Electronic Supplementary Information for

**Methylene Blue as a Far-red Light-mediated Photocleavable Multifunctional Ligand**

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1. Materials
2. Methods
   2.1. Synthetic schemes
   2.2. Light irradiation of test solutions
   2.3. Measurements of fluorescence emission and absorbance
   2.4. High performance liquid chromatography
   2.5. Determination of singlet oxygen generation
   2.6. Mass spectroscopy
   2.7. Cell study
3. Supplemental figures
4. Mass spectroscopy
5. Fourier Transform Infrared Spectroscopy
6. Reference
1. MATERIALS

Methylene blue (MB) hydrate 96+% and was obtained from Acros Organic (Pittsburgh, PA). 4-Nitrophenyl chloroformate, carbonyl diimidazole, ethylchloroformate, triethylamine, ethanolamine and sodium dithionite were purchased from Fisher Scientific (Hampton, NH). Primaquine diphosphate was purchased from Carbosynth (Berkshire, UK). \( p \)-nitrosodimethylaniline was obtained from Sigma-Aldrich (Saint Louis, MO).

HACAT cells and optimized DMEM media were purchased from Addexbio Technologies, CA, USA. Penicillin-Streptomycin (10,000 U/mL), Dimethyl sulfoxide and Trypsin-EDTA was purchased from Fisher Scientific, MA, USA. Thiazolyl Blue Tetrazolium Bromide (MTT reagent) was obtained from Sigma-Aldrich, MO, USA.

All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and used as received.

2. METHODS

2.1. Synthetic scheme

Scheme S1. Synthetic scheme of carbamate bond-containing MB derivatives MB-NP, MB-NB and MB-ET. Reagents and conditions: (1) MB, sodium dithionate, sodium carbonate, water/toluene, 50\(^\circ\)C, 1 h; (2), (3) and (4): leuco methylene blue, 4-nitrophenyl chloroformate (2) or ethyl chloroformate (3) or 4-nitrobenzyl chloroformate (4), triethylamine (TEA), dry toluene, 2-4\(^\circ\)C 1h, room temperature (RT) 24 h. The synthesis of MB-NB has been previously described\(^1\).
Scheme S2. Synthetic scheme of urea bond-containing MB derivatives. Reagents and conditions: (1) MB-NP, primaquine (PQ) base, anhydrous tetrahydrofuran (THF), TEA, 60°C reflux 24 h. (2) MB-NP, excess ethanolamine (EA), anhydrous THF, TEA, 60°C reflux overnight. (3) MB-EA, 4-nitrophenyl chloroformate, TEA, anhydrous dichloromethane (DCM), 0-4°C 1 h, RT 24 h. (4) MB-EA-NP, PQ base, anhydrous DCM, TEA, RT 24 h.

Detailed description of the synthesis.

Leuco-methylene blue (L-MB): Methylene blue monohydrate (1,200 mg, 3.2 mmol) was dissolved in 100 ml of deionized water. Toluene (150 ml) was added to the solution of MB followed by nitrogen flushing. After a constant nitrogen flow was established, sodium dithionate (1,116 mg, 6.4 mmol) followed by sodium carbonate anhydrous (680 mg, 6.4 mmol) was added to the reaction mixture while stirring at 50°C. After 15 min of vigorous stirring, the reaction mixture turned into yellow and both phases became clear indicating that leuco methylene blue was transferred into toluene phase.

MB-NP, MB-ET and MB-NB: Toluene phase containing L-MB was transferred into a nitrogen-discharged flask containing anhydrous sodium sulfate for drying under a nitrogen atmosphere via dropwise addition over 10 min. Resultant anhydrous L-MB solution in toluene was transferred into a solution of each chloroformate solution dissolved in 10 ml of toluene maintained in an ice bath; 4-nitrophenyl chloroformate (1,286 mg, 6.4 mmol) for MB-NP, ethyl chloroformate (646 mg, 6.4 mmol) for MB-ET and 4-nitrobenzyl chloroformate for MB-NB\textsuperscript{1}. Upon completion of transferring L-MB into each chloroformate solution, TEA (1,692 µl, 12.8 mmol) was added. The reaction was proceeded overnight at room temperature. Then, reaction mixture was collected after rinsing three times with each of the following solutions using a separatory funnel: saturated sodium bicarbonate, 0.01N hydrochloric acid solution, and brine. The obtained organic phase was dried, evaporated under reduced pressure and recrystallized in acetonitrile 2 times and yielded a bright orange solid crystal of MB-NP (450 mg, 31%), black solid crystal of MB-ET (300 mg, 26%) or dark red crystal of MB-NB\textsuperscript{1}. 
1H NMR spectrum of **MB-NP** in CDCl3.

