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**Title: CHIKV strains Brazil (wt) and Ross (lab-adapted) differ with regard to cell host range and antiviral sensitivity and show CPE in human glioblastoma cell lines U138 and U251**

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**Abstract**

Chikungunya virus (CHIKV), a (re)emerging arbovirus, is the causative agent of chikungunya fever (CHIKF). To date, no approved vaccine or specific antiviral therapy are available. CHIKV has repeatedly been responsible for serious economic and public health impacts in countries where CHIKV epidemics occurred. Antiviral tests *in vitro* are generally performed in Vero-B4 cells, a well characterised cell line derived from the kidney of an African green monkey. In this work we characterised a CHIKV patient isolate from Brazil (CHIKV<sup>Brazil</sup>) with regard to cell affinity, infectivity, propagation and cell damage and compared it with a high-passage lab strain (CHIKV<sup>Ross</sup>). Infecting various cell lines (Vero-B4, A549, Huh-7, DBTRG, U251 and U138) with both virus strains, we found distinct differences between the two viruses. CHIKV<sup>Brazil</sup> does not cause cytopathic effects (CPE) in the human hepatocarcinoma cell line Huh-7. Neither CHIKV<sup>Brazil</sup> nor CHIKV<sup>Ross</sup> caused CPE on A549 human lung epithelial cells. The human astrocyte derived glioblastoma cell lines U138 and U251 were found to be effective models for lytic infection with both virus strains and we discuss their predictive potential for neurogenic CHIKV disease. We also detected significant differences in antiviral efficacies regarding the two CHIKV strains. Generally, the antivirals ribavirin (RBV), hydroxychloroquine (HCQ) and T-1105 seem to work better against CHIKV<sup>Brazil</sup> in glioblastoma cells than in Vero-B4.

Finally, full genome analyses of the CHIKV isolates were done in order to determine their lineage and possibly explain differences in tissue range and antiviral compound efficacies.

Keywords: antivirals in vitro, CHIKV cell model, human cell line for CHIKV, U138, glioblastoma cell line, antivirals, efficacies, CHIKV strain comparison...

## 1 Introduction

### Taxonomy, Structure, Genome organisation, ecology and epidemiology

Chikungunya virus (CHIKV) is an arthropod borne (arbo-) virus of the *alphavirus* genus. Belonging to the “Old World” viruses, CHIKV is categorised as an arthritogenic alphavirus due to the primary site of disease manifestation, the joints [1].

To date three CHIKV phylogroups and one distinct sublineage are known. The phylogroups consist of the West African (WA), East-Central-South African (ECSA) and Asian genotype [2]. The Indian Ocean Outbreak, which started in Kenya in 2004, was caused by a mutated sublineage that is referred to as the Indian Ocean Lineage (IOL) and originated from of the ECSA isolates [3].

CHIKV is an enveloped virus and the virion contains single-standed, positive-sense RNA of about 11,800 nucleotides [4]. The virus has the general structure of all alphaviruses (for details on structure, epidemiology and pathogenesis see Hucke, Bestehorn-Willmann [5] and Hucke and Bugert [6]).

CHIKV is generally transmitted to humans by the bite of an infected mosquito from the *Aedes* family, mainly *Aedes aegypti* and *Aedes albopictus* [7]. After entering the skin, viral replication and amplification seem to occur mainly in dermal fibroblasts [8]. Dendritic cells capture virus particles, transport them to the nearest lymph nodes where blood monocytes and macrophages are infected. At this point viremia sets in [9]. Via blood stream CHIKV then reaches the muscles and joints. Infection of these sites causes the main symptoms of CHIKF – myalgia and arthralgia. Infection of the joints often results in cartilage degradation and bone loss [10], which explains the severe and debilitating arthralgia that are the hallmark of the disease and gave the virus its name. After the acute phase of the illness has passed, myalgia and arthralgia can go into a chronic state and last for months or even years, leaving the patient with a severely deteriorated quality of life.

