

Inhibition of herpes simplex virus type 1 replication by novel hsa-miR-7704 *in vitro*

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Abstract

Herpes simplex virus type 1 (HSV-1) infections are one of the most common diseases in human population. HSV-1 causes subclinical, mild to severe diseases, especially in immunocompromised patients. Acyclovir has been used to reduce manifestations of HSV-1 infections. The extensive use of this drug has led to the development of resistant strains. Thus, designing a novel anti-herpes drug with different mechanisms of action is urgently needed. Cellular microRNAs (miRNAs) have direct antiviral effects in addition to their regulatory functions. In this study we used a novel miRNA (hsa-miR-7704), expressed in macrophages, to inhibit HSV-1 lytic infection in HeLa cells. Synthesized hsa-miR-7704 mimics were transfected into HSV-1 infected HeLa cell. The inhibitory effects of the miRNA were evaluated by plaque assay, real time polymerase chain reaction and the viral titers were measured by the 50% tissue culture infective dose (TCID₅₀). The viral titer and cell cytopathic effect were dramatically decreased in HeLa cells transfected with hsa-miR-7704 (50 and 100 nM), compared with HSV-1 infected cells alone or transfected with the mock miRNA control. These results suggest that hsa-miR-7704 inhibits HSV-1 replication efficiently *in vitro*. This may provide an alternative mechanism to prevent HSV-1 infections.

Keywords: Antiviral therapy; HSV-1; Micro RNA; miR-has-7704; miR-SX1.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a linear double-stranded DNA and a neurotropic virus that infects mainly mucoepithelial cells. This virus belongs to the Herpesviridae family. HSV-1 establishes lifelong latency in the sensory ganglia (1). HSV-1 is a common communicable virus in human populations. In the Germany and United States, the prevalence of HSV-1 was approximately 50% and 75%, respectively (2,3). HSV-1 causes a wide range of diseases including stomatitis, encephalitis, and keratitis (4). the ability of the virus to spread to the central nervous system which leads to encephalitis, is the most serious complication of HSV-1 infection (5) Acyclovir and its derivatives have been used to reduce manifestations of HSV-1 infections (6). However, resistance to acyclovir is increasing in immunocompromised patients (7). This

drug has serious side effects, especially in renal failure (8). Therefore, development of new anti-HSV-1 drugs is significant.

MicroRNAs (miRNAs) are small, non-coding RNAs that play a regulatory role in genes expression by binding to the target mRNA. It has been shown that miRNAs are involved in virus-host interactions. microRNA has been widely used to manipulate gene expression, identify gene functions on a whole-genome scale, and develop antiviral strategies for the prevention and treatment of human viral diseases (9). To date, miRNA has been employed against several human pathogens including hepatitis B and C virus (10, 11).

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Increasing evidences have shown that interleukin-27 have antiviral effects against many viruses including HSV-1 (12). It has been reported that interleukin-27 differentiates monocytes to HIV-1, HIV-2, influenza, and simian immunodeficiency virus resistance macrophages (I-MAC). Investigation of miRNA expression profile in I-Mac revealed seven novel miRNA which are hsa-miR-7704 (-SX1), -7705 (SX-2), -7702 (SX-3), -6852 (-SX4), -SX-5, -7703 (-SX6), and -7706 (-SX7). It seems that I-MAC induces antiviral effect presumably through miRNAs expression. Microarray analysis demonstrated that some of this miRNAs potentially target the open reading frame of the gene of HSV-1, poliovirus, HSV2, HIV-1 (13). This finding suggests that part of virus resistant feature of macrophages might be related to the presence of such novel miRNAs. microRNA mimics are innovative molecules designed to mimic endogenous mature miRNA molecules, when transfected into cells (14).

In this study, we investigated the effect of overexpression of the novel hsa-miR-7704 (-SX1) on HSV-1 replication using HeLa cells. Although the effect of lots of miRNAs on HSV-1 infection has been evaluated, the role of miR-SX1 on HSV-1 replication has remained uninvestigated. These findings may provide a new insight to miRNA-based antiviral therapy.

