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Simvastatin and oncolytic vesicular stomatitis virus actions on HeLa cells

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ABSTRACT

Statins, the cholesterol-lowering drugs, are known to exert pleiotropic actions including anticancer and antiviral effects. The interaction between simvastatin and oncolytic vesicular stomatitis virus (VSV), a potential candidate for anticancer therapy, was investigated. HeLa cells in culture were exposed to simvastatin and/or VSV-green fluorescence protein (GFP) at different times and concentrations. VSV protein contents, virus infectivity, apoptosis (PE Annexin V), necrosis (7-aminoactinomycin D) and cell viability were analyzed by immunoblot, plaque-forming units/size, and flow-cytometry. VSV infection was characterized by the presence of infected cells (GFP-stained cells), glycoprotein (G), nucleocapsid/polymerase (N/P) and matrix (M) viral proteins in cell lysates and culture media, production of viable progeny, and induction of apoptosis, necrosis and cell death. The effects were directly related to the exposure time and multiplicity of infection (MOI) employed. Notably, large numbers of apoptotic and/or necrotic cells were GFP-negative (non-GFP stained). Simvastatin induced cell loss, apoptosis and necrosis; however, concentrations > 2 μM were required to observe significant cell damage. Simvastatin (0.25-2 μM; > 4 hours exposure) exerted a potent antiviral action, characterized by decreases in viral proteins, ability of viral progeny to induce plaque formation, number of infected, apoptotic and necrotic cells, and increased cell survival. Greater antiviral action was observed with higher concentrations. VSV-induced cell damage (cell loss + apoptosis + necrosis) was reduced by 79 ± 8% with 4 μM simvastatin ($P < 0.01$). Simvastatin inhibited VSV replication and infectivity, and markedly decreased VSV-induced cell damage, independently of its pro-apoptotic action. Exposure time and inoculum size are strong determinants of the mechanisms by which VSV induces cell death.

KEYWORDS: simvastatin, vesicular stomatitis virus, apoptosis, antiviral

INTRODUCTION

Statins, or 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are best known for their ability to effectively lower circulating levels of low-density lipoprotein cholesterol (LDL-C) [1]. In addition, statins exert actions that are independent of blood cholesterol-lowering, known as pleiotropic actions [2]. Among others, statins, such as simvastatin, fluvastatin and lovastatin, have been shown to induce apoptosis and exert antiviral actions. HIV [3], influenza A [4], hepatitis C and B [5, 6] and measles viruses [7], are some of the viruses inhibited by statins. However, statins have been reported to increase the cancer-killing properties of oncolytic adenovirus [8]. This finding is in contrast with previous studies on non-oncolytic

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viruses, where statins exerted a well-defined antiviral action. For this reason, we evaluated the effects of simvastatin, an effective antiviral statin, on the oncolytic vesicular stomatitis virus (VSV), an enveloped negative-strand RNA virus [9, 10]. In addition, we tested whether the pro-apoptotic action of simvastatin plays a role in determining VSV replication, infection and cell death. The effect of simvastatin on the oncolytic action of VSV was assessed employing immunoblot, viral titers, and flow cytometry methodologies. Simvastatin and VSV-induced cell damage was determined by quantification of cell loss, apoptosis and necrosis.

MATERIALS AND METHODS

The interaction between VSV-GFP and simvastatin was studied on HeLa cells in culture. HeLa cells were infected with VSV-GFP (gift from Dr. G. Barber, University of Miami, FL). Experimental conditions were optimized by employing several VSV MOIs and exposure times. The effects of simvastatin on VSV infection were studied by pretreating cells with simvastatin (EMD/Merck, NJ), and then exposing them to VSV-GFP. Different exposure times and simvastatin concentrations were tested. Cells were tested at 80-90% confluence.

