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Activity of a phenolic dibenzylsulfide against New World arenavirus infections

Brian B Gowen^{1,2,3,*}, Kie-Hoon Jung^{1,3}, Eric J Sefing^{1,3}, Min-Hui Wong^{1,3}, Jonna B Westover^{1,3}, and Donald F Sme^{1,3}

¹Department of Animal, Dairy, and Veterinary Sciences, Utah State University, Logan, UT, USA

²School of Veterinary Medicine, Utah State University, Logan, UT, USA

³Institute for Antiviral Research, Utah State University, Logan, UT, USA

Abstract

Background—Junín virus (JUNV) and several other Clade B New World arenaviruses cause human disease ranging from mild febrile illness to severe viral hemorrhagic fever (HF). These viruses pose a significant threat to national security and safe and effective therapies are limited outside of Argentina, where immune plasma is the standard of care for treating JUNV infection in cases of Argentine HF.

Methods—An *in vitro* screen of the Chemtura library identified several compounds with activity against Tacaribe virus (TCRV), a Clade B arenavirus closely related to JUNV. Of these compounds, D746, a phenolic dibenzylsulfide, was further pursued for additional *in vitro* studies and evaluated in the AG129 mouse TCRV infection model.

Results—D746 was found to act during an early to intermediate stage of the TCRV replication cycle and micromolar range activity was confirmed by virus yield reduction assays with both TCRV and JUNV. Although intraperitoneal twice daily treatment regimens were found to be highly effective when started 2 hours prior to TCRV challenge in AG129 mice, post-exposure treatment initiated 3 days after infection was not efficacious. Interestingly, despite the pre-exposure treatment success, D746 did not reduce serum or tissue virus titers during the acute infection. Moreover, D746 elicited ascites fluid accumulation in mice during, as well as independent of, infection.

Conclusions—Our findings suggest that D746 may be altering the host response to TCRV infection in AG129 mice in a way that limits pathogenesis and thereby protects mice from otherwise lethal infection in the absence of measurable reductions in viral burden.

1. Introduction

Several clade B New World arenaviruses (NWA) cause disease ranging from mild febrile illness to fatal viral hemorrhagic fever (HF) in humans. These arenaviruses are transmitted to humans typically through contact with rodent reservoirs or inhalation of aerosolized virus passed in their excreta. Junín virus (JUNV) is the etiologic agent of Argentine HF, a disease that can evolve into severe hemorrhagic and neurological manifestations in some patients with a 15–30% fatality rate in cases requiring medical attention [1]. The National Institute of Allergy and Infectious Diseases has classified JUNV and other pathogenic clade B viruses

*Corresponding author brian.gowen@usu.edu.

Disclosure statement

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as category A priority pathogens because they pose a significant risk to national security and public health [2].

The nonspecific antiviral, ribavirin, is the only licensed drug available to treat arenaviral HF under emergency provisions [3]. However, there is very limited data to support the use of ribavirin to treat severe infections with New World HF arenaviruses [1,4]. In addition, ribavirin is also associated with toxicity in the form of a reversible hemolytic anemia in humans [5,6], and teratogenic effects observed in rodents [7]. The threat of intentional release of highly pathogenic arenaviruses by terrorist groups combined with the risk of naturally occurring outbreaks in certain regions of the world underlines the need for the development of safe and more effective therapies.

Tacaribe virus (TCRV) is a clade B arenavirus that is closely related to JUNV and other pathogenic clade B arenaviruses [8], but is not considered a frank human pathogen [9,10]. Consequently, early stage drug discovery research is greatly facilitated as TCRV can be handled in biosafety level 2 (BSL-2) laboratories rather than in stringent BSL-4 maximum containment required for JUNV and other HF arenaviruses. To this end, we and others have previously reported on the use of cell culture-based cytopathic TCRV infection assays to identify and evaluate promising anti-arenavirus compounds [11,12].

With the objective of discovering and developing novel anti-arenavirus compounds, we used TCRV infection assays as a screen for the Chemtura Library, wherein we identified several compounds with antiviral activities against NWA. Here we report on one compound, a phenolic dibenzylsulfide referred to as D746, that consistently demonstrated activity against TCRV and other NWA in more rigorous virus yield reduction assays. Studies employing the TCRV AG129 mouse infection model, based on challenge of type I and II interferon (IFN) receptor knockout mice [13], revealed D746 activity as a pre-exposure prophylaxis, but not as a post-exposure intervention. Remarkably, D746 pre-treatment did not reduce viral titers, but did elicit accumulation of ascites fluid, suggesting that an immune mediated alteration of the host response may confer the observed protective effect.

