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Phenotypic and genotypic characterization of induced acyclovir-resistant clinical isolates of herpes simplex virus type 1

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ABSTRACT

Eleven strains of acyclovir (ACV)-resistant herpes simplex virus type 1 (HSV-1) were generated from HSV-1 clinical isolates by exposure to ACV. Genotype of the thymidine kinase (TK) and DNA polymerase (*pol*) genes from these mutants were further analyzed. Genotypic analysis revealed four non-synonymous mutations in TK gene associated with gene polymorphism and two to three non-synonymous mutations in DNA *pol* gene. Seven and six strains contained at least one resistance-associated mutation at TK and DNA *pol* gene, respectively. Resistance-associated mutations within the TK gene consisted of 64% of non-synonymous frameshift mutations within the homopolymer region of G's and C's, and 36% of non-synonymous nucleotide substitutions of the conserved gene region (C336Y, R51W and R222H), nucleotide that produced stop codon (L288Stop) and two amino acid substitutions outside the conserved region (E39G & L208F). There were 10 non-synonymous amino acid substitutions located outside the conserved region with the unclear significance to confer resistance observed. Resistance-associated mutations in DNA pol gene include insertion of G at the homopolymer region of G's (794–797) and amino acid substitutions inside (V621S) or outside (H1228D) the conserved region. *In silico* analysis of the mutated TK (C336Y, R51W and L208F), and DNA *pol* (V621S and H1228D) suggested structural changes that might alter the stability of these proteins. However, there were several mutations with unclear significance to confer ACV-resistance identified, especially mutations outside the conserved region.

1. Introduction

Herpes simplex virus type 1 (HSV-1) is one of the most common viral infections throughout the world. In patients with immunodeficiency, including HIV infection or transplant-associated infection, HSV-1 can lead to death (Stranska et al., 2004). Transmission occurs by contact with secretion from an infected person with either overt infection or asymptomatic excretion of the virus (Piret and Boivin, 2011).

This easy transmission causes HSV-1 related diseases to be classified as contagious disease (US National Library of Medicine, 2012). Currently, acyclovir (ACV) and foscarnet (FOS) are the specific and effective drug of choice. ACV is a guanosine analogue antiviral drug, primarily used as the treatment for herpes simplex virus infection, which is selectively converted into acyclo-guanosine monophosphate (acyclo-GMP) by viral thymidine kinase and then further phosphorylated to acyclo-guanosine triphosphate (acyclo-GTP) by the cellular kinase. Acyclo-GTP acts as a substrate that competes with deoxyguanosine triphosphate (dGTP) to viral DNA polymerase resulting in premature DNA chain termination (Elion, 1983). FOS is a pyrophosphate analogue against herpesviruses, human immune deficiency virus (HIV) and either RNA and DNA viruses. This drug acts via inhibition of viral polymerase, which interferes with the exchange of pyrophosphate from deoxynucleoside triphosphate during viral replication by binding to a site of HSV DNA polymerase (Crumpacker, 1992).

Prolonged ACV treatment and uncontrolled use of generic drugs were the main factors of ACV-resistant HSV-1 evolution. The emergence of ACV-resistant isolates increased tremendously in 10–15 years (Korovina et al., 2010). In immunocompromised patients, there were 4–7% of ACV-resistant (Andrei et al., 2007; Chen et al., 2000; Englund et al., 1990; Wade et al., 1983; Wright et al., 2003) while in immunocompetent population, there was approximately 0.1–0.7% of ACV-resistant isolated (Bacon et al., 2002; Christopher et al., 1998; Collins and Ellins, 1993). The increment of ACV-resistant HSV-1 isolates was not limited only to the patients but also in children. According to Wang et al. (2011), the prevalence rate of ACV-resistant HSV-1 in children was higher than predicted. Moreover, multiple mechanisms leading to the resistance were identified, in which suggested that new anti-herpetic drugs with different mechanisms of action should be explored.

ACV-resistant HSV-1 contains either mutated thymidine kinase (TK) or DNA polymerase (*pol*) gene, or both. TK-deficient gene re-

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sulted in the prevention of phosphorylation of acyclovir to become acyclo-GMP, in which further phosphorylated by cellular kinases to be acyclo-GTP in which acts as the competitor to deoxyguanosine triphosphate (dGTP) (Bush et al., 2011). On the other hand, DNA *pol* gene mutation prevented the DNA chain termination by reducing the affinity to acyclo-GTP (Darby et al., 1984). Therefore, natural dGTP will be used instead of acyclo-GTP as the substrate of choice during the DNA chain elongation. Previous studies showed that 95% of acyclovir-resistant HSV-1 is caused by mutation at TK gene (Morfin and Thouvenout, 2003; Stranska et al., 2004). The mutations usually involved single amino acid substitutions throughout the TK gene, insertions or deletions of 1 or 2 nucleotides, especially in homopolymer runs of G's and C's (Gaudreau et al., 1998; Griffiths, 2011; Sauerbrei et al., 2010), amino acid substitutions at the nucleoside-binding site, ATP-binding site and the conserved region (Stranska et al., 2004). Meanwhile, DNA *pol* gene mutations mainly involved amino acid substitutions in the conserved region of the enzyme (Bestman-Smith and Boivin, 2003; Stranska et al., 2004).

