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PHOTODYNAMIC TOXICITY OF PORPHYRINS AND CHLORINS FOR A HUMAN TUMOR CELL LINE: COMBINED LIGHT AND CONCENTRATION DOSE RESPONSES FOR THE RETAINED FRACTION

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In recent years porphyrins and related materials have been tested as antitumor agents. A technique was devised to obtain dose-response curves for the sensitizer fraction that resists one day of elution by tissue culture medium -- the retained fraction. We found a steep "threshold" dose response relationship that helps to explain tumor destruction without damage to normal tissues. The family of dose-response curves produced by a wide range of light exposures suggests that chlorins and porphyrins do not act by identical mechanisms. Moreover, they suggest that chlorins will prove superior in practical use.

While many dyes can be used to demonstrate photodynamic toxicity, contemporary interest centers on porphyrins and chlorins, since certain species are selectively retained in tumor tissue. They are also activated by red light, which penetrates tissue to a greater depth than shorter wavelengths. These sensitizers are thought to act through a photodynamic mechanism mediated by singlet oxygen (1). For a dose response study, sensitizer, light, oxygen, and quenchers are all important variables. This situation is more complex than traditional pharmacologic dose response studies.

A dose response curve in radiation biology is normally plotted as cloning efficiency versus radiation dose. In this communication we utilize

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a modified approach appropriate for porphyrins and related sensitizers. The selective antineoplastic effect depends on a retained fraction, since light exposure is delayed to allow clearance of unbound material. An in-vitro study of the dose-reponse relationship should allow an uptake interval and an elution interval to approximate the in-vivo phenomena. The initial sensitizer dose and the light exposure should both be varied; this requirement makes conventional technique laborious. We utilized 96-well microtest trays and estimated viable cells by reading the absorbance of a vital stain. Results strongly suggest that porphyrins and chlorins act by somewhat different mechanisms, and the observed differences favor chlorins in practical usage.

Materials and Methods

Hematoporphyrin Derivative was prepared by the method of Lipson et al. (2). Chlorin e_6 was purchased from Porphyrin Products (Logan, UT) and crude non-coppered sodium chlorophyllins (Chlorophyll MM®) was a gift of the Bush, Boake, and Allen company (London, U.K.). The cutoff filter was Hoya Optics (Fremont, CA) type R60. Neutral Red solution at 0.33% was Sigma Chemical (St. Louis, MO) Cat. N2889; Sigma also supplied Basal Medium Eagle (Cat. B9638). The human colon carcinoma HT-29 was obtained from the American Type Culture Collection (Rockville, MD). Fetal Bovine Serum from Hyclone (Logan, UT) was added to 5%. No antibiotics, organic buffers, or trace elements were added. Freedom from mycoplasma was tested by cultivation on agar. A Dynatech MR-580 plate scanner was used.

Microtest wells were seeded with 5,000 HT29 in 100 µl of medium. After one day, the medium was replaced with a 70 µl aliquot using an adjustable 8-channel pipetting device. Working under dim light, the medium in the first column was replaced with 239 µl of medium containing 100 µg/ml of sensitizer. Then 169 µl was transferred to the next column. The tips were changed and dilution continued to the tenth column, leaving two columns as controls. The dilution ratio of 239/169 is 1:1.4142 -- a 1:2 dilution step is obtained in every second column. Trays were inclubated in a 5% CO_2 atmosphere at 37° C, using covered metal pans to ensure darkness. The sensitizer aliquots were replaced with 100 µl of medium after 48 hours, and another day was allowed for elution of unbound material. After 72 hours trays were exposed through the bottom to filtered light (cutoff at 600 nm) from a 300-W slide projector. The light was projected through the inner glass door of an incubator and reflected upward to a tray on a glass plate. The

trays were exposed to light for 0, 4, 16, 64, or 256 minutes, and returned to incubation. After 96 hours viable cells were stained for two hours with Neutral Red diluted 1:100 into medium. It is important to use warm medium at about neutral pH, since otherwise the stain may form crystals during incubation. A brief spin in a bench-top centrifuge was included to remove seed crystals from the stain solution. After staining, excess Neutral Red was removed by two 180 µl rinses of Dulbecco's Phosphate Buffered Saline. Cell-associated stain was eluted with 150 µl of methanol buffered with 25% (v/v) of 100 mM citrate-sodium citrate at pH 4.2. The Dynatech reader wavelengths were 540 nm test and 630 nm reference.

