Selective estrogen receptor modulators limit alphavirus infection by targeting the viral capping enzyme nsP1

Rajat Mudgal[1], Chandrima Bharadwaj[2], Aakriti Dubey[1], Shweta Choudhary[1], Perumal Nagarajan[2], Megha Aggarwal[1], Yashika Ratra[2], Soumen Basak[2*], Shailly Tomar[1*]

[1]Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, India

[2]National Institute of Immunology, New Delhi, India

Correspondence:

***S. Tomar, Department of Biosciences and Bioengineering,**

Indian Institute of Technology Roorkee,

Roorkee 247667, Uttarakhand, India

Tel: +91 1332285849

E-mail: shailly.tomar@bt.iitr.ac.in

***S. Basak, Systems Immunology Laboratory,**

National Institute of Immunology,

Aruna Asaf Ali Marg, New Delhi 110067, India

Email: sobasak@nii.ac.in

Abstract

Alphaviruses cause animal or human diseases that are characterized by febrile illness, debilitating arthralgia, or encephalitis. Selective estrogen receptor modulators (SERMs), a class of FDA-approved drugs, have been shown to possess antiviral activities against multiple viruses, including Hepatitis C virus, Ebola virus, dengue virus, and vesicular stomatitis virus. Here, we evaluated three SERM compounds, namely 4-hydroxytamoxifen, tamoxifen, and clomifene, for plausible antiviral properties against two important alphaviruses, chikungunya virus (CHIKV) and Sindbis virus (SINV). In cell culture settings, these SERMs displayed potent activity against CHIKV and SINV at non-toxic concentrations with EC_{50} values ranging between 400 nM and 3.9 μM. Further studies indicated that these compounds inhibit a post-entry step of the alphavirus life cycle, while enzymatic assays involving purified recombinant proteins confirmed that these SERMs target the enzymatic activity of non-structural protein 1 (nsP1), the capping enzyme of alphaviruses. Finally, tamoxifen treatment restrained CHIKV growth in the infected mice and diminished musculoskeletal pathologies. Combining biochemical, cell culture-based studies, and *in vivo* analyses, we strongly argue that SERM compounds, or their derivatives, may provide for attractive therapeutic options against alphaviruses.

Keywords: Alphavirus, chikungunya virus, Sindbis virus, SERMs, antiviral, nsP1, murine model

Introduction

Alphaviruses are enveloped arboviruses and possess a positive-sense (+ve sense), single-stranded RNA genome. Symptomatic manifestations of alphavirus infection in humans vary from fever or rash to incapacitating polyarthralgia and intense asthenia persisting for months.[1] These viruses can be broadly classified into New World alphaviruses, such as Venezuelan equine encephalomyelitis virus (VEEV), and Old World alphaviruses, such as chikungunya virus (CHIKV) and Sindbis virus (SINV). While Old World viruses mainly cause polyarthritis, New World viruses are predominantly associated with encephalitis in the infected host.[2] In particular, numerous re-emergence cases of CHIKV infections were reported in the past 50 years; the most severe outbreak was observed in the year 2005 in La Réunion and parts of the Indian subcontinent resulting in significant morbidities, economic losses, and stress on the health care infrastructure.[3,4] On the other hand, SINV has been attributed to massive fatalities owing to recurrent outbreaks in Scandinavia, Africa, Europe, and the Middle East, since 1952.[5] Despite significant human health relevance, there are currently no licensed antiviral drugs or vaccines to combat the alphavirus menace. Treatment of infection is palliative and consists of the administration of analgesics or antipyretics to impart symptomatic relief.

Post-infection, the alphaviral genome is translated to produce a non-structural and a structural polyprotein. Proteolytic cleavage of these polyproteins generates functional viral polypeptides.[2,6] For e.g., proteolytic cleavage of the non-structural polyprotein generates four non-structural proteins (nsPs) viz. nsP1, nsP2, nsP3, and nsP4, which play essential roles in viral RNA (vRNA) synthesis within host cells.[7] In particular, nsP1 is a membrane-associated enzyme that directs the capping of genomic and subgenomic vRNAs involving methyltransferase (MTase) and guanylyltransferase (GTase) activities.[8] Capping of the vRNA is crucial for translation initiation,

protecting vRNAs, including genomic RNA, from host exonucleases, and masking viral nucleic acids from host-encoded pathogen recognition receptors, such as MDA5 and RIG-I.[9] Due to the distinctive and non-canonical capping mechanism, nsP1 is thought to be a promising antiviral target for alphaviruses.[10–12]

Identification and evaluation of novel antiviral therapeutics are challenging and time-consuming processes. However, repurposing drugs already in clinical use may expedite antiviral development against alphaviruses.[13] 4-hydroxytamoxifen (4-OHT), tamoxifen, and clomifene belong to the triphenylethylene class of molecules and are known estrogen receptor antagonists.[14,15] Tamoxifen is typically used as a maintenance agent in estrogen-receptor-positive tumor patients.[16] 4-OHT is a metabolite of tamoxifen.[17] Clomifene is used for treating female infertility. Both tamoxifen and clomifene have also found their off-label use in treating male infertility and idiopathic gynecomastia.[18,19] These compounds, along with their analogs, are generally referred to as selective estrogen receptor modulators (SERMs).[15] Previous reports revealed that SERMs are active against multiple viruses, including the hepatitis C virus (HCV), herpes simplex type 1 virus (HSV-1), Ebola virus, dengue virus, vesicular stomatitis virus, etc.[20–25] However, the plausible role of SERMs in limiting alphavirus infections has not been examined. Experimental mouse models have provided important mechanistic insights on alphavirus-induced immunopathologies. In particular, Gardner et al. (2010), described a footpad infection model involving adult wild-type mice that recapitulates arthritogenic phenotypes observed in human subjects infected with CHIKV.[26] Animal studies revealed that local inflammation orchestrated by macrophages and antigen-specific CD4 T cells leads to footpad swelling in CHIKV-infected mice.[26,27] Accordingly, targeting T cells or macrophages improved

musculoskeletal pathologies in these mice.[27–29] Moreover, these animal models are also accelerating the testing of potential antiviral drugs in preclinical settings.[30]

Here, we report potent antiviral activity of three SERMs, namely, 4-OHT, tamoxifen, and clomifene, against CHIKV and SINV in both cell culture-based settings and the preclinical mouse model. Our biochemical studies further established that SERMs are capable of inhibiting the enzymatic activity of nsP1 from multiple alphaviruses. Taken together, SERM compounds may offer an effective means for antiviral therapy against the alphavirus genus.

Results

SERMs inhibit alphaviral propagation

As SERMs were active against multiple viruses, we sought to examine if this family of drugs also display anti-alphaviral properties against alphaviruses. Prior to the evaluation of antiviral activity, cell viability of BHK-21 and Vero cells in presence of compounds was determined by MTT [3-(4,5- Dimethylthiazol- 2-yl)-2,5- Diphenyltetrazolium Bromide] assay (Figure S1A-C and S2A-C). An additional cytotoxicity assay, Lactate Dehydrogenase assay (LDH) assay, which assesses the membrane integrity of cells, was also performed in Vero cells (Figure S2D-F). The calculated half-maximal cytotoxic concentration (CC_{50}) values are presented in Table 1. A luciferase reporter system, SINV-FLuc, that has a firefly luciferase fused at the 3´end of the SINV E1 gene, was used for primary evaluation of antiviral activity of compounds (Figure S1D). The compounds were tested in BHK-21 cells at 2.5 μM concentration at which there was no observable cytotoxicity. All the three compounds showed a significant decrease in luciferase activity that was used as a surrogate of viral replication in the infected cells (Figure S1D).

