



## HIGHLIGHT

# Shades of phototoxicity in fluorescent imaging agents (that are not supposed to be phototoxic)

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### Funding information

National Science Foundation, Grant/Award Number: CHE-2154133

### Abstract

This article is a highlight of the paper by Huang et al. in this issue of *Photochemistry and Photobiology*. It describes shades of phototoxicity in fluorescent imaging agents that are not intended to be phototoxic. Phototoxicity was assessed using a modified neutral red uptake (NRU) in vitro assay with mean photo-effects (MPE) for the fluorescent agents IRdye800, indocyanine green (ICG), proflavine, and methylene blue (MB), with comparisons to known phototoxic agents benzoporphyrin derivative (BPD) and rose bengal (RB). The experimental conditions were aimed to mimic clinical settings, using not only visible light, but also near-infrared light for insight to photosafety and deep tissue damage. Molecular mechanisms underlying the phototoxicities were not sought, but IRdye800 and ICG were mainly deemed to be safe, whereas proflavine and MB would require precautions since phototoxicity can overshadow their utility as fluorescent imaging agents.

### KEYWORDS

fluorescence agents, mean photo-effects, neutral red uptake, photosafety, phototoxicity

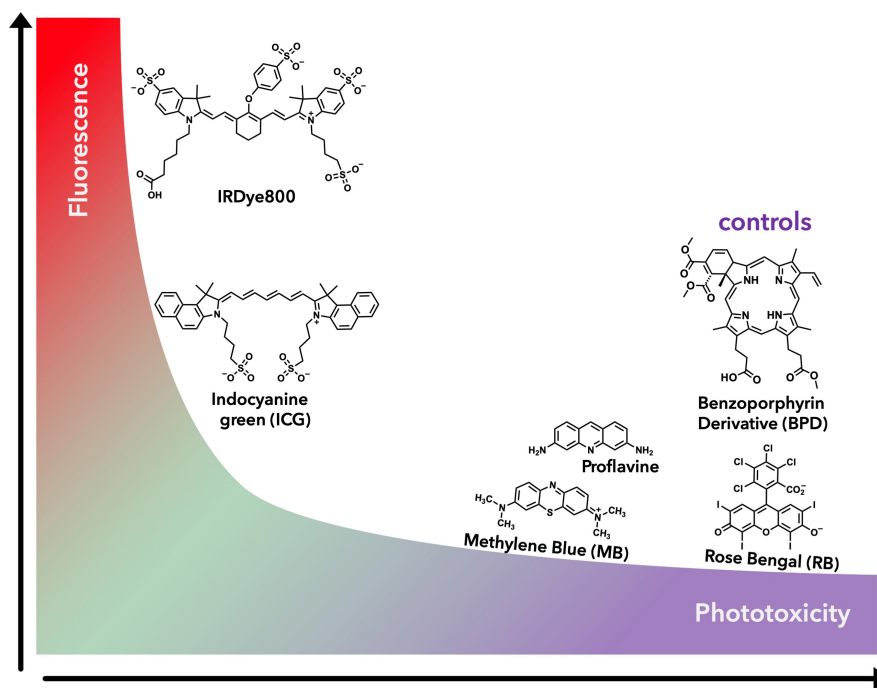
## COMMENTARY

Despite the common use of fluorescent imaging agents, underlying phototoxicity can be a problem. Efforts are usually not focused on testing of fluorescent agents' phototoxicity. But here, such testing has been carried out, as reported by Huang et al. in this issue of *Photochemistry and Photobiology*.<sup>1</sup> Shades of phototoxicity of fluorescent agents can arise, as is illustrated graphically in [Figure 1](#). Information is needed but often difficult to parcel out from literature<sup>2–6</sup> due to different conditions and light sources that cloud the view.

