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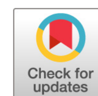
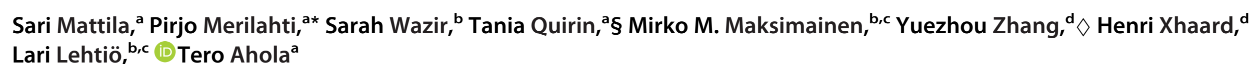
ANTIVIRAL AGENTS



# Macrodomain Binding Compound MRS 2578 Inhibits Alphavirus Replication



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**ABSTRACT** Alphaviruses are positive-strand RNA viruses causing febrile disease. Macrodomain-containing proteins, involved in ADP-ribose-mediated signaling, are encoded by both host cells and several virus groups, including alphaviruses. In this study, compound MRS 2578 that targets the human ADP-ribose glycohydrolase MacroD1 inhibited Semliki Forest virus production as well as viral RNA replication and replicase protein expression. The inhibitor was similarly active in alphavirus *trans*-replication systems, indicating that it targets the viral RNA replication stage.

**KEYWORDS** macrodomain, alphavirus, antiviral, chikungunya virus, virus replication

Alphaviruses are positive-strand RNA viruses typically delivered to vertebrate hosts by mosquito vectors. Several alphaviruses, most importantly chikungunya virus (CHIKV), cause febrile illness with joint pain, rash, and arthritis (1), which can progress to chronic arthralgia persisting for several months or even years (2). CHIKV has caused epidemics all around the world in over 50 countries (3). There are currently no commercial vaccines or specific antiviral treatments against alphaviruses (4).

The alphavirus genome encodes four nonstructural proteins (nsP1 to -4), which are essential components of the RNA replication complex (5). Inhibitors of the virus-encoded enzymes polymerase, protease, and RNA-capping enzyme have already been described (6). nsP3 functions as a mediator of interactions with a broad range of host proteins (7). The amino terminus of nsP3 harbors a macrodomain, an evolutionarily conserved domain of ~150 amino acids. Macrodomains are present in several cellular proteins and encoded in the genomes of coronaviruses, alphaviruses, rubella virus, and hepatitis E virus. Macrodomains function in ADP-ribose (ADPr) signaling. They can bind mono(ADP-ribose) or poly(ADP-ribose) and in many cases remove ADPr marks from target proteins (8, 9). In CHIKV, the macrodomain is essential for replication (10). Thus, viral macrodomains could be promising targets for antivirals (8).

This study builds on a set of compounds that were previously discovered as potential human ADP-ribose glycohydrolase MacroD1 ligands through computational screens (11). In the previous study, from a pool of 65 virtual hits that were experimentally tested, 11 compounds showed activity using an assay measuring the inhibition of ADPr binding to MacroD1. However, at the time, it was not possible to validate further their weak activities.

Here, we decided to test the antiviral properties of these compounds. Using infection with Semliki Forest virus (SFV), a close relative of CHIKV, we tested the effects of 10 compounds that were available for repurchase (see Fig. S1 in the supplemental material), at 10 $\mu$M and 50 $\mu$M. Only one compound, MRS 2578, showed significant antiviral activity at

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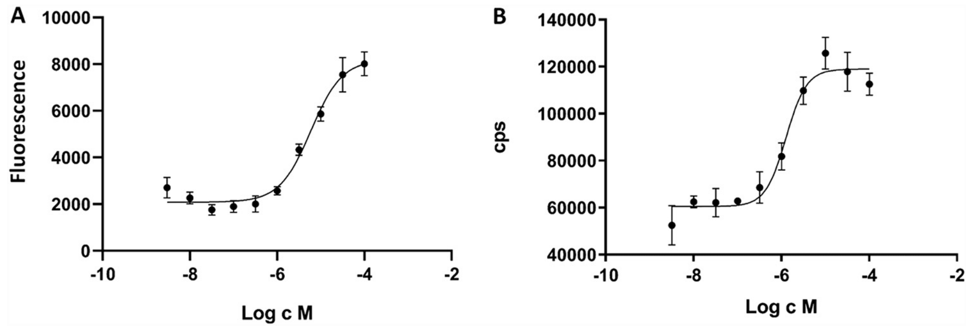


**FIG 1** MRS 2578 inhibits MacroD1 enzymatic activity. Concentration-response curves of the hydrolysis of $\alpha$-NAD$^+$ (A) and the removal of protein-linked ADP-ribose (B) are shown. Measurements were done by an activity-based fluorescence assay and an AlphaScreen-based assay, respectively. cps, counts per second.

a 50 $\mu$M concentration (Fig. S2). The compound MRS 2578 was previously found to inhibit the binding of MacroD1 (called LRP16 there) to poly(ADP-ribose) and several signaling proteins, with significant biological consequences in the cancer models studied (12).