The quantitative analysis of the antiviral activity of the compounds was done by the titer determination of the progeny virus released 24 hours post-infection (hpi) in the supernatant of the treated or untreated cells infected with CHIKV or SINV. All three compounds demonstrated a marked reduction in infectious virus production in a dose-dependent manner (Figure 1A). Selectivity index values calculated from EC_{50} and CC_{50} suggested that within an order of magnitude, these drugs displayed largely comparable selectivity against CHIKV and SINV (Table 1). The anti-CHIKV and anti-SINV activities of 4-OHT, clomifene, and tamoxifen were further validated by qRT-PCR. There was a significant reduction in intracellular vRNA in the cells treated with the tested compounds 24 hpi. (Figure 1B). Corroborating our plaque assay, 4-OHT was more potent against SINV as compared to CHIKV. Finally, immunostaining cells with an anti-alphavirus antibody confirmed that treatment of CHIKV- or SINV-infected cells with 4-OHT, tamoxifen, or clomifene substantially suppress the abundance of viral proteins at 36 hpi (Figure 1C).

Table 1: The CC_{50}, EC_{50}, and selectivity index values of 4-OHT, tamoxifen, and clomifene in BHK-21 and Vero cells. MTT: [3-(4,5- Dimethylthiazol- 2-yl)-2,5- Diphenyltetrazolium Bromide]; LDH: Lactate dehydrogenase; 4-OHT: 4-hydroxytamoxifen; CHIKV: chikungunya virus; SINV: Sindbis virus; CC_{50}: half-maximal cytotoxic concentration; EC_{50}: half-maximal effective concentration.

Compound	**BHK-21 cells**	**Vero cells**					
	CC_{50}-MTT (µM)	CC_{50}-MTT (µM)	CC_{50}-LDH (µM)	**CHIKV**		**SINV**	
				EC_{50} (µM)	SI[1]	EC_{50} (µM)	SI[2]
4-OHT	8.83	14.63	14.92	0.93	15.7	0.40	36.5
Tamoxifen	12.25	25.40	19.61	3.95	6.4	3.69	6.9
Clomifene	8.54	26.73	20.43	3.16	8.5	1.24	21.5

[1] Selective index (CC_{50}-MTT/ EC_{50} CHIKV)
[2] Selective index (CC_{50}-MTT/ EC_{50} SINV)

Inhibition of alphaviral replication at a post-entry step

To gather insight into the mode of action of these compounds, time-of-addition studies were performed (Figure 2A). When cells were pretreated with the compounds before virus infection, minimal inhibitory effect on the viral yield was observed. A cotreatment assay was performed to examine if the compounds can affect viral binding and entry into the cell. Viral yield remained largely unaffected when compounds were present during infection suggesting that compounds do not interfere with virus particle stability or its attachment to the host cell. Moderate dose-

dependent inhibition of viral replication observed in cotreatment assay can be attributed to the possible effects of the residual amount of inhibitors retained by the cells during cotreatment (Figure 2B). All three compounds were most effective against both CHIKV and SINV when they were added during an early post-entry phase of viral replication in the infected cells suggesting a similar antiviral mechanism of the inhibitors in these two alphaviruses (Figure 2C-D).

A SINV reporter replicon was designed to investigate the inhibitory effect of the compounds on viral protein synthesis (Figure 2E). The tested compounds showed a moderate inhibitory effect against SINV replicon indicating a weak suppression of protein synthesis (Figure 2E). Additionally, to examine if the compounds exert an inhibitory effect on vRNA replication, intracellular vRNA was quantified at 6 hpi to minimize the contribution of multiple rounds of viral infection. A statistically significant but modest decrease in cell-associated vRNA was observed 6 hpi for CHIKV and SINV (Figure 2F and Figure 2G). To further examine the mechanistic function of these SERMs against alphaviruses, SINV vRNA in the extracellular fractions was quantified (Figure 2H). Intriguingly, there was markedly less reduction in extracellular virus-associated vRNA as compared to the infectious virions released at 24 hpi (Figure 2H and Figure 1B).

Further, both SINV and CHIKV were serially passaged in the presence of gradually increasing concentrations of compounds. Viral-induced cytopathic effects (CPE) were monitored and no change in viral susceptibility to the inhibitors was observed even after multiple passages. Only partial resistance to CHIKV in presence of 4-OHT could be observed after 15 passages which suggested that a high resistance is indeed difficult to generate against these compounds. A plaque-purified strain also displayed an altered plaque phenotype compared to the wild-type virus (Figure 3A-B). The sequencing data of partially-resistant isolate revealed 4 synonymous

mutations in the nsP-coding region. Two mutations lie in the zinc-binding and macro domains of the nsP3-coding region, one is in disordered region of the nsP4-coding region, and one lies in the MTase/GTase domain of nsP1 (Figure 3B). While the mutations in nsP3 and nsP4 fall in the enzymatically-inactive regions, the point of mutation in nsP1 lies in the catalytic domain of the enzyme indicating that the likely molecular target of SERM compounds is alphaviral nsP1.

SERM compounds inhibit alphaviral nsP1 activity

Results of the studies described above prompted us to evaluate the effect of these compounds on the enzymatic activity of alphaviral nsP1. A capillary electrophoresis (CE)-based MTase assay that measures the amount of SAH generated during CHIKV and VEEV nsP1 MTase reactions was utilized.[12] Further, an enzyme-linked immunosorbent assay (ELISA)-based assay that measures the m7GMP-nsP1 adduct formed in guanylation reaction was used to determine the inhibitory activity of compounds on CHIKV nsP1.[31] We observed that the compounds inhibited the MTase activity of both CHIKV and VEEV nsP1 at 50 µM concentration (Figure 3C). The ELISA-based assay also showed a concentration-dependent reduction in CHIKV nsP1 enzymatic activity in the presence of tested SERMs (Figure 3D).

Tamoxifen alleviates CHIKV-induced pathologies *in vivo*

Next, we set out to examine if the anti-alphaviral property of SERMs was preserved *in vivo*. To this end, we adopted the previously published CHIKV footpad infection model involving adult wild-type mice that recapitulates certain arthritogenic phenotypes observed in human subjects.[26] As described earlier, subcutaneous CHIKV infection into the footpad caused a transient reduction in the body weight in these mice at day one post-infection that was accompanied by a bimodal footpad swelling peaking at day two and again at day six (Figure S3A-B).[26] Using

tamoxifen as a representative drug, we then examined the efficacy of SERMs in reducing these inflammatory musculoskeletal pathologies. We optimized a therapeutic regime where CHIKV-infected mice were successively injected with 20 mg/kg of tamoxifen on every alternate day beginning at day one post-infection. We found that tamoxifen instigated robust recovery of body weight in CHIKV infected mice (Figure 4A). Compared to the vehicle control, tamoxifen treatment also significantly improved the footpad swelling at both day two and six post-infection (Figure 4B and Figure 4C).

Because tamoxifen also directly impedes inflammatory pathways, we asked if the curative tamoxifen effect was indeed associated with reduced viremia *in vivo*.[32] We determined the load of infectious CHIKV particles in the infected footpad by $TCID_{50}$ assay and also scored the abundance of vRNA in CHIKV-infected mice by qRT-PCR. Consistent with the reduced musculoskeletal pathology, tamoxifen treatment led to a more than one log decrease in the titer of plaque-forming CHIKV in the infected footpad at both day two and day six post-infection (Figure 4D). Similarly, we captured an approximately five-fold decrease in the level of vRNA in the blood of tamoxifen-treated mice at day two (Figure 4E). In parallel, tamoxifen treatment ensured a close to one log reduction in the level of viral genome RNA in the footpad at both day two and day six (Figure 4F). As also described earlier, our histopathological analyses of tissues derived from the joint footpad revealed that CHIKV infection triggered marked subcutaneous edema, profound infiltration of mononuclear immune cells, and local necrosis at day six (Figure 4G).[26] Tamoxifen treatment regime considerably ameliorated these signatures of CHIKV-induced inflammation. In sum, our analyses confirmed that SERMs are capable of restraining viremia and mitigating immunopathologies in alphavirus-infected mice.