MATERIAL AND METHOD

Cell culture and virus

HeLa cell line (ATCC CCL-2) was obtained from the National Cell Bank of Iran, Pasteur Institute of Iran (Tehran, I.R. Iran) and maintained in Dulbecco's modified eagle's medium (DMEM, Gibco, USA)-high glucose supplemented with 10% fetal calf serum (FCS; Gibco, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C in 5% CO₂ (15). The HSV-1 strain was kindly provided from Virology Department, Iran University of Medical Sciences, Tehran, I.R. Iran. The HSV-1 stock was propagated on HeLa cells, titrated by 50% tissue culture infective dose (TCID₅₀) (16) and stored at -80 °C.

Cytotoxicity assay

Cytotoxicity of miR-SX-1 on HeLa cells was determined using 3-(4,5-dimethyl-2-thiazolyl) - 2,5-diphenyl - 2H - tetrazolium bromide (MTT) assays (17). HeLa cells (15×10^4 /well) were plated in 96-well plates. Cells were transfected with different concentrations of miRNA (50 and 100 nM) by polyfect (Qiagen, USA) according to the manufacturer's instruction and incubated for 24 h. Each transfection experiment was done in triplicate. After predetermined time, 10 µL MTT stock solution (5 mg/mL) was added to each well, and the plate was incubated for 4 h in the dark. Then, the MTT solution was discarded and 100 µL dimethyl sulfoxide (DMSO) was added to each well, and plates were gently shaken for 15 min at room temperature. The viability of cells was recorded at an absorbance of 570 and the reference wavelength of 630 nm by ELISA reader (BioRad, USA). The percentage of cell viability of each group was calculated by the following equation (17):

$$\text{Cell viability} = \left(\frac{\text{Absorbance of transfected cell}}{\text{Absorbance of control cells}} \right) \times 100$$

HSV-1 miR-SX1 mimics and transfection

The miR-SX-1 mimics used in this experiment, are double-stranded and chemically synthesized by Qiagen (USA) according to the mature nucleotide sequences of miR-has-7704 obtained from www.mirbase.org as 5'- CGGGGUCGGCGGCGACGUG - 3'(accession number MIMAT0030019). The mock, as a negative control, is a small RNA that does not target any known gene, were purchased from Exiqon, Denmark. HeLa cells were grown to 70-80% confluence and then transfected with miR-SX-1 (50 or 100 nM) and mock miRNA or without miRNA, by Polyfect. After 24 h post-transfection, the cells were infected with HSV-1 at multiplicity of infection (MOI) 0.1, 1, 5 for 1 h with gentle shaking 15 min intervals to allow viral adsorption. The cells were cultured with serum-free DMEM at 37 °C in 5% CO₂ incubator (18). The inhibitory effect of miRNA on HSV-1 replication was measured by comparing cytopathic effect (CPE) on transfected cells to no transfection or mock after 2- days posttransfection.

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Transfection efficacy

HeLa cells were seeded in 24-well cell culture plates. At 70-80% confluency FAM-labeled miRNA mimic control 5'UCACCGGGUAAUCACUUG (miRCURY LNA microRNA, Exiqon, Denmark) was transfected into HeLa cells at 50 and 100 nM concentrations. On 24, 48, and 72 h post-transfection, the transfection efficacy was assessed using fluorescent microscope (Nikon, Japan).

Plaque assays

HeLa cells were grown in 24-well plates to 70 to 80% confluence and then transfected with miR-SX1 mimics and infected with HSV-1 as described above and let to absorb for 1 h at 37 °C in 5% CO₂ incubator with gentle shaking at 15 min intervals. Then, cells were overlaid with 1 mL of a 1:1 mixture of Noble agar (Difco, USA) and 2 × DMEM/high glucose. After 48 h post-infection agarose was removed carefully and plates were fixed with 10% paraformaldehyde for 15 min and stained with 1% crystal violet for 20 min. Plaques were counted and photographed. The plaque reduction ratio was calculated (18).

