

Establishment of an antiviral assay system and identification of severe fever with thrombocytopenia syndrome virus inhibitors

Antiviral Chemistry and Chemotherapy
2017, Vol. 25(3) 83–89
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DOI: 10.1177/2040206617740303
journals.sagepub.com/home/avc



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Abstract

Aims: Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne infectious disease. SFTS is epidemic in Asia, and its fatality rate is around 30% in Japan. The causative virus severe fever with thrombocytopenia syndrome virus (SFTSV) is a phlebovirus of the family Phenuiviridae (the order Bunyavirales). Although effective treatments are required, there are no antiviral agents currently approved for clinical use. Ribavirin and favipiravir were examined for their anti-SFTSV activity and found to be selective inhibitors of SFTSV replication *in vitro*. However, their activity was not sufficient. Therefore, it is mandatory to identify novel compounds active against SFTSV. To this end, we have established a safe and rapid assay system for screening selective inhibitors of SFTSV.

Methods: The virus was isolated from SFTS patients treated in Kagoshima University Hospital. Vero cells were infected with SFTSV and incubated in the presence of various concentrations of test compounds. After three days, the cells were examined for their intracellular viral RNA levels by real-time reverse transcription-PCR without extracting viral RNA. The cytotoxicity of test compounds was determined by a tetrazolium dye method.

Results: Among the test compounds, the antimalarial agent amodiaquine was identified as a selective inhibitor of SFTSV replication. Its 50% effective concentration (EC₅₀) and cytotoxic concentration (CC₅₀) were 19.1 ± 5.1 and $>100 \mu\text{M}$, respectively. The EC₅₀ value of amodiaquine was comparable to those of ribavirin and favipiravir.

Conclusion: Amodiaquine is considered to be a promising lead of novel anti-SFTSV agents, and evaluating the anti-SFTSV activity of its derivatives is in progress.

Keywords

Bunyaviridae, inhibitors, replication

Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is a recently identified tick-borne disease caused by SFTS virus (SFTSV). SFTSV is a novel phlebovirus of the family Phenuiviridae (the order Bunyavirales), which is epidemic in Asia including Japan.^{1–3} Main clinical features of SFTS are high fever, fatigue, gastrointestinal symptoms, such as vomiting and diarrhea, thrombocytopenia, and leukopenia. The elevation of serum aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase is often observed.⁴ The case fatality rate of SFTS is around 10%, according to the previous report in China. However, it increases with increasing age of patients and can be as high as 30% in Japan. Therefore, it is desired to identify the novel compounds active against SFTSV.

The nucleoside analog ribavirin has a broad spectrum of antiviral activity against various emerging virus diseases.⁵ These include bunyavirus infections,

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such as Crimean-Congo hemorrhagic fever, Rift Valley fever, hemorrhagic fever with renal syndrome, and hantavirus pulmonary syndrome.⁶⁻⁸ Therefore, ribavirin was examined for its antiviral activity against SFTSV replication *in vitro* and found to be active.^{9,10} More recently, the nucleotide analog favipiravir was reported to selectively inhibit SFTSV replication *in vitro* and *in vivo*.^{11,12} Favipiravir has been licensed in Japan as a potent and selective inhibitor of influenza virus replication through the inhibition of viral RNA-dependent RNA polymerase activity.¹³ However, the antiviral activity of favipiravir appears to be optimized for influenza virus. Therefore, it is still mandatory to identify selective SFTSV inhibitors of which mechanism of action is different from that of favipiravir.

In the present study, we established a safe and rapid assay system for screening selective inhibitors of SFTSV *in vitro*. Using the assay system, we examined several compounds for their antiviral activity and found that the antimalarial drug amodiaquine and its derivatives were selective inhibitors of SFTSV replication.

