Photosensitization, Uptake, and Retention of Phenoxazine Nile Blue Derivatives in Human Bladder Carcinoma Cells

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ABSTRACT

The overall goal of our research is to develop effective new photosensitizers for tumor-selective photodynamic therapy. Phenoxazine dyes, including several Nile blue analogues, are known to localize selectively in animal tumors. Structural modifications yielded several series of analogues with substantially higher \(^1\)O\(_2\) yields and different photochemical and physicochemical properties. This study examined the photosensitization potency, cellular uptake, and retention of these derivatives in human bladder carcinoma cells (MGH-U1) in culture. Nile blue derivatives containing halogens and/or sulfur substituents were selected to exhibit different \(^1\)O\(_2\) yields, pKa values, and hydrophobicities.

The effectiveness of these derivatives in mediating photokilling of tumor cells in vitro corresponded well with the \(^1\)O\(_2\) yields of these compounds, indicating that structural modifications which resulted in increased \(^1\)O\(_2\) yields enhanced potency in mediating phototoxicity in vitro. Using derivatives (sat-NBS and sat-NBS-61) with the highest \(^1\)O\(_2\) quantum yield (0.35 and 0.821), over 90% cell kill was achieved at a sensitizer concentration of \(5 \times 10^{-4}\) M, about 3 orders of magnitude more effective than hematoporphyrin derivative, the only sensitizer currently effective PDT1 is an investigational cancer treatment procedure. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The overall goal of our research is to develop photosensitizers with high tumor selectivity. Our investigations have centered on a group of compounds related to the phenoxazine dye Nile blue. This investigation stems from results of early studies by Lewis et al. (9-11), who, in an extensive search for dyes that selectively stain tumors and retard tumor growth, found a number of phenoxazines, including several Nile blue analogues, which cause intense, diffuse staining of viable tumor cells but not of the tumor stroma or of surrounding tissues. These dyes exhibit relatively low systemic toxicity, and some of them temporarily retard tumor growth. These findings were further substantiated by studies of Riley (12) and of Bates and Kershman (13). The former tested the effect of 55 different dyes on transplantable mouse carcinomas and sarcomas and found that Nile blue stained tumors and retarded their growth. The latter investigators observed selective intense staining of Nile blue 2B on a number of transplanted and carcinogen-induced murine brain tumors. These early studies strongly suggested that phenoxazines may constitute a special class of dyes that are selectively localized in tumors.

More recently, it was shown that the tumor-localizing phenoxazines, such as Nile blue A and Nile blue 2B, have very low \(^1\)O\(_2\) yields because of inefficient intersystem crossing of excited states (14). Structural modifications of Nile blue A, including halogenation, sulfur substitution, and saturation of the angular D-ring, resulted in a number of derivatives with increased \(^1\)O\(_2\) yields (14, 15). These derivatives have a strong absorbance within the therapeutic window of light (600-1200 nm) and relatively low systemic toxicity and thus may be effective photosensitizers for PDT. Preliminary experimental results showed that several of these derivatives are effective in mediating the selective photokilling of tumor cells in culture (14). The combination of high photoactivity, strong absorption of red wavelengths, and possible high tumor selectivity makes these compounds promising candidates as photosensitizers for the selective PDT of tumors. Furthermore, these derivatives possess different physicochemical properties, such as pKa and hydrophobicity, and are capable of undergoing protonation-deprotonation conversion depending on the pH of the environment (15, 15). These compounds, therefore, may be useful in the investigation of the possible effects of these factors on cellular dye

INTRODUCTION

PDT3 is an investigational cancer treatment procedure. The therapy is effective against a wide range of solid tumors, including carcinomas of the skin, esophagus, lung, bladder, and breast (1-8). The theoretical basis of the treatment relies on a dual selectivity to achieve a high therapeutic ratio, the selectivity of the photosensitizer to localize in higher concentrations in malignant versus surrounding nonmalignant tissues and the selectivity of the treatment light to activate specifically the exogenous sensitizer and destroy the tumor. Currently, many investigators are searching for new photosensitizers to expand the utility of this treatment. Approaches for this search include dyes that are chemically well defined, with high photoactivity at excitation wavelengths above 600 nm and with high tumor selectivity (8).

