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Fluorometric Characterization of a Methylene Blue Derivative Sensitive to Reactive Oxygen Species (ROS)

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Fluorometric characterization of a methylene blue derivative sensitive to reactive oxygen species (ROS)

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ABSTRACT

Methylene blue (MB) has many uses within both microbiology and pharmacology. MB can treat disorders such as methemoglobinemia, malaria, Alzheimer's disease, and certain forms of cancer. MB is also useful for molecular imaging due to its off-on fluorescent capabilities. MB derivatives with a urea bond at the 10-N position have been cleavable by triggers such as light. However, I was interested in sensitivity to reactive oxygen species (ROS). In this study, I wanted to determine if the MB derivative MB-EA exhibited sensitivity to ROS. MB-EA was exposed to varying concentrations of hydrogen peroxide and MB release was measured. I concluded that MB-EA is increasingly sensitive to increasing concentrations of hydrogen peroxide, and exhibits sensitivity to ROS. This could allow for new MB therapeutics to be administered in diseases that produce ROS.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
L-MB	leuco-methylene blue
MB	methylene blue
MB-EA	MB ethanolamine
MB-NP	MB nitrophenyl
MB-PQ	MB primaquine
PDT	photodynamic therapy
NIR	near-infrared light
NTR	nitroreductase
ROS	reactive oxygen species
TEA	triethylamine
THF	tetrahydrofuran

Background

Methylene blue (MB) is a synthetic dye with extensive pharmacological activity and many biomedical applications. Its chemical structure can be seen in Figure 1.

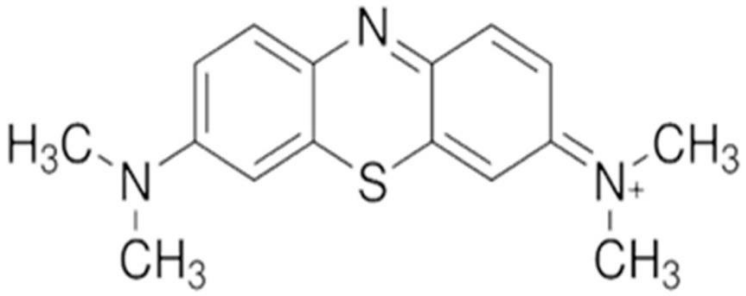


Figure 1. Structure of Methylene Blue

MB is FDA approved for the treatment of acquired methemoglobinemia. Methemoglobinemia is characterized by hemoglobin being unable to carry oxygen due to the ferrous component of the heme molecule being oxidized to a ferric state. MB corrects this issue by reacting within the red blood cell to reduce to leuco-methylene blue (L-MB), which is then able to reduce the ferric iron (Fe^{3+}) back to oxygen carrying ferrous iron (Fe^{2+}). [1] MB has also proven to be an effective antimalarial therapy. MB has shown substantial antimalarial activity against all forms of malaria in various endemic areas, and when combined with other antimalarial agents, it has shown activity against falciparum malaria in Africa. MB seems to act slowly against P. falciparum when administered alone; however, when administered with artemisinin the combination rapidly cleared parasites and decreased transmission from mosquitos. MB as an antimalarial therapeutic has become more researched as of late as P. falciparum is becoming more resistant to many common drug therapies such as chloroquine. [2] MB has also demonstrated that it can

be useful for slowing the progression of Alzheimer's disease (AD). MB has an inhibitory effect on the cGMP pathway, and it also affects many cellular and molecular events that are closely associated with the progression of AD, such as attenuating formations of amyloid plaques and neurofibrillary tangles and partially repairing impairments in mitochondrial function and cellular metabolism. MB also affects many neurotransmitter systems that are believed to play important roles in the pathogenesis of AD.[3]

