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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.

2.6. Mutant model building

Crystal structure modeling was performed using YASARA Version 13.2.21 (Krieger et al., 2002). Crystal structures of human HSV-1 thymidine kinase (TK) in complex with ACV (Protein Data Bank [PDB]: 2KI5) (Bennet et al., 1999), a complex of the C-terminal 36 residues of UL30 bound to residues 1–319 of UL42 (UL42–UL30) of HSV-1 (Protein Data Bank [PDB]: 1DML) (Zuccola et al., 2000) and herpes simplex virus 1 DNA polymerase (Protein Data Bank [PDB]: 2GV9) (Liu et al., 2006) were chosen as the template for the corresponding mutant modeling and energy changes calculation. Structural models of HSV-1 mutants were done using mutate single residue protocol and energy changes were carried out in FoldX as implemented in the YASARA program suite (Van Durme et al., 2011). The conformer isomerism analysis was done using three conformation isomers. Energy changes for each mutation were calculated after protein repair using RepairPDB (FoldX energy minimization) function.

3. Results

Replication of ACV-sensitive strain was strictly inhibited by the ACV, whereas the resistant strains with either mutated TK or DNA *pol* replicated and formed plaque in Vero cells overlaid with methylcellulose containing 2.5 μg/mL (11.0 μM) ACV. This ACV concentration was chosen as theoretically, HSV-1 that can grow in $\geqslant$2.0 μg/mL ACV concentration correlates directly between *in vitro* resistance to acyclovir and clinical response to acyclovir therapy (Field, 2001; Safrin et al., 1994; Swierkosz et al., 2004). For phenotypic test confirmation, all ACV-resistant isolates was infected to Vero cells grown in DMEM containing 2.5 μg/mL of ACV and proven to produce plaques (Fig. 1). Half maximum effective concentration (EC_{50}) of all ACV-resistant isolates showed variable results in which above the resistance cut-off (2.0 μg/mL) (Table 1; Nos. 1–11 and Table 2; Nos. 1–11) whereas the ACV-sensitive isolates showed the EC_{50} below 1.13 μg/mL. The viral DNA products of TK and DNA *pol* genes with approximate size of 1306 bp (Fig. 2) and 3950 bp (data not shown) were successfully amplified via PCR. The genotypic characterization, including polymorphism-associated mutations of eleven isolates demonstrated in Tables 1 and 2 were established by sequencing of the TK and DNA *pol* genes.

In the TK gene, all ACV-resistant and ACV-sensitive HSV-1 in this study contained four polymorphism-associated mutations (Table 1; Nos. 1–12). For resistance-associated mutations, frameshift and non-synonymous mutations occurred to seven HSV-1 isolates (Table 1; Nos. 1, 3, 6, 7, and 9–11). By contrast, four ACV-resistant HSV-1 isolate showed no resistance-associated mutation in the TK gene (Table 1; Nos. 2, 4, 5, and 8).

Eleven ACV-resistant HSV-1 isolate (Table 2; 1–11) in this study contain at least two polymorphism-associated mutations in the DNA *pol* gene. We detected the following mutations: V905M in all isolates; P1124H in all isolates; T1208A in 10. For resistance associated mutation, there were seven isolates involved. These include amino acid substitutions and insertion of nucleotide after the homopolymer region (Table 2; Nos. 1–5, 8, and 11): H1228D; V621S; insertion of G at the nucleotide 794–797; insertion of G after nucleotide 1787.

In silico analysis of the mutant model revealed the effects of certain amino acid substitutions to the TK and DNA *pol* protein stability (Table 3; Nos. 1, 2, 9, 10 and 11). Amino acid substitution R51W, C336Y (Fig. 3) and L208F in TK gene showed significant free energy changes in which severely destabilizing the structure. Likewise, van der Waals interaction clashes and disrupted hydrogen bonds were also observed. In contrast, amino acid substitutions R222H, M46K, V90L, M130I, E296K and E296Q proven to stabilize the TK protein structure by the addition of new hydrogen bonds (Table 3; Nos. 3–8). In DNA polymerase gene, mutation in the conserved region (V621S) and outside the conserved region (H1228D; Fig. 4) was proven to disrupt the protein with the significant result of average free energy changes (Table 3; Nos. 10 and 11) with disrupted hydrogen bond. However, there were no van der Waals interaction clashes observed.