Discussion

Based on the antiviral effect exhibited by multiple SERMs against other virus families, we attempted to characterize the anti-alphaviral activity of three SERMs: 4-OHT, tamoxifen, and clomifene. As evident by plaque reduction assay, RT-PCR studies, and immunostaining, all these three compounds effectively abrogated the replication of SINV and CHIKV in cell culture. Results of the time-of addition studies and sequence analysis of partial-4-OHT resistant phenotype suggested that SERM compounds likely targeted nsP1 capping enzyme, a component of the vRNA replication machinery. In the presence of SERMs, a marked difference was observed between the number of infectious virions released and extracellular vRNA. Capping of vRNA in the infected cells is orchestrated by a functional alphaviral nsP1 and inhibition of nsP1 by SERM compounds would release a significant number of noncapped virus particles. The discrepancy in the magnitude of effect on extracellular vRNA and infectious units released is supported by previous studies which demonstrated that noncapped vRNAs are infectious but at significantly lower levels as compared to the capped RNAs.[33,34] Though, SINV RNA has been shown to undergo translation and encapsidation without a cap structure, noncapped vRNAs are found to be highly unstable.[34] Concordantly, the enzymatic activity of CHIKV nsP1 and VEEV nsP1 was inhibited by all the three compounds tested in the MTase activity assay. A secondary orthogonal ELISA-based assay also validated the anti-nsP1 activity of the tested SERM compounds.

Replicon assay using SINV-REP which does not involve viral entry or release indicated that these SERMs interfere with the viral replication machinery and affect viral protein synthesis. The data from the SINV-FLuc virus also suggested a decrease in vRNA replication and/or translation when the infected cells were treated with these compounds. The observed decrease in RNA

translation can be a direct consequence of the reduction in capping activity by nsP1 or a result of perturbation of RNA replication machinery due to inhibitor binding to nsP1. We further demonstrated that these compounds marginally suppress vRNA replication in infected cells. Apart from vRNA capping, nsP1 has also been found to be involved in the synthesis of negative strand of vRNA.[28,29] However, we cannot rule out the possibility that reduction of vRNA levels or sugenomic translation is due to the degradation of noncapped vRNAs (generated because of the compound-mediated inhibition of nsP1) by host RNA decay machinery or plausible modulation of nsP1 interaction with other nsPs due to inhibitor binding.

Besides engaging estrogen receptor signaling, SERMs also modulate numerous other cellular factors and functions; they inhibit immune-regulatory NF-κB signaling, repress the protein kinase C activity, control the calmodulin pathway, and restrict acidification of lysosomes and endosomes.[32,35–37] Previous studies established antiviral activities of SERMs against a number of human pathogens. Interestingly, antiviral activities of SERMs were found to be mostly independent of estrogen receptors and involved other cellular or viral targets.[14,24,38,39] For example, tamoxifen inhibited the binding and penetration of HSV-1 by targeting the cell surface chloride channels.[24] Similarly, SERMs affected the interaction of HCV to the cells and endocytosis.[38] Endolysosomal calcium accumulation upon treatment with SERMs obstructed cellular entry by the Ebola virus.[23] On the other hand, tamoxifen diminished vesicular stomatitis virus infections involving type-1 interferons.[25] Lastly, estrogen receptor modulators raloxifene hydrochloride and quinestrol were shown to restrict translation and/or replication of dengue virus.[20]

We performed anti-CHIKV studies using Vero cells, which lack the expression of estrogen receptor α and produce only a low level of estrogen receptor β.[23] Of note, Vero cells also do not

secrete type-1 interferons but we do not rule out that SERMs also influence other host pathways or modulate other steps of the alphavirus life cycle. In particular, the relatively low frequency of SERM-resistant plaque mutants of CHIKV indicated the possible engagement of multiple viral targets. While our results assure that adaptive mutations may necessarily not pose a threat to the translation potential of SERM regimes, future studies ought to more extensively characterize the SERM-alphavirus interaction interface. Our *in vitro* enzymatic assays confirmed inhibition of enzymatic activity of purified CHIKV nsP1 and VEEV nsP1 proteins. Computational analysis of protein-ligand binding (including extensive molecular dynamic simulations) and site-directed mutagenesis combined with *in vitro* activity studies will be required to accurately assess the nsP1-SERM interaction and elucidate the binding preferences of similar compounds to the alphaviral capping enzyme. Nonetheless, our current work offers a proof of concept that SERM compounds can act as direct acting antivirals by impairment of alphaviral nsP1 thereby providing another estrogen receptor signalling-independent mechanism for antiviral effect of SERMs.

Local inflammation triggered upon CHIKV infection orchestrates the influx of leukocytes, including macrophages and CD4 T cells, in the infected tissue leading to musculoskeletal pathologies.[26] In our mouse infection studies, the therapeutic application of tamoxifen efficiently restricted CHIKV propagation and persistence *in vivo*. We further noticed a modest but significant improvement of the footpad swelling in CHIKV-infected mice administered with a moderate dose of tamoxifen (20 mg/kg). Importantly, alphaviruses engage the cellular NF-κB signaling pathway, which promotes inflammatory gene expressions.[40,41] It has been recently shown that besides promoting inflammation, NF-κB signaling also augments replication of SINV in infected neurons.[41] Curiously, tamoxifen, independent of estrogen receptor signaling, also potently suppresses the NF-κB pathway and restrains inflammation.[32,42] Taken together, we

argue that the therapeutic effect of tamoxifen observed in our preclinical model could have been attributed to its both anti-CHIKV activity and generic anti-inflammatory functions. It is also possible that anti-alphaviral SERMs activities relied, at least in part, on the down-modulation of NF-κB signaling. Further studies are warranted to fully dissect the contribution of host targets and viral factors in generating anti-alphaviral SERM effects.

In conclusion, this report identifies and characterizes SERMs as promising lead candidates for further development of targeted anti-alphaviral therapy. Dose-dependent inhibition of CHIKV and SINV by all three tested SERMs, and inhibition of CHIKV nsP1 and VEEV nsP1 enzymatic activity affirms the broad-spectrum antiviral activity of SERMs against alphaviruses. A number of SERMs are FDA-approved, have good oral availability, established pharmacological and safety profiles, and are already in clinical practice for half a century. With the effective inhibition of alphaviral replication in cell culture and mice model by SERM compounds, a possible new approach of using SERMs and their superior analogs can be further investigated for the identification and development of pan-alphavirus therapeutic agents.

Materials and Methods

Ethics statement

WT C57BL/6 mice were housed at the National Institute of Immunology (NII) and used adhering to the institutional guidelines (Approval number – IAEC 545/20).

Cells, animals, viruses, and compounds: Vero cells (NCCS, Pune, India) were maintained in DMEM (HiMedia, India) supplemented with 10% heat-inactivated FBS (Gibco), 100 units of penicillin, and 100 mg/ml of streptomycin at 37°C and in a 5% CO_2 incubator. Stocks of CHIKV

(accession number, KY057363.1; strain, 119067) and SINV were maintained using standard viral adsorption techniques.[43,44] (Z)-4-OHT, tamoxifen, and clomifene citrate were purchased from Cayman chemicals and dissolved in 100% dimethyl sulfoxide (DMSO).

Luciferase-based antiviral assay

The luciferase-based assay used a genetically engineered strain of SINV (SINV-FLuc) wherein the *firefly luciferase* gene is driven by an internal ribosome entry site element (IRES).[31,45] A reporter SINV replicon (SINV-REP) was constructed in which all the structural proteins downstream of subgenomic (SG) promoter were replaced with FLuc and IRES element at the 3′end.

BHK-21 cells were electroporated in a 0.2 cm cuvette (25 µF, 1.5 kV, and 200 Ω) with 10 µg of capped RNA transcripts for SINV-FLuc or SINV-REP. For SINV-REP, 4 h post-transfection, compounds or 0.1% DMSO were added to the transfected cells, and cell lysate was collected 6 h after the addition of compounds. For SINV-FLuc, media was collected 48 h post-transfection and assayed for titer determination. For inhibition assays, BHK-21 cells at a confluency of 80-90% in a 24-well plate were infected with SINV-FLuc at MOI of 0.1 for 1.5 h. Media containing compounds or 0.1% DMSO was added to the infected cells and lysate was collected at 12 hpi. Luciferase activity of lysates was measured using a firefly luciferase assay kit (Promega, USA) according to the manufacturer's instructions.

