human lung cancer cell lines and the normal human lung fibroblast cell line are shown in Table 1. NCI-H23, NCI-H841, NCI-H460, NCI-H520, and JMN were supplied by Dr. John Minna. A549 and CCL-210 were obtained from American Type Culture Collection (Rockville, MD). The malignant cell lines were grown in RPMI 1640 medium (Biofluids, Inc., Rockville, MD) supplemented with penicillin, streptomycin, glutamine, and 10% fetal calf serum. Cells were maintained in stock monolayer cultures at 37°C in a 5% carbon dioxide/95% air environment. The lines were passaged once per week except for NCI-H520 (squamous cell) which was passaged every 2 weeks. The media were changed between passes every 3–4 days. The normal human lung fibroblast line (CCL-210, American Type Culture Collection) was grown in Ham's F-12 medium (Biofluids, Inc.) supplemented with penicillin, streptomycin, glutamine, and 15% non-heat-inactivated fetal calf serum.

For the clonogenic cell survival assay and sensitizer uptake experiments, 6×10^5 to 1×10^6 cells were inoculated into 100-mm Petri dishes with 15 ml of growth media for all cell lines except NCI-H520, for which $1.5–2 \times 10^6$ cells were plated. After 48 h the media were removed and replaced with 10 ml of standard sensitizer media containing 25 μg/ml of Photofrin II and 1% fetal calf serum adjusted to pH 7.3. The cultures were handled in a dimly lighted room. The cells were incubated at 37°C for 2 h and then used for clonogenic cell survival assay and sensitizer uptake measurements. Controls for each experiment were cells exposed to light without sensitizer and cells exposed to sensitizer without light exposure.

Clonogenic Cell Survival Assay. Following a 2-h incubation in sensitizer, the media were removed, the plates were washed twice with PBS containing calcium and magnesium, and 10 ml of PBS was added to the plates. After light treatment, the PBS was removed and the cells were washed with PBS containing calcium and magnesium. The cells were trypsinized from the dishes, counted, diluted, and plated. Cells were plated in triplicate for each experimental point and each experiment was repeated a minimum of 3 times. The dilutions plated included 250 cells/dish, 5000 cells/dish, and 100,000 cells/dish. The dishes were incubated 7–21 days for macroscopic colony formation with a colony being defined as having 50 or more cells (11). The colonies were fixed

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[1] To whom requests for reprints should be addressed, at Head, Thoracic Oncology Section, Surgery Branch, National Cancer Institute, NIH, Building 10, Room 2B07, Bethesda, MD 20892.

[2] The abbreviations used are: PDT, photodynamic therapy; D_o, dose required to decrease surviving fraction to 37% along the exponential portion of the survival curve; Spearman correlation coefficient; *n*, extrapolation number; PBS, phosphate-buffered saline.

Table 1 *Cell line characteristics*

Cell Line	Histology	Plating[a] Efficiency (%)	Doubling Time (h)	Cell volume[b] (fl × 10^3)
A549	Adenocarcinoma	53 ± 4[c]	26.4	1.412 ± 0.069[c]
NCI-H23	Adenocarcinoma	14 ± 1	38.4	1.643 ± 0.025
NCI-H841	Small cell variant	21 ± 1	25.2	1.474 ± 0.081
NCI-H460	Large cell	25 ± 3	14.4	1.639 ± 0.060
JMN	Mesothelioma	40 ± 6[d]	24.0	2.359 ± 0.113
NCI-H520	Squamous cell	10 ± 2[d]	45.1	1.142 ± 0.076
CCL-210	Normal lung fibroblast	6 ± 1	30.0	1.967 ± 0.129

[a] Plating efficiency measured using clonogenic cell survival assay.
[b] Measured using a Coulter spectrum channel analyzer (P_2 = 0.002, Kruskal-Wallis test).
[c] Mean ± SEM.
[d] With addition of irradiated feeder cells to clonogenic cell survival plates to total cell density of 2 × 10^5.

in methanol:acetic acid mixed 3:1 and stained with crystal violet. Plating efficiency was defined as the ratio of the number of colonies counted to the number of cells plated. Surviving fraction was defined as the plating efficiency of treated cells divided by the plating efficiency of control cells. X-irradiated feeder cells were used with JMN (mesothelioma) and NCI-H520 (squamous cell carcinoma) because both lines exhibited poor plating efficiency at low cell density. A separate flask of non-PDT-treated cells (JMN or NCI-H520) was exposed to 20 Gy. The irradiated cells were then added to each serial dilution plate of the corresponding cell line so that the total cell density was 200,000 cells/dish. Irradiated cells plated alone in separate dishes resulted in no colony formation.