MB has also shown great results as a photodynamic therapeutic. Photodynamic therapy (PDT) involves a photosensitizer that is localized in the target tissue being illuminated with visible light. PDT has been used with positive results in treating several forms of cancer, and PDT provides many advantages over other cancer therapies. PDT works on almost all types of cancers, and PDT can be repeated several times if necessary because there are no cumulative toxic effects. Also, PDT is usually an outpatient procedure, and it can also be used for treating elderly patients or patients who are too sick for surgery due to its lower risk profile. Tardivo et al. conducted a study in which they treated several superficial tumors with MB and light and found promising results. Patients with basal cell carcinoma, metastatic melanoma, and Kaposi's sarcoma were treated, and a majority of patients experienced a complete response. PDT using MB has also shown good anti-bacterial activity. MB has shown photobacteriocidal effects on Enterococcus ssp as well as Staphylococcus aureus. MB's use as an anti-bacterial agent could become more prominent as bacterial resistance to other therapies increases.[4]

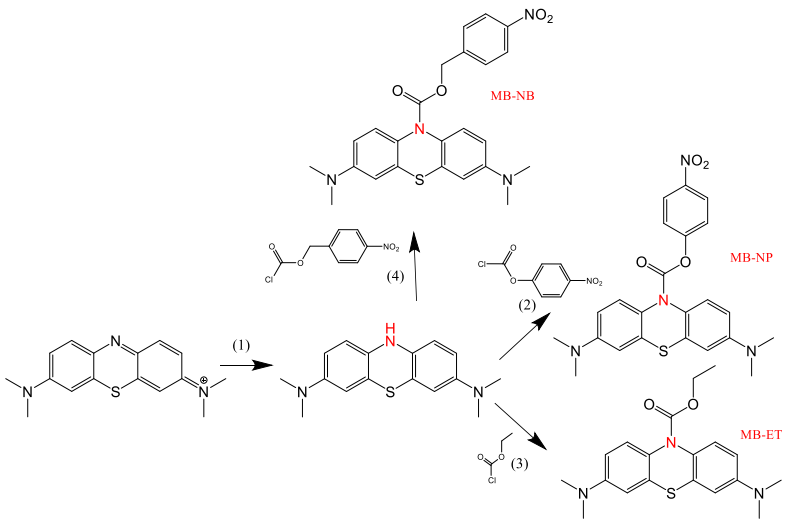
MB is also useful for molecular imaging due to its off-on fluorescence. When MB is chemically modified at the 10-N position, no fluorescence is observed. However, once the conjugated portion is cleaved from the 10-N position, MB's fluorescence is turned back on.[5]

This has proven to be beneficial for imaging and treatment skin-related diseases such as bacterial infections and skin cancer. MB's off-on fluorescence has also been used to create a near-infrared light (NIR) fluorescent probe for the detection of nitroreductase (NTR). This probe was created in a study conducted by Bae *et al.* by conjugating a p-nitrobenzyl group through a carbamate bond to MB at the nitrogen of the phenothiazine ring. In the presence of NTR, this p-nitrobenzyl group will be cleaved and MB's fluorescence will be turned on. This NTR induced activation renders p-nitrobenzyl methylene blue (p-NBMB) a satisfactory imaging sensor for the detection of bacteria expressing NTR.[6]

