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Antiviral Research 61 (2004) 141–151
Antiviral Research
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Review

Methylene blue photoinactivation of RNA viruses

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Received 16 September 2003; accepted 11 November 2003

Abstract

We present a review of the current status of the use of methylene blue (MB) photoinactivation of viruses starting with the first early observations up to its current use to inactivate HIV-1 in blood products. Basic mechanism of action studies conducted with model bacteriophages indicate that MB-photomediated viral RNA-protein crosslinkage is a primary lesion and that oxygen, specifically singlet oxygen, is very important also. Basic studies on the mechanism of action with HIV are lacking; however, we do show new data illustrating that viral reverse transcriptase inactivation per se cannot account for MB-mediated photoinactivation. We also show data illustrating that MB photomediates the inactivation of West Nile Virus, a flavivirus, which poses a significant new threat to the continental US. MB photoinactivation of viruses show significant promise because the technology not only offers significant potency but the history of safe MB use in human therapy makes it attractive also.
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Keywords: Methylene blue; Photoinactivation; RNA viruses; West Nile Virus; HIV

1. Introduction

MB in the presence of light potently inactivates RNA viruses. This technology is now being used in some blood centers in Europe to inactivate HIV-1 in blood and blood products. Aside from the effectiveness of this technology in viral inactivation, the fact that MB has been utilized in humans for many years in various treatments makes it additionally attractive. This review article provides a historical perspective on the early basic observations, which led to its eventual practical use. We will also examine the various scientific reports to provide a view of what is now understood about the mechanism of antiviral action, then present observations on its current use in treating blood and blood products and finally, provide data we have collected as background observations on MB-mediated photoinactivation of HIV-1, as well as some recent observations made on MB-mediated photoinactivation of West Nile Virus (WNV). The latter will be presented in the context of the seriousness of this new viral threat.

2. Historical use of MB in humans

MB has been used for various treatments in humans for nearly one century and currently is used for oral administration as an antiseptic, disinfectant, and antidote for nitrate poisoning. MB was first isolated as a new chemical in 1876. It is widely used in treatment of methemoglobinemia (Clifton and Leikin, 2003) and for validation of compliance of medicines (Kraus et al., 1986) and now used for treatment of blood products to photoinactivate HIV as discussed in detail later. MB has been tested in humans for treatment along with lithium for manic-depression psychosis where 300 mg was given per day for over 2 years (Naylor et al., 1986). MB has known biochemical actions one of which is its well-known ability to act catalytically to oxidize methemoglobin. Other known activities include its inhibitory action on guanylate cyclase (Arnold et al., 1977; Gruetter et al., 1981). The long period of safe use of MB in humans makes it much easier therapeutically to develop as an antiviral agent and it is one of the reasons why there is so much interest in it.

3. Very early observations of antiviral action of MB

The initial observations on the ability of MB to inactivate bacteriophages and viruses were recorded in the

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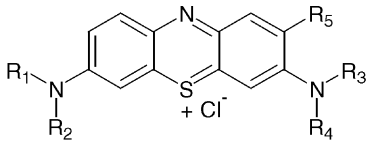
doi:10.1016/j.antiviral.2003.11.004

early 1930s (Perdrau and Todd, 1933). The bacteriophages and viruses utilized were not well characterized (fowl plague, louping-ill, herpes, vaccina, canine distemper, Borna disease, Fujinami Tumor, infectious ectsomelia, foot-and-mouth disease) and quantitative methods of the antiviral activity of MB were imprecise at that time. Nevertheless, it was noted that light as well as oxygen was important in MB-mediated viral inactivation. Additionally, data were presented on the effectiveness of MB and direct sunlight on in vivo inhibition of viral infection to recently inoculated (12–18 h) animals for viruses showing skin or eye lesions (Perdrau and Todd, 1933). A review of the photoinactivation of animal viruses was presented by Wallis and Melnick (1965). This 1965 review revealed very few advances since the 1930s except that more quantitative methods of viral inactivation and characterization were used and that toluidine blue, a chemical analogue of MB, was noted as being effective, however most of the research noted was done with proflavin as a photosensitizer. Observations by Heinmets et al. (1955) on the use of MB to inactivate various viruses in human blood plasma were not reviewed simply because these military-supported studies were not in the public information domain for over 30 years after they were done.

4. MB basic photochemistry

MB is commonly classed as a thiazine dye, but its formal chemical name is 3,7-bis(dimethylamino)phenothiazine-5-ium chloride. Its structure is presented in Fig. 1. Also presented is the formula for the more commonly available thiazine dye analogues. Clearly, many other chemical derivatives are possible and indeed others have been studied for their antiviral activity (see later).



DYE	R_1	R_2	R_3	R_4	R_5
Thionin	H	H	H	H	H
Azure C	H	CH_3	H	H	H
Azure A	H	H	CH_3	CH_3	H
Azure B	CH_3	CH_3	H	CH_3	H
Methylene Blue	CH_3	CH_3	CH_3	CH_3	H
Toluidine Blue	CH_3	CH_3	H	H	CH_3

Scheme illustrating chemical structure of the thiazine dyes

Fig. 1. Chemical structure of Thiazine dyes of which methylene blue is one of the most widely known.

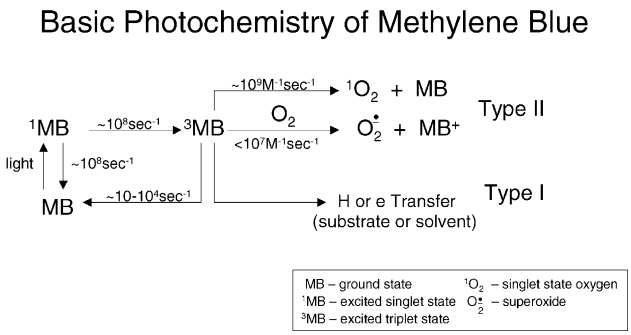


Fig. 2. Scheme illuminating the basic photochemistry of methylene blue (MB). MB is activated to the excited singlet state (^{1}MB) by light. ^{1}MB can decay with essential equal probability back to ground state or the excited triplet state ^{3}MB. ^{3}MB can react in Type II-mediated processes with oxygen to form singlet oxygen ($^1O_2$) and ground state MB or alternatively in a much slower process to form superoxide and MB. Alternatively, ^{3}MB can also react in Type I processes where hydrogen atom or electron transfer processes can occur with solvent or solute.