Materials and methods

Compounds

Amodiaquine was synthesized according to the method described by Burckhalter et al.¹⁴ 4-(7-Fluoroquinolin-4-ylamino)-2-diethylaminomethylphenol is a novel compound and was synthesized by the following procedure. Briefly, 4-amino-2-[(diethylamino)methyl]phenol dihydrochloride (133.6 mg, 0.500 mmol) was dissolved in dry ethanol (2.0 ml) and 4-chloro-7-fluoroquinoline (95.3 mg, 0.525 mmol) was added to the solution, and the reaction mixture was refluxed for a further 3 h. The solvent was removed under reduced pressure, and the residue was dissolved in distilled water. Careful addition of a dilute ammonium hydroxide (2%) liberated the free base as a solid. The solid was filtered off, washed with water, and dried for overnight under vacuum. Purification was achieved by recrystallization using methanol to give the compound. 4-(7-Bromoquinolin-4-ylamino)-2-diethylaminomethylphenol and 4-(7-iodoquinolin-4-ylamino)-2-diethylaminomethylphenol were synthesized according to the method described by Conroy et al.¹⁵ Ribavirin and favipiravir were purchased from Sigma-Aldrich (St. Louis, MO) and Selleckchem (Houston, TX), respectively. All compounds were dissolved in dimethyl sulfoxide at a concentration of 20 mM and stored -20°C until use.

Cells and virus

Human hepatoma-derived Huh-7 and monkey kidney-derived Vero cells were used for experiments. The cells were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin. A clinical strain of SFTSV was isolated from SFTS patients hospitalized and treated in Kagoshima University Hospital. The virus was propagated in Huh-7 cells, and its infectious titer was determined in a focus forming assay. Briefly, the cells were inoculated with serially diluted virus suspensions. After incubating for three days, viral foci were detected by immunostaining with an anti-SFTSV rabbit polyclonal antibody, as previously described.¹¹ The virus stocks were stored at -80°C until use. All experiments with infectious SFTSV were performed in a biosafety level 3 (BSL3) facility in Kagoshima University, according to the institutional biosafety operating regulations and procedures.

Results

Detection and isolation of SFTSV from patients

The serum samples were inoculated onto Vero cells. After incubating for three days, viral RNA was isolated from culture supernatants and amplified with reverse transcription (RT)-PCR using SFTSV-specific primer pairs.¹⁶ The cells were subjected to immunostaining with the anti-SFTSV antibody and observed microscopically (Figure 1). The culture supernatants were further inoculated onto Huh-7 cells to propagate the virus. After incubating for three days, the culture supernatants of infected Huh-7 cells were examined for their infectious titer by immunostaining and found to be 5×10^6 focus forming units (FFU)/ml (data not shown).

Establishment of anti-SFTSV assay system

Vero cells (2×10^4 cells/well) were cultured in a microtiter plate. After incubating for 24 h, the cells were infected with various dilutions of SFTSV and further incubated in the presence of various concentrations of test compounds. After three days, the cells were washed once with PBS. The intracellular SFTSV RNA was quantified with real-time RT-PCR using TaqMan Gene Expression Cells-to-CT™ Kit (Thermo Fisher Scientific, Waltham, MA) and SFTSV-specific TaqMan primers/probe.¹⁷ The primers (5'-GGGTCC CTGAAGGAGTTGTAAA-3' and 5'-TGCCTTCA CCAAGACTATCAATGT-3') and probe (5'-FAM-TTCTGTCTTGCTGGCTCCGCGC-TAMRA-3') were designed to target the conserved nucleic acid sequences