We then concentrated on MRS 2578. We confirmed the biochemical inhibition of MacroD1 enzymatic activity by MRS 2578 with an activity-based fluorescence assay measuring the hydrolysis of $\alpha$-NAD$^+$ (13) and an AlphaScreen-based assay measuring the removal of protein-linked ADP-ribose (14). $IC_{50}$ (50% inhibitory concentration) values of 6.8 $\mu$M ($pIC_{50} \pm$ SEM [standard error of the mean], 5.17 $\pm$ 0.12; $n$ = 3) and 2.2 $\mu$M ($pIC_{50} \pm$ SEM, 5.65 $\pm$ 0.12; $n$ = 3) were observed, respectively (Fig. 1). In luciferase-based antiviral assays with a luciferase-bearing derivative of SFV4 termed SFV-Rluc, MRS 2578 strongly inhibited SFV infection at a 50 $\mu$M concentration (Fig. 2).

The antiviral activity of MRS 2578 was validated with other approaches. SFV-Rluc was used in all virus-based experiments. Virus production was determined by a standard plaque assay in high-multiplicity (multiplicity of infection [MOI] of 10) and low-multiplicity (MOI of 0.01) infections in BHK-21 cells. Both experimental setups showed effective reductions in the production of infectious SFV with 25 $\mu$M MRS 2578 treatment (Fig. 3A), by over 1 log and by 3 log PFU/ml, respectively. This effect increased further at a 50 $\mu$M concentration. Next, the effect of MRS 2578 on SFV RNA and protein expression was studied in virus-infected BHK-21 cells. The expression of the viral replicase proteins nsP1 and nsP3 was remarkably reduced at the 25 $\mu$M concentration even at a high MOI of 10 at 5 h postinfection (Fig. 3B). RNA levels followed a similar pattern, but RNA production did not stall entirely (Fig. 3C).

Plasmid-based *trans*-replication systems of SFV and CHIKV can be used to specifically study the replication stage of alphaviruses, as the replication proteins and RNA templates are produced from transfected DNA plasmids (15, 16). In both the SFV and CHIKV systems (see Fig. S3 in the supplemental material for the specific constructs used), MRS 2578 reduced the expression levels of the marker protein *Renilla* luciferase

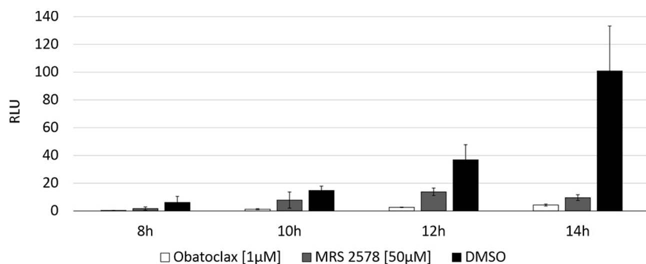


**FIG 2** MRS 2578 inhibits alphavirus infection. MRS 2578 was added at 50 $\mu$M at the same time as a luciferase-bearing derivative of SFV4 termed SFV-Rluc (luciferase fused with nsP3) (23) at a multiplicity of infection (MOI) of 0.01, and luciferase activity was measured at different time points. Obatoclax was used as a positive control (17). RLU, relative luminescence units; DMSO, dimethyl sulfoxide.