The effectiveness of these derivatives in mediating photokilling of tumor cells in vitro corresponded well with the \(^1\)O\(_2\) yields of these compounds, indicating that structural modifications which resulted in increased \(^1\)O\(_2\) yields enhanced potency in mediating phototoxicity in vitro. Using derivatives (sat-NBS and sat-NBS-61) with the highest \(^1\)O\(_2\) quantum yield (0.35 and 0.821), over 90% cell kill was achieved at a sensitizer concentration of \(5 \times 10^{-4}\) M, about 3 orders of magnitude more effective than hematoporphyrin derivative, the only sensitizer currently promising candidates as photosensitizers for the selective PDT of tumors. Furthermore, these derivatives possess different physicochemical properties, such as pKa and hydrophobicity, and are capable of undergoing protonation-deprotonation conversion depending on the pH of the environment (15, 15). These compounds, therefore, may be useful in the investigation of the possible effects of these factors on cellular dye
uptake and might provide insight into the mechanism for selective localization of compounds in tumors.

The main purposes of the current study were (a) to evaluate the photosensitization potencies of various Nile blue derivatives in mediating cell killing in vitro and to identify potentially effective photosensitizers for PDT and (b) to examine the cellular uptake and retention of derivatives with different chemical properties to shed light on the mechanism of selective localization of Nile blue in tumors. The results reveal that the effectiveness of these derivatives in mediating photokilling of tumor cells corresponded with their O2 yields, thus suggesting that those with high O2 yields can be effective photosensitizers and that O2 is probably a major mechanism of the phototoxic effect induced by these compounds. The uptake of Nile blue derivatives was rapid, was highly concentrative, was proportional to dye concentration in the medium, and could occur at temperatures below 2°C. Furthermore, uptake was not affected by the presence of serum, nor did it correlate with the pKa or hydrophobicity of the dyes. These results indicate that the uptake does not occur by endocytosis or through a carrier-mediated mechanism. Details of the process are yet to be defined.

MATERIALS AND METHODS

Nile Blue Derivatives. Previous reports (14–16) showed that structural modifications of Nile blue A resulted in derivatives with enhanced O2 yields and different physicochemical properties. Derivatives used in this investigation were available from previous studies (15, 16), and their O2 quantum yields, absorption maxima, pKa values, and partition coefficients in octanol and PBS are listed in Table 1.

Tumor Cells. The experimental system used for this study is a human bladder tumor cell line, MGH-U1, which is a subculture from a well-established bladder cancer cell line T-24 (17). MGH-U1 cells grow routinely in McCoy’s 5A medium supplemented with 5% fetal calf serum. Under this culture condition, the cells have a doubling time of 14 h and a colony formation efficiency of about 50%.

Determination of Photocytotoxicity. Because of the wide differences in photoactivity among various Nile blue derivatives to be tested, we varied the dye concentration rather than the light dose in the evaluation of the sensitizing effect of these dyes in mediating photocytotoxicity in vitro. MGH-U1 cells (2 × 10^5 in a 100-mm dish) were incubated with 10 ml of each of the derivatives, ranging from 10^-8 to 10^-3 M, for 30 min at 37°C. Extracellular dye was removed, and light treatment followed immediately. The treatment light source was a Polaroid projector with filters allowing light of 590–700 nm to pass through. The power density of this light source at a distance of 10 cm was 8–10 mW/cm². The light dose used for the treatment was 4.8 J/cm² over a treatment period of 8 to 10 min. The number of viable cells, as determined by the colony-forming assay, was used to assess the photo-dynamic effect. Controls for the study were untreated cells and those treated with light or sensitizer only.

