ISSN : 0974 - 7532 Volume 10 Issue 5

Research & Reviews in

BioSciences

Regular Paper

RRBS, 10(5), 2015 [166-172]

The proposed action of styrylpyrone derivative in hsv-1 infected vero cells by differential gene expression

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ABSTRACT

This study was done to identify and characterize specific cellular genes expression during infection of host with HSV-1 and treatment with styrylpyrone derivative (SPD). SPD showed antiviral activity with different modes of action against HSV-1. SPD was effective in inhibiting cell death when the substance was added at 2 hours to 4 hours post infection. Cell death was only observed when treatment was delayed to 5 and 6 hours post infection. Positive effect to this mode of action suggests that SPD were able to treat HSV-1 infected cells at two effective concentration of $1.563x10^{-7}$ µM and $7.813x10^{-8}$ µM in treatment mode [S+V]+SPD. Differential gene expression (DEG) method was used to determine and isolate the genes that are differentially expressed in HSV-1 infected Vero cells with and without treatment with SPD. Results from DEG analysis showed that a total of 177 genes were expressed differentially with 89 cDNAs candidates were induced and 88 cDNAs candidates were repressed. All the genes were determined by their nucleotide sequences that were compared with the database from Genbank via bioinformatic analysis. Eight markers from DEG analysis were chosen and their expressions were confirmed by using Real-Time PCR. Results from Real-Time PCR showed 100% correlation in the markers' expression profile showed by DEG method. The cDNA markers that were induced in their expression include mitogen-activated protein kinase, tapasin, carboxypeptidase M, RAB member RAS oncogen family, p53and G protein-coupled receptor. We found that SPD induced apoptosis, as measured by oncogene family gene expression and caspase activation. The apoptosis mediated by SPD in infected cells was associated with the activation of p53 and Bcl-2 protein via intrinsic pathway. SPD also exerts its anti-HSV-1 properties by activating an extrinsic pathway via caspase-8 activation in infected cells. From this study, the understanding on how SPD acted upon HSV-1 infected host cells during the infection process was proposed. In this study it was shown that SPD has a potential in controlling HSV-1 infection selectively without disturbing non-infected cells.

KEYWORDS

Goniothalamusumbrosus;
Styrylpyrone derivative;
Antivirus;
Differential gene expression;
Herpes simplex virus type-1.

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INTRODUCTION

Styrylpyrone derivative (SPD) is a plant-derived pharmacologically active compound extracted from *Goniothalamus sp*. The plant is grown by generations of traditional practitioners mostly for post-partum-related healthcare and family planning. Report by Azimahtolet al.[1,2] showed that styrylpyrone derivatives from *Goniothalamus sp.* are potent anti-tumor with profound anti fertility properties attributed by its progesterone and estrogen antagonistic activities. Previously reported that SPD inhibited the proliferation of MCF-7 human breast cancer cells by inducing apoptotic cell death, while having minimal effectson non-malignant cells[9].

Multiple strains of herpes viruses exist. It has been difficult to develop an agent that will not simultaneously injure or kill the host cell. Viruses can remain latent for years. Currently, there are no antiviral agents that can eliminate this latency. In addition, viruses canchange genetically, thereby making them resistant to antiviral agents over time[4]. Previous studies have shown that infection of Vero cells with HSV-1 will be treated with SPD. In this study, we determined the differential gene expression of Vero cells infected or non-infected with herpes simplex virus type-1 (HSV-1) and treated with SPD. From this study, the understanding on how SPD acted upon HSV-1 infected host cells during the infection process was proposed. In this study it was shown that SPD has a potential in controlling HSV-1 infection selectively without disturbing non-infected cells.

The differential display PCR is a powerful method with which is possible to identify altered gene expression at the mRNA level in any eukaryotic cell. This procedure allows for selection of differentially expressed genes at the molecular level as the fragments of cDNA obtained by PCR amplification are easily cloned and sequenced[5]. In these study, we apply the differential display technique to investigate the pattern of mRNA expressed in HSV-1 infected Vero cells with or without SPD treatment.

MATERIALS AND METHODS

Cell and viruses

Vero cells (monkey kidney cell line) and HSV-1 were obtained from the stock cultures of the Microbiology Laboratory, School of Bioscience and Biotechnology, Faculty of Science and Technology, UKM.

Plant collection

The plant was collected at an area of Sungai Kelantan, Kelantan, Malaysia anda voucher specimen was deposited in collectionSchool of Bioscience and Biotechnology, Faculty of Science and Technology, UKM. (Voucher No: NZS 3148).