***In vitro* antiviral assays:** Cytotoxicity of various compounds was evaluated in MTT assay and LDH assay (see the supplementary material). For inhibitor treatment of the infected cells, sub-confluent Vero cells were infected for 1.5 h at 1 MOI in a 24-well plate at a density of $1x10^5$ cells/well. The media was then removed, and compound dilutions, lower than their respective

toxic doses, prepared in low-serum media were added and cells were incubated at 37ºc. At 24 hpi, culture supernatant was harvested and subjected to plaque-forming assay (see a detailed description of the plaque assay in the supplementary material). The reduction in viral titer relative to DMSO-treated cells was measured and graphs were analysed using Graph Pad Prism for determining the half-maximal effective concentration (EC_{50}) values. The antiviral activity of various compounds was also examined in the indirect immunofluorescence assay, where intracellular viral particles were stained using anti-CHIKV antibodies (see the supplementary materials for details). Cells were harvested at various time points and the abundance of intracellular vRNA was quantified in the qRT-PCR assay. A detailed description of the qRT-PCR protocol and primers are available in the supplementary material.

nsP1 expression, purification, and enzymatic assays: CHIKV nsP1 and VEEV nsP1 were expressed and purified from *E. coli* as described.[12] Reaction conditions and data analysis for CE-based MTase assay and ELISA-based guanylation assay were described earlier.[12,31]

CHIKV infection in mice and tamoxifen treatment: As described, six weeks old male mice were subcutaneously (SC) injected on the ventral side of the right footpad with 10^6 PFU of CHIKV (strain no: 119067).[26] CHIKV-infected mice were injected with tamoxifen at day+1, day+3, and day+5 post-infection. Control mice were injected with an equal volume of oil. Infected mice were further examined for body weight changes at the indicated days post-infection. The swelling of the infected footpad was quantified by estimating the increase in the footpad upon infection using Insize digital Vernier caliper.[27] In certain sets, mice were sacrificed at day six, and tissues from decalcified footpads were subjected to H&E staining for scoring pathologies associated with musculoskeletal inflammation. At the indicated days post-infection, blood was collected through the retro-orbital route, and the footpad was dissected from the

infected mice. Total RNA was isolated from blood or tissue samples and the abundance of vRNA was determined by qRT-PCR using primers specific for the +ve sense genome.[46] Separately, we prepared a serial dilution of CHIKV stock, whose titer was determined using the standard plaque assay. RNA was isolated and qRT-PCR was performed using these serial diluents. By plotting C_T values against the known CHIKV titer, a standard curve was generated. We used this standard curve for estimating the number of copies of RNA genomes present in samples from infected mice. Alternatively, the viral titer in these tissues was determined by the $TCID_{50}$ method (see Supplementary Information).[47]

Acknowledgements

We thank Prof. Richard J Kuhn at Purdue University, USA for providing SINV-FLuc plasmid and Biotech Consortium India Limited, Department of Biotechnology (DBT), Govt. of India for assistance in filing the patent on "An antiviral composition against alphaviruses" (Patent file no. 201911049130, dated 29th November 2019). We thank V. Kumar, Systems Immunology Laboratory, NII for technical assistance. Graphical abstract was created with Biorender.com.

Author Contributions

Conceived the idea, R.M., S.T., and S.B.; Designed the experiments, R.M., S.C., S.T., C.B., and S.B.; Performed protein purification and enzymatic assays, R.M.; Performed viral inhibition assays: R.M., S.C, and A.D.; Performed murine experiments: C.B., Y.R., and P.N.; Analyzed the data: R.M., S.T., C.B., and S.B.; Wrote the Paper, R.M., S.C., A.D., S.T., C.B., and S.B.

Funding

This work was supported by a research grant ISRM/12(46)/2020 to S.T. from the Indian Council of Medical Research (ICMR). Virus research in the S.B.'s laboratory is funded by the S.N.

Ramachandran National Bioscience Award for Career Development award to S.B. by DBT, Govt. of India, and NII-core. R.M., S.C., and M.A. are thankful to CSIR for financial support. A.D. thanks Department of Biotechnology, Govt. of India, and Y.R. thanks Indian Council for Medical Research, Govt. of India for research fellowship.

Transparency declarations: None to declare.

References:

1. Mostafavi, H., Abeyratne, E., Zaid, A., and Taylor, A. (2019). Arthritogenic Alphavirus-Induced Immunopathology and Targeting Host Inflammation as A Therapeutic Strategy for Alphaviral Disease. Viruses *11*, 290.

2. Strauss, J.H., and Strauss, E.G. (1994). The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. *58*, 491–562.

3. Pialoux, G., Gaüzère, B.-A., Jauréguiberry, S., and Strobel, M. (2007). Chikungunya, an epidemic arbovirosis. Lancet Infect. Dis. *7*, 319–327.

4. Soumahoro, M.-K., Boelle, P.-Y., Gaüzere, B.-A., Atsou, K., Pelat, C., Lambert, B., Ruche, G.L., Gastellu-Etchegorry, M., Renault, P., Sarazin, M., et al. (2011). The Chikungunya Epidemic on La Réunion Island in 2005–2006: A Cost-of-Illness Study. PLoS Negl. Trop. Dis. *5*, e1197.

5. Adouchief, S., Smura, T., Sane, J., Vapalahti, O., and Kurkela, S. (2016). Sindbis virus as a human pathogen-epidemiology, clinical picture and pathogenesis. Rev. Med. Virol. *26*, 221–241.

6. Strauss, E.G., Rice, C.M., and Strauss, J.H. (1984). Complete nucleotide sequence of the genomic RNA of Sindbis virus. Virology *133*, 92–110.

7. Rupp, J.C., Sokoloski, K.J., Gebhart, N.N., and Hardy, R.W. (2015). Alphavirus RNA synthesis and non-structural protein functions. J. Gen. Virol. *96*, 2483–2500.

8. Ahola, T., and Kääriäinen, L. (1995). Reaction in alphavirus mRNA capping: formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP. Proc. Natl. Acad. Sci. *92*, 507–511.

9. Hyde, J.L., and Diamond, M.S. (2015). Innate immune restriction and antagonism of viral RNA lacking 2′-O methylation. Virology *0*, 66–74.

10. Tomar, S., Narwal, M., Harms, E., Smith, J.L., and Kuhn, R.J. (2011). Heterologous production, purification and characterization of enzymatically active Sindbis virus nonstructural protein nsP1. Protein Expr. Purif. *79*, 277–284.

11. Feibelman, K.M., Fuller, B.P., Li, L., LaBarbera, D.V., and Geiss, B.J. (2018). Identification of small molecule inhibitors of the Chikungunya virus nsP1 RNA capping enzyme. Antiviral Res. *154*, 124–131.

12. Inhibition of Chikungunya virus by an Adenosine Analog targeting the SAM□dependent nsP1 methyltransferase - Mudgal - - FEBS Letters - Wiley Online Library https://febs.onlinelibrary.wiley.com/doi/abs/10.1002/1873-3468.13642.

13. Pushpakom, S., Iorio, F., Eyers, P.A., Escott, K.J., Hopper, S., Wells, A., Doig, A., Guilliams, T., Latimer, J., McNamee, C., et al. (2019). Drug repurposing: progress, challenges and recommendations. Nat. Rev. Drug Discov. *18*, 41–58.

14. Montoya, M.C., and Krysan, D.J. (2018). Repurposing Estrogen Receptor Antagonists for the Treatment of Infectious Disease. mBio *9*, e02272-18.

15. Plouffe, L. (2000). Selective estrogen receptor modulators (SERMs) in clinical practice. J. Soc. Gynecol. Investig. *7*, S38-46.