The basic photochemistry of MB has been studied in chemical systems, i.e. see review by Foote (1976). The principles learned in these systems provide a basis to understand the action of MB in most biological systems. Fig. 2 illustrates the pertinent photochemical principles involved. Light excites ground state MB to an excited singlet state (^{1}MB) which has about equal probability (based on reaction rate) of going back to ground state or to an excited triplet state (^{3}MB). Excited triplet state MB can decay very slowly back to ground state or react by Type I or Type II reactions. Type II reactions occur in the presence of oxygen. The most rapid Type II reaction is with oxygen to produce singlet oxygen and ground state MB. It should be noted that this is a quantum mechanically allowed reaction because oxygen exists as a triplet state molecule in its ground state. Singlet state oxygen is 23 kcal higher energy state than ground state oxygen and is a very potent oxidizing agent. Excited triplet state MB can also react with oxygen to yield superoxide but its formation is at a much slower rate than singlet oxygen. Excited triplet state MB can also react with substrate or solvent to mediate H atom or electron transfer in Type I reactions. Type I reactions can occur in air-saturated aqueous solutions (Foote, 1976). Foote presents data illustrating that Type I and Type II reactions of ^{3}MB are of about equivalent importance in the reactions with guanosine monophosphate in O_2-saturated organic solvent or air-saturated aqueous solutions (Foote, 1976).

5. MB nucleic acid interactions and photochemistry

Much of the basic MB photochemistry has been done in dilute solutions. It is well known that MB binds strongly with DNA and specifically in G-C-rich regions. For this reason, basic photochemical processes of MB have been studied when it is present with DNA as well as G-G- and A-T-rich

oligomers (Kelly et al., 1987). The authors noted that the excited singlet state of MB is strongly quenched by MB binding to the G-C-rich regions but not in the A-T-rich regions. The decreased yield of excited singlet state caused a subsequent decrease in the amount of excited triplet state of MB. It was noted that the lifetime of ^{3}MB was much longer when O_2 was absent. This observation is in keeping with the energy transfer from ^{3}MB to oxygen forming singlet oxygen. There was much less singlet oxygen formed when MB was bound to G-C regions of DNA. This is as expected because the yield of ^{3}MB was decreased. This data calls into question the role of oxygen and singlet oxygen in the damage caused by light activated MB. However, as reviewed below, there is strong data to support the notion that oxygen and singlet oxygen are essential in the antiviral activity of MB. It is clear there are many mechanistic unknowns that account for the antiviral activity of MB. It is quite possible that sequential reactions are involved; first photochemical events independent of oxygen, followed by those involving singlet oxygen. It should be noted that higher levels of singlet oxygen were formed when MB was bound to A-T-rich regions (Kelly et al., 1987).

It has been known for some time that MB, in the presence of light, mediates nucleic acid strands breaks (Cadet et al., 1986; OhUigin et al., 1987; Muller et al., 1990) and that singlet oxygen is an important intermediate preferentially attacking guanine to produce many different oxidation products (Cadet et al., 1983). Singlet oxygen produced by decomposition of a chemical endoperoxide has been shown to cause strand breaks in single stranded DNA (Ribeiro et al., 1992). Singlet oxygen was considered but not definitely proven to be the cause for MB-mediated photoinduced DNA lesions in Salmonella typhimurium (Epe et al., 1989).

6. MB-photoinduced 8-hydroxy guanine formation in nucleic acids

We showed that MB-photoinduced 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation in DNA (Floyd et al., 1989, 1990; Schneider et al., 1990) and then 8-hydroxy-guanosine (8-OHG) in RNA, either in the isolated polymer (Schneider et al., 1993) or within the Qβ bacteriaphage (Schneider et al., 1998, 1999). Formation of 8-OHdG in isolated DNA required the presence of oxygen and the amount formed was enhanced by over three-fold when H_2O was replaced by D_2O. These observations strongly implicate the involvement of singlet oxygen. First, by the absolute requirement for oxygen, and second, because the lifetime of singlet oxygen is significantly prolonged by D_2O compared to H_2O. It should be noted however that 8-OHdG can be formed in DNA by the action of light upon riboflavin (Kasai et al., 1992). Cadet's group has also shown that MB photooxidizes 8-OHdG to form other products (Buchko et al., 1995). This may limit the yield of 8-OHdG in DNA; yet we have shown that as much as 4% of the total guanines are converted to 8-OHdG by MB in the presence of light (Schneider et al., 1990). We compared several thiazine dyes as to their ability to photomediate 8-OHdG formation in isolated DNA and showed the following order of effectiveness: MB > Azure B > Azure A > toluidine blue > thionin (Floyd et al., 1990).

Proximity of target to the singlet oxygen generator is important in antiviral effectiveness and action. MB has a positive charge, which among other properties, increases its affinity to bind to negatively charged RNA, in either isolated polymer (Schneider et al., 1993) or within the bacteriophage (Schneider et al., 1993; Jockusch et al., 1996). Rose Bengal (RB) is a well-known very potent photoinduced generator of singlet oxygen. We showed, when RB and MB are compared on a mole to mole basis with regards to their ability to generate 8-OHG in isolated RNA as well as with regards to bacteriophage photoinactivation, MB was at least 10^4 more effective than RB (Schneider et al., 1993). We also showed that MB-photomediated 8-OHG formation was insensitive to temperature changes in the 13–37° C range. This result was in contrast to hydroxyl free radical-mediated 8-OHG formation, which was very sensitive to temperature (Schneider et al., 1993). In the same study we showed in R17 bacteriophage that MB-mediated photoinactivation was also insensitive to temperature changes in the 13–37 °C range as well as to metal chelators. This result is again in keeping with singlet oxygen-mediated processes. The absolute requirement for oxygen in MB-photomediated Qβ phase inactivation is demonstrated by the data in Table 1. This is a summary of an extensive series of studies we conducted on the mechanism of MB photoinactivation of Qβ (Schneider et al., 1998). The data show that under illumination in the presence of 1 μM MB and under a nitrogen environment a loss of plaque forming units (Pfu) dropped from $\sim 8 \times 10^{10}$ to $\sim 3 \times 10^9$ in 17 min of treatment. In comparison in the presence of oxygen, active virions dropped from $\sim 5 \times 10^{10}$ to $\sim 5 \times 10^2$ in 14 min. The ratio of photoinactivation in the presence of oxygen versus nitrogen was approximately 4×10^6 more effective. Lee et al. (1997) also showed the absolute requirement of oxygen in MB-photomediated inactivation of Qβ and demonstrated a first order dependence to oxygen concentration in the lowest concentration range.

