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### Article

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1 Chemical features of the photosensitizers new methylene blue N and S137 influence their  
2 subcellular localization and photoinactivation efficiency in *Candida albicans*

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6 Gabriela Braga Rodrigues<sup>a,§</sup>, Guilherme Thomaz Pereira Brancini<sup>a,§</sup>, Sérgio Akira  
7 Uyemura<sup>a</sup>, Luciano Bachmann<sup>b</sup>, Mark Wainwright<sup>c</sup>, Gilberto Ubida Leite Braga<sup>a,\*</sup>

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11 <sup>a</sup>Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de  
12 Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto,  
13 SP 14040-903, Brazil

14 <sup>b</sup>Departamento de Física, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto.  
15 Universidade de São Paulo, Ribeirão Preto, SP 14040-903, Brazil

16 <sup>c</sup>School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University,  
17 Liverpool L3 3AF, United Kingdom

18

19 <sup>\*</sup>Corresponding author: Faculdade de Ciências Farmacêuticas de Ribeirão Preto,  
20 Universidade de São Paulo, Ribeirão Preto, São Paulo, 14040-903, Brazil.

21 E-mail adress: gbraga@fcfrp.usp.br.

22 <sup>§</sup>These authors contributed equally to this work.

23

24 **ABSTRACT**

25 Antimicrobial photodynamic treatment (APDT) has emerged as an effective  
26 therapy against pathogenic fungi with both acquired and intrinsic resistance to commonly  
27 used antifungal agents. Success of APDT depends on the availability of effective  
28 photosensitizers capable of acting on different fungal structures and species. Among the  
29 phenothiazinium dyes tested as photoantifungals, new methylene blue N (NMBN) and  
30 the novel pentacyclic compound S137 are the most efficient. In the present study we  
31 compared the effects of APDT with NMBN and S137 on the survival of *Candida albicans*  
32 and employed a set of fluorescent probes (propidium iodide, FUN-1, JC-1, DHR-123 and  
33 DHE) together with confocal microscopy and flow cytometry to evaluate the effects of  
34 these two chemically diverse photosensitizers on cell membrane permeability,  
35 metabolism and redox status, and mitochondrial activity. Taken together, our results  
36 indicate that, due to chemical features resulting in different lipophilicity, NMBN and  
37 S137 localize to distinct subcellular structures and hence inactivate *C. albicans* cells via  
38 different mechanisms. S137 localizes mostly to the cell membrane and, upon light  
39 exposure, photo-oxidizes membrane lipids. NMBN readily localizes to mitochondria and  
40 exerts its photodynamic effects there, which was observed to be a less effective way to  
41 achieve cell death at lower light fluences.

42

43 **Keywords:** antimicrobial photodynamic treatment, fungal photodynamic inactivation,  
44 phenothiazine photosensitizers, fluorescent probes, reactive oxygen species

45

## 46 1. Introduction

47 Several procedures in modern medicine, such as solid organ and hematopoietic  
48 stem cell transplantations, surgeries, autoimmune disease therapies, and uncontrolled  
49 HIV infection make millions of patients vulnerable to lethal fungal diseases (Köhler et al.  
50 2015; Limper et al. 2017). *Candida albicans*, usually a harmless commensal fungus, is  
51 also an opportunistic pathogen for immunocompromised people and the major human  
52 fungal pathogen in the USA and several other countries (Nishimoto et al. 2020). Today,  
53 fungal infections are among the most difficult diseases to treat in humans (Köhler et al.  
54 2015). One of the factors that makes treatment so difficult is the rapid acquisition of  
55 resistance to all of the only four major classes of antifungal agents clinically available:  
56 azoles, polyenes, echinocandins, and a nucleotide analog (Chang et al. 2019; Perlin et al.  
57 2017; Shor and Perlin 2015). Additionally, many species of *Candida*, such as *Candida*  
58 *auris* and *Candida glabrata* are intrinsically resistant to some antifungal classes (Chang  
59 et al. 2019; Nishimoto et al. 2020; Rhodes and Fisher 2019). Multidrug resistance can  
60 eliminate treatment options completely, which has a serious effect on patient survival  
61 (Perlin et al. 2017).

62 The emergence of resistance to currently used antifungals has promoted the  
63 development of novel antifungal approaches, such as ~~the~~ antimicrobial photodynamic  
64 treatment (APDT). The basic principle behind photodynamic antimicrobial inactivation  
65 is the combination three factors: (1) visible or near-infrared light, (2) molecular oxygen,  
66 and (3) a photosensitizer (PS). Light exposure excites the photosensitizer to a singlet state.  
67 Then, intersystem crossing results in a photosensitizer in an excited triplet state which  
68 can interact with molecular oxygen either via electron or energy transfer. Electron  
69 transfer, also called Type I reactions, usually results in the formation of radicals such as  
70 the superoxide radical anion. Conversely, energy transfer or Type II reaction results in

71 the formation of singlet oxygen. In either case, reactive oxygen species (ROS) such as  
72 singlet oxygen, superoxide radical anions, and hydroxyl radicals have a broad spectrum  
73 of activity and can damage several microbial targets ~~such as~~ among the various proteins,  
74 lipids, and nucleic acids encountered, therefore making selection of resistant strains  
75 unlikely (Brancini et al. 2016; Wainwright et al. 2017). Among photoantimicrobials  
76 evaluated as antifungals, the phenothiazinium dyes methylene blue and toluidine blue are  
77 the most commonly used, mainly due to their low toxicity and their long-established use  
78 for other clinical applications (Rodrigues et al. 2013; Wainwright et al. 2017).  
79 Phenothiazinium derivatives with improved photoantimicrobial activity against yeasts  
80 and filamentous fungi such as new methylene blue N (NMBN) and the novel pentacyclic  
81 compound S137, have been identified (Dai et al. 2011; Rodrigues et al. 2013). APDT  
82 with NMBN and S137 has been shown to be highly effective against fungi of the genera  
83 *Aspergillus* (de Menezes et al. 2014), *Candida* (Dai et al. 2011; Rodrigues et al. 2013),  
84 *Colletotrichum* (de Menezes et al. 2014), *Neoscytalidium* (Tonani et al. 2018), and  
85 *Trichophyton* (Rodrigues et al. 2012).

86 The most important factor determining the outcome of APDT is how a  
87 photosensitizer interacts with cells of the target microorganism, with its subcellular  
88 localization being of particular interest (Gonzales et al. 2017; de Menezes et al. 2014; de  
89 Menezes et al. 2016). This is because ROS have a short half-life and therefore exert their  
90 action in the vicinity of their production site (Castano et al. 2004). Cellular uptake and  
91 intracellular localization is determined by chemical and structural features of the PS (e.g.  
92 molecular mass, lipophilicity, charge distribution, number of H-bond donors and  
93 acceptors, etc.), the concentration of the PS, the incubation time, and the phenotypic  
94 characteristics of the target cells (Castano et al. 2004). PS characteristics such as charge

95 type and distribution as well as lipophilicity may be controlled by informed synthesis  
96 (Wainwright and Giddens 2003).

97 The use of confocal laser scanning fluorescence microscopy has made the  
98 determination of intracellular localization of PS much easier. Colocalization of  
99 subcellular organelle-specific fluorescent probes with differing fluorescence emission  
100 peak to that of the PS can be used to more closely identify the site of localization and  
101 these probes can also be used to identify sites of damage after illumination (Castano et al.  
102 2004).

103 The photosensitizers NMBN and S137 are chemically and structurally distinct,  
104 and consequentially present different outcomes when used in APDT. For instance, use of  
105 S137 usually results in cell damage even in the dark (dark toxicity) and its microbial  
106 photoinactivation tends to be higher at lower light fluences when compared to NMBN.  
107 As previously mentioned, PS subcellular localization can greatly influence the results of  
108 APDT. Therefore, here we compared NMBN and S137 by employing a set of fluorescent  
109 probes (propidium iodide, FUN-1, JC-1, DHR-123, and DHE) together with confocal  
110 microscopy and flow cytometry in order to evaluate potential PS subcellular localization  
111 as well as the mechanism behind APDT with these PS.