Extraction of SPD

The dried powdered bark of *G. umbrosus*(500 g) was extracted in a Soxhlet apparatus with light petroleum ether for 72 hours. Evaporation of the light petroleum ether resulted as a mixture of solid and thick brown oil (10 g). Recrystallisation of light petroleum yielded white crystals (1 g) which were identified as SPD[7].

Plate Processing Technique for Cytotoxicity Test and Antiviral Assay. MTT (Tetrazolium blue) assays were used to evaluate the reduction of viability of cell cultures in presence and absence of SPD. After 48 hour incubation, the microtitre plates were processed using the modified MTT assay[3,11]. Absorbance of released dye was recorded by using microplate reader (LabsystemsMulticanMultisoft) at ë=540 nm. Readout from each well was normalize against the absorbance from empty wells and data was presented as the percentage of survived cells compared to control cells Semple[12] and the LC_{50} valueswere calculated.

Cytotoxicity test

The cytotoxic effect of SPD and ACV towards Vero cells was investigated by the MTT method. The purified SPD (1 mg) was first reconstituted in 50 ìl DMSO and then further diluted with 950 ìl Minimal Essential Medium (MEM) with 5% fetal bovine serum (GIBCO)-MEM/FCS to obtain a $1x10^3$ µg/mL ($5x10^{-6}$ µM) stock solution. The cytotoxicity assays were carried out using 0.1 mL of cell suspension, containing $2.5x10^5$ cells/mL seeded in each well of a 96-well microtitre plate. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Confluent Vero cells were treated with SPD that was diluted from $1x10^3$ µg/mL ($5x10^{-6}$ µM) to $3.815x10^{-3}$ µg/mL ($1.907x10^{-11}$ µM). Control cells were incubated without test sample. The microtitre plates were incubated at 37 ºC in a humidified incubator with CO2 5% for a period of 48 h. Nineteen wells were used for each concentration of the

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test sample. The morphology of the cells was inspected daily and observed for microscopically detectable alterations.

Antiviral assay

The antiviral activity early screening was done to observe the antivirus activity effect of SPD towards HSV-1. SPD from $1x10^{3}$ µg/mL ($5x10^{-6}$ µM) to 1.953 µg/mL ($9.766x10^{-9}$ µM) were tested on four different virus concentrations which is $1CD_{50}$, $5CD_{50}$, $10CD_{50}$ and $50CD_{50}$. Three modes of treatments to detect anti HSV-1 activity in each of the fraction according to NurulAiniet al. (2006) and Nazlina et al. (2008) with some modification which is (i) cells were inoculated with virus 2, 4, 5, 6, 8 and 10 hours before treatment with SPD, (C+V)+SPD; (ii) the virus was inoculated to the cells one day after treatment with extract, (C+SPD)+V and (iii) the virus and extract were inoculated simultaneously to cells, C+(V+SPD). The viral concentrations used for cell inoculations were fixed at10CD_{50}. Antiviral activities were determined using the MTT assay as above. Acyclovir (ACV) (Sigma-Aldrich), $1.25x10^{3}$ mg/mL was used as a positive control.

Differential gene expression in cells infected with HSV-1 and treated with SPD

Vero cells were infected with HSV-1 at 2, 4, 5, 6, 8 and 10 hours. Then, SPD was added at 2, 4, 5, 6, 8 and 10 hours post infection. Total RNA was isolated from infected and treated Vero cells (Epicentre, USA) and subjected to RT-PCR using 20 universal primer pair (GeneFishing™DEG Premix Kit, Seegene). cDNAs fragments were then reamplified, cloned into yT&A cloning vector (Yeastern Biotech Co., Ltd.) and sequenced. Sequence results were analyzed by blastn to determine the gene function.

Analysis of relative gene expression data using real-time quantitative PCR

For LightCyclerusing Multicolor iCycler Real-time PCR mechine from BioRad, reaction a mastermix of the following reaction components was prepared: 1 ìL (100 ng) RNA, 5 ìL 2X Green RT-PCR PreMixMasterAmp™ Green Real-Time RT-PCR (Epicentre, USA), 0.5 ìL forward primer (10 ìM), 0.5 ìL reverse primer (10 ìM) (TABLE 1), 5 ìL free nuclease water and 0.5 ìL RT DNA Polymerase. The following LightCycler experimental run protocol was used: denaturation program (95ÚC for 1 min), amplification and quantification program repeated 35 times (95ÚC for 10 s, 62ÚC for 30 s, 72ÚC for 30 s with a single fluorescence measurement) mealting curve program (55ÚC ke 95ÚC) and finally a cooling step to 40ÚC. The change in expression of the target gene normalized to *â*-actin. The Ct values provided from real-time PCR then were analyzed using $2^{-\Delta\Delta C}T$ method[10].