Table 1
Effect of oxygen on methylene blue photoinactivation of Qβ phage

Atmosphere gas	Active virions[a]		
	Initial	Final	Fold-change
Nitrogen	8×10^{10}	3×10^9	2.7×10^1
Oxygen	5×10^{10}	5×10^2	1×10^8

[a] Data summarized from Schneider et al. (1998). The number of active virions are presented as plaque forming units per milliliter and are approximated from referenced Fig. 1. The small change experienced under nitrogen is most likely due to the difficulty of removing all the oxygen and preventing some from getting into the reaction mixture from the tubing and while removing the ten 22-μl samples to obtain time-course assays of the number of active virions present.

7. MB-photomediated viral RNA-protein crosslinkage

We have spent considerable research effort conducting experiments to understand the mechanisms involved in MB-photomediated inactivation of RNA viruses. Our most extensive effort has focused on the model viral systems R17 and Qβ (Schneider et al., 1998). At one time we had considered MB photomediation of 8-OHG formation in RNA was perhaps the key event involved. However, experiments performed on Qβ have convinced us that 8-OHG formation only accompanies the critical processes. Our results clearly indicate that photomediation of viral RNA-protein crosslinkage is probably the most important event in viral inactivation. Key results are summarized in Fig. 3. These results show that irradiation of Qβ phage in the presence of increasing concentrations of MB resulted in exponentially increasing amounts of viral RNA-protein crosslinkage products. We discovered that RNA recoverable by phenol extraction decreased as MB concentration increased and that the RNA lost from this fraction appeared at the water/phenol interface bound to viral proteins. The RNA-protein fraction was treated with proteinase K to digest the protein and this yielded the RNA that had been lost from the phenol fraction. The aqueous (phenol extractable) RNA along with that present in the RNA-protein crosslinkage, accounted for nearly all the total viral RNA as Fig. 3 shows. At this time the chemical nature of the crosslink and its target nucleotide remain to be determined. Fig. 3 also shows the result obtained on separate but similar experiments where the 8-OHG content of viral RNA was determined in relation to lethal hits calculated using the poisson distribution (Schneider et al., 1998). Clearly there is about one 8-OHG per seven or eight lethal hits throughout the MB concentrations tested. It should be noted that even at 0.5 μM under the 30 min light exposure used, each virion had already achieved two lethal hits. Therefore, the first lethal hit under our exposure conditions occurred at less than 0.5 μM MB, i.e. perhaps at 0.2 μM or less. This data clearly implicates that 8-OHG formation or oxidative damage of the RNA alone does not directly account for the lethal event of the virus. On the other hand, it does clearly implicate that viral RNA-protein crosslinks are of primary importance. We did a series of experiments directed toward a more rigorous evaluation of the importance of MB-photomediated RNA-protein crosslinkage as the lesion responsible for Qβ inactivation (Schneider et al., 1999). In these experiments, we took advantage of evaluating the lethal lesion using infectious RNA assays in a comparative basis with the more traditional phage inactivation assays where Qβ is plated on a F+ bacteria host (*Escherichia coli* Q 13). The infectious RNA assays utilized the extracted RNA from Qβ phage, which was then plated on spheroplasts from *E. coli* Q 13. Utilizing this approach we were able to more definitively evaluate the specific lesion responsible for MB-mediated Qβ photoinactivation. These series of experiments established several important facts; (A) The MB-photomediated lethal lesion does reside in the RNA genome of Qβ. This was directly demonstrated by the fact that Qβ phage inactivation (assessed by phage plaque assay)

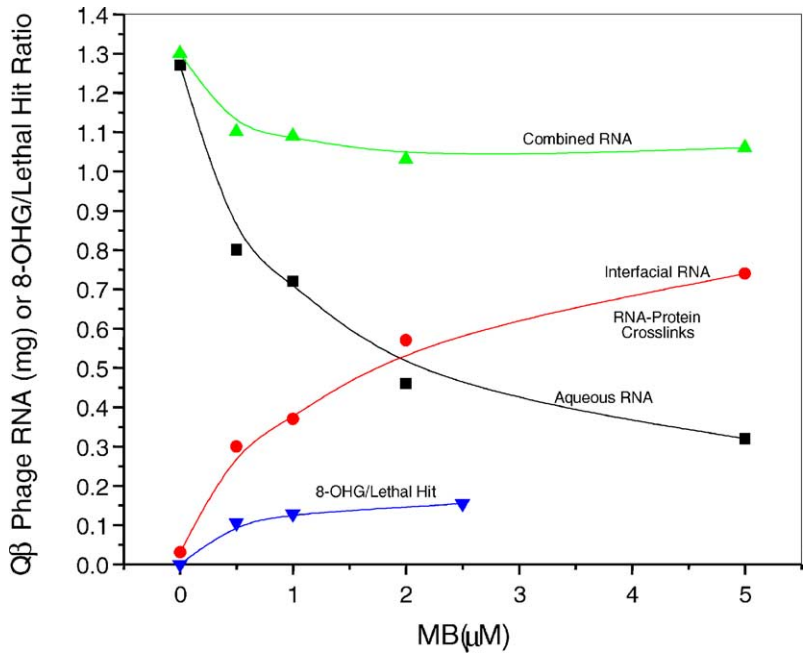


Fig. 3. Data illustrating that Qβ when illuminated with while light in presence of oxygen causes the mediation of viral RNA-protein crosslinks. Aqueous RNA represents that extractable with phenol. Combined RNA is the sum of that present in the aqueous fraction plus that in the interfacial fraction, i.e. RNA-protein crosslinks. Also presented is the amount of 8-OHG present as a ratio to lethal hits. The data presented are summarized from that presented in our published study (Schneider et al., 1998).