112

## 113 **2. Materials and Methods**

### 114 *2.1. C. albicans strain and growth conditions*

115 *C. albicans* strain ATCC 64548 was obtained from the American Type Culture  
116 Collection (ATCC) (Manassas, USA). Cells were grown on Sabouraud Dextrose Agar  
117 (SDA) medium (BD Difco, USA) in the dark, at 35 °C, for 48 h. Cells from isolated  
118 colonies were transferred to 150-mL Erlenmeyer flasks containing 50 mL of YPD  
119 medium [1% Yeast Extract (BD Difco, Sparks, USA), 2% Peptone (BD Difco) and 2%

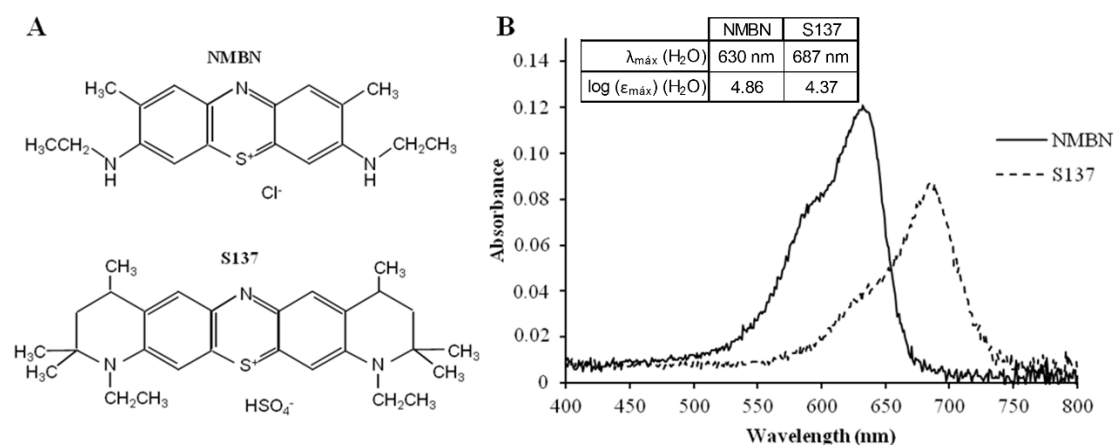
120 Dextrose (Vetec, Duque de Caxias, Brazil)]. Cultures were incubated in the dark at 35 °C  
 121 for 6 h under shaking (100 rpm). Cells were then washed in phosphate-buffered saline  
 122 (PBS, pH 7.4) (8,000 × g, 5 min) and cell concentration was adjusted by counting in a  
 123 hemocytometer and performing the appropriate dilutions in PBS.

124

## 125 2.2. Photosensitizers

126 New Methylene Blue N zinc chloride double salt (NMBN) was purchased from  
 127 Sigma-Aldrich (catalog number 202096; St. Louis, USA) (Fig. 1A). The pentacyclic  
 128 phenothiazinium photosensitizer S137 was synthesized as previously described  
 129 (Wainwright et al. 2011) (Fig. 1A). Stock solutions of the PS were prepared in water at a  
 130 concentration (500 μM) two hundred-fold greater than the concentration used in the  
 131 study. The solutions were stored in the dark at -20 °C for up to 2 weeks. Dilutions were  
 132 prepared in PBS. Absorption spectra of the PS were obtained with a Ultrospec™ 2100  
 133 Pro UV-visible spectrophotometer (GE Healthcare) in water (Fig. 1B).

134



135

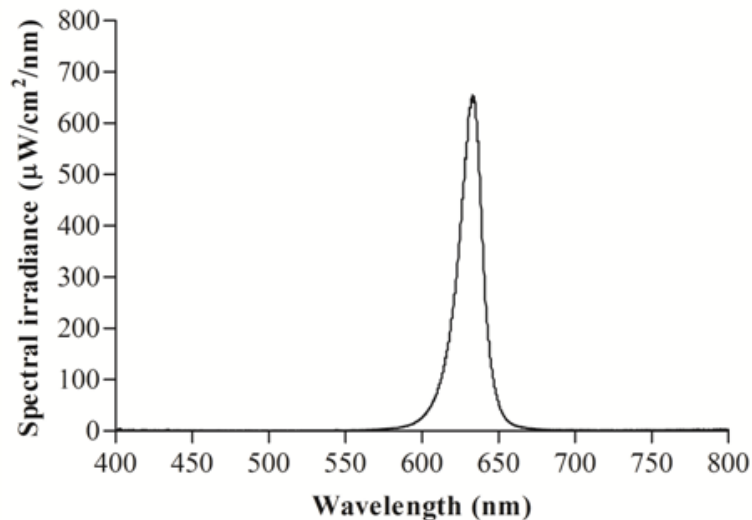
136 **Fig. 1.** Chemical structure (A) and absorption spectra (B) of the photosensitizers NMBN and S137

137

## 138 2.3. Light exposure



139 Light was provided by an array of 96 light-emitting diodes (LED) with peak  
140 emission at  $631 \pm 20$  nm and an irradiance of  $13.89 \text{ mW cm}^{-2}$ . Irradiance and emission  
141 spectrum (Fig. 2) were obtained with a USB spectroradiometer (Ocean Optics, Dunedin,  
142 USA) as previously described (Rodrigues et al. 2012).  
143



144  
145 **Fig. 1.** Irradiance spectrum of the red light source used in this study

146  
147 *2.4. Photodynamic treatment*

148 Five mL of the fungal cell suspension and 5 mL of the PS (NMBN or S137) were  
149 added to 15 mL tubes (TPP, Switzerland). Final concentrations of cells and PS in the  
150 mixture were  $2 \times 10^7$  cells  $\text{mL}^{-1}$  and  $2.5 \mu\text{M}$  of NMBN or S137. Tubes were kept in the  
151 dark for 30 min at  $28^\circ\text{C}$  and light exposure was performed under agitation in a 60-mm  
152 Petri dish. The fluences used were 3, 9, and  $14 \text{ J cm}^{-2}$  (obtained after 3.42, 10.28, and  
153 17.13 min, respectively). Relative cell survival after APDT was evaluated for each  
154 fluence used by counting colony-forming units (CFU). To do this, the initial suspensions  
155 were serially diluted tenfold in PBS to give dilutions of  $10^{-1}$  to  $10^{-3}$ . Fifty microliters were  
156 then spread on the surface of 5 mL of SDA medium in Petri dishes ( $60 \times 15$  mm). Three

157 replicate-dishes were prepared for each light treatment. The dishes were incubated in the  
158 dark at 35 °C. After 24 h, CFU were counted at 8× magnification daily for up to 4 days.  
159 A dark control group was obtained by treating cells with PS but never exposing them to  
160 light. A light control group was prepared by exposing cells alone (in the absence of PS)  
161 to light fluences of 3, 9, and 14 J cm<sup>-2</sup>. Absolute controls consisted of cells unexposed to  
162 either light or PS. Relative survival was calculated as the ratio of CFU of fungal cells  
163 treated only with light (light effect), only with PS (toxicity in the dark), and light and PS  
164 (APDT) to CFU treated with neither light nor PS. Three independent experiments were  
165 performed.

166

#### 167 *2.5. Propidium iodide (PI) staining and visualization*

168 After APDT with NMBN or S137, cell suspensions were washed with PBS to  
169 remove excess PS. Cells were then suspended in a 1.5 µM PI (Sigma-Aldrich, catalog  
170 number P4170) solution prepared in PBS immediately before being used. Flow cytometry  
171 was performed in a BD FACSCanto I equipment and BD FACSDiva software. In each  
172 experiment, ten thousand events were monitored with excitation at 488 nm and detection  
173 between 564 and 606 nm. Cells not treated with PS and cells treated with 70% ethanol  
174 were used as negative and positive controls, respectively. Three independent experiments  
175 were performed.