RESULTS AND DISCUSSION

Cytotoxicity Test. Cytotoxic assay was performed to determine the LC_{50} of SPD and of ACV. SPD ex-

TABLE 1: List of putative genes that were choose for real-time PCR analysis

	Primer Nucleotide sequences
Carboxypeptidase M	(F) 5' TTGTGTTGTTAGGGTTTGGAGGTATCT 3' (R) 5' TTTTCTTTCAAACGGAATCTTGCTCT 3'
Tapasin	(F) 5' TTGTGTCTGTATTCTTTAGGAGGGGC 3' (R) 5' GGGGTCACTTCAGGTGGCAGG 3'
Mitogen-activated protein kinase	(F) 5' ACCTCCCTTGTCTCAGATGGTGTGT 3' (R) 5' TGTTGGTTGGGTTGTTGGTTTCTAT 3'
Ras	(F) 5' GCTGAGGCTGGAGAATCGCTTGAAT 3' (R) 5' TTTGGTTGTGAGACAGGGGTTTGCT 3'
G protein-coupled receptor	(F) 5' CCTTGTTGTTTTGGTTTGTTGAGACAGAGT 3' (R) 5' TGCCTGTGAATCCCACGCTACTCG 3'
p53	(F) 5' GGGGGTGGCTACTGTCCG 3' (R) 5' GGTTCGTATGTTAGTAGTGACAGGGT 3'
Ubiquitin	(F) 5' TGCTCGGCGTGAGGGAGTATGG 3' (R) 5' CAACATCCTAACTCGTGCTCTGGGTGA 3'
Calmodulin	(F) 5' TTATCAGGCATCTCTCCAGTCAATCCCC 3' (R) 5' TGCGTGACCCCTTCTGAGCACAACA 3'
β-actin	(F) 5' TGGGTCAGAAGGATTCCTATGTG 3' (R) 5' TCGTCCCAGTTGGTGACGAT 3'

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hibited cytotoxic effect towards Vero cells in a concentration-dependent manner. The LC_{50} value of SPD towards Vero cells was 31.25 µg/mL ($1.563x10^{-7}$ µM)and the LC_{50} value of acyclovir towards Vero cells was $1.25x10^{9}$ µg/mL. This result indicated that SPD appeared to be least toxic than ACV. No significant changes in cellular morphology were observed in SPD-treated cells at a concentration of 31.25 µg/mL as compare to normal Vero cells. Results from cytotoxicity are important to determine the actual concentration of SPD wasused in the antiviral test.

Early Screening of SPD Antiviral Activity against HSV-1. SPD inhibited different concentration of HSV-1 infection in a concentration-dependent manner (Figure 2). At $1CD_{50}$ HSV 1 concentration, the EC_{50} value were 4.290 µg/mL, at $5CD_{50}$, the EC_{50} value were $1.188x10^{2}$ µg/mL, at $10CD_{50}$, the EC_{50} value were $2.125x10^{2}$ µg/mL and at $50CD_{50}$, the EC_{50} value were $2.375x10^{2}$ µg/mL.

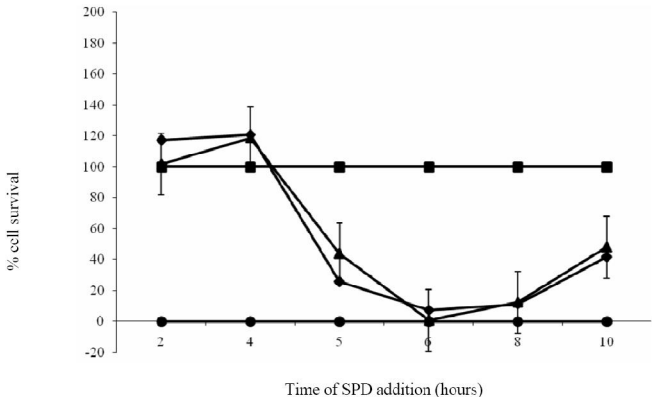

Figure 1 : Effect of the addition of the SPD against anti-HSV-1 activity in the first line of treatment (I = [S + V] + E) at concentrations of SPD (²%) 31.25 pg / mL and (♦) 15.63 pg / mL and the concentration of HSV-1 10CD50 after 2, 4, 5, 6, 8 and 10 pj. (■) represent control cells and (●) represents the negative control

