

## Methylene blue photoinactivation abolishes West Nile virus infectivity in vivo

James F. Papin<sup>a,1</sup>, Robert A. Floyd<sup>b</sup>, Dirk P. Dittmer<sup>c,\*</sup>

<sup>a</sup> Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, USA

<sup>b</sup> The Oklahoma Medical Research Foundation, USA

<sup>c</sup> Department of Microbiology and Immunology and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, CB# 7290, 804 Mary Ellen Jones Bldg, Chapel Hill, NC 27599-7290, USA

Received 1 March 2005; accepted 12 July 2005

### Abstract

The prevalence of West Nile virus (WNV) infections and associated morbidity has accelerated in recent years. Of particular concern is the recent demonstration that this virus can be transmitted by blood products and can cause severe illness and mortality in transfusion recipients. We have evaluated methylene blue (MB) + light as a safe and cost-effective means to inactivate WNV in vitro. This regimen inactivated WNV with an  $IC_{50}$  of 0.10  $\mu$ M. Up to  $10^7$  pfu/ml of WNV could be inactivated by MB + light with no residual infectivity. MB + light inactivated three primary WNV isolates from the years 1999, 2002 and 2003 and prevented mortality in a murine model for WNV infection. Since MB is already approved for human use at a dose of 100 mg/kg/day, we conjecture that MB + light treatment of blood products for high-risk patients will be efficacious and suitable for use in resource-limited settings.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** Methylene blue; West Nile virus

WNV belongs to the Flaviviridae, a family of over 70 related viruses (Murphy et al., 1995). WNV is an enveloped single-stranded positive sense RNA virus with a genome of approximately 11 kb encoding for three structural genes and seven non-structural genes. WNV is maintained in nature through an enzootic cycle involving mosquitoes and birds. Recent reports show that WNV can also be passed from human to human by blood transfusion (Iwamoto et al., 2003; Pealer et al., 2003). With the recent epidemic increase in WNV prevalence in the U.S. (CDC, 1999, 2002; Enserink, 2002; Lanciotti et al., 1999), we can expect a rise in WNV positive blood donors and a rising need for methods to inactivate WNV in blood products.

The drug Methylene Blue (MB) has seen limited use to inactivate HIV in blood products (Lambrecht et al., 1991;

Mohr et al., 2004). Here, we report the sensitivity of WNV to MB. MB (Sigma Inc. St. Louis, MO, USA) at 20  $\mu$ M was mixed with WNV virus (strain OK02) in 1 ml volume and after incubation in the dark for 20 m, the mixture was photoinactivated for 10 m with fluorescent white light (10 cm distance from a 40 W fluorescent white culture hood light at room temperature). Starting with a concentration of 20  $\mu$ M MB, 10-fold serial dilutions down to a concentration of 0.002  $\mu$ M were used to inactivate  $10^7$  pfu of WNV yielding an estimated  $IC_{50}$  of 0.10  $\mu$ M (Fig. 1A).

The inactivation of viral RNA by MB occurs in a light-dependent manner (reviewed in Floyd et al., 2004). Therefore, we compared 20  $\mu$ M MB-treated WNV with and without exposure to light. Fig. 1B shows two dilutions of OK02 WNV that were either subjected to light exposure as before or incubated in the dark and plated on Vero cells. These doses represent the upper limit of WNV concentrations found in human plasma to date (Pealer et al., 2003). Upon light exposure MB completely inactivated both dilutions of virus. Without light exposure, MB was unable to diminish WNV infectivity

\* Corresponding author. Tel.: +1 919 966 7960; fax: +1 919 962 8103.

E-mail address: [ddittmer@med.unc.edu](mailto:ddittmer@med.unc.edu) (D.P. Dittmer).

<sup>1</sup> Present address: International Laboratory of Molecular Biology for Tropical Disease agents, University of California at Davis, USA.