Immunoblots

Cells were lysed with the following buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 15 mM $MgCl_2$, 0.5% NP-40 detergent solution (Thermo Fisher Scientific, PA), and protease inhibitors cocktail (Sigma-Aldrich, MO). Lysates were kept on ice for 20 min, sonicated, and then centrifuged at 14000 × g for 30 min. Protein concentration was determined by the Lowry Method. Reducing agents were added to the samples, and immunoblot analysis was performed. Proteins were separated by 12% SDS-PAGE, and transferred into nitrocellulose membranes (GE Healthcare, NJ). Non-specific sites were blocked with 5% milk in PBS/0.05% NP-40 detergent solution. The membranes were incubated with primary antibodies: mouse polyclonal anti-VSV-GFP (gift from Dr. G. Barber, University of Miami, FL). The membranes were washed with PBS/0.05% NP-40 and incubated with secondary antibodies conjugated to horseradish peroxidase (American Qualex, CA). The VSV protein bands were detected by chemiluminescence using SuperSignal West Femto Substrate (Thermo-Fisher Scientific, PA). Quantitation of bands was performed using ImageJ software program (National Institutes of Health).

VSV titers

HeLa cells were infected with VSV, as described above. Baby hamster kidney cells (BHK-21) were seeded into six-well plates and cultured to 80% confluence. BHK-21 cells were infected with 300 µl of serially diluted viral samples from HeLa supernatants. After BHK-21 viral adsorption, the inoculum was removed and replaced with complete Dulbecco's Modified Eagle's Medium with 1% low-melting-point agarose (LMPA) (Sigma-Aldrich, MO). When isolated infections (plaques) were visible under the fluorescent microscope, approximately 50 images were obtained at random from each experimental condition. Relative plaque size was calculated using MetaMorph software. When plaques were visible, the LMPA was removed; BHK-21 cells were fixed and stained by the addition of 1 ml of Gentian violet diluted in methanol (0.01% w/v) (Sigma-Aldrich, MO). Plaque-forming units (PFU) were calculated (PFU/ml) using duplicate samples.

Flow cytometry analysis

Viral infection in the presence and absence of simvastatin, as well as the effects of simvastatin in non-infected cells, were also evaluated employing flow cytomery analysis. VSV effects were studied employing different MOIs (0.0001-1 MOI) and different exposure times (0-16 hours). Simvastatin effects were studied using 0.125, 1, 2, 4 and 8 µM of simvastatin for 24 hours. To study simvastatin-VSV interaction, cells were subsequently infected with VSV-GFP at 0.1 MOI for 12 hours. Apoptosis inducer camptothecin (8 µM) was used as a positive control. For these experiments, cells were cultured in 12-well plates until 60-80% confluence was reached, and then treated with simvastatin, VSV-GFP, camptothecin or vehicle (dimethyl sulfoxide, DMSO 0.1 µM). Subsequently, cells were trypsinized and washed with phosphate-buffered saline (PBS), using a Beckman refrigerated centrifuge at 1200 rpm. The pellets were then resuspended in 4% paraformaldehyde fixation solution and incubated on ice for 1 h. Cells were pelleted under the same centrifugation conditions and resuspended in 500 µL

of PE Annexin V/7-aminoactinomycin D (7-AAD) binding buffer (BD Pharmingen™). 100 µL (~1×10^5 cells) of resuspended cells were transferred to plastic 12 × 75 mm tubes and 5 µl of PE Annexin V and 5 µl of 7-AAD were added to each tube. Cells were gently vortexed and incubated for 15 min at room temperature (25 °C) in the dark. After incubation, 400 µl of 1X Binding Buffer was added to each tube and subsequently analyzed by flow cytometry within 1 h in a BD C6 Accuri Flow Cytometer (BD Pharmingen™). Data acquisition was established at 10,000 cells, and the volume required for data acquisition was used to estimate cell number.

Statistics

The data was reported as means of 3-5 independent experiments of samples run in triplicates. Student's *t*-test and one-way analysis of variance (ANOVA) with post-hoc Tukey's test were employed for continous variables, and non-parametric Mann-Whitney *U* test was used when normality criteria were not met (StatSoft Inc., OK, USA). Significance was assessed at $P < 0.05$.

RESULTS

Characterization of simvastatin effects on HeLa cells

Table 1 depicts the effects of increasing concentrations of simvastatin on HeLa cells using flow cytometry. Simvastatin induced apoptosis, necrosis and cell loss, in a concentration-dependent manner. Significant increases in apoptosis and necrosis were observed with concentrations > 2 µM. With 2 µM simvastatin, the percentage of apoptotic and/or necrotic cells averaged 2.6 ± 0.8% and total number of cells damaged (cell loss + apoptotic + necrotic) averaged 13 ± 2% (Table 1). With 4 and 8 µM simvastatin, total cell damage averaged 21 ± 4 and 32 ± 6%, respectively.