Sensitizer Uptake/Fluorescence. Following a 2-h incubation in sensitizer, the media were removed and the plates were washed twice with PBS containing calcium and magnesium, trypsinized, counted, and centrifuged at 300 × *g* for 5 min. The cells were disrupted by vigorous vortexing and the fluorescence of the pellet was extracted using acetic acid and saturated sodium acetate in a 3:1 mixture (12). Cellular debris was separated from the supernatant by centrifugation at 2300 × *g* for 20 min and the fluorescence of the supernatant read using a fluorescence spectrophotometer (Model 650–10S; Perkin Elmer, Norwalk, CT). Relative fluorescence was converted to mg of sensitizer/10^6 cells by comparison with a standard curve determined from known concentrations of sensitizer.

Protein Assay. For each cell line 1 × 10^6 cells/ml were disrupted by sonication for 10 s (4710 Series Ultrasonic homogenizer; Cole Parmer, Chicago, IL). The homogenate was centrifuged at 1100 × *g* for 5 min and the protein of the supernatant measured using the Bradford technique (13).

Cell Volume. Cell volume was measured using a cell size frequency analyzer (Channelyzer 256; Coulter Electronics, Hialeah, FL).

Statistics. All results are expressed as means ± standard error of the mean. The clonogenic cell survival curves for each cell line were fitted to the single hit/multitarget model using the Albright program (14). The light dose required for 1% survival (2 log cell kill) was calculated from these parameters. Survival parameters of the different cell lines were compared using a χ^2 test.

Individual group means were compared using the Kruskal-Wallis test (15). All *P* values reported are two sided. The degree of correlation between light dose required for 1% survival and each of the measured parameters was assessed using r_s (16). The degree of correlation between sensitizer uptake and cell protein and volume was similarly examined.

RESULTS

Clonogenic Cell Survival Curves. Survival curves for each cell line are shown in Fig. 1 and the calculated parameters for the single hit/multitarget model and the dose for 1% survival are shown in Table 2. χ^2 analysis of the survival parameters showed significant differences among the cell lines with A549 (adenocarcinoma) being the most resistant cell line and CCL-210 (normal lung fibroblast) the most sensitive line, with the other cell lines having intermediate sensitivities. Kruskal-Wallis test of the dose for 1% survival confirmed a significant difference among the cell lines (P_2 = 0.003). The relative resistance of A549 is not a general property of adenocarcinoma as NCI-H23, also an adenocarcinoma, was more sensitive to PDT than A549. NCI-H23 had a sensitivity comparable to several of the other cell lines (NCI-H841, NCI-H460, NCI-H520).

Sensitizer Uptake. The amount of sensitizer taken up by the cells after a 2-h sensitizer incubation is shown in Table 2. Sensitizer uptake was expressed in 3 ways: amount of sensitizer/10^6 cells, amount of sensitizer/mg cell protein, and amount of sensitizer/μm^3 cell volume. Kruskal-Wallis test of the sensitizer uptakes confirmed that there was a significant difference among the cell lines. CCL-210, which was the most sensitive cell line, took up the most sensitizer. However, there was no significant difference in sensitizer uptake between A549 and NCI-H460 (P = 0.16), yet H460 was much more sensitive to PDT. Therefore, PDT sensitivity did not correlate well with sensitizer uptake.

To statistically verify the lack of correlation between PDT sensitivity and sensitizer uptake, the r_s was calculated comparing the dose at 1% survival for each cell line *versus* sensitizer uptake per million cells, per mg protein, or per μm^3 cell volume. The values of r_s ranged from 0.43–0.57, none of which were statistically significant. Hence, no good correlation was seen between these parameters and sensitivity to PDT. Also, no correlation was seen between sensitizer uptake at 2 h and cell protein content (r_s = 0.32) or between sensitizer uptake and cell volume (r_s = 0).

