Phototoxic effects of 635-nm light on canine transitional cell carcinoma cells incubated with 5-aminolevulinic acid

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Objective—To determine whether transitional cell carcinoma (TCC) cells incubated in media containing 5-aminolevulinic acid (ALA) would produce sufficient protoporphyrin IX (PpIX) to cause lethal phototoxic effects when exposed to 635-nm light.

Sample Population—Canine TCC cells (K9TCC).

Procedure—Cultured K9TCC cells were exposed to graded doses of ALA, and PpIX concentrations were determined. Cells then were exposed to various doses of 635-nm light from a diode laser, and cell viability was assayed.

Results—Production of PpIX was dependent on time and dose of ALA. The K9TCC cells incubated with ALA produced sufficient PpIX to cause lethal phototoxic effects when exposed to 635-nm light. Phototoxic effects were dependent on time and dose of ALA. Increasing laser power density and energy density decreased cell survival.

Conclusions and Clinical Relevance—ALA is an effective photosensitizer for in vitro photodynamic treatment of K9TCC cells. Further studies are warranted to assess the safety and efficacy of ALA as a photosensitizer for use in treating dogs with TCC.

Impact for Human Medicine—On the basis of this study, dogs with TCC may be useful in the development of protocols for ALA-based photodynamic therapy of humans affected with muscle-invasive bladder cancer. (*Am J Vet Res* 2003;64:131–136)

Transitional cell carcinoma (TCC) is the most common malignancy of the urinary bladder in dogs, but these tumors represent only 2% of all cancers in dogs.[1] Conventional treatment for dogs with TCC, including surgery, chemotherapy, and radiation therapy, fails to induce durable remission in affected dogs, although palliation of clinical signs is possible.[1-4] A nonsteroidal anti-inflammatory drug, piroxicam, has been introduced as an alternative treatment for dogs with TCC, resulting in a median survival time of 181 days.[5]

Because TCCs in dogs share many characteristics of invasive bladder carcinomas in humans, they are becoming recognized as a tumor model for evaluation of novel treatment methods.[6,7] One potentially useful method for treatment of humans with bladder carcinoma is **photodynamic therapy (PDT)**, which has been used to treat a limited number of humans with bladder cancer.[8-10] However, PDT has not been widely embraced because of variable tumor responses,[11] documenting the need for a good model in animals that can be used to develop efficacious treatment protocols. Because dogs are of sufficient size to allow use of the same equipment and dosimetry as that used for people, they are good models for preclinical evaluations of PDT protocols.[12]

Photodynamic therapy typically involves administration of a tumor-localizing photosensitizer followed by activation with light of the appropriate wavelength.[13] The ensuing photochemical reaction generates oxidative damage sufficient to directly kill tumor cells, causes vascular collapse and ischemic tumor death, and incites an inflammatory reaction that results in nonspecific killing of tumor cells.[14,15] Variability in localization of photosensitizer within tissues has been reported,[16] and bladder fibrosis and decreased bladder capacity have been observed in healthy dogs after whole-bladder PDT, presumably as a result of nonspecific photosensitizer reactions.[17] Another potential complication of PDT in the urinary bladder is full-thickness necrosis of the bladder wall. Therefore, it is important that photochemical reactions be confined to the tumor during bladder PDT.

A concept that may prove useful for bladder PDT is stimulating tumor cells to synthesize a photosensitizer intracellularly. This has been accomplished by exogenous administration of **5-aminolevulinic acid (ALA)**, a naturally occurring component of the heme biosynthetic pathway.[18,19] Although ALA does not react to photostimulation, it is rapidly converted by malignant epithelial cells to **protoporphyrin IX (PpIX)**, a highly efficient photosensitizer.[20]

The ALA-induced accumulation of PpIX is minimal in mesenchymal tissues[21,22]; therefore, the likelihood of unwanted photochemical reactions in the muscularis layer resulting in full-thickness necrosis is decreased, making ALA a good candidate for urinary bladder PDT. Furthermore, PpIX is retained intracellulary,[18] contrary to the perivascular accumulation of some preformed photosensitizers.[16] Similarly, ALA can be administered topically, IV, or parenterally.[23] In addition, ALA has been cursorily evaluated for use as a photosensitizer in cats.[24,25]

The purpose of the study reported here was to determine in vitro whether cultured canine TCC cells

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accumulated measurable amounts of PpIX after exposure to ALA and whether this PpIX accumulation was sufficient to cause lethal phototoxic effects when exposed to 635-nm laser light. This study represented the first step in determining whether dogs with naturally developing TCC can serve as models for the development of clinically useful ALA-based PDT protocols for use in humans.

