Electronic Supplementary Information for

Insight into hydroxyl radical-mediated cleavage of caged methylene blue: the role of Fenton's catalyst for antimalarial hybrid drug activation

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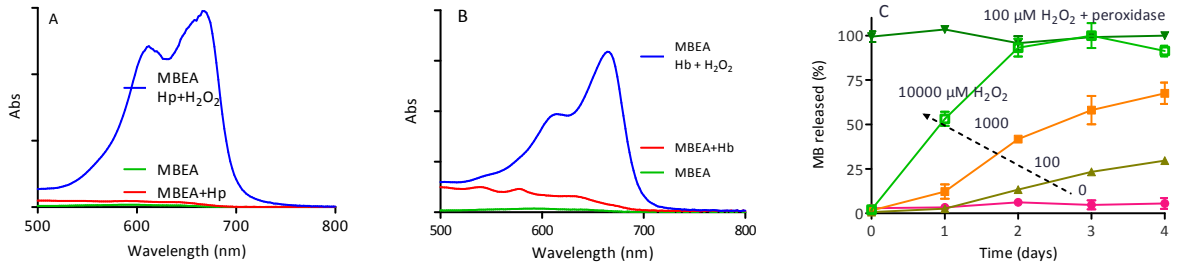


Fig. S1. Absorbance spectra of MB-EA solution subjected to the treatment with ˙OH species (Fenton reagent, H_2O_2, (a) horseradish peroxidase, and (b) hemoglobin, at pH 7.4. (c) The profile of MB release from MB-EA upon the incubation with H_2O_2.

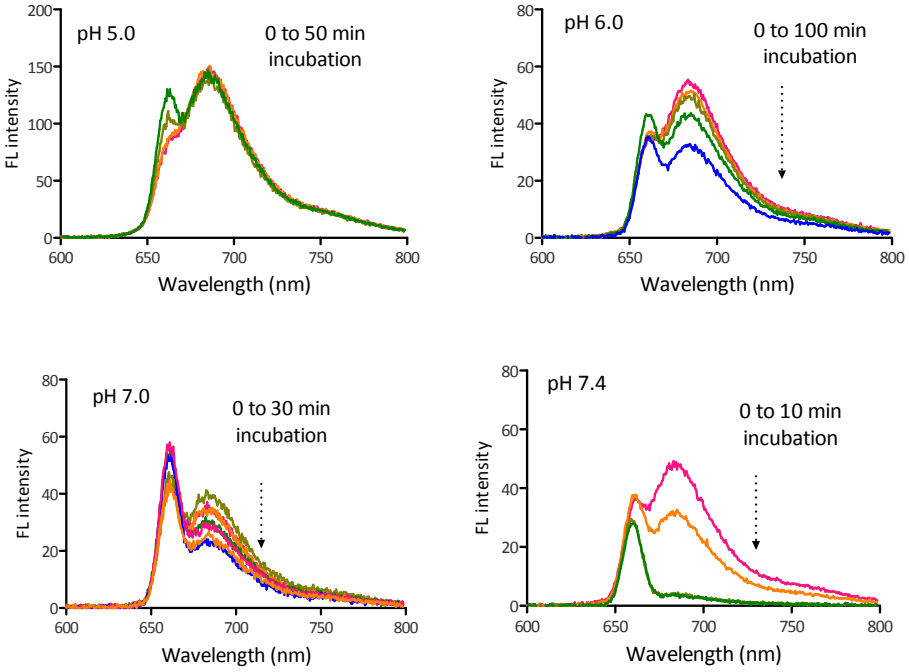


Fig. S2. Fluorescence scan of MB-EA solutions incubated with $FeSO_4.7H_2O$ at different pH values, for an increasing amount of time.