Finally, the colony-forming ability of each cell line was evaluated against three measures of PDT sensitivity, the light dose at 1% surviving fraction, the extrapolation number *n*, and the survival curve D_0 (the inverse of the terminal slope of the PDT survival curve). The results of these comparisons are shown in Fig. 2. There was a much better correlation between the plating efficiency and light dose at 1% survival (R = 0.81) when compared to the extrapolation *n* (R = 0.46) or the D_0 (R = 0.37). Despite the disparity among these comparisons, the general tendency was that the cell lines with the lowest plating efficiency were the most sensitive to PDT treatment.

DISCUSSION

Our results demonstrate differences among human lung cancer lines and their sensitivity to PDT under controlled conditions. Although the finding that such differences exist is not surprising, little work has been done to examine the relative PDT sensitivities of human cell lines. Cohen *et al.* (10) examined the PDT sensitivity of 12 explanted human central nervous system tumors by measuring the sensitizer concentration required for 90% cytotoxicity at a constant light dose. After a 1-h incubation with hematoporphyrin derivative, a 5-fold variation among the cell lines in the amount of sensitizer required for 90% cytotoxicity was observed. Melloni *et al.* (15), using rhodamine 123 as a sensitizer in a 24-h incubation, showed significant differences in sensitivity among human colon, breast and melanoma cell lines to the sensitizer alone and in combination with light. None of these studies, however, have attempted to correlate inherent characteristics of the cells with PDT sensitivity.

Bohmer and Morstyn (9) have shown that different amounts of sensitizer were taken up by different cell lines under identical conditions. These differences were dependent upon the cell size, with larger cells taking up more sensitizer than small cells. Williams and Runge (8) have shown that different cell lines will

