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In Vitro Induction of PDT Resistance in HT29, HT1376 and SK-N-MC Cells by Various Photosensitizers[¶]

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ABSTRACT

Our approach to examine the mechanism(s) of action for photodynamic therapy (PDT) has been *via* the generation of PDT-resistant cell lines. In this study we used three human cell lines, namely, human colon adenocarcinoma (HT29), human bladder carcinoma and human neuroblastoma. The three photosensitizers used were Photofrin, Nile Blue A and aluminum phthalocyanine tetrasulfonate. The protocol for inducing resistance consisted of repeated *in vitro* photodynamic treatments with a photosensitizer to the 1–10%-survival level followed by regrowth of single surviving colonies. Varying degrees of resistance were observed. The three induced variants of the HT29 cell line were the most

extensively studied. Their ratios of increased survival at the LD90 level range between 1.5- and 2.62-fold more resistant.

INTRODUCTION Return to TOC

Photodynamic therapy (PDT)[†] is based on the photoactivation of the photosensitizer, *e.g.* Photofrin. Photofrin has been the most characterized and leading photosensitizer; its subcellular effects and localization continue to be extensively studied (1). Following short incubations it has been shown to localize in the plasma membrane, nuclear membrane and cytoplasm (2). After longer incubations it is found bound in the nuclear membrane, other organelles and especially the mitochondria that have been repeatedly implicated as the primary subcellular site of porphyrin localization (3–5). There are now many classes of second-generation photosensitizers with improved biochemical, pharmacological or photobiological properties.

Selection of aluminum phthalocyanine tetrasulfonate ($AlPcS_4$), Nile Blue A and Photofrin as the three photosensitizers to be used for the generation of induced-resistant cell lines was based on the localization properties for each of the different photosensitizers. Since the intracellular localization sites are unique, consequently the target(s) for direct tumor phototoxicity are also expected to be unique for each photosensitizer. As such, the selection of these distinct photosensitizers will generate tools to allow for the determination of the mechanisms involved in conferring induced resistance to each sensitizer.

The specific aims of this current project were: (1) to generate resistant variants; and (2) to elucidate the mechanism(s) by which PDT resistance is induced by the three photosensitizers listed above in each of three human tumor cell lines. The three tumor cell lines selected were human neuroblastoma (SK-N-MC), human colon adenocarcinoma (HT29) and human bladder carcinoma (HT1376). These cell lines were chosen because induced resistance in human tumor cell lines is of clinical relevance, and each tissue type is suitable for PDT. Multiple cell lines were used because there is a large body of information indicating that there are large differences in inherent sensitivity for different organs, tissues and individuals. By comparing these three cell lines in both their inherent sensitivity as well as their ability to become resistant it is hoped that general principles may be extracted concerning the mechanisms and degrees of possible induced resistance.

The use of multiple photosensitizers, combined with multiple cell lines, will allow for the identification of cell line–specific or sensitizer-specific changes involved in resistance. The generation of resistant variants will enable investigators to understand the molecular mechanism(s) of sensitivity to various photosensitizers based on inherent and induced resistance in various cell lines. This is the first report of a comprehensive study of the generation of various resistant variants to three unique photosensitizers.

MATERIALS AND METHODS Return to TOC

Materials. Fetal calf serum, 10× trypsin/ethylenediamine-tetraacetic acid (EDTA), alpha-minimal essential medium, penicillin and streptomycin were purchased from GIBCO BRL (Mississauga, Ontario, Canada). Nile Blue A (purity >88%) was purchased from Sigma (St. Louis, MO). Photofrin® was from Quadralogic Technologies Inc. (Vancouver, British Columbia, Canada). Photofrin is a purified porphyrin mixture. $AlPcS_4$ (purity >97%) was from Porphyrin Products (Logan, UT).

Cells and culture conditions. HT29, HT1376 and SK-N-MC were obtained from ATCC (Rockville, MD). They were grown as monolayers in alpha–minimum essential medium plus deoxyribonucleoside and ribonucleosides, supplemented with 10% fetal calf serum, 1% penicillin and streptomycin. All the cells were maintained in an incubator at 37°C with 5% CO_2 at 90% humidity. Doubling times for all the cells were obtained from the linear portion of their growth curves. Cell cultures used for all experiments were 48 h cultures, at which time exponential growth was achieved.

