Evaluation of broad-spectrum antiviral compounds against chikungunya infection using a phenotypic screening strategy [version 1; peer review: 2 approved with reservations, 1 not approved]

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Abstract
Chikungunya fever is an emerging disease and a significant public health problem in tropical countries. Recently reported outbreaks in Brazil in 2015 drew attention to the need to develop prevention and treatment options, as no antiviral chemotherapy or vaccines are currently available for this disease. Two strategies have been proved to accelerate the discovery of new anti-infectives: phenotypic screening and drug repurposing. Phenotypic screening can support the fast interrogation of compounds without the need for a pre-validated drug target, which is not available for the chikungunya virus (CHIKV) and has the additional advantage of facilitating the discovery of antiviral with novel mechanism of action. Drug repurposing can save time and resources in drug development by enabling secondary uses for drugs that are already approved for human treatment, thus precluding the need for several of the mandatory preclinical and clinical studies necessary for drug approval. A phenotypic screening assay was developed by infecting the human hepatoma Huh-7 cells with CHIKV 181/25 and quantifying infection through indirect immunofluorescence. The compound 6-azauridine was used as a positive control drug. The screening assay was validated by testing a commercial library of 1,280 compounds, including FDA-approved drugs, and used to screen a panel of broad-spectrum antiviral compounds for anti-CHIKV activity. A high content assay was set up in Huh-7 cells-infected with CHIKV. The maximum rate of infection peaked at 48 hours post-infection, after which the host cell number was greatly reduced due to a strong cytopathic effect. Assay robustness was confirmed with Z'-factor values >0.8 and high
correlation coefficient between independent runs, demonstrating that the assay is reliable, consistent and reproducible. Among tested compounds, sofosbuvir, an anti-hepatitis C virus drug, exhibited good selectivity against CHIKV with an EC$_{50}$ of 11 µM, suggesting it is a promising candidate for repurposing.

**Keywords**
Chikungunya, High content screening, drug discovery, antivirals

This article is included in the *Neglected Tropical Diseases* collection.
**Introduction**

Chikungunya virus (CHIKV) is an arthropod-borne virus that belongs to the *Alphavirus* genus of the Togaviridae family. Alphaviruses are positive-sense, single-stranded RNA viruses that can produce severe encephalitis, such as in the infections caused by Ross River virus (RRV), Western- (WEE), Eastern- (EEE) and Venezuelan-equine encephalitis (VEE) virus. Alphaviruses can also be arthritogenic, such as in the case of CHIKV, Mayaro virus (MAYV), and O’nyong’nyong virus (ONNV)5. CHIKV was responsible for several recent (re)emerging outbreaks in humans24. Nowadays, approximately one billion people around the globe, especially in the tropics, are estimated to live in risk areas of CHIKV outbreaks45. In the Americas, CHIKV was first detected in 2013, in St. Martin, an island in the Caribbean, and quickly spread to other countries, including Brazil46. CHIKV produces an acute disease with high fever, headache, nausea, vomiting and conjunctivitis. Patients also develop severe joint pain, which eventually evolves into an arthritogenic syndrome that can last from weeks to years2. Recently, CHIKV infection has also been associated with neurological complications1. There are no antiviral drugs or vaccines available for CHIKV, and the supportive care treatment aims at reducing symptoms and include analgesics, anti-inflammatory and antipyretic drugs.

Some anti-CHIKV molecules have been discovered as a result of antiviral screening campaigns, such as a harringtonine, a plant alkaloid that reduced CHIKV replication by interfering with protein translation in vitro5; D-N4-hydroxycytidine (NHC), a nucleoside analogue, that inhibits RNA synthesis by targeting replication complex10; and barberry, abamectin and ivermectin, which all also reduce viral RNA synthesis11. Most assays were based on replicon systems, a classic way to evaluate drugs that interfere with the viral replication phase, but which cannot account for drugs that might inhibit other steps of the viral cycle, such as cell entry or virion assembly and release. Thus, alternative assays that deploy infectious viral particles, such as those that are based on measurement of cellular infection by high-content screening (HCS)12,13, enable the investigation of compounds that may interact with different stages of infection and lead to the to discover of new classes of antivirals.

**Methods**

**Cells**

Huh-7 hepatocellular carcinoma cells were cultivated in DMEM F-12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 units/ml Penicillin (GIBCO) and 100 µg/ml Streptomycin (GIBCO) at 37°C, 5% CO₂. The Vero cell line derived from the kidney of an African green monkey were cultivated in DMEM high glucose (GIBCO) and 100 µg/ml Streptomycin (GIBCO) at 37°C, 5% CO₂. CHIKV-MHS was obtained from this blood.

**Compounds and commercial library**

The compounds 6-azauridine as well as non-infected cells treated with vehicle

**Production of mouse hyperimmune sera (MHS)**

Mouse hyperimmune sera was obtained from previously prepared stocks16. Briefly, to prepare these stocks mice (*Mus musculus*) received 4 weekly inoculations of 0.2 ml of brain macerate suspensions from newborn mice infected with CHIKV in PBS, by the intraperitoneal route. At 5 days after the last immunization, the animals were anesthetized and underwent intracardiac puncture for blood collection. CHIKV-MHS was obtained from this blood.