Quantitative real-time polymerase chain reaction analysis

HeLa cells were grown in 12-well plates to 80 to 90% confluency and then transfected with various miRNA (50, 100 nM) by polyfect. After 24 h, cells were infected with HSV-1 at the indicated MOI. DNA was extracted from HSV-1 infected cells. The cells were washed three times with PBS and then digested in 500 µL lysis buffer (10 mM tris-HCL pH 7.4, 0.1 M EDTA, 0.5% SDS). DNA was extracted from HSV-1 infected cells by 3 times freeze and thawing followed by phenol-chloroform.

The DNA was precipitated with 100% ethanol in the presence of 0.3 M sodium acetate. After washing with 70% ethanol, the pellet were dried and suspended in 100 µL of sterile tris-EDTA buffer and stored at -20° C (19).

To detect the relative expression level of HSV-1 DNA, SYBR Green based real time polymerase chain reaction (RT-PCR) was

carried out. Relative expression levels of HSV-1 DNA were assessed by amplification of viral UL47 gene.

The primer pairs used to detect UL47 were as follows: forward, 5'GACGTACGCGATGAGATCAA3' and reverse, 5'GTTACCGGATTACGGGGACT3' (20). Human beta-globin was used as internal control gene (21). All RT-PCRs were done in 20 µL volume containing Real Q plus master mix green (Ampliqon, Denmark). The reaction conditions were 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 65 °C for 20 s, and 72 °C for 30 s, and 72 °C for 5 min by ABI Step-one (Applied Biosystems, USA). Each run was completed with a melting curve analysis to confirm the specificity of the amplification.

Statistical analysis

Data are demonstrated as mean ± standard deviation (SD). Kolmogorov-Smirnov's test and based on the normal distribution of the data, independent sample t-test were used to analyze the data with SPSS, Version 20. The significance level was considered to be less than 0.05.

RESULTS

MiR-SX-1 effects on cell viability

To determine the effect of miR-SX-1 on the viability of cells, we used MTT assay (Fig. 1). The viability of HeLa cells in different concentrations (50 and 100 nM) of miR-SX-1 remained unchanged ($P > 0.05$).

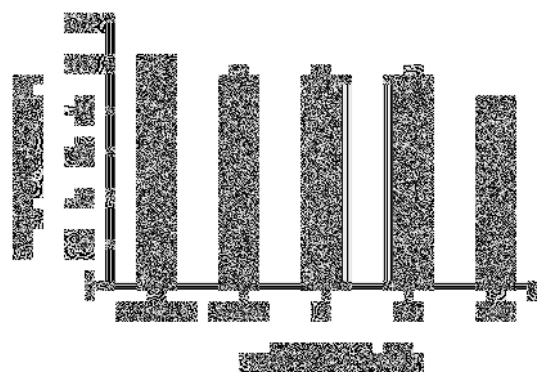


Fig. 1. Cell viability with miR-SX1 mimics and mock at different concentrations assessed by MTT assay. Each sample was analyzed in triplicate ($P > 0.05$).

Transfection efficacy

The transfection efficiency, appropriate concentration, and optimum expression condition of miR-SX1 in HeLa cells were determined by fluorescent microscopy. As shown in Fig. 2, the transfection efficiency was up to 85% on the first day. The percentage of transfection was maintained about 80% up to 48 h after transfection but decreased 72 h later.

MiR-SX-1 inhibits cytopathic effects of HeLa cells infected with HSV-1

HeLa cells were transfected with 50 and 100 nM concentrations of miR-SX-1 mimics or mock miRNA. Twenty-four h post

transfection HeLa cells were infected with HSV-1 at MOI of 0.1, 1, and 5 as described above. Cellular morphological changes were observed 6 and 24 h post infection by inverted microscope and photographed. At 6 h post-infection, there were no obvious differences in cellular morphology among cells transfected with miR-SX-1, the mock, without miR-SX-1, and control cells (data not shown). Infected cells usually show clusters. At 24 h post-infection, miR-SX-1 reduced CPE in a dose-dependent manner with full inhibition at 100 nM miR-SX1 in MOI of 1, and 5. Although, CPE increased clearly in HSV-1 infected cells alone or cells transfected with the mock miRNA control (Fig. 3a-3c).

