African Journal of Microbiology Research Vol. 6(27), pp. 5715-5719, 19 July, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1011
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Evaluation of antiviral effect of atorvastatin on H1N1 infection in MDCK cells

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Accepted 5 June, 2012

Influenza virus causes annual epidemics and occasional pandemics despite developing new vaccines and antiviral drugs, thus presents significant public health concerns. For efficient treatment of influenza, researchers have shown interest on drugs with pleiotropic effects. Clinical efficacy of all conventional drugs in this case is ambiguous. Atorvastatin as a common drug used to treat hypercholestrolemia can block downstream processes in cholesterol biosynthesis pathway such that some of them are important supporting factors for the virus infectivity. To study antiviral potential of atorvastatin, the cells were treated with effective concentration (EC_{50}) of atorvastatin in vicinity of 100 tissue culture infectious dose ($100TCID_{50}$) of the virus sample during infection in different exposure types and the cell viability and the viral titers were examined. The results showed that atorvastatin in combination treatment with virus, reduced the virus infectivity by decreasing the virus titer and increasing the cell viability in comparison with the virus treatment. The experiment demonstrated significant anti-influenza properties of atorvastatin in cell culture probably by inhibiting the early stage of multiplication. Atorvastatin might be a good option as alternative to other chemical drugs in upcoming pandemics.

Key words: Atorvastatin, influenza virus, antiviral potential, MTT assay, hemagglutination assay.

INTRODUCTION

For hundreds of years, influenza virus has brought about annual epidemics and occasional pandemics despite improving in vaccine and antiviral drugs technologies, which is due to genetic modifications. Thus presents significant public health problems associated with considerable economic consequences (Fedson, 2008; Frost et al., 2007; Wilschut, 2009). Recently, researchers have been motivated to work on common in use drugs with emphasis on new pathways of their effects which are beneficial to treat this evasive infection. Conventional drugs such as Oseltamivir and zanamivir are recommended to prevent the release of viral particles by blocking neuraminidase. Amantadine and rimantadine also have been used against influenza to inhibit its penetration/uncoating (Pathumwadee et al., 2008; Sarah and Hong, 2008). For all these drugs, resistance is widespread (Pathumwadee et al., 2008) that the Centers for Disease Control and Prevention (CDC) in the US has warned about its continued use (Fiore et al., 2008).

Atorvastatin as a common drug to treat hypercholesterolemia (Goldman-Levine and Bohlman, 2005) is a member of the inhibitors of the hydroxyl methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme (Armstrong and Safo, 2005). This enzyme controls the cholesterol biosynthesis pathway (Endo, 1992). By this inhibiting properties, atorvastatin can block some downstream molecules in cell construction that some of them are key factors for the virus infectivity (Sun and Whittaker, 2003).

So, for this preliminary study that involved MTT cytotoxicity method and Hemagglutination assay, it can

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Table 1. Cytotoxicity of atorvastatin on MDCK cells.

Concentration (µM)	Mean ± SD
20	0.53±0.30*
15	0.60±0.26*
10	0.82±0.04
5	0.85±0.05
2	0.90±0.06
1	0.92±0.03
0	1.00±0.00

Optical densities (mean ± SD) from different concentrations of atorvastatin exposed to the cell. Values are averages of 4 independent examinations. *: Significantly different from untreated sample (p≤0.05).

be suggested that atorvastatin as a representative of statins family might be introduced as an effective alternative or supplementary therapy to antiviral agents. But, more detailed investigations are needed in ongoing studies to evaluate the localization of function of this representative and other members of this family.

MATERIALS AND METHODS

Virus sample and cell culture

The influenza virus used in this study was influenza A H1N1/New Jersey/8/76 (VR-897™) obtained from ATCC (The Global Bioresource Center). It was propagated in Madin-Darby Canine Kidney (MDCK) cells in the presence of 1 µg/ml of Tosylamide, Phenylethyl Chloromethyl Keton-treated Trypsin (Trypsin_TPCK) (Sigma, St. Louis, Missouri, USA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) from Mediatech Cellgro (Northbrook, Illinois, USA) supplemented by 10% Fetal Bovine Serum (FBS) GmbH (Pasching, Austria) and 100 Units/ml Penicillin G and 100 µg/ml Streptomycin (Sigma). At the time of virus inoculation, FBS was removed and media was supplemented by Trypsin_TPCK.

Atorvastatin

Atorvastatin base was purchased from Sigma as a white powder. To prepare the stock, 10 mg of the powder was dissolved in 1 ml DMSO and filter-sterilized through 0.22 µm syringe filter. Prior to its use, this stock solution was diluted.

