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The *in vitro* and *in vivo* antiviral properties of combined monoterpene alcohols against West Nile virus infection

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ABSTRACT

West Nile Virus (WNV) is a mosquito-borne flavivirus that can cause neuroinvasive disease in humans and animals for which no therapies are currently available. We studied an established combination of monoterpene alcohols (CMA) derived from *Melaleuca alternifolia*, against WNV infection. The *in vitro* results show that CMA exhibits virucidal activity, as well as reduces the viral titres and percentage of infected cells. The antiviral mechanism of action of CMA was studied. We found that CMA did not alter the intracellular pH, neither induced apoptosis, but did induce cell cycle arrest in the G0/G1-phase although that was not the antiviral mechanism. Furthermore, we tested CMA *in vivo* using IRF $3^{-/-}/7^{-/-}$mice and it was found that CMA treatment significantly delayed morbidity due to WNV infection, reduced the loss of body weight and reduced the viral titres in brain. These findings suggest that CMA could be a therapeutic agent against WNV infection.

1. Introduction

The Flavivirus genus, in the family *Flaviviridae*, includes highly pathogenic positive stranded RNA viruses such as West Nile virus (WNV), dengue virus (DENV), Zika Virus (ZIKV), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV) (Mackenzie et al., 2004; Gubler, 2001; Gould and Solomon, 2008; Hayes, 2009). Currently, flaviviral infections constitute a major international health problem with approximately 50 million cases per year for DENV infection alone (WHO, 2014). In the case of WNV, its recent emergence in the Americas and spread in Africa, Middle East, the Mediterranean, Australia, the West and Central Asia has presented a considerable concern as well (WHO, 2011). In the United States from 1999 to 2012, over 36,000 cases of WNV infection were reported to the Centre for Disease Control and Prevention (CDC) with 49% resulting in hospitalization and 4% death, having an approximate cumulative cost of US $778 million (Barrett, 2014). Despite the clinical impact that flaviviral infections have, there are no approved therapies for human use against WNV or DENV.

In the case of DENV, humans are the only known natural host, thus an *in vivo* model of dengue disease has been a challenge and the understanding of DENV pathogenesis has been limited (Simmons et al., 2012). Although several animal models including mice, rats, rabbits, dogs, pigs and non-human primates (NHP) have been tested, when infected with DENV, none of these has shown disease similar to what occurs in humans (Zompi and Harris, 2012; Herrero et al., 2013). In contrast, WNV provides a more convenient model of flaviviral infections as their replication cycle is similar and the disease symptoms of infected mice closely resemble that of human infection (Samuel and Diamond, 2006). Therefore, by studying flaviviral infections using WNV as a model, it may be possible to identify broad-spectrum inhibitors with prophylactic and/or therapeutic activity against several flaviviruses (Diamond, 2009).

The flavivirus genome is approximately 11 kb and encodes a single polyprotein, that it is cleaved by virus and host-encoded proteases into 3 structural and 7 non-structural (NS) proteins (Lindenbach et al., 2007). The structural proteins: capsid (C), transmembrane (prM) and envelope (E) constitute the virion. The non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are involved in viral RNA replication, virus assembly and modulation of the host cell responses (Lindenbach et al., 2007; Diamond, 2003).

An established combination of monoterpene alcohols (CMA) was obtained from the essential oil of the Australian native plant *Melaleuca alternifolia,* commonly known as Tea Tree oil (TTO) (Low

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et al., 2015). This particular CMA has been approved by the Australian Therapeutic Goods Administration for human use as topical[2] and oral[3] herbal medicines known as *Melaleuca alternifolia* concentrate (MAC). The CMA used in this study is similar to TTO (Table 1), except that 85% of the lipophilic monoterpene hydrocarbons contained in TTO have been removed, thereby enriching the content of the monoterpene alcohols such as Terpinen-4-ol, the main component of both TTO and the CMA (Low et al., 2015; Carson et al., 2006; Mondello et al., 2006). *In vitro* studies have proposed that certain essential oils (TTO) and monoterpenes (Terpinen-4-ol) derived from essential oils, have antiviral activity against Herpes Simplex virus (HSV) (Astani et al., 2010) and Influenza viruses (Garozzo et al., 2011). However, whether the CMA has antiviral activity against WNV and the mechanisms of action of the CMA against WNV are unknown. This study demonstrated that the CMA has an effective *in vitro* antiviral and virucidal activity against the non-pathogenic Australian strain of WNV, Kunjin (WNV_{KUNV}) and the pathogenic American strain New York 99 (WNV_{NY99}). Also, this study investigated mechanisms by which the CMA could exert its antiviral effects and provides preliminary evidence for its possible use as an antiviral therapy *in vivo*.

2. Methods

2.1. Cells and virus preparation

African green monkey kidney (Vero) and Baby hamster kidney (BHK 21) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 5% heat inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin sulphate. Both cell lines were grown at 37 °C in a 5% CO_2 incubator.