4. Discussions

This study reports on the phenotypic and genotypic characterization of 11 ACV-resistant isolates derived from ACV-sensitive clinical HSV-1 strain. ACV-sensitive isolate was also studied to assess heterogeneity within the TK and DNA *pol* gene and help to determine the frequency of polymorphism not likely to be related to ACV resistance.

Eleven isolates that can replicate in Vero cells supplemented with 2.5 μg/mL ACV contained mutated TK or DNA *pol* genes. This prevented the phosphorylation of ACV or DNA chain elongation (Safrin et al., 1994; Swierkosz et al., 2004). This study confirmed several polymorphisms-associated mutations within the TK gene of HSV-1 unlikely to be directly associated with ACV-resistance namely N23S, K36E, R89Q and A265T. These mutations have been described by Burrel et al. (2010), Chibo et al. (2004), Kudo et al. (1998), Morfin et al. (2000), Sauerbrei et al. (2010).

In contrary, we identified six amino acid substitutions in ACV-resistant isolates in which directly to be associated with ACV-resistance namely E39G, R51W, C336Y, R222H, L208F and L288Stop. Amino acid substitution E39G in UKM-6 has been previously reported as causing resistance to ACV (Bestman-Smith et al., 2001; Sasadeusz et al., 1997). However, Chibo et al. (2004) reported that this mutation was present in ACV-sensitive strain and suggests its

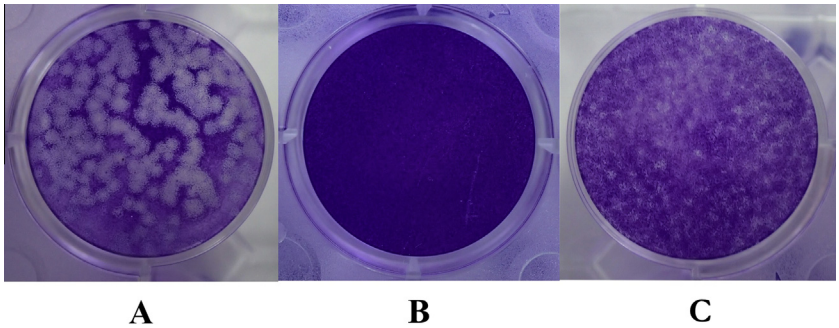

Fig. 1. Plaque formation of Vero cells infected with HSV-1 at 200 pfu/well. (A) Clinical isolates with no acyclovir treatment, (B) clinical isolates with 2.5 μg/mL acyclovir and (C) ACV-resistant isolates with 2.5 μg/mL.

Table 1
Phenotypic and genotypic analysis of HSV-1 thymidine kinase (TK) genes from acyclovir (ACV)-resistant (Nos. 1–11) and ACV-sensitive (No. 12) HSV-1. Novel mutations are in bold. Mutations with unclear significance are in Italics.

