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In Vitro Optimization of EtNBS-PDT against Hypoxic Tumor Environments with a Tiered, High-Content, 3D Model Optical Screening Platform

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Abstract

Hypoxia and acidosis are widely recognized as major contributors to the development of treatment resistant cancer. For patients with disseminated metastatic lesions, such as most women with ovarian cancer (OvCa), the progression to treatment resistant disease is almost always fatal. Numerous therapeutic approaches have been developed to eliminate treatment resistant carcinoma, including novel biologic, chemo, radiation, and photodynamic therapy (PDT) regimens. Recently, PDT using the cationic photosensitizer EtNBS was found to be highly effective against therapeutically unresponsive hypoxic and acidic OvCa cellular populations *in vitro*. To optimize this treatment regimen, we developed a tiered, high-content, image-based screening approach utilizing a biologically relevant OvCa 3D culture model to investigate a small library of side-chain modified EtNBS derivatives. The uptake, localization, and photocytotoxicity of these compounds on both the cellular and nodular levels were observed to be largely mediated by their respective ethyl side chain chemical alterations. In particular, EtNBS and its hydroxyl-terminated derivative (EtNBS-OH) were found to have similar pharmacological parameters, such as their nodular localization patterns and uptake kinetics. Interestingly, these two molecules were found to induce dramatically different therapeutic outcomes: EtNBS was found to be more effective in killing the hypoxic, nodule core cells with superior selectivity, while EtNBS-OH was observed to trigger widespread structural degradation of nodules. This breakdown of the tumor architecture can improve the therapeutic outcome and is known to synergistically enhance the antitumor effects of front-line chemotherapeutic regimens. These results, which would not have been predicted or observed using traditional monolayer or *in vivo* animal screening techniques, demonstrate the

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ASSOCIATED CONTENT

Supporting Information

Supplementary Movie 1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

powerful capabilities of 3D *in vitro* screening approaches for the selection and optimization of therapeutic agents for the targeted destruction of specific cellular subpopulations.

Keywords

hypoxia; acidosis; 3D cancer culture models; photodynamic therapy; high-content screening; microscopy

INTRODUCTION

The treatment of advanced, disseminated cancers is one of the major clinical challenges today. Though therapeutic intervention can initially appear successful, patients often enter remission while still harboring subclinical, metastatic disease that eventually becomes resistant to front-line therapies. This is highlighted by the fact that it is the metastases, and not the primary solid tumors, that are known to cause 90% of cancer deaths.¹ Peritoneal disseminated metastatic cancers, such as epithelial ovarian cancer (OvCa), are particularly problematic, as the lack of early and accurate detection methods leads to 75% of patients presenting with advanced stage metastatic ovarian carcinomatosis. Though many OvCa patients who have undergone “debulking” surgery and subsequent chemotherapy cycles do not display any clinical signs of disease at the cessation of treatment, 30–50% of these patients will experience relapse with chemoresistant OvCa.² There are few, if any, proven second-line therapies for patients who present with treatment resistant OvCa, leading to a dismal overall survival rate of 10 to 30% for patients with advanced stage disseminated metastatic disease.³

Part of the challenge in treating patients suffering from ovarian carcinomatosis is the lack of information regarding metastatic lesions on the microscale throughout the treatment process. This inability to follow detailed spatiotemporal cellular treatment response in real time presents a major difficulty in the design and optimization of therapeutic regimens. This is especially true in our need to understand the complex interplay between therapeutic regimens and environmental factors that ultimately lead to treatment failure, in particular hypoxia and acidosis.

Hypoxia is widely recognized as a major player in the tumor microenvironment that is associated with poor prognosis and patient survival rates. In fact, about 60% of human tumors exhibit severe hypoxia or anoxia.⁴ In addition to the selective pressure it exerts on cells, hypoxia also causes the upregulation of a wide range of genes controlling processes such as proliferation, extracellular matrix (ECM) remodeling, angiogenesis, anti-apoptotic regulation, invasion, and migration.⁵ Hypoxia has been implicated in cellular resistance to front-line chemotherapeutics such as doxorubicin,⁶⁻⁸ cisplatin,^{7,9} carboplatin, and paclitaxel.⁹ As radiation therapy is oxygen-dependent, hypoxic tumor regions are additionally protected from the DNA damage caused by ionizing radiation.

Acidosis in tumors, a consequence of hypoxia and the Warburg effect, also contributes to poor therapeutic response. The intra/extracellular pH gradient within tumor microenvironments can inhibit the uptake and partitioning of weakly basic therapeutic compounds.¹⁰⁻¹² For example, Reichert et al. found that acidosis reduces the therapeutic efficacy of the frontline OvCa therapeutic cisplatin.¹³ In addition, acidosis can also trigger changes in cellular export processes: it has been demonstrated that acidosis in tumor cells increases the activity of the ABC transporter P-glycoprotein 1 (Pgp/MDR1) in the active export of the chemotherapeutic agent doxorubicin.¹⁴

Designing and evaluating therapeutics that can both reach hypoxic and acidic tumor compartments and overcome these physiological factors is challenging, especially for OvCa where the metastatic disease is microscopic and widespread. Animal models, especially when used in conjunction with *in vivo* imaging tools such as microendoscopy, can be helpful in examining treatment effects.^{15,16} These imaging approaches still only provide snapshots throughout the course of therapy, with limited ability to correlate treatment response with physiological factors like hypoxia. Although samples derived from patients are instructive, they are also episodic, and do not necessarily provide dynamic treatment response information.

Biologically relevant, *in vitro* model platforms offer the ability for real-time study, screening, and selection of optimal therapeutic compounds.¹⁷ These models, which fill a critical niche between cellular and animal experiments, can serve important roles in understanding how human-derived samples respond to treatment. *In vitro* tumor models, such as the recently developed metastatic 3D OvCa culture system,¹⁸⁻²⁰ display many of the features present in disseminated OvCa micrometastatic nodules *in vivo*. 3D culture systems restore many of the architectural cues, cell–cell adhesions, cell–matrix contacts, and associated downstream cell signals that are critical for accurately modeling the growth and treatment response of tumors *in vivo*. For example, a study using 3D tumors plated on Matrigel found that simple monolayer cultures considerably overestimated therapeutic efficacy, while 3D tumor cultures demonstrated treatment response more similar to that observed *in vivo*.²⁰ As nodules grow, they can additionally take on many of the heterogeneous microenvironmental properties found in patients, including acidic, hypoxic regions that are unresponsive to standard therapy and extracellular matrix secreted by cancer cells which can impair the uptake of therapeutics.^{21,22} Importantly, this model is compatible with advanced imaging techniques for long-term monitoring of treatment^{18,23} and high-content imaging assays to assess therapeutic efficacy on the microscale.^{19,20,23} The 3D OvCa overlay culture system has been found particularly capable at revealing the detailed spatiotemporal therapeutic response of cells in normally resistant hypoxic and acidic tumor microenvironments.²⁴