In vitro photosensitization and colony-forming assay. Preconfluent cells were removed from the culture dish by 5 min incubation in 5×; trypsin/EDTA. After the cells were spun at 1000 rpm for 5 min, they were resuspended and counted, and appropriate dilutions were made to obtain 4×10^2 cells/mL. One milliliter of this cell suspension was added to wells of 6-well tissue-culture plates (Falcon, North Yorkshire, UK) containing 1 mL of medium. Cells were allowed to adhere for 4–6 h, at which time the various doses of Nile Blue A, Photofrin or $AlPcS_4$ in 1 mL of medium were added; the control cells received 1 mL of medium only. Cells were drugged and after 1 h (Nile Blue A) or 18 h (Photofrin or $AlPcS_4$), the medium was removed and replaced with fresh medium. The cells were then irradiated on a 100×50 cm^2 light-diffusing surface, illuminated by a bank of fluorescent tubes (Philip type TL/83), filtered with red acetate filters (Rossolux No. 19; ROSCO, CA) to provide wide-band illumination above 585 nm. The energy fluence rate was 9.2 W/m^2 in the wavelength 525–700 nm, representing 12% of the total filtered output. Irradiation for 15 min (Nile Blue–treated cells), 5 min (Photofrin-treated cells) and 10 min ($AlPcS_4$-treated cells) resulted in an incident energy fluence of 8.1×10^3, 2.7×10^3 and 5.4×10^3 J/m^2, respectively. All procedures were carried out in minimal ambient light condition after plating the cells. After 5 days of undisturbed growth in the dark the colonies were stained with 0.08% methylene blue in 70% methanol. Colonies containing >20 cells were counted under an inverted microscope. For Photofrin II and $AlPcS_4$ PDT the percentage survival is expressed relative to the average of three controls, namely, no drug no light, drug no light and no drug light. Nile Blue A showed considerable dark toxicity, and therefore two separate control conditions were used for each experiment (no drug no light as well as drug no light) instead of the averaged control. (All three of these controls did not differ significantly from each other for Photofrin or $AlPcS_4$.)

Protocol for inducing resistance. In brief, HT29, HT1376 and SK-N-MC cells were subjected to series of incubations with Nile Blue A, Photofrin or

$AlPcS_4$ and light irradiation treatment as described above. Multiple cultures from single surviving colonies were harvested and regrown as separate cultures that served as stock for subsequent treatment–regrowth cycles. Each cycle of the treatment was aimed at achieving survival levels in the 1–10% range, based on survival assays performed in the previous cycle. Multiple cultures from single surviving colonies were grown and tested at each selection cycle. PDT-resistant variants were selected according to colony-forming assay. This protocol is also explained in detail elsewhere (6).

Cycles of PDT treatments to induce resistance in HT29 cells are summarized in Table 1, and similar strategies were used in PDT induction in HT1376 and SK-N-MC cells. For the first five cycles of PDT induction the strategy was to use a lower dose of drug and a longer incubation time. After five cycles the drug dose was increased, but the incubation period was shortened.

Statistical analysis. All values reported are mean ± standard error of mean or standard deviation. A Paired Student's *t*-test was used to determine statistical significance. *P* values <0.05 are considered significant. The D_{10} (dose of 10% cell survival) was calculated using the second-degree polynomial fit: $Dc = Bo + B1(S) + B2(S)^2$, where Dc is the drug concentration, Bo, B1 and B2 are constants and S is the percent survival. The D_{10} ratio (resistant/parental cells) was then determined. Cells with over 1.5-fold increase in PDT resistance were considered as PDT-resistant variants.

RESULTS Return to TOC

Intrinsic sensitivity

The three human cell lines used to induce PDT resistance were first characterized for their intrinsic resistance to treatment. We examined their relative intrinsic sensitivity to various photosensitizers in comparison to each other. Figure 1a shows representative clonogenic survival curves for the three parent cell lines, where survival is plotted as a function of Nile Blue A concentration for 1 h incubation and fixed-light fluence. Nile blue A showed considerable dark toxicity, and therefore two separate control conditions were used for each experiment (no drug no light as well as drug no light). HT29 was found to be the most resistant to treatment for all the doses tested. The HT1376 cells had slightly greater resistance than the SK-N-MC cells which were the most sensitive to PDT using this sensitizer.