Apart from these well-known sites of infection, CHIKV has been known to infect a wide range of secondary organs which may cause severe complications in patients [7]. Although CHIKV has originally not been classified as a neurotropic virus, the La Reunion outbreak recorded an increased number of neurological complications (*e.g.* meningitis, encephalitis, febrile seizures, Guillain Barré syndrome, neuro-ocular diseases), especially in the elderly and the very young [11, 12]. It was demonstrated that CHIKV is able to replicate in neurons, astrocytes, oligodendrocytes and microglia cells [13].

To date, no approved vaccine or specific antiviral therapies are available. Considering the time it takes to fully recover from CHIKV disease, an effective antiviral is of utmost importance. A variety of antivirals curb CHIKV infection *in vitro* but lack efficacy *in vivo* [6]. Well established antivirals for *in vitro* assays are chloroquine/hydroxychloroquine, ribavirin, and favipiravir, although they show significant differences in their efficacy depending on the virus strain and cell line [6].

So far, little focus has been given on which human cell lines are suitable for *in vitro* studies with CHIKV. Also the question on whether different virus strains show different cell affinities in relevant human cell lines has not been addressed properly. Furthermore, antivirals might have different efficacies depending on the cell line and the virus strain. There is the possibility that high-passage, laboratory adapted strains (such as CHIKV<sup>Ross</sup>) are able to replicate in cell lines which are not affected by wt CHIKV infection. This raises the question to which extent such high-passage reference strains are still comparable to field strains in regard of antiviral efficacies. To shed light on these questions, two different CHIKV strains, the high-passage Ross strain, isolated in 1953 (CHIKV<sup>Ross</sup>), and a field isolate from Brazil, isolated in 2015 (CHIKV<sup>Brazil</sup>) were compared with regard to cell affinity and drug sensitivity towards well established antiviral substances. Finally, a whole genome sequence comparison of both strains was performed to try to explain differences in cell affinity or drug sensitivities on a genomic level.

## 2 Materials and Methods

### 2.1 Cells and cell culture

Vero-B4 cells (ATCC® CCL-81™) [14], A549 cells (ATCC® CCL-185™) [15], Huh-7 cells (JCRB0403) [16], the glioblastoma cell line DBTRG-05MG (ATCC® CRL-2020™) [17], were obtained from ATCC while the human glioblastoma cell lines U138 (aka U-138 MG, ATCC® HTB-16™) and U251 (aka U-251 MG, ATCC® HTB-17™; formerly known as U-373 MG) were a gift of R. Brack-Werner, Institute of Virology, German Research Center for Environmental Health (GmbH).

Dulbecco's Modified Eagle Medium (DMEM(1X) + GlutaMAX™-I medium, Thermo Fisher Scientific Ltd, UK), with either 1 g/L of D-glucose (in the following referred to as "Low Glucose" (LG)) or with 4.5 g/L of D-glucose ("High Glucose" (HG)) were used. 5% heat inactivated foetal bovine serum (FBS; Sigma-Aldrich, Hilden, Germany) was added. U138 and U251 cells were kept on DMEM HG medium while Vero-B4, A549, DBTRG and Huh-7 were kept on DMEM LG.

### 2.2 Antiviral substances

The antiviral compound T-1105 was provided by the School of Pharmacy and Pharmaceutical Sciences of the Cardiff University, UK. T-1105 is a direct nucleoside (purine) analogue and the defluorinated analogue of favipiravir (T-705).

The compound was provided as a solid powder and was dissolved in DMSO to create a 10 mM solution.

Other antiviral substances used as controls were ribavirin (RBV), and hydroxychloroquine (HCQ) (both from Sigma-Aldrich). RBV and HCQ were dissolved in purified water to create stock solutions of 100 mM and 10 mM respectively. For further dilutions DMEM LG was used.

### **2.3 Virus**

Viruses used in this study are part of the BSL3 reference collection of the Bundeswehr Institute of Microbiology (IMB), Munich. The wildtype CHIKV strain L3-4497 originates from a patient isolate from Brazil (CHIKV<sup>Brazil</sup>; 2015). Subpassaged samples of the initial cultivation (Vero-B4) were used to establish a working stock of CHIKV (also grown on Vero-B4). In this study the wildtype CHIKV strain used had previously been passaged twice on Vero-B4 cells after its isolation. GenBank accession number: Banklt2561907 Chikungunya\_Brazil\_4497 ON009842.