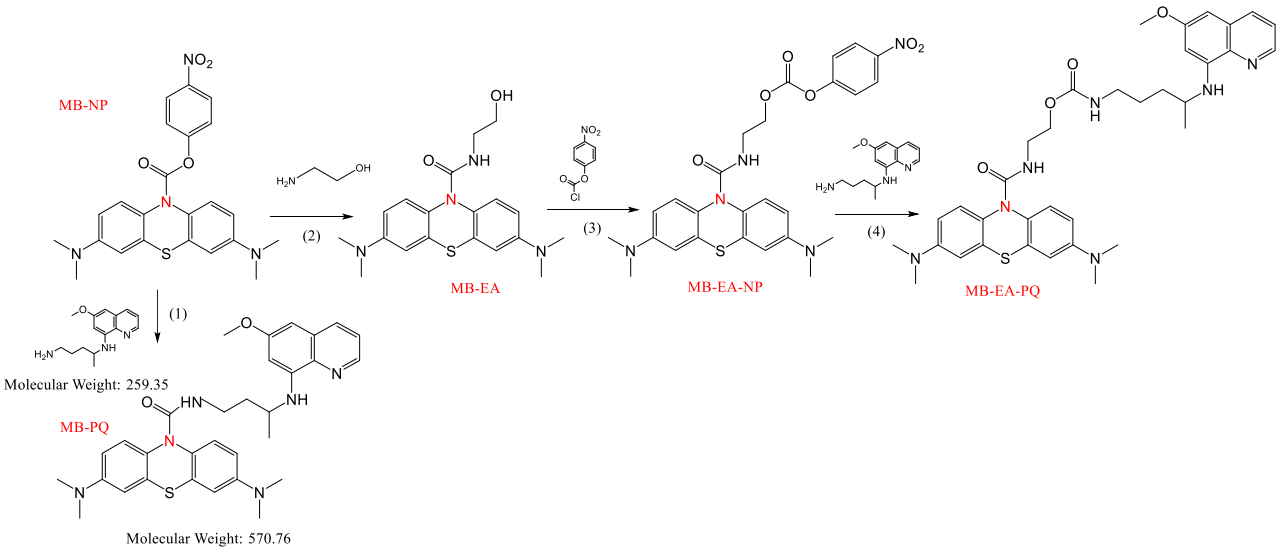
Methods

In a few of the aforementioned applications and therapies of MB, reactive oxygen species (ROS) were addressed as a possible variable in experimental or clinical outcomes. The goal of my study was to determine ROS sensitivity of the MB derivative MB-ethanolamine (MB-EA). MB-EA was synthesized from MB-nitrophenyl (MB-NP), and MB-NP was derived from L-MB, which is easily generated from MB. To provide a little more detail, 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^{o}C. After 15 min of vigorous stirring, the reaction mixture faded from yellow and both phases became clear, indicating that L-MB was transferred into toluene phase. This L-MB is then used to form MB-NP. 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. Upon completion of transferring L-MB into each chloroformate solution, triethylamine (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. MB-NP (450 mg, 1.0 mmol) was dissolved in anhydrous THF and added dropwise into 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^{o}C for 24 hours. Upon completion of the reaction, the mixture was collected, evaporated under reduce pressure, dissolved in dichloromethane 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 blue powder of MB-EA. Scheme 1 shows the process from MB to L-MB and then to MB-NP and other carbamate bond containing derivatives, while scheme 2 shows the production of MB-EA and other urea bond containing MB derivatives from MB-NP.



Scheme S1. Synthetic scheme of carbamate bond-containing MB derivatives MB-NP. 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)**, triethylamine (TEA), dry toluene, 2-4°C 1h, room temperature (RT) 24 h. The synthesis of MB-NB has been previously described[1].

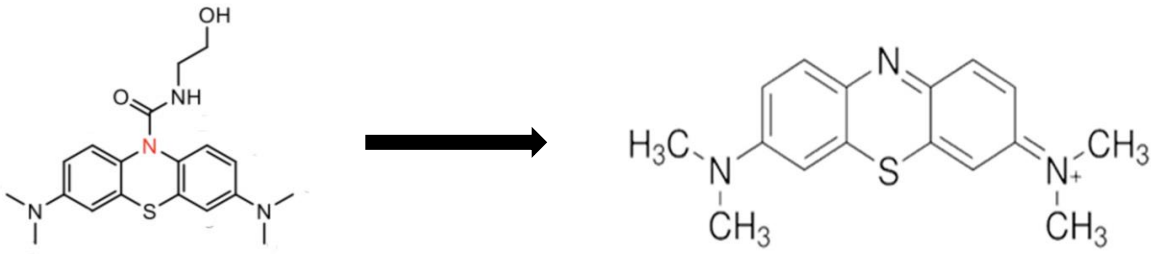


Scheme S2. Synthetic scheme of urea bond-containing MB derivatives. Reagents and conditions: **(1)** MB-NP, primaquine (PQ) base, anhydrous tetrahydrofuran (THF), TEA, 60°C reflux 24 h. **(2)** MB-NP, excess ethanolamine (EA), anhydrous THF, TEA, 60°C reflux overnight.