One treatment approach that has shown particular promise against therapeutically resistant OvCa is the widely used, FDA-approved modality known as photodynamic therapy (PDT).²⁴⁻²⁶ PDT utilizes molecules known as photosensitizers that preferentially accumulate in tumorous tissues and impart cellular cytotoxicity when irradiated with specific wavelengths of light. Photosensitizers can react with their environment through two complementary photochemical mechanisms: a “type I” mechanism where the molecule directly creates a variety of radical species and an oxygen-dependent “type II” mechanism where the photosensitizer generates singlet oxygen radical species that mediate cytotoxicity. The vast majority of photosensitizers currently used in the clinic operate via the type II mechanism. As opposed to the majority of cancer therapeutics that target DNA and cellular replication checkpoints, PDT imparts cytotoxicity through the direct destruction of organelles such as lysosomes and mitochondria, circumventing many oncogenic anti-apoptotic mechanisms. PDT has been successfully employed to treat cancer cells with resistance to front-line chemotherapeutics^{27,28} and has been demonstrated to synergistically enhance the efficacy of combinatorial treatment with the front-line agent carboplatin.²⁰

Like other therapeutic modalities, PDT is not without limitations, and there is no single photosensitizer that can optimally treat every type of lesion. This is particularly true in the case of hypoxic tumors, where low pO₂ is highly detrimental to type II photosensitizers that require molecular oxygen to mediate their cytotoxic effects. This lack of efficacy in hypoxic tumor microenvironments has led to a search for new photosensitizers that can effectively combat therapeutically unresponsive lesions. Recently, the molecule EtNBS (5-

ethylamino-9-diethyl-aminobenzo[*a*]phenothiazinium chloride)²⁹⁻³¹ was found to be a potent photosensitizer for the specific treatment of hypoxic tumor cell populations.²⁴ EtNBS was observed to penetrate and selectively accumulate into the difficult-to-reach acidic and hypoxic cores of large model OvCa tumor nodules, even through thick layers of extracellular matrix. Moreover, unlike PDT agents currently in clinical use, EtNBS is able to impart cytotoxicity through both type I and II mechanisms, allowing it to kill cells across a wide range of oxygen tensions and even under severe hypoxia. While our initial study found EtNBS to be a powerful antitumor agent, more optimized molecules for the treatment of acidic, hypoxic tumor environments undoubtedly exist.

In the studies reported here, we developed an optical imaging-based, tiered screening methodology for the characterization and identification of optimal hypoxia-selective side-chain-modified derivatives of EtNBS. This was accomplished by combining the *in vitro* 3D OvCa model with both standard techniques and high-content, quantitative imaging assays to create a screening approach capable of selectively evaluating therapeutics against cells in a biologically relevant hypoxic tumor environment. As the EtNBS derivatives were developed to retain the same photochemical properties as the parent compound,³² this screen was able to investigate and select optimal therapeutics based on their individual pharmacological parameters. Using this approach, we identified a derivative of EtNBS that not only kills hypoxic tumor cells but also triggers the widespread structural degradation of tumor nodules. This structural unpacking of tumor nodules can improve oxygenation and drug penetration, and has been linked to enhanced therapeutic efficacy and synergy.²⁰ The methodology developed here can be readily applied to the development of a wide range of therapeutic regimens and modalities targeting specific cellular subpopulations in a variety of cancers.

MATERIALS AND METHODS

Derivative Synthesis, Purification, and Characterization

The compounds shown in Figure 1a–c were synthesized according to procedures previously described.³² The synthesis of additional new compounds shown in Figure 1d,e are described below.

2-Carboxy-N-(9-(diethylamino)-5H-benzo[*a*]-phenothiazin-5-ylidene)ethanaminium Chloride (Figure 1d) (EtNBS-2C-COOH)—Bunte salt³² (276 mg, 1.0 mmol) and silver carbonate (606 mg, 2.2 mmol) were added to a refluxing solution of 3-(naphthalen-1-ylamino)propanoic acid (430 mg, 2.0 mmol), and the resulting deep blue reaction mixture was stirred for 30 min. After this time, the reaction mixture was filtered through a pad of Celite and concentrated to near dryness with the aid of a rotary evaporator. The resulting dark blue solid was portioned between 25 mL of dichloromethane and 10 mL of saturated sodium carbonate solution. The organic layer was separated, acidified with ~0.4 mL concentrated HCl, and allowed to dry overnight in a fume hood. The crude product was purified on a silica column, using 10% methanol in dichloromethane as the mobile phase: ¹H NMR (CD₃OD) δ 9.07 (d, *J* = 8.7 Hz, 1H), 8.31 (d, *J* = 8.7 Hz, 1H), 8.05 (d, *J* = 12 Hz, 1H), 7.9 (dt, *J* = 9.0 Hz and *J* = 1.5 Hz, 1H), 7.84 (dt, *J* = 9.0 Hz and *J* = 1.5 Hz, 1H), 7.45 (dd, *J* = 9.0 Hz and *J* = 3 Hz, 1H), 7.39 (br, s, 1H), 7.26 (d, *J* = 3 Hz, 1H), 4.0 (t, *J* = 6.9 Hz, 2H), 3.75 (q, *J* = 6.9 Hz, 4H), 2.97 (t, *J* = 6.9 Hz, 2H), 1.41 (t, *J* = 6.9 Hz, 6H). MALDI-MS calculated for C₂₃H₂₄N₃O₂S: 406.1; found 406.0. HPLC retention time = 17.7 min (C-18 column, 4.6 mm × 250 mm) with 20–100% acetonitrile in water (0.1% TFA) within 25 min.

2-Amino-N-(9-(diethylamino)-5H-benzo[a]phenothiazin-5-ylidene)ethanaminium Chloride (Figure 1e) (EtNBS-NH₂)—Bunte salt³² (276 mg, 1.0 mmol) and silver carbonate (606 mg, 2.2 mmol) were added to a refluxing solution of *tert*-butyl (2-(naphthalen-1-ylamino)ethyl)carbamate (570 mg, 2.0 mmol), and the resulting deep blue reaction mixture was stirred for 30 min. After this time, the reaction mixture was filtered through a pad of Celite and concentrated to near-dryness with the aid of a rotary evaporator. The resulting dark blue solid was portioned between 25 mL of dichloromethane and 10 mL of saturated sodium carbonate solution. The organic layer was separated, acidified with ~0.4 mL of concentrated HCl, and allowed to dry overnight in a fume hood. The crude product was purified on a C-18 preparatory HPLC column using acetonitrile and water as mobile phases: ¹H NMR (CD₃OD) δ 9.16 (d, *J* = 7.8 Hz, 1H), 8.37 (d, *J* = 8.1 Hz, 1H), 8.12 (d, *J* = 9.6 Hz, 1H), 7.94 (dt, *J* = 7.8 Hz and *J* = 1.5 Hz, 1H), 7.84 (dt, *J* = 7.8 Hz and *J* = 1.5 Hz, 1H), 7.52 (dd, *J* = 7.8 Hz and *J* = 3 Hz, 1H), 7.43 (br, s, 1H), 7.40 (d, *J* = 3 Hz, 1H), 4.0 (t, *J* = 6.6 Hz, 2H), 3.75 (q, *J* = 6.9 Hz, 4H), 3.41 (t, *J* = 6.6 Hz, 2H), 1.35 (t, *J* = 6.9 Hz, 6H). MALDI-MS calculated for C₂₂H₂₅N₄S: 377.1; found 377.0. HPLC retention time = 16.1 min (C-18 column, 4.6 mm × 250 mm) with 20-100% acetonitrile in water (0.1% TFA) within 25 min.

