Methylene blue-mediated photodynamic inactivation as a novel disinfectant of enterovirus 71

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Objectives: We tested whether methylene blue, an inexpensive and safe photosensitizer, is feasible for photodynamic inactivation of enterovirus 71 (EV71) in the environment.

Methods: By escalating light doses and photosensitizer concentrations, photoinactivation of EV71 and other enteroviruses was examined in vitro. Viral transmission in the environment was simulated with a neonatal mouse model in vivo. Possible mechanisms were analysed with alterations of viral DNA and proteins after treatments.

Results: Photodynamic inactivation of EV71 in suspensions occurred in a dose-dependent manner. The optimal condition for photoinactivating EV71 required a light dose of 200 J/cm² in the presence of methylene blue. This photodynamic condition was also able to inactivate other enteroviruses, including poliovirus 1 and coxsackieviruses A2, A3, A16 and B3. In an imitation environment, EV71 spread on a solid surface was inactivated by methylene blue-mediated photodynamic inactivation and prevented EV71 transmission to mice. Western blot and RT–PCR analysis indicated that both the viral proteins and the genome were disrupted after photodynamic inactivation.

Conclusions: Methylene blue-mediated photodynamic inactivation may provide a novel way to eliminate environmentally contaminated sources of EV71 to prevent infection.

Keywords: EV71, PDT, photodynamic therapy

Introduction

Enterovirus 71 (EV71) is a single positive-stranded RNA non-enveloped virus that belongs to the Enterovirus genus of the Picornaviridae family. Most EV71 infections are mild, such as hand, foot and mouth disease and herpangina in young children. However, CNS infections with life-threatening pulmonary and cardiac complications may occur.1–2 EV71 has been regarded as the most important neurotropic enterovirus since the effective control of the poliovirus.3 More than a dozen severe EV71 outbreaks have been reported worldwide4–8 since it was first recognized in California in 1969.8

EV71 has been isolated from sewage, groundwater10 and bathing water.11 The virus is resistant to 70% alcohol disinfection, and it is believed to be transmitted through the faecal–oral route.1,12 Frequent hand washing is encouraged to prevent EV71 infection in daycare facilities, pre-schools and kindergartens. However, such practice is by no means able to reduce the organisms in the environment. Peracetic acid and chlorine compounds are the most commonly used disinfectants of solid surfaces in public places and hospitals. These agents are potentially genotoxic and/or carcinogenic, and are environmentally toxic to humans, especially to toddlers who crawl on the floor and lick their hands.
Photodynamic treatment (PDT) has been applied to destroy cancer cells and a variety of microorganisms including viruses and bacteria by combining light and photosensitizing agents in the presence of oxygen. Methylene blue, a thiazine compound, has been used for decades for cell staining, delineating tumor margins during operations and as an antidote for nitrate poisoning and methaemoglobinemia. Methylene blue has also been used as a photosensitizer in PDT. Methylene blue absorbs visible light between 620 and 670 nm. After photodynamic activation, methylene blue generates singlet oxygen and free oxygen radicals, which inactivate bacteria and viruses by damaging and cross-linking the nucleic acid and proteins. Methylene blue-mediated PDT (MB-PDT) has been applied in the sterilization of blood products because of a number of advantages including its high safety profile, low cost, minimal leftovers and low reduction of coagulation factors.

In this study, we investigated the possibility of using MB-PDT as an environment disinfectant by examining the efficacy of methylene blue photoinactivation of EV71 in suspension and on a solid surface.

Materials and methods

Cells and viruses

Rhabdomyosarcoma (RD) cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin. Stock viruses of EV71/Tainan/4643/98 (GenBank accession number AF304548), mouse-adapted EV71 strain MP4, coxsackie A2 virus (CA2), coxsackie A3 virus (CA3), coxsackie A16 virus (CA16), coxsackie B3 virus (CB3) and poliovirus type 1 (Department of Virology, National Cheng Kung University Hospital, Tainan, Taiwan) were grown in RD cells. Both EV71/4643 and MP4 strains were tested by anti-EV71 mAb (mAb979; Chemicon, Temecula, CA, USA) indirect immunofluorescence staining of infected RD cell cultures. CA2, CA3, CA16, CB3 and poliovirus were confirmed by standardized serum pool typing (ATCC). Viral RNA was extracted from viral suspension (Trizol reagent; Invitrogen, California, USA) with an optical power meter (Nova II-Laser Power/Energy Meter; Ophir Optronics, Israel). An electric fan was placed on the side and turned on during irradiation to avoid a heating effect. Viable virus was recovered as described above.

