



UNIVERSITI PUTRA MALAYSIA

***STATINS MODULATION OF INFLUENZA VIRUS H1N1 INFECTION
IN MDCK CELLS BY MOLECULAR SIGNALING PATHWAYS***

PARVANEH MEHRBOD

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By

PARVANEH MEHRBOD

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

May 2013

*It is an honor for me to dedicate this thesis to my
parents and brothers for their love, endless support
and encouragement
and
affectionate thanks to my supervisors and friends*

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

STATINS MODULATION OF INFLUENZA VIRUS H1N1 INFECTION IN MDCK CELLS BY MOLECULAR SIGNALING PATHWAYS

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May 2013

Chair: Professor Aini Bt Ideris, PhD

Faculty: Institute of Bioscience

Influenza virus infection still remains a major cause of morbidity and mortality. Recently, to lessen influenza infection and increase its efficient treatment, researchers worldwide are developing novel drugs as well as evaluating existing drugs with pleiotropic effects that are able to inhibit the replication and life cycle of influenza viruses.

Influenza A virus is a multi-step infectious agent that can induce or inhibit different pathways inside the host cell by recruiting the host machinery system to support the virus replication and induce tissue destruction. One of these pathways is cytokine over-expression following infection which causes hypercytokinemia. This process affects different GTPase proteins isoprenylation. These GTPase proteins are involved in different pathways such as endocytosis and actin cytoskeleton polymerization. Another

important pathway is autophagy and lysis, where activation of autophagy could also be an effective way of eliminating influenza A virus.

Anti-inflammatory and immunomodulatory agents might be beneficial as effective alternative to vaccines and antivirals against influenza virus. Statins as anti-inflammatory agents are competitive inhibitors of the enzyme HMG-CoA reductase, which is the enzyme responsible for the rate determining step early in the cholesterol biosynthesis. By inhibiting HMG-CoA reductase, statins block the synthesis of isoprenoid intermediates, which serve as lipid attachments for GTPase signaling molecules. Therefore, the inhibition of their proper membrane localization may play an important role in biological effects of statins to affect downstream molecular signaling pathways.

The present study was designed to determine whether statins could affect viral cellular infection via their effect on cholesterol biosynthesis and inhibition of membrane GTPase molecules prenylation and if they can inhibit endocytosis and induce autophagy. Common detection methods used in this study like HA, MTT, ELISA, q-PCR and SDS-PAGE along with Western blotting and immunofluorescence techniques indicated statins inhibitory effects on influenza replication. Statins decreased the HA titer and increased the cell viability in different combined treatments with the virus ($P \leq 0.05$ & $P \leq 0.01$) as compared to the influenza virus treatment. They also induced morphological changes on the virus. Data also showed that statins can limit immune system over expression significantly by decreasing the inflammatory reactions based on inhibitory

effects on two important proinflammatory cytokines; IL-6 and TNF- α . The level of viral and cellular target genes copy numbers from statins and virus combined treatments obtained from q-PCR absolute quantification assay showed substantial decrements in majority of the combined treatments ($P \leq 0.001$). The quantification of cytokines by q-PCR was confirmed by ELISA. The results even indicated the potential of influenza A virus to induce the pro-inflammatory cytokine secretions in CrFK cells at higher levels than MDCK cells which support the growth of certain influenza virus strains to some extent.

The inhibitory effect of statins was verified by detecting Rabs and RhoA proteins prenylation. The expression level of prenylated form of Rabs and RhoA proteins in statin and combined treatments of H1N1 detected through Western Blotting were significantly different from H1N1 treatment ($P \leq 0.001$). Statins promoted delocalization of Rabs and RhoA proteins from membranous fraction to cytosol. By contrast, influenza A virus promoted their localization from cytosol to membrane.

Statins also induced remarkable morphological changes in the assembly of actin cytoskeleton by inhibiting RhoA protein isoprenylation which is an important factor for actin filaments polymerization. The results detected by fluorescent staining showed that, despite the virus inoculation which caused condensation of the actin filaments, statins induced destruction in their assembly which is effective on the virus transportation inside the cell. In addition; the effect of statins was hindered by pre-exposure of cells to statin inhibitors namely FPP and GGPP as downstream intermediates of cholesterol

biosynthesis pathway. Additionally, the fluorescent staining study showed that both statins and virus exposure to the cells caused increments in the lysosomal mass but not significantly different from control ($P \geq 0.05$). Statins and influenza A virus have similar effect on autophagy but in different ways. Statins act directly on LC3 lipidation as a reliable marker of autophagosome formation and increase autophagosome formation or delivery to lysosomes, however, influenza A virus acts through its M2 protein channel to inhibit the maturation of autophagosome during autophagy. Hence, statins can act as double-edged swords which enhance autophagy by decreasing the cholesterol synthesis; however, this event may cease intracellular destruction of virus due to inability of autophagosome to undergo maturation.