Photosensitization in D2O and in Hypoxia. To determine whether D2O can enhance the photodynamic effect of Nile blue derivatives, cells were permitted to take up the photosensitizer as above (NBA-6I, 5 × 10^-7 M; NBS-6I, 2 × 10^-7 M) and were incubated with D2O/PBS for 30 min at 37°C to allow exchange of D2O with the cellular water. Light treatment was performed in the presence of D2O at light doses ranging from 1.2 to 7.2 J/cm², and a colony-forming assay was used to assess the result of the treatment. A hypoxic condition was created as described (18), by placing the cell culture plate in an air-tight chamber and perfusing the chamber with humidified nitrogen. The oxygen tension in the chamber was reduced to 21 ± 2 (SD) ppm after about 30 min of gassing. Oxygen tension was monitored from the effluent gas flow for the duration of the hypoxia using an oxygen tension meter (Thermox 1; Thermo Laboratory Instrument, Inc.). Light treatment proceeded while the cells in the chamber were maintained at this reduced oxygen tension by continuous gassing during photoirradiation. The colony assay was used to assess the results of the treatment. Controls for both D2O and hypoxia experiments were cells treated with photosensitizer and light without D2O and those treated under atmospheric oxygen tension.

Dye Extraction and Quantitation. For quantitation of dye in cells, culture plates were washed twice with Dulbecco’s PBS to remove excess dyes. Cells were removed from plates with 0.1% EDTA, dissolved in concentrated HCl, and extracted with chloroform:methanol (1:1) acidified with 0.5% glacial acetic acid. Concentrations of the dye in the extracts were measured by fluorescence spectroscopy in acidic chloroform:methanol at the maximal excitation and emission wavelengths for each derivative: NBA, 620 nm, 660 nm; NBA-6I, 610 nm, 670 nm; NBS, 650 nm, 690 nm; NBS-6I, 660 nm, 690 nm; sat-NBS, 620 nm, 660 nm; sat-NBS-6I, 650 nm, 670 nm. In this study, the overall dye recovery from the cellular experiments was usually between 40 and 50%. Up to about 50% of the dyes were bound to the plastic surface and could not be recovered.

Uptake Studies. The patterns of uptake of the Nile blue derivatives by MGH-U1 cells were determined by placing 2 × 10^6 cells per 60-mm dish and allowing them to attach overnight. Dyes (2 ml) at 2.5 × 10^-4 M in phenol red-free and serum-free McCoy’s 5A medium were added to the cells, and cellular dye concentrations were determined at different time intervals for up to 80 min while the cells were incubated at 37°C.

To examine the effect of dye concentration on the uptake of Nile blue derivatives, two experiments were performed. In the first, the uptake of NBA-6I over time by cells was determined at 4 different dye concentrations in the medium: 0.156, 0.625, 2.5, and 10 µM. In the second, the uptake of NBA-6I at 30 min was examined at various dye concentrations ranging from 10^-7 to 10^-4 M.

To determine whether cellular uptake of Nile blue derivatives can proceed at temperatures below 2°C, cells in culture, washing buffer, and dye-containing medium were precooled on ice to reach a temperature between 0 and 2°C before the uptake was allowed to proceed on ice for various intervals.

Efflux Studies. The patterns of efflux of Nile blue derivatives from the cells were determined by first plating the cells at 2 × 10^4 per 60-mm dish and permitting them to attach overnight. The cells were then incubated with serum-free McCoy’s 5A medium containing 2.5 × 10^-4 M dye for 30 min at 37°C to allow uptake of the dye. The dye-containing medium was removed, and the cell culture plates were washed twice and overlaid with the serum-free, dye-free medium. Cellular dye concentrations were determined at various time intervals for up to 6 h. To reduce reuptake of the effluxed dye, fresh dye-free medium was replaced every hour throughout the efflux period.