To observe ROS sensitivity of MB-EA, I incubated four vials containing MB-EA 4 micrograms/ml and introduced varying concentrations of hydrogen peroxide (H_2O_2), a reactive oxygen species, to each vial. Hydrogen peroxide was chosen due to its increased stability over other ROS. The vials were given H_2O_2 concentrations of 0 micromolar (control), 100 micromolar, 1,000 micromolar, and 10,000 micromolar. Each vial was then wrapped in aluminum foil and placed inside of a refrigerator. This eliminated all exposure to light and prevented any cleavage that could have occurred at room temperature. The vials were then checked daily for four days and the fluorescence of each vial was measured using a fluorometer. Fluorescence measurements were performed using a LC500 Perkin Elmer fluorescence spectrophotometer at 660 nm excitation wavelength, 600 to 800 nm emission wavelength, 10nm excitation slit, and 10 nm emission slit. Exactly 1 ml of the tested solution was loaded into the fluorescence cell and sealed with a paraffin film to prevent solvent evaporation during the irradiation process. The vials were checked and the fluorescence was measured at the same time each day so that there was a uniform 24 hours between each measurement. After being measured, the vials were wrapped in foil again and returned to the refrigerator until the next measurement.

Results

I hypothesized that as the concentration of H_2O_2 increased, the amount of MB released would also increase. This result is due to H_2O_2 cleaving MB-EA, thus releasing MB from the bound ethanolamine group and restoring its fluorescence. The proposed off- on cleavage of MB-EA is demonstrated in Figure 2.



Fluorescence off

Fluorescence on

Figure 2. MB-EA off-on fluorescence

The data gathered from the fluorometer was organized and formatted into a graph (Figure 3) that displays MB released over time. The graph demonstrates that as the H_2O_2 concentration was increased, the amount of MB released was also increased. The vial with the highest concentration of H_2O_2, 10,000 micromolar, exhibited 100% MB release after 3 days. The 1,000 micromolar and 100 micromolar vials both showed increases in MB released each day; however, neither concentration produced full MB release buy the end of the four days. The 1,000 micromolar H_2O_2 vial showed greater MB release than the 100 micromolar H_2O_2 vial. The control vial containing no H_2O_2 did not release any MB during the four days. The data shows that MB-EA is relatively stable in the absence of H_2O_2, but demonstrates significant sensitivity to increasing concentrations of H_2O_2. To further demonstrates that MB-EA is cleaved to release fluorescent MB, MB-EA was incubated with H_2O_2 1,000 micromolar for 10 days. The fluorescent intensity of the cleaved MB over time is displayed in Figure 4.