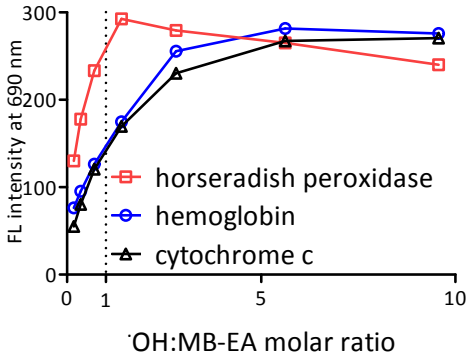


Fig. S3. Fluorescence intensity at 690 nm, stemming from freed MB in MB-EA solution, after the treatment with ·OH (Fenton reagent, H_2O_2 using hemoproteins as the catalyst). The concentration of ·OH is assumed to be equal to the concentration of H_2O_2

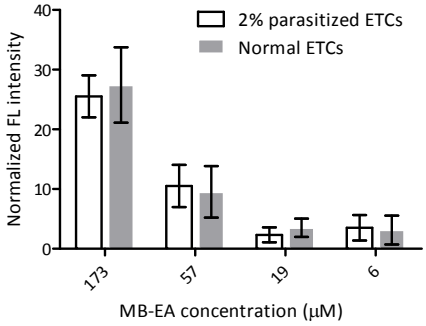


Fig. S4. FL intensity of MB-EA treated culturing wells that contained 2% parasitized and normal ETCs. (n=4). No statistically significance was observed between the groups.

Table S1. Half maximal inhibitory concentration (IC_{50}) of MB, PQ, physical mixture, urea bond containing and carbamate bond containing MB derivatives against *Plasmodium falciparum D6* and *W2*, and VERO cells.

IC 50 (nM)	P. f D6	P. f W2	VERO
MB	13.1	12.8	1531.3
PQ	4324.5	1882.8	>10453.7
MB+PQ	26.8	42.1	1176
MB-EA	26.8	25.2	9823.1
MB-EA-NP	46.1	53.4	2722.1
MB-EA-PQ	32.1	29.2	5995.5
MB-PQ	31.9	35.4	8339.8
MB-NP	9257.3	9516.3	>10565.8
MB-NB	1219.9	1351.7	>10246.7
MB-ET	3785.8	1736.4	>13315.8

Table S2. List of HOCl probes and associated protocol to generate hydroxyl radical (˙OH)