Plaque assay

Confluent monolayers of RD cells were prepared in 24-well plates (2–5 × 10^5 cells/well). Cells were infected with serial dilutions of virus suspension, overlaid with 1.5% methylcellulose in DMEM containing 2% FBS and incubated at 37°C for 3 days before the plaques were visualized with crystal violet.

SDS–PAGE and western blotting

To examine how PDT affects the viral proteins, methylene blue was added to the viral suspension to obtain a mixture with a final content of 12 or 60 μg of viral protein and 0, 0.5, 5.0 or 50 μM methylene blue. Specifically, 12 μg of EV71 viral protein (31.5 μL) was incubated with 3.5 μL of methylene blue (0, 5, 50 or 500 μM) for 10 min or 60 μg of viral protein (157.5 μL) was incubated with 17.5 μL of 50 μM methylene blue for 10 min before irradiation (PDT-1200). After irradiation, 20 μL of the mixture (containing 7 μg of EV71 viral protein) was mixed with loading buffer and incubated at 90°C for 15 min before being separated by 12.5% SDS–PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Temecula, CA, USA). The membrane was then blocked with 3% skimmed milk and stained with EV71 immune serum (1:500 dilution) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution; Jackson ImmunoResearch, Philadelphia, PA, USA). The reaction was developed using a 3,3’,5-diaminobenzidine (DAB) peroxidase substrate kit (Vector, Burlington, Ontario, Canada). Plaque assay showed that no viable virus was detected after the MB-PDT.

RT–PCR

Viral RNA was extracted from viral suspension (Trizol reagent; Invitrogen, Carlsbad, CA, USA), with or without MB-PDT, and converted into cDNA (ImProm-II reverse transcription system; Promega, Madison, WI, USA). PCR amplification was performed on a 3 μL cDNA sample. The PCR cycle rate was set corresponding to the light dose. For example, the virus was exposed to 100 J/cm^2 at 100 mW/cm^2 and 150 J/cm^2 at 150 mW/cm^2 to maintain a constant irradiation time. Controls included a virus exposed to light without methylene blue (light control) and a virus kept in the dark with methylene blue (dark control). Viral titre was determined by plaque assay after MB-PDT as described below.
added to a 75 mL flask before the addition of 1 mL of methylene blue.

Transmission of surface-bound EV71 to mice

To mimic EV71 transmission in the environment, 1 mL of EV71 viral suspension (3 × 10^7, 3 × 10^6 and 3 × 10^5 pfu) or culture medium was pre-mixed with Tween 20 (0.1% final concentration; Merck, Schuchardt, Germany) and was evenly spread on the bottom of a 75 mL flask (Nunc, Roskilde, Denmark) by gentle rotation. The top side of the flask was removed for handling of the animals and irradiation purposes. One-day-old ICR mice (specific-pathogen-free; Laboratory Animal Center, National Cheng Kung University College of Medicine, Tainan, Taiwan) were placed into the flasks (six per flask) after fasting for 3 h. The flask was wrapped up with Saran wrap on top and loosely capped for air exchange. The animals were kept in the flasks for 4 h at room temperature, returned to their dams thereafter and observed daily for weight changes and survival. All the animals were killed 1 or 2 weeks later with an overdose of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL, USA). Blood samples were collected from a solid surface to mice, 1-day-old mice were exposed to surface-bound EV71 with or without MB-PDT. Briefly, 1 mL of EV71 viral suspension (3 × 10^7 pfu) or culture medium (all pre-mixed with Tween 20) was added to a 75 mL flask before the addition of 1 mL of methylene blue (0.05 mM). The flask was immediately irradiated by the LED light source (27.8 mW/cm^2) followed by the introduction of animals for 4 h. All procedures were approved by the University Animal Care Committee and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Neutralization test and virus titration