The lab attenuated CHIKV Ross strain L3-3950 (CHIKV<sup>Ross</sup>; NH177) has been isolated from an outbreak in Tanzania in 1953 [18-20]. GenBank accession number: Banklt2561907 Chikungunya\_Ross\_NH177 ON009843.

Both virus strains belong to the ESCA genotype.

#### **2.3.1 Virus stock production**

Vero-B4 cells were cultivated in a T75 flask in DMEM LG with 5% FBS until they reached 80% confluence. After removal of supernatant and a one-time washing with DMEM LG, 500 µL of the original virus stock suspension from the L3 reference stocks were added to the T75 cell culture flask and canted gently to ensure the virus reached the entire cell layer. After one minute, 20 mL of DMEM LG with 5% FBS were added to the bottle and subsequently flasks were incubated at 37°C and 5% CO<sub>2</sub> until maximal cytopathic effect (CPE) was observed via microscope (Zeiss Axiovert25, Germany).

Two to three days post infection the supernatant of the bottle was collected, FBS was added to a final concentration of 20%, and the virus solution was aliquoted into 1 mL cryotubes with 500 µL of CHIKV suspension each and stored at -70°C. Virus stock titres were evaluated via plaque assay.

#### **2.3.2 Virus titting via plaque assay**

One mL of Vero-B4 and U138 cells ( $1.2 \times 10^5$  cells/mL) were seeded into a 24-well plate and allowed to settle overnight. The next day, the supernatant of the cells was removed and cells were infected with 200 µL of a 10-fold serial dilution (DMEM LG) of CHIKV<sup>Brazil</sup> or CHIKV<sup>Ross</sup> (Vero-B4 cells only).

The plate was gently swayed and incubated for 30 min at 37°C and 5 % CO<sub>2</sub>.

Then, 800  $\mu\text{L}$  of 0,8 - 1 % carboxymethylcellulose (CMC) (Sigma-Aldrich, Schnellendorf, Germany) dissolved in MilliQ water,sterilized by autoclaving, mixed with DMEM and 2.5% FBS, was carefully added to each well using a multipette (Eppendorf, Germany). The plate was then incubated at 37°C with 5%  $\text{CO}_2$  and observed daily for CPE with a microscope. Three to four days p.i. the cells were fixed and dyed by adding 1 mL of crystal violet (aqueous solution with 0.2% certified crystal violet and 20% formaldehyde (both from Merck, Darmstadt, Germany)) directly to each well. The plate was then incubated in the fridge at 4 °C overnight. Plates were then gently washed with distilled water until all the CMC and superfluous dye had been removed. Plaque assays with Vero-B4 cells were repeated at least 3 times independently. Assays with U138 cells were repeated twice.

## **2.4 Cell viability assay with MTS and data evaluation**

Unless stated otherwise, cells were seeded at a density of  $1 \times 10^4$  cells/100  $\mu\text{L}$ /well in DMEM with 5% FBS in clear 96-well plates and allowed to settle overnight. The plates were incubated at 37°C with 5%  $\text{CO}_2$  and 95-99% relative humidity. For treatment 50  $\mu\text{L}$  of compound dilution were added to the corresponding wells. Virus infection was done with 50  $\mu\text{L}$  of CHIKV dilution one hour after treatment. Toxicity assays and untreated non-infected (Mock) control were done adding 50  $\mu\text{L}$  of medium instead of virus dilution. Final FBS concentration in the treated/infected wells was 2.5%. The the plates were then incubated for four days.

All cell viability assays were done using the CellTiter 96®Aqueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, USA) according to the manufacturer's protocol with the difference that 20  $\mu\text{L}$  MTS solution were used per 200  $\mu\text{L}$  of experimental volume. Absorbance was measured at 490 nm with a reference wavelength of 620 nm using an ELISA plate reader (iMark™ Mikroplate Reader). Apart from  $\text{IC}_{50}/\text{CC}_{50}$  evaluation, the Optical Density (OD) values obtained were put into relation to Mock control with Microsoft Excel. Mock thus represents 100% viable cells in the column graphs. All graphs were prepared using GraphPad Prism6 Software.