VSV infection and expression in HeLa cells: effects of simvastatin

VSV infection was characterized by the presence of three major bands corresponding to glycoprotein (G), nucleocapsid (N)/viral polymerase (P), and matrix (M) viral proteins present in supernatants (cell media with viral progeny) and lysates (intracellular

Table 1. Effects of increasing concentrations of simvastatin on HeLa cells.

	All cells N (%)	**Apoptotic and/or necrotic cells N (%)**	**Total cell damage (cell loss + apoptosis + necrosis) N (%)**
DMSO control	614 ± 56 (100)	2 ± 4 (0.1 ± 0.2)	2 ± 4 (0.1 ± 4)
SIM 0.1 µM	625 ± 59 (102 ± 10)	1 ± 4 (0.3 ± 2)	0 (0.3 ± 2)
SIM 1 µM	613 ± 45 (100 ± 9)	7 ± 4 (1.2 ± 6)	7 ± 4 (1.2 ± 6)
SIM 2 µM	546 ± 51* (88.9 ± 8)*	14 ± 5* (2.6 ± 0.8)	73 ± 9* (13 ± 2)*
SIM 4 µM	523 ± 57* (85.7 ± 7)*	35 ± 8* (7 ± 2)**	129 ±16* (21 ± 4)*
SIM 8 µM	472 ± 59** (76.9 ± 10)	54 ± 6* (12 ± 3)**	199 ± 24* (32.4 ± 6)**

Results from flow cytometry experiments. HeLa cells were exposed to different concentrations of simvastatin for 24 hours, each. Number of cells (N values) must be multiplied by 1000 to get the estimated number of cells/ml. Shown are mean values ± SEM of cell numbers or percentages of at least 3 experiments of samples run in triplicates. Statistically significant from DMSO vehicle control at *$P < 0.05$ and **$P < 0.01$. SIM: simvastatin.

replication) (Figure 1). Concentrations of simvastatin as low as 0.25 µM significantly reduced VSV N/P, M and G protein levels, both in supernatants and lysates (Figure 1 A, B and C). Marked inhibition was observed with 1 µM simvastatin (Figure 1). Pretreatment times of at least 4 hours were required for simvastatin to induce significant inhibition of VSV protein levels (Figure 1C). Interestingly, simvastatin-induced inhibition of VSV protein levels (replication) was observed with concentrations of simvastatin at which it did not produce significant cell loss, apoptosis and/or necrosis; of HeLa cells (Table 1).

We compared the number and size of PFUs when BHK-21 cells were exposed to supernatants (media) of VSV-infected HeLa cells (MOI 1.0) pretreated with simvastatin (8 hours) or the statin-vehicle (Figure 2). In the absence of simvastatin, VSV titers averaged 8.75×10^6 ($\pm$ 1.05×10^6 SEM) PFU/ml, and plaques had a relative radius equivalent to 61593 ± 5431