16. Legha, S.S. (1988). Tamoxifen in the Treatment of Breast Cancer. Ann. Intern. Med. *109*, 219–228.

17. Squirewell, E.J., Qin, X., and Duffel, M.W. (2014). Endoxifen and Other Metabolites of Tamoxifen Inhibit Human Hydroxysteroid Sulfotransferase 2A1 (hSULT2A1). Drug Metab. Dispos. *42*, 1843–1850.

18. Homburg, R. (2005). Clomiphene citrate—end of an era? a mini-review. Hum. Reprod. *20*, 2043–2051.

19. Wibowo, E., Pollock, P.A., Hollis, N., and Wassersug, R.J. (2016). Tamoxifen in men: a review of adverse events. Andrology *4*, 776–788.

20. Eyre, N.S., Kirby, E.N., Anfiteatro, D.R., Bracho, G., Russo, A.G., White, P.A., Aloia, A.L., and Beard, M.R. (2020). Identification of Estrogen Receptor Modulators as Inhibitors of Flavivirus Infection. Antimicrob. Agents Chemother. *64*, e00289-20, /aac/64/8/AAC.00289-20.atom.

21. Watashi, K., Inoue, D., Hijikata, M., Goto, K., Aly, H.H., and Shimotohno, K. (2007). Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with viral RNA polymerase NS5B. J. Biol. Chem. *282*, 32765–32772.

22. Tohma, D., Tajima, S., Kato, F., Sato, H., Kakisaka, M., Hishiki, T., Kataoka, M., Takeyama, H., Lim, C.-K., Aida, Y., et al. (2019). An estrogen antagonist, cyclofenil, has anti-dengue-virus activity. Arch. Virol. *164*, 225–234.

23. Johansen, L.M., Brannan, J.M., Delos, S.E., Shoemaker, C.J., Stossel, A., Lear, C., Hoffstrom, B.G., DeWald, L.E., Schornberg, K.L., and Scully, C. (2013). FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. Sci. Transl. Med. *5*, 190ra79-190ra79.

24. Zheng, K., Chen, M., Xiang, Y., Ma, K., Jin, F., Wang, X., Wang, X., Wang, S., and Wang, Y. (2014). Inhibition of herpes simplex virus type 1 entry by chloride channel inhibitors tamoxifen and NPPB. Biochem. Biophys. Res. Commun. *446*, 990–996.

25. Cham, L.B., Friedrich, S.-K., Adomati, T., Bhat, H., Schiller, M., Bergerhausen, M., Hamdan, T., Li, F., Machlah, Y.M., Ali, M., et al. (2019). Tamoxifen Protects from Vesicular Stomatitis Virus Infection. Pharmaceuticals *12*, 142.

26. Gardner, J., Anraku, I., Le, T.T., Larcher, T., Major, L., Roques, P., Schroder, W.A., Higgs, S., and Suhrbier, A. (2010). Chikungunya virus arthritis in adult wild-type mice. J. Virol. *84*, 8021–8032.

27. Teo, T.-H., Chan, Y.-H., Lee, W.W.L., Lum, F.-M., Amrun, S.N., Her, Z., Rajarethinam, R., Merits, A., Rötzschke, O., Rénia, L., et al. (2017). Fingolimod treatment abrogates chikungunya virus–induced arthralgia. Sci. Transl. Med. *9*.

28. Miner, J.J., Cook, L.E., Hong, J.P., Smith, A.M., Richner, J.M., Shimak, R.M., Young, A.R., Monte, K., Poddar, S., Crowe, J.E., et al. (2017). Therapy with CTLA4-Ig and an antiviral monoclonal antibody controls chikungunya virus arthritis. Sci. Transl. Med. *9*, eaah3438.

29. Rulli, N.E., Rolph, M.S., Srikiatkhachorn, A., Anantapreecha, S., Guglielmotti, A., and Mahalingam, S. (2011). Protection From Arthritis and Myositis in a Mouse Model of Acute Chikungunya Virus Disease by Bindarit, an Inhibitor of Monocyte Chemotactic Protein-1 Synthesis. J. Infect. Dis. *204*, 1026–1030.

30. Parashar, D., and Cherian, S. (2014). Antiviral Perspectives for Chikungunya Virus. BioMed Res. Int. *2014*, e631642.

31. Kaur, R., Mudgal, R., Narwal, M., and Tomar, S. (2018). Development of an ELISA assay for screening inhibitors against divalent metal ion dependent alphavirus capping enzyme. Virus Res.

32. Takada, Y., Bhardwaj, A., Potdar, P., and Aggarwal, B.B. (2004). Nonsteroidal anti-inflammatory agents differ in their ability to suppress NF-κB activation, inhibition of expression of cyclooxygenase-2 and cyclin D1, and abrogation of tumor cell proliferation. Oncogene *23*, 9247–9258.

33. Rice, C.M., Levis, R., Strauss, J.H., and Huang, H.V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J. Virol. *61*, 3809–3819.

34. Sokoloski, K.J., Haist, K.C., Morrison, T.E., Mukhopadhyay, S., and Hardy, R.W. (2015). Noncapped Alphavirus Genomic RNAs and Their Role during Infection. J. Virol. *89*, 6080–6092.

35. Wiseman, H., Laughton, M.J., Arnstein, H.R., Cannon, M., and Halliwell, B. (1990). The antioxidant action of tamoxifen and its metabolites. Inhibition of lipid peroxidation. FEBS Lett. *263*, 192–194.

36. O'Brian, C.A., Housey, G.M., and Weinstein, I.B. Specific and Direct Binding of Protein Kinase C to an Immobilized Tamoxifen Analogue. 5.

37. Tamoxifen inhibits acidification in cells independent of the estrogen receptor | PNAS https://www.pnas.org/content/96/8/4432.short.

38. Murakami, Y., Fukasawa, M., Kaneko, Y., Suzuki, T., Wakita, T., and Fukazawa, H. (2013). Selective estrogen receptor modulators inhibit hepatitis C virus infection at multiple steps of the virus life cycle. Microbes Infect. *15*, 45–55.

39. Fan, H., Du, X., Zhang, J., Zheng, H., Lu, X., Wu, Q., Li, H., Wang, H., Shi, Y., Gao, G., et al. (2017). Selective inhibition of Ebola entry with selective estrogen receptor modulators by disrupting the endolysosomal calcium. Sci. Rep. *7*, 41226.

40. Bais, S.S., Ratra, Y., Khan, N.A., Pandey, R., Kushawaha, P.K., Tomar, S., Medigeshi, G., Singh, A., and Basak, S. Chandipura Virus Utilizes the Prosurvival Function of RelA NF-κB for Its Propagation. J. Virol. *93*, e00081-19.

41. Yeh, J.X., Park, E., Schultz, K.L.W., and Griffin, D.E. NF-κB Activation Promotes Alphavirus Replication in Mature Neurons. J. Virol. *93*, e01071-19.

42. Behjati, S., and Frank, M.H. (2009). The effects of tamoxifen on immunity. Curr. Med. Chem. *16*, 3076–3080.

43. Singh, H., Mudgal, R., Narwal, M., Kaur, R., Singh, V.A., Malik, A., Chaudhary, M., and Tomar, S. (2018). Chikungunya virus inhibition by peptidomimetic inhibitors targeting virus-specific cysteine protease. Biochimie *149*, 51–61.

44. Kaur, R., Neetu, null, Mudgal, R., Jose, J., Kumar, P., and Tomar, S. (2019). Glycan-dependent chikungunya viral infection divulged by antiviral activity of NAG specific chi-like lectin. Virology *526*, 91–98.

45. Snyder, J.E., Kulcsar, K.A., Schultz, K.L., Riley, C.P., Neary, J.T., Marr, S., Jose, J., Griffin, D.E., and Kuhn, R.J. (2013). Functional characterization of the alphavirus TF protein. J. Virol., JVI-00449.