176 Confocal fluorescence microscopy was used to visualize PI entry into cells. After  
177 APDT and PI staining, cells were centrifuged (10,000 × g, 2 min) and the supernatant was  
178 discarded. Three microliters of 2% Ultra Pure low-melting-point agarose (Invitrogen) and  
179 3 µL of Fluoromount (Sigma) were added to 3 µL of cell pellet and the mixture was used  
180 to mount the slide. Confocal microscopy was performed on a Leica DMI 6000 CS,  
181 scanner TCS SP8 with a 63× objective lens (f/1.4) and using oil immersion. For PI

182 visualization, excitation was performed with an Optically Pumped Semiconductor Laser  
183 at 488 nm and detection at 597-637 nm.

184

#### 185 *2.6. FUN-1 staining and visualization*

186 After APDT with NMBN or S137, cells were washed with 10 mM HEPES pH 7.2  
187 (Sigma-Aldrich) supplemented with 2% glucose (hereinafter referred to as GH buffer) to  
188 remove excess PS. Cells were then suspended in a 0.5  $\mu$ M FUN-1 solution (Molecular  
189 Probes, Life Technologies, Eugene, OR, USA) prepared in GH buffer. Cells were  
190 incubated in the dark under shaking (300 rpm) at 30 °C for 30 min. The  
191 spectrofluorimetric analysis was performed in black 96-well plates with a Synergy 2  
192 equipment (BioTek<sup>®</sup>, Winooski, USA). Excitation was set to 475-495 nm and detection  
193 to 518-538 nm (green fluorescence) and 580-600 nm (red fluorescence). Three  
194 independent experiments were performed.

195 For confocal microscopy, FUN-1-stained cells were centrifuged (10,000  $\times$  g, 2  
196 min) and slides were mounted and visualized as described above for PI. Laser excitation  
197 was set to 488 nm and detection to 530-560 nm (green fluorescence) and 604-636 nm (red  
198 fluorescence).

199

#### 200 *2.7. JC-1 staining and visualization*

201 After APDT with NMBN and S137, cells were washed (10,000  $\times$  g, 2 min) with  
202 GH buffer to remove excess PS. Cells were then suspended in a 5  $\mu$ M JC-1 (Molecular  
203 Probes, Life Technologies, USA) solution prepared in GH buffer and incubated in the  
204 dark under shaking (300 rpm) at 35 °C for 30 min. Then, cells were washed twice with  
205 GH buffer and flow cytometry was performed as described previously. A total of 10,000  
206 events were monitored. Excitation was set to 488 nm and detection to 515-545 nm (green

207 fluorescence) and 564-606 nm (red fluorescence). Three independent experiments were  
208 performed.

209 Confocal microscopy was performed as described previously. Laser excitation  
210 was set to 488 nm and detection to 505-550 nm (green fluorescence) and 575-630 (red  
211 fluorescence).

212

### 213 *2.8. Dihydrorhodamine-123 (DHR-123) staining and visualization*

214 After APDT with NMBN or S137, cells were washed ( $10,000 \times g$ , 2 min) with  
215 GH buffer to remove excess PS. Cells were then suspended in a  $5 \mu\text{g mL}^{-1}$  DHR-123  
216 solution (Sigma-Aldrich, catalog number D1054) prepared in GH buffer and incubated in  
217 the dark at  $25^\circ\text{C}$  for 120 min. Flow cytometry was performed as described previously. A  
218 total of 10,000 events were monitored. Excitation was set to 488 nm and detection to 515-  
219 545 nm. Three independent experiments were performed.

220 Confocal microscopy was performed as described previously. Laser excitation  
221 was set to 488 nm and detection to 501-570 nm.

222

### 223 *2.9. Dihydroethidium (DHE) staining and visualization*

224 After APDT with NMBN or S137, cells were washed ( $10,000 \times g$ , 2 min) with  
225 GH buffer to remove excess PS. Cells were then suspended in a  $20 \mu\text{M}$  DHE (Sigma-  
226 Aldrich, catalog number D7008) solution prepared in GH buffer and incubated in the dark  
227 at  $25^\circ\text{C}$  for 45 min. Flow cytometry was performed in a Guava EasyCyte 8HT (Merck  
228 Millipore, Darmstadt, Germany). In each experiment, a total of 30,000 events were  
229 analyzed using the red filter. Three independent experiments were performed.

230 Confocal microscopy was performed as described previously. Laser excitation  
231 was set to 552 nm and detection to 556-624 nm.

232

233 *2.10. PS lipophilicity prediction*

234 Lipophilicity of NMBN and S137 (as expressed by logD as a function of pH) was  
235 calculated with the MarvinJS logD Predictor software (ChemAxon). PS structures used  
236 in the predictions are those depicted in Fig. 1.

237

238 *2.11. Statistical analysis*

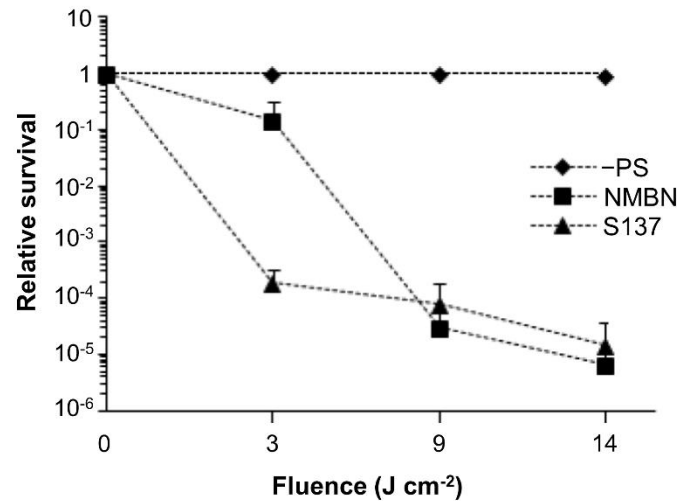
239 Differences between means were analyzed via ANOVA with Tukey's post-test.  
240 Significance threshold was set to  $P < 0.05$ . Statistical analyses were performed with SAS®  
241 9.2 software (SAS Analytics, USA).

242

243 **3. Results**244 *3.1. C. albicans survival after APDT*

245 The PS NMBN and S137 were compared in terms of cell mortality after APDT  
246 with fluences of 3, 9, and 14 J cm<sup>-2</sup>. Importantly, treatment with PS alone or light exposure  
247 alone did not result in cell mortality (Fig. 3). At 3 J cm<sup>-2</sup>, S137 was a much more effective  
248 PS, reducing cell viability by 99.98% (3.70 log<sub>10</sub>) whereas NMBN achieved only 85.2%  
249 (0.83 log<sub>10</sub>) under the same conditions (Fig. 3). Increasing fluence to 9 and to 14 J cm<sup>-2</sup>  
250 allowed NMBN and S137 to achieve similar cell mortality, which was above four orders  
251 of magnitude for both PS (Fig. 3).