Neutralizing antibody titre of the serum samples collected from the animals exposed to surface-bound EV71 with or without MB-PDT was determined using a microassay. Briefly, 50 μL of serial serum dilutions was mixed with 50 μL of 100-fold tissue culture infectious dose 50 (TCID50) EV71 in a 96-well plate, and RD cell suspensions (final concentration 8 × 10^3 cells) were added 2 h later. After incubation for 6 days at 37°C, the neutralizing antibody titre was determined as the highest dilution of serum that inhibited virus growth. Mouse hyper-immune serum with a neutralizing antibody titre of 2^8 against EV71 was used as a positive control in all of the assays. The hyperimmune serum was generated in adult mice immunized with live EV71 (2 × 10^6 pfu per 200 μL, two subcutaneous injections and one intraperitoneal injection at 3 week intervals). For viral titration, the tissue samples were inoculated onto monolayers of RD cells. These cells were inspected daily for a minimum of 14 days for CPE. Viral titres were expressed as log pfu per mg of tissue. The lower limit of virus detection was 20 pfu.

Statistics

One-way analysis of variance (ANOVA) was performed to determine whether there were significant differences between the different test conditions. The Bonferroni correction was applied for pairwise comparisons. Data were analysed using SigmaStat™ (Systat Software Inc., San José, CA, USA) version 3.11. A P value of <0.05 was considered significant. Data were calculated from at least three independent experiments.

Table 1. Sequences of the primer pairs for gene fragments of EV71, the position and size of the amplicons, and the regions that the primer pairs spanned

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence</th>
<th>nt position</th>
<th>Size (kb)</th>
<th>Region spanned</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’ 1623: CCTATTAGCCCGTTAGATT 3’ 1933: TTCCCGGCTTGACGTCGAG</td>
<td>1623–1933</td>
<td>0.31</td>
<td>1B–1C</td>
</tr>
<tr>
<td>2</td>
<td>5’ 2449: GTGGCGATGTGATGAGAG 3’ 2780: GTTAGTCTAGTTACGGAGT</td>
<td>2449–2780</td>
<td>0.33</td>
<td>1D</td>
</tr>
<tr>
<td>3</td>
<td>5’ 3356: CATATCTAGGGGCCAATT 3’ 3833: TCTCTTTGACACTGCNGTC</td>
<td>3356–3833</td>
<td>0.47</td>
<td>2A–2B</td>
</tr>
<tr>
<td>4</td>
<td>5’ 5022: CTATACAGGAGTATAACA 3’ 5537: ACTATA TTGTGTTCAACCC</td>
<td>5022–5537</td>
<td>0.52</td>
<td>2C–3C</td>
</tr>
<tr>
<td>5</td>
<td>5’ 6135: CTGCATGAACTGATGAGT 3’ 6655: AACACACTGGGCTGAGAC</td>
<td>6135–6655</td>
<td>0.52</td>
<td>3D</td>
</tr>
<tr>
<td>6</td>
<td>5’ EcoRI site primer: CGCGGGAAATTCAATCTTCCCCACGCTGGTGACC 3’ 2307: TACACTACAGGTGTTACGATA</td>
<td>1–2307</td>
<td>2.30</td>
<td>1A–1C</td>
</tr>
<tr>
<td>7</td>
<td>5’ 5569: GGAGTTAGTAGAGCAACA 3’ 7423: TAAATTGTTATACAGAATGCGCCGCGAAAA</td>
<td>5569–7423</td>
<td>1.85</td>
<td>3C–3D</td>
</tr>
<tr>
<td>8</td>
<td>5’ 2140: TACCCA AGGCCGCGGACGACCA 3’ 5630: GCTCACACTGGTAACTGTTGATAC</td>
<td>2140–5630</td>
<td>3.49</td>
<td>1C–3C</td>
</tr>
</tbody>
</table>
Results