Materials and Methods

Sample population—A **canine TCC cell line (K9TCC)**[26,a] was used for all experiments. Cells were maintained in logarithmic-phase monolayer culture in standard medium[b] supplemented with 10% **fetal bovine serum (FBS)**[c] and 2mM L-glutamine.[d] Cells were grown at 37°C in an environment of 95% air-5% CO_2 in a humidified incubator.

Accumulation of PpIX—To quantify the conversion of ALA into PpIX, 5×10^6 K9TCC cells were inoculated into standard media in 25-cm^2 culture flasks. Twenty-four hours later, graded doses of ALA[e] in low-serum media (1% FBS) were added to the flasks, and cells were incubated for various intervals of 0 to 24 hours. Untreated flasks served as control samples, and all experiments were performed in triplicate. After incubation, flasks were rinsed with cold PBS solution, and 500 µL of 1% Triton X-100 was added. Flasks were frozen and thawed, after which the PpIX was extracted in acidified methanol, as described elsewhere.[24] Extracts were clarified by centrifugation, and PpIX fluorescence was measured with a luminescence spectrometer[f] at an excitation wavelength of 405 nm and an emission wavelength of 604 nm. To generate a standard curve, untreated cells in flasks were washed with cold PBS solution, pure PpIX[g] was added, and PpIX was extracted in a similar manner.

Assessment of phototoxic effects—To determine the in vitro phototoxic effects of ALA-induced PpIX, 1×10^4 cells were seeded into 35-mm-diameter culture dishes. Twenty-four hours later, graded doses of ALA in low-serum media were added to the dishes. Dishes were incubated in the dark for 0 to 24 hours, irradiated with varying doses of 635-nm light from a diode laser,[h] and incubated for an additional 48 hours. Laser wavelength was confirmed by use of a spectrometer with a charged-couple device,[i] and power output of the laser was confirmed by use of a thermopile power meter.[j] Cells exposed to light alone or ALA alone, as well as cells that were not exposed to ALA or light, served as control samples, and all experiments were performed in triplicate. Forty-eight hours after laser irradiation, cell survival was calculated by use of a resazurin fluorescence assay[k] for viable cells.[27,28] Briefly, cells were incubated with media supplemented with 10% resazurin, which is reduced to a fluorescent form by viable cells. Fluorescence (excitation wavelength, 560 nm; emission wavelength, 590 nm), which is directly proportional to the number of viable cells, was determined after cells had been incubated with resazurin for 4 hours.

Results

Pure PpIX was used to generate a standard curve. Results revealed a linear relationship between PpIX concentration and fluorescence (**Fig 1**).

Incubation of K9TCC cells in ALA-containing media resulted in the conversion of ALA to measurable quantities of PpIX. After an initial lag phase of approximately 1 hour, accumulation of PpIX within K9TCC incubated with 2.0mM ALA increased as a function of time (**Fig 2**). A slight plateau in PpIX production was observed between 6 and 8 hours, but PpIX accumulation continued to increase throughout the 24-hour study period. The PpIX accumulation in K9TCC cells after 4 hours of incubation with ALA was directly proportional to ALA concentrations up to 0.6mM, but mean PpIX concentrations did not increase beyond 117pM even when ALA concentrations were between 1.2 and 5.0mM (**Fig 3**).

Incubation of K9TCC cells in ALA-containing media produced sufficient PpIX that cells were lethally photosensitized to exposure to 635-nm laser light. After an initial 1-hour lag phase, survival of K9TCC cells decreased after exposure to 2.0mM ALA and 635-nm laser light (power density, 18 mW/cm^2; energy density 15 J/cm^2) as a function of ALA-incubation time (**Fig 4**). Maximum killing of cells was evident between 4 and 6 hours after onset of ALA incubation and did not change appreciably between 6 and 24 hours. Survival of K9TCC cells also decreased after a 4-hour exposure to varying concentrations of ALA and 635-nm laser light (power density, 18 mW/cm^2; energy density, 15 J/cm^2; **Fig 5**). The most precipitous decrease in cell survival was detected when ALA concentrations were between 0.15 and 0.6mM. However, incubation with 5mM ALA and subsequent irradiation were sufficient to kill 90% of K9TCC cells.