As yet, however, there have been no studies for TK and DNA *pol* genes of acyclovir-resistant HSV-1 that were derived from clinical isolates in Malaysia. In this study, a clinical HSV-1 isolate was subjected to the several concentrations of acyclovir treatment to obtain ACV-resistant HSV-1. The genotypic characterization of eleven isolates was determined by amplification and sequencing of TK and DNA *pol* genes, and the stability of the TK and DNA polymerase protein of amino acid substitutions were further analyzed by *in silico* analysis.

2. Materials and methods

2.1. Virus and cells

African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (ATCC) (Rockville, USA). The viruses were grown and titrated using monolayers of the Vero cells. Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) (Flowlab, Australia) supplemented with 5% fetal bovine serum (FBS: Junior Scientific Inc, USA), penicillin–streptomycin (Sigma–Aldrich, UK), amphotericin B (PAA Labs, GmBH) and non-essential amino acid (Thermo Scientific, UK) incubated at 37 °C in a humidified air containing 5% CO_2. Clinical isolate of HSV-1 was obtained from virus stock culture, School of Biosciences & Biotechnology, Universiti Kebangsaan Malaysia (UKM). The DMEM medium was used without FBS for viral propagation.

2.2. Antiviral test compounds

The ACV (Sigma–Aldrich, GmbH) was used as the antiviral drug for the selection of drug resistant isolates and the phenotypic characterization of HSV resistance.

2.3. Multiple resistance selection assay

Eleven independently prepared pools of HSV-1 clinical isolates were individually incubated with 2 μg/mL of ACV in DMEM containing 5% FBS. After 1 h of incubation, the ACV-treated viruses were infected to Vero cells and overlaid with methylcellulose containing similar treatment. Isolated plaque from each pool was picked and further plaque purified with increasing ACV concentrations (2.0–2.5 μg/mL).

2.4. Phenotypic characterization of ACV resistance

Antiviral testing for ACV resistance was performed in 24-well plate by plaque reduction assay (PRA) in Vero cells culture, as previously described with some modifications (Swierkosz et al., 2004). Vero cells were seeded at density of 5×10^4 and incubated for two days. After the viral infection of 50 pfu/well and incubated for 1–2 h, infected Vero cells were overlaid by methylcellulose containing ACV compounds at a serial dilution over a range between 1.13 and 5.63 μg/mL. Infected Vero cells, in which overlaid by methylcellulose without ACV compound acts as virus control. There were four replicates were done for each ACV concentration tested. The plates were incubated at 37 °C in a humidified air containing 5% CO_2. After 2–3 days, infected Vero cells were fixed and stained with 0.4% crystal violet solution in a mixture of formalin (3% *v*/v) and ethanol (1.67% v/v) in distilled water for 40 min. Half maximum effective concentration (EC_{50}) for each isolates determined from the dose–response curves.

2.5. Genotypic characterization of ACV resistance

The genotypic analysis of resistance of HSV-1 was carried out by the amplification of DNA fragments of the viral TK and DNA *pol* genes as well as subsequent sequencing. The oligonucleotide primers were based on the previous research (Sauerbrei et al., 2010) which were constructed using reference HSV-1 strain 17 (GenBank accession No. X14112) (McGeoch et al., 1985). The TK gene of resistant isolates was amplified as one fragment and sequenced in 2 fragments, whereas the DNA *pol* gene was divided into 4 and 5 fragments, respectively. After isolation of DNA from the supernatant of virus-infected Vero cells using Viral Nucleic Acid Extraction Kit II (Geneaid, UK), viral DNA was amplified by polymerase chain reaction (PCR). Proofreading Phusion DNA polymerase enzyme (Thermo Scientific, UK) was used. Standard PCR mixture contained 0.5 μM of each primer plus approximately 50 ng DNA template. The reaction mixture was supplemented with 3% DMSO to improve target product specificity and yield during PCR amplification. After an initial denaturation step for 30 s at 98 °C, reaction mixtures were cycled 35 times through denaturation at 98 °C for 10 s and polymerization at 72 °C for 30 s followed by a final extension step at 72 °C for 10 min. Amplified DNA fragments were purified using the Gel/PCR fragments extraction kit (Geneaid, UK). Then, viral DNA was quantified spectrophotometrically. An amount of 150 ng DNA/μL was used for sequencing. Sequencing reactions of purified PCR products were performed using DNA using BigDye® Terminator V 3.1 cycle sequencing kit (Life Technologies, Malaysia) and 10 mM oligonucleotide primers. Sequencing reaction mixture with a total volume of 10 μL consisted of 1–5 μL of the purified viral DNA, 0.5–0.75 μL primers, and 2 μL sequence reaction mix containing DNA *pol* and labeled ddNTPs were further analyzed using 3730 × l DNA Analyzer (Applied Biosystem, USA). After initial incubation at 95 °C for 3 min to denature the template DNA, the thermal conditions of amplification followed were 25 cycles of 95 °C for 20 s, 50 °C for 15 s, and 60 °C for 60 s. A final extension step was done at 60 °C for 10 min. Sequences were analyzed by DNA Sequencing Analysis Software (Applied Biosystem, USA). Primary DNA sequence assembly and analysis were performed, and sequencing results were compared with published sequences of the reference strains HSV-1 strain 17 using the software BioEdit Sequence Alignment Editor Version 7.1.9 (Hall, 1999). All specified sequence positions of single nucleotide polymorphism corresponded to nucleotide positions in the reference strains.