**Assay development**

Huh-7 cells were seeded in black polystyrene 384-well assay plates (Greiner Bio-One) at 3,000 cells/well in 40 µl DMEM-F12 supplemented with 10% FBS and incubated overnight. Cells were infected with 10 µl of inoculum of CHIKV 181/25 at different multiplicities of infection (MOIs) of 0.5, 0.05 and 0.01. Plates were fixed at different periods of time (36, 48 and 72 hours) and submitted to the immunofluorescence assay (described below) and images are acquired using an InCell Analyzer 2200 (GE Life Sciences).

**Primary screening and assay validation**

A library stock plate containing the aforementioned compounds at 2 mM in DMSO was used to prepare the intermediate plate by a 16.6-fold dilution in DPBS, to a concentration of 60 µM and 3% DMSO. Then, 10 µl of the intermediate plate content was transferred onto the cell-containing plate. The final concentration of library compounds in the assay plate was 10 µM, with 0.5% DMSO. Controls were placed in lateral columns in all plates. Positive controls were infected cells treated with 50 µM of 6-azauridine as well as non-infected cells treated with vehicle.
(0.5% DMSO in DPBS). Negative controls were infected cells treated with vehicle. Cells were infected by 10 µl of CHIKV 181/25 at MOI 0.05. Plates were incubated for 48 h at 37°C, 5% CO2, under humidified atmosphere, and then fixed with 4% (w/v) PFA for 15 min at room temperature and washed twice with DPBS. Then, plates were incubated with CHIKV-MHS (mouse hyperimmune sera) diluted 1:1500 (v/v) prepared in blocking buffer (DPBS containing 5% FBS) for 30 min. Each plate was washed twice with DPBS, followed by incubation at room temperature for 30 min with the AlexaFluor488-conjugated goat anti-mouse IgG (Cat. No. A-11001, Thermo-Scientific) diluted 1:2000 (v/v), and 5 µg/ml of 4’,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in DPBS. Each plate was washed twice with DPBS. All plates were filled up with 50 µl of PBS/well. Images were acquired using a confocal microscope High-Content System InCell Analyzer 2200 (GE Life Sciences) and processed by InCell Investigator v.1.6.1 software (GE, USA). Four different images were acquired from each well at x20 magnification. Automated image analysis was performed cell-by-cell through a defined mask based on fluorescence signal measurement and cell morphology. Cell segmentation parameters defined the analysis performed by the Investigator software. The nuclei were segmented from the DAPI staining images and each nucleus was determined as a minimum area of 50 µm. Total cells were filtered from the AlexaFluor488 channel and were defined as minimum area of 100 µm. The mean AlexaFluor488 fluorescence signal of infected cells were defined from the cytoplasm mask, with values based on means signal from fluorescence of wells from infected cells showing a six-times higher value compared to the mean of Alexa488 fluorescence signal of non-infected cells. Images were treated using image analysis program ImageJ v1.51 to set up colors and merge image channels to final visualization. The validation of screening was conducted in two independent experiment.

Data normalization
Infection ratio (IR) was defined as the ratio between (i) the total number of infected cells, and (ii) the total number of cells. Data were normalized with the negative (DMSO-treated, infected cells) and positive (infected cells treated with 50 µM 6-azauridine) controls. Normalized activity was calculated as described by Pascoalino et al.2. Cell survival was expressed as the percentage of the total cell number from test sample divided by the average total cell number from the positive control wells: Cell number test sample/Avg. cell number of positive control) × 100. Normalized activity and cell survival values were processed with the GraphPad Prism software version 7. Representative graphs of sample distribution were obtained by plot data at TIBCO Spotfire 7.0 software. Plates were also submitted to quality control measurement of Z’-factor as described by Zhang et al.17.

Dose-response assay
For dose-response curves, drugs were prepared as described8. The initial test concentrations were 100 nM for IFN-α2A, 50 nM for bafilomycin, 120 µM for chloroquine and mycophenolic acid, and 100 µM for sofosbuvir, daclatavir, ledipasvir and 5-fluorouracil. Dose response assay were calculated based on percentage of normalized activity and cells survival for each concentration tested. Data were plotted with GraphPad Prism software version 7. The sigmoidal dose-response curve (variable slope) function were used to calculate the effective concentration that inhibited 50% of infection (EC50), and the concentration of compound that presented a 50% reduction in cell number in comparison to the controls (CC50). The ratio between CC50 and EC50 determines the selective index (SI).

Statistical analysis
Two-way analysis of variance (ANOVA) with Sidak’s test, a multiple comparison test, was conducted to calculate statistical significance (P < 0.05) of cell numbers from non-infected and infected CHIKV 181/25 at different multiplicity of infection and incubation time experiment. The coefficient of determination (R2) test were used to determine statically coefficient of variation between screening replicates from normalize activity data. All data were plotted using GraphPad Prism Software version 7.