Yield = 31%; 1H NMR (400 MHz, CDCl3) δ ppm: 8.24 (2H, d), 7.44 (2H, d), 7.37 (2H, d), 6.71 (2H, d), 6.64 (2H, d), 2.95 (12 H, s)).

**MB-PQ and MB-EA:** MB-NP (450 mg, 1.0 mmol) was dissolved in anhydrous THF and added dropwise into PQ base (1030 mg, 2.0 mmol) or ethanolamine (620 mg, 11.11 mmol). Later TEA (259 µl, 2.0 mmol) in anhydrous THF solution was added to the solution. The reaction mixture was protected from light during reaction and refluxed at 60°C for 24 hours. Upon completion of the reaction, the mixture was collected, evaporated under reduce pressure, dissolved in DCM and washed three times with each of the following solutions: saturated sodium bicarbonate, and water. The obtained organic phase was dried, evaporated under reduced pressure and subjected to flash column chromatography using hexane:ethylacetate 1:1, and later 100% acetone. The eluted solution was evaporated under reduced pressure to yield a light green powder of MB-PQ (490 mg, 85%) or light blue powder of MB-EA (230 mg, 75%).
$^1$H NMR spectrum of **MB-EA** in CDCl₃.

Yield = 75%; 1H NMR (400 MHz, CDCl₃) δ ppm: 7.33 (2H, d), 6.70 (2H, d), 6.62 (2H, d), 5.40 (NH, s), 3.69 (2H, t), 3.36 (2H, q), 3.27 (OH, s), 2.94 (12 H, s).

$^1$H NMR spectrum of **MB-PQ** in CDCl₃.

Yield = 85%; 1H NMR (400 MHz, CDCl₃) δ ppm: 8.55 (1H, d), 7.95 (1H, d), 7.33 (2H, d), 7.30 (1H, t), 6.70 (2H, d), 6.59 (2H, d), 6.35 (1H, s), 6.30 (1H, s), 6.00 (1H, d), 4.99 (NH, s), 3.90 (3H, s), 3.64 (NH, s), 3.28 (2H, t), 2.94 (12H, s).
**MB-EA-NP**: 4-Nitrophenyl chloroformate (400 mg, 2.0 mmol) was dissolved in anhydrous DCM and added dropwise into MB-EA (350 mg, 1.0 mmol) solution in 10 ml anhydrous DCM in an ice bath. After reacting 30 min, the reaction was run at room temperature for another 8 hr. The reaction mixture was collected after washing three times with each of the following solutions: saturated sodium bicarbonate, and brine. The obtained organic phase was dried, evaporated under reduced pressure and subjected to flash column chromatography using hexane:ethylacetate 2:1, and later 100% acetone. The eluted solution was evaporated under reduced pressure to obtain a light brown powder (230 mg, 80%).

**MB-EA-PQ**: MB-EA-NP (200 mg, 0.372 mmol) was dissolved in 10 ml anhydrous DCM and added dropwise into PQ base (115 mg, 0.446 mmol), TEA (186 mg, 1.86 mmol) in DCM solution. The reaction mixture was protected from light during the reaction and kept at room temperature for 24 hrs. The obtained organic phase was dried, evaporated under reduced pressure and subjected to flash column chromatography using hexane:ethylacetate 1:1, and 100% ethylacetate. The eluted product solution was evaporated under reduced pressure yielding a light green powder of MB-EA-PQ (220 mg, 90%).

\[1\text{H NMR spectrum of MB-EA-NP in CDCl}_3.\]

Yield = 80%; 1H NMR (400 MHz, CDCl3) δ ppm: 8.25 (2H, d), 7.32 (2H, d), 7.28 (2H, d), 6.70 (2H, d), 6.63 (2H, d), 5.29 (NH, s), 4.34 (2H, t), 3.58 (2H, t), 2.94 (12H, s)).
1H NMR spectrum of **MB-EA-PQ** in CDCl3.