252



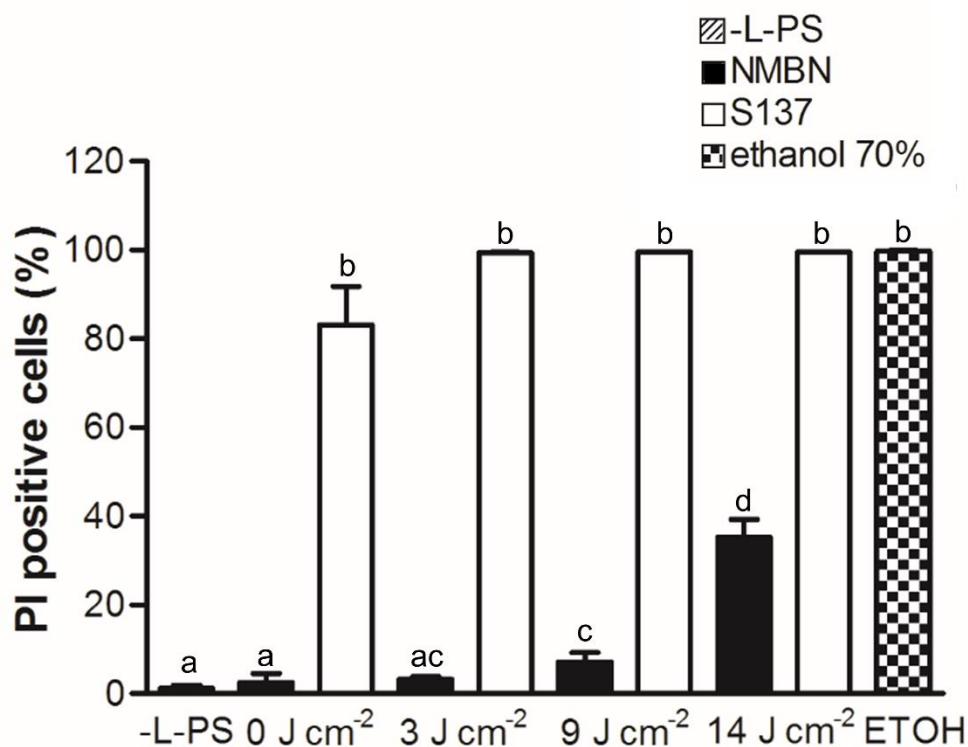
253

254 **Fig. 3.** Relative survival of *Candida albicans* after antimicrobial photodynamic treatment  
 255 with NMBN and S137 as a function of light fluence. Control groups were either treated  
 256 with light alone (-PS) or photosensitizer alone (fluence = 0 J cm<sup>-2</sup>). Error bars are the  
 257 standard deviation from three independent experiments.

258

### 259 3.2. Propidium iodide staining and visualization

260 Staining with PI was used to study fungal membrane disturbance caused by the  
 261 PS both in the dark and after APDT. In the dark, NMBN caused little to no PI labeling as  
 262 evaluated by flow cytometry whereas S137 caused about 80% of cells to become PI-  
 263 positive (Fig. 4). The percentage of PI-positive cells achieved 100% for S137 already at  
 264 the lowest fluence used (3 J cm<sup>-2</sup>) whereas this number was only about 40% for NMBN  
 265 even at the highest fluence (14 J cm<sup>-2</sup>) (Fig. 4).



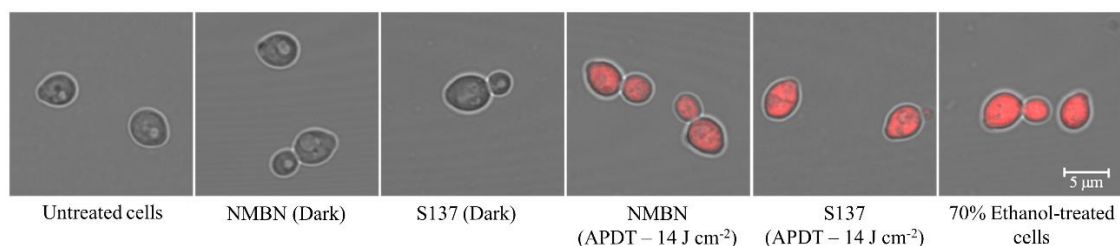
266

267 **Fig. 4.** *Candida albicans* Propidium iodide staining as evaluated by flow cytometry. Cells  
 268 were treated with either NMBN or S137 and control groups received neither light nor  
 269 photosensitizer (-L -PS). Different lower case letters indicate that means are statistically  
 270 different. Error bars are the standard deviation from three independent experiments.

271

272 Although adding S137 resulted in PI permeability already in the dark in flow  
 273 cytometry experiments, confocal fluorescence microscopy could not distinguish between  
 274 NMBN and S137 in the dark (Fig. 5). At 14 J cm<sup>-2</sup>, both NMBN- and S137-treated cells  
 275 were stained (Fig. 5).

276



277

278 **Fig. 5.** *Candida albicans* propidium iodide staining as evaluated by confocal fluorescence  
 279 microscopy. Control cells were not treated with either photosensitizer or light. NMBN

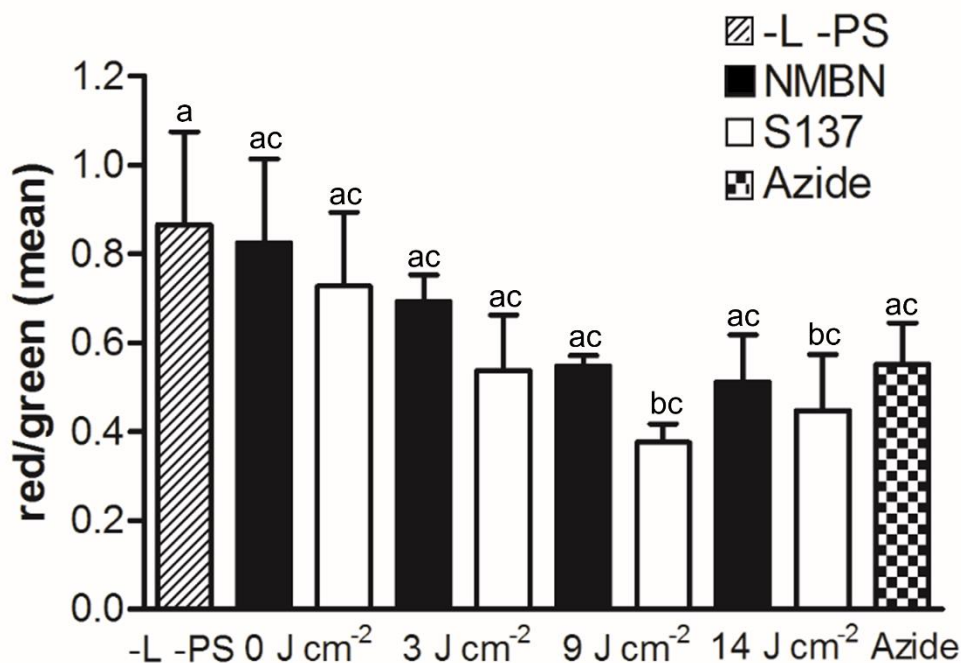
280 and S137 were used either in the dark or under light at a fluence of 14 J cm<sup>-2</sup>. Images are  
 281 representative of three independent experiments.

282

### 283 3.3. FUN-1 staining and visualization

284 FUN-1 is a dye that diffuses inside fungal cells and stains them green irrespective  
 285 of viability. However, in viable cells, further processing of the dye results in the  
 286 appearance of red fluorescent spots accompanied by reduced green fluorescence.  
 287 Therefore, the red/green fluorescence ratio is used as a marker of cell viability in flow  
 288 cytometry experiments. Cells treated with either NMBN or S137 in the dark were not  
 289 significantly different from untreated cells (Fig. 6). After APDT, the red/green  
 290 fluorescence ratio decreased proportionally with increasing fluences and both PS were  
 291 similar in this regard, although the majority of differences were not statistically  
 292 significant (Fig. 6).

293



294

295 **Fig. 6.** *Candida albicans* FUN-1 staining as evaluated by spectrofluorimetry. Cells were  
 296 treated with either NMBN or S137 and control groups received neither light nor



297 photosensitizer (-L -PS). Different lower case letters indicate that means are statistically  
 298 different. Error bars are the standard deviation from three independent experiments.