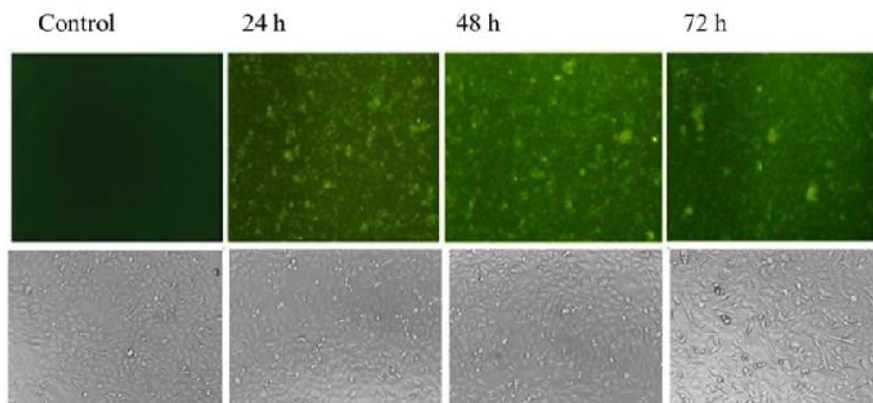


Fig. 2. Transfection efficiency. FAM-labeled miRNA at 100 nM was transfected into HeLa cells. The transfection efficiency of the miRNA was detected by fluorescent microscopy and brightfield at 24, 48, and 72 h after transfection. Magnification, 400 \times .

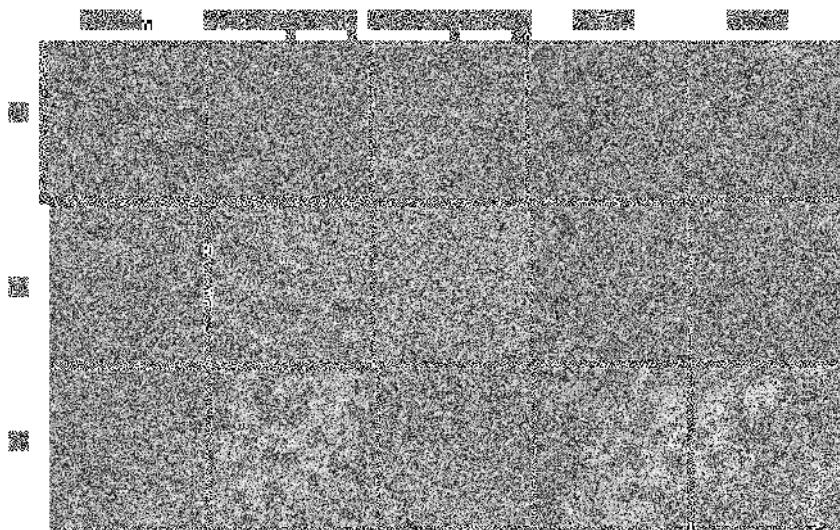


Fig. 3. MiR-SX1 effects on HSV-1 production in HeLa cells. HeLa cells were transfected with miR-SX1 mimics at 50, 100 nM or mock at 100 nM, followed by HSV-1 infection with MOI (a) 0.1, (b) 1, and (c) 5, 24 h post-transfection. The cells were imaged at 24 h post-infection. CPE clearly increased in the cells transfected with mock and HSV-1 infected cells alone, although CPE dramatically decreased in cells treated with miR-SX1 mimics. Magnification, 400 \times . miR, microRNA; HSV-1, herpes simplex virus type 1; CPE, cytopathic effect.