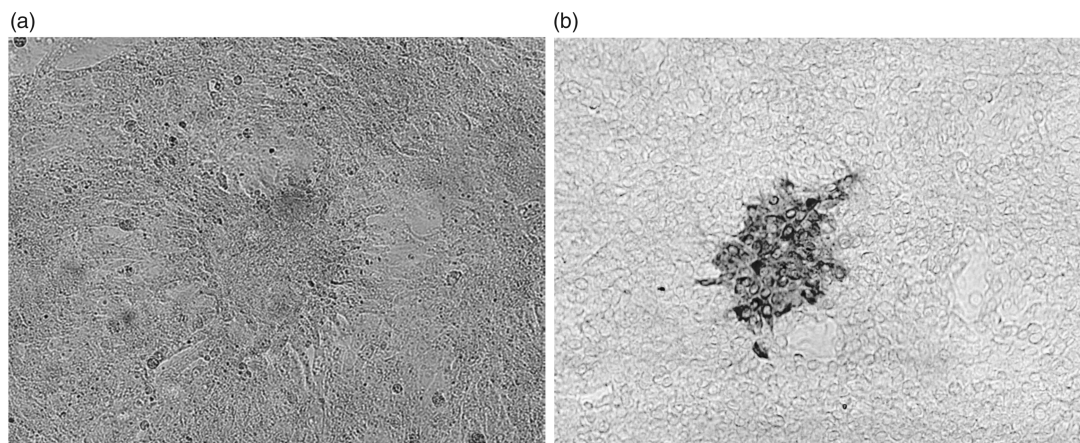


Figure 1. SFTSV-infected Vero cells. (a) Microscopic observation (100 \times) and (b) immunostaining (100 \times).

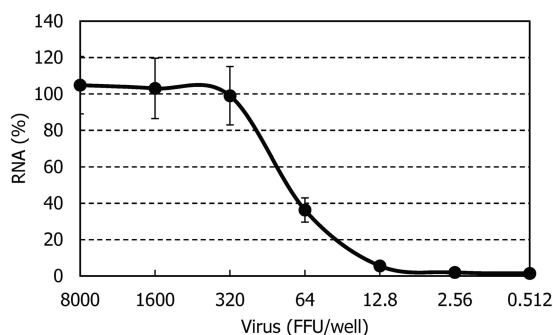


Figure 2. Dose-dependent viral RNA amplification signal curve. Vero cells (2×10^4 /well) were cultured in a 96-well plate for 24 h in the absence of compounds. After incubation, the cells were infected with the indicated amount of SFTSV and further incubated. After three days, the amount of intracellular viral RNA was determined by the real-time RT-PCR method using TaqMan Gene Expression Cells-to-CTTM Kit. The experiment was carried out in triplicate and means \pm SD are shown.

in the S segment. Figure 2 shows a dose-dependent viral RNA amplification signal curve. The amplification signal was saturated, when the viral input was 320 FFU or higher. No signal was identified, when the input was less than 12.8 FFU. The amplification signal curve decreased with decreasing the input between 320 and 12.8 FFU. Therefore, 200 FFU (multiplicity of infection=0.01) was used for the anti-SFTSV assay of compounds.

The cytotoxicity of compounds was examined in parallel with their antiviral activity, which was based on the viable cell number of mock-infected cells determined by a tetrazolium dye method. Briefly, Vero cells (2×10^4 cells/well) were cultured in a microtiter plate. After incubating for 24 h, the cells were exposed to various concentrations of test compounds and further incubated. After three days, 100 μ l of culture medium was removed from each well, and 10 μ l of water-soluble

tetrazolium dye solution (Dojindo, Kumamoto, Japan) was added. After incubating for 2 h, the absorbance was read at two wavelengths (450 and 690 nm) with a microplate reader.

Anti-SFTSV activity of amodiaquine derivatives

Several nucleoside and nonnucleoside compounds were examined for their anti-SFTSV activity in Vero cells. Ribavirin and favipiravir were used as reference compounds. As previously reported, both compounds reduced the intracellular SFTSV RNA level in a dose-dependent manner (Figure 3(a) and (b)). Ribavirin and favipiravir did not reduce the viability of mock-infected Vero cells at concentrations up to 100 μ M. These results indicate that the present assay method functions for evaluating the anti-SFTSV activity of compounds. Among the test compounds, the antimalarial agent amodiaquine was identified as a selective inhibitor of SFTSV replication (Figure 3(c)). The anti-SFTSV activity of amodiaquine was almost equivalent to that of favipiravir. The 50% effective concentrations (EC_{50}) of ribavirin, favipiravir, and amodiaquine were 40.1 ± 16.3 , 25.0 ± 9.3 , and 19.1 ± 5.1 μ M, respectively (Table 1). Their 50% cytotoxic concentrations (CC_{50}) were > 100 μ M, indicating amodiaquine is a selective inhibitor of SFTSV replication.