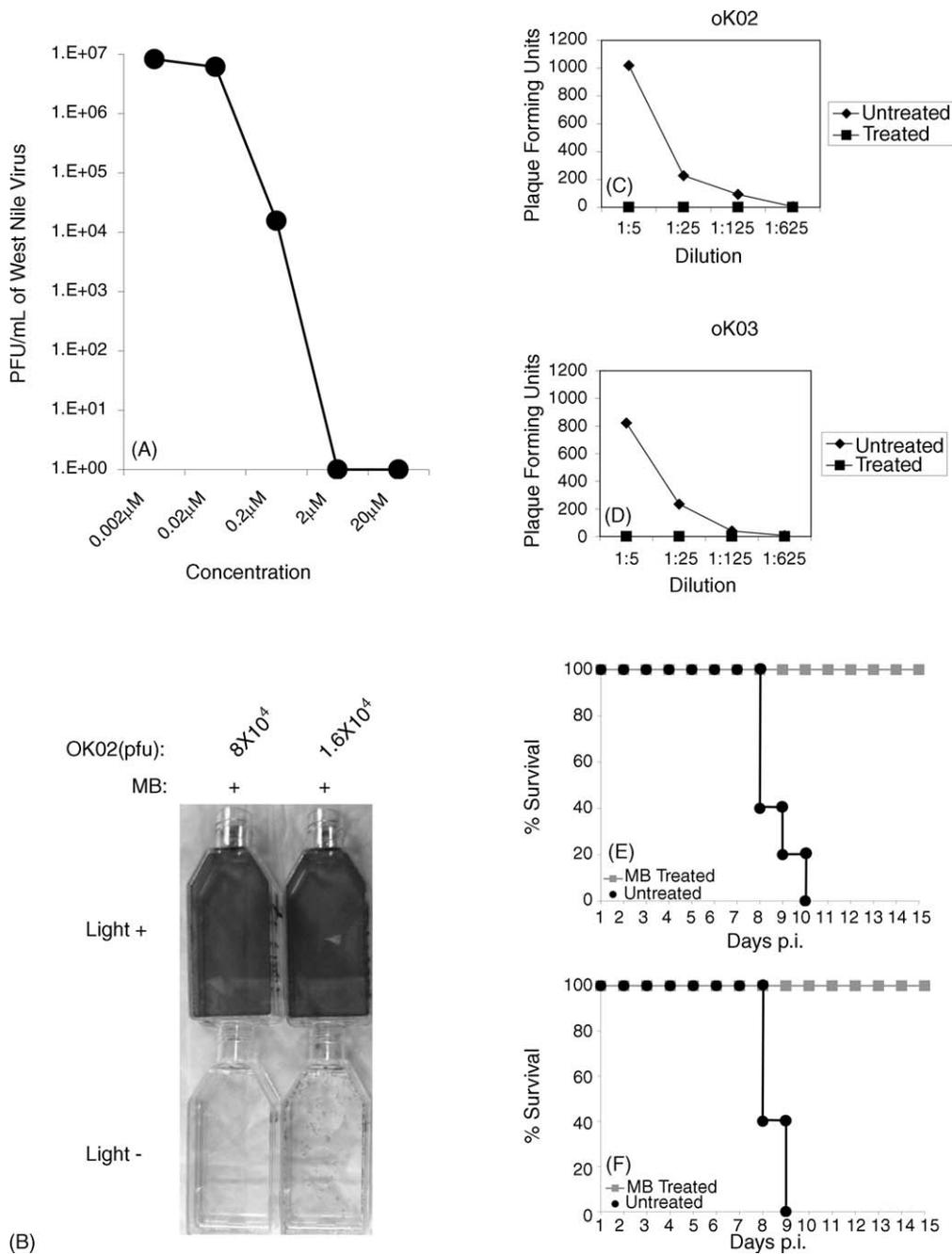


Fig. 1. (A) Titration of MB + light activity against West Nile Virus. Plaque-forming units ( $10^7$  pfu) of West Nile virus were treated with the indicated concentration of MB. Shown is the yield of infectious West Nile virus (y-axis) plotted against the concentration of MB used for treatment of the virus (x-axis). The limit of detection of the plaque-assay for WNV is 1 pfu/ml. (B) Picture of Vero cell monolayers in T25 flasks stained with Geimsa after infection with two different amounts of West Nile virus ( $8 \times 10^4$  and  $1.6 \times 10^4$  pfu) that had been mixed with 20  $\mu$ M MB and exposed (upper row) or not exposed (lower row) to white light for 10 min. OK02 (C) and OK03 (D) strains of West Nile virus were either mock-treated (◆) or treated with 2  $\mu$ M MB (■), diluted as indicated on the x-axis and placed onto confluent monolayers of Vero cells in T25 flasks. Shown is the yield of infectious virus (y-axis) plotted against the dilution (x-axis) of the treated an untreated samples. (E) Immunocompetent BalbC/j and (F) immunodeficient (C.B.17-SCID) mice were infected with either  $10^4$  pfu of WNV i.p. (■), or with  $10^4$  WNV previously treated with 20  $\mu$ M MB i.p. (●). The plot shows the percent survival at each day post-inoculation ( $n = 5$  for each group).

and the cellular monolayer was completely destroyed by the virus.