Alterations in laser irradiation also affected survival of K9TCC cells incubated with ALA. Decreased cell survival was associated with increasing energy density, but survival decreased minimally beyond 10 J/cm^2 (**Fig 6**). Decreased cell survival was also associated with increasing power density throughout the range

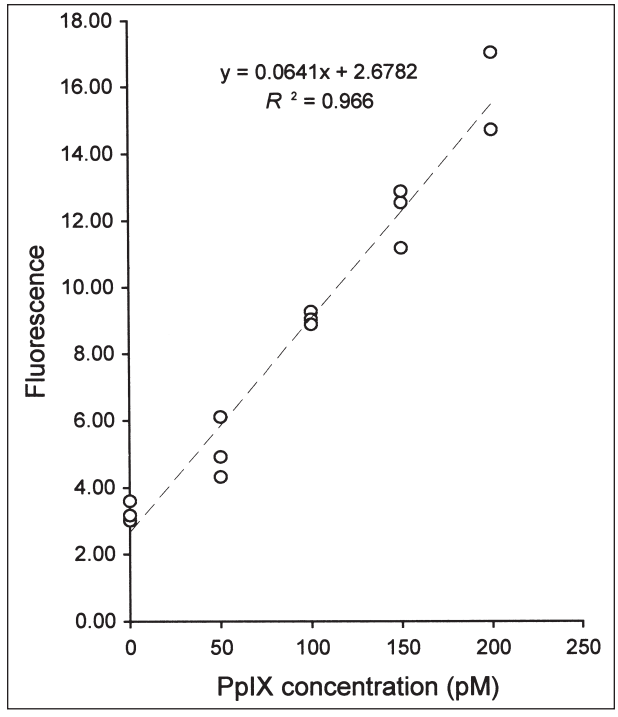

Figure 1—Graph of the relationship between fluorescence and the amount of protoporphyrin IX (PpIX) added to cultured canine transitional cell carcinoma (TCC) cells and then extracted in acidified methanol. Experiments were performed in triplicate. The dashed line represents the line of best fit. Fluorescence is reported in arbitrary units.

tested (**Fig 7**). None of the laser irradiations was sufficient to increase the temperature within the culture dishes (data not shown).

Within hours after exposure to laser light at 635 nm, K9TCC cells incubated with ALA underwent morphologic changes consistent with cell death, including increases in cellular vacuoles and membrane blebs, and numerous cells were observed in various stages of detachment from the surface of the culture dishes. Exposure of K9TCC cells to light alone or ALA alone did not have an effect on cell proliferation, survival, or morphologic characteristics.

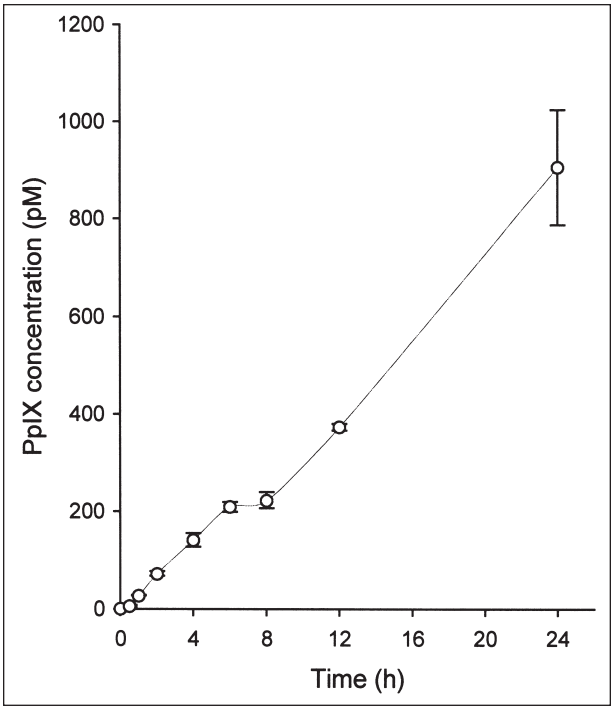

Figure 2—Accumulation of PpIX concentrations in cultured canine TCC cells incubated with 2mM 5-aminolevulinic acid (ALA) for varying time periods. Results represent mean ± SD concentrations of PpIX.