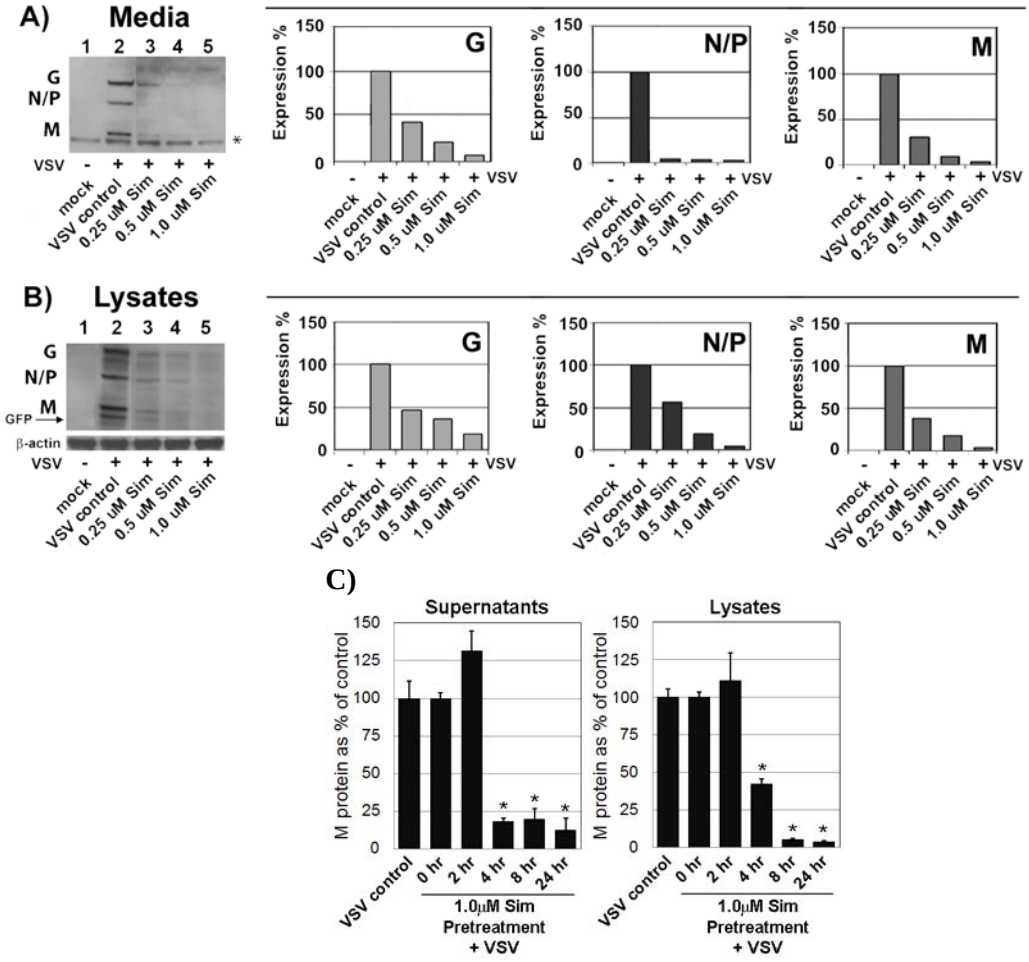
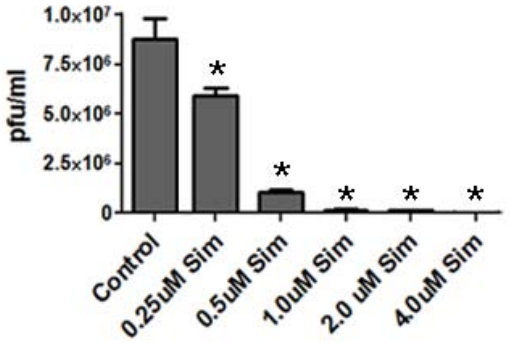


Figure 1. Effects of VSV on Hela cells: interaction with simvastatin. To study the presence of viral proteins as a marker of infection, cells were infected with VSV-GFP for 16 hours at an MOI of 1. Additionally, cells were pretreated with simvastatin for 8 hours prior to infection with VSV-GFP. Viral proteins were detected by immunoblots performed with anti-VSV-GFP antibodies. Detectable VSV protein bands are indicated by G (Glycoprotein), N/P (Nucleocaspid/Polymerase), and M (Matrix protein). In the media (A), a non-specific crossreacting band is indicated by an asterisk and served as loading control. In the lysates (B), protein bands were normalized against β-actin expression. Also in cell lysates, an extra VSV band was detected, corresponding to the expression of GFP protein. (C): effects of simvastatin pretreatment times on M-protein expression in supernatants and lysates. Corresponding bar graphs were normalized to the loading controls and to VSV control infections, arbitrarily set to 100%. *P < 0.01.



Pretreatment	Aver. Titer	Std. Error
VSV Control	8.75×10^6	1.05×10^6
0.25μM Sim	5.90×10^6	4.00×10^5
0.5μM Sim	1.04×10^6	1.55×10^5
1.0μM Sim	1.65×10^5	1.50×10^4
2.0μM Sim	1.40×10^5	1.00×10^4
4.0μM Sim	1.80×10^4	3.00×10^3

Figure 2. VSV titers: effects of simvastatin. HeLa cells were pretreated with the indicated concentrations of simvastatin for 8 hours prior to VSV-GFP infection at MOI of 1.0 for 16 hours. Samples of media containing progeny VSV were collected and assayed for titers of infectious VSV as described in the 'Materials and Methods' section. Shown are mean values ± SEM. Data were analyzed for statistical significance using one-way ANOVA and post-hoc Tukey's test; *P < 0.01.