While MB + L can inactivate many RNA viruses (Floyd et al., 2004), using phage Qbeta as a model system, we concluded that MB + L caused the cross-linking of viral RNA to viral proteins in a singlet oxygen-mediated process (reviewed

in Floyd et al., 2004). Basic mechanistic studies using isolated RNA and isolated viral protein showed that the dsRNA region can also be cross-linked to the viral protein by MB + L. At this point it is unresolved whether MB + L exhibits sequence preferences or specificity. Hence, we considered the possibility that the inactivation of one particular strain of WNV may

not necessarily reflect the efficacy of MB against other strains of WNV. To test the ability of MB + L to inactivate multiple WNV strains, MB was also tested against WNV strains OK02 and OK03 representing recent primary U.S. isolates. WNV ( $5 \times 10^3$  pfu) were incubated with  $2 \mu\text{M}$  MB + L (Fig. 1C and D) and treated and mock-treated virus plated at indicated dilutions. MB photoinactivated both strains of WNV. MB + L also inactivated the prototypical NY99 strain of WNV (data not shown).

Next, we infected one group of BalbC/j mice ( $n = 5$ ) with a dose of  $10^4$  pfu/animal of WNV strain OK02. By day 9 only 40% of the mice were alive. By day 10 all mice (100%) had succumbed to infection, yielding a mean survival of time of 9.4 days (Fig. 1, panel E). We then tested the ability of MB to block WNV strain OK02 lethal infection in mice. Reinforcing the results obtained tissue culture-based assays for WNV infectivity, 100% of the mice infected with  $10^4$  pfu of  $20 \mu\text{M}$  MB-treated WNV were still alive at day 15 (Fig. 1, panel E). The difference in survival was significant to  $p \leq 0.0015$  at day 15 using Log-Rank test.

An active immune system within the BalbC/j mice could contribute to blocking infection in the MB-treated group. It is possible that if only a few infectious particles survived the MB photoinactivation then the host immune response would impede the disease. To rule out this possibility, we repeated the experiment using severe-combined immune deficient (SCID) mice. Two groups of C.B.17-SCID mice ( $n = 5$  per group) were infected i.p. with  $10^4$  pfu of MB-treated or mock-treated virus. Sixty percent of the untreated group succumbed to infection by day 8, and all mice in this group were dead by day 9 (Fig. 1, panel F). This yields a mean survival of 9.4 days, which was almost identical to that of the BalbC/j mice. Similar to the BalbC/j mice animals injected with MB-treated WNV survived to day 15 and beyond. At day 15 p.i., we calculated  $p \leq 0.0031$  by Log-Rank test of survival. This result rules out the possibility that host immunity played a role in stopping WNV disease in these mice.

These experiments demonstrate the efficacy of MB to photoinactivate WNV in tissue culture and, for the first time, demonstrate the absence of residual infectivity in an animal model of WNV infection. This result is consistent with prior work on MB (Mohr et al., 2004) and extends those studies in important ways: (i) MB + light was able to inactivate multiple independent low-passage isolates of WNV from recent outbreaks 2002 and 2003; (ii) we determined the  $\text{IC}_{50}$  to be  $0.10 \mu\text{M}$ ; when a concentration of  $20 \mu\text{M}$  MB was used a reduction of  $10^7$  pfu was achieved, which is higher than any WNV titer found in human blood products to date; (iii) MB + light-inactivated virus was no longer infectious in an animal model of WNV infection. Laboratory mice are very sensitive to WNV infection (Beasley et al., 2002, 2003; Kramer and Bernard, 2001; Perelygin et al., 2002; Samuel, 2002). As little as 1 pfu/animal can be lethal and  $10^3$  pfu causes mortality in 100% of infected animals within 7–8 days. MB + L treatment completely block-associated morbidity and mortality at challenge doses of  $10^3$  (data not shown)

and  $10^4$  pfu (Fig. 1) per animal. MB has been used in the treatment of humans for many years. It is safe with the longest reported oral use for up to 19 months at  $100 \text{ mg/kg}$  ( $\sim 50 \mu\text{M}$  in blood) twice daily with no reported side effects (Naylor et al., 1986). DiSanto and Wagner (1972) report that MB is absorbed orally and has a half-life of about 10 h. The in vivo half-life for MB + L-inactivated WNV still remains to be established, but our animal experiments imply that MB + L-inactivated WNV particles have no toxic side effects either. Use of this technology to inactivate a wide range of viruses in blood products will help to lessen the ever-increasing threat of viral infection from blood transfusion. It should also be noted that while blood is currently tested for infectious agents such as HIV-1, hepatitis C, and WNV; the blood units testing positive cannot be used. MB + L technology could combat blood shortages by rendering these once useless blood samples useful again. Using MB + L treatment to disinfect blood and blood products is currently under evaluation, though some damage to platelets has also been reported (Wagner and Skripchenko, 2003; Dodd, 1994).