**MB-PDT inactivated EV71 in suspension**

Methylene blue (0.05 mM) alone had no effect on the viability of EV71 after incubation for up to 120 min in the dark (Figure 1). However, viral titres were reduced 1.5 log after exposing the virus to 100 J/cm² of red light at 100 mW/cm² (Figure 1). The photoinactivation was not related to incubation time. We therefore incubated the virus with methylene blue for 10 min in subsequent experiments. Pre-activation of methylene blue with 100 J/cm² at 100 mW/cm² before the addition of EV71 did not affect the viability of the virus (data not shown). EV71 exposed to 100 J/cm² in the presence of 0.05 mM methylene blue resulted in a 1.5 log inhibition (Figure 2a). Increased light dose (150 and 200 J/cm²) provided better inhibition (~3 log) with the same methylene blue concentration (Figure 2b and c). No virus was detected at 0.5 mM methylene blue and 150 J/cm² (Figure 2b) or ~0.1 mM methylene blue and 200 J/cm² (Figure 2c). PDT provided nearly an 8 log-fold killing. The 50% inhibition concentration (IC50) of MB-PDT for EV71 was ~150 J/cm² of red light with 0.05–0.1 mM methylene blue. The blue methylene blue faded to nearly a clear colour under this PDT condition [Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. Further experiments showed that other enteroviruses, including poliovirus 1, CA2, CA3, CA16 and CB3, were completely inactivated after exposure to 200 J/cm² at 200 mW/cm² in the presence of 0.1 mM methylene blue (a 6.5–9.5 log inhibition of viral growth), indicating that MB-PDT has a broad anti-enterovirus spectrum [Figure S2, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)].

**MB-PDT damaged the viral proteins and genome**

To determine whether disruption of macromolecules was involved in the mechanism of the photoinactivation of EV71, viral proteins were immunoblotted with mouse anti-EV71 immune serum. The protein profile was altered considerably as characterized by smearing and disappearing of several protein bands including those of the structural and non-structural proteins after a low dose of MB-PDT (50 J/cm² at 50 mW/cm², methylene blue concentration ≥0.5 μM). Strikingly, all viral protein bands disappeared after virus was exposed to 50 μM methylene blue with light (Figure 3d). On the other hand, two...
20 kDa new protein bands (arrowheads, Figure 3a and b) appeared after PDT and their intensity increased with light dose (Figure 3b). Eight EV71-specific primer pairs were used to monitor the integrity of the genome after PDT (0.05 mM methylene blue and 200 J/cm²). Four of the primer pairs, 5022–5537, EcoRI–2307, 5569–7423 and 2140–5630, which were flanked with a sequence ≥0.52 kb, showed no amplification after irradiation (Figure 3c), indicating that the corresponding nucleotides might have been altered.

**MB-PDT inhibited the persistence of EV71 on a solid surface**

The titre of surface-bound EV71 decreased ~1 log-fold per day within 48 h under RH of 70% and 90%, and only 0.02% of the virus survived after incubation for 3 days under RH of 90% (Figure 4a). The virus titre declined even more quickly at 50% RH. No virus was detected on day 3 under RH of 50% and 70%. MB-PDT effectively inactivated surface-bound EV71 (Figure 4b). Virus exposed to 25 J/cm² with 0.05 mM methylene blue provided a 3.5 log reduction in viral titre. No detectable virus was noted after increasing the light dose to 50 J/cm². Methylene blue in the dark did not alter the viral count. Viral titre was decreased with light exposure in the absence of methylene blue, which was probably related to a local drying effect by air blowing from the LED electric fan.
Table 2. MB-PDT reduced the transmission of surface-bound EV71 to mice

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>EV71 exposure (pfu)</th>
<th>medium</th>
<th>$3 \times 10^3$</th>
<th>$3 \times 10^4$</th>
<th>$3 \times 10^5$</th>
<th>$3 \times 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-PDT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>7</td>
<td>4.28 ± 0.37</td>
<td>4.27 ± 0.59</td>
<td>4.10 ± 0.27</td>
<td>3.30 ± 0.24*</td>
<td>4.45 ± 0.47**</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>6.30 ± 0.24</td>
<td>6.15 ± 0.31</td>
<td>5.59 ± 0.35</td>
<td>5.00 ± 0.30*</td>
<td>6.23 ± 0.25**</td>
</tr>
<tr>
<td>Numbers of live animals (%)</td>
<td>7</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
<td>5/6 (83)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
<td>3/6 (50)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>Serum neutralizing antibody titre (log2)</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.26 ± 0.10</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are means ± SEM.

*One-day-old ICR mice (n=6–12) were exposed to EV71 with/without MB-PDT as described in the Materials and methods section.

