Photodynamic Therapy of Medically Important RNA Virus and Virus-infected Cells

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ABSTRACT

In this current study, a novel nano-photosensitizer in photodynamic therapy is explored as a potential anti-viral technology. Dengue 2 viruses were mixed with the ZnPC-nanoparticle and exposed to near-infrared (NIR) light at varying distances and durations. Viral plaque assay revealed that virus inactivation increased with higher ZnPC-nanoparticle concentrations, longer NIR light illumination and shorter exposure distance. Close interaction of the ZnPC-nanoparticles and dengue 2 viruses was shown through electron microscopy. Photo-inactivation of dengue 2 virus-infected human hepatocyte cells (HepG2) with ZnPC-nanoparticles also showed effects of virus inactivation. Using ZnPC-nanoparticles that were conjugated with antibody specific for dengue 2 virus, immunofluorescent imaging further revealed the specificity of ZnPC-nanoparticles in targeting dengue virus-infected HepG2 cells. Cell viability assay (MTT) indicated that the cytotoxic effect by NIR light on HepG2 cells was only minimal. Therefore, this nano-photosensitizer photodynamic therapy could be a feasible and novel approach for inactivation of enveloped viruses and therapy of virus-infected cells.

INTRODUCTION

Photodynamic therapy has been traditionally applied in antibacterial and antifungal studies since the 1900s (O’Riordan et al, 2005). Recently, it has been researched for oncological treatment purposes. It requires excitation of a photosensitizer to the triplet state by certain light wavelengths. Energy that is re-emitted when it returns to the ground state is taken up by oxygen molecules close-by to produce singlet oxygen radicals, which can damage molecules like nucleic acid. Common photosensitizers are dyes or dye derivatives. However, these are activated by ultraviolet light that is harmful to cells. In this study, a novel photosensitizer that utilizes NIR wavelength for excitation had been designed to overcome this problem. The photosensitizing agent, ZnPC is coated onto a nanoparticle and its anti-viral effect on medically important viral pathogen (dengue virus) and virus-infected cells are being assessed in this study.

MATERIALS AND METHODS

Cell lines and virus

Mosquito C6/36 cells were used for the cultivation of dengue virus (serotype 2) pool. Human hepatocyte cell line (HepG2) was used for photo-inactivation treatment and baby hamster kidney cells (BHK-21) was used for titering of infectious virus using plaque assay.

Zinc-phthalocyanine (ZnPC) nano-photosensitizer

The ZnPC nano-photosensitizer (nanoparticle) used in this current study was kindly provided by A/P Zhang Yong, Department of Bioengineering, NUS. In brief, the ZnPC-nanoparticle was synthesized through a hydrothermal synthesis procedure, producing PEI / NaYF4:Yb3+, Er3+ nanoparticles, with ZnPC adsorbed onto the surface (Figure 1).

Sample preparation for transmission electron microscopy

Dengue 2 virus that was grown, harvested and concentrated using a centrifuge device was further purified with 25% sucrose cushion by ultracentrifugation. Neat dengue 2 virus alone and
a virus mix with ZnPC-nanoparticles were negatively stained with 2% phosphotungstic acid and viewed under the transmission electron microscope.

**Photo-inactivation of virus suspension**
Dengue 2 virus (10^5 PFU in 100µL), mixed with ZnPC-nanoparticles was irradiated with NIR light under various experimental conditions (Table 1). All experiments sets were carried out in triplicates and analyzed for infectious virus titer via plaque assay.

**Photo-inactivation of dengue virus-infected cells**
HepG2 cells were infected with dengue 2 virus (Multiplicity of Infection = 10) for two days and incubated with ZnPC-nanoparticles (concentrations of 22 µg/mL, 110 µg/mL, 220 µg/mL, 330 µg/mL and 440 µg/mL, 550 µg/mL) for 2 hours. Photo-inactivation was carried out with NIR light at a distance of 10 cm for 5 minutes. The treated cells were left to recover for 2 days and the culture supernatants were collected for plaque assay to determine the infectious virus titers.

**Conjugation of ZnPC-nanoparticles with antibody**
Monoclonal antibody specific for dengue 2 virus was conjugated to ZnPC-nanoparticles and stored at 4 ºC. These will be referred to as antibody-conjugated ZnPC-nanoparticles in this abstract.

**Immunofluorescence microscopy**
Dengue virus-infected HepG2 cells (3 days post-infection) grown on coverslips were incubated with either ZnPC-nanoparticles or antibody-conjugated ZnPC-nanoparticles for 2 hours before fixing the cells with ice cold methanol. For staining of dengue virus-infected cells, the primary antibody used was mouse monoclonal anti-dengue envelope protein antibody and the secondary antibody used was goat anti-mouse FITC. DAPI was used to stain the nuclei of the cells. Conventional optical fluorescence microscopy with excitation and emission wavelengths for FITC (480 nm, 550 nm), for DAPI (359 nm, 461 nm) and for ZnPC / Texas Red (596 nm, 629 nm) was used to view the specimens under 20x and 40x objectives.