46. Agarwal, A., Singh, A.K., Sharma, S., Soni, M., Thakur, A.K., Gopalan, N., Parida, M.M., Rao, P.V.L., and Dash, P.K. (2013). Application of real-time RT-PCR in vector surveillance and assessment of replication kinetics of an emerging novel ECSA genotype of Chikungunya virus in Aedes aegypti. J. Virol. Methods *193*, 419–425.

47. Sourisseau, M., Schilte, C., Casartelli, N., Trouillet, C., Guivel-Benhassine, F., Rudnicka, D., Sol-Foulon, N., Roux, K.L., Prevost, M.-C., Fsihi, H., et al. (2007). Characterization of Reemerging Chikungunya Virus. PLoS Pathog. *3*.

Figure Legends

Figure 1: Antiviral activity of SERMs against CHIKV and SINV in cell culture. (A) Plaque assay revealing changes in CHIKV (top three panels) or SINV (bottom three panels) titer on the treatment of virus-infected cells with an increasing concentration of 4-OHT, tamoxifen, and clomifene. The viral titer in the culture supernatant was determined at 24 h post-infection. The viral titer in corresponding vehicle-treated cells was considered 100%. Data were normalized using Graph pad's non-linear regression curve fit and the calculated 50% effective concentration (EC_{50}) values are summarized in Table 1. Values are mean from three independent experiments and error bars are standard deviation. (B) Barplots showing fold-reduction in the abundance of vRNA in CHIKV or SINV infected Vero cells treated with SERMs compared to vehicle control (VC). At 24 h post-infection, RNA was isolated and analyzed by qRT-PCR for the expression of the E1 region of the viral genome. Statistical significance of the difference between intracellular vRNA in treated cells and vehicle control-treated cells was assessed by one-way ANOVA test and Dunnett's posttest.***, $p<0.001$. Values are mean and error bars are standard deviation (n=3). (C) Immunofluorescence microscopy of CHIKV infection in Vero cells upon treatment with SERMs. Virus infection and treatment with SERMs were performed as mentioned in the supplementary section (S1). At 36 h post-infection, the infected cells were fixed and stained using an anti-alphavirus primary antibody and FITC-conjugated secondary antibody. DAPI was used for counter-staining cell nuclei. The image represents three independent biological replicates. Bars, 200 µM.

Figure 2: Characterizing SERM-mediated inhibition of viral growth. (A) Schematic representation of the time course of viral infection and treatment with SERM compounds. Vero cells were treated with compounds at different time points (in hours) before or after virus

infection and the viral titer (PFU/ml) was determined for CHIKV (B and C) and SINV (B and D). For Cotreatment studies, compounds were added simultaneously with the virus inoculum. For pre-treatment assay, Vero cells were treated with the inhibitors 2 h prior to viral adsorption. For post-treatment assay, cells were incubated at 37˚C with viral infection medium which was replaced by maintenance medium at different time-points containing 5 μM 4-OHT or 7.5 μM tamoxifen/clomifene. Supernatants were collected 24 h post-infection for the quantification of viral titer by plaque assay. 0.1% DMSO was used as vehicle control. For all the experimental groups, Vero cells were infected with CHIKV or SINV at an MOI of 1. VC: vehicle control; hpi: hours post-infection; PFU: plaque-forming unit. (E) SINV minigenome replicon assay capturing the luciferase activity in lysate derived from BHK21 cells transfected with capped RNA encoding the SINV-REP reporter replicon. Cells were subjected to 6 h of treatment with VC or 2.5 μM of the indicated SERMs. A schematic diagram of SINV-REP has also been presented (top). (F and G) Changes in vRNA levels in CHIKV and SINV infected cells at 6 h post-infection. Vero cells were infected with CHIKV and SINV at MOI of 5 followed by treatment with 5 μM of the compounds. vRNA levels were assessed by qRT-PCR and the fold reduction in vRNA relative to VC-treated cells is plotted. Actin: endogenous control; E1: target gene. (H) Quantitative determination of extracellular SINV vRNA levels at 24 h post-infection. Vero cells were infected with SINV at MOI of 1 and were treated with indicated concentrations of the SERM compounds. Quantification of the vRNA in the culture supernatant was done using qRT-PCR as discussed in the material and methods section. Statistical significance of the difference in viral titer/luciferase activity/vRNA levels between treated and vehicle control-treated cells was assessed by one-way ANOVA test and Dunnett's posttest. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$. Values are mean and error bars are standard deviation from triplicate experiments.

Figure 3: nsP1 capping enzyme is implicated in alphavirus inhibition by SERMs. Altered plaque phenotype of the partially-resistant variant of CHIKV against 4-OHT. (A) Plaques of the untreated virus after 48 h post-infection and virus passaged 15 times under the selection pressure of 4-OHT. (B) Non-synonymous mutations in the nsP-coding region of partially-resistant CHIKV variant. nsP: non-structural proteins. (C) nsP1 activity inhibition by tested compounds. SERM compounds were tested at 50 μM concentration in the CHIKV and VEEV nsP1 MTase reaction mixture. The reaction was carried out as described previously by Mudgal et al., 2020. The MTase activity is shown relative to the enzyme activity in the reaction mixture in the presence of vehicle control. (D) Dose-dependent inhibition of guanylation of nsP1 in the presence of SERMs. Relative guanylation by CHIKV nsP1 was determined using an ELISA assay that measures the amount of m7GMP-nsP1 adduct formed in the reaction. Reaction conditions were similar to those described earlier by Kaur et al., 2018. Data points represent mean value and error bars are standard deviation from two independent experiments.

Figure 4: Testing therapeutic efficacy of tamoxifen in CHIKV-infected mice. CHIKV-infected mice were injected successively with 20 mg/kg of tamoxifen or vehicle control at day one, three, and five post-infection. Subsequently, changes in the body weight (A) and the footpad area (B), were measured at the indicated days. (C) Representative image of CHIKV infected joint footpad of mice subjected to tamoxifen therapeutic regime. (D) Dot plots showing the load of infectious virus particles in the infected footpad at the indicated days post-infection, as determined by $TCID_{50}$ method. Graphs revealing the abundance of vRNA in the blood (E) and the infected footpad (F) at the indicated day post-infection. (G) Representative immunohistochemistry images revealing H&E stained sections of the joint footpad. Mice were either uninfected (left panel) or infected with CHIKV six days prior to image

acquisition (middle panel) or infected similarly with CHIKV and then subjected to a therapeutic regime (right panel). Footpad sections from two animals per set and three fields/sections were examined. E denotes edema; * denotes infiltrates; B signifies bone. Images are representative of three mice per group.