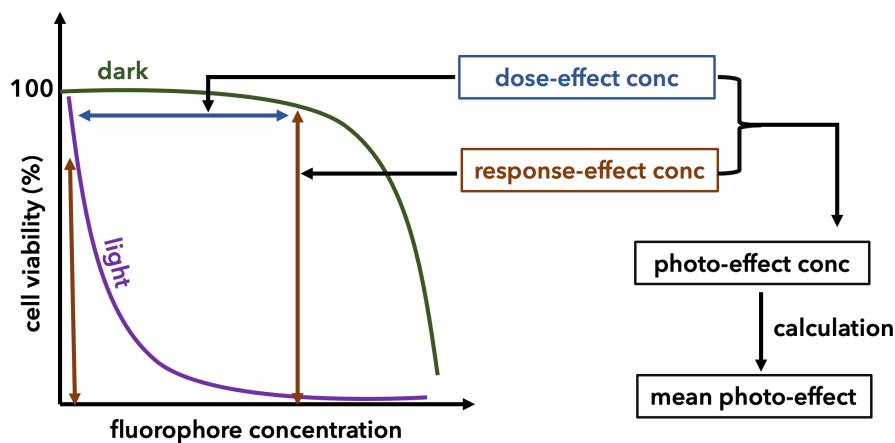
Parsing out of phototoxicity from supposed nontoxic fluorescent agents is needed. But despite advances in fluorescence imaging, this deconvolution of fluorescence from phototoxicity is challenging. Unwanted photoproduction of oxygen radicals and radical ions (type I) and singlet oxygen (<sup>1</sup>O<sub>2</sub>, type II)<sup>7,8</sup> and even photothermal processes can be damaging and hinder fluorescent agents' utility.<sup>9,10</sup> Understanding how phototoxic processes arise can help researchers design them only as fluorescence agents rather than as *double agents*. Such double agency is of concern clinically, indeed there is a need for facile photosafety evaluation of clinically important fluorescent

**Abbreviations:** BPD, Benzoporphyrin derivative; BODIPY, Dipyrromethene boron difluoride; ICG, Indocyanine green; MPE, Mean photo-effects; MB, Methylene blue; NIR, Near infrared; NRU, Neutral red uptake; OECD, Organization for Economic Cooperation and Development; ROS, Reactive oxygen species; RB, Rose bengal; <sup>1</sup>O<sub>2</sub>, Singlet oxygen.

This article is part of a Special Issue dedicated to the topic of Critical Issues and Recent Advances in the Clinical Translation of Photobiology and Photomedicine



**FIGURE 1** Hazy shades of phototoxicity in the fluorescent agents listed here. Benzoporphyrin derivative and rose bengal were control phototoxic compounds with which the others were compare to.



**FIGURE 2** Simplified illustration of dose–response curves and the calculation of mean photo-effects.

agents. Again, such insight would help researchers tailor fluorescent compounds to minimize phototoxic damage.

Huang et al. sought insight on phototoxic damage by fluorescent compounds in their work.<sup>1</sup> A modified in vitro 3T3 neutral red uptake (NRU) viability assay was used to show whether cells incorporate the neutral red dye,<sup>11,12</sup> to help deduce membrane permeability and lysosome activity. Organization for Economic Cooperation and Development testing guidelines were used.<sup>13</sup> Dose–response curves were plotted in the presence or absence of light (Figure 2). Mean photo-effects (MPE) were calculated from the dose–response curves also using Phototox software.<sup>14</sup> In a nutshell, photo-effect concentrations,

dose-effect concentrations, and response-effect concentrations were obtained, labeled in Figure 2 as photo-effect-conc, dose-effect-conc, and response-effect-conc, respectively, where MPE values  $<0.1$  are non-phototoxic,  $\geq 0.1$  to  $<0.15$  are equivocally phototoxic, and  $\geq 0.15$  are phototoxic. Fluences ranged from 0.2 to 6.0 J/cm<sup>2</sup> (a typical non-phototoxic threshold is 5 J/cm<sup>2</sup>) viewing not only <sup>1</sup>O<sub>2</sub> production factors, but also varying irradiance. Irradiance was varied along with excitation wavelengths ranging from 450 to 785 nm, striving for clinically relevant insight to a series of fluorescent agents.