Results

Absorbance readings are given as the percent survival, with control wells as 100%. It has long been known that neutral red is taken up in proportion to cell number (3). Kull and Cuatrecasas found that neutral red is appropriate for assays of proliferation and survival (4).

Conventional studies depict biological response versus the $\log_{10}$ of agent concentration over a wide dose range. Here, even 1:2 dilutions are too wide, so square-root steps were devised to locate the threshold of lethality. Vital staining shows a sharp threshold for both crude porphyrins (figure 1) and pure or crude chlorins (figures 2 and 3). Dark toxicity is less abrupt. A modest growth stimulation at subtoxic concentrations is often seen in this system.

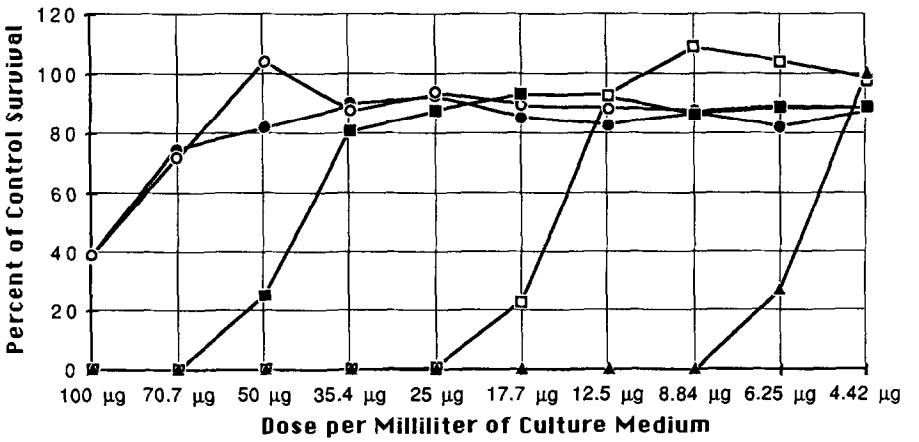

Figure 1. Dose responses for Hematoporphyrin Derivative. Trays were left dark (●), exposed for 4 minutes (○), 16 minutes (■), 64 minutes (□), or 256 minutes (▲).

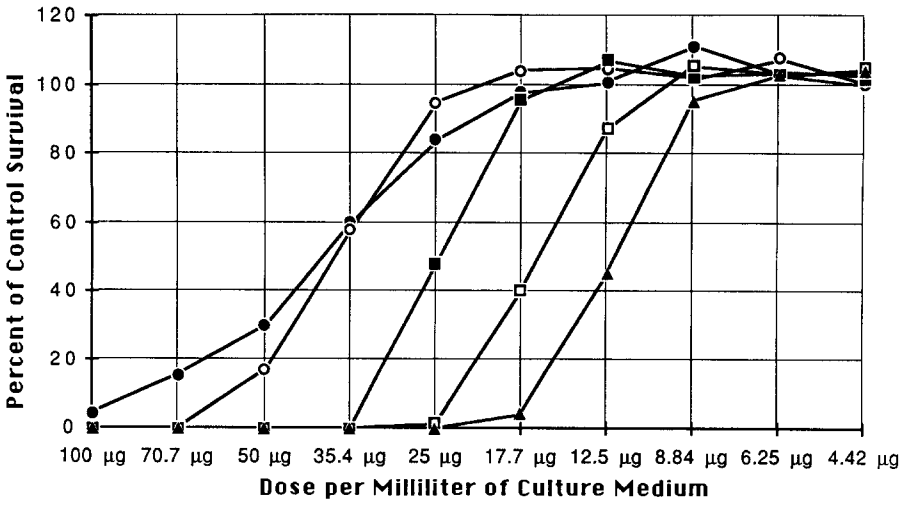

Figure 2. Dose responses for Chlorin e_6. Symbols as in figure 1.