To gain more active inhibitors, several amodiaquine derivatives were synthesized and examined for their activity. When the chloride of amodiaquine was substituted for another halogen molecule, such as fluorine, bromine, and iodine, all derivatives also displayed the anti-SFTSV activity (Table 2). Among the derivatives, the iodine derivative appeared to be the most active. Its EC_{50} and CC_{50} were 15.6 ± 4.9 and > 100 μ M, respectively.

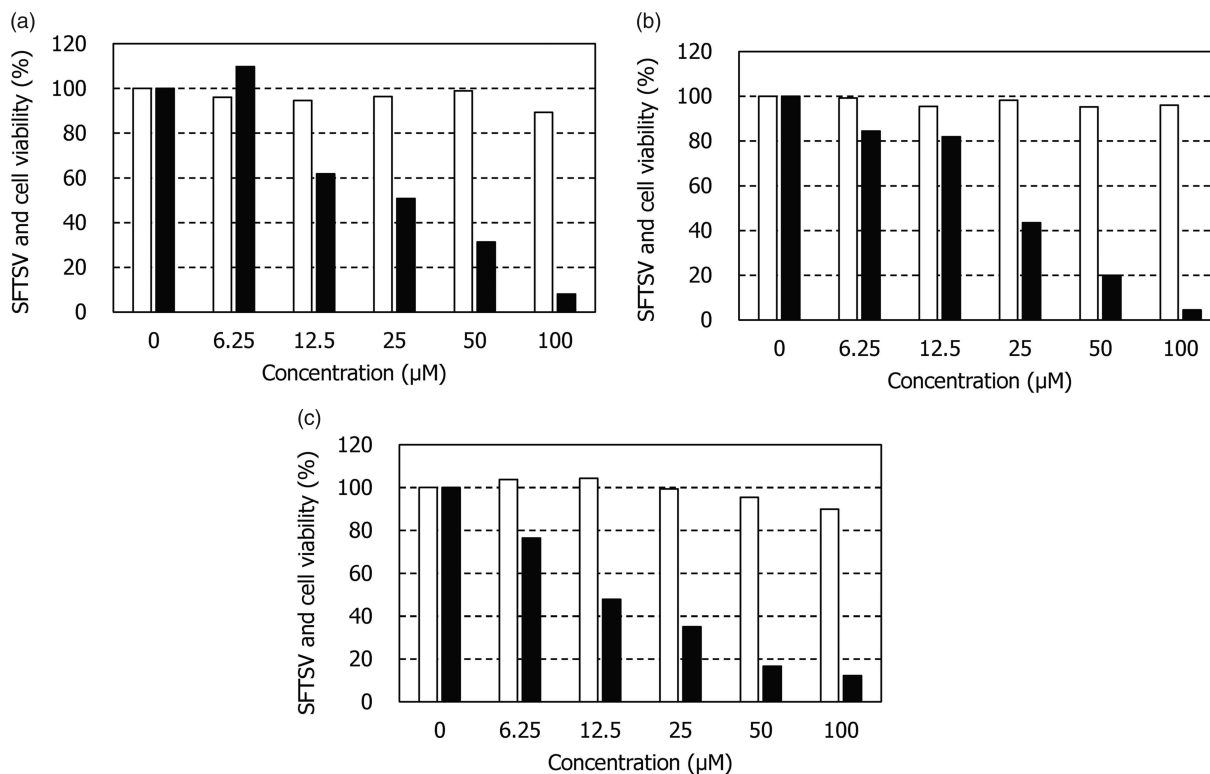


Figure 3. Anti-SFTSV activity and cytotoxicity of ribavirin, favipiravir, and amodiaquine. Vero cells (2×10^4 /well) were cultured in a 96-well plate for 24 h in the absence of compounds. After incubation, the cells were mock-infected or infected with SFTSV at a multiplicity of infection of 0.01 and further incubated in the presence of various concentrations of (a) ribavirin, (b) favipiravir, and (c) amodiaquine. After three days, the number of viable mock-infected cells (open columns) and the intracellular viral RNA levels of infected cells (closed columns) were determined by the tetrazolium dye and real-time RT-PCR methods, respectively. The experiment was carried out in triplicate, and representative results are shown. SFTSV: severe fever with thrombocytopenia syndrome virus.

Table 1. Anti-SFTSV activity and cytotoxicity of ribavirin, favipiravir, and amodiaquine in Vero cells.

Compound	EC ₅₀ (μM)	CC ₅₀ (μM)
Ribavirin	40.1 ± 16.3	>100
Favipiravir	25.0 ± 9.3	>100
Amodiaquine	19.1 ± 5.1	>100

Note: All data represent mean ± SD for three separate experiments. EC₅₀: 50% effective concentration; CC₅₀: 50% cytotoxic concentration; SFTSV: severe fever with thrombocytopenia syndrome virus.

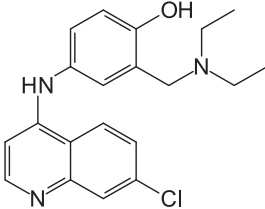
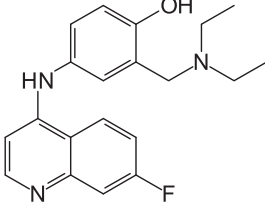