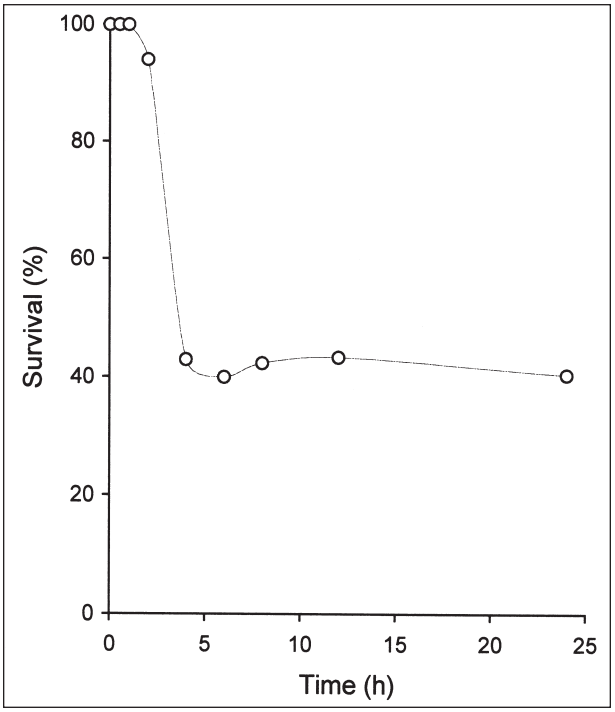

Figure 4—Survival of canine TCC cells after incubation with 2mM ALA for varying time periods and exposure to 635-nm laser light (power density, 18 mW/cm^2; energy density, 15 J/cm^2).

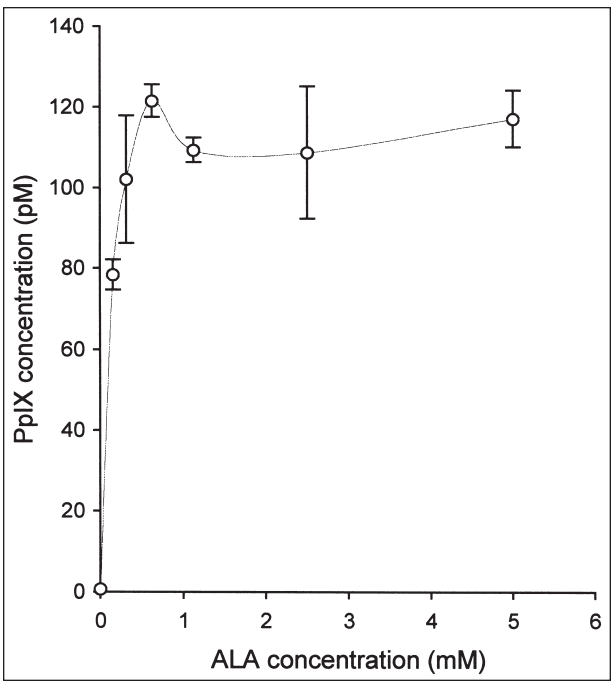

Figure 3—Accumulation of PpIX in cultured canine TCC cells incubated for 4 hours with varying concentrations of ALA. Results represent mean ± SD concentrations of PpIX.

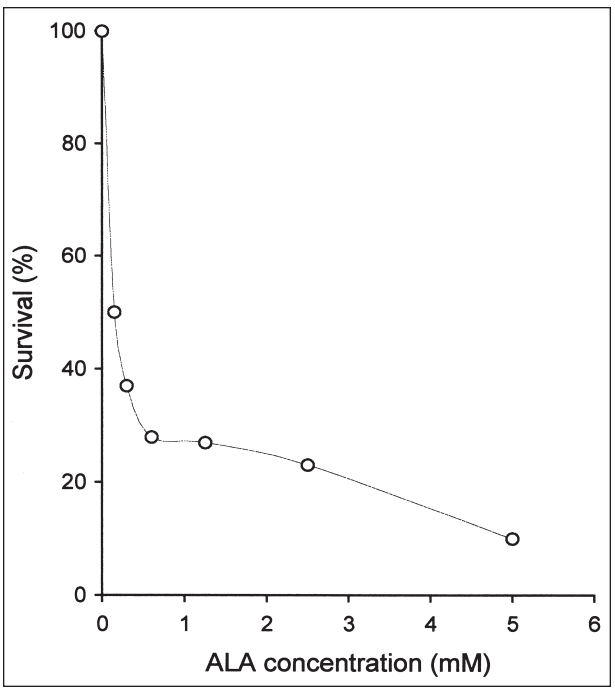

Figure 5—Survival of canine TCC cells after a 4-hour incubation with varying concentrations of ALA and exposure to 635-nm laser light (power density, 18 mW/cm^2; energy density, 15 J/cm^2).