Antiviral Activity of SPD at Different Times of Addition. To study the inhibitory effect of SPD on the stage of HSV-2 infection, the compound was added at different periods which is before, during and after of virus infection. Results showed that SPD effectively inhibited HSV-1 infection in the first treatment, I= (C+V)+SPD which is SPD was added after 2, 4, 5, 6, 8 and 10 hours post-infection (Figure 1). These times of treatment were choosing according to complete life cycle of HSV which is about 12 hours (Ward & Stevens 1975). The effectiveness of SPD in treating infected cells was gained through this treatment method. Our result demonstrated that SPD reacted in early stage of HSV-1 life cycle from 2 to 4 hours post infection before the percentage of cells survival reduced on 5 and 6 hours post infection. Maximum antiviral activity of SPD is 118.7% cells survival on 31.25 µg/mL ($1.563x10^{-7}$ µM) concentration of SPD on 4 hours post infection comparing with 120.7% on 15.63 µg/mL ($7.813x10^{-8}$ µM) SPD concentration. At 2 to 4 hours prior to virus infection, SPD showed their antiviral activity according to the percentage of cell survival compare to infected cell without any treatment with SPD. Antiviral activity of SPD occurred in the early stage of HSV-1 replication cycle after the adsorption and penetration was completed. At 2 to 4 hours prior to virus infection, SPD showed their antiviral activity according to the percentage of cell survival compare to infected cell without any treatment with SPD. This result suggested that antiviral activity of SPD occurred in the early stage of HSV-1 replication cycle after the adsorption and penetration was completed because in herpes virus infection, adsorption and penetration usually occurred in first 2 hours after infection[6].

Pre-treatment of cell with extract provides a protection against the infecting virus. Treatment of cells for 24 hours with SPD before viral infection in treatment (C+SPD)+V represents the capability of the SPD to protect the cells from viral attachment and hence giving us the idea of prophylactic effect of the compound. We suggested that SPD have capability in changing receptor on cells preface or stimulate cell protection mechanism. Besides that, SPD also have possibility in competed with virus glycoprotein to bind on cell membrane. Our result showed that pra-treatment cells with 31.25 µg/mL ($1.563x10^{-7}$ µM) SPD caused 39.3% cells survival compared with 44.7% for post-treatment cells. Meanwhile pra-treatment cells with 15.63 µg/mL ($7.813x10^{-8}$ µM) SPD caused of 10.2% cell survival compared 15% for post-treatment cells (Figure 2). SPD was found not to have prophylactic effect as demonstrated this treatment mode.

Simultaneous addition of virus and test compounds to cell culture represent whereas that anti-HSV-1 SPD caused by it capability to act directly on viral particle such as modification of free viruses surface's that inhibits viral attachment to host cells[13]. Test was carried out when SPD were added simultaneously into virus incubation within 0, 1, 2, 4 and 6 hours. Result showed that

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SPD was not found reacted as antiviral agent in protected cells from virus infection but SPD showed capable to treated disease cause by the virus infection (Figure 3).

Differential gene expression in cells infected with HSV-1 and treated with SPD. Results from DEG analysis showed that a total of 177 genes were expressed differentially with 89 cDNAs candidates were induced and 88 cDNAs candidates were repressed. All the genes were determined by their nucleotide sequences that was compared with the database from Genbankvia bioinformatic analysis. Eight markers from DEG analysis were chosen and their expressions were confirmed by using Real-Time PCR. Results from Real-Time PCR showed 100% correlation in the markers' expression profile showed by DEG method (TABLE 2). HSV-1 could alter host cell gene expression through the action of virion proteins or by synthesis of new viral proteins after infection. HSV-1 infection has been shown to perturb cell cycle progression, which leads to changes in gene expression. Viral factors, induced cellular factor and changes in cell cycle progression have the potential to exert profound effects on host cells gene expression[14]. Many cellular gene have been identified whose activity changes after HSV-1 infection and after SPD pre-treatment. From the time of infection and treatment with SPD study conducted, the following mechanism of SPD action is proposed. Antiviral activity of SPD occurred in the early stage of HSV-1 replication cycle that is after the adsorption and penetration. At 2 hours post infection (pi), the virus starts to repress host cell protein productivity by repressing the ribosome biogenesis protein. However, the SPD overcomes this action. At 4 hr and 5 hr pi, adenine nucleotide translocator was expressed which is indicated by viral nucleic acid replication that consumes the host cells energy. Cell activity was stopped during this time. But this action was however not allowed by SPD. At 5 hr pi and continues to 10 hr pi, the gene that control cell death through apoptosis were expressed such as mitogen-activated protein kinase, p53, famili oncogene RAS, caspase-8and G protein-coupled receptor in cell treated with SPD but not in infected cells. HSV-1 inhibits apoptosis