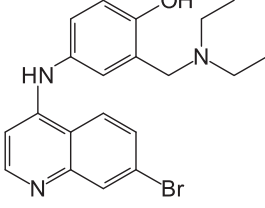
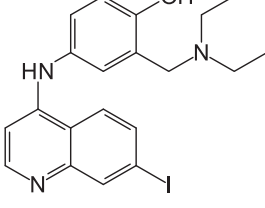
Discussion

Since the identification of the first case of SFTS in 2012, more than 250 cases have been reported so far in Japan.^{18,19} In particular, Kagoshima prefecture, where our university is located, is one of the most epidemic areas of SFTS in Japan. Therefore, we attempted to isolate SFTSV from patients and establish a simple and safe assay system for screening of compounds against the virus. SFTSV can grow in a variety of cell lines, including Vero and Huh-7 cells. However, it was

reported that SFTSV hardly induced cytopathic effect in infected cells, except for the macrophage cell line DH82.¹ In fact, we also observed that our clinical isolates could propagate well in Vero and Huh-7 cells without apparent morphological change (Figure 1(a) and data not shown). Therefore, immunostaining with an anti-SFTSV antibody was necessary for detecting the infected cells (Figure 1(b)).

To evaluate the antiviral activity of compounds, several assay methods have been established and used for their screening. For anti-SFTSV assays, the viral yield reduction assay is most commonly used. It is based on the measurement of infectious virus titer of culture supernatants in the absence or presence of compounds by a focus forming assay.⁹⁻¹¹ This method is reliable and reproducible but not suitable for screening of compounds, since serial dilution of culture supernatants, overlay with agar or methylcellulose, and immunostaining with a virus-specific antibody are required to complete the assay. It is also possible to measure SFTSV RNA in culture supernatants by real-time

Table 2. Anti-SFTSV activity and cytotoxicity of amodiaquine derivatives in Vero cells.