(area of pixel counts) (Figure 2). Simvastatin effectively decreased VSV titers in a concentration-dependent manner (Figure 2). Simvastatin IC 50% (concentration needed to reduce PFU values to half of control) averaged 0.278 ± 0.03 μM. A greater effect was seen with higher concentrations (> 2 log-units with 2-4 μM; P < 0.0001). Pretreatment with 1.0 μM simvastatin reduced the plaque radius by nearly half (from 61593 ± 5431 to 30371 ± 2218; P < 0.01). Experiments conducted with a lower viral load (MOI of 0.01 and 0.1) and a lower concentration of simvastatin (1 μM, for 24 hours), further confirmed the reduction in viral titers (data not shown).

VSV-induced HeLa cell infection, apoptosis and cell death: effects of simvastatin

Flow cytometry assays showed that VSV infection was associated with cell death (fewer remaining cells), presence of GFP-stained cells (GFP-positive), and of apoptotic and necrotic cells (Figure 3). Most apoptotic cells (PE Annexin V-positive) were also positive for 7-AAD (marker for necrosis); therefore, results were presented as the sum of apoptotic cells, necrotic cells and of cells that were both apoptotic and necrotic. The number of cells lost, of infected cells (GFP-positive) and of apoptotic and/or necrotic cells, increased in direct proportion with the virus inoculum (MOI) and the time of exposure to VSV (Figure 3). Significant decreases in cell loss were observed with 0.1 and 1 MOI and exposure times of 10 hours or longer (Figure 3). The percentage of GFP-positive cells increased from 14.4 ± 4% with a 0.001 MOI to 70 ± 8% with an MOI of 1 (P < 0.001) (Figure 3). Interestingly, not all apoptotic and necrotic cells were GFP-positive. This was more evident at lower inoculum and short exposure times, where most of the apoptotic and/or necrotic cells were GFP-negative. However, even at an MOI of 1 and a 16-hour exposure time, nearly half of all apoptotic and necrotic cells were GFP-negative (Figure 3).

VSV-induced HeLa cell damage (cell loss + apoptosis + necrosis) was studied in cells pretreated with simvastatin (Figure 4 and table 2). Cells were pretreated with simvastatin (0.125-8 μM) followed by 12 hours of exposure to VSV at 0.1 MOI. Simvastatin decreased VSV-induced cell loss and increased the number of remaining viable cells. In addition, simvastatin reduced the percentage of infected (GFP-positive) cells and of GFP-positive cells that were apoptotic and necrotic. Significant increases in cell survival were observed with 1 μM simvastatin. With 4 μM simvastatin, VSV-induced total cell loss was reduced from 228345 ± 16843 ml^{-1} to 67387 ± 3601 ml^{-1} (P < 0.01); the number of viable cells increased from 288142 ± 22181 to 448142 ± 31004 ml^{-1} (P < 0.01). The percentage of infected cells was reduced by 34.6% ± 4% (P < 0.01), and the percentage of infected cells that were apoptotic and/or necrotic was reduced by 37.9 ± 6%. In summary, simvastatin significantly inhibited VSV-induced HeLa infection and death (Figure 4, table 2).

DISCUSSION

VSV effectively replicated, infected and killed HeLa cells in culture. The effects of VSV were