No.	Viral strain	Polymorphism-associated mutation (*ul23*)	Resistance-associated mutation (*ul23*)	Acyclovir (ACV) susceptibility (EC_{50}; μg/mL)
1.	UKM-1	N23S, K36E, R89Q, A265T	Homopolymer C region nt 666–669 (insertion of C), **L288Stop**, *E296K*, C336Y (cysteine active site), *E273C*	3.61
2.	UKM-2	N23S, K36E, R89Q, A265T	–	>5.63
3.	UKM-3	N23S, K36E, R89Q, A265T	*E296Q*, Homopolymer C region nt 883–887 (insertion of C)	>5.63
4.	UKM-4	N23S, K36E, R89Q, A265T	–	2.82
5.	UKM-5	N23S, K36E, R89Q, A265T	–	3.19
6.	UKM-6	N23S, K36E, R89Q, A265T	*G35R*, R51W(ATP-binding site), *K45E*, *M46K*, E39G, *V90L*, *V140I*, Homopolymer G region nt 182–185 (insertion of G)(ATP-binding site)	2.25
7.	UKM-7	N23S, K36E, R89Q, A265T	*L159F*, **L208F**, R222H (conserved region), Homopolymer G region nt 430–436 (insertion of G) Homopolymer C region nt 462–466 (insertion of C) Homopolymer C region nt 548–553 (insertion of C) (nucleoside-binding site) Homopolymer C region nt 666–669 (insertion of C) Insertion of C after nt 15	2.81
8.	UKM-9	N23S, K36E, R89Q, A265T	–	4.32
9.	UKM-10	N23S, K36E, R89Q, A265T	Homopolymer G region nt 430–436 (insertion of G)	>5.63
10.	UKM-11	N23S, K36E, R89Q, A265T	*M130I*	>5.63
11.	UKM-12	N23S, K36E, R89Q, A265T	Homopolymer G region nt 275–279 (insertion of G) Homopolymer G region nt 182–185 (insertion of G)	3.66
12.	Acyclovir-sensitive strain	N23S, K36E, R89Q, A265T	–	<1.13

Table 2
Phenotypic and genotypic analysis of HSV-1 DNA polymerase (*ul30*) genes from acyclovir (ACV)-resistant (Nos. 1–11) and ACV-sensitive (No. 12) HSV-1. Novel mutations are in bold.

No.	Viral strain	Polymorphism-associated mutation (*ul30*)	Resistance-associated mutation (*ul30*)	Acyclovir (ACV) susceptibility (EC_{50}; μg/mL)
1.	UKM-1	V905M P1124H T1208A	**H1228D**	3.61
2.	UKM-2	V905M P1124H T1208A	**H1228D** Insertion of G after nt 1787	>5.63
3.	UKM-3	V905M P1124H T1208A	**H1228D**	>5.63
4.	UKM-4	V905M P1124H T1208A	Homopolymer G region nt 794–797 (insertion of G) **H1228D**	2.82
5.	UKM-5	V905M P1124H T1208A	**H1228D**	3.19
6.	UKM-6	V905M P1124H T1208A	–	2.25
7.	UKM-7	V905M P1124H T1208A	–	2.81
8.	UKM-9	V905M P1124H T1208A	**H1228D** **V621S** (Delta C Exo III)	4.32
9.	UKM-10	V905M P1124H T1208A	–	>5.63
10.	UKM-11	V905M P1124H T1208A	–	>5.63
11.	UKM-12	V905M P1124H T1208A	**H1228D**	3.66
12.	Acyclovir-sensitive strain	V905M P1124H T1208A	–	<1.13

role may be indirect. Besides that, amino acid substitution R51W (ATP-binding site) in the same isolate involved in contributing ACV resistance, correlates with previous research (Andrei et al., 2005; Chibo et al., 2004; Duan et al., 2009; Frobert et al., 2008; Stranska et al., 2004). This mutation causes TK gene to be unable to transfer a phosphate group from ATP to an unphosphorylated