2. Materials and methods

2.1. Animals

AG129 IFN receptor deficient mice were bred at Utah State University under specific pathogen free conditions. They were fed irradiated mouse chow and autoclaved water *ad libitum*. Five to ten-week old mice were used for the experiments. All animal procedures used in this study complied with guidelines set by the USDA and Utah State University Animal Care and Use Committee.

2.2. Viruses

TCRV, strain TRVL 11573, was obtained from American Type Culture Collection (ATCC; Manassas, VA). The virus stock used for *in vitro* experiments was derived from clarified cell lysates produced by three passages in Vero 76 African green monkey kidney cells. The virus stock used for mouse studies was from clarified liver homogenates from AG129 mice challenged with the *in vitro* stock. The JUNV Candid #1 strain was obtained from Dr. Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX). The Candid #1 virus used was passaged once in B-SC-1 epithelial African green monkey kidney cells and once in Vero cells. Pichindé virus (PICV), strain An 4763, was provided by Dr. David Gangemi (Clemson University, Clemson, SC). The PICV used was passaged once through hamsters and once in Vero cells.

2.3. Test compounds

D746 was provided by Chemtura Corporation (Guelph, Ontario, Canada). For *in vitro* activity experiments, D746 was dissolved in DMSO then further diluted in sterile minimal essential medium (MEM, Thermo Scientific Hyclone, Logan, UT) to final DMSO concentrations of less than 0.5%. For efficacy studies in mice, D746 was dissolved in ethanol, then further diluted in Cremaphor® (Sigma-Aldrich, St. Louis, MO) and saline (CES) at a ratio of 1:1:8. Ribavirin was supplied by ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and was prepared in sterile saline. All treatments were administered by intraperitoneal (i.p.) injection of 0.1 ml.

2.4. Cell culture antiviral assays

Vero cells were obtained from ATCC and maintained in MEM supplemented with 0.18% NaHCO_3 and 10% fetal bovine serum (FBS, Hyclone Thermo Scientific). Viruses were titrated in MEM culture medium containing 2% FBS to identify the optimal infectious dose that produced maximal cytopathic effect (CPE) in less than 9 days. Varying concentrations of D746 and ribavirin (positive control) were added to test wells containing 70–80% confluent Vero cells at the time of infection with viruses at multiplicity of infection (MOI) of approximately 0.001. Plates were incubated at 37°C, 5% CO_2 , until virus-infected control wells were observed to have maximal viral CPE (7–8 days), at which time the plates were processed to determine virus yield reduction (VYR) as previously described [11]. Concentrations of compound that reduced virus yield by 1 $\log_{10}$ (EC_{90}) were determined by regression analysis. For toxicity determinations done in parallel, compounds were added to uninfected Vero cells and the drug concentrations that reduced cell viability by 50% (CC_{50}) were also determined by regression analysis. Selectivity index (SI) values were calculated as $\text{CC}_{50}/\text{EC}_{90}$ for the VYR assays.

For time-of-addition assays, subconfluent Vero cell monolayers in 24-well plates were inoculated with TCRV at a MOI of 0.02 and incubated at 37°C, 5% CO_2 for 1 hour. Triplicate wells were incubated with varying concentrations of D746 or MEM culture medium at –2–0, 0–3, 3–6, and 6–9 h relative to the time of infection. Following the 2–3 h treatment periods, the media was replaced with new growth media devoid of D746. Plates were frozen at –80°C 24 h post-infection and viral CPE was determined for calculation of cell culture 50% infectious dose (CCID_{50}) as previously described [14].

