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Inhibition of enterovirus 71 infection by antisense octaguanidinium dendrimer-conjugated morpholino oligomers

Chee Wah Tan [a], Yoke Fun Chan [a,b], Yi Wan Quah [c], Chit Laa Poh [c,*]

[a] Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Tropical Infectious Disease Research and Education Center, University of Malaya, 50603 Kuala Lumpur, Malaysia
[c] Faculty of Science and Technology, Sunway University, 46150 Petaling Jaya, Selangor, Malaysia

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ABSTRACT

Enterovirus 71 (EV-71) infections are generally manifested as mild hand, foot and mouth disease, but have been reported to cause severe neurological complications with high mortality rates. Treatment options remain limited due to the lack of antivirals. Octaguanidinium-conjugated morpholino oligomers (vivo-MOs) are single-stranded DNA-like antisense agents that can readily penetrate cells and reduce gene expression by steric blocking of complementary RNA sequences. In this study, inhibitory effects of three vivo-MOs that are complementary to the EV-71 internal ribosome entry site (IRES) and the RNA-dependent RNA polymerase (RdRP) were tested in RD cells. Vivo-MO-1 and vivo-MO-2 targeting the EV-71 IRES showed significant viral plaque reductions of 2.5 and 3.5 $\log_{10}$PFU/ml, respectively. Both vivo-MOs reduced viral RNA copies and viral capsid expression in RD cells in a dose-dependent manner. In contrast, vivo-MO-3 targeting the EV-71 RdRP exhibited less antiviral activity. Both vivo-MO-1 and 2 remained active when administered either 4 h before or within 6 h after EV-71 infection. Vivo-MO-2 exhibited antiviral activities against poliovirus (PV) and coxsackievirus A16 but vivo-MO-1 showed no antiviral activities against PV. Both the IRES-targeting vivo-MO-1 and vivo-MO-2 inhibit EV-71 RNA translation. Resistant mutants arose after serial passages in the presence of vivo-MO-1, but none were isolated against vivo-MO-2. A single T to C substitution at nucleotide position 533 was sufficient to confer resistance to vivo-MO-1. Our findings suggest that IRES-targeting vivo-MOs are good antiviral candidates for treating early EV-71 infection, and vivo-MO-2 is a more favorable candidate with broader antiviral spectrum against enteroviruses and are refractory to antiviral resistance.

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1. Introduction

Enterovirus 71 (EV-71) is a single-strand, positive-sense RNA virus. EV-71 usually cause mild hand, foot and mouth disease (HFMD) characterized by fever with papulovesicular rash on the palms and soles (Ooi et al., 2010). In recent years, EV-71 infections were also associated with neurological complications with high mortalities among infants and young children < 6 years old (Solomon et al., 2010). To date, no effective antiviral agent is available for clinical use (Shang et al., 2013b; Tan et al., 2014). Thus, there is an urgent need to develop effective antiviral agents to treat EV-71 infection.

Considering the morbidity caused by EV-71, new approaches to the development of therapeutics are needed. A number of promising RNA-based therapeutics designed to inhibit EV-71 infections have shown promising results, including siRNA and shRNA (Deng et al., 2012; Sim et al., 2005; Tan et al., 2007a,b; Wu et al., 2009). However, the limitations of RNA-based therapeutics are short half-life and it required a delivery agent which might be toxic to the host. Therefore, nucleic acid-based therapeutics should be designed to possess favorable pharmacological properties such as in *vivo* stability and low toxicity.

A phosphorodiamidate morpholino oligomer (PMO) is a single-stranded DNA-like compound that has the ability to bind to the mRNA and inhibit gene expression by steric blockage of complementary RNA. PMOs are highly nuclease-resistant and do not require the RNase H or other catalytic proteins for their activity (Kole et al., 2012; Summerton, 1999). PMOs have been conjugated with various cell-penetrating compounds such as cell-penetrating peptides and octaguanidinium dendrimers which are able to enhance their uptake by cells (Moulton and Jiang, 2009). Peptide conjugated-PMOs (PPMO) have been demonstrated to inhibit various viral infections, including Ebola virus (Warfield et al., 2006), West Nile virus (Deas et al., 2005), dengue virus (Kinney et al., 2005), sindbis virus (Paessler et al., 2008), coronavirus (Neuman

* Corresponding author. Tel.: +60 3 7491 8622x3837; fax: +60 3 5635 8633.
E-mail address: pohcl@sunway.edu.my (C.L. Poh).

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et al., 2004), herpes simplex virus 1 (Moerdyk-Schauwecker et al., 2009), porcine reproductive and respiratory syndrome virus (Opriessnig et al., 2011; Patel et al., 2008), foot-and-mouth disease virus (Vagnozzi et al., 2007), poliovirus, rhinovirus (Stone et al., 2008), and coxsackievirus B3 (Yuan et al., 2006).

In this study, three octaguanidinium dendrimer conjugated-morpholino oligomers (vivo-MOs) targeting the EV-71 internal ribosome entry site (IRES) core sequence and the RNA-dependent RNA polymerase (RdRP) were tested for their inhibitory effects against EV-71. We demonstrated that the two vivo-MOs targeting the IRES core sequence showed significant inhibition of EV-71 infection.

2. Materials and methods

2.1. Cells and viruses

Rhabdomyosarcoma (RD, ATCC) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS). EV-71 strains 41 (GenBank accession number: AF316321), BrCr (GenBank accession number: AB204852) and UH1/97 (GenBank accession number: AM396587); coxsackievirus A16 (CV-A16), PV and chikungunya virus (CHIKV) strain MY/08/065 (GenBank accession number: FN295485) were propagated in RD cells.