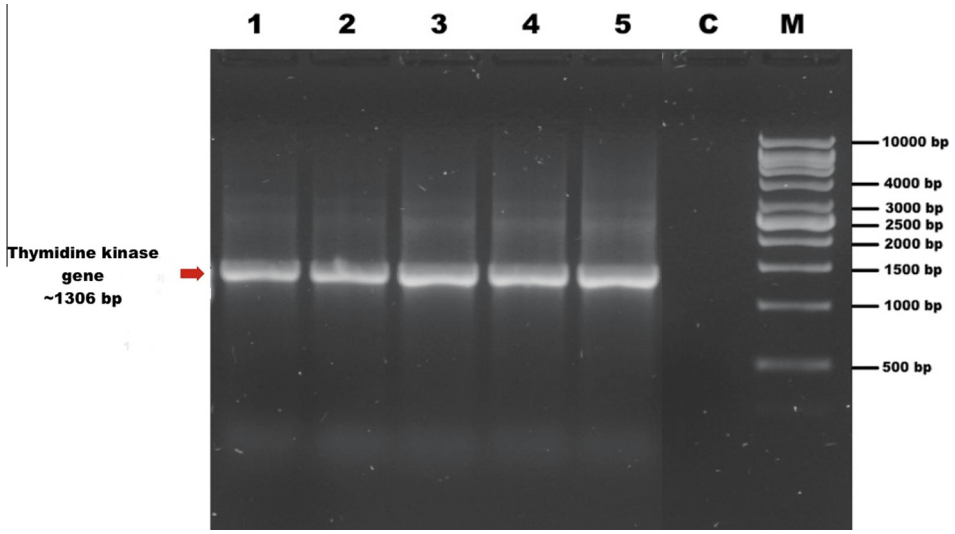

Fig. 2. Amplified TK gene (~1306 bp) from UKM-1 to UKM-5 isolates. Well 1: UKM-1, 2: UKM-2, 3: UKM-3, 4: UKM-4, 5: UKM-5, C: No DNA, M: 1 kb DNA ladder.

Table 3
Average free energy changes, van der Waals interaction clashes and disrupted hydrogen bonds in thymidine kinase (TK) (Nos. 1–9) and DNA polymerase (*pol*) (Nos. 10–11).

No.	Mutations	Average free energy changes[a] (kcal/mol)	van der Waals interaction clashes (residue involved)	Disrupted hydrogen bonds
Thymidine kinase (TK)				
1.	R51W	16.90	Yes (Trp51, Phe161, Leu159, Leu49 & Ile361)	Yes
2.	C336Y	13.53	Yes (Tyr336, Tyr329 & Gln331)	Yes
3.	R222H	-23.47	No	Yes
4.	M46K	-22.94	No	Yes
5.	V90L	-24.45	No	Yes
6.	M130I	-22.21	No	Yes
7.	E296K	-23.65	No	Yes
8.	E296Q	-23.27	No	Yes
9.	L208F	41.62	Yes (Phe208 & Tyr329)	Yes
DNA polymerase (pol)				
10.	V621S	85.57	No	Yes
11.	H1228D	51.87	No	Yes

[a] Free energy changes ($\Delta\Delta G$(change) calculated based on three protein models runs. $\Delta\Delta G$(change) > 0.5 kcal/mol: the mutation is destabilizing, $\Delta\Delta G$(change) < −0.5 kcal/mol: the mutation is stabilizing.

thymidine (dT) (Brown et al., 1995). Consequently, ACV could not be phosphorylated by TK to form ACV-GMP and prevented the termination of chain elongation. *In silico* analysis that was performed consolidated this finding by destabilization of the TK protein structure via van der Waals interaction clashes and disrupted hydrogen bond (Table 3; No. 1). Likewise, the mutation in the cysteine-active site (C336Y) was proven to confer ACV-resistance in HSV-1 isolate (UKM-1) (Duan et al., 2009; Saijo et al., 2002; Sergerie and Boivin, 2006; Stranska et al., 2004). Crystal structure of this mutant revealed van der Waals interaction clash between Tyr 336, Tyr 329 and Gln 331 (Fig. 3C). According to Berg et al. (2002), as the atoms with van der Waals interactions were too close to each other, the potential energy due to repulsion was exceptionally high and weaken the structure. This finding was in agreement with the calculated energy changes (Table 3; No. 2). This significant energy change also correlated with the finding by Evans et al. (1998) which suggested that cystein at or very near to position 336 is strongly conserved, and any amino acid substitution at this site will destabilize the three-dimensional structure of the active site.