Yield = 90%; 1H NMR (400 MHz, CDCl3) δ ppm: 8.55 (1H, d), 7.95 (1H, d), 7.33 (2H, d), 7.30 (1H, t), 6.70 (2H, d), 6.59 (2H, d), 6.35 (1H, s), 6.30 (1H, s), 6.00 (1H, d), 5.26 (2H, t), 4.99 (2H, t), 4.75 (NH, s), 3.90 (3H, s), 3.64 (NH, s), 3.28 (2H, t), 2.94 (12H, s)).

### 2.2. Light irradiation of test solutions

A LumaCare® LC-122A equipped with 24W halogen lamp (non-coherent light source) and a 660 nm optic fiber probe with 325 mW/cm² light intensity at the fiber tip was used for all far-red irradiation experiments. The light intensity at the irradiated samples surface was estimated based on the distance from the fiber tip to the sample. Generally, sample placing 25.42 mm from the optic fiber tip will be subjected to a 25 mW/cm² incident light intensity.

An Ultra-LUM UV crosslinker chamber, model AEX-800 was used for all UV irradiation experiments. The 6 x 6-watt mercury lamps mounted on the ceiling emit 365 nm wavelength light. The intensity of light inside the chamber varied from 1.5 to 2.0 mW/cm² during the operating time.

### 2.3. Measurements of fluorescence emission and absorbance

Fluorescence measurements were performed using a LC500 Perkin Elmer fluorescence spectrophotometer at 660 nm excitation wavelength, 600 to 800 nm emission wavelength, 10 nm excitation slit and 10 nm emission slit. MB derivatives were completely dissolved and diluted in a solvent mixture of acetone and 1 mM pH 7.4 phosphate buffer at a ratio of 50:50 unless otherwise stated. The sample concentration was kept below 10 µM to warrant the linear relationship between fluorescence intensity and MB concentration without MB aggregation. Exactly 1 ml of the tested solution is loaded into a fluorescence cell and sealed with a paraffin film to prevent solvent evaporation during the irradiation process.

UV absorbance was scanned using a Genesys 8 UV-VIS spectrophotometer. The sample concentrations were kept at around 50 µM since the detection sensitivity of UV absorbance is lower than that of fluorescence emission.
2.4. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was performed using a Waters Breeze system equipped with a UV detector at 265 nm, C18 100 x 4.6 5 µm column, at a flow rate of 1 ml/min. A mixture of acetonitrile and 0.1% triethylamine in water adjusted to pH 3.0 at a ratio of 70:30 was used as the mobile phase and injection volume was 10 µl.

MB derivatives dissolved in DMSO (100 µl) was spiked on top of 2 ml 0.01 M pH 7.4 phosphate buffered saline (PBS) or simulated gastric fluid. Simulated gastric fluid was prepared so that the final solution contains 0.2 % w/v sodium chloride and 0.7% v/v hydrochloric acid, adjusted pH to be in 1.0-1.2.

2.5. Determination of singlet oxygen generation

Singlet oxygen generation from far-red light irritated urea bond-containing MB derivatives was monitored by oxidation and subsequently bleaching of p-nitrosodimethylaniline (RNO). A solution mixture of MB-EA-PQ (50 µM) or MB (50 µM), RNO (200 µM) and histidine (20 µM) was prepared in 10 mM pH 7.4 phosphate buffer:acetone 50:50. Test solutions were irradiated with far-red light (660 nm, 24 mW/cm2).

2.6. Mass Spectroscopy

LC-MS/MS analysis was conducted using a Waters Acquity BEH Shield RP18 column (100 mm 2.1 mm i.d., 1.7 µm) on a Waters Acquity UPLC system (Waters, Milford, MA) that consists of a binary solvent manager, sample manager, heat controlled column compartment, photodiode array (PDA) detector, and Xevo G2-S QToF mass spectrometer. The instrument was operated by MassLynx NT 4.1. The column and sample temperature were maintained at 40°C and 10°C, respectively. The mobile phases were composed of 0.05 % formic acid of water (A) and acetonitrile containing 0.05 % formic acid (B) using gradient elution at a flow rate of 0.3 mL/min as follows: 0-8 min, 5 % B to 95 % B; 8-10 min, 95 % to 100 % B. The analysis was followed by a 3 min washing procedure with 100 % B and re-equilibration period of 3.5 min with initial condition. A strong needle wash solution (90/10; acetonitrile/water, v/v) and weak needle wash solution (10/90; acetonitrile/water) were used. The injection volume was 2 µL.