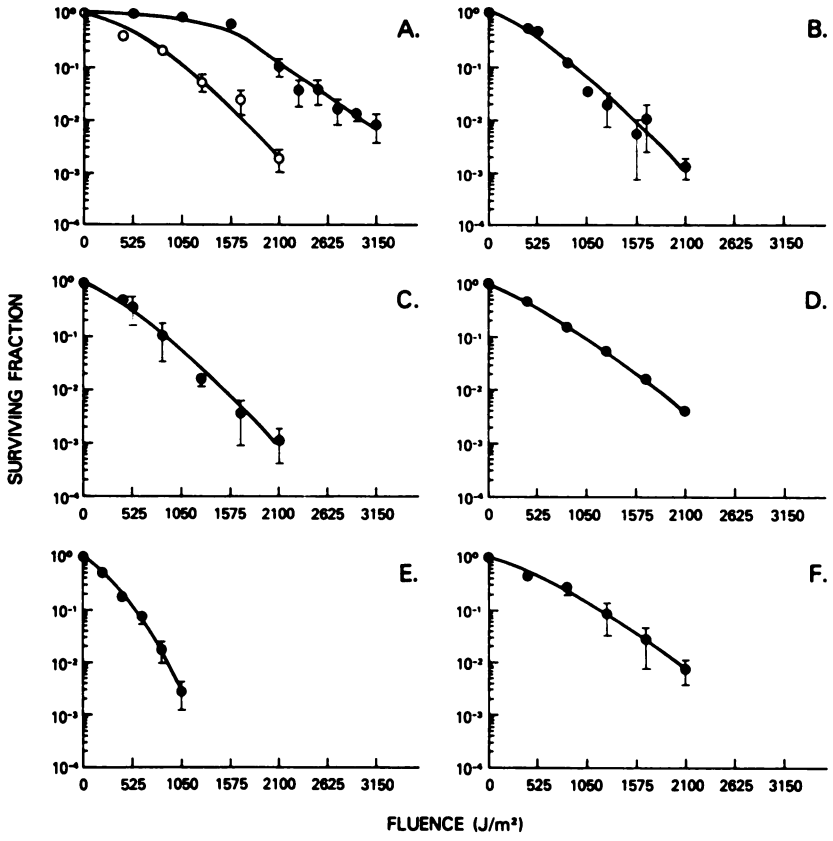

Fig. 1. Clonogenic cell survival curves for each cell line. *A*, A549 (●) and NCI-H23 (○), adenocarcinoma; *B*, NCI-H841, small cell variant; *C*, NCI-H460, large cell; *D*, JMN, mesothelioma; *E*, CCL-210, normal lung fibroblast; *F*, NCI-H520, squamous cell. *Bars*, the SEM of a minimum of 3 experiments. Significant differences are apparent among the cell lines. Although an adenocarcinoma (A549) was the most resistant to PDT, this is not a general property of adenocarcinoma as the NCI-H23 line was more sensitive than A549 (*A*).

Table 2 *Cell survival and fluorescence versus cell characteristics*

Cell line	n[a]	D_o (J/m²)[b]	Dose for 1% survival[c]	μg Sensitizer/10⁶ cells[d]	ng Sensitizer/mg protein[e]	ng Sensitizer/(μm)³ volume
A549	51.13 ± 37.00[f]	341 ± 31	2602 ± 147	0.98 ± 0.13	3.83 ± 0.52	0.72 ± 0.10
NCI-H23	11.96 ± 8.30	194 ± 19	1192 ± 109	1.68 ± 0.20	5.75 ± 0.68	1.03 ± 0.12
NCI-H841	2.64 ± 1.10	259 ± 16	1036 ± 41	1.80 ± 0.20	6.91 ± 0.77	1.26 ± 0.14
NCI-H460	4.45 ± 2.20	224 ± 15	1078 ± 78	0.69 ± 0.06	2.76 ± 0.24	0.43 ± 0.04
JMN	1.24 ± 0.17	378 ± 11	1503 ± 17	1.45 ± 0.13	4.41 ± 0.40	0.63 ± 0.06
NCI-H520	5.65 ± 2.30	353 ± 20	1121 ± 194	1.80 ± 0.24	8.67 ± 0.11	1.66 ± 0.22
CCL-210	2.38 ± 1.10	175 ± 11	473 ± 26	3.16 ± 0.17	9.32 ± 0.51	1.66 ± 0.09

[a] Extrapolation number (extrapolation of terminal slope of the survival curve to the y axis).
[b] Cells were incubated in 25 μg/ml of Photofrin-II for 2 h and then treated with 630 nm light to total energies of 0–3150 J/m². Viability was assessed at 7–21 days using the Albright program (14). D_o, dose to reduce survival to 37% along the terminal exponential portion of the survival curve.
[c] $P_2 = 0.003$ for at least one difference among the means (Kruskal-Wallis test).
[d] Cells were incubated in 25 μg/ml of Photofrin-II for 2 h. The sensitizer was extracted using acetic acid/saturated sodium acetate and read using a spectrophotometer ($P_2 = 0.004$, Kruskal-Wallis test).
[e] Protein measured using Bradford method (13) ($P_2 = 0.004$, Kruskal-Wallis test).
[f] Mean ± SEM.

take up different amounts of sensitizer. Our study showed differences between the PDT sensitivities of different human lung cancer lines. We then attempted to correlate these differences in sensitivity with measurable cell parameters such as sensitizer uptake, expressed per cell, per mg protein, and per cell volume. Although significant differences were noted among the cell lines and sensitizer uptake per million cells, per mg protein, and per cell volume, these differences did not correlate with differences in PDT sensitivity. It should be noted that such comparisons should be made with considerable caution, in that sensitizer uptake measurements were made using the entire population of cells, while the PDT sensitivity assessments represent only the proportion of cells that actually form colonies. The greatest concern lies with the cell lines that have low plating efficiencies. For these cell lines it is conceivable that the sensitizer uptake in the proportion of cells capable of forming colonies (those cells used to determine PDT sensitivity) differs from "average" sensitizer uptake of the entire population. Ideally, the appropriate way to correlate sensitizer uptake with PDT sensitivity would be to use only cell lines with 100% plating efficiencies, a rare characteristic of most human tumor cell lines in general. Another way to potentially resolve this issue would be to evaluate sensitizer uptake and PDT sensitivity in the same cell line under different conditions that yield a low and high plating efficiency. To our knowledge, at present there are few, if any, procedures to accomplish this objective.