The family of curves show that more light is required to reach the threshold as sensitizer is reduced, and an obvious difference between porphyrins and chlorins appears in this relationship. To reach the threshold with chlorins requires sixteen times more light when the concentration is reduced by one-half. This fourth-power change means that the light dose required rises very sharply as the chlorin dose declines. Hematoporphyrin Derivative is distinctly different. The relationship is more complex -- between linear and second-power.

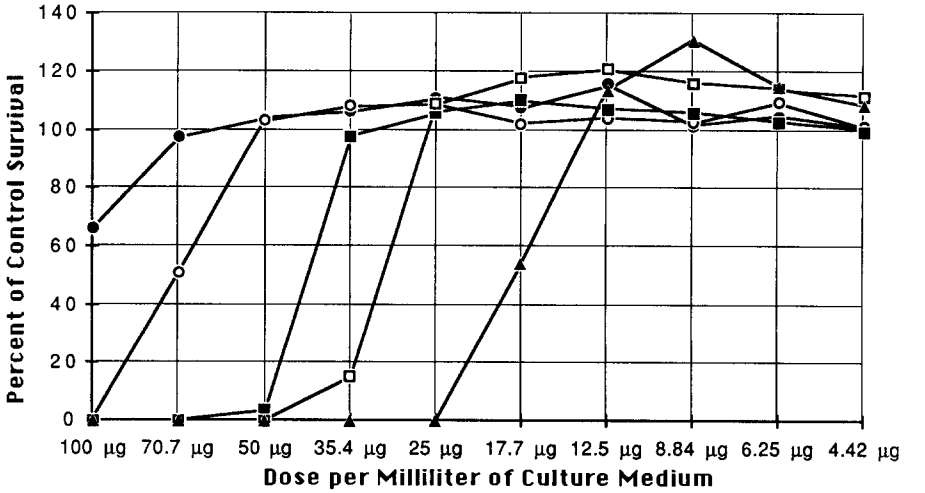

Figure 3. Dose responses for crude chlorophyllins. Symbols as in figure 1.

Discussion

A technique with automation potential is needed to study the variables that control photodynamic toxicity. Our approach uses a 5 step sequence -- seeding, sensitizing, eluting, exposing, and reading -- and requires 96 hours of incubation. The initial 5000 cells grow throughout this period. The results represent duplicate or triplicate rows.

Chlorin e_6 is the parent of NPe6, a candidate drug. The structure is given in a recent report that also shows excellent murine tumor cures without cutaneous photosensitization (5). We compared the pure chlorin with crude non-coppered sodium chlorophyllins, which were shown to be a complex mixture by high-performance liquid chromatography (data not shown). The similar dose-response data suggests that chlorin e_6 is not unique -- such behavior may be generic to chlorins.

A selective antineoplastic effect by either class would be facilitated by the threshold dose response. After time allowed for clearance, even relatively small differences in retention would allow tumor destruction without exceeding the toxic threshold for surrounding tissue. The threshold probably represents the limit of cellular quenching or repair activity.

We do not advance a mechanistic explaination for the complex agent dose vs. light dose relationship revealed in this study. The results do suggest that porphyrins and chlorins act by different mechanisms. Since cutaneous photosensitization is a problem in clinical use, the results imply that, if other aspects are equal, chlorins are superior. The fourth power increase in the light required for toxicity means that chlorin clearance would pass below the level that can be activated by sunlight long before porphyrins.

We are now extending these findings to other cell lines and culture conditions with altered oxygen or quencher concentrations. The microtest approach will allow structure-activity relationship studies that include the important variables of photodynamic toxicity. With appropriate

development, it should be possible to perform in-vitro studies that model phenomena observed in-vivo.

References

1. Thomas, J. P., Hall, R. D., and Girotti, A. W. (1987) Can. Lett. 35; 295-302.
2. Lipson, R. L., Balden, F. J., and Olsen, A. M. (1961) J. Nat'l. Can. Inst. 26; 1-11.
3. Pidot, A. L. R. (1971) Appl. Microb. 22; 671-677.
4. Kull, F. C., and Cuatrecasas, P. (1983) Appl. Biochem. Biotechnol. 8; 97-103.
5. Nelson, J. S., Roberts, W. G., and Berns, M. W. (1987) Can. Res. 47; 4681-4685.