RESULTS

Photocytotoxicity and O2 Yield. Nile blue dyes, in general, are not highly photoactive and therefore are not effective photosensitizers (14, 16). The purposes of this part of our investigation were to confirm the previous preliminary finding that structural modifications to increase O2 yields could enhance the photosensitizing ability in mediating cell killing in vitro (15) and to examine whether the photosensitizing potency in vitro correlates with the O2 yields of these derivatives. Results of the study (Fig. 1) show that the photosensitizing potency of these derivatives in vitro paralleled the O2 quantum yields of the compounds. Derivatives (such as NBA and NBA-6Br) that have low O2 yields of 0.005 and 0.007, respectively, were not efficient sensitizers, whereas those with high O2 yields (such as sat-NBS and sat-NBS-6I), with O2 quantum yields of 0.35 and 0.82, respectively, were very effective in mediating the photocytotoxic effect. The E50 (the extracellular sensitizer concentration needed to cause a 50% cell kill upon photoradiation) was more than 10 µM for NBA, about 2.5 µM for NBA-6Br,
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Table 1 Structures and photochemical and physicochemical properties of Nile blue derivatives

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Structure</th>
<th>(1^\text{O}_2) yield*</th>
<th>(\lambda_{\text{max}}) (nm)*</th>
<th>(\epsilon^a)</th>
<th>pKa*</th>
<th>P*</th>
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<td>NBA</td>
<td></td>
<td>0.005</td>
<td>623</td>
<td>75,300</td>
<td>10.0</td>
<td>173</td>
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<tr>
<td>NBA-6Br</td>
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<td>0.007</td>
<td>643</td>
<td>80,600</td>
<td>8.0</td>
<td></td>
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<tr>
<td>NBS</td>
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<td>0.025</td>
<td>645</td>
<td>67,500</td>
<td>10.0</td>
<td>356</td>
</tr>
<tr>
<td>NBA-6I</td>
<td></td>
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<td>642</td>
<td>79,000</td>
<td>6.6</td>
<td>5.625</td>
</tr>
<tr>
<td>NBS-6I</td>
<td></td>
<td>0.23</td>
<td>660</td>
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<td></td>
<td>0.35</td>
<td>628</td>
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<td>109</td>
</tr>
<tr>
<td>sat-NBS-6I</td>
<td></td>
<td>0.82</td>
<td>637</td>
<td>79,300</td>
<td>9.5</td>
<td>1,752</td>
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* Values of \(1^\text{O}_2\) yield, \(\lambda_{\text{max}}\), \(\epsilon\), and pKa are taken from Refs. 13–15.

Fig. 1. The photocytotoxic effect on MGH-U1 cells mediated by Nile blue derivatives with different \(1^\text{O}_2\) yields. MGH-U1 cells (2 \times 10^6 in a 100-mm dish) were incubated with 10 ml of each of the derivatives, ranging from 10^-10 to 10^-3 M, for 30 min at 37°C. Extracellular dye was removed, and light treatment followed immediately. Photoirradiation was carried out with a broad band light source of 590–700 nm and a light dose of 4.8 J/cm². Each point is the mean of 6 determinations. Bars, SD. The \(1^\text{O}_2\) yields of the derivatives are: NBA, 0.005; NBA-6Br, 0.007; NBS, 0.025; NBA-6I, 0.036; NBS-6I, 0.23; sat-NBS, 0.35; sat-NBS-6I, 0.82.

0.15 \(\mu\)M for NBA-6I and NBS, 0.04 \(\mu\)M for NBS-6I, and about 0.003 \(\mu\)M for sat-NBS and sat-NBS-6I. Relative photosensitizing efficiencies of these derivatives were calculated, by taking into consideration dye uptake (from Fig. 3) and extinction coefficient, with the formula:

Relative photosensitizing efficiency = \(1/EC_{50} \times 1/IC_{30} \times \epsilon\)

where IC_{30} is the intracellular dye concentration in nanomoles/10^6 cells with 30 min of uptake (from Fig. 3) and \(\epsilon\) is the extinction coefficient (from Table 1). As listed in Table 2, compounds with high \(1^\text{O}_2\) yields tend to have high relative photosensitizing efficiencies. Although other biological factors and factors such as cellular distribution of the dye may be important and other mechanisms may also be involved, these
data suggested that the generation of $^{13}O_2$ is probably a major mechanism mediating the photocytotoxic effect. The result also indicates that structural modifications that increase $^{13}O_2$ yields effectively enhance the in vitro photosensitizing potency of Nile blue derivatives, suggesting that some of the derivatives can be effective photosensitizers for tumor therapy.