2.5. *In vivo* D746 efficacy studies

In all experiments, AG129 mice were sorted into treatment groups of 10–22 mice to minimize gender and age differences and challenged i.p. with 0.2 ml of TCRV prepared in MEM. D746, ribavirin, and CES vehicle placebo treatments were administered i.p. twice daily to achieve the stated mg/kg/day dose. The duration of treatment for all experiments was 7 days. For the prophylactic efficacy experiments, treatment was started 2 h prior to challenge with 150 CCID_{50} of TCRV. For the evaluation of post-exposure efficacy, D746 treatment was initiated 3 days after challenge with 450 CCID_{50} of TCRV. The increase to 450 CCID_{50} was to achieve uniform lethality which was not observed at the lower dose. In two of the experiments, groups of 4–5 animals were sacrificed on day 9 of infection for analysis of serum and tissue virus titers. The remaining animals were observed for 3–4 weeks for morbidity and mortality. Body weights were assessed every 3 days starting on day 0, relative to time of challenge.

2.6. Serum and tissue virus titers

Virus titers were assayed using an infectious endpoint dilution cell culture assay as previously described [11]. Briefly, a specific volume of tissue homogenate or serum was

serially diluted and added to triplicate or quadruplicate wells of Vero 76 cell monolayers in 96-well microplates. The viral CPE was determined after a 10-day incubation, and the 50% endpoints were calculated as described [14]. The assay detection limits are designated by the lowest point on the Y axis of the virus titer graphs.

2.7. Statistical analysis

Two-way analysis of variance (ANOVA) with Bonferroni post-test was used to analyze the time-of-addition data. The Mantel-Cox log-rank test was used for analysis of Kaplan-Meier survival curves. One-way ANOVA with the Newman-Keuls post-test was performed to analyze virus titer differences. All statistical evaluations were done using Prism® (GraphPad Software, La Jolla, CA).

3. Results

3.1. *In vitro* antiviral activity of D746

Following the initial “hit” of the phenolic dibenzylsulfide, D746 (structure shown in Figure 1), against TCRV infection during the screening of the Chemtura library, the inhibitory effect of the compound was confirmed using more rigorous secondary testing assays. As shown in Table 1, D746 was active in the μ M range against TCRV, JUNV, and PICV by VYR assays, with SI values ranging from 5 to 21. Notably, the D746 TCRV inhibition dose-response curve was shallow compared to ribavirin (Figure 2A). The curve representing cell cytotoxicity of the compound relative to ribavirin is shown in Figure 2B. D746 was inactive against the MP-12 vaccine strain of Rift Valley fever virus, dengue virus (serotype 2), West Nile virus, Yellow fever virus, Venezuelan equine encephalitis virus, SARS coronavirus, and several strains of influenza virus in primary testing (data not shown). These results demonstrate the specificity of D746 towards arenaviruses.

3.2. D746 time-of-addition effect on TCRV infection

Time-of-addition studies were conducted to provide insights into the mechanism by which D746 inhibits arenavirus infection. Vero cell monolayers were treated by the addition of D746 at various times prior to and during TCRV infection and the reduction in virus production relative to the untreated cultures was assessed at 24 h post-infection. The greatest inhibition of TCRV replication was observed when the cells were pretreated, with diminishing dose-responsive activity seen out to the 3–6 h treatment time (Figure 3). There was no significant inhibition seen when cells were treated 6 hours after infection. We have previously reported that 14 h or more are required for the detection of infectious TCRV particles in Vero culture supernatants [15], suggesting that virus detected after 24 h in culture is from the initial infection cycle. Thus, our findings suggest that the effects of D746 target an early to intermediate stage of the virus life cycle, with inhibition of virus adsorption being likely.

3.3. Prophylactic efficacy of D746 against TCRV infection in mice

Considering the anti-arenavirus activity of D746 in cell culture, we next evaluated the compound in the TCRV AG129 mouse infection model. For the first efficacy study, we selected a 7-day treatment regimen consisting of daily doses of 100 and 10 mg/kg, administered by i.p. injections every 12 h starting 2 h prior to TCRV challenge. These doses were well tolerated by uninfected AG129 mice in an initial mean tolerated dose study (data not shown). As seen in Figure 4, the 100 mg/kg/day dose was highly effective, protecting 6 of 8 (75%) infected AG129 mice from mortality compared to 4 of 22 (18%) survivors in the placebo group that received CES vehicle. There were no significant differences in survival curve analysis or mean day of death comparison between the groups treated with 10 mg/kg/

day D746 versus placebo. Unexpectedly, ribavirin, routinely included as a positive control, failed to protect TCRV-challenged mice at a daily total dose (50 mg/kg/day) previously reported to be effective [13]. In the aforementioned studies, ribavirin was given as a once daily (50 mg/kg) dose, whereas in the present study, it was divided into two 25 mg/kg doses every 12 hours. It is conceivable that the larger bolus dose reached more effective concentrations in critical areas, thereby resulting in a measurable protective effect. Further, the added stress due to twice daily injections may have also contributed to the ineffectiveness observed in this initial experiment with D746.