The series of fluorescent agents studied were: IRDye800 (e.g., conjugatable to antibodies for use in head and neck

cancer and glioblastoma),<sup>15</sup> indocyanine green (ICG, e.g., used in image-guided surgery),<sup>16</sup> methylene blue (MB, e.g., used for breast and uterine tumor imaging),<sup>17,18</sup> and proflavine (e.g., oral cancer imaging).<sup>19</sup> The irradiation wavelength of 785 nm was used for IRdye800 and ICG, to reach further into the red region of the spectrum concomitant with deeper absorption in tissue. IRdye800 and ICG are among fluorophores coming into current use as they are absorbers in the NIR.

Huang and co-worker's report forms part of an increasing appreciation of underlying phototoxicity of commonly used fluorophores. IRdye800 is non-phototoxic with MPE below 0.1 under the conditions. ICG can wreak havoc and is phototoxic at higher fluences (e.g., MPE of 0.2 at 6 J/cm<sup>2</sup>) and concentrations especially given its effectiveness as a photothermal agent.<sup>20–24</sup> While MB is minimally phototoxic at 1 J/cm<sup>2</sup>, it is clearly phototoxic at 6 J/cm<sup>2</sup>. MB efficiently produces <sup>1</sup>O<sub>2</sub>,<sup>25</sup> so that caution is needed not only for higher fluences, but concentration as a fluorescent imaging agent. Like MB, proflavine is phototoxic, indeed even at 0.6 J/cm<sup>2</sup> (MPE above 0.15), proflavine is known to form oxygen radicals by type I processes and <sup>1</sup>O<sub>2</sub> by a type II process but to a lesser extent,<sup>26</sup> where proflavine's quantum yield for singlet oxygen formation is only 0.12.<sup>27,28</sup> Furthermore, MB and proflavine have not only light-dependent, but also appreciable dark toxicities, which is a further negative aspect in their use as fluorescent agents. The above results were compared against two known phototoxic compounds, BPD<sup>29</sup> and RB<sup>30</sup> that bear phototoxicity as low as 0.2 J/cm<sup>2</sup> from the MPE calculations. Thus, Huang et al.<sup>1</sup> reveal shady degrees of phototoxicity in some of the fluorescent imaging agents and many more could be tested.

The tested fluorescent imaging agents<sup>1</sup> fit well in the clinical area. Huang and co-worker's data are useful where the NRU assay, dose–response curves, and MPE calculations provided a view of the phototoxicity for relative comparisons. One drawback is that the phototoxicity mechanism of action is not readily available. A question is: *How can fluorescent agents be screened for prompt phototoxicity assessment including insight to the phototoxicity mechanism? Can methods be devised for rapid throughput in this vein?* This is easier suggested than done, where a do's and don'ts guide<sup>31</sup> has described good practices to assess photo-generated ROS and intermediates, as well as time-resolved methods for monitoring <sup>1</sup>O<sub>2</sub> at very low levels.

## CONCLUSION

There is no denying that measuring phototoxicity with evaluation of mechanism of action will require future improvements. Issues to address in future studies as we look forward include compound phototoxic damage deep in

tissue, with information to extrapolate to intraoperative fluorescent imaging processes that are also needed. Huang et al.'s<sup>1</sup> modified NRU assay can be used in not only cyanine- and xanthine-type fluorescent agents, but also for a wide range of other fluorescent agents. The phototoxicity of BODIPY and phthalocyanine fluorescent agents could also be probed.<sup>32–34</sup> For extrapolation to various biological conditions, future work could also focus on deconvoluting fluorescence imaging from the identity of the cell-damaging reactive oxygen species or photothermal mechanism.

## ACKNOWLEDGMENTS

Support from the National Science Foundation (CHE-2154133) is acknowledged. We thank Leda Lee for assistance with the graphic arts.

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**How to cite this article:** Essang S, Greer A. Shades of phototoxicity in fluorescent imaging agents (that are not supposed to be phototoxic). *Photochem Photobiol.* 2024;100:1694-1697. doi:10.1111/php.13856