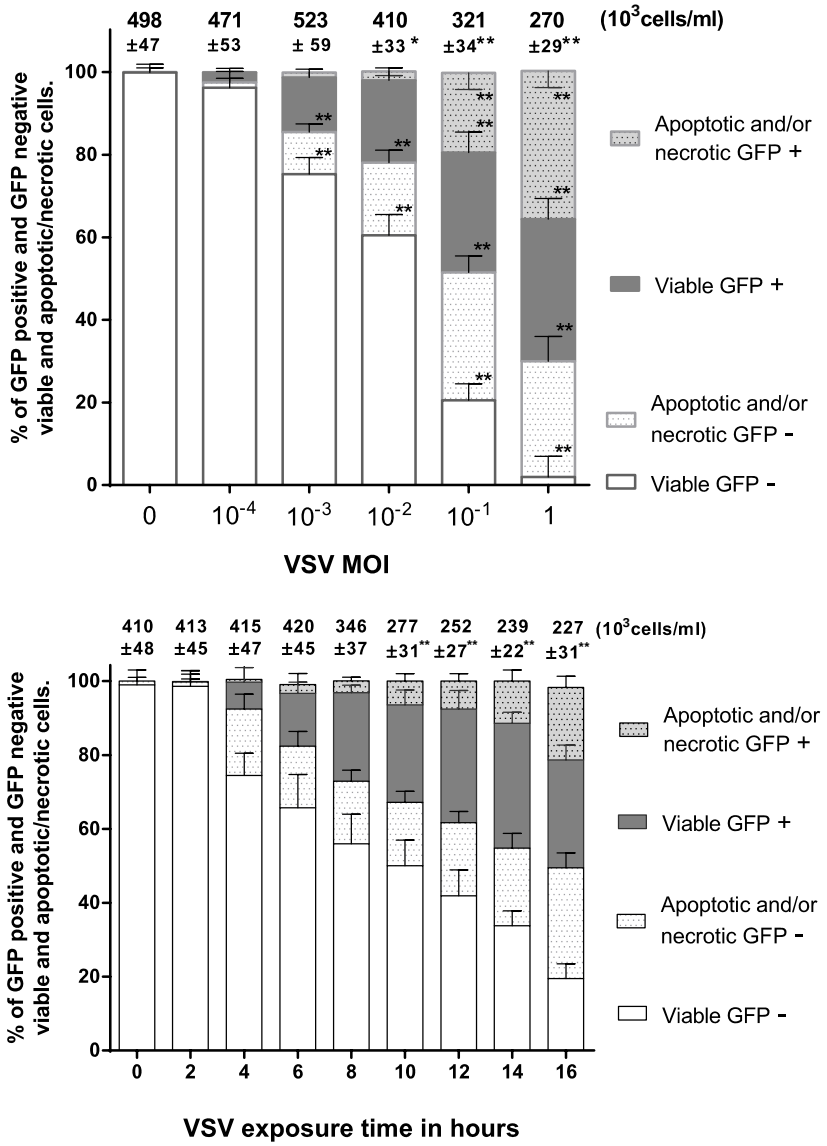


Figure 3. Effects of increasing VSV inoculum and exposure times: analysis by flow cytometry.
Top: HeLa cells were exposed to increasing MOIs of VSV for 12 hours each. Numbers above the columns represent: (the total number of cells on the dish/ml) × 1000. Ordinate: % of GFP-positive and GFP-negative cells, either viable (negative for apoptosis and necrosis markers) or positive for apoptosis, necrosis or both (damaged). Abscissa: VSV MOI used.
Bottom: HeLa cells were exposed to 0.1 MOI VSV for different exposure times. Numbers above the columns represent: (the total number of cells on the dish/ml) × 1000. Ordinate: % of GFP-positive and GFP-negative cells, either viable (negative for apoptosis and necrosis markers) or positive for apoptosis, necrosis or both (damaged). Abscissa: Exposure times to VSV.
Data were analyzed for statistical significance using one-way ANOVA and post-hoc Tukey's test (GraphPad Prism 5 software). Statistically significant from control (cells not exposed to VSV) at $^{*}P < 0.05$, $^{**}P < 0.01$.

found to be dependent on exposure time and inoculum size; i.e., higher inoculi required shorter exposure times to induce significant cell death (present study). Several mechanisms seemed to determine VSV-induced apoptosis and necrosis. In fact, it was of interest to note the abundance of non-GFP-stained apoptotic and necrotic cells during VSV infection, mostly at low viral loads and short incubation times. It is possible that these apoptotic and necrotic GFP-negative cells represent early stages of VSV