Because significant protection was afforded by prophylactic D746 treatment in the first experiment, a second study that included higher doses of compound and additional animals for determining reductions in virus titer was conducted. All 10 mice treated with the highest dose of 200 mg/kg/day D746 survived the TCRV challenge, compared to 25% (3 of 12) in the placebo group (Figure 5A). In addition, significant protection (70% survival) was also observed at D746 doses of 100 and 50 mg/kg/day, confirming the results from the first experiment with the 100 mg/kg/day dose. Half of the mice that received the higher dose of ribavirin given for the second experiment survived the infection; however, due to the 25% survival rate in the placebo group, the difference was not significant. Doses of up to 200 mg/kg/day were also evaluated in parallel in uninfected animals with some weight loss observed, but all mice ultimately recovered following the cessation of treatment (data not shown).

In general, mean group mouse weights dropped dramatically from day 9 to day 12 (Figure 5B). The mice that received the 200 mg/kg/day dose of D746 started to recover by day 15. Mice treated with the 100 and 50 mg/kg/day doses began to show a collective increase in weight on day 21 and day 24, respectively. The placebo- and ribavirin-treated mice had the greatest mean weight loss by the end of the observation period, with the ribavirin group actually showing a brief improvement prior to a second decrease in animal weights. However, variability in weight change was most pronounced at the end of the experiment due to continued illness in some mice while others were in recovery.

Predetermined cohorts of infected and treated mice were sacrificed on day 9 for analysis of serum and tissue virus titers. Unexpectedly, significantly higher serum, liver, and lung titers were present in the D746-treated mice when compared to the placebo-treated animals, and the effect appeared to be dose-dependent; however, there was no effect on spleen titers (Figure 5C–F). Ribavirin reduced virus titers in the serum, liver, and spleen. In general, virus titers in the placebo-treated animals were lower than expected, which was likely due to the disease developing more slowly in this particular experiment. Considering the survival data, the increased virus burden in the mice treated with D746 was unexpected.

Notably, during the sacrifice, we observed large volumes of ascitic fluid and fibrous material surrounding the liver. The effect was dose-dependent, with the mice in the 200 mg/kg/day D746 treatment group clearly having the most ascites fluid accumulation (data not shown). This phenomenon was restricted to the D746 groups as the ribavirin and placebo animals were unremarkable upon opening of the peritoneal cavity for tissue collection. This suggested that the i.p. injections of D746 were causing this fluid accumulation.

3.4. Efficacy of D746 as a post-exposure treatment in TCRV-challenged mice

Having confirmed pre-exposure prophylactic efficacy, we next evaluated the ability of D746 to protect TCRV-challenged mice when administered starting 3 days post-exposure. We initiated treatment on day 3 based on previous data suggesting that day 4 is when virus is first observed in the spleen during TCRV infection [13]. This would allow for the evaluation of the compound prior to the appearance of substantial viral titers. Due to limiting amounts

of compound, we evaluated D746 at a dose of 100 mg/kg/day. In contrast to the efficacy observed when given pre-exposure, D746 treatment did not prevent mortality when administered after infection (Figure 6A). Only ribavirin was effective post-exposure. Collectively, the ribavirin data from the 3 mouse experiments suggests that shifting the treatment schedule so that mice receive the drug through day 9 of infection (experiment 3), as opposed to day 6 (experiments 1 and 2), dramatically improves survival. It is possible that allowing the immune system to respond to the natural progression of the virus for the initial 3 days resulted in better neutralization of the virus, which combined with ribavirin treatment extending further into the course of infection, protected 90% of the mice from a lethal TCRV challenge.