MiR-SX1 reduces HSV-1 titer in HeLa cells

HeLa cells were transfected with miR-SX1 (50 and 100 nM) followed by infecting HSV-1 into the cells at different MOI (0.1, 1, and 5), after 24 h post transfection. At 24 h post-infection, the titer of virus in miR-SX1 transfected cells was much lower than in the mock and non-transfected cells (Fig. 4). The virus production is reduced more than 94% at 100 nM of miR-SX-1 in MOI of 5 ($P < 0.001$)

Evaluation of miR-SX1 on HSV-1 infection was performed by plaque assay, as a gold standard phenotypic method for evaluation of HSV-1 susceptibility to antiviral drugs (18). miR-SX1 at 100 nM reduced plaque numbers about 75, 94, and 96 % at MOI 0.1, 1, and 5,

respectively compared with the control virus (Fig. 5).

MiR-SX1 decreased HSV-1 genome load

RT-PCR is reported to be a sensitive method for determination of antiviral susceptibility of HSV-1 (22). Because genome replication makes the virus pathogenic, the antiviral activity of miR-SX1 was investigated by quantification of the viral genome. Primers targeting UL 47 was used for viral quantification. As shown in Fig. 6, HeLa cells transfected with miR-SX1 at 100 nM, could result in a noteworthy decrease of viral DNA in cells compared with the virus control group ($P < 0.001$).