One isolate (UKM-7) had amino acid substitution in the conserved region within the gene, R222H. Duan et al. (2009) reported that the same amino acid substitution involved in ACV-resistance. Although the average energy change was proven to stabilize the structure (Table 3; No. 3), Wang et al. (2011) reported that amino acid substitution in this residue reduced the ACV-binding pocket and renders ACV-resistant HSV-1. Interestingly, a novel amino acid substitution located outside the conserved region, L208F also showed significant destabilization of TK protein structure (Table 3; No. 9). This happened due to hydrogen bond and van der Waals interaction destruction. In UKM-1, L288Stop resulted in a stop codon that signals a termination of translation in UKM-1 isolate. A considerable part of amino acid substitutions could be defined as resistance-associated in connection with the phenotypic findings. However, both of novel substitution L208F and L288Stop have not yet been reported in any previous studies.

There were 10 out of 16 amino acid substitutions identified located outside the conserved region of TK gene (Table 1; Nos. 1, 3, 6, 7, 10). These substitutions include E296K, E296Q E273C, G35R, K45E, M46K, V90L, V140I, L159F and M130I, which have not been documented previously. Although the average energy changes calculated of certain amino acid substitutions (R222H, M46K, V90L, M130I, E296K and E296Q) revealed that these mutations stabilized the TK protein structure. The presence of these mutations does not necessarily rule out their role singly in ACV-resistant, and require additional experiments for clarification. In order to validate whether these mutations were resistance-associated mutation, more HSV-1 isolates to be examined for the polymorphism of TK gene in further studies to facilitate the interpretation of mutations