Figure 1b shows representative clonogenic survival curves for these three parental cell lines, where survival is plotted as a function of Photofrin concentration for 18 h incubation and fixed-light fluence. The 100% survival was the average of three control conditions: no Photofrin and no light; 15 μg/mL Photofrin and no light; and no Photofrin and light There was no significant difference between these three controls. HT1376 cells were the most resistant to the treatment for all the doses tested. An intriguing finding showed that in comparing HT29 and SK-N-MC, at doses lower than LD90, the SK-N-MC cells were more resistant, but at higher doses their relative

resistance was the reverse. Nevertheless, both were reduced to a survival level of 0.1 at a dose of 15 μg/mL.

A representative clonogenic survival curve for 18 h incubation in varying concentrations of $AlPcS_4$ is shown in Fig. 1c. Here again the 100% survival is the average of the three control conditions cited above. For this photosensitizer SK-N-MC cells showed the greatest colony-forming ability for all the doses tested, HT29 cells were found to have intermediate resistance, while HT1376 cells were the most sensitive to the treatment.

Figure 1a–c highlights the variation intrinsic to *in vitro* photosensitization. For each sensitizer used a different cell line was found to be the most resistant. These experiments were conducted numerous times in order to test the reproducibility of these results. Although there was considerable experiment-to-experiment variation in the survival curves, the resistance patterns represented in the graphs were consistently observed in every experiment.

Induction of resistance

Resistant variants of HT29, HT1376 and SK-N-MC were developed to the three photosensitizers, namely, Nile Blue A, Photofrin and $AlPcS_4$ in each of the three cell lines. For every experiment each condition was measured in triplicate, and each experiment was repeated three times. Again, experiment-to-experiment variation was found, but the displayed resistance patterns were consistently observed in every experiment. The 100% survival in each case was the average of three different control conditions: no drug and no light; drug and no light; and no drug and light. There were no significant differences found between these three controls for Photofrin and $AlPcS_4$; however, considerable drug toxicity was found in the cells treated with Nile Blue A. For this reason there are two Nile Blue A survival curves; Fig. 2a,c,e shows the effect of drug incubation alone, while Fig. 2b,d,f shows the colony-forming ability after *in vitro* photosensitization.

For PDT induction in HT29 cells a progressive and incremental change in the clonogenic response was observed with most cycles of treatment and regrowth. The final PDT-induced variants were designated HT29/N8, HT29/P10 and HT29/A11. These were obtained after 8, 10 and 11 cycles of PDT using Nile Blue A, Photofrin and $AlPcS_4$ in HT29 cells, respectively. Colony-forming assay was carried out to quantify the difference of PDT resistance between these variants and their parental HT29 cells. The cells were PDT treated using increasing concentrations of Nile Blue A (Fig. 2a,b), Photofrin (Fig. 3a) or $AlPcS_4$ (Fig. 3b). It was found that for each PDT dose studied the survival of *in vitro* photosensitized variants was greater than in HT29 cells. Colony-forming assays using parental and induced variants were analyzed, and the determination of relative resistance at the 10%-survival level (LD90) for the parental cells showed that the HT29/N8, HT29/P10 and HT29/A11 variants all meet our criteria for designation as “resistant.” To be termed resistant the cells had to show a 1.5-fold increase in resistance relative to the parental line. The HT29/N8 line showed the highest level of induced

resistance and had a 2.62-fold increase in clonogenic survival, the HT29/P10 cells had a 1.5-fold increase and the HT29/A11 cells were found to be 2.16-fold more resistant than the HT29 cells to PDT. Thus, all the HT29 variants are considered PDT resistant and as such are currently being used for further analysis.