The effect of the delayed treatments on reducing viral titers was also evaluated on day 9 in subsets of mice infected and treated in parallel to those observed for mortality. D746 was again ineffective at reducing viral titers; however, in contrast to the second experiment, viral loads in D746-treated groups were not significantly elevated compared to the placebo group (Figure 6B–D). Consistent with the survival data (Figure 6A), the most dramatic reduction of virus titers was observed with ribavirin, with significant reductions in the serum and liver (Figure 6B, C). One death occurred in the ribavirin group on day 8 of infection, prior to the time of sacrifice, resulting in fewer animals for the analysis of serum and tissue virus titers. Because this occurred prior to when we would expect death due to the virus infection, this animal is believed to have died from other complications. Notably, the production of ascites fluid seen in the previous experiment in the D746-treated mice was confirmed during the harvesting of tissues from infected mice. Moreover, uninfected animals also had ascites fluid accumulation in the peritoneum, suggesting that the compound alone is inducing an inflammatory reaction at the site of treatment.

4. Discussion

The present study describes the identification of a phenolic dibenzylsulfide, D746, with *in vitro* activity against TCRV and JUNV, and prophylactic activity in a mouse model of TCRV infection. The apparent specificity for arenaviruses in cell culture may be due to inhibition of host cell functions that specifically support arenaviral replication. The precise mechanism by which D746 inhibits replication in cell culture is not clear, but time-of-addition studies indicate that an early to intermediate step in the virus life cycle is being disrupted. In particular, because treatment of cells prior to virus infection caused virus inhibition, this suggests that D746 may attach to cells and block subsequent virus adsorption. Such was the case with a previously reported dibenzamido molecule that inhibited cytomegalovirus adsorption to cells [16]. Further studies would need to be performed with D746 to validate this hypothesis.

Interestingly, despite highly reproducible inhibitory effects in cell culture experiments, the compound failed to reduce viremia or tissue viral loads in mice. A similar disconnect has been reported previously with the aristeromycin derivative, MY-24, which was able to inhibit viral replication in cell-based arenavirus assays, but not in mice infected with TCRV [13]. Notably, however, MY-24 did not elicit ascites and was effective both as a pre-treatment and therapeutically when administered starting 5 days after virus challenge. These differences suggest that MY-24 acts in a distinct manner from D746.

In the case of D746, we hypothesize that the peritoneal cavity fluid elicited by the i.p. treatment is indicative of an inflammatory response that may alter the disease process in a way that promotes survival of the mice when treatment begins prior to challenge, despite no reductions on systemic or tissue viral titers measured on day 9 of the infection. This beneficial effect was lost when treatment began after challenge, as there was still evidence

of ascites fluid accumulation, but the mice were not protected from lethal TCRV infection. In the second pretreatment efficacy experiment, we actually found significantly higher serum, liver, and lung titers in the D746-treated mice compared to the placebo group. We presume that when D746 was given prior to infection the changes resulting from the inflammatory response enhanced the production of virus, perhaps through the recruitment of more specific target cells that amplify the virus, while ameliorating host disease processes that likely contribute to the demise of the animals. When treatment was initiated after TCRV infection, the efficacy of the compound was lost and the increase in virus titers relative to the placebo treatment group was no longer observed. An alternative explanation of the higher titers in the D746-treated mice in the second pre-exposure treatment experiment could be that the placebo group may have had atypically lower viral titers than usual. Unfortunately, we did not have sufficient test compound available to validate whether D746 elevates viral titer under pretreatment conditions. But it is clear that D746 does not reduce viral titers in treated animals.

Presently, there are limited options for the treatment of severe arenaviral infections [1,17]. Despite active research towards identifying new treatments to counter arenaviral HF syndromes, only a limited number of therapies have been evaluated in animal models [18]. Although D746 was efficacious when it was administered prophylactically, it is questionable whether further development of the compound as an anti-arenavirus therapy is warranted since 1) its activity *in vitro* was only modest, 2) it was only active when administered prior to infection, 3) treatment was not associated with reductions on viral titers, and 4) excess fluid recruited into the peritoneal cavity was linked to i.p. administration of the compound. Nevertheless, the findings reported describe a novel antiviral scaffold and underscore the challenges associated with translating *in vitro* results into animals.