2.2. Vivo-MOs

All vivo-MOs were synthesized by Gene Tools LLC (USA). The 23-mer vivo-MOs were designed to be complementary to the EV-71 (strain 41) IRES stem-loop V-VI and the RdRP gene (Table 1, Fig. 1). All the vivo-MOs were dissolved in phosphate buffer saline (PBS) at concentration of 0.5 mM. The cytotoxicity of the vivo-MOs were evaluated using Cell Titer 96 AQueous cell proliferation reagent (Promega) according to the manufacturer's instructions.

Table 1
Sequence of the 23-mer vivo-MOs and target locations in EV-71 RNA.

Vivo-MOs	Sequence (5'–3')	Target location in EV-71 RNA (nucleotide position)
1	CAGAGTTGCCCATTACGACACAC	IRES core (512–534)
2	GAAACACGGACACCCAAAGTAGT	IRES core (546–568)
3	AAACAATTCGAGCCAATTTCTTC	3D Pol gene (7303–7325)
Control	CCTACTCCATCGTTCAGCTCTGA	–

2.3. In vitro inhibitory effects of vivo-MOs in RD cells

RD cells were seeded at 1.5×10^4 cells or 1.5×10^5 cells within each well of a 96-well plate or 24-well plate, respectively and incubated overnight at 37 °C in 5% CO_2. After overnight incubation, the growth medium was removed and replaced with EV-71 inoculum with a multiplicity of infection (MOI) of 0.1 (PFU per cell) and incubated at 37 °C for 1 h. After incubation, the inoculum was removed and replenished with maintenance medium (DMEM with 2% FBS) with or without vivo-MOs. The inhibitory effects of the vivo-MOs were evaluated by plaque assay, TaqMan real-time RT-PCR and western blot analysis 24 h post-infection (hpi) as previously described (Tan et al., 2012, 2013). The western blot signal was enhanced using SuperSignal® western blot enhancer (Pierce Biotechnology).

2.4. Time of addition assay

Vivo-MOs were added to RD cells at various time points relative to viral inoculation. RD cells were pre-incubated with vivo-MOs at final concentration of 5 μM for 4 h before EV-71 inoculation at a MOI of 0.1. In concurrent studies, both vivo-MOs and EV-71 were added into RD cells for 1 h followed by replacement of medium without vivo-MO. For post-infection studies, RD cells were infected with EV-71 for 1, 2, 4 and 6 h before vivo-MOs were applied. The viral titers for each experiment were quantitated 24 hpi by plaque assays.

2.5. In vitro inhibitory effects of vivo-MOs against various enteroviruses

To evaluate the efficacy of vivo-MOs against different enteroviruses including CV-A16 and PV, RD cells were pre-incubated with vivo-MOs at the final concentration of 5 μM for 4 h before viral

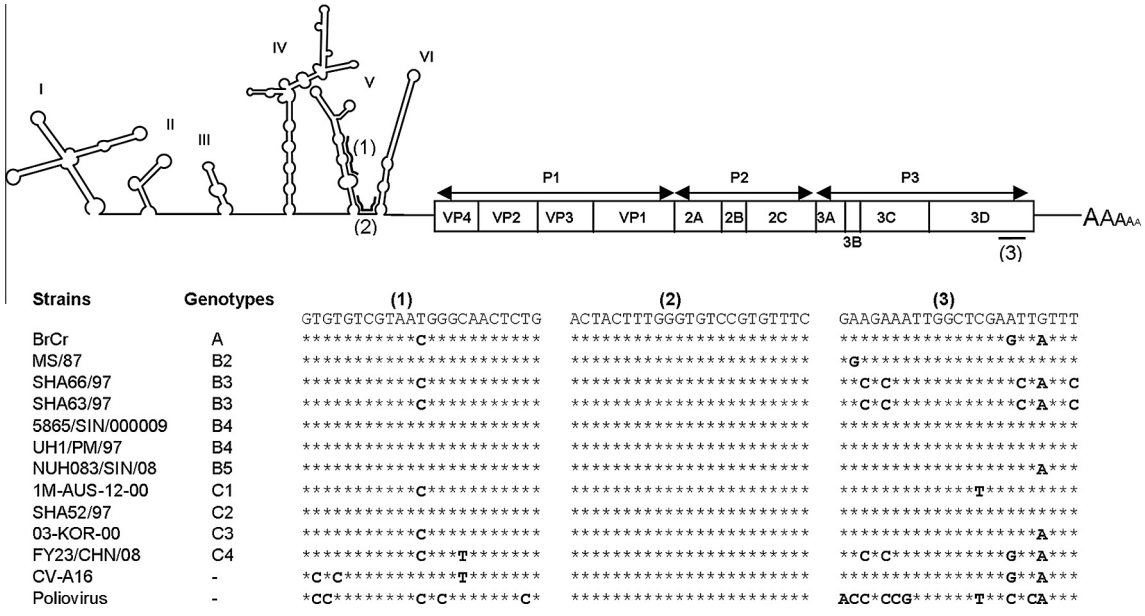

Fig. 1. Schematic illustration of the EV-71 genomic structure. Three genomic vivo-MOs target sequences (5' to 3') are indicated as (1)–(3) within the proposed secondary structures of the IRES region and the RdRP gene of EV-71 RNA. The sequences of these three targeted regions were aligned across all EV-71 genotypes, CV-A16 and PV.

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