The HT1376 cells were treated identically to the HT29 cells in an attempt to generate resistant variants. Only one resistant variant could be induced using this line. The resistant variant HT1376/N8 was obtained after eight cycles of PDT using Nile Blue A and can be seen in Fig. 2c,d. It must be noted that the induced resistance appears to be toward the drug itself and not necessarily toward photosensitization. Measurements at the 10%-survival level (LD90) showed HT1376/N8 cells to be 2.81-fold more resistant than HT1376 cells to *in vitro* photosensitization. Attempts to induce resistance in the variants HT1376/P10 and HT1376/A8 were stopped at 10 and 8 cycles of *in vitro* photosensitization using Photofrin or $AlPcS_4$, respectively. The survival curves for these two variants and the parental line HT1376 are displayed in Fig. 3c,d. Neither cell line had an increase in resistance of 1.5-fold, necessary for their designation as resistant variants.

Finally all three of the variants derived from the SK-N-MC parent cell line went through nine cycles of PDT in an attempt to induce resistance. These cells are called SK-N-MC/N9, SK-N-MC/P9 and SK-N-MC/A9. The photosensitizers used were Nile Blue A, Photofrin and $AlPcS_4$, respectively. After these nine cycles of PDT all three of the variants had failed to show a 1.5-fold increase in resistance, and therefore investigation into this cell line and its variants was terminated. Representative clonogenic survival curves for these cells using Nile Blue A can be seen in Fig. 2e,f. The survival curves for these cells using Photofrin and $AlPcS_4$ can be seen in Fig. 3e,f, respectively.

Table 2 shows the LD90 ratio (resistant *versus* parental cells) for each of the nine variants that were developed. An asterisk denotes the four resistant variants.

DISCUSSION Return to TOC

The primary goal of this work was to derive PDT-resistant HT29, HT1376 and SK-N-MC cells for future use as a tool in the ongoing study of PDT mechanisms and resistance. This has been achieved for the HT29 cell line and partially for the HT1376 cell line. The degree of resistance in most of these variants is of clinical relevance and may potentially yield valuable information in elucidating the mechanism of action for various photosensitizers.

In characterizing the intrinsic levels of resistance in the three parental cell lines large variations were found. Variability in sensitivity to a single photosensitizer for different cell lines is not surprising. However, the different relative rankings with respect to resistance are very interesting and highlight

the importance of appropriate photosensitizer selection. Moreover, it correlates with our current understanding that the mechanisms and pathways of cellular death are sensitizer specific. In a recent review the relative survival for various cell lines was also shown to be dependent on the sensitizer used (1).

For the HT29 cell line we were able to create resistant variants for all three photosensitizers. It is not apparent from either the initial clonogenic survival curves or any properties intrinsic to this cell line why resistance was possible here and not in the other two cell lines. HT29 was significantly more resistant to Nile Blue A–mediated PDT but displayed intermediate sensitivity to the other sensitizers. As such, we were unable to identify any characteristics of the three cell lines that were predictive of their ability to generate resistant variants upon repeated PDT action.

The levels of resistance generated ranged from a 1.5- to 2.62-fold increase in survival at the LD90 level. It is interesting that the two largest inductions of resistance were found using Nile Blue A. Since just four resistant variants (HT29/N8, HT29/A11, HT29/P10 and HT1376/N8) were generated in total, it would not be prudent to attempt to extrapolate from this interesting finding. It is difficult to pinpoint why some cell lines did not change in their levels of sensitivity with the repeated cycles of PDT. It must be assumed that there was no significant variation in cellular sensitivity within the population, which could have been exploited by our repeated treatments. Moreover, no mutations occurred that conferred a selective advantage during our repeated treatments. Conversely, it is apparent that either a variation within the population or a selectively advantageous mutation is what facilitated the development of the resistant variants.

Previously, Gomer's group (7) developed radiation induced fibroblast (RIF) variants that were 1.2–1.4-fold more resistant, whereas our group generated RIF cells with an approximately two-fold increase in resistance (6). Both these numbers are in the same order as these new human resistant variants. From these three independent experiments it appears that the generation of resistance, although possible, is not usually very large.

In conclusion, we have demonstrated that the relative resistance to PDT for these three human tumor cell lines is photosensitizer specific. Moreover, resistance to PDT *in vitro* can be achieved in some human tumor cell lines. The different variants of the HT29 cell line that have been developed are now being used to make comparative measurements to investigate the specific mechanisms of photodynamic action *in vitro.* Future work will include more in-depth characterization of the cell lines, studies of drug targeting and binding, differential displays and comparisons of stress-response pathways in both the sensitive and the resistant cell lines.