The MS experiments were carried out on a Xevo G2-S QToF mass spectrometer that was connected to the UHPLC system via an ESI interface. The MS parameters was operated in the positive ionization mode as follows: 0.8 kV capillary voltage; 15 V cone voltage; 85 and 450 °C for ion source and desolvation temperature, respectively; 50 and 800 L/h for cone and desolvation gas flows, respectively. Leucine-enkephalin was used for the lock mass at a concentration of 2 ng/mL and flow rate of 5 µL/min. Ions [M+H]+ (m/z 556.2771 Da) and a fragment ion (m/z 278.1141 Da) of leucine-enkephalin were employed to ensure mass accuracy during the MS analysis. The lock spray interval was set at 30 s, and the data was averaged over three scans. The mass spectrometer was programmed two steps between low (10 eV) and elevated (15-40 eV) collision energies on the gas cell, used a scan time of 0.1 s per function over a mass range of m/z 100–1500 Da.

2.7. Cell study

The HACAT cells were cultured and maintained at 37 °C in a humidified atmosphere of 5% CO2 and 90% relative humidity. The optimized DMEM supplemented with 10% fetal bovine serum, penicillin (100 mg/mL) and streptomycin (100 mg/mL) were used for cell growth. The medium was replaced every other
day in flask and when the cells reached 80-90% confluence, cells were detached using Trypsin-EDTA. The detached cells were plated at a density of 20,000 cells/well in 48-well culture plates.

On attaining cell confluence of 90–95%, media were aspirated from plates. Three 48-well culture plates were treated with different concentration (20 and 10 µM) of MB, MB-EA and dissolved in serum free media. After incubation for 24 h, 2 plates were exposed to far-red light. One of the two irradiated plate was used for fluorescence microscopic examination and the other plate was incubated for another 24 h. Third plate was incubated for 48 h after MB, MB-EA and EA treatment. The cell viability in second and third plate was estimated using MTT assay.

The HaCaT cell viability in plates were estimated using Thiazolyl Blue Tetrazolium Bromide (MTT) reagent. After the incubation of second and third cultured plates, the media was aspirated and replaced with fresh serum-free media containing 0.5 mg of MTT reagent. These MTT reagent treated plates were incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO2 and 90% relative humidity. After incubation of cell culture plates, the media was aspirated. Formazan crystals formed by live cells were dissolved in DMSO. Synergy H1, microplate reader was used to record the absorbance in 48-well culture plates. The absorbance of formazanin DMSO solution was recorded at 570 nm. The absorbance at wavelength of 630 nm (contributed by excess of cell debris) was recorded and subtracted with absorbance recorded at 570 nm. The percentage cell viability was calculated by comparing the absorbance recorded from untreated cells as the negative control.
3. SUPPLEMENTAL FIGURES

Fig. S1 (a) to (c): Fluorescence spectra and (d) to (e) absorption spectra of MB-EA (4.825 µM), MB-EA-NP (4.825 µM) and MB-PQ (4.825 µM) solutions, respectively in acetone:10 mM pH 7.4 phosphate buffer 70:30. Samples were irradiated with far-red light (660 nm, 8.21 mW/cm², 5.08 cm from fiber optic tip, 1 cm² irradiated area). The inserts show the change in emissive fluorescence intensity at 685 nm over time representing the rate of MB release. The fluorescence and absorbance spectra shows an increase in 600-700 nm absorbance range corresponding to fluorescent MB. The red line represents fluorescence or absorption spectra of freshly prepared sample that shows negligible fluorescence emission or absorption at visible to near infra-red wavelength region. All scans were done in triplicate and average values were plotted. (g) to (i): Absorption spectra of carbamate bond-containing MB derivatives MB-NP, MB-NB and MB-ET (30 µM) after far-red light irradiation (660 nm, 8.21 mW/cm², 5.08 cm from fiber optic tip, 1 cm² irradiated area) show negligible change in the higher wavelength absorption band.
Fig. S2. HPLC chromatogram of (a) dissolved solid MB-PQ after UV light irradiation for 30 min (b) MB-PQ solution in gastric fluid in the dark environment (c) MB-PQ solution in 0.01 M pH 7.4 phosphate buffered saline in the dark environment. The chromatograms do not show any changes in peak intensity and retention time.