and lesioned total RNA extracted from treated phage correlated with the activity assessed by infections RNA assay. (B) The lethal lesion in the phage RNA was directly correlated with the amount of RNA-protein crosslinkage formed and even resided in the infectious RNA after proteinase K treatment. This data implicate that the specific residual amino acid—RNA lesions caused loss of activity. (C) Directly treating extracted phage RNA with MB and light did cause a loss of activity in the infectious RNA assay but there was a much greater loss of activity if the phage RNA was treated with MB and light in the phage per se. These results then demonstrated that Qβ RNA infectious activity is significantly ($\sim 10^3$-fold) more sensitive to MB photoinactivation in its protein-associated virion state as compared with its purified isolated polymer state (Schneider et al., 1999).

A rigorous study of the effect of MB-photoinduced RNA-protein crosslink formation was conducted by Liu et al. (1996) using isolated RNA and isolated specific proteins. A comparison was made between MB and the more traditional method of UV light-mediated RNA-protein crosslinks. In this study, two proteins, human protein kinase R and Drosophila staufen protein, both known to bind to double stranded RNA (dsRNA), were utilized. Various RNA constructs were also utilized, including some with double strand regions (dsRNA) as well as those having only single strand regions (ssRNA). The research demonstrated that; (A) MB was much more effective in photoinduced dsRNA-protein crosslink formation than UV, (B) MB was less effective in mediating RNA-protein crosslinkage when ssRNA was used when compared to dsRNA, (C) that UV was more effective in mediating ssRNA-protein crosslinks, and (D) that MB did not mediate RNA-RNA crosslinks. The effectiveness of MB when dsRNA was utilized was attributed to its well-known preference to bind to double stranded regions of RNA. Utilizing the information this study provides, it is likely then that MB-photomediated RNA crosslinkage with viral proteins probably occurs with those regions of Qβ RNA which contain double stranded regions. At present, it is not known which Qβ protein is crosslinked. It should also be noted that the mechanism of action of MB photoinactivation of HIV or WNV has not been studied in detail enough to indicate that viral RNA-protein crosslinkage is the lethal lesion.

8. Light spectral studies in MB-photomediated activity

There is a paucity of very high-quality quantum efficiency studies on specific MB-photomediated processes of biological importance. The absorption spectrum of MB in water shows a major peak in the red region with a sharp peak at 664–666 nm and one shoulder at 610–640 nm. An absorbency of MB at 665 nm of $\varepsilon = 81{,}600\ \mathrm{cm}^{-1}\ \mathrm{M}^{-1}$ is used (Liu et al., 1996). There are also less intense peaks in the UV to near UV region and especially at 284–300 nm region. The intense absorptions in the red region provides a major benefit for the use of MB in biological systems because light in this region is minimally absorbed by biological molecules hence allowing it to penetrate deeper. Also, light in the red region does not experience as much Raleigh scattering as does light in the UV-near UV region again allowing the light to penetrate better. When MB binds to nucleic acids in the virions it experiences slight spectral shifts (Jockusch et al., 1996). Those studies of the assessment of the type of MB binding to virus were evaluated by fluorescence derived from 532 nm laser pulses to excite MB (Jockusch et al., 1996). Specific studies on the spectroscopy of MB-nucleic acid interactions with Poly GC and Poly AT regions have used 308 nm excitation laser pulses (Kelly et al., 1987). Most studies, including our own, where model virus systems were studied utilized white light. Theoretically it is expected that the most effective wavelength for MB-photomediated processes should be at 665 nm but that has not been rigorously proven.

9. Use of MB to photoinactivate HIV in blood products

The MB photoinactivation technology is now being used to inactivate HIV in blood products in some European blood centers. This has been brought about largely due to early work of Mohr's group demonstrating the potential of phenothiazine dyes to photoinactivate several model viruses as well as HIV-1 in human fresh frozen plasma (Lambrecht et al., 1991). Within 1 year after this report for example, over 40,000 units of treated plasma were used in lower Saxony (FRG) between February and October of 1992 (Mohr et al., 1993). Follow up research and further developments since then have been summarized in several brief reports by Mohr's group (Mohr, 1998; Corash, 1998; Mohr et al., 1995, 1997). Wagner has recently reviewed this field in a broader more general context where the potential of other phenothiazine dyes including dimethylmethylene blue was also discussed (Wagner, 2002).

The potential for the use of MB photoinactivation technology in blood products faces challenges. Fig. 4 presents the results of some studies we have done using MB to inactivate HIV in blood products. The results illustrate that HIV is readily photoinactivated by 1 μM MB when the virus is present in blood plasma and platelet-rich plasma but it becomes more difficult in whole blood per se. Several hurdles must be overcome. These include; light penetration problems in the dense fluid and possible photoinduced damaging effect on red cells per se as well as blood proteins. Wagner has conducted the most extensive research on the challenges involved in the potential use of MB in whole blood (Wagner et al., 1993, 1994, 2002).