Compound	Structure	EC ₅₀ (μM)	CC ₅₀ (μM)
1 (Amodiaquine)		19.1 ± 5.1	>100
2		36.6 ± 9.3	>100
3		31.1 ± 16.8	>100
4		15.6 ± 4.9	>100

Note: All data represent mean ± SD for three separate experiments.

1: 4-(7-Chloroquinolin-4-ylamino)-2-diethylaminomethylphenol; 2: 4-(7-Fluoroquinolin-4-ylamino)-2-diethylaminomethylphenol; 3: 4-(7-Bromoquinolin-4-ylamino)-2-diethylaminomethylphenol; 4: 4-(7-Iodoquinolin-4-ylamino)-2-diethylaminomethylphenol; EC₅₀: 50% effective concentration; CC₅₀: 50% cytotoxic concentration; SFTSV: severe fever with thrombocytopenia syndrome virus.

RT-PCR.¹⁰ However, the extraction of viral RNA from each culture supernatant is necessary, suggesting that the method is not realistic to deal with a large number of assay samples. Furthermore, considering the high virulence of SFTSV, which has to be handled in the BSL3 facility, the infectious virus particles in assay samples should be inactivated at an early step of the assay procedures.

Based on the above, we have decided to directly measure the intracellular viral RNA level without extracting RNA. The infected cells exposed to various concentrations of test compounds can be treated with lysis buffer after washing with PBS and subjected to real-time RT-PCR without extracting RNA, when TaqMan Gene Expression Cells-to-CT™ Kit is used for the assay.

The treatment of the cells with lysis buffer inactivates infectious virus particles, so that the subsequent experiments can be performed without the BSL3 facility. In addition, all manipulations from cell lysis to real-time RT-PCR can be carried out in a microplate, which has already been applied to our anti-hepatitis C virus assay in replicon cells.^{20,21} Only four days are required to complete the assay, even when the cells are infected with the virus at a low multiplicity of infection (0.01). Furthermore, culture medium change or replenishment is not necessary during the assay period. Taken together, it is clear that the established anti-SFTSV assay is rapid, sensitive, and safe.

Using the assay system, we have examined a number of compounds for their anti-SFTSV activity in vitro.

Among the compounds, amodiaquine was identified as a selective inhibitor of SFTSV (Figure 3(c)). Amodiaquine is a drug approved for treatment of malaria in clinic, while it is also known to inhibit dengue virus and Ebola virus replication in vitro and in vivo, respectively.^{22,23} Sakurai et al.²⁴ recently showed that 14 derivatives including amodiaquine itself were potent and selective inhibitors of Ebola virus replication in vitro. We also tested several amodiaquine derivatives and found to be active against SFTSV replication (Table 2). Although the mechanism (target molecule) of amodiaquine for inhibition of SFTSV replication remains unknown, it is possible that amodiaquine inhibits SFTSV replication through a mechanism similar to that against dengue virus.²² Further studies, such as time-of-addition experiments and combination with other antivirals, are in progress to elucidate the mechanism of action of amodiaquine.

In conclusion, we have established a safe and rapid assay system for screening selective inhibitors of SFTSV. Using this system, we have found that amodiaquine and its derivatives are selective inhibitors of SFTSV replication in vitro. Thus, the compounds should be further pursued for their clinical potential as anti-SFTS agents.

Acknowledgements

The authors are thankful to the patients and staff at the Hematology and Immunology, Kagoshima University Hospital.

Authors' contribution

MB and MT carried out the experimental work. MB and MO analyzed the data. NS synthesized amodiaquine derivatives. NA collected sera from SFTS patients, while MS provided the anti-SFTSV rabbit polyclonal antibody as well as useful information of the virus. MB wrote the manuscript.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: MB, MT, and NS are the inventors of a patent application on amodiaquine derivatives as anti-SFTSV agents.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported in part by JSPS KAKENHI Grant number JP26460559 and Center for Clinical and Translational Research of Kyushu University (2016, A-77).

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