Most of the derivatives have negligible dark toxicities within the range of dye concentrations tested in this study. At the highest concentration tested, NBA (5 μM), NBS (2 μM), NBA-6I (1 μM), sat-NBS (0.15 μM), and sat-NBS-6I (0.1 μM) have dark toxicities of less than 5%, whereas NBA-6Br (2 μM) and NBS-6I (0.2 μM) have dark toxicities of 27.5 ± 3.5% and 24 ± 4%, respectively.

Effects of D$_2$O and Hypoxia on Photosensitization. To examine the possibility that $^{13}O_2$ is the main mechanism of photocytotoxicity, photoirradiation treatment was performed under two conditions that would alter the $^{13}O_2$ yield: (a) in the presence of D$_2$O, which increases the $^{13}O_2$ lifetime and thereby enhances $^{13}O_2$-mediated photocytotoxicity (19, 20), and (b) under a hypoxic condition, which reduces $^{13}O_2$ production and concomitantly decreases $^{13}O_2$-mediated photocytotoxicity (18).

As shown in Fig. 2, photoirradiation in the presence of D$_2$O enhanced the photokilling mediated by either NBA-6I or NBS-6I by about 2-fold, whereas photoirradiation under the hypoxic condition virtually eliminated the cytotoxic effect of the sensitizers. Although a two-fold enhancement of cytotoxicity by D$_2$O is much less than the potential increase in $^{13}O_2$ decay lifetime, this is in accordance with similar values reported for other $^{13}O_2$-mediated photosensitizers (20–23). One of the explanations for the lower than theoretical enhancement is from inadequate exchange of cellular water with D$_2$O (20). Although the overall results do not exclude the involvement of other mechanisms, they do support the hypothesis that singlet oxygen is a major cytotoxin for the light-induced inactivation of cells mediated by Nile blue derivatives.

Uptake of Nile Blue Derivatives. Preliminary experiments using either trypsinization or EDTA treatment to remove cells from culture plates yielded similar quantitative results on dye uptake. This suggests that there was minimal nonspecific binding of dyes to the surface of the cells. Quantitation of dye concentrations in cells showed that all derivatives exhibited similar uptake patterns (Fig. 3), with a rapid initial uptake followed by a more gradual increase in the intracellular concentration. The accumulation of dye in the cells was highly concentrative. At the end of the 80-min uptake period, dye concentrations of NBS and NBA-6I were intracellularly 0.7 and 1.2 nmol/10$^6$ cells and extracellularly 0.17 and 0.06 nmol/ml, respectively. Based on the assumption that cell volume is in the order of 3 μl/10$^6$ cells, these represent intra/extracellular concentration ratios of 1380 (NBS) to 6470 (NBA-6I). Cellular dye uptake did not correlate directly with the pKa values or partition coefficients of the derivatives. For example, the dye with the highest uptake was NBA-6I, which has a pKa of 6.6. However, a dye with a similar pKa of 6.5, NBS-6I, had a much lower uptake. Furthermore, NBS-6I has an uptake value similar to those of dyes with high pKa values.
Retention of Nile Blue Derivatives. The dye efflux study (Fig. 4) showed that, with the exception of sat-NBS, over 80% of the cellular dyes were retained after 6 h of efflux. The low efflux rates together with the highly concentrative uptake observed suggested that the derivatives were held within the cell by mechanisms that could include dye aggregation, binding to cellular components, and sequestering in intracellular compartments. Also, the fact that the majority of the dyes were recovered from the cells suggested that, during the course of the experiment, there was no significant irreversible metabolic degradation of these compounds in the intracellular environment, which is an important requirement for an effective photosensitizer.