Our studies presented in Fig. 4 were conducted with white light utilized to excite MB. As noted earlier MB absorbs light maximally at 660 nm. Since hemoglobin and other plasma proteins do not absorb at this wavelength,

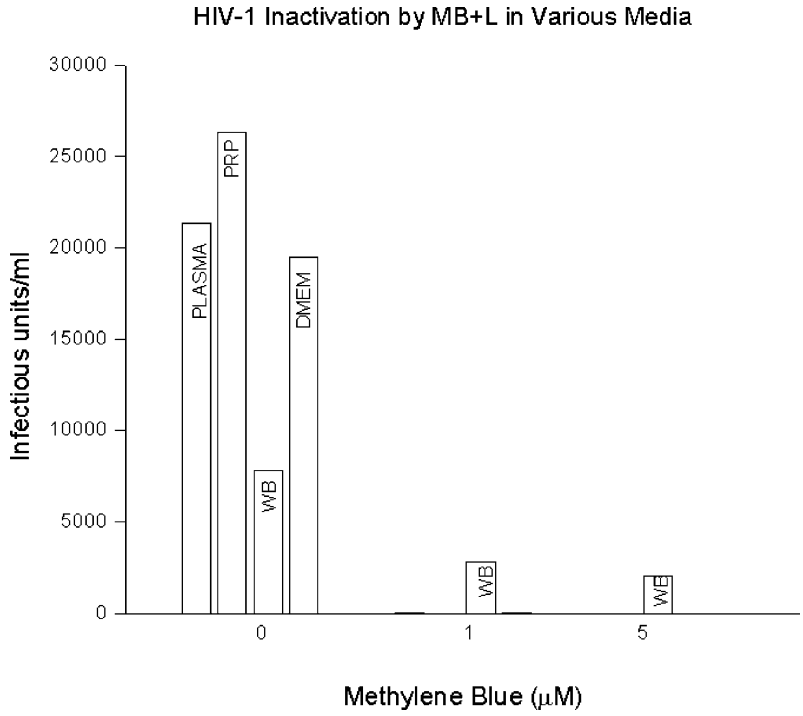


Fig. 4. Effect of MB on HIV inactivation in whole blood (WB), platelet-rich plasma (PRP), blood plasma, and DMEM media. Approximately $\sim 3 \times 10^5$ active virions of HIV/ml was added to each of these media and after incubation for about 15 min with mild agitation an aliquot was removed to assay for active virions. To other fractions of the inoculated media MB was added to a final concentration of 1 or 5 μM. The MB treated inoculated media were incubated in the dark for 15 min, then added to a depth of 0.5 cm in 4 cm diameter petri plates and exposed to light from a 100-W soft white light (Sylvania Tungsram 7) at room temperature for 30 min. In order to remove the infrared (heat generating) rays the light was passed through a 0.5 cm water filter. The 30 min light exposure corresponds to 2.9×10^4 J/m^2 (1025 fc in the 400–700 nm range as measured by an Extech model 401025 fc/lx m; foot candle conversion is 0.001496 J/s/ft^2). HIV-1 infectious particles were measured by injecting HeLa-CD4-LTR-β-gal cells, which contain the gene that encodes the CD4 receptor and a single copy of truncated HIV LTR region containing the tat binding region joined to the lac z gene (encoding β-galactosidase). A nuclear transport signal sequence in the β-galactosidase protein transports this enzyme to the nucleus after it has been synthesized following infection of the cell by HIV. HIV titer determinations were made by addition of dilutions of the virus suspensions to HeLa-CD4-LTR-β-gal cells growing as monolayers in 96- or 24-well plates. The culture medium was removed from the monolayer cells immediately prior to addition of 20–40 μl of the diluted virus suspension; the monolayer-virus combination was incubated for 2 h at 37 °C in 5% CO_2 followed by addition of fresh medium and further incubation for 40 h. The monolayer cultures were fixed and histochemically stained for the presence of blue-colored nuclei resulting from the activity of β-galactosidase on X-Gal. Each cell, cell doublet (a recently divided cell) or syncytium containing blue-colored nuclei was counted as one infectious center which, in turn, represented one infectious unit in the original suspension. Cell free stocks of HIV-1 were produced by harvesting the supernatants of productively infected H9 cells in liquid media. HIV-1 obtained as HTLV-IIIb/H9 (cat. no. 398) was from Robert Gallo; HeLa-CD4-LTR-β-gal cell line (cat. no. 1470) was from Michael Emerman; H9 cell line (cat. no. 87) from Robert Gallo; and HeLa cell line (cat. no. 153) from Richard Axel. Regents were obtained from the AID's Research and Reference Reagent Program Division of AID's, NIAID, NIH.

then light at this wavelength would be expected to penetrate well and also be more effective than white light in MB-mediated photo processes. Mohr et al. (1997) conducted a study on vesicular stomatitis virus (VSV) spiked fresh frozen plasma where white light, green-yellow light, and red light were compared on MB-mediated viral inactivation as well as damage to plasma proteins. White light was provided by a bank of fluorescent tubes yielding incidence light at 42 W/cm^2. Green-yellow light was provided by low-pressure sodium lamps providing high-intensity light at 590 nm with incidence intensity of 165 W/cm^2. Red light at 660 nm with a band width of 22 nm was provided by light-emitting diodes delivering incidence intensity of 272 W/cm^2. Thus the white light was much less intense than the red or green-yellow light. VSV was rapidly inactivated by the red or green-yellow light but slower by the white light. Plasma protein damage was much less when red or green-yellow light was used even though they were much more intense than the white light.

Wagner et al. (1993) utilized red fluorescent bulbs (General Electric F20T12-R) having maximum emission at 650 nm to irradiate, at 9 W/m^2 incidence, human red cell suspensions. Vesicular stomatitis virus (VSV) and 5 μM MB were added to the red cells to assess not only the antiviral potential but also alterations in parameters important in cell storage. Red light given at a total dose of 1.8×10^4 and 3.2×10^4 J/m^2 inactivated VSV by 6 and $\geq 7 \log_{10}$, respectively, and it was completely dependent of the presence of MB and light irradiation. Light in the absence of MB was ineffective. MB and light irradiation at a total dose of 3.2×10^4 J/m^2 in 30% red cell suspensions caused an increase in hemolysis and increased loss of electrolytes

and a decrease in ATP content and some changes in 2,3-diphosphoglycerate. These parameters were changed about 15–25% in comparison to controls after 42 days of cell storage at 4 °C. In a follow-up study, Wagner et al. (1994) examined MB-mediated photoinactivation of other viruses in 30% red cell suspensions as well as potassium leakage. They noted HIV was inactivated by over 3 $\log_{10}$ with 1.4×10^3 J/m^2 red light in the presence of 5 μM MB. Sindbis virus was very effectively photoinactivated but encephalomyocarditis virus (a nonenveloped picornavirus) was not affected at all. They also showed that the MB phototreatment caused increased potassium leakage from cells under storage. In this study, as well as the previous one the red cell suspension sample thickness was 1.9 mm. Banks of fluorescent bulbs were positioned above and below the sample.