Nevertheless, the results obtained in this study support the potential and capacity of statins as new strategy and effective regimen to control influenza A virus infection. However, further studies are recommended to define more involved pathways and underlying mechanisms of statins as anti-influenza agents and its clinical application in treating humans infected with influenza virus.

Key words: Influenza virus, Statins, Cytokine, Endocytosis, Autophagy

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**PEMODULATAN STATIN JANGKITAN H1N1 DALAM SEL MDCK OLEH
LALUAN ISYARAT MOLEKUL**

Oleh

PARVANEH MEHRBOD

Mei 2013

Pengerusi: Profesor Aini Bt Ideris, PhD

Fakulti: Institut Biosains

Jangkitan virus influenza masih menjadi punca utama morbiditi dan kematian. Baru-baru ini, usaha untuk mengurangkan jangkitan influenza dan menyediakan rawatan yang cekap telah mendorong penyelidik di seluruh dunia untuk membangunkan ubat baru atau menilai ubat sedia ada yang mempunyai kesan pleiotropik ke atas replikasi dan kitaran hidup virus influenza

Virus influenza A adalah agen berjangkit pelbagai peringkat yang boleh mendorong atau menghalang laluan yang berbeza di dalam sel tuan rumah dengan merekrut sistem perkakas perumah untuk menyokong replikasi virus dan mengaruh kerosakan tisu. Salah satu laluan itu ialah ekspresi sitokin secara berlebihan selepas jangkitan yang mengakibatkan hipersitokinemia. Proses ini mempengaruhi protein GTPase yang berbeza melalui isoprenilasi. GTPase protein terlibat dalam laluan berbeza seperti endositosis dan polimerisasi sitoskeleton aktin. Satu lagi laluan penting yang terlibat ialah autofagi

dan lisis di mana pengaktifan autofagi juga boleh menjadi cara yang berkesan untuk menghapuskan virus influenza A.

Agen anti-radang dan imunomodulasi kemungkinan bermanfaat sebagai alternatif yang berkesan untuk vaksin dan antiviral terhadap virus influenza. Statin sebagai agen anti-radang adalah perencat kompetitif bagi enzim 'HMG-CoA reductase', yang merupakan enzim yang bertanggungjawab untuk kadar penentuan pada peringkat awal dalam biosintesis kolesterol. Dengan menghalang HMG-CoA reductase, statin menghalang sintesis perantaraan isoprenoid, yang bertindak sebagai lampiran lipid untuk isyarat molekul kumpulan GTPase. Oleh itu, perencatan untuk penempatan membran yang betul boleh memainkan peranan yang penting dalam kesan biologi statin untuk menjejaskan laluan hiliran isyarat molekular.

Kajian ini telah direka untuk menentukan sama ada statin boleh menjejaskan jangkitan selular virus melalui kesannya pada biosintesis kolesterol dan perencatan membran prenilasi molekul GTPase samaada mereka boleh menghalang endositosis dan mendorong autofagi. Kaedah pengesanan yang biasa digunakan dalam kajian ini seperti HA, MTT, ELISA, qRT-PCR, SDS-PAGE bersama-sama dengan pemedapan Western dan imunopendarfluor menunjukkan bahawa statin di samping kesan asas mereka pada laluan kolesterol boleh menghadkan ekspresi sistem imun yang ketara dan mengurangkan tindak balas keradangan dengan merencat penghasilan IL-6 dan TNF- α yang disebabkan oleh jangkitan influenza ($P \leq 0.001$). Statin mengurangkan titer HA dan meningkatkan keupayaan hidup sel dalam campuran rawatan yang berbeza dengan virus

($P \leq 0.05$ & $P \leq 0.01$) berbanding rawatan dengan virus influenza. Ianya juga mencetus perubahan morfologi pada virus. Data juga menunjukkan bahawa statin berupaya menghadkan sistem imun terhadap ekspresi berlebihan dengan mengurangkan tindak balas inflamasi berdasarkan kepada kesan perencatan ke atas dua sitokin proinflamasi penting; IL-6 dan TNF- α . Tahap bilangan salinan gen sasaran virus dan selular daripada gabungan rawatan statin dan virus yang didapati daripada asai pengkuantitian mutlak q-PCR menunjukkan pengurangan yang ketara dalam kebanyakan gabungan rawatan tersebut ($P \leq 0.001$). Pengkuantitian sitokin melalui q-PCR telah disahkan dengan ELISA. Malahan kajian juga menunjukkan keupayaan virus influenza A untuk mencetus pembesaran sitokin proinflamasi dalam CrFK pada tahap yang lebih tinggi berbanding sel-sel MDCK yang menyokong pertumbuhan sesetengah strain virus influenza pada tahap tertentu.