Cell Culture

The OVCAR5 human ovarian cancer cell line (Fox Chase Cancer Institute) was used for all experiments. This cell line had been previously characterized by microsatellite marker analysis and was kept between passage numbers 9 and 21 for all experiments. Cells were maintained in RPMI 1640 medium (mod.) 1X with L-Glutamine (CellGrow, MediaTech) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen) and 1% 5,000 IU/mL penicillin/streptomycin (CellGrow, MediaTech). Trypsinization of cells was carried out using 0.05% Trypsin (CellGro, MediaTech) following incubation with DPBS lacking calcium and magnesium (CellGro, MediaTech).

Subcellular Localization

For live cell imaging, 35 mm glass-bottom dishes (D35-20-0-N, In Vitro Scientific) were seeded with 200,000 OVCAR5 cells 24 h prior to experiments. For labeling of the mitochondria or endoplasmic reticulum (ER), cells were first incubated with 500 nM EtNBS or EtNBS derivative in 2 mL of complete medium for 1.5 h, and then washed with DPBS (CellGrow, MediaTech). This concentration of 500 nM was found to be optimal for the administration of EtNBS in previous studies.²⁴ Cells were labeled according to the manufacturer's protocol using MitoTracker Green FM (Invitrogen) or ER-Tracker Green glibenclamide BODIPY FL (Invitrogen). Lysosomal labeling was instead achieved using the manufacturer's protocol for LysoTracker Red DND-99 (Invitrogen), followed by a 1.5 h incubation in 500 nM photosensitizer solution in complete medium. Prior to imaging, dye or photosensitizer containing solutions were replaced with warmed complete medium.

During imaging, cells were maintained at 37 °C in an environmental chamber heating apparatus (DH35i, Warner Instruments). Samples were imaged using an inverted Olympus FV1000 hyperspectral laser scanning confocal microscope equipped with an Olympus UPlanSApo 60× 1.2 NA water immersion objective. The MitoTracker Green FM and ER-Tracker Green dyes were pumped using the 488 nm light from an argon ion laser, with their fluorescence emission collected between 500 and 600 nm. LysoTracker Red was visualized by excitation with a 543 nm HeNe laser, with emission collected between 555 and 655 nm. EtNBS and its derivatives were imaged using a 635 nm diode laser with the near-IR emission collected between 650 and 750 nm. Transmission images were collected simultaneously along with the fluorescence data. As EtNBS is a potent photosensitizer, great care was taken not to trigger photodamage and the subsequent rupture of lysosomes and the

ER, which causes the release and redistribution of EtNBS into the cytosol. Cells to be imaged were identified using the microscope eyepiece and brought into focus as quickly as possible using the “4× scanning” short pixel dwell time focus mode on the FV1000. Cells were imaged with a low 635 nm laser power of 50 μ W so as not to induce photodamage during image acquisition.

Images were analyzed using the ImageJ software package and the LOCI bioformats library. In order to optimize image data for qualitative analysis, image contrast and brightness were adjusted using the “HiLo” look-up table in the ImageJ software package. MitoTracker fluorescence images were processed using the smooth (twice) and sharpen (twice) functions in ImageJ to enable improved qualitative analysis of structural features.

Cellular Extraction of EtNBS and Its Derivatives

Cellular extraction of photosensitizers followed a modified protocol developed by Cincotta et al.³⁰ Uptake experiments were carried out in suspension to prevent the binding of EtNBS and its derivatives to the plastic flask surface, as had been previously reported in studies using structurally similar compounds.²⁹ Briefly, cells were trypsinized and 3,000,000 cells were pelleted for five minutes at 1,000 rpm. After the supernatant had been discarded, the cell pellet was resuspended in 6 mL of 500 nM photosensitizer in complete medium and then vortexed to ensure a uniform suspension. Cells were then incubated for a duration of either 45 min or 1.5 h at 37 °C and 5% CO₂, during which time samples were vortexed every ten minutes to prevent adherence of cells to the test tube. Following incubation, cells were pelleted by centrifugation at 1,000 rpm for five minutes. Free photosensitizer was then twice washed from cells by resuspending the pellet in DPBS, repelleting cells at 1,000 rpm for five minutes, and discarding the supernatant. The photosensitizer was extracted from cells by resuspending the pellet in methanol weakly acidified with HCl. Samples were alternately vortexed and sonicated in an ultrasonic bath (2510R-MT, Branson) until cellular debris was no longer detectable by eye.

Extraction samples were diluted in methanol weakly acidified with HCl. A fluorometer (FluoroMax-3, HORIBA) set to excite samples at 632 nm was used to acquire the fluorescence emission spectra between 650 and 800 nm. Fluorescence emission was preferentially utilized over absorbance for the determination of concentration, as fluorescence is more sensitive for low concentration measurements and less susceptible to issues related to scattering caused by cellular debris in the diluted samples. The collected fluorescence emission spectra were plotted and integrated using the Igor Pro software package to calculate the total fluorescence area under the curve (AUC). Background subtraction of all spectra was performed using a spectrum collected from a reference sample containing untreated cells processed identically to account for instrument noise as well as any contributions from endogenous cellular fluorophores. The integrated values for each derivative were normalized to the integrated fluorescence of the EtNBS-treated cell sample.

Monolayer PDT Efficacy

OVCAR5 cells were plated at a density of 50,000 cells per well in 24-well plates (353047, BD) 24 h prior to therapy. To prevent treatment light crosstalk between adjacent wells, only the four corner wells of each plate were used for each experiment. Each photosensitizer was evaluated in two separate experiments, with each experiment utilizing four wells per plate per condition for a total of $N=8$. Cells were incubated in 1 mL of 500 nM photosensitizer solution in complete medium for 1.5 h at 37 °C and 5% CO₂. Photosensitizer containing medium was aspirated from each well and replaced with fresh complete medium prior to PDT. Irradiation was carried out with 100 mW/cm² light from a 660 nm centered high-power diode source (M660L2, ThorLabs) delivered to the bottom of culture wells using a

custom-built, timed illumination system. Precise illumination timing was accomplished using a fast shutter (VS25S2ZM0, Uniblitz) and programmable shutter driver (T132, Uniblitz). Following PDT, cells were returned to the 37 °C incubator before evaluation of cellular viability 24 h post-PDT.