Viral stocks of the Australian strain of West Nile Virus, Kunjin MRM61C (WNV_{KUNV}) (Khromykh et al., 1999) and NY99 strain (WNV_{NY99}) were generated from corresponding plasmids encoding either full-length cDNAs [FLSDX for WNV_{KUNV} (Khromykh et al., 1998)] or two large cDNA fragments that were then joined by ligation for WNV_{NY99} (Audsley et al., 2011). Full-length viral RNAs were transcribed *in vitro* from either linearised plasmid DNA (WNV_{KUNV}) or in vitro ligated and linearised DNA (WNV_{NY99}) and electroporated into BHK cells to recover viruses. Both WNV_{KUNV} and WNV_{NY99} were then passaged in Vero cells to produce virus stocks. Stocks were titrated by plaque assay using Vero cells. Infected cells were maintained in DMEM supplemented with 2% FBS (DMEM-2%).

2.2. Reagents

The CMA and the vehicle (TPGS: d-alpha tocopheryl polyethylene glycol 1000 succinate) were kindly provided by Prof. Max Reynolds (CEPH, Griffith University, Australia and 98 Alive™ PO Box 82 Underwood, QLD 4119 Australia). Terpinen-4-ol (Fluka/Sigma-Aldrich) and Tea Tree Oil (Thursday Plantation, Australia) were purchased. Lovastatin (LOV) (Mevinolin-Abcam Biochemicals), was diluted in DMSO and used as a control for inducing G0/G1 cell cycle arrest (Javanmoghadam Kamrani and Keyomarsi, 2008). Nocodazole (NOCO) (Sigma-Aldrich), was diluted in DMSO and used as a control for inducing G2/M cell cycle arrest (Chulu et al., 2010).

[2] Patent WO2008010188 A2.
[3] Patent WO2009044241 A1.

2.3. Cytotoxicity assays

Vero cells were seeded in triplicate in 96-well plates at a density of 2.5×10^4 cells/well in DMEM supplemented with 2% FBS (DMEM-2%). After removal of the medium, serial dilutions of CMA, Terpinen-4-ol, TTO, vehicle (TPGS), LOV and NOCO in DMEM-2% were added to the cells. Cytotoxic (100% dead) controls were obtained by treating the cells with Nonylphenol ethoxylate, Tergitol NP-9 (NP-9) (Sigma) at a final concentration of 0.01% v/v and non-treated (viable) controls with DMEM-2% alone. To determine cell viability, Sytox Green (Life Technologies) was added at a final concentration of 1 μM. Sytox Green fluorescent dye uptake was determined at the indicated times using a Flex Station plate spectrophotometer. The percentage of viable cells in samples was determined based on the relative percentage of dead cells compared to the 100% dead control value. Based on the cytotoxicity results, the maximal effective dose (ED) was the highest level of drug alone (MAC, Terpinen-4-ol or TTO) that showed no cytotoxicity. The tissue culture half maximal toxicity doses ($TCCD_{50}$) were also determined.

2.4. Inhibition of WNVKUNV-induced cytopathic effect

Vero cells were seeded in triplicate in 96-well plates in DMEM-2% and infected with WNV_{KUNV} at an MOI of 1, for 2 h at 37 °C. After washing with PBS to remove the viral inoculum, cells were treated with DMEM-2% containing: 0.0075 or 0.000375% v/v CMA, 0.004% v/v Terpinen-4-ol, 0.002% v/v TTO or 0.000375% v/v vehicle (TPGS). DMEM-2% was added to the non-treated control. Plates were incubated at 37 °C and cell viability was measured at the indicated times using the previously described Sytox Green method.

2.5. Viral replication kinetics

Vero cells were seeded in 96-well plates and infected with WNV_{KUNV} or WNV_{NY99} at an MOI of 1 for 2 h at 37 °C. After washing with PBS to remove the viral inoculum, treatments dissolved in DMEM-2% were added at concentrations equal to 0.0075 or 0.000375% v/v CMA, 0.004% v/v Terpinen-4-ol, 0.002% v/v TTO or 0.000375% v/v vehicle (TPGS). DMEM-2% was added to the non-treated control. Plates were incubated at 37 °C for 3 days. Culture supernatants were harvested every 24 h post-infection (h.p.i) and viral titres were determined immediately after harvesting using a standard plaque assay on Vero cells.

2.6. Virucidal assays

1×10^4 PFU of WNV_{KUNV} or WNV_{NY99} were incubated with the CMA or vehicle at the final concentrations of 0.0075 or 0.000375% v/v, respectively. DMEM-2% was added to the non-treated control. After 5 min, 30 min, 2 h, 5 h and 15 h of incubation in the dark at 37 °C, samples were diluted and immediately quantified using a standard plaque assay on Vero cells.

2.7. Flow cytometry, NS1 expression and cell cycle analysis

Vero cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well, infected with WNV_{KUNV} or WNV_{NY99} at an MOI of 1 and treated as described in the viral replication kinetics section. Every 24 h.p.i, cells were analysed for NS1 expression and/or DNA content/cell cycle. Cells for NS1 expression were harvested using 0.5% Trypsin-EDTA, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 (Sigma) in 4% formaldehyde, blocked with PBS containing 2% BSA and probed with mouse primary anti-NS1 (4G4) before rat anti-mouse conjugated with Alexa®488 IgG