Effect of Dye Concentration on Uptake. To examine whether the accumulation of Nile blue derivatives is a saturable process, two uptake experiments with varying dye concentrations were performed. In the first, the uptake of NBA-6I over time was examined at four dye concentrations ranging from 0.156 to 10 μM. The results (Fig. 5, left) indicate similar uptake patterns for all dye concentrations studied and increased uptakes with higher concentrations of dye in the medium. In the second experiment, the 30-min uptake of NBA-6I was examined over a range of dye concentrations from 10^{-7} to 10^{-4} M. The result (Fig. 5, right) indicates that the uptake of NBA-6I by the cells was a linear function of dye concentration over the entire range examined. Thus, there was no apparent saturation of uptake of the dye by the cells within the concentration range examined.

This apparently conflicts with the uptake patterns (Fig. 3), showing reduced uptake after the initial phase. This contradiction may be explained by the depletion of dye in the medium rather than by the saturation of cell capacity in dye retention. To verify this hypothesis, dye was added to the medium after an initial 20-min uptake period, after the rate of uptake had waned significantly. Data in Fig. 6 show that repeated additions of NBA-6I after 20 min of uptake resulted in further accumulations of the dye, thus confirming that the reduction of uptake after the initial phase was due to dye depletion in the medium rather than any saturation of the cellular capacity to retain dye.

Effect of Serum and Low Temperature on Uptake. In contrast to the anionic photosensitizer, hematoporphyrin derivative or Photofrin, which exhibits reduced uptake in the presence of 10% fetal calf serum to the medium had no effect on uptake. As shown in Fig. 7, the addition of 10% fetal calf serum to the medium had no effect on uptake over the wide range of dye concentrations examined. The effect of lowering the temperature below 2°C was examined to determine whether cellular uptake of the dye occurred by means of pinocytosis, which cannot take place at these low temperatures (25-27). To maintain uptake conditions below 2°C, cell plates, washing buffer, and dye-containing medium were precooled on ice to a temperature between 0 and 2°C before the uptake was allowed to proceed on ice. Uptake of three Nile blue derivatives was examined at this temperature. The results (Fig. 8) indicated significant dye uptake (69 ± 7% for NBA, 76 ± 2% for NBA-6I, and 53 ± 9.5% for sat-NBS) occurring at this low temperature, ruling out pinocytosis as a likely mechanism of uptake for Nile blue derivatives. Inasmuch as most carrier-mediated mechanisms of membrane transport are temperature-dependent, the result also suggested that carrier mediation is probably not the mechanism for the uptake of these compounds.

DISCUSSION

For PDT to become a useful tool for cancer therapy with intent to cure, it is essential that it be able to treat diffuse, multiple, infiltrating, and even invisible tumors. Improved tumor selectivity of the photosensitizer is a key requirement in achieving this goal. A photosensitizer with high tumor selectivity will yield low skin photosensitivity and low systemic toxicity and have a high treatment efficiency due to a high concentration of sensitizer in tumors. The overall benefit would be a higher therapeutic ratio between the tumors and their surrounding tissues and, consequently, the possibility of expanding the utility of PDT to treat life-threatening infiltrating and invisible tumors. The goal of our investigation is to develop Nile blue derivatives as effective photosensitizers for the selective destruction of malignant tumors. In the present study, we examined the photosensitizing potency, cellular uptake, and retention of these derivatives, aiming to identify potentially effective photosensitizers and to provide a better understanding for the basis of selective localization of these dyes in tumors.