Monolayer Cellular Viability Assay

To measure the therapeutic response of OVCAR5 cells to the EtNBS derivative library, the MTT assay was selected to report cellular viability 24 h post-PDT. The MTT assay relies on the reduction of methylthiazolyldiphenyltetrazolium bromide by mitochondrial enzymes to an insoluble purple-colored formazan to report cellular viabilities. This colorimetric assay is used to report relative living cell number, and has been found to correlate well with the clonogenic assay for many cell lines and therapy regimens,³³⁻³⁵ including PDT.^{36,37} In addition, the MTT assay has been used in numerous PDT studies utilizing the OVCAR5 cell line.^{26,38,39} As the cellular response to therapy was found to be essentially complete at 24 h post-illumination, it was determined that this time point would be ideal for administration of the MTT assay.

Twenty-four hours following illumination, cells were incubated in 400 μ L of 1 mg/mL MTT solution (Sigma) in complete medium for 60 min at 37 °C and 5% CO₂. The MTT medium solution was aspirated completely from all wells and replaced with 400 μ L of DMSO for the extraction of cellular formazan. Cells were incubated in subdued light for 15 min at room temperature, and the absorbance of formazan containing DMSO at 570 nm was measured using a plate reader (SpectraMax M5, Molecular Devices).

The average absorbance was calculated for each photosensitizer and treatment condition, with the average absorbance from untreated cells used to define 100% viability. The average absorbances for each treatment condition were normalized to this no treatment (NT) control. An outlier analysis was carried out, with outliers defined as values lying beyond ± 2 standard deviations. Final cellular viabilities were calculated by averaging the outlier-rejected, normalized absorbances. The error was calculated by taking the standard deviation of the averaged, normalized experimental values.

The *in Vitro* 3D Hypoxic Micrometastatic OVCAR5 Tumor Model

3D cultures were plated in either 35 mm glass-bottom plates (P35G-0-14-C, MatTek) or 24-well glass-bottom plates (P24G-0-13-F, MatTek) based on protocols described previously.¹⁸⁻²⁰ It should be noted that 3D cultures were plated in every other well of 24-well plates to prevent treatment light crosstalk between wells. Using this configuration, trial experiments found no evidence of treatment crosstalk between culture wells.

Time-Lapse Uptake Imaging and Analysis of Uptake Kinetics

Thirteen day old 3D cultures in 35 mm dishes were placed in an environmental chamber (DH35i, Warner Instruments) and maintained at 37 °C and 5% CO₂ for the duration of imaging sessions. The 3D cultures were imaged using an inverted Olympus FV1000 hyperspectral confocal beams scanning microscope configured with a programmable motorized stage. Multiple positions within each culture dish were selected and programmed into the automated software package prior to the addition of photosensitizer. Once the program was ready, cultures were then treated with 2 mL of 500 nM photosensitizer in complete medium and imaging was initiated immediately. To avoid triggering photodamage, a short dwell time of 2 μ s per pixel was used along with a very low 635 nm laser power of 13 μ W. Time-lapse images were acquired every 30 min for 5 h with an initial time point immediately following administration of the photosensitizer. Images were acquired using an Olympus UPlanSApo 10 $\times$ 0.4NA objective with the photosensitizer fluorescence collected

between 650 and 750 nm. Transmission images were simultaneously collected along with the fluorescence data.

Postacquisition image analysis was carried out with the ImageJ and Matlab (Mathworks) software packages using the open-source LOCI bioformats library. ImageJ and the Keynote presentation program (Apple) were used to build the time-lapse uptake movie presented in Supplementary Movie 1 in the Supporting Information.

Cryosectioning of 3D OvCa Cultures

Cryosectioning was carried out using a previously published protocol.²⁴ Sections were immediately adhered to a slide and covered with a coverslip to prevent drying.

Staining for Hypoxia with Pimodinazol in 3D Nodules

Staining for hypoxia in day 13 OVCAR5 nodules was carried out using a previously reported protocol,²⁴ derived, in part, from Debnath et al.^{18,40} AlexaFluor 568 goat anti-mouse secondary antibodies (Invitrogen) were used to label the anti-pimodinazole antibodies bound to hypoxic regions.

PDT of 3D OvCa Cultures

Day 13 3D cultures in 24-well plates were incubated in 1 mL of 500 nM photosensitizer solution in complete medium for 4.5 h at 37 °C and 5% CO₂. Photosensitizer containing medium was aspirated from wells and replaced with 2% GFR Matrigel-supplemented complete medium, followed immediately by PDT. Irradiation of tumor nodules was carried out on the custom-built illumination system using the output of a 670 nm fiber coupled diode laser (model 7401, HPD) at an irradiance of 130 mW/cm². This slightly higher irradiance than used in the monolayer PDT experiments was chosen based on measurements of laser power loss due to scattering of the treatment light passing through the Matrigel plates. Following treatment, plates were returned to the 37 °C incubator for 24 h before evaluation of viability using the Live/Dead assay.

High-Content Live/Dead Imaging Assay and Quantitative Analysis

To quantify both the overall cytotoxicity and the spatial patterns of cell killing within individual nodules, the Live/Dead viability/cytotoxicity assay kit (L3224, Invitrogen) was used for the high-content imaging assay as previously described.^{18,19} The major advantage of this high-content approach is that it generates hundreds of images containing thousands of individual nodules per photosensitizer and treatment condition that can be mined for detailed treatment response information. Images collected with the confocal microscope were processed using ImageJ and the open source LOCI bioformat package. The high-content Live/Dead data was processed using custom-developed Matlab routines and the LOCI bioformats package. The total viability values were calculated using a modified version of a Matlab script described previously.²⁴ Each live or dead fluorescence image was background subtracted using a calculated average live or dead channel background value calculated from no treatment (NT) control wells. As the exact same imaging parameters were used for every experiment, all results could be directly and quantitatively compared between experiments and treatment conditions.

To find the core cell killing efficiency, a custom Matlab routine was used to first create a binary mask using Otsu's method from a combined background-subtracted, fluorescence image composed of both live and dead fluorescence channels. To define a peripheral region with uniform depth, an outline was then drawn around the outside of each nodule with a thickness of approximately 105 μ m. This outlined region was then compared to the initial binary mask using a binary XOR to generate a nodule core mask. This mask was then

applied to the live and dead images, and the individual pixel intensities were summed to tabulate nodule core live and dead fluorescence intensities. These intensities were then converted into viabilities (viability = live signal/(live + dead signals)) and normalized to NT control values.