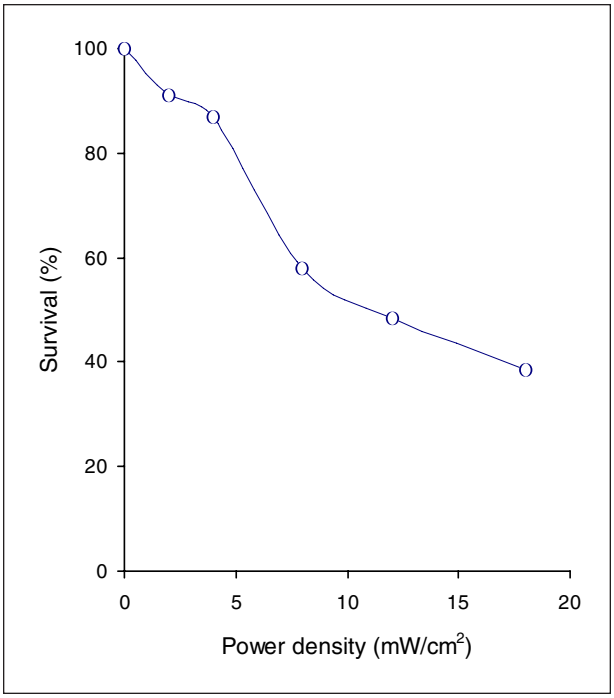

Figure 6—Effect of power density on survival of cultured canine TCC cells that were incubated for 4 hours with 2mM ALA and then exposed to 635-nm laser light (energy density, 10 J/cm^2).

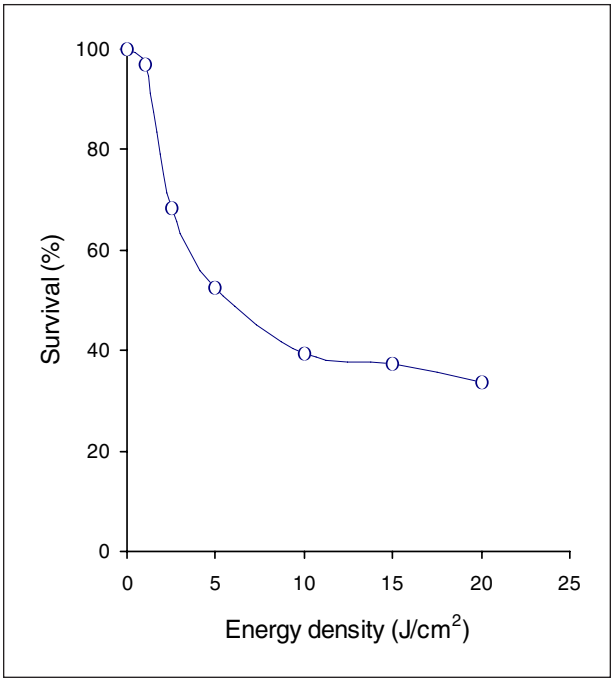

Figure 7—Effect of energy density on survival of cultured canine TCC cells that were incubated for 4 hours with 2mM ALA and then exposed to 635-nm laser light (power density, 18 mW/cm^2).

Discussion

In the study reported here, ALA induced PpIX accumulation within cultured K9TCC cells. This is similar to ALA metabolism reported in human TCCs in vitro.[29] Accumulation of PpIX in cultured K9TCC cells was a time-dependent event. However, there was no apparent advantage in incubating K9TCC with ALA for longer than 4 to 6 hours. This is consistent with a preclinical investigation[30] that documented peak PpIX concentrations in urinary bladder epithelium 4 hours after administration of ALA. The lag phase observed in PpIX accumulation and in the development of phototoxic effects is likely the result of the time needed for transport of the hydrophobic ALA molecule across the plasma membrane. Esterified ALA derivatives, which rapidly diffuse across the cell membrane, cause a more rapid accumulation of PpIX than ALA in cultured carcinoma cells[31] and may represent alternatives for stimulating the in situ production of photosensitizers for PDT.