In subsequent studies, Wagner et al. (2002) and Skripchenko and Wagner (2000) utilized a flow cell system where 670 ± 13 nm peak intensity illumination was provided by two adjustable intensity red light-emitting diodes. The sample thickness was 1 mm and the flow system was designed such that the red cell suspension was pumped at 0.317 ml/s with a mean residence time within the flow cell of 13.1 s. The 30% red cell suspension was spiked with VSV and 6 μM dimethylmethylene blue. Log of VSV inactivation was linearly dependent on the intensity of red light illumination. ATP decrease and potassium loss from red cells after 42 days of storage were enhanced by the phototreatment. This study highlights production-oriented approaches to the application of the MB photoinactivation technology.

10. MB photoinactivation of HIV—more development needed

The studies on effectiveness of various light wavelength and sample thickness highlights the recognized need for further development in this area. To this end, we have conducted new experiments which provide areas for consideration. First is the question of MB; is this the most effective thiazine dye or would a close relative be more effective? There have been few if any head to head comparison studies of MB with other dyes. Fig. 5 shows data we have collected where toluidine blue O (TBO) is compared on a head to head basis with MB on HIV inactivation. TBO is significantly more effective as the results show. This may be due to several factors, including better penetration of TBO into the enveloped virus. Perusal of the chemical structures in Fig. 1 shows that TBO has one less CH_3 group, however its positioning is not as a quaternary ammonium linkage as do the other four CH_3 groups on MB. Dimethylmethylene blue (DMMB) has two extra CH_3 group when compared to MB and this does make it more available for intracellular photo initiated processes as noted by Skripchenko and Wagner (2000). They saw extensive damage to T-cell DNA when DMMB was compared to MB, which caused very little if any damage.

Another question we have considered for possibly improving the MB photoinactivation technology is the delivery of the light in short intense pulses. The rationale is that the brief pulses of light will allow specific dark processes to occur between the pulses that are critical to virus inactivation yet minimizing the processes that are involved in

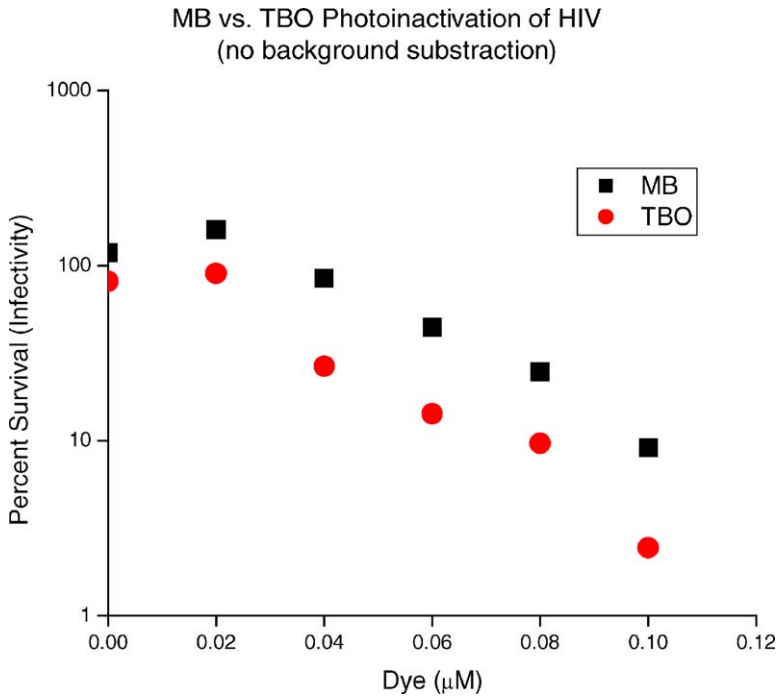


Fig. 5. HIV at about 3×10^5 virions/ml were dispersed into well of 96-well plates and exposed to light as described in Fig. 4 for 30 min in the presence of toluidine blue O (TBO) or MB at the concentration indicated. HIV inactivation was determined as noted in Fig. 4.

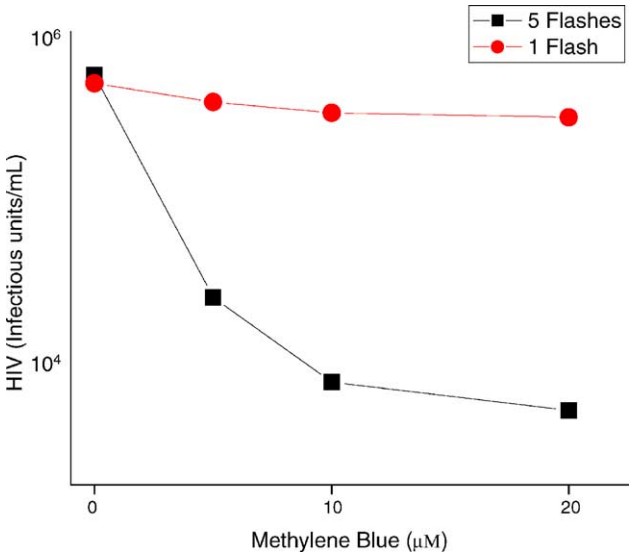


Fig. 6. Photodynamic inactivation of HIV as function of number of strobe light flashes. Light consisted of exposure of the plate to two (one Morris and one JTL Studio Systems) AC slave light flashes, one above and one below the 96-well culture plate. The flash units were offset diagonally with respect to the other's position so as to provide complementary exposure. The light path distance was measured from the surface of the AC slave unit to the middle of the sample. Additionally, the bulb of each unit is inset approximately 0.5 cm from the surface. Since the surface of each unit is of reflective material the samples are exposed to reflected light in addition to the light coming directly from the bulb. Flashes were initiated by pressing the test button on one of the units, which caused it as well as the opposite unit to flash simultaneously. Samples (20 μl) were removed from the wells following the first flash, then the plate was repositioned for an additional four flashes. The plate was exposed to the flashes with the cover in place, but there was no additional filter (i.e. water layer, as was used in 30-min exposures). Light path = 0.5 cm.