Figure 1

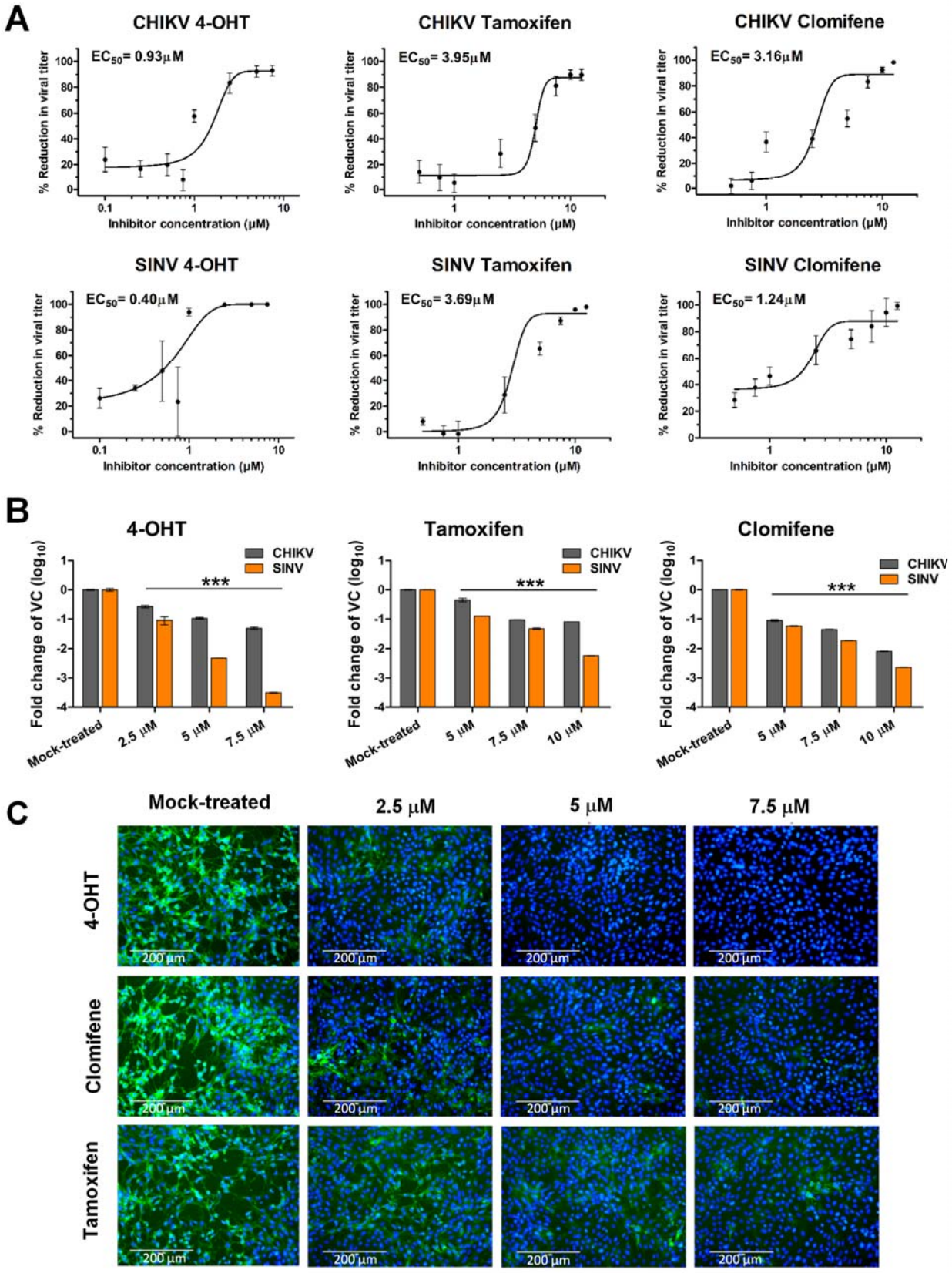

A
CHIKV 4-OHT
EC50= 0.93μM
CHIKV Tamoxifen
EC50= 3.95μM
CHIKV Clomifene
EC50= 3.16μM
SINV 4-OHT
EC50= 0.40μM
SINV Tamoxifen
EC50= 3.69μM
SINV Clomifene
EC50= 1.24μM
% Reduction in viral titer
Inhibitor concentration (μM)
B
4-OHT
Tamoxifen
Clomifene
CHIKV
SINV
***
Fold change of VC (log10)
Mock-treated
2.5 μM
5 μM
7.5 μM
10 μM
C
Mock-treated
2.5 μM
5 μM
7.5 μM
4-OHT
Clomifene
Tamoxifen
200 μm

Figure 2

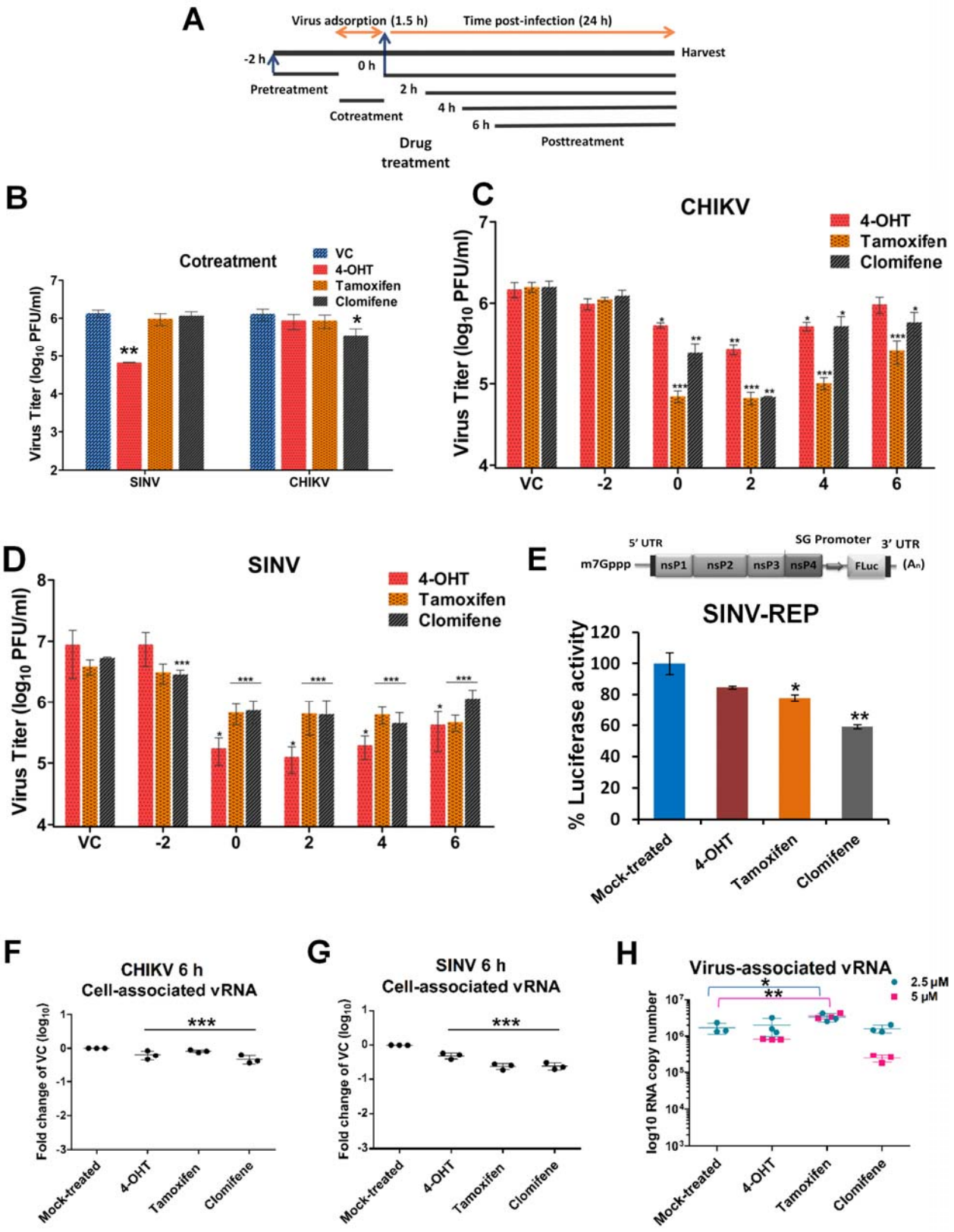
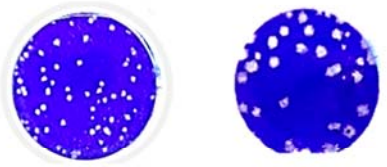

A
Virus adsorption (1.5 h)
Time post-infection (24 h)
-2 h
0 h
Harvest
Pretreatment
2 h
Cotreatment
4 h
6 h
Drug treatment
Posttreatment
B
Cotreatment
VC
4-OHT
Tamoxifen
Clomifene
Virus Titer (log10 PFU/ml)
SINV
CHIKV
C
CHIKV
4-OHT
Tamoxifen
Clomifene
Virus Titer (log10 PFU/ml)
VC
-2
0
2
4
6
D
SINV
4-OHT
Tamoxifen
Clomifene
Virus Titer (log10 PFU/ml)
VC
-2
0
2
4
6
E
5' UTR
SG Promoter
3' UTR
m7Gppp
nsP1
nsP2
nsP3
nsP4
FLuc
(An)
SINV-REP
% Luciferase activity
Mock-treated
4-OHT
Tamoxifen
Clomifene
F
CHIKV 6 h
Cell-associated vRNA
Fold change of VC (log10)
Mock-treated
4-OHT
Tamoxifen
Clomifene
G
SINV 6 h
Cell-associated vRNA
Fold change of VC (log10)
Mock-treated
4-OHT
Tamoxifen
Clomifene
H
Virus-associated vRNA
2.5 μM
5 μM
log10 RNA copy number
Mock-treated
4-OHT
Tamoxifen
Clomifene