Results
Assay development
A high-content screening assay was developed to evaluate compounds activity against CHIKV infection in vitro. The first step consisted on defining the cell model to support viral infection. A range of cell lines has been reported as being susceptible to CHIKV infection, such as Vero, human fetal lung fibroblast (MRC-5), baby hamster kidney (BHK), human embryonic kidney 293 (HEK-239T) and Huh-718,19. The Huh-7 cell line was selected as it has desirable features for high content imaging, such as adherent monolayer growth, and is human cell line, meaning it is a more representative in vitro model than would be cells of another species. The second step was determining the optimal multiplicity of infection (MOI) and the necessary period of time for the efficient viral infection in 384-well plates. Cells were infected at three different MOI (0.5, 0.05 and 0.01) and incubated for different periods of time (36, 48 and 72 hours). The total cell number and the IR were determined. When cells were plated and infected concomitantly, even the lowest MOI tested showed high cytopathic effect (data not shown). Thus, cells were plated 24 h before infection (Figure 1A). For the highest MOI, CHIKV infection decreased cell number by 70% at 48 hours and by almost 100% at 72 hours compared with non-infected cells. There was no significant difference in cell number between non-infected cells and infected cells for both 0.05 and 0.01 MOIs at 36 hours. Compared to non-infected cells, a decrease in cell number by 42% and 22% at MOIs 0.05 and 0.01, respectively, was observed at 48 hours (Figure 1A). After 36 hours of incubation, the IR reached 0.99 for all MOIs, but the lowest MOI gave high variation in IR between replicate wells. Therefore, with the aim of testing drugs, a 0.05 MOI at 48 hours of incubation was selected for further experiments to achieve the longer time of exposure to drug treatment possible under these conditions, a high ratio of infection with minor variability,
Figure 1. Development of CHIKV high content assay. (A) Huh-7 cells were infected with different MOIs (0.5, 0.05 and 0.01) of CHIKV. The cell number and the infection ratio (defined as the ratio between total cell number and number of infected cells per sample), were evaluated after 36, 48 and 72 hours of infection. Error bars represent the standard deviation of 48 wells. Quantification of total cell number was comparable between non-infected and CHIKV 181/25-infected cells (p < 0.05). (B) General scheme of CHIKV high content assay. On day 1, Huh-7 cells were plated onto 384-well plates at 3000 cells/well. Then, after 24 hours (day 2), compounds were added, followed by addition of virus diluted at MOI 0.05. The plates were incubated for 48 hours up to immunofluorescence assay and high content analysis on day 4.

Assay validation
To validate the assay, high-content screening was run using a commercial library of compounds. Cell infection was determined by indirect CHIKV immunofluorescence detection. Figure 2A shows a raw image and software segmentation analysis of the same image. The 6-azauridine compound was previously reported to have activity against CHIKV, and was chosen as the reference compound in this assay. The activity of 6-azauridine was assessed using a dose-response curve (Figure 2B). The EC\textsubscript{50} of 0.65 µM 6-azauridine and EC\textsubscript{100} of 50 µM 6-azauridine were determined against CHIKV. In order to validate the assay reproducibility and robustness, a commercial library composed of 1,280 compounds was tested at a single concentration (10 µM). A good window between positive and negative controls was observed.
As a result, the mean for all plates Z’-factor values were 0.86±0.09, indicating that the established assay is reliable. Additionally, there was a high correlation coefficient between runs (Coefficient of determination R²: 0.86), which was determined using normalized activity of each single well between the first (R1) and the second (R2) screens, including compounds and controls (Figure 2D).

Evaluation of known antivirals against CHIKV

A set of compounds with known antiviral activity were evaluated against CHIKV. A total of 9 compounds were tested in dose-response curves. Figure 3 lists the name, molecular structures and dose-response curve plots for all compounds. Interferon α2A (IFN-α2A) and mycophenolic acid have reported activity against CHIKV. The HCS assay confirmed their reported activity, demonstrated by EC₅₀ values of 0.7 nM and 0.8 µM and high SI of >14 and 8.25, for IFN-α2A and mycophenolic acid, respectively. In order to evaluate compounds with previously reported activity against CHIKV, bafilomycin A1 and chloroquine were tested, giving an EC₅₀ of 0.01 µM and 21 µM, respectively; however, they were cytotoxic in HuH-7 cells, with low SI values (5 and 0.3, respectively). The antiviral activity of daclatasvir, an anti-hepatitis C virus (HCV) drug, against CHIKV was associated with high cytotoxicity, and it had a low SI value of 1.3. Ledisparir, also an anti-HCV compound, and 5-fluorouracil, which has reported activity against ZIKV, did not present anti-CHIKV activity in the HCS assay. Sofosbuvir is an FDA-approved compound against HCV, and has been recently described as an active compound against other flaviviruses, including dengue and Zika. Sofosbuvir demonstrated dose-dependent activity against CHIKV (EC₅₀ 11 µM) with no cytotoxicity in HuH-7 cells. Representative images of sofosbuvir activity, alongside 6-azauridine, are displayed in Figure 4.