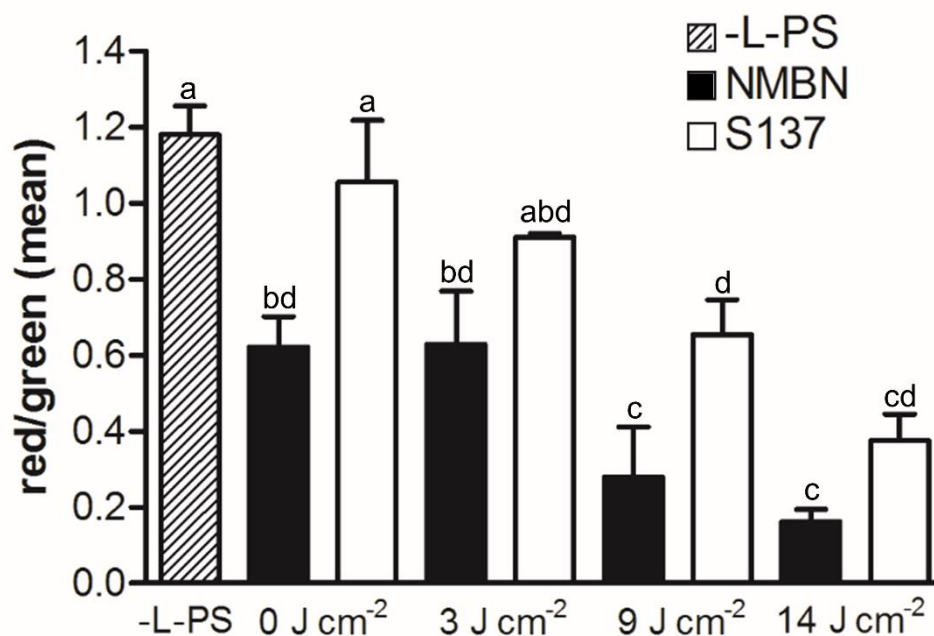
299

300 As expected, confocal fluorescence microscopy showed the accumulation of  
 301 vacuolar-like red fluorescence in untreated cells and those that were treated with either  
 302 PS in the dark, indicating normal viability (Fig. S1). After APDT with  $14 \text{ J cm}^{-2}$ , these  
 303 red spots were lost and cells stained yellow (Fig. S1).

304

### 305 3.4. JC-1 staining and visualization

306 JC-1 is a dye that accumulates in mitochondria in a membrane potential-dependent  
 307 manner. This accumulation is indicated by a red-to-green fluorescence shift. The loss of  
 308 mitochondrial membrane potential (depolarization) reduces the red/green fluorescence  
 309 ratio. Treating cells with S137 in the dark resulted in no mitochondria depolarization.  
 310 However, NMBN caused considerable loss of membrane potential in the dark (Fig. 7).



311

312 **Fig. 7.** *Candida albicans* JC-1 staining as evaluated by flow cytometry. Cells were treated  
 313 with either NMBN or S137 and control groups received neither light nor photosensitizer  
 314 (-L -PS). Different lower case letters indicate that means are statistically different. Error  
 315 bars are the standard deviation from three independent experiments

316

317 Mitochondrial membrane potential decreased upon light exposure for both PS,  
318 even though S137 required a fluence of  $9 \text{ J cm}^{-2}$  to achieve a statistically significant  
319 difference from the control (Fig. 7).

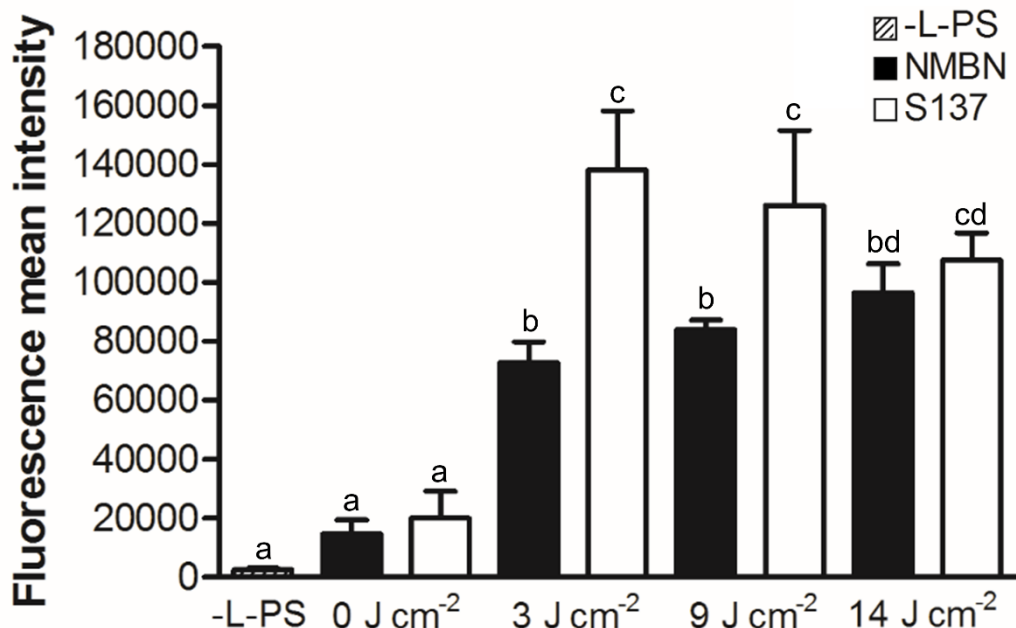
320 Although flow cytometry experiments showed that NMBN can reduce  
321 mitochondrial membrane potential already in the dark, fluorescence microscopy did not  
322 indicate the same result as both NMBN and S137, when used in the dark, were very  
323 similar to untreated cells (Fig. S2). Upon light exposure ( $14 \text{ J cm}^{-2}$ ), the expected decrease  
324 in red/green fluorescence ratio was observed for both PS. However, loss of red  
325 fluorescence was higher for NMBN when compared to S137 (Fig. S2), which reflects  
326 flow cytometry experimental data (Fig. 7).

327

### 328 *3.5. Dihydrorhodamine-123 (DHR-123) staining and visualization*

329 DHR-123 is an uncharged and membrane-permeant compound that, upon  
330 oxidation, is converted to the mitochondrial dye rhodamine-123, emitting green  
331 fluorescence. Treating cells with either PS in the dark did not result in a significant  
332 increase in green fluorescence. Light exposure at a fluence of  $3 \text{ J cm}^{-2}$  revealed that S137  
333 generated more DHR-123-oxidizing species than did NMBN (Fig. 8), which was also  
334 observed for the fluence of  $9 \text{ J cm}^{-2}$ . At  $14 \text{ J cm}^{-2}$ , both PS leveled off and produced about  
335 the same amount of oxidizing species (Fig. 8).

336



337

338 **Fig. 8.** *Candida albicans* Dihydrorhodamine-123 staining as evaluated by flow  
 339 cytometry. *Candida albicans* cells were treated with either NMBN or S137 and control  
 340 groups received neither light nor photosensitizer (-L -PS). Different lower case letters  
 341 indicate that means are statistically different. Error bars are the standard deviation from  
 342 three independent experiments.

343

344 Fluorescence microscopy, as expected, showed no green fluorescence in untreated  
 345 cells and cells treated with either PS (Fig. S3). Strong green fluorescent emission was  
 346 observed at 14 J cm<sup>-2</sup>, which was similar for NMBN and S137 (Fig. S3).