To quantify the degree of nodular structural breakdown post-PDT, a Matlab routine was developed to count the number of individual small particles created by treatment. First, a total fluorescence image for each well was calculated by summing background-subtracted live and dead images together. This combined image was then processed using a 2D circular averaging filter of approximately 30 μm in diameter to smooth out fine image features. This blurred image was then subtracted from the total fluorescence image to remove large image features and isolate particles 30 μm and smaller in diameter. The number of these features was then counted and averaged across each treatment condition.

Statistical Analysis

Statistical analysis was accomplished using the JMP 9 software package (SAS). Pairwise Student *t* tests were employed to calculate statistical significance.

RESULTS

Cellular Uptake Is Related to Net Derivative Charge

The first tier of the screen was focused on selecting photosensitizers that demonstrated cytotoxicity against monolayer OvCa cultures. The uptake of photosensitizers into OVCAR5 cells in suspension was determined after both 45 and 90 min incubation periods to look for derivative-dependent saturation effects. An advantage of using photosensitizers in this tiered screen is that they are themselves fluorescent, allowing for direct quantification of uptake and cellular concentration. No appreciable derivative-dependent uptake rates at these two time points were observed, indicating that the rate of uptake is consistent over a 90 min incubation period for all derivatives (Figure 2).

Cellular uptake was found to be dominated by the net molecular charge of the derivative species. Intracellular levels of EtNBS, EtNBS-OH, and EtNBS-NH₂ were found to be quite similar, while the two zwitterionic carboxylic acid-terminated derivatives, EtNBS-2C-COOH and EtNBS-COOH, had drastically reduced intracellular concentrations after incubations of 45 and 90 min. Structurally, these two compounds differ only in the length of the carbon linker between the conjugated ring system and the terminal carboxylic acid. It is worth noting that there is a statistically significant difference in uptake between the two acid compounds (0.012 ± 0.005 , EtNBS-2C-COOH, and 0.089 ± 0.023 , EtNBS-COOH, 90 min, $p < 0.007$), which is likely due to the difference in molecular charge distribution.⁴¹

EtNBS Derivatives Have the Same Subcellular Localization Patterns

The subcellular localization of different EtNBS derivatives was definitively identified by coadministration and live cell imaging with fluorescent markers for the ER, lysosomes, or mitochondria. Due to the potency of EtNBS as a photosensitizer, great lengths were taken during imaging to prevent cellular photodamage and the associated redistribution to the cytosol, cytomembranes, and other intracellular structures as has been previously reported.⁴² Through colocalization analysis, the subcellular localization of all side-chain-modified derivatives was found to be identical to that of EtNBS.

All of the derivatives exhibited the same pattern of punctate perinuclear lysosomal localization, as well as the more diffuse localization to the ER, as the parent EtNBS compound (Figure 3). At a concentration of 500 nM, none of the derivatives were observed

to localize to the mitochondria. Although the intracellular concentrations of the EtNBS carboxylic acid-terminated derivatives were low, derivative fluorescence was detected and observed to have the same subcellular localization as the parent compound.

This result suggests that, while the side-chain modifications influence cellular uptake and retention, subcellular localization is dominated by the derivatives' shared benzo[*a*]-phenothiazinium conjugated ring system. This also indicates that all derivatives are likely to affect the same subcellular structures, and thus impart cellular cytotoxicity via the same therapeutic mechanisms. This result, along with the knowledge that the photochemical properties of EtNBS are orthogonal to side-chain modifications,³² enabled the direct comparison of derivative-dependent therapeutic effects.

Cationic EtNBS Derivatives Demonstrate Greater Efficacy Than Their Neutral Counterparts

The photocytotoxicities of EtNBS derivatives were evaluated at a concentration of 500 nM with irradiations of 1, 5, 10, 15, and 20 J/cm² delivered to the bottom of wells. It is worth noting that some light power was lost due to scattering that occurred as light passed through the bottom of the plates. Not surprisingly, the PDT efficacy of each EtNBS derivative was found to be correlated with its degree of cellular uptake (Figure 2); derivatives with the highest degrees of uptake were found to have the greatest cellular cytotoxicity (Figure 4). Despite their side-chain modifications, derivatives that displayed uptake comparable to that of EtNBS were found to have the same level of dark (photosensitizer only, no light) toxicity.

EtNBS-2C-COOH was the only derivative that did not display any photocytotoxicity at any of the light doses administered. Interestingly, treatment with EtNBS-2C-COOH appeared to stimulate cell proliferation in a light-dose-dependent manner as measured using the MTT assay. This is consistent with previous reports using low light dose Visudyne (BPD)-PDT.¹⁹ EtNBS-COOH experienced a less drastic reduction in PDT efficacy, with a calculated normalized cellular viability of 0.60 ± 0.13 after 20 J/cm² treatment. EtNBS-OH and EtNBS-NH₂ displayed PDT-induced cell killing that were essentially identical to that of EtNBS for all of the light doses administered.

As it was the only derivative that did not display any photocytotoxicity, EtNBS-2C-COOH was not carried into the next tier of screening.

Quantitative Confocal Time-Lapse Imaging of Derivative Uptake Shows Distinct Spatial and Kinetic Patterns in 3D Tumor Nodules

Time-lapse confocal microscopy was employed in the next screening tier to quantitatively visualize the uptake parameters of EtNBS and its derivatives in the hypoxic 3D OvCa model. Identical image settings were used for each time-lapse uptake experiment to enable direct comparison between EtNBS derivative uptake data sets.

EtNBS was observed to rapidly concentrate into the large metastatic OvCa nodules over a 4.5 h period, as previously reported (Figure 5a).²⁴ The addition of a hydroxyl-terminated alkyl chain did not seem to significantly alter this uptake pattern, with the EtNBS-OH derivative observed to localize throughout the tumor nodules (Figure 5b). EtNBS-COOH, on the other hand, appeared to have substantially reduced nodular uptake (Figure 5c), consistent with the reduced cellular uptake observed in monolayer experiments. Interestingly, EtNBS-NH₂ was observed to have a very different pattern of spatial uptake, with its localization almost exclusively restricted to the periphery of 3D tumor nodules (Figure 5d). Despite the altered uptake pattern, the strong level of EtNBS-NH₂ fluorescence intensity in the nodule periphery indicates a local concentration comparable to that of EtNBS (Figure 5a).

Comparison of the derivative uptake time-lapse movies reveals that the side-chain modifications also influenced photosensitizer uptake kinetics and spatial localization patterns. Time-lapse movies taken at select well positions are shown in Supplementary Movie 1 in the Supporting Information. Both EtNBS and EtNBS-OH are observed to have very similar uptake kinetics, with the fluorescence of both photosensitizers observed to plateau after 4.5 h. Although the initial rates of nodular uptake of EtNBS-NH₂ and EtNBS-COOH are comparable to that of EtNBS, after about 30 min the uptake of both compounds begin to approach an asymptote. EtNBS-NH₂ uptake into the periphery of nodules plateaus after approximately 120 min, as the limited penetration depth likely served to prevent further accumulation. On the other hand, EtNBS-COOH was observed to penetrate through the entirety of nodules, but its lack of nodular uptake and accumulation is thought to be due to its low retention on the cellular level (Figure 2).