Figure 2. Validation of CHIKV high content assay. (A) Interface of image processing and analysis. (B) Dose-response curve for reference compound 6-azauridine. Left y-axis: Normalized activity values (black squares and curves); Right y-axis: Cell survival values in percentage (red dots and curves); x-axis: Log of molar compound concentrations. Data points are means, and error bars represent standard deviations from two independent experiments. (C) Left graphic: Representative scatter plot of the infection ratio showing controls separation. Dots represent each single tested well and colors represent different treatments, where: 0.5% DMSO negative control (yellow); non-infected cells (red); 50 µM 6-azauridine positive control (green). Z’-factor value of 0.87 was obtained from total data controls from two independent runs for screening validation, between inter-replicates and intra-replicates plates. The continuous line represents the mean of each control, the dotted line represents 3 standard deviations from the mean of the negative and positive controls. Right graphic: Data correlation (normalized activity) from two screening runs of a small library; dots represent each single tested well and colors represent different treatments, where: 0.5% DMSO negative control (yellow); non-infected cells (red); 50 µM 6-azauridine positive control (green); and tested compounds (gray). The coefficient of determination of R square (R² 0.86) was calculated using GraphPad Prism software.
| Compound          | Structure | Dose Response Curve |
|-------------------|-----------|---------------------|
| Interferon α2A    | Protein   | ![Dose response curve for Interferon α2A](image) |
| Sofosbuvir        | ![Sofosbuvir structure](image) | ![Dose response curve for Sofosbuvir](image) |
| Daclatasvir       | ![Daclatasvir structure](image) | ![Dose response curve for Daclatasvir](image) |
| Ledispavir        | ![Ledispavir structure](image) | ![Dose response curve for Ledispavir](image) |
| Bafilomycin A1    | ![Bafilomycin A1 structure](image) | ![Dose response curve for Bafilomycin A1](image) |
| Chloroquine       | ![Chloroquine structure](image) | ![Dose response curve for Chloroquine](image) |
| 5-fluorouracil    | ![5-fluorouracil structure](image) | ![Dose response curve for 5-fluorouracil](image) |
| Mycophenolic Acid | ![Mycophenolic Acid structure](image) | ![Dose response curve for Mycophenolic Acid](image) |

**Figure 3.** Dose response and molecular-structure of antiviral drug. Dose response curves of interferon α2A, sofosbuvir, daclatasvir, ledispavir, bafilomycin A1, chloroquine, 5-fluorouracil and mycophenolic acid as anti-CHIKV activity (black) or the effect on Huh-7 survival (Red). Values of EC\(_{50}\) means effective concentration of 50% infection inhibition, and SI means selective index (SI) based on CC\(_{50}\) concentration of compounds of 50% cytotoxicity (not showed). ND, non-determined values.
Currently, most assays available for drug screening against CHIKV are based on cell viability methodologies, which evaluate the compounds capacity to prevent cell lysis. Such approaches have the advantage of being of lower in cost and higher-throughput than image-based phenotypic assays. However, background noise interference in quality and usage of counter-screening assays to assess compound cytotoxicity and support conclusions should be considered. Conversely, HCS assays provide multi-parametric evaluation of both viral infection and cytotoxicity in same assay. Therefore, in the present study we propose the development of a reproducible, phenotypic HCS for CHIKV, in order to trial drugs with antiviral activity.

Different approaches have been described to assess drugs in a high-throughput screening (HTS) format against CHIKV, including measurement of cell viability using a resazurin assay, replicon-based assay using a Renilla luciferase reporter, which targets only replication-process-interfering compounds, or a HCS assay using BHK cells. In this study, we opted for the Huh-7 cell line as this has been used for HCS for antiviral discovery by our group and others for hepatitis C, dengue and Zika. Moreover, it is reported that Huh-7 cells are permissive to CHIKV infection. The CHIKV viral cycle usually happens in a short period of time, between 8 and 16 hours, following high cytopathic effect. In this manner, we opted to use a relatively low MOI (0.05) to prevent high cell lysis, and it can be expected that multiple infection cycles happen during the assay duration (48 h). Thus, all potential targets during the whole viral cycle can be exposed to the compounds. The developed assay also proved to be robust and reproducible.

IFN-2α, bafilomycin A1, chloroquine and mycophenolic acid had all been previously reported as active against CHIKV in vitro, and their antiviral activity was confirmed under the conditions used in this study. However, bafilomycin A1 and chloroquine were cytotoxic, resulting in low SIs (<5). Bafilomycin A1, an inhibitor of mammalian vacuolar-type H(+) -ATPase, prevents the acidification of the endosomal compartment, where the low pH allows the fusion of viral capsid followed by the entrance in the cytoplasm, thus preventing a crucial early step of the CHIKV virus cycle. The same inhibition mechanism was observed in vitro during for the infection of sindbis virus, a prototype alphavirus. Previous studies have reported the cytotoxicity of bafilomycin A1 in HEK123 cells, as was also observed here in Huh-7 (Figure 3). The activity of chloroquine, an antimalarial compound, was extensively investigated against CHIKV, although studies in vivo with infected mice showed inefficient activity. In addition, clinical trials comparing double-blinded placebo groups and patients with CHIKV infection group did not present convincing data regarding chloroquine treatment efficacy. Studies in CHIKV-infected Vero cells suggested that chloroquine exerts antiviral activity by preventing CHIKV internalization. The chloroquine EC50 values observed in this study (21 µM) in Huh-7-infected cells are in accordance with values previously reported for Vero cells (17 µM)22. However, the cytotoxicity of chloroquine seems to vary depending on the cell type or assay conditions, as chloroquine showed greater cytotoxicity in Huh-7 cells (with values of CC50 of 56 µM) than Vero cells (CC50 >100 µM). Mycophenolic acid inhibits inosine monophosphate dehydrogenase (IMPDH), an essential enzyme in de novo biosynthesis of guanine, and has been reported to have antiviral activity for both single strand RNA negative and positive viruses, for instance, against influenza virus.