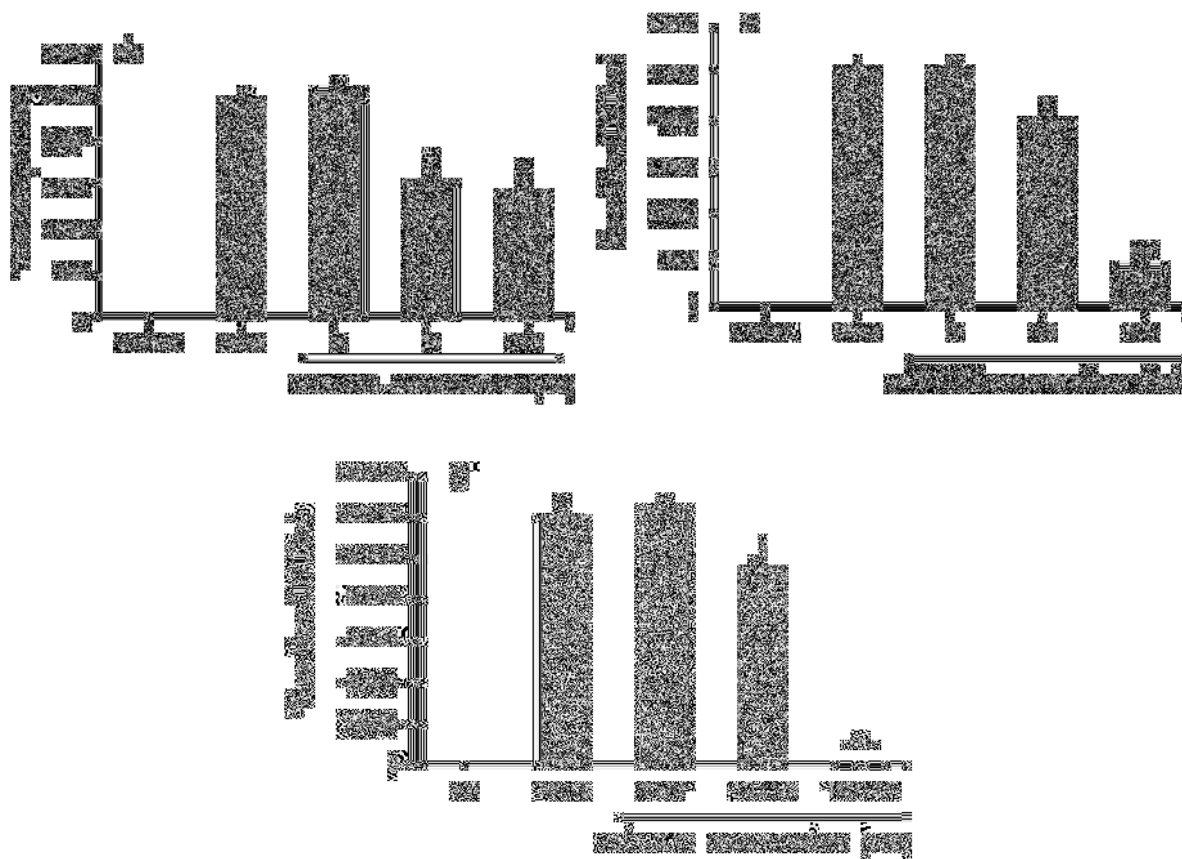


Fig. 4. Virus titer in HeLa cells transfected with miR-SX1 mimics. HeLa cells were transfected with miR-SX1 at 0, 50, and 100 nM and mock RNA or without miRNA. After 24 h post-transfection, the cells were infected with HSV-1 with MOI (a) 0.1, (b) 1, and (c) 5. After 24 h samples were harvested for TCID₅₀. Control group are cells without treatment and virus infection. TCID₅₀ showed that treatment with miRNA mimics at 100 nM decreased the titer of the virus in different MOIs compared to the mock and HSV-1 infected cells, * $P < 0.05$ and *** $P < 0.001$. miR, microRNA; HSV-1, herpes simplex virus type 1; TCID₅₀, 50% tissue culture infective dose ; MOI, multiplicity of infection.

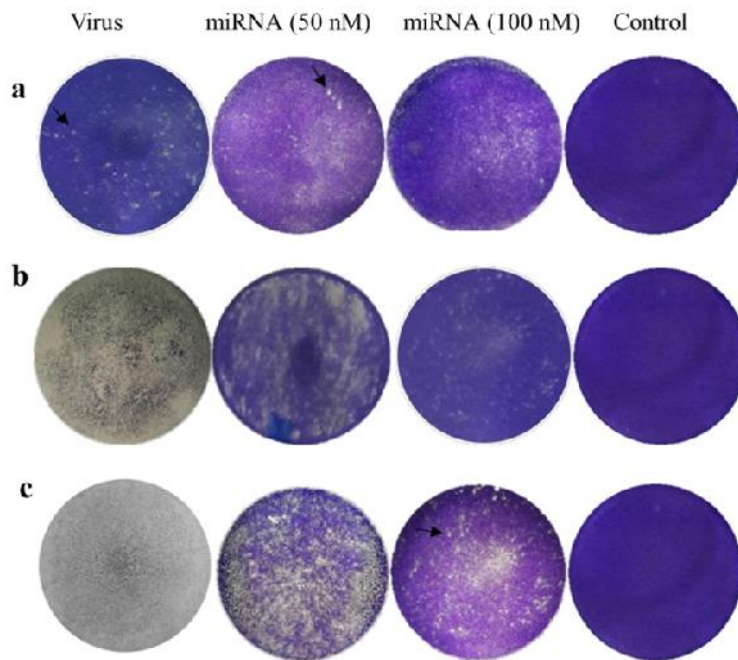


Fig. 5. Effect of miR-SX1 on plaque formation. HeLa cells were transfected with miRNAs at specified concentration and infected with HSV-1 at different MOI (a, 0.1; b, 1; and c, 5). Plaques were counted 72 h later compared with the virus control group. Arrows indicates virus plaque. miR, microRNA; MOI, multiplicity of infection.

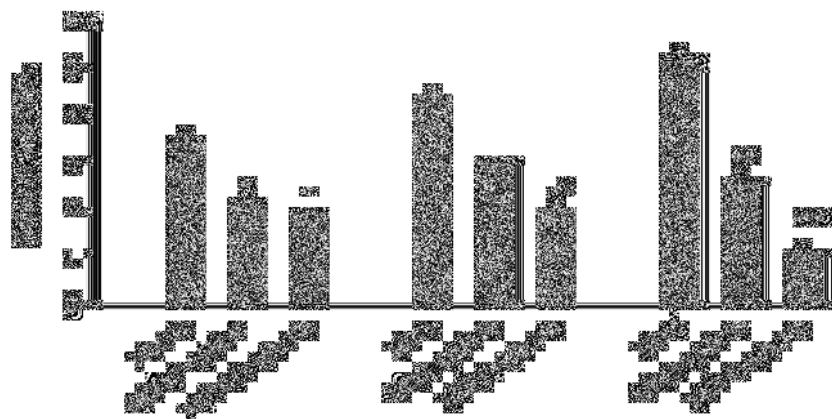


Fig. 6. Effect of miR-SX1 on viral genome load. HeLa cells were transfected with 50 and 100 nM of miR-SX1 and infected with HSV-1 at MOI 0.1, 1, and 5. At 18 h post-infection the DNA was extracted and relative level of viral DNA was quantified. Data are analyzed as the mean of three individual experiments. Significant differences were observed in comparison with corresponding control group (viral group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