**Measurement of cellular cytotoxicity using MTT assay**
Cell viability (MTT) test was performed on mock-infected and dengue virus-infected HepG2 cells that were subjected to photo-inactivation with ZnPC-nanoparticles, anti-dengue virus antibody alone or antibody-conjugated ZnPC-nanoparticles.

**RESULTS AND DISCUSSION**
In the first part of the study, we have successfully optimized the protocol for the concentration and purification of dengue virus using ultracentrifugation. This enabled us to prepare the dengue virus particles for negative staining and visualization under the electron microscope. Under the transmission electron microscope, dengue 2 virus particles appeared as white rough spheres (arrows) against the stained background and had an average diameter of 50 nm (Figure 2A). The surface proteins (spike-like) could be clearly observed. ZnPC-nanoparticles were observed as dark particles (arrows) due to its high electron density nature (Figure 2B). The diameter of the nanoparticles also averaged around 50 nm. Close proximity interaction between the ZnPC-nanoparticles (arrows) and dengue virus particles (arrowheads)
that had been incubated together for one hour at room temperature was observed (Figure 2C). These observations suggest that the application of photodynamic inactivation technology is feasible due to the close interaction between the virus and ZnPC-nanoparticles.

When different concentrations of ZnPC-nanoparticles (Table 1) were added to the dengue 2 virus and followed by photo-activation with NIR light, there was a dosage-dependent reduction in the infectious dengue virus titer (Figure 3). At the lowest ZnPC-nanoparticle concentration of 2 µg/mL, longer light exposure (10 minutes) caused virus titer to be effectively reduced but not at shorter light exposure duration (5 minutes). The strongest inactivation of dengue virus occurred when a combination of the shorter distance between the specimen and the NIR light (5 cm) as well as a longer light exposure of 10 minutes was used.

Following the effectiveness of photodynamic therapy directly on virus, the possibility of virus inactivation in a virus-infected cellular system was further investigated. Light distance of 10 cm and duration of 5 minutes were used. Since at 2200 µg/mL, dengue virus particles were effectively inactivated, a lower concentration range (22 µg/mL to 550 µg/mL) of the ZnPC-nanoparticles was used for photo-inactivation of dengue virus-infected HepG2 cells. Our results revealed a greater reduction of infectious dengue virus titer that was released by HepG2 cells when ZnPC-nanoparticles concentration was increased (Figure 4). However, within this range of concentrations, there was no complete inactivation of dengue virus from the infected cells.

To further enhance the specific delivery of ZnPC-nanoparticles to dengue virus-infected HepG2 cells, ZnPC-nanoparticles were conjugated to anti-dengue virus antibody. This may serve to enhance the directed localization of ZnPC-nanoparticles on dengue virus particles in suspension or onto dengue virus-infected cells, which could result in specific inactivation of virus. By improving the target specificity of the ZnPC-nanoparticles, the chances of them clustering on non-infected cells, and causing off-target damages upon photo-activation, could also be reduced. The specificity of antibody-conjugated ZnPC-nanoparticles onto dengue virus-infected HepG2 cells was revealed through immunofluorescence assay. Dengue 2 virus-infected HepG2 cells (3 days post-infection) as shown in Figure 5 were incubated with antibody-conjugated ZnPC-nanoparticles (Figure 5A-D) or ZnPC-nanoparticles (Figure 5E-H) for 2 hours to allow sufficient time for cellular localization. The cell nuclei were stained blue with DAPI (specific nucleus stain, Figure 5A & E), the expression of dengue virus antigen in the infected cells were stained green (Figure 5B & F) while the red fluorescence displayed the distribution of the ZnPC-nanoparticles (Figure 5C & G). Antibody-conjugated ZnPC-nanoparticles showed specific localization onto virus-infected cells only (Figure 5D). An uninfected cell at the lower right corner was free from ZnPC-nanoparticles, while there were considerable overlaps of the green and red fluorescence in the infected cells, appearing as yellow fluorescence (Figure 5D). In contrast, ZnPC-nanoparticles are localized to all cells infected by dengue virus (Figure 5H).

Despite its anti-viral effectiveness, the cellular cytotoxicity induced by the ZnPC-nanoparticles is a concern. Thus, a cellular cytotoxicity test (MTT assay) was performed to address the effect that photo-activation by the ZnPC-nanoparticles, antibody-conjugated ZnPC-
nanoparticles and anti-dengue virus antibody alone, has on cell viability. Mock-infected cells that were photo-activated with ZnPC-nanoparticles or antibody-conjugated ZnPC-nanoparticles showed high survival percentage (approximately 100%), with only a slight reduction in cellular viability when 110 µg/mL ZnPC-nanoparticles was used. For dengue-virus infected cells, photo-activation with ZnPC-nanoparticles resulted in 80-100 % cellular viability and with antibody-conjugated ZnPC-nanoparticles, there were 40-80 % cell survival (data not shown).

CONCLUSION

The novel ZnPC-nanoparticle based photodynamic therapy effectively inactivates viruses in cell-free and in cellular systems, even at low concentrations. It can be developed as an anti-viral technology platform for other enveloped and non-enveloped viral pathogens.

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REFERENCES