**Figure 4. Sofosbuvir activity against CHIKV.** Panel of representative images. First line of images shows nuclei staining with DAPI. Second line of images shows immunofluorescence against CHIKV (AlexaFluor488). Third line of images is the merge between the lines one and two. First column displays non-infected cells, followed by infected cells treated with 0.5% DMSO, 50 µM 6-azauridine, 12.5 µM sofosbuvir and 25 µM sofosbuvir.
dengue virus\(^{34}\), and the alphavirus VEEV\(^{34}\). CHIKV activity was previously reported to have EC\(_{50}\) values of 0.1 µM in Vero cells\(^{35}\), and here was observed in Huh-7 EC\(_{50}\) of 0.8 µM, confirming values in similar levels (Figure 3) albeit with lower selectivity. Nevertheless, a human cell model, such as the Huh-7 cell line, should be preferred to screen antiviral candidates to promote a more representative values of activity and cytotoxicity, which may diverge when compared to non-human cells lines\(^{36}\).

Sofosbuvir, 5-fluorouracil, daclatasvir and ledispavir are all FDA-approved drugs with reported activity against flaviviruses. The nucleoside analog 5-fluorouracil is used to treat neoplastic disease\(^{37}\), and we have recently shown its antiviral activity \textit{in vitro} against ZIKV infection\(^{38}\). However, 5-fluorouracil presented no activity against CHIKV in our assay, suggesting selectivity for flaviviruses. Comparable results were obtained for ledispavir, which did not show inhibition against CHIKV, even at the highest concentration tested. Ledispavir, daclatasvir and sofosbuvir are direct-acting antiviral agents and have been successfully used to treat HCV-infected subjects. Those compounds target NS5A and NS5B, two HCV non-structural proteins\(^{39}\). NS5A presents three domains, which are responsible for genome replication, virus assembly through production of infection virus particles, and regulation of viral genome replication, from direct interaction of NS5A domain II with NS5B. NS5B is an RNA-dependent RNA-polymerase (RdRp) that directs the RNA synthesis in the HCV replication cycle\(^{40}\). Ledispavir and daclatasvir are NS5A inhibitors, while sofosbuvir, an uridine nucleoside analog that targets NS5B that is usually administrated in combination with daclatasvir\(^{41}\). Besides HCV, recent studies have demonstrated that sofosbuvir can inhibit infection by other flavivirus \textit{in vitro} and in mice\(^{25}\). Our results demonstrated sofosbuvir elicited a concentration-dependent inhibition of CHIKV infection (Figure 3 and Figure 4), suggesting that this drug might have a broader antiviral spectrum than previously known.

Differently from HCV, which the genome organization consisted in five non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A\(^{30}\)), CHIKV possess four non-structural protein (nsP1, nsP2, nsP3 and nsP4), being the RdRp domain localized at nsP4. Alignment sequence of RdRp has demonstrated highly conserved regions between CHIKV and other flaviviruses. More specifically, the motif B region, which is a functional domain of viral RdRp coding region, and the R1 motif, which has a role in nucleoside triphosphate binding during viral RNA synthesis, are highly conserved. Besides, CHIKV RdRp forms similar structures to the RdRp of other RNA viruses\(^{31}\). The search for direct-target compounds against CHIKV have focused on nsP2, due to its multifunctioning domains, which acts as helicases to form RNA secondary structures, as triphosphates responsible for RNA capping enzyme and removing terminal phosphate from new RNA template, and as proteases responsible for processing non-structural polyproteins\(^{32}\). In addition, its well-known structure makes nsP2 a suitable target for drug design\(^{33}\). However, few studies have focused on the search for compounds that target RdRp for CHIKV\(^{34,35}\). A compound that targets RdRp would be attractive, as RdRp acts on a viral process, is essential for replication of the viral genome and does not affect host cells\(^{33}\).

In conclusion, the phenotypic high content analysis established herein revealed that sofosbuvir is a promising candidate for use against CHIKV infection. Further studies should be performed in order to elucidate the exact mechanism related to CHIKV RdRp inhibition by sofosbuvir.

**Data availability**

Dataset 1. All raw data from the present study. Raw data are separated according to the figure in which they are presented; a guide to the data is available as a .docx file. DOI: https://doi.org/10.5256/f1000research.16498.d221905\(^{36}\).