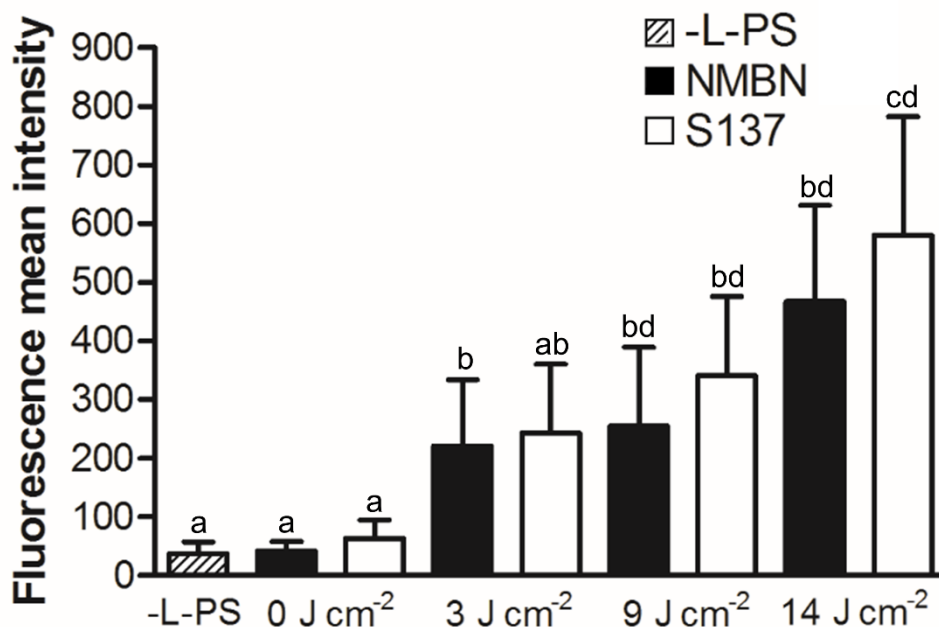
347

### 348 3.6. Dihydroethidium (DHE) staining and visualization

349 DHE is widely regarded as an indicator of superoxide anion radical (O<sub>2</sub><sup>•-</sup>)  
 350 production because DHE oxidation by O<sub>2</sub><sup>•-</sup> gives 2-hydroxyethidium, which emits red  
 351 fluorescence. However, unspecific oxidation of DHE by other ROS results in ethidium,  
 352 which also emits red fluorescence and is hard to distinguish from 2-hydroxyethidium.  
 353 Therefore, we employed DHE as a general indicator of ROS and not specifically of O<sub>2</sub><sup>•-</sup>.  
 354 Neither NMBN nor S137 leads to ROS production in the dark when compared to

355 untreated cells (Fig. 9). ROS production increased upon light exposure, although we  
 356 observed no difference between NMBN and S137 (Fig. 9).

357



358

359 **Fig. 9.** *Candida albicans* dihydroethidium staining as evaluated by flow cytometry. Cells  
 360 were treated with either NMBN or S137 and control groups received neither light nor  
 361 photosensitizer (-L -PS). Different lower case letters indicate that means are statistically  
 362 different. Error bars are the standard deviation from three independent experiments.

363

364 Confocal fluorescence microscopy reflected flow cytometry results: no red  
 365 fluorescence was observed in the dark for either PS and red fluorescence was observed at  
 366 14 J cm<sup>-2</sup> that was indistinguishable between NMBN and S137 (Fig. S4).

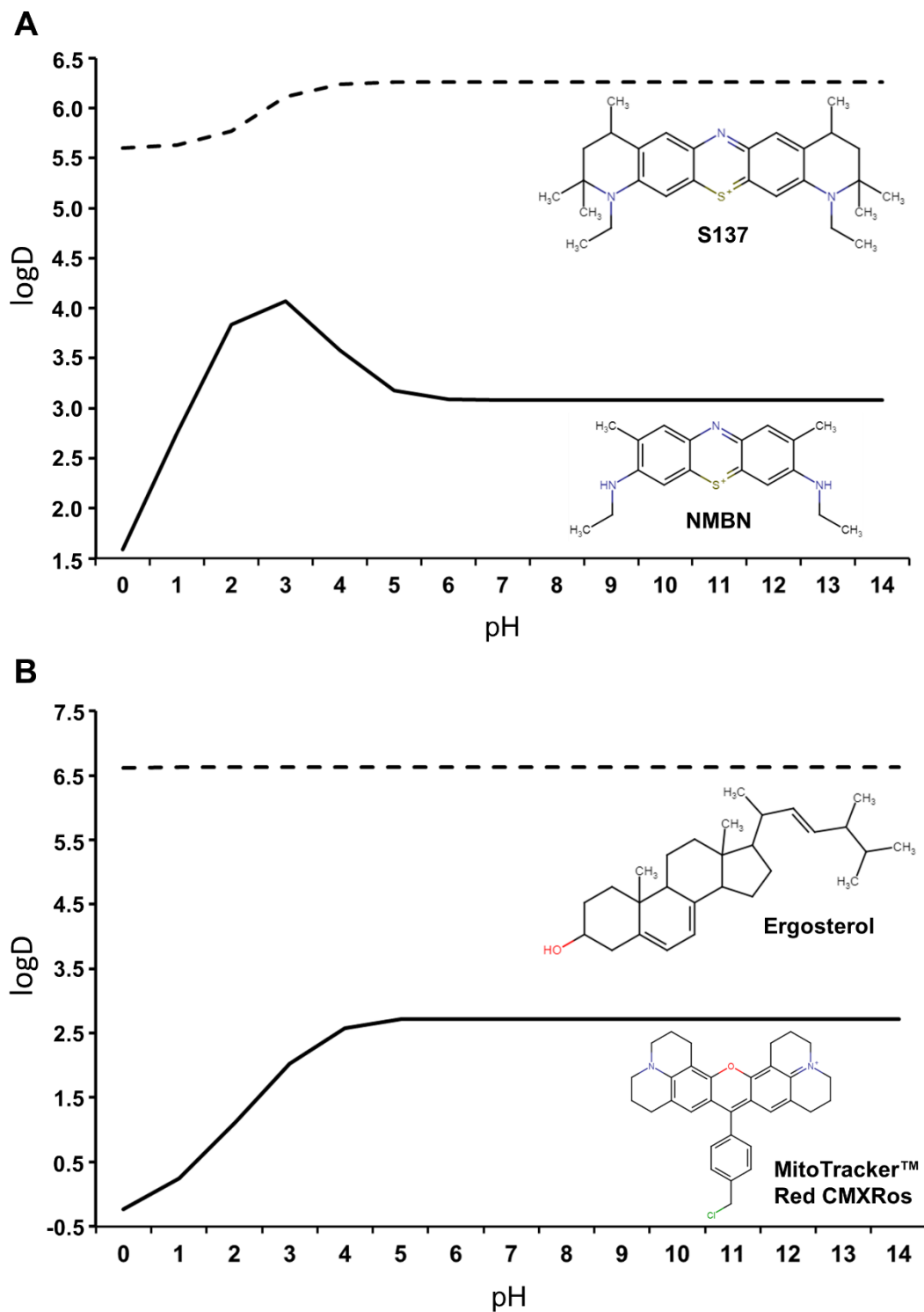
367

### 368 3.7. NMBN and S137 lipophilicity prediction

369 In the dark, S137 caused extensive membrane damage (Fig. 4) and NMBN  
 370 reduced mitochondrial membrane potential (Fig. 7). These observations prompted an  
 371 investigation of PS lipophilicity. Predicting logD as a function of pH for both PS revealed  
 372 that whereas NMBN is of moderate lipophilicity (logD = 3.08 at pH 7), S137 is highly

373 lipophilic ( $\log D = 6.26$ ) (Fig. 10A). For comparison, we also calculated  $\log D$  values for  
374 the membrane component ergosterol ( $\log D = 6.63$ ) and the mitochondria-specific dye  
375 MitoTracker™ Red CMXRos ( $\log D = 2.72$ ) (Fig. 10B).

376



377

378 **Fig. 10.** Prediction of lipophilicity (logD) as a function of pH for the photosensitizers NMBN and  
 379 S137 (A), and membrane-associated ergosterol and mitochondrial dye MitoTracker™ Red  
 380 CMXRos (B).