	DOI and reference	Chemical platform	˙OH generation protocol	Sensitivity claim
1	10.1039/c7sc03784h Peng Wei et. al.[1]	Methylene blue (Phenothiazine)	pH 7.2 10 mM PBS $FeCl_2$: H_2O_2 : probe (100 : 10 : 1 mol. equiv.) [˙OH] assumed equal to [H_2O_2]	Exclusively sensitive to HOCl
2	10.1002/anie.201813648 Peng Wei et. al.[2]	Methylene blue	pH 7.2 10 mM PBS $FeSO_4$: H_2O_2 : probe (40: 4 : 1 mol. equiv.) [˙OH] assumed equal to [H_2O_2]	Exclusively sensitive to HOCl
3	10.1039/c8ra01037d Xin Huang et. al.[3]	Methylene blue	pH 7.4 10 mM PBS ˙OH generation protocol not described	Exclusively sensitive to HOCl
4	10.1016/j.dyepig.2020.108308 Yu Chen et. Al.[4]	Methylene blue	Not described	Exclusively sensitive to HOCl
5	10.1016/j.dyepig.2020.108404 Wubin Zheng[5]	Phenoxazine	pH 7.4 PBS FeSO4	Exclusively sensitive to HOCl
6	10.1021/acs.iecr.7b00381 Jing Lv[6]	Fluorescein	pH 7.4 10 mM PBS Fe^{2+} : H_2O_2 : probe (10 : 10 : 1 mol. equiv.) Type of ferrous salt not specified	Exclusively sensitive to HOCl
7	10.1021/jacs.5b01881 Jun Jacob Hu[7]	Tetrafluoro fluorescein	pH 7.4 10 mM PBS $FeCl_2$: H_2O_2 : probe (1000 : 10 : 1 mol. equiv.)* [˙OH] assumed equal to [Fe(II)]	66-100 fold more sensitive towards $O_2^{\cdot-}$ than ˙OH
8	10.1039/c8cc03963a Yin Jiang[8]	Thiocarbamate Naphthalene	pH 7.4 10 mM PBS $FeCl_2$: H_2O_2 : probe (1000 : 10 : 1 mol. equiv.)[a] [˙OH] assumed equal to [Fe(II)] [a]Follows protocol from #5	Exclusively sensitive to HOCl
9	10.1039/c5sc03855c Jun Jacob Hu[9]	Fluorescein	pH 7.4 0.1 M PBS $FeCl_2$: H_2O_2 : probe (10 : 1 :1 mol. equiv.) [˙OH] assumed equal to [Fe(II)]	>83 times more sensitive toward HOCl than ˙OH
10	10.1002/anie.201500537 Yin Jiang[10]	-nitro-benzo [1,2,5] oxadiazole	pH 7.4 10 mM PBS Fe2+ : H2O2 : probe (500 : 100 : 1 mol. equiv.) [˙OH] is assumed to be equal to [H2O2][b] [b]Follows protocol from Chang's Group[11]	5 times more sensitive toward HOCl than ˙OH
11	10.1039/c3cc44463e Mustafa Emrullahoğlu[12]	BODIPY – Ox	pH 7.2 buffer $FeCl_2$: H2O2 : probe (20 : 200 : 5 mol. equiv.)	Exclusively sensitive to HOCl
12	10.1021/ja505988g Hao Zhu[13]	BODIPY	pH 7.4 buffer X : H_2O_2 : probe (X : 10 : 1 mol. equiv.) No information about iron species found	Exclusively sensitive to HOCl
13	10.1039/c3cc47864e Guanghui Cheng[14]	Selenomorpholine	pH 7.4 20 mM PBS No information found	Exclusively sensitive to HOCl
14	10.1039/c4cc07411d Hongde Xiao[15]	Phenothiazine ring	pH 7.3 10 mM PBS 100 : 1 (OH : probe)	Exclusively sensitive to HOCl
15	10.1039/c4cc02673j Ji-Ting Hou[16]	Benzoyl acetohydrazide	pH 7.4 20 mM PBS $Fe(ClO)_4$: H_2O_2 : probe (100 : 200 : 1 μM)	Exclusively sensitive to HOCl
16	10.1021/acs.analchem.6b01738 Qingling X[17]		pH 7.4 50 mM PBS $FeCl_2$: H_2O_2 : probe (200 : 400 : 2 μM)	Exclusively sensitive to HOCl
17	10.1039/C8AN00586A Xiaoyi Wang[18]	Porphyrin	*OH was freshly prepared *OH : probe (100 : 1) No information found	Exclusively sensitive to HOCl

2. MATERIALS

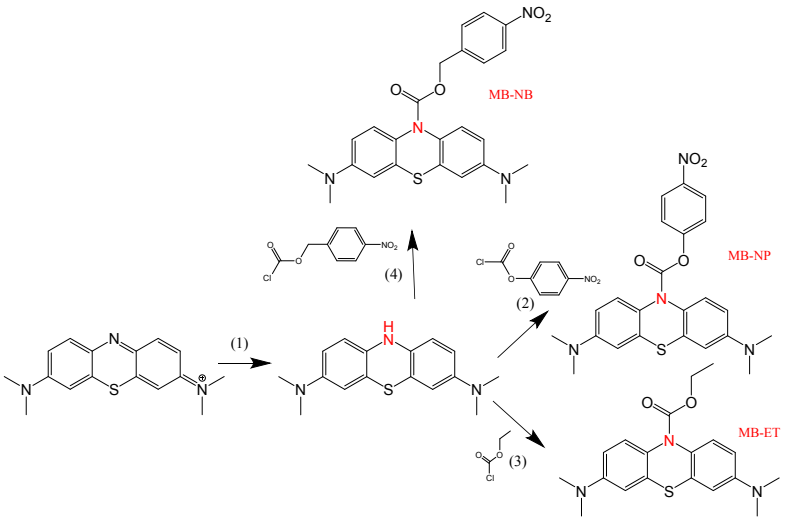
Methylene blue (MB) hydrate 96+% and was obtained from Acros Organic (Pittsburgh, PA). 4-Nitrophenyl chloroformate, carbonyl diimidazole, ethylchloroformate, triethylamine, ethanolamine and sodium dithionite were purchased from Fisher Scientific (Hampton, NH). Primaquine diphosphate was purchased from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

Rabbit erythrocytes cells were purchased from Innovative Reseach, MI, USA.