## References

- Beasley, D.W., Davis, C.T., Guzman, H., Vanlandingham, D.L., Travassos da Rosa, A.P., Parsons, R.E., Higgs, S., Tesh, R.B., Barrett, A.D., 2003. Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States. *Virology* 309, 190–195.
- Beasley, D.W., Li, L., Suderman, M.T., Barrett, A.D., 2002. Mouse neuroinvasive phenotype of West Nile virus strains varies depending upon virus genotype. *Virology* 296, 17–23.
- CDC, 1999. Outbreak of West Nile-like viral encephalitis—New York, 1999. *MMWR Morbid., Mortal. Wkly. Rep.* 48, 845–849.
- CDC, 2002. West Nile Virus Activity—United States, July 31–August 7, 2002, and Louisiana, January 1–August 7, 2002. *MMWR Morbid., Mortal. Wkly. Rep.* 51, 681–683.
- DiSanto, A.R., Wagner, J.G., 1972. Pharmacokinetics of highly ionized drugs. II. Methylene blue—absorption, metabolism, and excretion in man and dog after oral administration. *J. Pharm. Sci.* 61, 1086–1090.
- Dodd, R.Y., 1994. Viral inactivation in platelet concentrates. *Transfus. Clin. Biol.* 1, 181–186.
- Enserink, M., 2002. West Nile's Surprisingly Swift Continental Sweep. *Science* 297, 1988–1989.
- Floyd, R.A., Schneider Jr., J.E., Dittmer, D.P., 2004. Methylene blue photoinactivation of RNA viruses. *Antiviral Res.* 61, 141–151.
- Iwamoto, M., Jernigan, D.B., Guasch, A., Trepka, M.J., Blackmore, C.G., Hellinger, W.C., Pham, S.M., Zaki, S., Lanciotti, R.S., Lance-Parker, S.E., et al., 2003. Transmission of West Nile virus from an organ donor to four transplant recipients. *N. Engl. J. Med.* 348, 2196–2203.
- Kramer, L., Bernard, K., 2001. West Nile virus infection in birds and mammals. *Ann. NY Acad. Sci.* 951, 84–93.
- Lambrecht, B., Mohr, H., Knuver-Hopf, J., Schmitt, H., 1991. Photoinactivation of viruses in human fresh plasma by phenothiazine dyes in combination with visible light. *Vox. Sang.* 60, 207–213.
- Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., et al., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* 286, 2333–2337.
- Mohr, H., Knuver-Hopf, J., Gravemann, U., Redecker-Klein, A., Muller, T.H., 2004. West Nile virus in plasma is highly sensitive to methylene blue-light treatment. *Transfusion* 44, 886–890.

- Murphy, F., Fauquet, C., Bishop, D., Ghabrail, S., Jarvis, A., Martelli, G., Mayo, M., Summers, M., 1995. Virus Taxonomy, classification and nomenclature of viruses. *Arch. Virol.* 10, 1–58.
- Naylor, G.J., Martin, B., Hopwood, S.E., Watson, Y., 1986. A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manic-depressive psychosis. *Biol. Psychiatry* 21, 915–920.
- Pealer, L.N., Marfin, A.A., Petersen, L.R., Lanciotti, R.S., Page, P.L., Stramer, S.L., Stobierski, M.G., Signs, K., Newman, B., Kapoor, H., et al., 2003. Transmission of West Nile virus through blood transfusion in the United States in 2002. *N. Engl. J. Med.* 349, 1236–1245.
- Perelygin, A.A., Scherbik, S.V., Zhulin, I.B., Stockman, B.M., Li, Y., Brinton, M.A., 2002. Positional cloning of the murine flavivirus resistance gene. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9322–9327.
- Samuel, C.E., 2002. Host genetic variability and West Nile virus susceptibility. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11555–11557.
- Wagner, S.J., Skripchenko, A., 2003. Investigation of photosensitizing dyes for pathogen reduction in red cell suspensions. *Biotech. Biochem.* 78, 171–177.