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Figure 1. Chemical structure of D746, a phenolic dibenzylsulfide

Figure 2. Effects of D746 and ribavirin on virus yield and cell viability

A) Virus titers were determined by endpoint titration of cell lysates from Vero cell cultures infected with TCRV (MOI 0.01) and treated with various concentrations of D746 and ribavirin. Cell lysates were from 7-day incubations. B) Cytotoxicity in confluent monolayers of uninfected cells treated in parallel was determined by neutral red dye uptake to measure cell viability. Data are presented as the percent cell viability after a 7-day incubation compared to untreated controls and are representative of multiple experiments. Means and standard deviations are shown. Untreated controls, mean = 6.56 ± 0.2 CCID₅₀/ml. CCID₅₀, 50% cell culture infectious dose.

Figure 3. Inhibitory effects of D746 added during specified times relative to TCRV infection
Vero cells were treated with varying final concentrations of D746 during the indicated time intervals (x axis) relative to the time of TCRV infection, and cultured for 24 h. Supernatants were collected and virus yields determined by endpoint dilution. The error bars represent the range from duplicate wells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to respective 0 μ M control.

-- D746 100 mg/kg/day
--- D746 10 mg/kg/day
— Ribavirin 50 mg/kg/day
— Placebo

Figure 4. Prophylactic efficacy of D746 in the AG129 mouse TCRV infection model

Mice ($n = 10$ for drug treatment groups, $n = 22$ for placebo group) were treated i.p. twice daily for 7 days with the indicated amounts of D746, ribavirin, or CES vehicle placebo starting 2 h prior to challenge with 150 CCID₅₀ of TCRV and observed for 25 days. ** $P < 0.01$ compared to CES placebo-treated animals.

Figure 5. Effect of D746 pretreatment on disease outcome and virus titers in AG129 mice challenged with TCRV

A) Survival outcome in mice (n = 10–11 for drug treatment groups, n = 12 for placebo group) treated i.p. twice daily for 7 days with the indicated doses of D746, ribavirin, or placebo, starting 2 h prior to challenge with 150 CCID₅₀ of TCRV. B) Individual mouse weights were measured at 3-day intervals during the efficacy study and are represented as the group mean and standard deviation of the percent change in weight relative to their starting weight on the day of challenge. Groups of mice (n = 4 per drug treatment group, n = 5 in placebo group) treated and challenged in parallel to those observed for morbidity and mortality were sacrificed on day 9 post infection for analysis of C) serum, D) liver, E) lung

and F) spleen viral titers. One mouse in the 100 mg/kg D746 group succumbed prior to the time of sacrifice. Unique symbols are used for each treatment group and represent values for the same animal across all tissues. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to animals receiving placebo.

Figure 6. Effect of post-exposure treatment with D746 on survival outcome and virus titers in AG129 mice challenged with TCRV

Mice (n = 10 for treatment groups, n = 15 for placebo group) were treated by i.p. injection with the indicated doses of D746, ribavirin or placebo twice daily for 7 days starting 3 days after challenge with 450 CCID₅₀ of TCRV. A) Percent survival is shown. Mice (n = 5 per group) were treated in parallel to the animals observed for mortality and sacrificed on day 9 post infection for analysis of B) serum, C) liver and D) spleen virus titers. Unique symbols in each treatment group represent values for the same animal across all parameters. ****P* < 0.001 compared to animals receiving placebo.

Table 1

Summary of *in vitro* anti-arenavirus activity of D746 and ribavirin.^a

Virus	VYR ^b					
	D746			Ribavirin		
	CC ₅₀	EC ₉₀	SI ^c	CC ₅₀	EC ₉₀	SI ^c
TCRV	21 ± 4.4	1.7 ± 0.9	12	540 ± 141	5.0 ± 0.7	108
JUNV	19 ± 3.6	0.9 ± 0.3	21	660 ± 330	6.3 ± 3.4	105
PICV	13.5 ± 7.4	3.0 ± 0.9	5	465 ± 219	13.8 ± 8.8	34

^aData are expressed as the mean and standard deviation of 3 independent experiments in Vero cells.

^bVirus yielded reduction (VYR) assay; CC₅₀ (50% cell cytotoxic dose) and EC₉₀ (90% effective dose) are in μM.

^cSelectivity index (SI) = CC₅₀/EC₉₀.

TCRV, Tacaribe virus; JUNV, Junín virus; PICV, Pichindé virus.