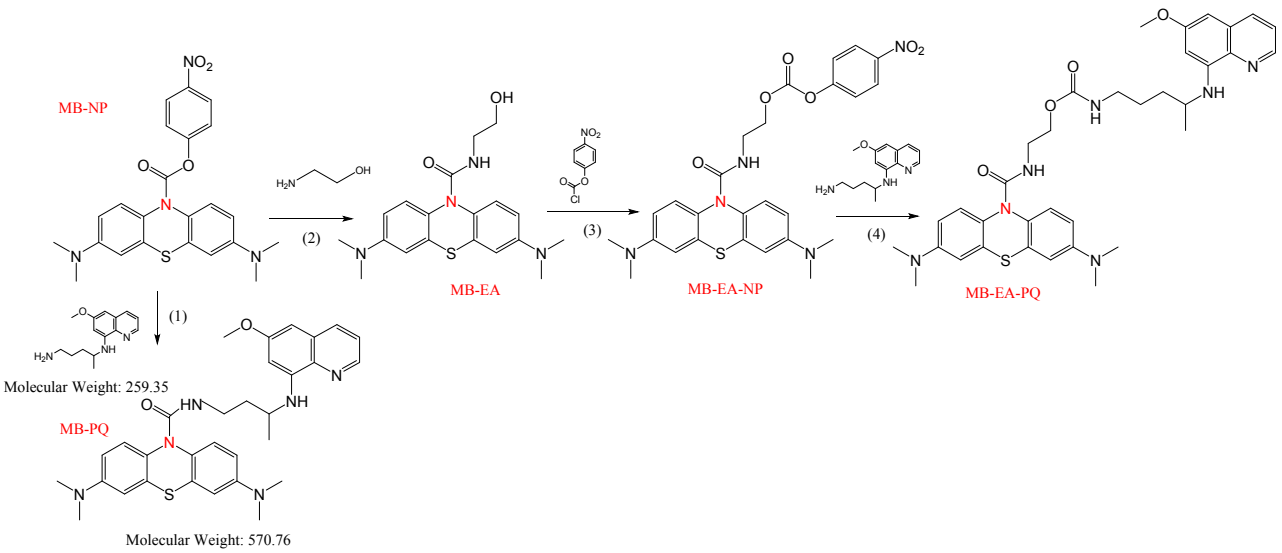
All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and used as received.

3. METHODS

3.1. 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°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°C 1h, room temperature (RT) 24 h. The synthesis of MB-NB has been previously described[19].



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.

Detailed description of the synthesis, ^{1}H NMR, FTIR and LC MS/MS characterizations were previously published by our research group. Interested readers are advised to search for the following article:

Huy M. Dao, et. al., Methylene blue as a far-red light-mediated photocleavable multifunctional ligand, Chem. Commun., 2020,56, 1673-1676

3.2. Preparation of solutions of MB derivatives and analytes

Stock solution of MB derivative (1 mM) were prepared in 1% acetonitrile in water. The test solutions of MB derivatives (5 µM) in 10 mM PBS solutions pH 5.0 to 8.0 were prepared by diluting 10 µl of stock solutions with corresponding PBS buffers.