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References

1. Strauss JH, Strauss EG: The alphaviruses: gene expression, replication, and evolution. Microbiol Rev. 1994; 58(3): 491–562. PubMed Abstract | Free Full Text
2. Ardéchiga-Caballos N, Aguilar-Sedién A: Alphaviral equine encephalomyelitis (Eastern, Western and Venezuelan). Rev Sci Tech. 2015; 34(2): 491–601. PubMed Abstract | Publisher Full Text
3. Lewande OW, Obanda V, Buchi G, et al: Global emergence of Alphaviruses that cause arthritis in humans. Infect Ecol Epidemiol. 2015; 4(1): 29853. PubMed Abstract | Publisher Full Text | Free Full Text
4. Paul BJ, Sadanand S: Chikungunya Infection: A Re-emerging Epidemic. Rheumatol Ther. 2018; 1–10. PubMed Abstract | Publisher Full Text
5. Plaixue G, Guizière BA, Jauréguby S, et al: Chikungunya, an epidemic arbovirus. Lancet Infect Dis. 2007; 7(5): 319–327. PubMed Abstract | Publisher Full Text
6. Morrison TE: Reemergence of chikungunya virus. J Virol. 2014; 88(20): 11644–7. PubMed Abstract | Publisher Full Text | Free Full Text
7. Gar Hassan VK, Duan B, Reid SP: Chikungunya Virus: Pathophysiology, Mechanism, and Modeling. Viruses. 2017; 9(12): pii: E368. PubMed Abstract | Publisher Full Text | Free Full Text
8. Agarwal A, Vbna D, Srivastava AK, et al: Guillain-Barre syndrome complicating chikungunya virus infection. J Neurovirol. 2017; 23(3): 504-607. PubMed Abstract | Publisher Full Text

9. Kaur P, Thiruchelvan M, Lee RC, et al: Inhibition of chikungunya virus replication by harringtonine, a novel antiviral that suppresses viral protein expression. Antimicrob Agents Chemother. 2013; 57(1): 155-67. PubMed Abstract | Publisher Full Text | Free Full Text

10. Elteneh M, Xiao S, Zandi K, et al: Characterization of d-N4-Hydroxyacetylene as a Novel Inhibitor of Chikungunya Virus. Antimicrob Agents Chemother. 2017; 61(4): pii: e02395-16. PubMed Abstract | Publisher Full Text | Free Full Text

11. Varghese FS, Kaukinen P, Gikas S, et al: Discovery of berberine, abacatinib and irinotecan as antivirals against chikungunya and other alphaviruses. Avantir. Res. 2016; 126: 117–24. PubMed Abstract | Publisher Full Text

12. Pascoalino B3, Courtmarche G, Condore MT, et al: Zika antiviral chemotherapy: identification of drugs and promising starting points for drug discovery from an FDA-approved library (version 1; referees: 2 approved). F1000Res. 2016; 5: 3523. PubMed Abstract | Publisher Full Text | Free Full Text

13. Cruz DJ, Koshi AC, Taniguchi JB, et al: High content screening of a kinase-focused library reveals compounds broadly-active against dengue viruses. PLoS Negl Trop Dis. 2013; 7(2): e2073. PubMed Abstract | Publisher Full Text | Free Full Text

14. Levitt NH, Ramsburg HH, Hasly SE, et al: Development of an attenuated strain of chikungunya virus for use in vaccine production. Vaccine. 1986; 4(3): 157–62. PubMed Abstract | Publisher Full Text | Free Full Text

15. Nakao E, Hotta S: Immunogenicity of purified, inactivated chikungunya virus in monkeys. Bull World Health Organ. 1973; 48(5): 559–602. PubMed Abstract | Publisher Full Text | Free Full Text

16. Figueiredo LT: The use of Aedes albopictus C6/36 cells in the propagation and ivermectin as antivirals against chikungunya and other alphaviruses. Antiviral Res. 2013; 99: e11479. PubMed Abstract | Publisher Full Text | Free Full Text

17. Zhang JH, Chung TD, Oldenberg KR: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen. 1999; 4(3): 67–72. PubMed Abstract | Publisher Full Text | Free Full Text

18. Roberts GC, Zothner C, Remenyi R, et al: Evaluation of the central role of mammalian and mosquito cells line for use in Chikungunya virus research. Sci Rep. 2017; 7(1): 14641. PubMed Abstract | Publisher Full Text | Free Full Text

19. Wikan N, Sakornwatanyo P, Ulbo S, et al: Chikungunya virus infection of cell lines: analysis of the East, Central and South African lineage. PLoS One. 2012; 7(1): e31178. PubMed Abstract | Publisher Full Text | Free Full Text

20. Briolat S, Garin D, Scaramozzino N, et al: Discovery of ledipasvir (GS-5885): a potent, once-daily oral NS5A inhibitor for the treatment of hepatitis C virus infection. Daclatasvir based high throughput screening assay to identify novel hepatitis C virus antivirals. Antiviral Res. 2013; 97(7): 611-17. PubMed Abstract | Publisher Full Text | Free Full Text

21. Karlas A, Berre S, Couderc T, et al: Development of a robust cytopathic screen for Chikungunya virus replication in Vero cells. PLoS ONE. 2011; 6(12): e28923. PubMed Abstract | Publisher Full Text | Free Full Text