It should be noted that, in such large tumor nodules (>300 μ m diameter), confocal microscopy naturally suffers from signal loss due to depth-dependent scattering and absorbance. To visualize the true, non-optically influenced uptake patterns, nodules incubated with the different derivatives were cryosectioned.

Cryosectioning Reveals Detailed Microscale Uptake Patterns of EtNBS Derivatives in the 3D *in Vitro* Nodules

Fluorescence microscopy of 35 μ m thick physical sections taken from nodules treated with photosensitizer clearly showed derivative-dependent uptake patterns. It should be noted that images of the physical sections were acquired and processed to optimize the spatial uptake pattern contrast. Thus, unlike images collected during the time-lapse confocal imaging experiments above, fluorescence intensities in the physically sectioned images should only be qualitatively compared.

As in previous studies, EtNBS was found to selectively concentrate predominantly into the acidic, hypoxic cores (Figure 6a) of large model metastatic tumor nodules.²⁴ Similarly, EtNBS-OH is also observed to accumulate into the core of the OvCa nodules, but with one very important difference: EtNBS-OH was found to localize in a more diffuse pattern over a greater spatial region than the parent compound, as indicated by the fluorescence distribution observed throughout a much larger volume in the nodule center (Figure 6b). This difference was consistently observed in nodules across many slices, indicating a strong trend in localization behavior. Interestingly, EtNBS-COOH was also observed to diffusely localize throughout the core of the OvCa nodules (Figure 6c), albeit at much lower uptake concentrations (Figure 5c).

Physical sections of nodules incubated with EtNBS-NH₂ showed uptake only in the outer three to five cell layers of nodules, as suggested by the time-lapse imaging experiments (Figure 6d). Interestingly, unlike the other derivatives that were observed to evenly distribute throughout nodules, EtNBS-NH₂ localized almost exclusively to those cells in contact with the incubation medium; cells in the nodule that were seated within the Matrigel bed (right half of Figure 6d) did not take up EtNBS-NH₂. Due to its poor uptake into the hypoxic and acidic nodule cores, EtNBS-NH₂ was excluded from the next screening tier.

High-Content Image Analysis of Derivative-Induced PDT Efficacy in Large, Hypoxic Spheroids

In the final tier of this study, the remaining photosensitizers were screened for their photocytotoxicity against the naturally hypoxic, 3D OvCa nodule culture system. Day 13 OvCa nodules were incubated for 4.5 h with 1 mL of 500 nM concentrations of EtNBS, EtNBS-OH, and EtNBS-COOH, and then irradiated for PDT. A 500 nM dose and 4.5 h

incubation time were found to be ideal for EtNBS-PDT in previous studies.²⁴ A high-content viability assay based on the Live/Dead kit was used to analyze the photodynamic therapy response. In order to directly compare photodynamic treatment efficacy of each derivative, identical imaging parameters were used for all Live/Dead imaging assay experiments. Previous studies have shown that apoptosis is the primary method of cell death 24 h following EtNBS-PDT at the concentration, incubation duration, and range of light doses used in these experiments.²⁴

The zwitterionic carboxylic acid compound EtNBS-COOH was found to display very limited PDT killing efficacy, with a normalized post-PDT cellular viability of 0.88 ± 0.07 . EtNBS and EtNBS-OH were observed to have statistically similar overall PDT killing efficacies (0.64 ± 0.08 and 0.68 ± 0.08 , respectively; $p = 0.22$), which was not entirely surprising given their similar nodular uptake rates and localization patterns. There were no statistically significant differences between the overall dark toxicities associated with any of the three compounds evaluated (Figure 7). Examination of the EtNBS and EtNBS-OH Live/Dead images, however, revealed major differences that required further investigation.

One of the distinct advantages of high-content image-based viability assays, such as the fluorescent Live/Dead assay used here, is the capability to revisit and mine the collected data using advanced image analysis routines. Time-lapse imaging and physical sectioning revealed that EtNBS had the greatest concentration of any photosensitizer in the hypoxic and acidic cores of the OvCa nodules (Figures 5a, 6a), leading us to hypothesize that this molecule would have greater core photocytotoxicity than its alcohol-terminated derivative. To directly assess the viability of cellular subpopulations in the treated nodules, a Matlab routine was developed to parse the imaging data and selectively extract post-PDT, tumor nodule core viabilities. As predicted, EtNBS was indeed determined to have better killing efficiency within the nodule core than EtNBS-OH after treatment at 26 J/cm^2 ($p < 0.0025$). In addition, the difference between the core dark toxicities was also shown to be statistically significant ($p < 0.025$), with EtNBS having a greater dark toxicity than EtNBS-OH (Figure 8). These results confirm that the greater core localization of EtNBS leads to its enhanced ability to selectively kill hypoxic and acidic core cells.

Beyond local changes in cellular viability, significant visual differences were observed between the EtNBS and EtNBS-OH Live/Dead killing patterns. One consequence of PDT that has received a great deal of interest for its clinical and therapeutic relevance is PDT-induced disruption of tumor architecture.²⁰ In this mechanism, structural degradation leads to enhanced penetration of oxygen and therapeutics into previously hypoxic and acidic tumor environments. A major difference in unpacking and fragmentation can be seen in Figure 9, where nodules treated with 26 J/cm^2 EtNBS-OH displayed far greater degradation than nodules treated at the same light dose with EtNBS. To quantitatively evaluate nodular architecture disruption, a Matlab routine was developed to spatially filter and count the number of unique nodular fragments smaller than $30 \mu\text{m}$. EtNBS-OH was found to cause statistically significantly greater PDT-induced disruption than EtNBS after both 13 J/cm^2 ($p < 0.02$) and 26 J/cm^2 ($p < 0.0001$) treatments (Figure 9c).

DISCUSSION

This multistage, imaging-based 3D *in vitro* screening platform was successful in evaluating a small library of photosensitizer derivative compounds against hypoxic and acidic tumor environments. We found that side-chain modifications can readily tune the behavior of EtNBS as a therapeutic agent, allowing for customization of spatial uptake rates and localization patterns. Using 3D tumor nodule cultures to examine pharmacological parameters including uptake, uptake rate, localization patterns, and therapeutic efficacy

offers considerable advantages over traditional screening methods while retaining the simplicity of standard *in vitro* approaches. For example, while a traditional screen utilizing monolayer cultures would have identified the cationic EtNBS derivatives as optimal, it likely would not have predicted the poor nodular uptake of EtNBS-NH₂. At the same time, it would have been extremely difficult to capture the same spatiotemporal pharmacokinetic, cytotoxicity, and structural change information obtained here when using *in vivo* animal model systems. The complex structural degradation differences found between EtNBS and EtNBS-OH, for example, would be most challenging to observe and quantify in a murine model of ovarian cancer.⁴³ High-content 3D model screens have the capability of being synergistic with traditional monolayer culture and xenograft model approaches, as *in vitro* 3D models can be used to weed out ineffective compounds and examine detailed long-term therapeutic responses that would be difficult or otherwise impossible to observe *in vivo*.