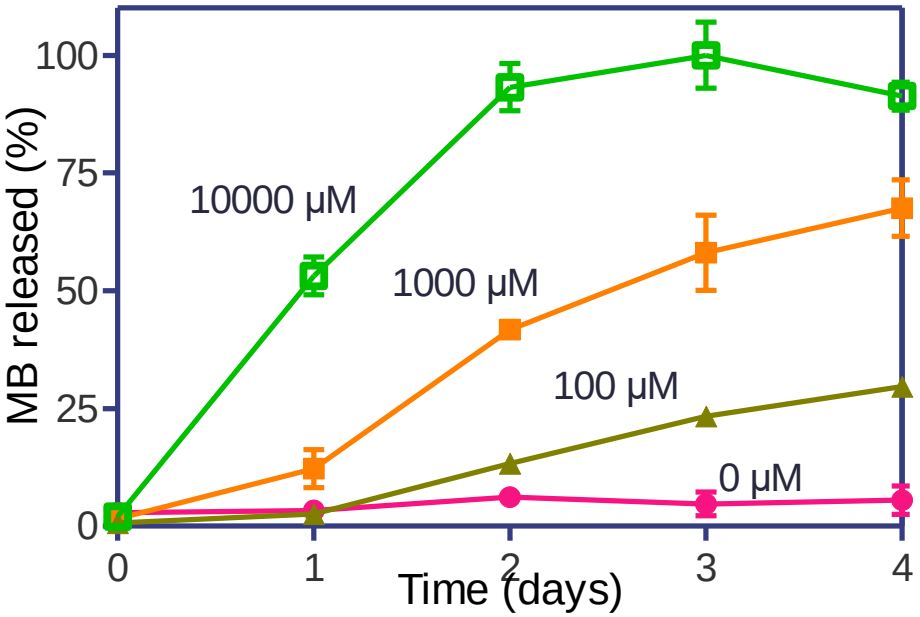


Figure 3. MB release over time with varying H_2O_2 concentrations

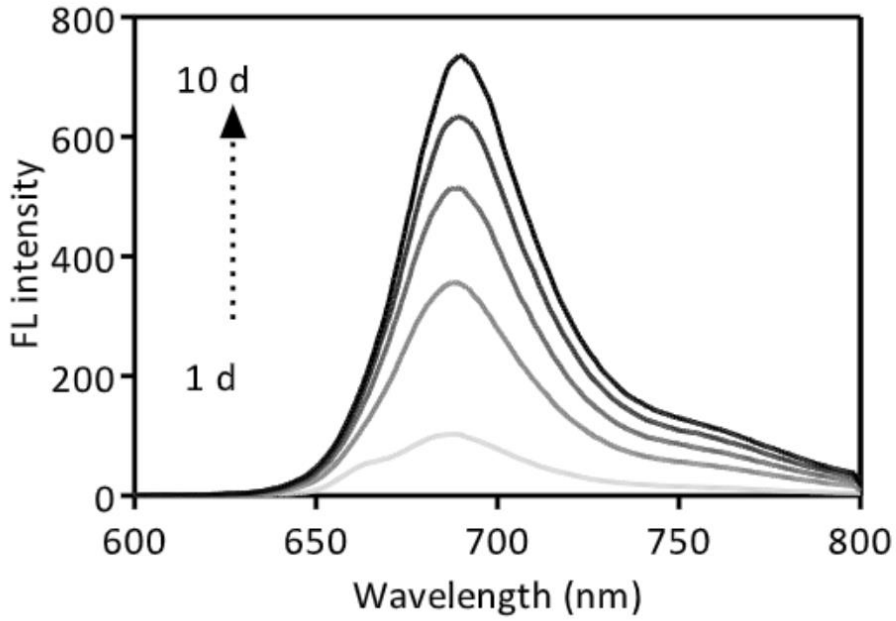


Figure 4. MB fluorescence overlay

Discussion

In this study, MB-EA was incubated with varying concentrations of H_2O_2, a reactive oxygen species, in order to evaluate the sensitivity of MB-EA to ROS. Upon addition of H_2O_2 to MB-EA solution, MB-EA was cleaved and MB was released. This result has clinical and experimental implications. Many disease states, such as cancer, place the body under oxidative stress, and this causes the production of ROS. The cleavage of MB derivatives by ROS can allow for the binding of other drugs or imaging agents to MB to be cleaved by interacting with ROS in diseased tissues. From a research perspective, studies in which researchers are trying to cleave MB from another bound molecule by any method other than ROS must be sure that the environment is devoid of ROS in order to obtain accurate results. If ROS is present, then it will be quite difficult to determine if MB was cleaved by their desired mechanism or if MB was cleaved by ROS. Researchers and clinicians should always be mindful of ROS when dealing with MB derivatives. One limitation of the study is that only one ROS and one MB derivative were used in the experiment. This provided very specific data, and more research would need to be done to expand the data to other types of ROS and MB derivatives. Another limitation is that ROS concentrations in diseased tissues were not gathered in this study, and these would be useful for clinical applications. There are a few directions for future research. First, more research should be conducted to test MB sensitivity to other ROS, such as hydroxy radical, superoxide, and nitric oxide, in order to find specificity. Research should also be done to test the stability of MB derivatives in different biological fluids and at different pH levels. This will provide data that would allow researchers to predict how MB derivatives would act in different areas of the body. Lastly, future research should also focus on possible conjugation of MB

derivatives with drug molecules or imaging probes to determine if this could provide an improvement over other therapies and diagnostics.

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