381

382

383

#### 384 4. Discussion

385           Understanding the mechanism behind microbial photoinactivation with different  
386 PS is a key step in improving the efficiency of APDT and in selecting the most appropriate  
387 PS based on target microorganism and condition. APDT of *C. albicans* with the PS  
388 NMBN and S137 revealed that the latter achieves increased cell mortality at lower  
389 fluences when compared to the former (Fig. 3). Under the experimental conditions used  
390 here, NMBN is expected to produce more singlet oxygen compared to S137 as its peak  
391 absorption (630 nm, Fig. 1B) essentially matches that of the light system used (631 nm).  
392 Furthermore, NMBN has a higher molar absorption coefficient (Fig. 1B). Indeed, recent  
393 observations from our group have shown that singlet oxygen quantum yield of NMBN is  
394 higher than that of S137 (De Menezes et al., in preparation). Therefore, the different  
395 efficiency in APDT between NMBN and S137 at  $3 \text{ J cm}^{-2}$  cannot be explained by  
396 photophysical properties alone. To better understand this phenomenon, we employed a  
397 set of fluorescent dyes analyzed by both flow cytometry (or spectrofluorimetry in the case  
398 of FUN-1) and confocal fluorescence microscopy.

399           Initially, we used FUN-1 and PI as vital dyes. FUN-1 was not capable of  
400 distinguishing the difference between APDT with NMBN and S137 at  $3 \text{ J cm}^{-2}$  (Fig. 6),  
401 showing that it is not an adequate dye to evaluate cell mortality after APDT. Results  
402 obtained with PI showed that S137 caused extensive membrane permeabilization even in  
403 the dark whereas NMBN could only permeabilize the membrane at higher light fluences  
404 (Fig. 4). Interestingly, membrane permeabilization by S137 was unrelated to cell survival  
405 as this PS caused no mortality in the dark (Fig. 3). This is in agreement with prior works  
406 showing that membranes of stressed yeast and conidia of filamentous fungi can become  
407 permeable to PI without loss of cell viability (Davey and Hexley 2011; de Menezes et al.  
408 2016; Tonani et al. 2018).

409           The above-mentioned increase in PI permeability after S137 treatment in the dark  
410 was easily quantified by flow cytometry (Fig. 4) but was not observed by confocal  
411 fluorescence microscopy (Fig. 5). This discrepancy between the two techniques is likely  
412 a consequence of differences in sensitivity. Flow cytometry is more sensitive than  
413 confocal microscopy because, in the latter, out-of-focus image signals are ignored by the  
414 confocal system, rendering this technique inadequate for faint fluorescence probes (Basiji  
415 et al. 2007). On the other hand, flow cytometry sacrifices all imaging capabilities in favor  
416 of greater sensitivity. In fact, flow cytometry can detect as few as 100 fluorescent  
417 molecules per cell (Basiji et al. 2007), making it the method of choice for quantitative  
418 measurement of a heterogeneous population of cells. Therefore, we can hypothesize that  
419 confocal microscopy could not detect the difference between NMBN and S137 in the dark  
420 for PI because the number of PI molecules entering the cell in S137-treated samples is  
421 insufficient to produce a fluorescence signal that is strong enough to be detected by  
422 confocal microscopy. Further evidence of this difference in sensitivity is that for other  
423 fluorescent probes (such as DHR-123) confocal microscopy fails to detect any fluorescent  
424 signal for both PS in the dark whereas flow cytometry detects a weak signal.

425           The increased uptake of PI by S137-treated cells prompted us to investigate PS  
426 lipophilicity. Predicting logD for S137 and NMBN revealed that the former is about  
427 1,500-fold more lipophilic than the latter at pH 7 (Fig. 10A). Indeed, S137 has a logD  
428 value comparable to that of ergosterol (Fig. 10B). These results indicate that S137 mainly  
429 accumulates at the cell membrane, potentially disturbing it and subsequently increasing  
430 PI permeability.

431           On the other hand, NMBN is only moderately lipophilic, which, combined with  
432 its positive charge, makes the PS a good candidate for mitochondria targeting (Rashid  
433 and Horobin 1990). Accordingly, the lipophilicity of NMBN is comparable to that of the



434 mitochondria-specific dye MitoTracker™ Red CMXRos (Fig. 10B). Use of the  
435 mitochondrial membrane potential indicator JC-1 revealed that NMBN caused  
436 considerable membrane depolarization already in the dark whereas S137 treatment was  
437 not different from untreated cells (Fig. 7). The fact that S137 is also a lipophilic cationic  
438 compound could indicate that it also targets mitochondria. However, extremely lipophilic  
439 compounds can take as long as hours or even days to diffuse through the lipid bilayer, a  
440 task that is achieved within minutes for moderately lipophilic molecules (Baláz 2000;  
441 Rashid and Horobin 1990). Therefore, under our experimental conditions in which cells  
442 and PS were allowed to interact for 30 min, the most likely outcome is that NMBN  
443 accumulates in mitochondria while S137, owing to its very high lipophilicity, is trapped  
444 at the cell membrane.

445         The reduced mitochondrial membrane potential observed after NMBN treatment  
446 could affect the outcome of some commonly used fluorescent dyes for monitoring  
447 reactive species production. This is the case for DHR-123. Oxidation of DHR-123  
448 produces rhodamine-123 which localizes to mitochondria. However, rhodamine-123  
449 accumulation is dependent on mitochondrial status: loss of membrane potential reduces  
450 dye accumulation and therefore washes away the fluorescent signal (Scaduto and  
451 Grotyohann 1999). In our experiments, rhodamine-123 signal was increased for S137 at  
452  $3 \text{ J cm}^{-2}$  when compared to NMBN, which would be a plausible explanation for the higher  
453 mortality achieved by S137 (Fig. 8). However, this result needs to be interpreted with  
454 care. Because NMBN caused mitochondrial membrane depolarization already in the dark,  
455 then rhodamine-123 accumulation and signal could be hindered in NMBN-treated cells.  
456 In support of this hypothesis, rhodamine-123 fluorescence did not increase for either S137  
457 or NMBN when fluence increased (Fig. 8), probably as a consequence of the reduced  
458 mitochondrial membrane potential at higher fluences (Fig. 7).

459 To overcome this limitation, we used DHE as a marker for the production of  
460 reactive species as it does not depend on mitochondrial status. The fact that NMBN and  
461 S137 produced approximately the same amount of reactive species at all light fluences  
462 tested (Fig. 9) indicates that it is most likely PS subcellular localization, and not the  
463 amount of reactive species generated, the deciding factor for APDT efficiency under our  
464 experimental conditions. In support of this hypothesis, prior work evaluating a series of  
465 photophysically similar porphyrin PS reported that photodynamic efficiency increases  
466 with increasing membrane binding and is only partially dependent on mitochondria  
467 localization (Pavani et al. 2009). Also, for PS targeting mitochondria, loss of membrane  
468 potential resulted in decreased binding (Pavani et al. 2009), a feature that could affect the  
469 outcome of APDT employing mitochondria-targeting PS such as NMBN.

470

## 471 **5. Conclusion**

472 Taken together, our results indicate that S137 and NMBN localize to different  
473 subcellular structures and hence inactivate *C. albicans* cells via different mechanisms.  
474 S137 localizes mostly to cell membrane and, upon light exposure, photo oxidizes  
475 membrane lipids, which in turn could change membrane permeability to S137 itself and  
476 allow the PS to reach other intracellular sites (Bocking et al. 2000). On the other hand,  
477 NMBN readily localizes to mitochondria and exerts its photodynamic effects there, which  
478 was observed to be a less effective way to achieve cell death at lower fluences. Finally,  
479 while using a combination of fluorescent dyes allowed us to better comprehend APDT  
480 with two distinct PS, the use of individual stains could be problematic: FUN-1 as a vital  
481 stain could not tell apart the differences in survival between S137 and NMBN at  $3 \text{ J cm}^{-2}$ ,  
482 <sup>2</sup>, DHR-123 depends on mitochondrial status which was affected by NMBN in the dark;  
483 and DHE is only a general indicator of reactive species production and cannot take into

484 account that the same species could differently affect survival depending on where it is  
485 generated.