22. Ashworth AW, Lentscher AJ, Zamora PF, et al: Antagonism of the Sodium-Potassium ATPase Impairs Chikungunya Virus Infection. mBio. 2016; 7(3): pii: e00693-16. PubMed Abstract | Publisher Full Text | Free Full Text

23. Kim HY, Li X, Jones CT, et al: Development of a multiplex phenotypic cell-based high throughput screening assay to identify novel hepatitis C virus antivirals. Antiviral Res. 2013; 97(7): 59-61. PubMed Abstract | Publisher Full Text | Free Full Text

24. Barrows NJ, Campos RK, Powell ST, et al: A Screen of FDA-Approved Drugs for Inhibitors of Zika Virus Infection. Cell Host Microbe. 2016; 20(2): 259-270. PubMed Abstract | Publisher Full Text | Free Full Text

25. Sousse M, Schilte C, Casaretto N, et al: Characterization of reemerging chikungunya virus. PLoS Pathog. 2007; 3(6): e89. PubMed Abstract | Publisher Full Text | Free Full Text

26. Khan M, Dhawanri R, Patro J, et al: Cellular IMPDH enzyme activity is a potential target for the inhibition of Chikungunya virus replication and virus induced apoptosis in cultured mammalian cells. Avantir. Res. 2011; 8(1): 1–8. PubMed Abstract | Publisher Full Text | Free Full Text

27. Brackman RM, Vanci C, et al: Role of the vacular ATPase in the Alphavirus replication cycle. PLoS ONE. 2010; 5(7): e11479. PubMed Abstract | Publisher Full Text | Free Full Text

28. Schuchman RM, Vanci R, Paper PA, et al: Characterization of reemerging chikungunya virus into mammalian cells: role of clathrin and early endosomal compartments. PLoS ONE. 2010; 5(7): e11479. PubMed Abstract | Publisher Full Text | Free Full Text

29. Maheshwark R, Srikantam V, Bhartiy D: Chloroquine enhances replication of Semliki Forest virus and encephalomyocarditis virus in mice. J Virol. 1991; 65(2): 992-995. PubMed Abstract | Publisher Full Text | Free Full Text

30. De Lamballerie X, Boisson V, Reymer JC, et al: On chikungunya acute infection and chloroquine treatment. Vector Borne Zoonotic Dis. 2008; 8(6): 837-839. PubMed Abstract | Publisher Full Text | Free Full Text

31. Te KK, Mao YK, Chan AS, et al: Mycophenolic acid, an immunomodulator, has potent and broad-spectrum in vitro antiviral activity against pandemic, seasonal and avian influenza viruses affecting humans. J Gen Virol. 2016; 97(8): 1807–17. PubMed Abstract | Publisher Full Text | Free Full Text

32. Lundberg L, Brahms A, Hooper I, et al: Characterization of reemerging chikungunya virus. PLoS Pathog. 2007; 3(6): e89. PubMed Abstract | Publisher Full Text | Free Full Text

33. Takhampunya R, Ubol S, Houng HS, et al: Discovery of berberine, abamectin and chloroquine as antivirals. Antiviral Res. 2013; 97(7): 59-61. PubMed Abstract | Publisher Full Text | Free Full Text

34. Vicenti I, Boccuto A, Giannini A, et al: Comparative analysis of different cell systems for Zika virus (ZIKV) propagation and evaluation of anti-ZIKV compounds in vitro. Virus Res. 2016; 244: 64–70. PubMed Abstract | Publisher Full Text | Free Full Text

35. Gaijar KK, Vora HH, Kobawala TP, et al: Deciphering the potential value of 5-fluorouracil metabolic enzymes in predicting prognosis and treatment response of colorectal cancer patients. In J Biol Markers. 2018; 32(2): 180–188. PubMed Abstract | Publisher Full Text | Free Full Text

36. Görtt M, Feld JD: Direct-acting antiviral agents for hepatitis C: structural and mechanistic insights. Nat Rev Drug Discov. 2016; 15(6): 308–318. PubMed Abstract | Publisher Full Text | Free Full Text

37. Hughes M, Griffin S, Harris M: Domain III of NS5A contributes to both RNA replication and assembly of hepatitis C virus particles. J Gen Virol. 2009; 90(Pt 12): 2923-1538. PubMed Abstract | Publisher Full Text | Free Full Text

38. Keating GM, Vaidya A: Sofosbuvir: first global approval. Drugs. 2014; 74(2): 213–22. PubMed Abstract | Publisher Full Text | Free Full Text

39. Penin F, Dubousson J, Rey FA, et al: Structural biology of hepatitis C virus. Hepatology. 2004; 39(1): 5–19. PubMed Abstract | Publisher Full Text | Free Full Text

40. Rupp JC, Sokoloski J, Gebhart NN, et al: Alphavirus RNA synthesis and non-structural protein functions. J Gen Virol. 2015; 96(9): 2483-500. PubMed Abstract | Publisher Full Text | Free Full Text

41. Saha A, Acharya BN, Priya R, et al: Development of an nsP2 protease based cell
free high throughput screening assay for evaluation of inhibitors against emerging Chikungunya virus. Sci Rep. 2018; 8(1): 10831. PubMed Abstract | Publisher Full Text | Free Full Text