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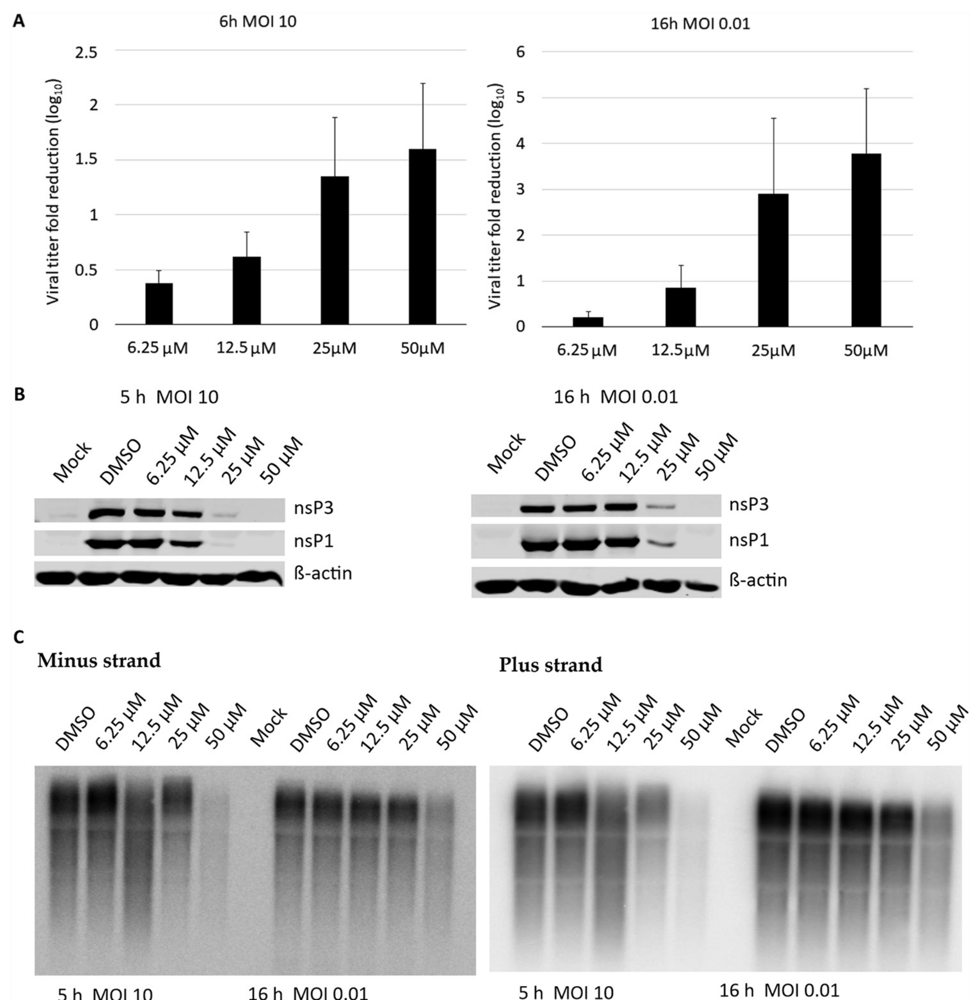


**FIG 3** Antiviral effects of MRS 2578 during virus infection. (A) SFV production in the presence of MRS 2578 was analyzed in infected cell culture medium at an MOI of 10 at 6 h postinfection and at an MOI of 0.01 at 16 h postinfection. The values are averages of the titers obtained from three experiments. The error bars represent standard errors. MRS 2578 was present at the indicated concentrations. (B) SFV nsP1 and nsP3 protein expression was detected by Western blotting. BHK-21 cells were incubated with SFV-Rluc (infection at an MOI of 10 or 0.01) and MRS 2578 for 5 h or 16 h. Actin was used as a control protein. (C) SFV RNA synthesis was analyzed by Northern blotting under the same conditions as the ones described above for panel B. Virus-specific RNAs were detected with probes complementary to the luciferase sequence in the viral genome (24), recognizing the minus strand and plus strand. Western and Northern blot experiments were reproduced twice, with similar results. The compound was added at the same time as the virus in all experiments.

(Rluc), placed under the control of the genomic promoter in place of the nsPs (Fig. 4A). The results indicate that MRS 2578 specifically inhibits the RNA replication stage, as the system bypasses virus entry. Virus replicase protein translation was investigated using the SFV *trans*-replication system: 2 h of transfection was followed by 14 h of incubation with different concentrations of MRS 2578. Surprisingly, Western blot analysis showed that the presence of nsP3 remarkably increased with the highest concentration of MRS 2578 (25 $\mu$M), indicating increased transcription and/or translation of plasmid-derived mRNA or decreased viral protein turnover (Fig. 4B). This increase in expression is not linked to viral RNA synthesis since the nsPs in this system are produced from a separate nonreplicating RNA in *trans*. This result further confirms that viral RNA replication is disrupted, even in the presence of the macrodomain-containing viral protein nsP3, during MRS 2578 treatment. To further exclude defects in entry and initial translation, we used a temperature-sensitive virus, SFV-ts9-Rluc, which is replication defective at the restrictive temperature but still undergoes entry and initial translation (17). MRS 2578



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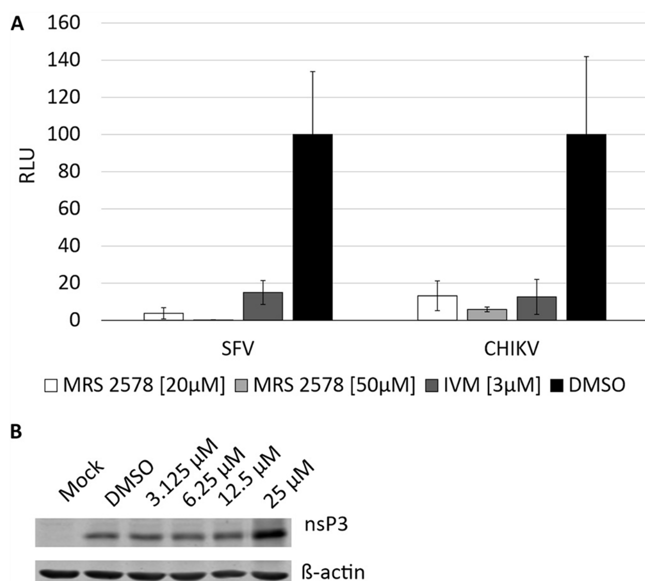