Fig. S3. (a) Fluorescence spectra and (b) fluorescence intensity at peak-emission of MB solutions showing bathochromic shift and fluorescence self-quench effect as MB concentration increase. The linear relationship between MB fluorescence intensity and concentration was determined to be from 20 to 3500 ng/ml (11.21 µM).

Fig. S4. (a) Rate of MB release from MB-EA-PQ solutions with different solvent compositions that range from 0 to 50% water in acetone, 100% water (MB-EA-PQ was solubilized using hp-β-cyclodextrin) and human serum. (b) Rate of MB release from MB-EA-PQ solution after far-red light irradiation demonstrating enhancement of photolysis at higher pH. (c) Photo-triggered fluorescence restoration from MB-EA, after treatment with a reducing agent, ascorbic acid. The graph showed a lag time which is equal to the time required for complete photolysis of MB-EA but similar pattern.
Fig S5. Fluorescence spectra of MB-PQ (~ 5.0 µM) solution subjected to (a) far-red light irradiation (~1.5 mW/cm²) and (b) UV light irradiation (1.5 mW/cm²). (c) Fluorescence intensity at 680 nm of the MB-PQ solution after far-red and UV light irradiation.

Fig S6. HPLC chromatogram of MB-PQ (50 µM) solution recorded after far-red light irradiation. The chromatogram shows gradual photolysis by the disappearance of the peak at long retention time representing intact MB-EA-PQ and the appearance of peaks at low retention time representing MB and PQ.
Fig. S7. (a) Extracted-ion chromatogram (EIC) at 175 Da of standard PQ solution (5 μg/ml) with 2.87 min peak corresponding to PQ. (b) Mass spectroscopy (MS) spectra of the 2.87 min peak shows PQ-related fragment at 175.08 Da. (c) EIC at 658 Da of standard MB-EA-PQ solution (5 μg/ml) with 6.82 min peak corresponding to MB-EA-PQ. (d) MS spectra of the 6.82 min peak shows characteristic fragments corresponding to MB (284 Da), EA-PQ (373 Da) and MB-EA-PQ (658 Da). (e-f) EIC at 658 Da of fully light-degraded MB-EA-PQ solution (5 μg/ml) overlaps with EIC at 658 Da of the methanol solution that serves as background chromatogram. (g) EIC at 175 Da of fully light-degraded MB-EA-PQ solution (5 μg/ml) with 3.41 min peak. (h) MS spectra of the 3.41 min peak show characteristic fragments corresponding to −EA-PQ (373 Da), PQ (286 Da) and quinoline ring (175 Da). (i-k) EIC of MB solution, and MS spectra of the 3.20 min peak. (l-m) EIC of fully light-degraded MB-EA-PQ solution, and MS spectra of the 3.26 min peak shows that MB was released.
Fig S8. Phase contrast microscope images of HaCaT cells treated with (a) 0.01 M pH 7.4 PBS buffer, (b) MB, (c) MB-EA and (d) EA after far-red light irradiation for 20 min at 660 nm and at a total irradiated energy of 9.8 J/cm². The pictures show the overwhelming presence of round-shape cells in MB (positive reference) and MB-EA treated groups and demonstrate the phototoxicity of MB and MB-EA. On the contrary, the pictures show little presence of the round-shape cells in PBS buffer and EA treated groups.

Fig. S9. Singlet oxygen generation from (a) 50 µM MB solution, (b) 50 µM MB-EA solution and (c) solution without MB and MB-EA was monitored using p-nitrosodimethyleaniline upon far-red light irradiation at a wavelength of 660 nm. Various irradiation times ranging from 0 to 40 min were used to determine the effect of irradiation time on singlet oxygen production. The results show that both MB and MB-EA solutions were able to generate singlet oxygen.
4. **MASS SPECTROSCOPY**


Estimated m/z
372.16
373.17
371.16

(Top) MS/MS spectra and (bottom) MS spectra of MB-EA at 4.273 m retention time.
(Top) MS/MS spectra and (bottom) MS spectra of MB-EA-PQ at 6.824 m retention time.
(Top) MS/MS spectra of MB-PQ and (bottom) MS spectra of MB-PQ at 7.325 m retention time.
5. FOURIER TRANSFORMED INFRA-RED SPECTROSCOPY

ATR-FTIR spectra of MB-EA.

ATR-FTIR spectra of MB-NP.
ATR-FTIR spectra of MB-EA-PQ and MB-PQ.

6. References