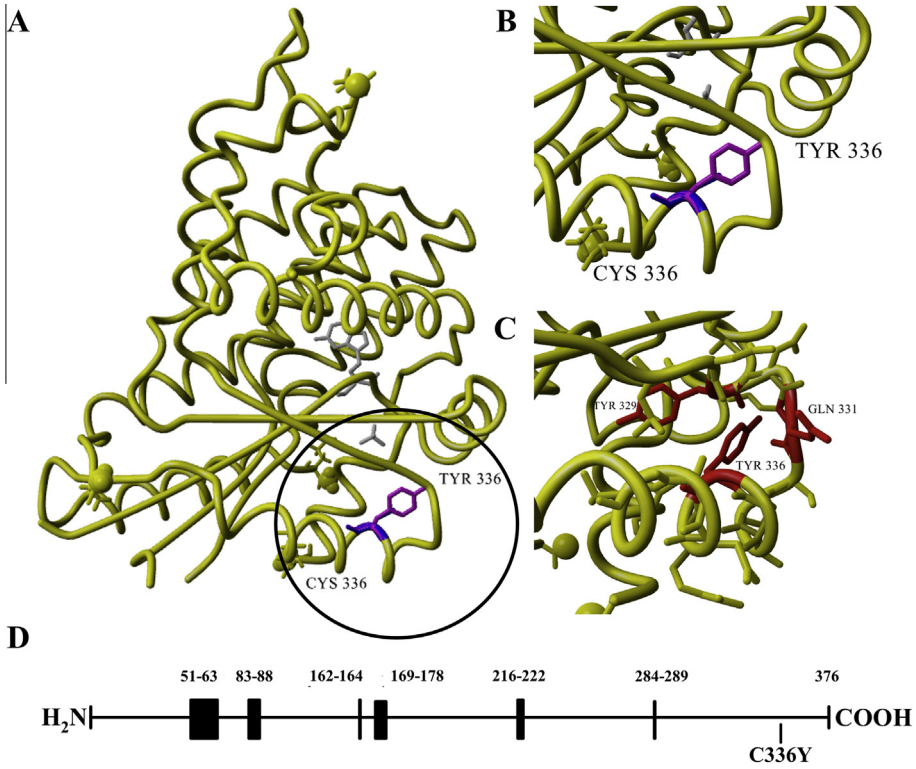

Fig. 3. Structure modelling of HSV-1 thymidine kinase (TK) (yellow) in complex with ACV (grey) (Protein Data Bank [PDB] accession No. 1DML (Zuccola et al., 2000). (A) Stereoscopic tube diagram of C336Y mutation. The wild type (blue) and mutant (magenta) are shown. (B) Zoomed-in view of C336Y mutation. (C) Zoomed-in view on the mutated C336Y shows van der Waals interaction clashes (red-coloured atom) between Tyr336, Gln331 and Tyr329. (D) The position of C336Y mutation in the TK protein. The conserved regions are indicated as black blocks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

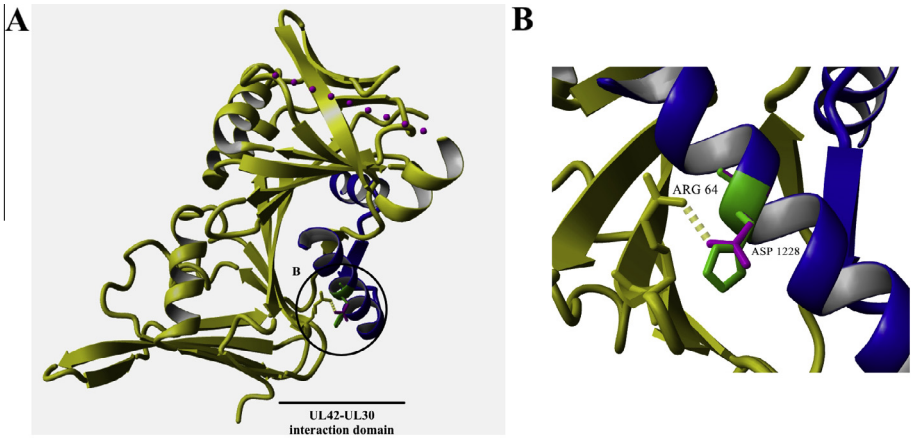

Fig. 4. Protein–protein interaction between UL42 processivity factor and UL30 DNA polymerase of HSV. (A) Ribbon diagram of the H1228 D mutation in UL42 (yellow)/UL30 (blue) complex. (B) Zoomed-in view of H1228D shows intermolecular hydrogen bond between R64 residue from UL42 processivity factor and H1228 (wild type) (green) or D1228 (mutant) (magenta) residues from UL30 DNA polymerase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

connected with resistance. However, there are six resistance-associated mutations involving the insertion of nucleotide at the homopolymeric region and two insertions of nucleotide throughout the TK gene in which the most common mechanisms to confer resistance. These alterations, most often located in homopolymer C regions at nucleotide positions 548–553, 462–466, 666–669 and 883–887, as well as homopolymer G region at nucleotide positions 182–185, 275–279 and 430–436, were well described in the literature (Chibo et al., 2004; Duan et al., 2009; Frobert et al., 2008; Morfin et al., 2000; Saijo et al., 2002; Sauerbrei et al., 2010; Stranska et al., 2005). These mutations result in the formation of a premature stop codon downstream from the shift, a truncated protein with no TK activity (Sasadeusz et al., 1997) or production of low level TK (Griffiths, 2011) and elongated TK polypeptide (Saijo et al., 2002, 1999). UKM-7 isolate demonstrated the most homopolymeric mutations (Table 1; No. 7). However, according to Sasadeusz et al. (1997), majority of ACV-resistant clinical isolates contains frameshift mutation, which functioned as the hot spot within the HSV TK gene. For this reason, we can conclude that treatment with acyclovir during the isolation and purification

of acyclovir-resistant HSV-1 indirectly induced the frameshift mutation at several sites of this isolate.