54. Delang L, Segura Guerrero N, Tas A, et al.: Mutations in the chikungunya virus non-structural proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. J Antimicrob Chemother. 2014; 69(10): 2770–84. PubMed Abstract | Publisher Full Text

55. Wada Y, Orba Y, Sasaki M, et al.: Discovery of a novel antiviral agent targeting the nonstructural protein 4 (nsP4) of chikungunya virus. Virology. 2017; 506: 102–12. PubMed Abstract | Publisher Full Text

56. Bonotto RM, Souza-Almeida G, Badra SJ, et al.: Dataset 1 in: Evaluation of broad-spectrum antiviral compounds against chikungunya infection using a phenotypic screening strategy. F1000Research. 2018. http://www.doi.org/10.5256/f1000research.16498.d221905
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Paban Kumar Dash
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Amrita Saha
Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, MA, USA

The authors have developed high content image-based screening and tested a commercial library of 1,280 compounds, including FDA-approved drugs for antiviral activity against Chikungunya virus. The authors found the drug sofosbuvir to be effective against chikungunya virus and proposed it to be a promising candidate for repurposing.

- High content screening (HCS) assay developed seems to be of medium throughput due to the cumbersome steps involved. Since this homogeneous cell-based assay involves multiple step additions, therefore number of steps in assay could be minimized either by using reporter virus, labelled virus or fluorophore conjugated antibodies. The assay robustness should be further validated by calculating signal-to-noise ratio, CV, inter-plate, intra-plate and day to day variability. The assay performance should be consistent from day to day; ideally the assay signal should not vary with passage and cell density.

- The authors should attempt to further validate the activity of Sofosbuvir, using different assays. Further, in this study, the experiments are performed with a low MOI of 0.05, would Sofosbuvir be effective when cells are infected with higher MOI of virus? MOI of 1 and 10 should be evaluated too, though the authors pointed out about highly cytotopathic nature of the virus. As such, the CHIV titre is very high in human patients.

- Furthermore, from the figure 3 data (sofosburvir), it might be possible the drug has had a slight proliferative effect on the Huh-7 cells and may have slightly increased the SI (cell viability curve, proliferation may affect infection rates). Please provide evidence indicating that proliferation has not occurred.

- The authors should provide information about the remaining compounds that didn't work against Chikungunya virus in their assay. At least tabulate the name of compounds; reporting of which will be extremely useful in dissemination of important information among researchers.

Is the rationale for developing the new method (or application) clearly explained?
Yes

**Is the description of the method technically sound?**
Partly

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Virology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

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Mukesh Kumar
Georgia State University, Atlanta, GA, USA

The manuscript entitled “Evaluation of broad-spectrum antiviral compounds against chikungunya infection using a phenotypic screening strategy” by Bonotto and colleagues evaluated an image-based phenotypic assay for high-throughput screening of anti-CHIKV compounds. The screening assay was validated by testing a commercial library of 1,280 compounds, including FDA-approved drugs. The research topic is interesting and has potential significance. However, there are major gaps in the depth of the information reported in this manuscript that make publication of the findings in its current form problematic. Moreover, results in this manuscript are poorly presented and inadequate information is provided.

In this manuscript, the screening assay was validated by testing a commercial library of 1,280
compounds. Where is the data on these compounds? Any anti-CHIKV activity by new compound? Not sure why these compounds were screened.

It is not clear what are the advantages of using this image-based assay over other established assays for drug screening. IFA is expensive and time-consuming. Discussion is more about efficacy of anti-viral compounds tested rather than use of IFA-based screening strategy.

The anti-CHIKV activity of Sofosbuvir must be validated by using another gold-standard assay such as plaque-assay.

Is the rationale for developing the new method (or application) clearly explained?
Partly

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 November 2018

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Rana Abdelnabi
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The article describe the optimization of a high throughput screening assay for CHIKV compounds
and its validation using a commercial library of 12800 compounds. The assay was developed in Huh7 cells and the endpoint was detected using indirect immunofluorescence staining with CHIKV specific antibodies. The author showed that the assay is working and they confirmed that using a couple of known CHIKV inhibitors. However, there are some concerns regarding the used assay:

1. The readout of this assay depends on IFAs which: i) require several steps of washing, fixation and staining and ii) are expensive compared to the ordinary colorimetric methods (e.g. MTS/PMS) because of the use of AlexaFlour antibody. Therefore, the use of this assay for high throughput screening would be time and money consuming. It will be more beneficial and technically sound to optimize high throughput assays using reporter CHIKV. This will eliminate the need for IFAs, which is the main drawback of this assay.
2. It is not addressed clearly in the manuscript what is the added value of the developed assay over the already established CHIKV assays.
3. Furthermore, the authors did not mention whether they found any new hits in the screened library (12800 compounds) using their developed assay. Were all the tested compounds inactive or there are some new hits? This part needs clarification.

Is the rationale for developing the new method (or application) clearly explained?
Partly

Is the description of the method technically sound?
No

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
No

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.
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