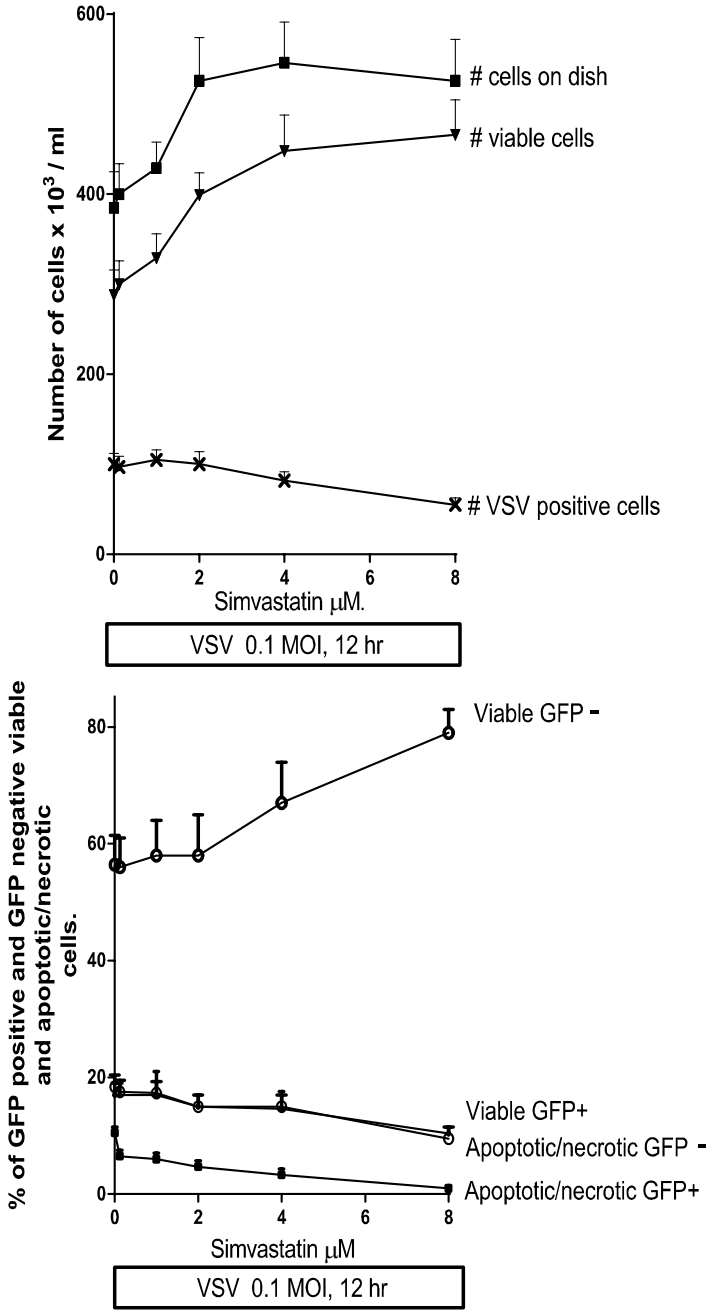


Figure 4. Effects of simvastatin on VSV-induced HeLa cell loss, viability, infection, apoptosis and necrosis. HeLa cells were exposed to the vehicle or simvastatin for 24 hours and subsequently infected with 0.1 MOI VSV for 12 hours. Results were analyzed by flow cytometry. Ordinates: **Top graph:** total number of cells, viable cells and infected cells (GFP-positive cells). Cell number must be multiplied by 1000 to obtain total number of cells/ml. **Bottom graph:** % of GFP-positive and negative viable and of damaged cells. Abscissa: Simvastatin concentrations.

infection (i.e., the virus has penetrated but has not replicated enough to be shown as a GFP-positive cell) and/or cells affected through paracrine mechanisms. The former is supported by the observation that programmed cell death, in the absence of replication, may be observed at early stages of VSV infection after the uncoating of virions, but prior to the synthesis of viral proteins [11, 12]. Therefore, we propose

Table 2. Flow cytometry analysis of VSV-simvastatin interaction.