**P<0.05 compared with the same viral dose group without MB-PDT.

**P<0.05 compared with the medium group.

**MB-PDT reduced viral transmission from a solid surface to neonatal mice**

Table 2 shows that PDT reduced environmental EV71 transmission to neonatal mice. In agreement with Wang et al.20 that EV71 was able to transmit from infected mice to non-infected cage mates, 1-day-old mice that had been in contact with high concentrations of EV71 $(3 \times 10^7$ pfu) for 4 h exhibited serum conversion, weight loss and death. They also had viable virus in their intestines. On the other hand, no evidence of infection was observed in mice exposed to the same dose of virus after MB-PDT (0.05 mM, 25 J/cm$^2$), indicating the inactivation of surface-bound EV71.

**Discussion**

This study demonstrated that MB-PDT can inactivate EV71 both in suspension and on a solid surface. The results suggest the potential for using MB-PDT as an environmentally friendly disinfectant for EV71.

Enveloped viruses including human immunodeficiency virus (HIV),23 dengue virus,24 vesicular stomatitis virus, simian virus 40 and calcivirus19 are more sensitive to PDT and all could be inactivated with 1 μM methylene blue. Non-enveloped viruses are more resistant to photodynamic killing than enveloped viruses.19 Nevertheless, MB-PDT worked effectively for EV71 in suspension with a higher PDT dose (0.1 mM methylene blue and 200 J/cm$^2$) in our study. Other enteroviruses including poliovirus 1, CA2, CA3, CA16 and CB3 were also inactivated under these conditions.

Methylene blue has high affinity for nucleic acid and protein, and PDT induces the cross-linking of protein–protein and protein–nucleic acid.16,26 Our data strongly suggested that photodynamic damages to the viral genome and proteins were responsible for EV71 inactivation. The generation of free radicals might be responsible for this damage. The fact that photodynamic effects were not affected by methylene blue pre-activation or prolonged methylene blue incubation, and that photoinactivation occurred almost immediately after PDT, support this hypothesis.

Although EV71 has been isolated from bathwater and groundwater,10,11 it is not known how the virus survives on solid surfaces. We showed that high humidity favours the survival of EV71 on solid surfaces. The virus can survive for 3 days in 90% RH. The results support the idea that saliva and/or body fluid and discharge on hands and fluid-contaminated toys may be a transmission source of the virus. Interestingly, MB-PDT worked for EV71 on a surface as efficiently as in suspension. Surface-bound EV71 could be totally eradicated by a low dose of MB-PDT (0.05 mM methylene blue and 50 J/cm$^2$) with an LED light source. An LED has a relatively narrow excitation wavelength $(635 ± 20$ nm) that might be more effective in activating methylene blue than a broad-spectrum light source. More importantly, such treatment was sufficient to prevent virus transmission to neonatal mice from the environment. A light dose of 50 J/cm$^2$ of red light exposure is easy to achieve. The average fluence rate of the red region $(620–670$ nm) of white light is $\sim 1$ mW/cm$^2$.24 Thus, a 7 h exposure of ambient light from household fluorescent lamps with an application of 0.05 mM methylene blue would achieve the IC$_{50}$ for EV71. A shorter duration of light exposure with a higher methylene blue concentration may be effective as well. An effective infection with EV71 depends on the viral load. Complete eradication of the virus may not be necessary to prevent viral transmission. Nonetheless, further studies are required to confirm this hypothesis.

There are many advantages of MB-PDT. First, the incubation time of methylene blue for photodynamic action is brief (minutes) as compared with other available photosensitizers, which require hours of incubation before irradiation.24–26 As shown in this study and in previous studies,24–26 methylene blue binds to microorganisms almost immediately and thus irradiation can be performed without any delay. Second, MB-PDT has a high safety margin.16 Third, methylene blue is cheaper than other available photosensitizers, including Photofrin, aminolevulinic acid and Ce6. For practical use as a...
disinfectant, methylene blue dye should not stain after application. Our experiments showed that at an effective working concentration (0.05 mM), methylene blue was barely visible after PDT.

In summary, the results suggested that low-dose MB-PDT may be used as an appealing alternative disinfection system for EV71 virus.

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**Transparency declarations**

None to declare.

**Supplementary data**

Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**