The low plating efficiencies of some cell lines evaluated prompted yet another comparison. When the plating efficiency of the various cell lines was compared against PDT sensitivity, a general trend was observed. Cell lines with low plating efficiencies were found to be the most sensitive to PDT; however, this relationship was best correlated with the drug dose to yield 1% survival as opposed to the survival curve D_o or n values. The reason for this discrepancy is unclear. Whether this observation is a uniform finding among lung cancer cell lines or other human tumor cell lines will require further study. There is

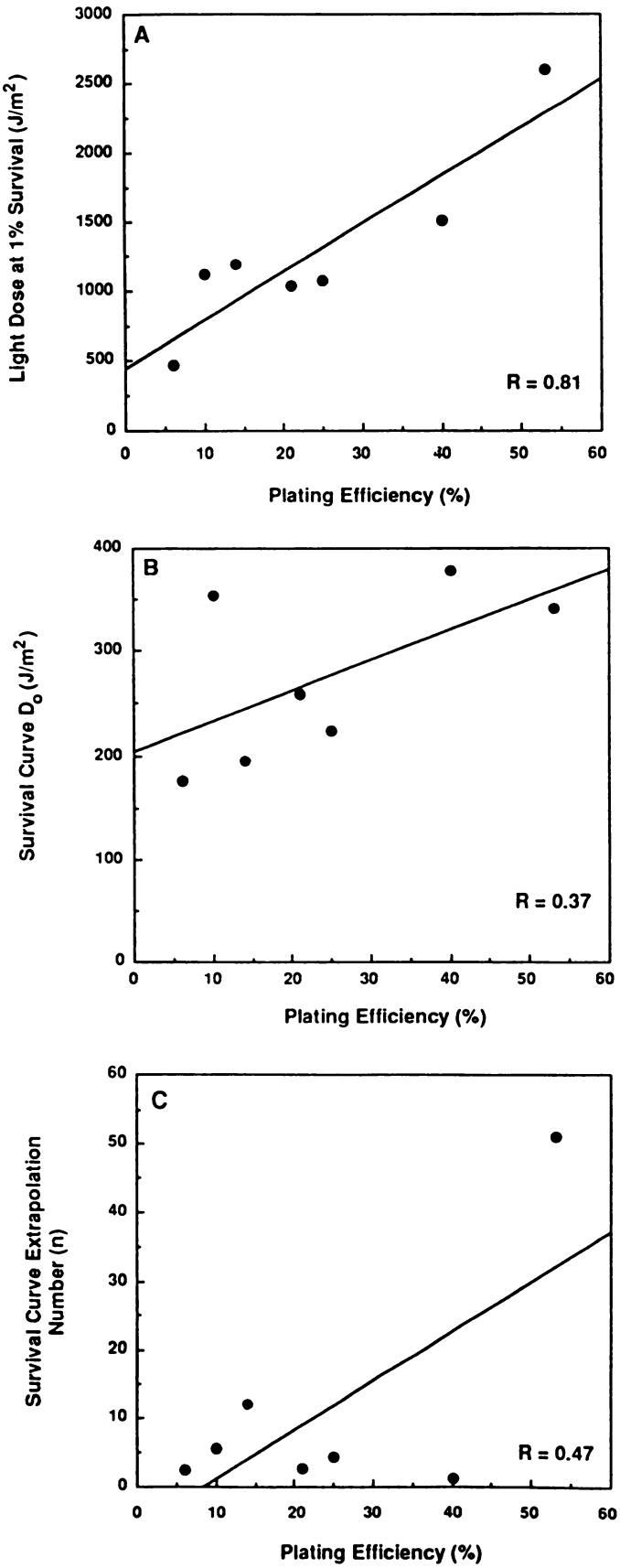

Fig. 2. Correlation of cell line plating efficiency with survival parameters after *in vitro* PDT including correlation with light dose at 1% survival (*A*, correlation with D_0 (*B*), and correlation with survival curve extrapolation *n* (*C*). Good correlation was noted with the light dose to yield 1% survival as an index of PDT effect. *R*, linear regression analysis correlation coefficient.