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TABLES Return to TOC

Table 1. Cycles of PDT treatment to induce resistance in HT29 cells

Nile Blue A (light fluence of 8.1 × 10^3 J/m^2)			Photofrin (light fluence of 2.7 × 10^3 J/m^2)			$AlPcS_4$(light fluence of 5.4 × 10^3 J/m^2)		
Cycle no.	Conc. (μg/mL)	Incubation time (min)	Cycle no.	Conc. (μg/mL)	Incubation time (h)	Cycle no.	Conc. (μg/mL)	Incubation time (h)
1–3	1	60	1–3	10	18	1–3	25	18
4, 5	1.5	60	4, 5	16	18	4, 5	30	18
6, 7	1.8	30	6, 7	30	4	6, 7	35	4
8	3	30	8, 9	60	4	8, 9	40	4
			10–14	100	4	10, 11	60	4

Table 2. LD90 ratio (resistant *versus* parental cells). Ratio ± SE for three experiments

Cell lines	LD90 ratio	Sensitizer used in PDT
HT29, HT29/N8	2.62 ± 0.265*	Nile Blue A
HT29, HT29/P10	1.502 ± 0.117*	Photofrin
HT29, HT29/A11	2.16 ± 0.42*	$AlPcS_4$
HT1376, HT1376/N8	2.81 ± 0.36*	Nile Blue A
HT1376, HT1376/P10	1.15 ± 0.04	Photofrin
HT1376, HT1376/A8	0.99 ± 0.02	$AlPcS_4$
SK-N-MC, SK-N-MC/N9	1.08 ± 0.07	Nile Blue A
SK-N-MC, SK-N-MC/P9	0.99 ± 0.032	Photofrin
SK-N-MC, SK-N-MC/A9	1.11 ± 0.041	$AlPcS_4$

*Cells that display greater than 1.5-fold increase in survival and were therefore denoted as resistant.

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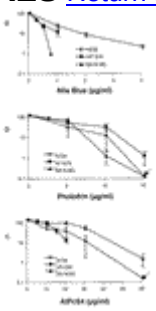

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Figure 1. Intrinsic sensitivity to PDT in HT29, HT1376 and SK-N-MC cells. Colony-forming assay was performed in HT29 ( ), HT1376 ( ) and SK-N-MC ( ) cells. Clonogenic survival curves represent the percentage survival in response to PDT using (a) Nile Blue A–; (b) $AlPcS_4$-; and (c) Photofrin-mediated PDT in these cells. The arrow ( ) indicates zero surviving colonies. Each data point is the average and SD from one experiment done in triplicate. Each experiment was repeated three times

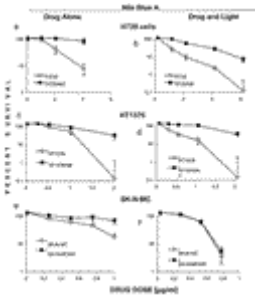

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Figure 2. Clonogenic survival curves for HT29, HT1376 and SK-N-MC and their resistant variants. PDT-sensitive ( ) and -resistant variant ( ). Cells were treated with varying drug concentrations of the three photosensitizers before exposure to light. Each data point is the average and SE of one experiment done in triplicate. Each experiment was repeated three times.

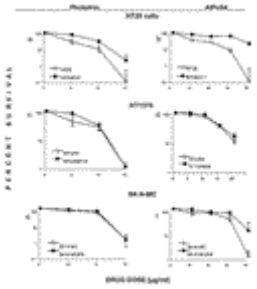

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Figure 3. Clonogenic survival curves for HT29, HT1376 and SK-N-MC and their resistant variants. PDT-sensitive ( ) and -resistant variant ( ). Cells were treated with varying drug concentrations of the Photofrin or $AlPcS_4$ photosensitizers before exposure to light. These two photosensitizers did not have significant dark toxicity (not shown). Each data point is the average and SD of one experiment performed in triplicate. Each experiment was repeated four times

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†*Abbreviations:* $AlPcS_4$, aluminum phthalocyanine tetrasulfonate; EDTA, ethylenediamine-tetraacetic acid; HT1376, human bladder carcinoma; HT29, human colon adenocarcinoma; PDT, photodynamic therapy; SK-N-MC, human neuroblastoma.