As has been observed in past studies utilizing 3D culture systems,^{20,24,44} a large difference in therapeutic response was observed between 2D and 3D cultures, with adherent 2D cell cultures displaying far greater cellular death than 3D cultures under similar treatment conditions. This treatment response difference is thought to arise from the artificial vulnerability of cells plated in simple 2D cell cultures, which lack many of the critical environmental and cellular signaling cues found *in vivo* that promote cell survival.⁴⁵ 3D culture systems, which partially restore these important signals through architectural, cell–cell, and cell–matrix signaling, display elevated treatment resistance and likely better model treatment response *in vivo*.⁴⁶ Additionally, even though the photosensitizers screened in this study could concentrate into the acidic and hypoxic nodule cores of large nodules, the cellular programs that act to protect cells in these harsh environments may have still played a pro-survival role,^{6,7,9,10} reducing overall therapeutic efficacy.

On the cellular level, we observed clear molecular chargemediated effects on the uptake of our side-chain-modified EtNBS analogues. Cationic photosensitizers were found to have high cellular retention, likely due to their rapid uptake into subcellular organelles. The zwitterionic carboxylic acid derivatives, on the other hand, were found to have reduced retention in cells, matching the behavior of other zwitterionic dyes.⁴¹ The charge distribution within a zwitterion has a very strong effect on its retention in cells and tissues, with more evenly, spherically distributed charges leading to reduced binding and retention.⁴¹ EtNBS-COOH, with a five-carbon alkyl linking chain, has a larger relative charge dipole than EtNBS-2C-COOH and showed increased relative retention, and thus efficacy, in cells. Another possible reason for the reduced retention of the zwitterionic derivatives may be the involvement of membrane transport proteins, which have been found to be active in pumping small molecules and chemotherapeutics out of cells.

Our results are consistent with previous studies that have determined the dominant mechanism of cellular uptake of EtNBS and similar molecules to be passive diffusion across cellular membranes. While the conjugated ring system of EtNBS and its derivatives holds a +1 positive charge, this charge is highly delocalized. Past studies with lipophilic, chargedelocalized cations, such as tetraphenyl borate analogues, have demonstrated that the capacity for passive diffusion across cellular membranes directly corresponds to the area over which the charge is delocalized.^{47,48} Further evidence supporting a passive diffusion mechanism comes from low-temperature cellular uptake studies of benzophenoxazines that found active transport and endocytosis to be non-dominant pathways for cellular uptake.²⁹ Once in the cell, EtNBS and its derivatives are seen to localize primarily to lysosomes, as has been observed previously.^{29,49}

Another possible parallel mechanism of uptake, albeit with a far lesser contribution to overall uptake, are electrostatic interactions of EtNBS and its analogues with cellular

polyanions, and hydrophobic interactions with lipophilic membrane constituents.²⁹ These interactions may contribute to the observed localization and accumulation of these compounds to the ER and lysosomes respectively, as the potential exists for internalization of the dyes via constitutive membrane recycling through the endocytic network and the recycling and processing of membrane-bound glycoproteins in the ER.²⁹ This mechanism may play a more important role in the uptake of EtNBS derivatives into the 3D tumor nodules.

The parent compound, EtNBS-OH, and EtNBS-COOH were all observed to localize to the core of 3D OvCa nodules, although with different kinetic rates. The exception was the primary aliphatic amine derivative EtNBS-NH₂, which was found to not penetrate more than three to five cell layers into nodules. One potential explanation for this limited uptake may be that the side-chain primary amines underwent non-specific hydrogen bonding to cellular and extracellular matrix components, thus limiting the ability of EtNBS-NH₂ to passively diffuse deep into the tumor nodules.⁵⁰ Additionally, it is worth noting that, at neutral and acidic pH levels, the primary amine of EtNBS-NH₂ is predominantly protonated, yielding EtNBS-NH₃⁺ with a localized positive charge of +1 on the side chain. This point charge, unlike the delocalized cationic charge of the core benzo[*a*]phenothiazinium molecule, has the potential for strong electrostatic interactions with polyanionic ECM and membrane constituents, potentially leading to the reduced penetration observed in the OvCa nodules. This surface localization may actually be useful for certain cancer therapeutic applications targeting the tumor surface or parenchyma. In the treatment of OvCa, for example, EtNBS-NH₂ could be co-delivered along with core-localizing EtNBS for spatially targeted peripheral nodule cytotoxicity.

The most interesting finding of this tiered image-based study was that, while both EtNBS and EtNBS-OH were found to have similar uptake, localization, and overall cytotoxicities, their therapeutic effects led to significantly different treatment outcomes. Both the parent and EtNBS-OH derivative localized into the core region of nodules, which is thought to be driven by diffusion of the cationic compounds along the nodular pH gradient.²⁴ However, EtNBS is predominantly located in the most acidic portion of the nodule core, while EtNBS-OH is observed to be more delocalized, likely due to the derivative's hydrophilic terminal hydroxyl group. This decentralized localization of EtNBS-OH led to the generation of reactive radical species throughout the interior of tumor nodules, which induced large-scale nodular structural degradation.

CONCLUSIONS

Tumor structural degradation has numerous therapeutic benefits and is especially apt for the many combination treatment regimens used to treat cancer in the clinic. By structurally decomposing a tumor nodule, many of the cellular junctions and cell layers that normally slow the diffusion of both oxygen and therapeutics into tumors are disrupted. This can provide oxygen to previously hypoxic core cells, reduce intertumoral acidity, and open channels for the delivery of cytotoxic agents that normally would not penetrate deeply into solid tissue. Front-line agents that either fail in low oxygen environments or do not partition into cells in acidic compartments can gain considerable boosts in therapeutic efficacy. For example, a recent PDT study utilizing the photosensitizer BPD found that structural degradation conferred a significant synergistic enhancement of carboplatin efficacy in OvCa tumor models.²⁰ As EtNBS-OH shares many of the same properties as EtNBS, such as efficacy against hypoxic cells, overall cellular cytotoxicity, rapid and deep penetration into the hypoxic, and acidic cores of tumor nodules, and has the additional capability of causing widespread nodular structural degradation, we feel that it will be an excellent tool for the treatment of OvCa and other hypoxic, disseminated cancers. This result would have been

difficult or impossible to find *in vivo*, and would not have been predicted by monolayer culture experiments.