**FIG 4** Antiviral effect of MRS 2578 in SFV and CHIKV *trans*-replication systems. (A) Luciferase assay. The compound was added at 20 $\mu$M and 50 $\mu$M concentrations 2 h after transfection, and luciferase activity was measured 14 h after the addition. Ivermectin (IVM) was used as a positive control (25). RLU, relative luminescence units. (B) SFV nsP3 protein expression was detected by Western blotting in the same experimental setup, except that MRS 2578 was present at 3.125, 6.25, 12.5, and 25 $\mu$M concentrations.

had no effect on the luciferase expression of SFV-ts6-Rluc at 20 $\mu$M and only a minor effect at 50 $\mu$M (Fig. S4 in the supplemental material).

Based on SFV-Rluc luciferase assay data and plaque titrations, $IC_{50}$ values were calculated for SFV infections, and Rluc was used to calculate $IC_{50}$ values in *trans*-replication. Dose-response curves of the replicates ($n$ = 3) are presented in Fig. S5. BHK-21 and BSR-T7 cell toxicity (50% cytotoxic concentration [$CC_{50}$]) values were 98 $\mu$M and 66 $\mu$M, respectively (Table 1). The highest $IC_{50}$ value of 34 $\mu$M from SFV-Rluc infection experiments was approximately three times higher than the other measured $IC_{50}$ values. An explanation might be that for this virus, luciferase translation starts directly from the viral genome and thus does not require initial RNA replication. However, virus titration experiments show that virus production is inhibited at lower concentrations, giving $IC_{50}$ values comparable to those for the *trans*-replication systems (Table 1). In these experiments, the selectivity index (SI) values are >5, indicating that the antiviral effect of MRS 2578 is not due to toxicity.

We show here by using SFV and CHIKV *trans*-replication systems that the compound MRS 2578 inhibited the replication phase of the virus life cycle. It is possible that MRS 2578 also targets the essential viral macrodomain of nsP3, although it was initially identified by computational screening against the human protein MacroD1. We were unable to determine the biochemical inhibition of viral macrodomains, as the

**TABLE 1** Antiviral activity of MRS 2578 against SFV and CHIKV replication

| Cell line | Assay | Mean antireplication[a] $IC_{50}$ ($\mu$M) (±SEM) | Mean toxicity[b] $CC_{50}$ ($\mu$M) (±SEM) | SI[c] |
|---|---|---|---|---|
| BHK-21 | SFV-Rluc infection (luciferase) | 34.29 (±1.21) | 98.31 (±2.48) | 2.87 |
| | SFV-Rluc infection (plaque titrations) | 9.71 (±1.88) | 98.31 (±2.48) | 10.13 |
| BSR-T7 | SFV *trans*-replication (luciferase) | 11.37 (±1.96) | 65.54 (±7.92) | 5.77 |
| | CHIKV *trans*-replication (luciferase) | 11.99 (±2.62) | 65.54 (±7.92) | 5.47 |

[a]$IC_{50}$, concentration causing 50% inhibition of replication (± standard error of the mean).
[b]$CC_{50}$, cytotoxic concentration causing 50% inhibition of cell survival (± standard error of the mean).
[c]SI, selectivity index (ratio of the toxicity $CC_{50}$ to the antiviral $IC_{50}$).



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recombinant protein derived from CHIKV was not active in the two types of hydrolysis assays used, and thus, more work and assay development are required with the viral proteins. It seems unlikely that inhibition of MacroD1 would affect cytoplasmic RNA replication since MacroD1 is a mitochondrial protein (18–20), but we cannot exclude that the antiviral effect of MRS 2578 could be based on cellular targets.

Recently, two studies analyzed potential CHIKV macrodomain-targeting molecules identified computationally. Shimizu et al. (21) found two antivirals, with $IC_{50}$ values similar to those of MRS 2578, after computational screening of 48,750 small molecules followed by biological analysis of 12 compounds. Their selectivity indices were 3.1 and 3.2, suggesting that the cytotoxicity of the compounds could be an issue (21). Zhang et al. (22) screened small molecular fragments virtually and by X-ray crystallography. One fragment that bound nsP3 showed an antiviral effect at an $IC_{50}$ of 23 $\mu$M, with an uncertain SI (22). Also, in those two studies, the target causing the antiviral effect remained uncertain.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

## ACKNOWLEDGMENTS

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