Results of the photosensitizing study showed that the effectiveness of Nile blue derivatives in mediating the photocytotoxicity of tumor cells in vitro corresponds closely with their \( ^1 \mathrm{O}_2 \) quantum yields. Structural modifications such as halogenation, sulfur substitution, and saturation of the angular D-ring, which substantially increase the \( ^1 \mathrm{O}_2 \) yields of Nile blue derivatives, were effective in increasing the in vitro photodynamic potency of these dyes. For derivatives such as sat-NBS and sat-NBS-6I, the combined effects of high \( ^1 \mathrm{O}_2 \) quantum yields, strong ab-
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Fig. 5. Effect of dye concentration on the uptake NBA-6I by MGH-U1 cells. Experiments were performed as described in Fig. 3 except with varying dye concentrations in the medium. Left, uptake of NBA-6I at 4 different dye concentrations of 0.156, 0.625, 2.5, and 10 μM. Points, means of 3 determinations. Right, uptake of NBA-6I at dye concentrations varying from 10⁻⁷ to 10⁻⁵ M. Only the 30-min interval cellular dye concentrations were determined. Points, means of 4 plates. Bars are SDs when they exceed symbol size.

Fig. 6. Uptake of NBA-6I by MGH-U1 cells upon repeat additions of dye into the medium. Experiments were performed as described in Fig. 3, with 2 x 10⁶ cells/60-mm dish and 2 ml of dye at 2.5 x 10⁻⁵ M (5 nmol). After 20 min of uptake, 100 μl of medium containing 5 nmol of dye was added to the plates (arrow), and cellular dye concentrations were determined at different intervals. This step was repeated once (arrow). Points, means of 3 determinations; bars, SD.

Fig. 7. Effect of 10% fetal calf serum (FCS) in the culture medium on the cellular uptake of NBA-6I. The experiment was performed as for Fig. 5 (right) except with 10% FCS added to the medium. Data for uptake without FCS (●) are also presented for comparison. Points, means of 4 determinations. All SDs do not exceed symbol size.

sorbances of light over 600 nm, and high cellular uptakes produced enhanced in vitro photodynamic potencies. The concentration needed to effect a 90% cell kill with these sensitizers was 5 x 10⁻⁸ M, which is about 3 orders of magnitude lower than that with hematoporphyrin derivative with a similar light dose and the same cell line (28, 29). This suggests that some of the Nile blue derivatives could potentially be effective photosensitizers for PDT.

Although a comparison of the ¹⁰⁰₂ quantum yields with the dye-mediated cell killing (Table 2) shows that photosensitizing potency tends to parallel ¹⁰⁰₂ yields, a perusal of the data indicates that this correlation is only a general trend. For example, sat-NBS and sat-NBS-6I have similar photosensitizing efficiencies, yet the former has a ¹⁰⁰₂ yield of 0.35 as compared to 0.82 for the latter. The fact that an absolute correlation is not observed for the dyes described herein is not surprising because other factors undoubtedly affect dye-mediated cell killing. Among these are intracellular dye distribution and possible dye aggregation in the cellular environment. Thus, ¹⁰⁰₂ quantum yields for dyes, measured in methanol, may not translate directly into ¹⁰⁰₂ yields in the cells.

All of the Nile blue derivatives examined had a similar cellular uptake pattern. The maximal difference in cellular concentrations among the various derivatives when photolysis was performed was less than two-fold. Thus, the difference in cellular dye concentrations does not constitute a major factor in determining the photodynamic potency of the derivatives. Rather, the concordance between photosensitizing potency and ¹⁰⁰₂ quantum yields indicates that ¹⁰⁰₂ yield is an important determinant of the photosensitizing activity of these dyes. This also suggests that ¹⁰⁰₂ is probably a major mechanism mediating the photocytotoxic action. Results showing enhanced photocytotoxicity of NBA-61 and NBS-61 by D₂O and reduced photocytotoxicity under hypoxic conditions substantiate this hypothesis. Although these data agree with findings on other photosensitizers, where the generation of ¹⁰⁰₂ has been shown to be the cytotoxic agent for photodynamic actions (18–23, 30), they do not rule out the participation of other mechanisms, such as the generation of free radicals or other oxygen-dependent processes in the cytotoxic effect.