Cytotoxicity assay

Confluent MDCK cells in 96-well plate were exposed to different concentrations of atorvastatin prepared by DMEM in different time intervals (24, 48 and 72 h). MTT assay as a colorimetric method was performed according to Mehrbod et al. (2009). Briefly, the media was removed, then 100 µl of 1X MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) was added to each well. Following incubation at 37°C for 2-3 h, formazan was solubilized by adding 100 µl of DMSO and mixing thoroughly to release the color. The optical density of the treatments was analyzed by ELISA reader machine (BioTek EL 800, USA) at 540 nm. Following this, 50% cytotoxic concentration (CC_{50}) of atorvastatin causing visible morphological changes in 50% of the cells with respect to cell control, effective concentration (EC_{50}) of atorvastatin which is the concentration required to achieve 50% protection against virus induced cytopathic effect, and viability of the cells were determined.

Anti-influenza effect of atorvastatin

Influenza virus ($100TCID_{50}$/0.1 ml) was added to the cells in different exposures with EC_{50} of atorvastatin and incubated at 37°C for 1 h. Following one hour incubation, unabsorbed viruses were washed and TPCK-containing medium was added (100 µl/well). After 48 h incubation the viability of the infected and non-infected cells were evaluated by MTT method. Meanwhile, the virus titer was determined by HA assay. The amount of virus used in each experiment was based on infected target cells of 0.5 multiplicity of infection (MOI) for both the viruses to produce 50% MTT formazan products as in uninfected control cells (Chattopadhyay et al., 2009).

Percent protection

Percent protection of atorvastatin on the cells was calculated as follows:

Percent protection = [(ODT)V-(ODC)V] / [(ODC)M-(ODC)V] × 100

That (ODT)V, (ODC)V and (ODC)M mean absorbance of the sample treated, the virus-infected control (no compound) and negative control, respectively (Shigeta et al., 1997).

Hemagglutination assay

The virus antigen titer in different treatments was determined by hemagglutination test. To the 50 µl of the serially diluted virus solutions in a U-shape 96 well plate (Nunc, Denmark), 50 µl of PBS and 50 µl of the chicken red blood cell (cRBC) suspension (0.5% volume in PBS) were added. After 60 min incubation, the endpoint of hemagglutination was evaluated by direct observation (Mehrbod et al., 2009).

Statistical analysis

The data expressed as mean ± SD was analyzed by analysis of variance (ANOVA) (SPSS 18.0) LSD post-hoc test. Sample values with p≤0.05 and p≤0.01 were considered statistically significant and highly significant, respectively.

RESULTS

Cell cytotoxicity

Cytotoxicity of atorvastatin in different concentrations and different time intervals was determined by MTT method reading optical densities (ODs) at 540 nm. It was found that atorvastatin was toxic at concentration of 15 µM and higher. EC_{50} of this compound was calculated by two-way ANOVA (LSD post-hoc test) at 10 µM that had no significant cytotoxic effect on the cells as in control. Data are shown in Table 1.

Table 2. Optical density at 540 nm for different exposure types of atorvastatin and virus.

Treatment	Mean ± SD
Virus-treated sample	0.184 ± 0.027
Co-penetration treatment	0.474 ± 0.039**
Pre-penetration treatment	0.413 ± 0.009**
Post-penetration treatment	0.237 ± 0.020

Different exposure types showed increments in OD values, but it was highly significant in Co- and pre- penetration treatments with p≤0.01 (mean ± SD) analyzed by SPSS, LSD post-hoc test. Data are averages of 4 independent tests, **: Highly significantly different from values obtained for co- & pre-penetration treatments compared to the virus untreated sample (p≤0.01).

Atorvastatin anti-viral effect on the virus

In this experiment, after running MTT assay, optical densities were measured in different exposure types in comparison with virus sample. All mean OD values measured, showed increments but it was highly significant in Co- and pre-penetration treatments (p≤0.01). Data are presented in Table 2.

Percent protection results

The percent protection of atorvastatin on the cell viability was calculated in combination with virus inoculation using viabilities of the cells obtained from formazan absorbance at 540 nm after 48 h exposures through related formula. The results shown in Figure 1 represent the higher protection in Co- and pre-treatments.

Hemagglutination assay results

Antiviral activity of atorvastatin against influenza virus in different experiments was assessed by hemagglutination endpoint test. Its inhibitory effect was shown by highly significant decrements in $\log_{10}$ HA titer in all types of combination treatments. Data are shown in Figure 2.

DISCUSSION

Influenza A virus is strongly associated with epidemics and pandemics and varies greatly in pathogenicity. In any given year, the seasonal influenza epidemic can be associated with thousands of deaths worldwide (Lamb and Takeda, 2001). Therapeutic vaccines failed to protect recipients from recurrences. Regarding this characteristic of this infectious agent that has made obstacles to design new drugs and vaccines, there is unmated and urgent need for cheap, readily available, less toxic alternate agents to control and prevent this infection. Therefore, researchers have moved towards working on common drugs with looking for their new pathways of effects which can be favorable to treat this serious infection.