As these results demonstrate, tiered drug screening platforms taking advantage of 3D *in vitro* models have the capability to play important roles in current and future drug development studies. Such platforms can be easily adapted to accommodate a wide variety of cancer models, and can be used for the development and optimization of a range of therapeutic compounds and combinatorial treatment regimens for the treatment of select cellular subpopulations. For example, although the EtNBS parent compound and EtNBS-OH derivative were found ideal when screened against our hypoxic OvCa model, their success is likely particular to the tumor microenvironment modeled by the culture system. *In vitro* cultures replicating different or more complex cancers, such as prostate, pancreatic, or lung, with varied environmental conditions including high interstitial pressure or leaky neovasculature could find different derivatives more successful.

High-content, image-based 3D model screens enable unique mechanistic insights along with the ability to monitor time-dependent processes that would be impossible to elucidate in simple monolayer *in vitro* models, at a level of detail that would be difficult or impossible to achieve *in vivo*. With the advent of low-cost, automated imaging and computational analysis systems,¹⁷ these approaches have the potential to reduce the cost, time, and number of animals typically consumed by conventional screening methods for evaluating the properties of novel compounds, leading to better, faster cures in the fight against cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

AUC	area under the curve
DPBS	Dulbecco's phosphate buffered saline
DMSO	dimethyl sulfoxide
DT	dark, no light, toxicity
ECM	extracellular matrix
ER	endoplasmic reticulum
EtNBS	5-ethylamino-9-diethylaminobenzo[<i>a</i>]phenothiazinium chloride
EtNBS-OH	5-(3'-hydroxypropylamino)-9-diethylaminobenzo[<i>a</i>]phenothiazinium chloride
EtNBS-COOH	5-(4'-carboxybutylamino)-9-diethylaminobenzo[<i>a</i>]phenothiazinium chloride
EtNBS-2C-COOH	5-(2'-carboxyethylamino)-9-diethylaminobenzo[<i>a</i>]phenothiazinium chloride

EtNBS-NH₂	5-(2'-aminoethylamino)-9-diethylaminobenzo-[a]phenothiazinium chloride
GFR	growth factor reduced
¹H NMR	proton nuclear magnetic resonance spectroscopy
HPLC	high performance liquid chromatography
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
MDR1	multidrug resistance transporter 1
MTT	methylthiazolyldiphenyltetrazolium bromide
NT	no treatment
OCT	optimal cutting temperature compound
OvCa	ovarian cancer
PDT	photodynamic therapy
Pgp	P-glycoprotein 1
RPMI	Roswell Park Memorial Institute medium
TFA	trifluoroacetic acid

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Figure 1.
Chemical structures of EtNBS and its derivatives: (a) EtNBS; (b) EtNBS-OH; (c) EtNBS-COOH; (d) EtNBS-2C-COOH; (e) EtNBS-NH₂.

Figure 2.

Cellular uptake of EtNBS and its derivatives into cells after 45 and 90 min incubations. The +0, +1, and +2 labels report the net charge of each derivative at neutral pH. Uptake of the photosensitizers into cells was quantified using fluorescence, with all values normalized to the fluorescence level of EtNBS. The cationic EtNBS, EtNBS-OH, and EtNBS-NH₂ displayed the highest degree of cellular uptake. The difference between these uptake values was not determined to be statistically significant. The zwitterionic EtNBS-COOH and EtNBS-2C-COOH compounds were found to have substantially reduced cellular uptake.

Figure 3. Subcellular localization of EtNBS. Cells were incubated with EtNBS along with fluorescent markers for subcellular organelles. The fluorescent markers for each organelle are presented in green, while the fluorescence of EtNBS is presented as red. EtNBS is observed to localize into the (a) endoplasmic reticulum and (b) lysosomes, but not into (c) mitochondria.

Figure 4.

Normalized viabilities of monolayer cultures treated with EtNBS and its derivatives across different treatment conditions. All viabilities were determined using the MTT assay, with results normalized to a no treatment (NT) control. The cationic EtNBS, EtNBS-OH, and EtNBS-NH₂ are observed to have nearly identical therapeutic efficacies across all light doses. While some therapeutic effect can be observed from EtNBS-COOH, EtNBS-2C-COOH is not observed to kill cells. LO: light only treatment. DT: dark toxicity treatment.

Figure 5.

Time-lapse confocal uptake imaging of 3D *in vitro* OvCa tumor nodules reveals spatial uptake and localization dynamics. Day 13 3D tumor nodules were incubated with photosensitizer for a period of 5 h and imaged every 30 min using identical parameters. Figure 5a–d shows the uptake and localization fluorescence intensity of each derivative at 5 h postadministration. The uptake intensity of each photosensitizer is shown in false color to enhance contrast: (a) EtNBS, (b) EtNBS-OH, (c) EtNBS-COOH, and (d) EtNBS-NH₂. A time-lapse movie presenting the entire uptake time-series is available as Supplementary Movie 1 in the Supporting Information.

Figure 6.

Physical sectioning and fluorescence microscopy reveal the uptake and localization pattern of EtNBS and its derivatives. Day 13 3D OvCa tumor nodules were incubated with EtNBS and its derivatives for 4.5 h before being frozen and subsequently sliced into 35 μm thick sections. Sections were imaged via fluorescence on a confocal microscope and are shown here in false color to enhance contrast: (a) EtNBS, (b) EtNBS-OH, (c) EtNBS-COOH, and (d) EtNBS-NH₂. (e) Two-photon image of a pimodinazole-stained 3D tumor nodule shown here in false color to visualize hypoxic tumor cells contained inside the large nodules.

Figure 7. Normalized viabilities 24 h following the PDT of 3D tumor cultures as measured using the high-content Live/Dead assay. Nodule cultures were treated with EtNBS, EtNBS-OH, or EtNBS-COOH across different light doses. All viabilities were normalized to the no treatment (NT) control. DT: dark toxicity treatment.

Figure 8.

Box plot of 3D tumor culture core cell viabilities following EtNBS and EtNBS-OH PDT. Images collected using the Live/Dead viability assay (used to generate Figure 7) were processed using a Matlab script to specifically assess the viabilities of nodule core regions. Calculated viabilities were normalized to the no treatment (NT) control. EtNBS was found to be statistically significantly more cytotoxic to nodule core cells at a treatment light dose of 26 J/cm². DT: dark toxicity treatment.

Figure 9.

EtNBS-OH-PDT causes significant nodule structural degradation 24 h following treatment.

(a) Transmission image of EtNBS-PDT nodules treated with 26 J/cm² light. (b)

Transmission image of EtNBS-OH-PDT nodules treated at the same light dose showing

considerably greater structural degradation in all nodules. (c) Box plot of particle counts

generated using a custom Matlab script. EtNBS-OH was found to cause a statistically

significant enhancement in structural degradation over EtNBS at both 13 J/cm² ($p < 0.02$)

and 26 J/cm² ($p < 0.0001$).