Kesan perencatan statin telah disahkan dengan pengesanan preniliasi protein-protein.

Tahap ekspresi bentuk protein-protein Rabs dan RhoA yang telah diprenilasi dalam rawatan statin dan rawatan gabungan H1N1 yang dikesan melalui pemedapan Western adalah sangat berbeza dengan rawatan H1N1 ($P \leq 0.001$). Statin mempromosi penyahsetempatan protein-protein Rabs dan RhoA dari pecahan membran ke sitosol. Sebaliknya, virus influenza A mempromosi penempatan mereka dari sitosol ke membran. Statin juga menggalakkan perubahan morfologi yang ketara dalam penyusunan sitoskeleton aktin dengan menghalang isoprenilasi pada RhoA protein yang merupakan faktor penting bagi polimerisasi filamen aktin. Keputusan yang dikesan dari pewarnaan pendarfluor menunjukkan bahawa walaupun inokulasi virus menyebabkan

kondensasi filamen aktin, statin merencat kemusnahan dalam penyusunannya yang berkesan terhadap pengangkutan virus di dalam sel. Sebagai tambahan, kesan statin telah dihalang oleh pra pendedahan sel dengan perencat statin seperti FPP dan GGPP sebagai perantaraan aliran laluan biosintesis kolesterol. Seterusnya, pewarnaan pendarfluor menunjukkan bahawa kedua-dua pendedahan statin dan virus terhadap sel-sel menyebabkan kenaikan dalam jisim lisosom tetapi tiada perbezaan yang ketara daripada kawalan ($P \geq 0.05$), statin dan virus influenza A mempunyai kesan yang sama terhadap autofagi tetapi dalam cara yang berbeza. Statin bertindak secara langsung pada lipidasi LC3 sebagai penanda pembentukan autofagosom dan meningkatkan pembentukan autofagosom atau penghantaran ke lisosom, walau bagaimanapun, virus bertindak menerusi saluran protein M2 virus influenza untuk merencat kematangan autofosome semasa autofagi. Disebabkan itu, statin boleh bertindak sebagai pedang bermata dua di mana ianya meningkatkan autofagi dengan mengurangkan sintesis kolesterol; walau bagaimanapun, keadaan ini berupaya menghalang kemusnahan intrasel oleh virus disebabkan ketidakupayaan autofagosom menjalani kematangan.

Walaupun, keputusan dari kajian ini menyokong potensi dan keupayaan statin sebagai strategi baru dan kaedah berkesan untuk mengawal jangkitan virus influenza A. Walau bagaimanapun, kajian lanjut diperlukan bagi lebih menjelaskan tentang laluan-laluan yang terlibat dan mekanisme dasar statin sebagai agen anti-influenza dan aplikasi klinikalnya dalam merawat manusia yang dijangkiti dengan virus influenza.

Kata kunci: Virus influenza, statin, sitokin, endositosis, autofagi

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APPROVAL

I certify that an Examination Committee has met on Date to conduct the final examination of Parvaneh Mehrbod on her PhD thesis entitled —Statins Modulation of Influenza Virus H1N1 Infection in MDCK Cells by Molecular Signaling Pathways| in accordance with Universiti Pertanian Malaysia Act 1980 and Universiti Pertanian Malaysia regulations 1981. The Committee recommends that the student be awarded the (PhD degree).

Members of the Examination Committee were as follows:

Saleha Abdul Aziz, PhD
Professor
Universiti Putra Malaysia
(Chairman)

Mohd Azmi Mohd Lila, PhD
Professor
Universiti Putra Malaysia
(Internal Examiner)

Siti Suri Arshad, PhD
Associate Professor
Universiti Putra Malaysia
(Internal Examiner)

Sagar M. Goyal, PhD
Professor
University of Minnesota
(External examiner)

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 June 2013

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Aini Ideris, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rahman Omar, PhD

Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

Mohd Hair-Bejo, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Tengku Azmi Tengku Ibrahim, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

PARVANEH MEHRBOD

Date: 13 May 2013

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