always a concern when conducting cell survival studies, regardless of the modality, when plating efficiencies are low. Whether the sensitivity to a given modality of the few percent of cells that actually are able to form colonies is representative of the entire population of cells is a question most difficult to answer. As mentioned above, the best way to gain insight into this potential problem would be to compare the PDT sensitivity of the same cell line under conditions that yield both high and low plating efficiencies. Resolution of this issue will require more work and perhaps refinement and improvement of cell culture techniques for human tumor cell lines. Alternatives to this experimental design to investigate the role of sensitizer concentration and inherent PDT differences could be performed. Instead of using a constant incubation time for each cell line, one could incubate each cell line for an appropriate interval with sensitizer concentrations to ensure that the final sensitizer concentration is equal in all cell lines. By exposing the cells to equal light energy, any resulting differences in survival could be attributed to "inherent differences." This approach, however, would not address the important issue of multiple cellular PDT targets (*i.e.*, nuclear membrane, plasma membrane, mitochondria, and lysosomes), and although total cellular fluorescence may be the same, differences in cellular organelle sensitizer uptake may account for survival differences. We have demonstrated this phenomenon of survival differences despite equal total cellular fluorescence in A549 cells when plateau levels of sensitizer uptake is reached (17). Thus, it would be necessary to define the organelle-sensitizer kinetics in such a study, possibly by using video fluorescence-imaging techniques with image analysis.

For the NCI-H460 cell line, it could be surmised that there may be a modest correlation between PDT sensitivity and sensitizer uptake. When examining the characteristics of this line, we noted that this cell line has a short doubling time compared with the other cell lines and it was a possibility that different distribution of cells in the cell cycle might influence the PDT sensitivity. Whether cell cycle variations influence the PDT response, however, remains an unanswered question. Moan *et al.* (18) showed that, for human NIHK 3025 cells, S phase cells were more sensitive (100-fold) than cells in G_1. However, Gomer and Smith (19) did not observe significant variation in PDT sensitivity with the cell cycle for Chinese hamster ovary cells. Hence, further work would be necessary to elucidate growth fraction differences with regard to these cell lines and the influence on PDT efficacy.

It is known that tumors *in situ* retain more hematoporphyrin derivative than normal tissue and for the most part all lung cancer types have been responsive to PDT (20); however, the degree of response (partial or complete) and duration of response are not well documented at present. These clinical findings are somewhat in conflict with the present *in vitro* findings. The normal lung fibroblast used as a control in this study exhibited the greatest dihematoporphyrin ether uptake and relative PDT sensitivity of all the cell lines studied. This cell line also had the lowest plating efficiency of the cell lines studied. While it may be desirable in comparative *in vitro* studies to include a "normal tissue" cell line, perhaps a lung fibroblast cell line is not representative of normal tissues evaluated thus far in clinical PDT. Unfortunately, there are no data evaluating normal thoracic organs for dihematoporphyrin ether retention and PDT sensitivity *in vivo*. Despite the qualitative clinical observation that all lung cancer cell types respond similarly to PDT, one cannot surmise that differential inherent tumor PDT sensitivities fail to exist *in vivo*. The fact that PDT treatment

opens "tumor-obstructed" airways does not address the ultimate histologic tumor response and the duration of PDT response. As clinical trials continue and protocols become more uniform, differences in PDT response among lung cancer histologies as measured by conventional oncological parameters may be revealed.

The findings of the present study show inherent differences in PDT sensitivities among different lung tumor types; however, these differences do not take into account that *in vitro* PDT sensitivity among different cell lines may relate to different targets (mitochondria or membranes) or differences in sensitizer receptor number or binding affinity, specifically for low density lipoprotein receptors (21). Moreover, the importance of *in vitro* PDT sensitivity studies, with their inability to have consistent predictive indicators of PDT efficacy, are difficult to extrapolate to *in vivo* data since differences in tumor vasculature (22) or the tumor extracellular milieu (23) can markedly influence PDT tumor response. Resolution of these issues will hopefully come as more is understood about the basic mechanisms of PDT action for both *in vitro* and *in vivo* systems.

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