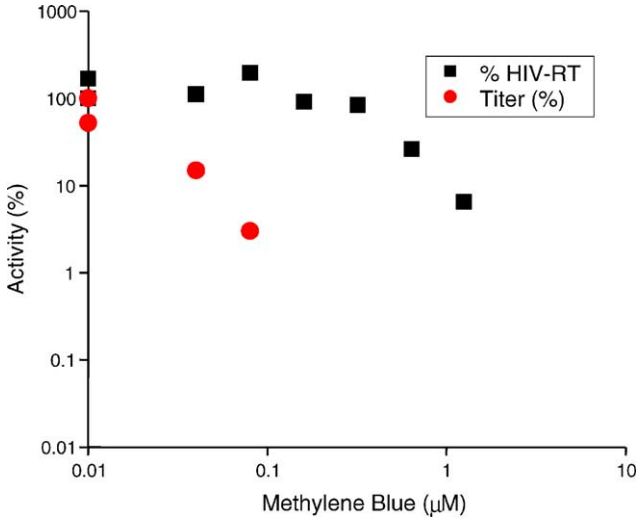


Fig. 7. HIV infectivity and reverse transcriptase activity as influenced by MB + L at different MB concentrations. The log of activity is plotted verses log of MB concentration. The reverse transcriptase activity was measured utilizing an ELISA kit (Boehringer Manheim Ca # 1468120) to ascertain if this enzyme was a target of MB photoinactivation. The infectivity of HIV was measured by the method described in Fig. 4 legend. The light exposure to $\sim 3 \times 10^5$ virions in DMEM media was conducted similar to that described in Fig. 2.

ancillary damage. Additionally the dark period would allow re-accumulation of oxygen at specific sites where it may have been depleted in crucial light-mediated reactions. Results of exploratory experiments are presented in Fig. 6. The results demonstrate that five strobe light flashes of ~250 ms each caused a 2 log loss in HIV activity. Thus in approximately 1.25 s total light time HIV was inactivated by 2 log. Clearly if pulsating light is to be used in practice, many more that five flashes will be needed to reduce the active viral count to much lower levels before it could be considered of practical value. Nevertheless these striking results illustrate the possible potential benefit of this approach. In future studies, it would be important to define total light energy delivered, specificity of wavelength, and the amount of ancillary damage to cells and plasma proteins that this approach would impart versus continuous illumination.

11. MB photoinactivation of HIV—mechanism of action

There have been very few studies published on the mechanism of MB photoinactivation of HIV. As noted earlier studies with model viruses indicate: (A) MB-photomediated viral RNA-protein cross linkage as a crucial lethal lesion and (B) the prime importance of oxygen, specifically singlet oxygen, as a key intermediate. We have done some basic research in this area with HIV-1. As illustrated in Fig. 7, MB-photomediated HIV-1 inactivation significantly precedes the inactivation of RNA reverse transcriptase (RT) activity within the treated virus. In prior research with isolated HIV-1 RT, we showed that it was relatively insensitive to MB-photomediated inactivation (Floyd and Schinazi, 2002). The results in Fig. 7 demonstrate that virion associated RT is also quite insensitive to MB photoinactivation. These results indicate that if MB photomediates RNA-protein crosslinkage in HIV-1, as was shown to be the case for Qβ, it is unlikely that a significant fraction of RT molecules become covalently bound to the viral RNA; or if so, the complex continues to function in vivo. More basic studies are needed to define the lethal lesions found in HIV by MB phototreatment.

12. West Nile virus

In the last 4 years, West Nile Virus (WNV) has become a significant threat to humans, livestock, and wild animals, especially birds in the United States. In this section, we present a background detailing the important threat the virus imposes and present results of our initial studies demonstrating that MB will photoinactivate WNV. It should be noted that WNV is an enveloped RNA virus belonging to the flavivirus family that contains at least 70 known viruses

including Hepatitis C virus, Japanese Encephalitis virus, Dengue virus and Yellow Fever virus. These flaviviruses raise concern because of their effects in humans and their increasing incidence. For instance, the World Health Organization reports about 0.5 million new cases annually of Dengue hemorrhagic fever (DHF), caused by Dengue virus, and estimates that about 50 million people are infected annually by Dengue virus (Clarke, 2002).

WNV was first reported in a Uganda woman in 1937 (Brinton, 2002). WNV has only recently invaded the Western Hemisphere but it is endemic in parts of Africa, the Middle East and Western Asia, particularly India (Brinton, 2002). WNV has shown outbreaks in Europe notably France, Romania and Russia (Brinton, 2002). Seroprevalence data indicate WNV activity in southern Romania as early as the 1960s. In the same region in 1996, a serious epidemic of WNV encephalitis/meningitis hospitalized 393 resulting in 17 deaths (Campbell et al., 2001). Neurological alterations resulting from WNV infections can appear as the most serious complications in the reported many infection cases where otherwise mild symptoms usually occur (Sampson and Armbrustmacher, 2001).