Currently-approved classes of conventional drugs to inhibit neuraminidase and M2 channel of the virus are oseltamivir and Amantadine, respectively, which are recommended in emergency situations (Pathumwadee et al., 2008; Sarah and Hong, 2008), but resistance to both classes is a common problem (Pathumwadee et al., 2008) which is the outcome of influenza virus genome evolving through proliferation and no long-time usage is recommended by the Centers for Disease Control and Prevention (CDC) in the US (Fiore et al., 2008). Therefore, search for other inexpensive easily-accessible drugs with improved clinical benefits is highly demanded to control the clinical health problems associated with influenza infections.

So, in recent studies, atorvastatin which is commonly used to treat hypercholesterolemia (Goldman-Levine and Bohlman, 2005) has attracted more interests. It is a member of the selective competitive inhibitors of hydroxyl methylglutaryl-coenzyme A (HMG-CoA) reductase (Armstrong and Safo, 2005). This enzyme is critical in cholesterol biosynthesis pathway (Endo, 1992), which is blocked by atorvastatin. Consequently, many other downstream molecules in cell construction which are necessary for the viral infectivity are blocked or stopped too (Sun and Whittaker, 2003). Statins have shown pleiotropic effects beyond their lipid lowering ability (Terblanche et al., 2007), however, little is known about their molecular pathway of effects against influenza virus infectivity.

Conclusion

In this study, which is a preliminary research, we used MTT cytotoxicity method and Hemagglutination assay to evaluate anti-influenza activity of atorvastatin against influenza A virus vaccine strain H1N1, New Jersey/8/76 infection in cell line. The CC_{50} of atorvastatin in MDCK cells was obtained as 15 µM and through calculation by SPSS 18.0 (two-way ANOVA, LSD post-hoc test) it was found that 10 µM concentration of atorvastatin as a non-toxic concentration can be introduced as EC_{50} to use for antiviral treatments. Data from different combination treatments of atorvastatin and virus sample, either by MTT method or HA assay, was analyzed by Microsoft excel 2010 and SPSS 18.0 and analysis of variance (ANOVA) was performed. It was found that, Co- and pre-penetration exposure types were more significantly effective against virus infection with p≤0.01. From these experimental but not clinical results, it can be suggested that atorvastatin as one of the HMG-CoA reductase inhibitor family members might be introduced as an effective supplementary medication or alternative to

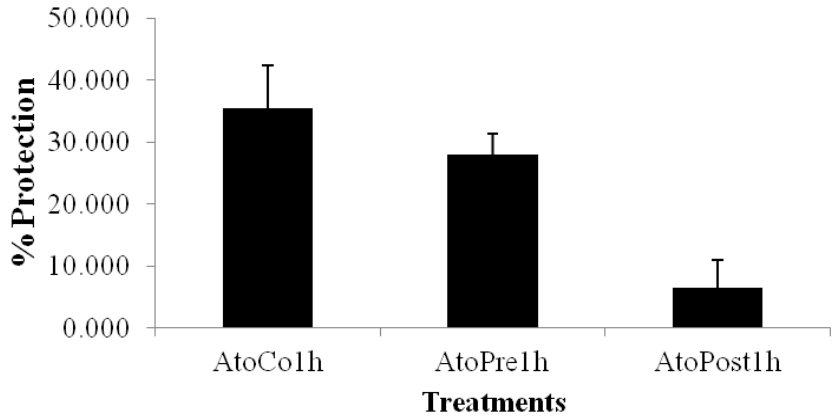

Figure 1. The graph shows atorvastatin percent protection on the cell viability from MTT results. Values are percentage averages of 4 independent experiments. It was found that Co- and pre-penetration exposures were more protective on the cell viability against the virus CPE. (AtoCo1h: Atorvastatin Co-penetration 1 h, AtoPre1h: Atorvastatin Pre-penetration 1 h, AtoPost1h: Atorvastatin Post-penetration 1 h).