Figure 3

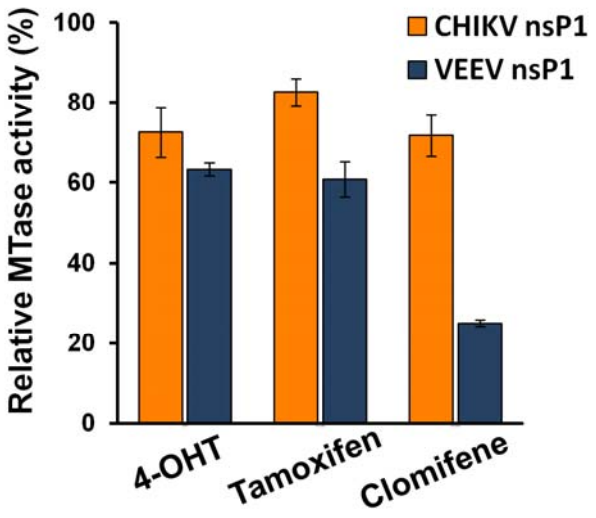
A



B

Non-synonymous mutations	Region
T155A	**nsP1**- MTase/GTase domain
Q280H	**nsP3**- Macro Domain
M490L	**nsP3**- Zinc Binding Domain
K39E	**nsP4**- Disordered Region

C



D

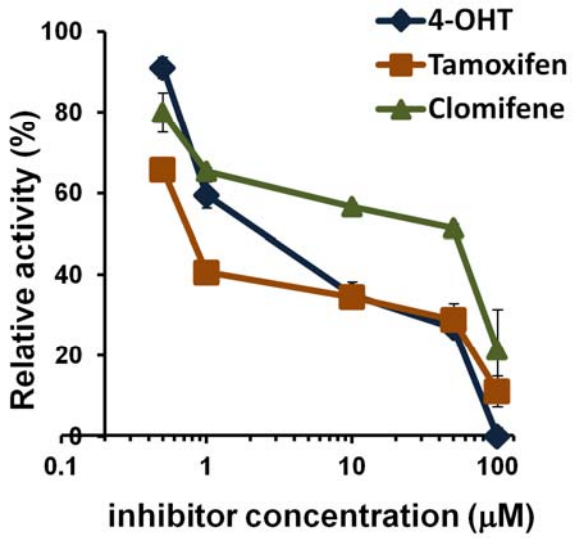
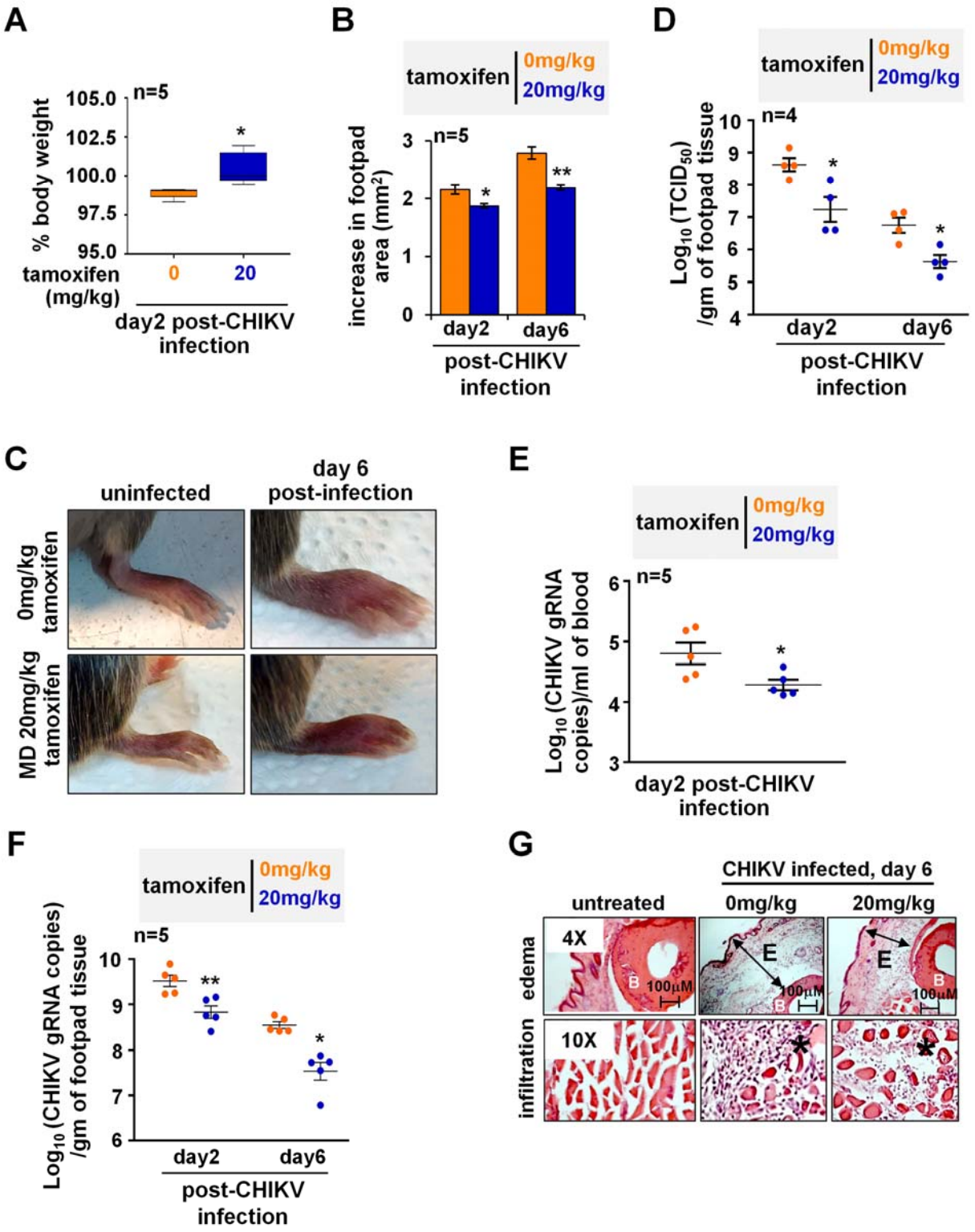


Figure 4


A
% body weight
105.0
102.5
100.0
97.5
95.0
n=5
*
tamoxifen (mg/kg)
0
20
day2 post-CHIKV infection
B
tamoxifen
0mg/kg
20mg/kg
increase in footpad area (mm²)
n=5
*
**
day2
day6
post-CHIKV infection
D
tamoxifen
0mg/kg
20mg/kg
Log10 (TCID50) /gm of footpad tissue
n=4
*
*
day2
day6
post-CHIKV infection
C
uninfected
day 6 post-infection
0mg/kg tamoxifen
MD 20mg/kg tamoxifen
E
tamoxifen
0mg/kg
20mg/kg
Log10 (CHIKV gRNA copies)/ml of blood
n=5
*
day2 post-CHIKV infection
F
tamoxifen
0mg/kg
20mg/kg
Log10 (CHIKV gRNA copies) /gm of footpad tissue
n=5
**
*
day2
day6
post-CHIKV infection
G
CHIKV infected, day 6
untreated
0mg/kg
20mg/kg
edema
infiltration
4X
10X
B
E
100µM