Several studies have shown miRNAs are important molecules that can regulate HSV-1 replication. Therefore, it can be concluded that some viral disease could be regulated or treated by inducing or controlling miRNAs. It has been demonstrated that miR-H6 encoded from HSV-1 genome targets ICP4 to help maintain latency (23). It has shown that the

synthesized ICP4-targeting siRNA exhibits excellent antiviral activity in inhibiting HSV-1 replication in retinal pigment epithelial cells (18). Also, siRNAs targeting VP5 (HSV-1 major capsid protein) confers excellent antiviral activity by inhibiting HSV-1 replication in cells (24). Because the effect of the novel miR-SX1 on HSV-1 has not yet been investigated, we focused to study the effect of miR-SX1 mimics on HSV-1 replication in

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HeLa cells. As we have shown, less CPE and lower viral titer were detected in HSV-1 infected cells with miR-SX1 compared with mock-treated cells or untreated controls. We found that miR-SX1 mimics inhibits HSV-1 CPE in HeLa cells. Similar results were reported by Umbach *et al.* and Duan *et al.* who found that viral miR-H6 inhibits expression of ICP4 in 293 cells and HCE (23,25). Cytotoxicity levels of miR-SX1 were very low even at high concentrations (data not shown). In addition, inhibitory effects were related to the concentration of the miRNA, such that 100 nM miRNA was, to some degree, more effective than 50 nM (Fig. 4). The concentration dependency of inhibitory effects of miRNA have also been reported in other virus infections. The inhibitory effect of miR-SX1 was investigated with different MOI of HSV-1. We showed that at different MOI, CPE decreased dramatically, while multinucleated giant cells could be seen in non-transfected infected cells. The course of infection will be really different between lower and higher MOI where at higher MOI the effect of HSV-1 infected cells can be seen while it will be difficult to see the effects derived from reinfections virus produced in cells infecting others. We found that miR-SX1 could inhibit infecting of other cells at low MOI (Fig. 3). In our present study, we used HeLa cells to study the effect of miR-SX1 mimics on replication of HSV-1. Recently, it has shown that these novel miRNAs, except miR-SX5, are expressed in low levels in HeLa cells (26). Therefore, the phenotypic effect of mimic miRNA-SX1 on HSV-1 infected HeLa cells is directly related to the exogenously transfected miRNA. The reduction of viral yields by RT-PCR validates the antiviral activity of miR-SX1 in HeLa cells. Targets of miRNA have a significant role in viral pathogenesis and host defense mechanisms. The potential putative virus targeted by miR-SX1 were identified by scanning of viral reference sequences from NCBI (13). The alignment binding position nucleotides (start position 66169 and 68339) would be UL30 gene which is involved in the DNA replication. One possible reason is that the inhibition of DNA replication might be a

strong candidate for the antiviral effect of miR-SX1 in the host cells. This is the first time any phenotypic effect of this novel miRNA is examined. More studies on functional aspects of the novel miR-SX1 such as identification of its key molecular targets would have a greater effect on our understanding of the antiviral mechanism of hsa-miR-7704 (-SX1).

CONCLUSION

We provided evidences for antiviral effect of novel hsa-miR-7704 (-SX1) on HSV-1 replication in HeLa cells. The identification of antiviral miRNAs provides beneficial evidence to develop our therapeutic strategies to control virus infections. Further studies are warranted to characterize the effect of this miRNA on various viral infections and cell types to define the role of miRNA on viral pathogenicity and host defense.

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