Accumulation of PpIX and phototoxic effects in K9TCC cells increased in a dose-dependent manner for ALA, which is consistent with other in vitro studies.[24,32-34] Unfortunately, this in vitro observation does not translate directly into clinical use. Intravenous administration of ALA at dosages that are > 100 mg/kg induced toxic effects in cats,[24] and administration at a dosage of 100 mg/kg caused death in a dog.[35] Nonlinear accumulation of PpIX in cultured K9TCC cells suggests there is a maximal effective concentration of ALA and that concentrations greater than the maximal effective concentration will overwhelm the heme biosynthetic pathway. This nonlinear relationship between PpIX accumulation and ALA concentration is reflected in the curve for cell survival. However, phototoxic effects seemingly increased disproportionately to PpIX production at the highest ALA concentrations.

The ALA-induced PpIX resulted in the dose-dependent toxic effects of 635-nm light on cultured K9TCC cells. Increases in energy density and power density caused a decrease in cell survival. However, none of the alterations in irradiation resulted in death of all cells. This may be a function of light distribution within the culture dishes, where cells at the edges of the dishes potentially received a lower light dose than those in the center of the dishes. Increases in energy density were associated with a nonlinear decrease in cell survival. Light doses > 10 J/cm^2 resulted in only a small percentage decrease in cell survival. This suggests that the accumulated PpIX in cultured K9TCC cells may have undergone photobleaching, rendering it ineffective.[36] Alternating light and dark intervals can overcome the effects of photobleaching, thereby enhancing the toxic effects of PDT,[37] which may have increased the observed killing of cells in the study reported here.

Power density was associated with an almost linear decrease in survival of K9TCC cells exposed to ALA. Because we did not detect an increase in temperature with the laser irradiation used in our study, hyperthermia did not contribute to the observed cell death. In this study, 18 mW/cm^2 represented the maximum power density attainable by the diode laser and 35-mm culture dishes. Power density can be increased by irradiating a smaller surface, such as single wells of a multiple-well tissue culture plate. However, light can scatter among the wells, making it difficult to determine dosimetry, and multiple-well plates offer variations in growing conditions for the cultured cells, compared with growing conditions in individual 35-mm

dishes. To maintain consistency, 35-mm dishes were used for all experiments, which precluded higher power densities that may have been more efficacious.

In other in vitro studies,[38-40] ALA proved to be an effective prophotosensitizer for PDT of human and murine bladder carcinoma cells. Normal urothelium responds in a manner similar to that for control cells in ALA-based PDT studies in vitro, suggesting that this is potentially an extremely selective method for treating bladder cancer in vivo. The use of ALA for PDT of the urinary bladder is attractive for several reasons. It can be given orally, parenterally, or instilled into the bladder, which allows flexibility in designing effective treatment protocols.

On the basis of results reported here, K9TCC cells are susceptible to ALA-based PDT in vitro. It could be argued that the observed survival of 40 to 50% of cells may not translate into a successful clinical treatment for TCCs. However, in addition to direct killing of cells, PDT in vivo causes other events, such as vascular stasis leading to ischemic cell death and inflammation with nonspecific killing of cells, that result in tumor control. Therefore, additional studies of ALA-based PDT for the treatment of dogs with TCCs are warranted to determine the safety and efficacy of this treatment modality.

[a]Provided by Dr. Deborah Knapp, Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, Ind.
[b]RPMI-1640, without phenol red, without L-glutamine, Sigma Chemical Co, St Louis, Mo.
[c]Fetal bovine serum, Gibco Inc, Grand Island, NY.
[d]L-glutamine, Gibco Inc, Grand Island, NY.
[e]5-aminolevulinic acid hydrochloride, Fisher Scientific International Inc, Hampton, NH.
[f]Model LS 55, Perkin-Elmer Inc, Norwalk, Conn.
[g]Protoporphyrin IX, Porphyrin Products Inc, Logan, Utah.
[h]Ceralas, CeramOptec Industries Inc, Longmeadow, Mass.
[i]WaveStar-V, Ophir Optronics Ltd, Boston, Mass.
[j]Vector H410, Scientech Inc, Boulder, Colo.
[k]Alamar Blue, BioSource International Inc, Camarillo, Calif.

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