The time-drive experiments were carried-out by placing 10 µl stock ferrous-ions containing solutions ($FeSO_4.7H_2O$ or hemoproteins) into caged MB derivatives test solution (5 µM) to achieve desired molar ratio. Subsequently, the solution was incubated for a cETCain period of time (0 to 100 min). Upon the addition of H_2O_2 to trigger the Fenton reaction, the intensity of fluorescence emission at 690 was recorded. As the precautions to prevent Fe^{2+} to Fe^{3+} oxidation, all $FeSO_4.7H_2O$ stock solutions were slightly acidified and purged with nitrogen for 5 min.

The fluorescence intensity of the solutions at different pHs were normalized using standard MB solutions at the respective pH. The conversion from fluorescence intensity to percentage of MB release was calculated based on the assumption that MB derivatives (5 µM) were completely cleaved to release 100% MB moieties when incubated with H_2O_2 (50 µM) and horseradish peroxidase (5 µM) for 5 min.

3.3. 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 10 mM pH 5.0-8.0 PBS at a ratio of 5:95 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.

3.4. 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.

3.5. Red blood cell study

Rabbit erythrocytes (ETCs) was obtained and use within 14 days due to its perishable nature. The stock ETCs was diluted 10 times and treated with different concentration (2 to 20 µM) of MB-EA. After incubation for 4 hrs, the ETCs suspensions were centrifuged and the supernatant was discarded. The ETCs were resuspended in 0.01 M pH 7.4 PBS solution. Subsequently, the ETCs were treated with 50 µM H_2O_2 to simulate elevated ROS concentration in disease model. Synergy H1 (Biotek, USA), microplate reader was used to record the absorbance in 48-well culture plates. Fluorescent microscopy (Olympus, TX, USA) equipped with a Texas red filter was utilized to capture the fluorescent microscopic image. All images are taken at the same exposure time of 1.4 s to enable the comparison of fluorescence intensity of different samples.

3.6. Assay for screening antimalarial activity and cytotoxicity

The antimalarial activity is determined against chloroquine sensitive (D6) and chloroquine resistant (W2) strains of P. falciparum by measuring plasmodial LDH activity according to the procedure of Makler and Hinrichs. The assay is performed in 96-well tissue culture-treated plates. A suspension of red blood cells infected with D6 or W2 strain of P. falciparum (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) is added to the wells of a 96- well plate containing 10 µL of serially diluted samples (plant extracts, column fractions or pure compounds). The plate is incubated at 37 O C, for 72 h in a modular incubation chamber with 90% N 2 , 5% O 2 , and 5% CO 2 . Parasitic LDH activity is determined by mixing 20 µL of the incubation mixture with 100 µL of the Malstat reagent and incubating at room temperature for 30 min. Twenty microliters of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) is then added and the plate is further incubated in the dark for 1 h. The reaction is then stopped by adding 100 µL of a 5% acetic acid solution and the absorbance is read at 650 nm. Artemisinin and chloroquine are included as the drug controls. IC 50 values are obtained from the dose response curves of growth inhibition. The in vitro cytotoxicity of samples to mammalian cells is

also determined in order to calculate the selectivity index of the antimalarial activity. Vero cells (monkey kidney fibroblasts) are seeded to the wells of 96-well plate at a density of 25,000 cells/well and incubated for 24 h. Upon confluency, test samples at different concentrations are added and cells are further incubated for 48 h. Cell viability is determined using a tetrazolium dye (WST-8). IC 50 values are obtained from dose response curves. Doxorubicin is included as drug control for cytotoxicity.

3.7. Measurements of FL intensity induced by elevated ROS levels in parasitized ETCs

To evaluate the uncaging of MB-EA and resultant FL stemmed from the freed MB, *P. falciparum* infected ETCs (4% parasitemia) and normal ETCs were incubated with presence of increasing concentration of MB-EA (0.97, 2.93, 8.81, 26.44, 79.33, and 238 ng/ml) for 24 and 48 h at 37^{o}C. After the incubation, plate was centrifuged at 2000 g for 5 min and supernatant was transferred to a new clear bottom 96 well plate. FL intensity of the supernatant was measured at 660 excitation wavelength and 690 emission wavelength using Spectramax-M5 plate reader (Molecular Decives, Sunnyvale, CA).

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