The mechanism dictating the localization of photosensitizers in tumors is not well understood (31). The higher localization of anionic photosensitizers, such as hematoporphyrin derivative and tetraphenylporphine sulfonate, in tumors (32) may be related not to certain properties of the tumor cells but rather to factors such as leaky tumor neovasculature, poor lymphatic
drainage of the tumor, and binding of photosensitizers to new tumor collagen (31, 33). For cationic compounds, such as rhodamine 123, it has been postulated that the delocalized positive charge of these molecules enables them to penetrate the hydrophobic cell membrane. The membrane potential is the driving force for the high retention of these dyes in the cell. A two-step process is postulated for this action: the electrochemical potential of the plasma membrane enables a higher uptake of these dyes into the cell, and the mitochondrial potential further concentrates the dyes into this organelle (34, 35).

The mechanism for the selective localization of Nile blue in tumors, as observed in early studies (10-13), is also unknown. Lewis et al. (11) indicated that there is a correlation of dye structure and selectivity of tumor staining; all compounds that stained tumors had amino or substituted amino groups in both the 5- and 9-positions. Varying the nature of the substituted amino groups in these positions resulted in compounds with marked differences in tumor staining ability. For example, monosubstitution in the 5-amino group with the benzyl radical yielded Nile blue 2B, which stained tumor tissue more intensely and was less toxic than NBA, in which the 5-amino group is unsubstituted. Foley et al. (16), in analyzing Lewis' data, pointed out that further structural features seemed to be essential for certain benzophenoxazines to stain tumors selectively. These include dialkylation of the 9-amino group and at least one hydrogen atom attached to the 5-amino group. Cincotta et al. (15) listed several structural and physical parameters that may be important to the tumor-staining ability of these compounds, including pKa, hydrophobicity, and the ability to undergo protonation-deprotonation reactions to expedite transport across the cell membrane. In the present study, we attempted to correlate the uptake and retention of Nile blue derivatives with pKa and hydrophobicity. Results of this study indicated similar overall patterns of uptake for all Nile blue derivatives examined and no apparent correlation between these properties and dye accumulation in cells. The difference between derivatives with the highest (NBA-6I) and lowest (NBS and sat-NBS-6I) uptake was only approximately two-fold. Structural modifications of Nile blue A that enhanced the $O_2$ yields of the derivatives apparently did not alter the properties of the dyes in a way that substantially changed their uptake. The fact that most of the dye can be recovered within 6 h of efflux suggests that there is no significant irreversible metabolic degradation of these compounds in cells, which is an important requirement for an effective photosensitizer.

Although the highly concentrative uptake of Nile blue derivatives indicates an active mechanism for the cellular accumulation of these dyes, the linear retention over a wide range of dye concentrations and the uptake that can proceed at low temperatures preclude pinocytosis and a carrier-mediated process as the mechanism of uptake. Several possible mechanisms may explain this phenomenon. One is the formation of molecular aggregates of the dyes when they enter the cells. Another is partitioning of the dyes in cellular lipids, as the dyes are highly lipophilic. Yet another possibility is sequestering of the dyes into certain subcellular organelles. All of these mechanisms can explain entrapment of the dye in the cell, preventing its efflux and achieving a high dye accumulation without an active transport mechanism at the plasma membrane level.

Thus, the potential mechanism for uptake and distribution of Nile blue derivatives in tumor cells may involve a simple diffusion of the dyes across the plasma membrane followed by distribution of the dyes into one or a number of intracellular compartments through different processes. These sites may include cytomembranes and subcellular organelles such as mitochondria and lysosomes. The localization of the dyes in the cytomembranes should rely on the lipophilicity of the dye to partition in lipid bilayers of the plasma and intracellular membranes. This generally will be an energy-independent process, and the steady-state concentration will be dependent on extracellular dye concentration. Sequestering of dyes in subcellular organelles such as mitochondria and lysosomes relies on the electrochemical gradient of the mitochondrial or lysosomal membranes, and these are maintained by energy-dependent processes. Studies are currently under way to define and delineate more precisely the mechanisms of uptake and distribution of these dyes in the cells.

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Photosensitization, Uptake, and Retention of Phenoxazine Nile Blue Derivatives in Human Bladder Carcinoma Cells

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