WNV is carried in an unusual wide array of mosquito species, primarily Culex and is passed on to wild birds, many of which are very susceptible; develop high-level viremia and die. They act as vectors to pass the virus to other mosquitoes, so-called bridge vectors, which feed on birds as well as people and domesticated animals (Campbell et al., 2003; Dohm et al., 2002; Prowse, 2003). Adult mosquitoes can pass the virus on to their progeny (Dohm et al., 2002). In roughly 4 years after the appearance of WNV in New York City in 1999, it has now been reported in most regions of the country except for the extreme West Coast and these states are expected to report it this year. From 1999 when it was first noted until November 2002, 3475 symptomatic infected people were reported of whom 201 died (Prowse, 2003). Older people seem to suffer more neurological complications than younger ones once they are infected (Prowse, 2003). It has been demonstrated that WNV is also passed from human to human through transfusion of infected blood (Prowse, 2003) and as the prevalence of WNV in the wild increase, so can we expect an increase in WNV positive blood donors. At this point, it is unclear whether the WNV form present in the United States is the more seriously debilitating form and probably arose from the eastern Mediterranean area (Lanciotti et al., 2002). Also, much is yet to be learned about its mode of infection and propagation in the human host (Diamond, 2003).

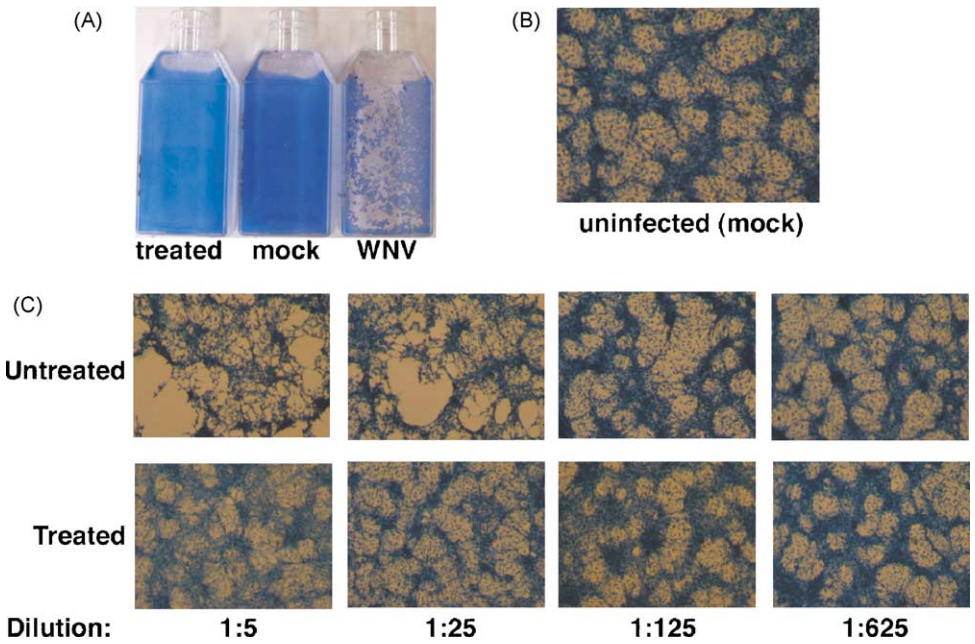


Fig. 8. Demonstration that MB photoinactives WNV. MB (20 μM) was incubated with viral stock solution of WNV for 20 min in the dark then exposed to the white light provided by a 40-W laboratory hood fluorescent bulb at a distance of 10 cm for 10 min. A comparable viral stock was also exposed to the light, however, it contained no MB. After light exposure the two viral stock solutions were placed onto Vero cells, grown to confluence, and then incubated at 37 °C for 5 days and then results evaluated. WNV exerts a cytopathic effect (CPE) on the Vero cells thus killing them. Inactivation of WNV by MB protects the cells from a CPE. (A) Three flasks of Giemsa-stained Vero cells. The right flask shows that Vero cells exposed to untreated WNV resulted in a large cytopathic effect causing the appearance of large areas of flask containing no cells. The left flask shows cells treated with WNV which had been exposed to 20 μM MB and then illuminated with white light as described above. Clearly, there was significantly less (if any) CPE on these cells. The center flask shows cells, which were not exposed to WNV. (B) A higher magnification of the untreated Giemsa-stained Vero cells. Individual cells are clearly visible. The images in panel (C) illustrate the results obtained where the WNV was treated with light only (top panel) or with WNV treated with MB plus light (bottom panel) and then the viral stock diluted by 1:5, 1:25, 1:125, and 1:625, respectively. Cytopathic effects on the cells are clearly apparent at 1:125 and possibly higher viral dilutions.

13. WNV—photoinactivation by MB

We have shown in preliminary experiments that MB photoinactivates WNV (Fig. 8). The WNV utilized (strain OKC02) has been plaque purified, thoroughly characterized and was isolated from a dead local bird. A viral stock solution ($\sim 10^8$ PFU/ml) was incubated with 20 μM MB for 20 min in the dark then exposed to at 40-W fluorescent bulb at a distance of 10 cm for 10 min. A comparable viral stock solution was exposed to light in a similar fashion but no MB was present. Perusal of the results in Fig. 8 (extensive details given in the legend) clearly shows that MB + L potently inactivates WNV. We estimate conservatively that the MB technology reduced the infectivity of WNV by about 3 logs. More extensive studies are now underway.

14. Future directions

In recent years, economic globalization leading to enhanced human mobility has brought about increased world wide exposure and enhanced distribution of previously localized strains of viruses that have evolved under unique conditions. Additionally the threat of the use of biological agents or possibly new strains developed specifically for the purpose to inflict pain and suffering on targeted populations has grown also. Concomitantly, there is also an increasing need for blood products for elective medical procedures and emergencies. The safety of blood products has been and will continue to become an evermore-pressing issue in light of the increasing threat being imposed. The MB photoinactivation technology offers significant potential for protection from several viruses in most blood products. The potency of the technology plus the past history of safe use of MB for various human therapy makes it unusually attractive. There is, however, a major need for not only more basic studies in this area but for active development of improved production-type methodology to enhance the antiviral action while minimizing the ancillary damage to the blood products per se.

Acknowledgements

We thank Mr. Quentin Pye for help with experiments concerning HIV and Mr. James Papain with those involving West Nile Virus. Funding support is acknowledged from NIH EB00983 (Dirk Dittmer) and NIH HL53585 (Robert A. Floyd).

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