486

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495

#### 496 **REFERENCES**

- 497 Baláž, Š., 2000 Lipophilicity in trans-bilayer transport and subcellular pharmacokinetics.  
498 *Perspectives in Drug Discovery and Design* 19:157-177.
- 499 Basiji, D.A., W.E. Ortyń, L. Liang, V. Venkatachalam, and P. Morrissey, 2007 Cellular  
500 image analysis and imaging by flow cytometry. *Clin Lab Med* 27 (3):653-670,  
501 viii.
- 502 Bocking, T., K.D. Barrow, A.G. Netting, T.C. Chilcott, H.G. Coster *et al.*, 2000 Effects  
503 of singlet oxygen on membrane sterols in the yeast *Saccharomyces cerevisiae*.  
504 *Eur J Biochem* 267 (6):1607-1618.
- 505 Brancini, G.T., G.B. Rodrigues, M.S. Rambaldi, C. Izumi, A.P. Yatsuda *et al.*, 2016 The  
506 effects of photodynamic treatment with new methylene blue N on the *Candida*  
507 *albicans* proteome. *Photochem Photobiol Sci* 15 (12):1503-1513.
- 508 Castano, A.P., T.N. Demidova, and M.R. Hamblin, 2004 Mechanisms in photodynamic  
509 therapy: part one—photosensitizers, photochemistry and cellular localization.  
510 *Photodiagnosis Photodyn Ther* 1 (4):279-293.
- 511 Chang, Z., V. Yadav, S.C. Lee, and J. Heitman, 2019 Epigenetic mechanisms of drug  
512 resistance in fungi. *Fungal Genet Biol* 132:103253.
- 513 Dai, T., V.J. Bil de Arce, G.P. Tegos, and M.R. Hamblin, 2011 Blue Dye and Red Light,  
514 a Dynamic Combination for Prophylaxis and Treatment of Cutaneous *Candida*  
515 *albicans* Infections in Mice. *Antimicrob Agents Chemother* 55 (12):5710-5717.
- 516 Davey, H.M., and P. Hexley, 2011 Red but not dead? Membranes of stressed  
517 *Saccharomyces cerevisiae* are permeable to propidium iodide. *Environ Microbiol*  
518 13 (1):163-171.
- 519 de Menezes, H.D., A.C. Pereira, G.T.P. Brancini, H.C. de Leao, N.S. Massola Junior *et*  
520 *al.*, 2014 Furocoumarins and coumarins photoinactivate *Colletotrichum acutatum*

- 521 and *Aspergillus nidulans* fungi under solar radiation. *Journal of Photochemistry*  
522 *and Photobiology B-Biology* 131:74-83.
- 523 de Menezes, H.D., L. Tonani, L. Bachmann, M. Wainwright, G.U. Braga *et al.*, 2016  
524 Photodynamic treatment with phenothiazinium photosensitizers kills both  
525 ungerminated and germinated microconidia of the pathogenic fungi *Fusarium*  
526 *oxysporum*, *Fusarium moniliforme* and *Fusarium solani*. *J Photochem Photobiol*  
527 *B* 164:1-12.
- 528 Gonzales, J.C., G.T.P. Brancini, G.B. Rodrigues, G.J. Silva-Junior, L. Bachmann *et al.*,  
529 2017 Photodynamic inactivation of conidia of the fungus *Colletotrichum*  
530 *abscissum* on *Citrus sinensis* plants with methylene blue under solar radiation. *J*  
531 *Photochem Photobiol B* 176:54-61.
- 532 Köhler, J.R., A. Casadevall, and J. Perfect, 2015 The Spectrum of Fungi That Infects  
533 Humans. *Cold Spring Harb Perspect Med* 5 (1).
- 534 Limper, A.H., A. Adenis, T. Le, and T.S. Harrison, 2017 Fungal infections in HIV/AIDS.  
535 *Lancet Infect Dis* 17 (11):e334-e343.
- 536 Nishimoto, A.T., C. Sharma, and P.D. Rogers, 2020 Molecular and genetic basis of azole  
537 antifungal resistance in the opportunistic pathogenic fungus *Candida albicans*. *J*  
538 *Antimicrob Chemother* 75 (2):257-270.
- 539 Pavani, C., A.F. Uchoa, C.S. Oliveira, Y. Iamamoto, and M.S. Baptista, 2009 Effect of  
540 zinc insertion and hydrophobicity on the membrane interactions and PDT activity  
541 of porphyrin photosensitizers. *Photochem Photobiol Sci* 8 (2):233-240.
- 542 Perlin, D.S., R. Rautemaa-Richardson, and A. Alastruey-Izquierdo, 2017 The global  
543 problem of antifungal resistance: prevalence, mechanisms, and management.  
544 *Lancet Infect Dis* 17 (12):e383-e392.
- 545 Rashid, F., and R.W. Horobin, 1990 Interaction of molecular probes with living cells and  
546 tissues. Part 2. A structure-activity analysis of mitochondrial staining by cationic  
547 probes, and a discussion of the synergistic nature of image-based and biochemical  
548 approaches. *Histochemistry* 94 (3):303-308.
- 549 Rhodes, J., and M.C. Fisher, 2019 Global epidemiology of emerging *Candida auris*. *Curr*  
550 *Opin Microbiol* 52:84-89.
- 551 Rodrigues, G.B., M. Dias-Baruffi, N. Holman, M. Wainwright, and G.U. Braga, 2013 In  
552 vitro photodynamic inactivation of *Candida* species and mouse fibroblasts with  
553 phenothiazinium photosensitizers and red light. *Photodiagnosis Photodyn Ther* 10  
554 (2):141-149.
- 555 Rodrigues, G.B., L.K. Ferreira, M. Wainwright, and G.U. Braga, 2012 Susceptibilities of  
556 the dermatophytes *Trichophyton mentagrophytes* and *T. rubrum* microconidia to  
557 photodynamic antimicrobial chemotherapy with novel phenothiazinium  
558 photosensitizers and red light. *J Photochem Photobiol B* 116:89-94.
- 559 Scaduto, R.C., and L.W. Grotyohann, 1999 Measurement of mitochondrial membrane  
560 potential using fluorescent rhodamine derivatives. *Biophys J* 76 (1 Pt 1):469-477.
- 561 Shor, E., and D.S. Perlin, 2015 Coping with Stress and the Emergence of Multidrug  
562 Resistance in Fungi. *PLoS Pathog* 11 (3).
- 563 Tonani, L., N.S. Morosini, H. Dantas de Menezes, M.E. Nadaletto Bonifacio da Silva, M.  
564 Wainwright *et al.*, 2018 In vitro susceptibilities of *Neoscytalidium* spp. sequence  
565 types to antifungal agents and antimicrobial photodynamic treatment with  
566 phenothiazinium photosensitizers. *Fungal Biol* 122 (6):436-448.
- 567 Wainwright, M., and R.M. Giddens, 2003 Phenothiazinium photosensitizers: choices in  
568 synthesis and application. *Dyes and Pigments* 57 (3):245-257.
- 569 Wainwright, M., T. Maisch, S. Nonell, K. Plaetzer, A. Almeida *et al.*, 2017  
570 Photoantimicrobials-are we afraid of the light? *Lancet Infect Dis* 17 (2):e49-e55.

- 571 Wainwright, M., K. Meegan, and C. Loughran, 2011 Phenothiazinium photosensitisers  
572 IX. Tetra- and pentacyclic derivatives as photoantimicrobial agents. *Dyes and*  
573 *Pigments* 91 (1):1-5.