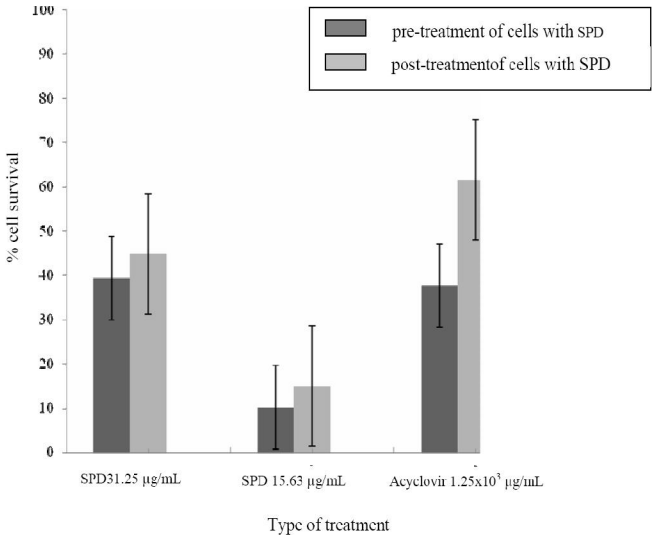

Figure 2 : Effect ofHSV-1 on thepre-treatment of cells versuspost-treatment of cellswith theSPDandacyclovir

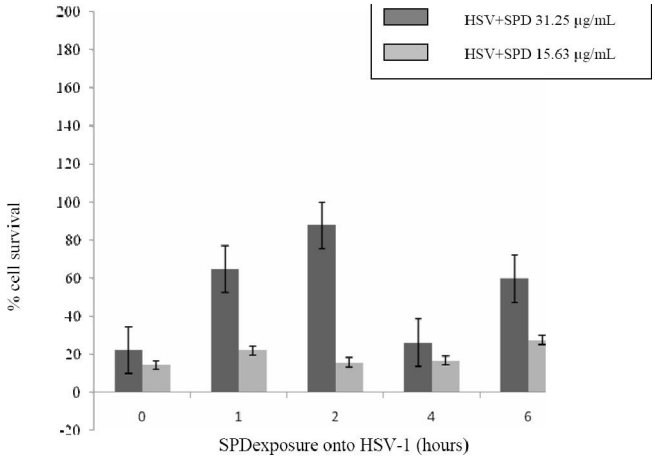

Figure 3 : Effect of SPD actions directly exposed to HSV-1 for 0, 1, 2, 4 and 6 hours

TABLE 2 : Comparision between differential gene expression (DEG) result and real time PCRanalysis

Predicted gene function	Accession number	Expression in infected cells	Expression in infected cells + SPD	Time post infection and treatment	Real-time mean fold change	Agree with Gene Fishing result?
Carboxypeptidase M	XR023655.1	Repress	Express	0 hr + SPD	21.6	Yes
Mitogen-activated protein kinase	NM004759.3	Repress	Express	3 hrs + SPD	19.0	Yes
Ras	XM001154629.1	Repress	Express	8 hrs + SPD	2.0	Yes
G protein-coupled receptor	XR 024003.1	Repress	Express	6 hr + SPD	24.8	Yes
p53	XM 001167426.1	Repress	Express	8 hrs + SPD	17.0	Yes
Ubiquitin	XM 001098907.1	Express	Repress	6 hrs + no SPD	-1.9	Yes
Calmodulin	XM 512771.2	Express	Repress	0 + no SPD	-16.1	Yes

of infected cells, presumably to ensure that the infected cell survives long enough to allow completion of viral replication.