Heterogeneity within HSV DNA polymerase (*pol*) has not previously been assessed extensively. There were no polymorphisms associated mutations in the conserved region observed. Several polymorphism-associated mutations were identified in this study, including V905M, P1124H, T1208A, which were located outside the conserved region. This finding correlated with the report published previously by Sauerbrei et al. (2010). By contrast, Wang et al. (2011) reported that V905M contributed to the resistance of clinical isolate HSV-1 from children. However, there remains possibility that this polymorphism associated mutations may be contributed to ACV-resistant in complement with other mutations. We identified amino acid substitutions and homopolymer G insertion in both conserved and non-conserved regions of DNA *pol* of four ACV-resistant isolates with wild-type TK sequences (Table 2; Nos. 2, 4–5, 8). This involved a new amino acid substitution which located outside the conserved region and have never been described as part of polymorphism. These include H1228D in all four isolates, a novel amino acid substitution V621S in the conserved region (Exo III motif) in UKM-9, insertion of G after nt 1787 in UKM-2, and insertion of G at homopolymer region nucleotide 794–797 in UKM-4 isolate. Although H1228D mutation has not extensively described as in the literature, crystal structure of this mutant showed that amino acid substitution at this site directly disturbed the hydrogen binding between R64 from UL42 and H1228 from UL30 (Fig. 4). According to Zuccola et al. (2000), this hydrogen binding is crucial for the side chain-side chain interaction between UL42 and UL30. Therefore, any disturbance of hydrogen binding at this specific site might affect the UL42-UL30 electrostatic and hydrophobic interaction. Bridges et al. (2001) reported that this residue was the binding site for UL42 (processivity subunit) to ensure optimal function of DNA *pol* gene. Thus, this finding has really consolidated the initial idea by Darby et al. (1984) in which suggested that DNA polymerase mutant reduced the affinity of DNA polymerase for acyclo-GTP and confer the ACV resistance. Likewise, viruses carrying mutations within the Exo II and Exo III motif have been previously associated with more than 40% ACV resistance in herpes simplex virus (Morfin and Thouvenout, 2003). Besides that, Gibbs et al. (1991) documented that Exo III mutations can result in viruses displaying ACV resistance. This suggests that UKM-9 isolate, which contains amino acid substitution V621S in Exo III conserved region, may have influences on drug resistant phenotype. *In silico* analysis for V621S exhibited massive DNA polymerase protein structure destabilization by disruption of hydrogen bond (Table 3; No. 10). Therefore, all of our findings related to protein stability did correspond with the finding by Pakula and Sauer (1989), which disruption of hydrogen bond or electrostatic interaction can contribute to protein destabilization, although the destabilization caused by this substitution smaller compared to severe core mutations.

Our results demonstrated the diversity of the mechanisms involved in HSV-1 resistance to ACV. Most mutations conferring resistance to ACV derived from HSV-1 clinical isolates occurred in the TK gene, usually in the homopolymer region of G's and C's (64%). These findings correlate with the previous research (Frobert et al., 2008; Gaudreau et al., 1998; Griffiths, 2011; Hwang et al., 1994; Sasadeusz et al., 1997; Sauerbrei et al., 2010; Stranska et al., 2005, 2004). Nucleotide substitutions producing stop codons in novel locations or amino acid substitutions were also identified (36%). Furthermore, mutations in TK active sites (C336Y, R51W and R222H) and outside the conserved region (L208F, E39G and L288Stop) were also found. In conserved and non-conserved regions within the DNA pol gene, amino acid substitutions in novel location (H1228D and V621S), and mutations in the homopolymer region were identified. In conclusion, recombinant phenotyping of herpes simplex virus is crucial for future research using co-transfection of overlapping cosmids and plasmids into Vero cell (Bestman-Smith and Boivin, 2003) or cloning of mutant genome into a plasmid as bacterial artificial chromosomes (BACS) (Gierasch et al., 2006) especially for those mutations located outside the conserved region of TK and DNA *pol* gene.

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