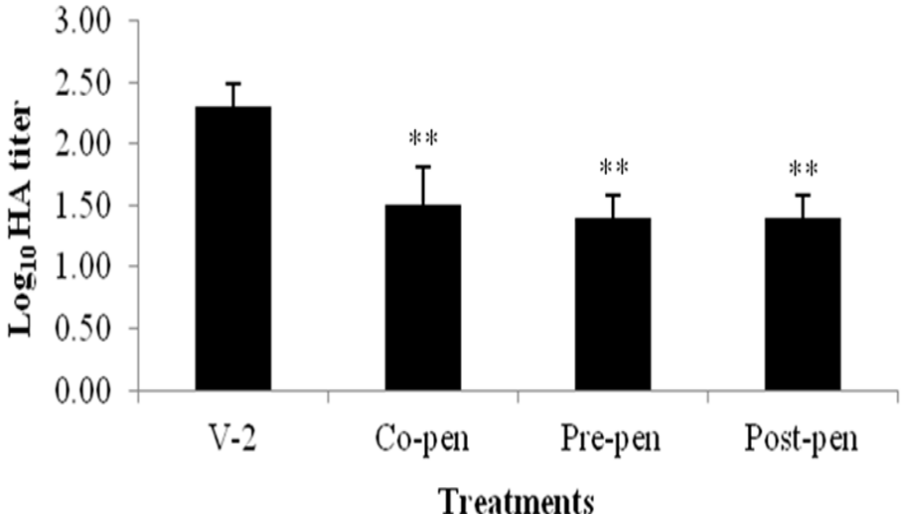

Figure 2. The illustration shows the inhibitory effect of atorvastatin on HA titer of influenza A virus at different exposures. Data are averages of 4 independent experiments analyzed by SPSS, LSD post-hoc test. (V-2: virus dilution 10^{-2}, Co-pen: Co-penetration, Pre-pen: Pre-penetration, Post-Pen: Post-penetration), **: Highly significantly different from value obtained for virus untreated sample compared to combination treatments ($p \leq 0.01$).

antiviral agents. More detailed investigations on the other viral and cellular pathways are under research by the authors to confirm and evaluate the localization of function of atorvastatin and other members of this family.

ACKNOWLEDGEMENT

This work is funded by the Ministry of Science, Technology and Innovation, Government of Malaysia under Grant No. 614800.

REFERENCES

Armstrong EJ, Safo PK (2005). Pharmacology of cholesterol and lipoprotein metabolism, Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy, Lippincott Williams & Wilkins, Baltimore. pp. 369-389.

Chattopadhyay D, ChawlaSarkar MC, Chatterjee T, Sharma Dey R, Bag P, Chakrabarty S, Khan MT (2009). Recent advancements for the evaluation of anti-viral activities of natural products. N Biotechnol., 25(5): 347-368.

Endo A (1992). The discovery and development of HMG-CoA reductase inhibitors. J. Lipid Res., 33(11): 1569-1582.

Fedson DS (2008). Confronting an influenza pandemic with inexpensive

generic agents: can it be done? Lancet. Infect. Dis., 8(9): 571-576.
Fiore A, Shay D, Broder K, Iskander J, Uyeki T, Mootrey G, Bresee J, Cox N (2008). Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). Recomm. Rep., 5: 71-60.
Frost FJ, Petersen H, Tollestrup K, Skipper B (2007). Influenza and COPD mortality protection as pleiotropic, dose-dependent effects of statins. Chest, 131(4): 1006-1012.
Goldman-Levine JD, Bohlman LG (2005). Ezetimibe/simvastatin (vytorin) for hypercholesterolemia. Am. Fam. Phys., 72(10): 2081-2083.
Lamb RA, Takeda M (2001) Death by influenza virus protein. Nat. Med., 7: 1286-1288.
Mehrbod P, Motamed N, Tabatabaian M, Soleimani Estyar R, Amini E, Shahidi M, Kheiri MT (2009). *In vitro* antiviral effect of "Nanosilver" on influenza virus. DARU, 17(2): 88-93.
Pathumwadee I, Chittima L, Thanyada R, Arthorn L, Maturos M, Panita D, Ornjira A, Krit C, Nopphorn K, Pornthep S, Somsak P, Supot H (2008). How amantadine and rimantadine inhibit proton transport in the M2 protein channel. J. Mol. Graph Model, 27: 342-348.
Sarah CD, Hong M (2008). Amantadine-induced conformational and dynamical changes of the influenza M2 transmembrane proton channel. PNAS, 105(5): 1483-1488.
Shigeta S, Shuichi M, Junko W, Shu S, Kauzo T, Yamase T (1997). Synergistic anti-influenza virus A (H1N1) activities of PM-523 (Polyoxomatalate) and Ribavirin *in vitro* and *in vivo*. J. Antimicrob. Ag. Chemother., 41(7): 1423-1427.
Sun X, Whittaker GR (2003). Role for influenza virus envelope cholesterol in virus entry and infection. J. Virol., 77(23): 12543-12551.
Terblanche M, Almog Y, Rosenson RS, Smith TS, Hackam DG (2007). Statins and sepsis: multiple modifications at multiple levels. Lancet. Infect. Dis., 7: 358-368.
Wilschut J (2009). Influenza vaccines: the virosome concept. Immunol. Lett., 12: 2118-121.