Mechanism of Action of SPD against HSV-1 Infection. The number of cells in a tissue is not only controlled by cell proliferation but also by programmed cell death of apoptosis. Apoptosis plays important roles both during development and in mature tissues. We found that SPD induced apoptosis, as measured by oncogene family gene expression and caspase activation. The apoptosis mediated by SPD in infected cells was associated with the activation of p53 and Bcl-2 protein via intrinsic pathway. SPD also exerts its anti-HSV-1 properties by activating an extrinsic pathway via caspase-8 activation in infected cells. The activation of apoptosis from outside the cell is caused by the association of procaspase-8 with the cytoplasmic domain of death receptor such as Fas. This association is mediated by adaptor proteins that bind both Fas and procaspase-8. The aggregated procaspase-8 molecules cleave each other, initiating the caspase cascade that leads to apoptosis. We suggested that SPD play the important roles in induce production of Fas ligand by T cells and also prevent production of survival factors in HSV-1 infected cells. The reason of this suggestion is because of in the immune system, one mechanism by which T cells kill target cells is to produce a protein called Fas ligand. Fas ligand binds its receptor, Fas, on target cells. Binding of Fas ligand to Fas induce apoptosis. There also exist extracellular proteins, call survival factors which prevent apoptosis by binding to their receptors on the surface of the cells.

When cells are stressed for example because of hypoxia or damage cause by unrepaired DNA damage they can also activate apoptosis from inside the cell by triggering procaspase aggregation and activation. In response to stress or damagen, pro-apoptotic signals induce mitochondria to releate cytochrome c into the cytosol, where it binds and activates an adaptor protein called Apaf-1. This complex activates a caspase called procaspase-9, which leads to triggering of the caspase cascade. The proapoptoticcaspases can bw divided into the group of initiator caspases including procaspases-2, -8, -9 and -10, and into the group of executioner caspases including procaspases-3, -6 and -7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomain, containing death effector domains (DED) in the case of procaspases-8 and -10 or caspase recruitment domain (CARD) as in the case of procaspase-9 and procaspase-2. In extrinsic apoptosis pathways, procaspase-8 is recruited by its DEDs to the death inducing signaling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family (Sartorius 2001).

The release of cytochrome c from the mitochondria is tightly controlled by members of the Bcl-2 family of proteins. Within this family of proteins, there are two sub-classes: proteins that promote apoptosis or proapoptotic such as Bad, Bax and PUMA and proteins that antagonize apoptosis or antiapoptotic such as Bcl-2 and Bcl-x_L. The antiapoptotic family members can directly bind to the proapoptotic family members and inhibit them. In the absence of the antiapoptotic family members, the proapototic family members, such as Bad and Bax, form channels in the mitochondrial membrane that results in the release of cytochrome c into the cytoplasm where it can activate procaspase-9 via Apaf-1.

The cell cycle is controlled at certain stages by checkpoints. These are biochemical mechanisms that stop the cell cycle if certain conditions are not met. One checkpoint is the G1 DNA damage checkpoint. If cells contain unrepaired damage to their DNA, the cell cycle is arrested in G1. This arrest requires a key transcription factor, p53, which is activated by DNA damage. There are three components to the system which is a DNA damage sensor, the Mdm2 protein which normally causes p53 to be degraded and the p53 itself. DNA damage causes phosphorylation of p53 and blocks the binding of Mdm2. This leads to the stabilization and accumulation of p53. p53 can then bind to the promoter of the p21 CDK inhibitor described earlier and activate its transcription. Inhibition of cyclin-dependent kinases (Cdks) leads to cell cycle arrest.
If p53 activation continues for a prolonged period of time, apoptosis ensues. This process kills cells with damaged DNA that remain unrepaired and serves to remove cells from tissues that may otherwise accumulate mutations that would be passed on to their daughter cells. p53 probably activates apoptosis by increasing the transcription of several genes. One target gene is the proapototic Bcl-2 family member Bax, whose

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gene directly activated by p53. Recent research indicates that p53 is more than a transcription factor. Unexpectedly, some p53 associates with mitochondria to directly promote the release of cytochrome c from mitochondria. This cytoplasmic population of p53 is inhibited by the anti-apoptotic Bcl-2 family member Bcl-$x_{L,}$ which directly binds to p53. One of the transcription targets of p5, PUMA releases p53 from Bcl-$x_{L.}$ This in turn allows p53 to bind to Bax which activates cytochrome c release and apoptosis ensues.

CONCLUSION

From this study, the understanding on how SPD acted upon HSV-1 infected host cells during the infection process was proposed. In this study it was shown that SPD has a potential in controlling HSV-1 infection selectively without disturbing non-infected cells.

ACKNOWLEDGEMENT

We wish to thank Ministry of Higher Education (UKM-ST-01-FRGS0051-2006) and International Foundation for Science (STGL-005-2007) for their financial supports in this project.

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