	All cells N (%)	GFP-negative viable N (%)	GFP-negative apoptotic and/or necrotic N (%)	GFP-positive viable N (%)	GFP-positive apoptotic and/or necrotic N (%)	Total cell damage (cell loss + apoptosis + necrosis) (%)
DMSO control	614 ± 43 (100)	611 ± 41 (99.9 ± 4)	4 ± 3 (0.2 ± 4)			(0.2 ± 0.2)
SIM 4 µM	526 ± 49 (85 ± 5)[a]	491 ± 41[a] (93 ± 6)[a]	36 ± 4[a] (7 ± 3)[a]			(21 ± 4)[a]
VSV	385 ± 41[a,b] (62.3 ± 5)[a,b]	217 ± 28[a,b] (56.4 ± 4)[a]	65 ± 13[a] (17.4 ± 3)[a,b]	71 ± 8 (18.5 ± 2)	29 ± 4[a,b] (7.4 ± 2)[b]	(53.1 ± 6)[a,b]
SIM 4 µM + VSV	547 ± 46 (89 ± 4)[a]	368 ± 15[a] (67 ± 6)[a]	80 ± 12[a] (14.6 ± 3)[a]	81 ± 6 (14.6 ± 0.4)	18 ± 3 (3.2 ± 1.5)	(27 ± 3)[a]

Cells were treated with vehicle, simvastatin, VSV, or simvastatin + VSV. Simvastatin pretreatment preceded VSV exposure. Shown are mean values ± SEM of cell numbers or percentages of at least 3 experiments of samples run in triplicates. Cell counts (N) should be multiplied by 1000 to obtain the estimated total number of cells/ml.
[a]Significantly different from control (DMSO) at $P < 0.05$. Significantly different from VSV at $P < 0.05$;
[b]Significantly different from simvastatin + VSV at $P < 0.05$.

that the pro-apoptotic oncolytic action of VSV may be achieved by a combination of mechanisms: a) cell damage by replicating inside cells affecting RNA transport (GFP-positive cells), b) entering the cells and triggering apoptosis without replication (GFP-negative), and c) activation of stress-responsive pathways in infected cells, leading to apoptosis in non-infected neighboring cells (GFP-negative).

Contrary to the report on oncolytic adenovirus, where statins increased the adenovirus oncolytic action [8], simvastatin inhibited oncolytic VSV-induced cell damage (cell loss + apoptotic + necrotic cells), greatly increasing the number and percentage of viable cells (present study). Simvastatin-induced inhibition of VSV was evidenced by large decreases in viral protein content and in the ability of VSV to form viral plaques. Consequently, oncolytic VSV adds to the list of viruses that are sensitive to the effects of statins. The marked decrease in nucleocapsid (N) and viral polymerase (P) proteins involved in viral genome replication and packing, may explain simvastatin-induced interference with VSV replication. Simvastatin-induced decrease in VSV matrix (M) protein and glycoprotein (G) levels, known to interfere with host mRNA export and protein synthesis [13, 14], may explain the reduced cytopathic action of the virus. We propose that the anti-VSV activity of simvastatin results from its effects on gene expression in the host cell. This is based on the following findings: a) HeLa cells had to be exposed to simvastatin for at least 4-6 hours prior to infection in order to observe anti-VSV activity; similar results were obtained with the hepatitis C virus [5], and b) simvastatin inhibits the replication of a multitude of viruses with different mechanisms of action.

Simvastatin has been reported to induce apoptosis and cell death of cancer cells [15-16]; however, in HeLa cells, apoptosis was observed only at high concentrations of simvastatin. In fact, fewer than 10% of the HeLa cells were found apoptotic and/or necrotic with concentrations of ≤ 4 µM simvastatin, and total cell damage (cell loss + apoptosis + necrosis) was also less than 15%. Concentrations of simvastatin at which it did not induce apoptosis or cell death, exerted marked anti-VSV effects. Even with 0.25 and 0.5 µM there were marked reductions in viral protein levels, both in cell lysates and supernatants, as well as on the ability of VSV to generate viable progeny (present study). Concentrations of 1 and 2 µM simvastatin, at which there was little to no pro-apoptotic action, significantly protected the HeLa cells from infection, and from VSV-induced cell loss, apoptosis and necrosis. Therefore, the possibility that the antiviral action of simvastatin resulted from a reduced number of viable cells available to be infected, or from an interaction between the virus and simvastatin pro-apoptotic mechanisms is unlikely.

CONCLUSION

In summary, simvastatin inhibited VSV replication, infectivity and VSV-induced apoptosis, necrosis and cell death of cancerous HeLa cells. These effects most likely result from simvastatin effects on gene expression, and seemed to be independent of the pro-apoptotic actions of simvastatin.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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