For comparisons of the different virus strains ordinary one-way ANOVA tests were done (GraphPad). Probabilities of the test results are given with p-values.

Raw data values were put into relation with Mock control (Mock = 100%) and the positive control (untreated infected cells = 0%) in Excel. For calculation of  $\text{IC}_{50}$  and  $\text{CC}_{50}$  values a dose-response curves equation (using raw data) of GraphPad Prism6 was applied. The programm then calculated the relative  $\text{IC}_{50}$  value in relation to the raw data values of the most efficient compound concentration. Goodness of fit and plausible range are given by  $R^2$  and 95% Confidence Interval (95% CI). If a raw data value deviated more than 20% from the mean of the repeats, this particular value was omitted.

### **2.4.1 Kill curves**

Apart from Huh-7 cells, all cells were seeded at a density of  $1 \times 10^4$  cells/100  $\mu$ l/well in DMEM with 5% FBS in 96-well plates. Huh-7 cells were seeded with only  $5 \times 10^3$  cells/100  $\mu$ l/well, due non-linear readout with CellTiter 96®AQ<sub>ueous</sub> Non-Radioactive Cell Proliferation Assay at higher concentration. After settling overnight, the cells were infected with 50  $\mu$ L of virus dilutions ranging from 0 to  $10^{-5}$  and incubated for 30 minutes. Then 50  $\mu$ L of DMEM were added. Kill curve infection experiments were repeated at least thrice independently, with three technical replicates. Cell viability was evaluated using MTS.

#### **2.4.2 Comparison of compound efficacy**

RBV, HCQ, and T-1105 were used in concentrations previously published to inhibit wt CHIKV in Vero cells [21], [22]. The concentration used in our experiments were thus: RBV at 410  $\mu$ M, HCQ at 10  $\mu$ M, T-1105 at 10  $\mu$ M and 50  $\mu$ M.

As T-1105 was dissolved in DMSO, final DMSO concentration in all wells of the assay was uniformly 0.1% (Mock and positive control as well) to make sure the controls were unbiased by the solvent.

Treatment and infection of the cells were done as described in 2.4.3 with the difference that multiplicity of infection (MOI) was 0.64. Each compound concentration had three or six technical replicates and the experiments were repeated at least thrice independently.

#### **2.4.3 IC<sub>50</sub>/CC<sub>50</sub> evaluation of RDV, HCQ and T-1105 in Vero-B4 and U138 cells**

For IC<sub>50</sub>/CC<sub>50</sub> evaluation Vero-B4 and U138 cells were used. Serial dilutions of the compounds (RBV, HCQ, and T-1105) were prepared in assay medium (DMEM LG). To avoid precipitation of T-1105, a final concentration of 0.3% DMSO was kept in all wells containing this compound (and in the corresponding control wells). Serial dilutions of RBV ranged from 10  $\mu$ M to 500  $\mu$ M in U138 cells and 200  $\mu$ M to 1000  $\mu$ M in Vero-B4 and the toxicity assays. Serial dilutions of HCQ and T-1105 ranged from 1  $\mu$ M to 100  $\mu$ M. 50  $\mu$ L of the compound dilution was added to the cells. Infection was done at a MOI of 0.355 with the CHIKV strain Brazil. As T-1105 had DMSO as a supplement to ensure solubility, two different kind of Mock and positive control (untreated infected cells) were run along, one with 0.3% of DMSO and the other without. Each compound was repeated at least thrice independently with three technical replicates.

### **2.5 Whole Genome Sequencing of Chikungunya Virus L3-4497 strain Brazil and Ross L3-3950 from InstMikroBio BW**

For sequencing one vial of the respective stock solutions of CHIKV<sup>Brazil/Ross</sup> was used and the total RNA was purified using the Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manual. For Library preparation the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® was used according to the manufacturer's

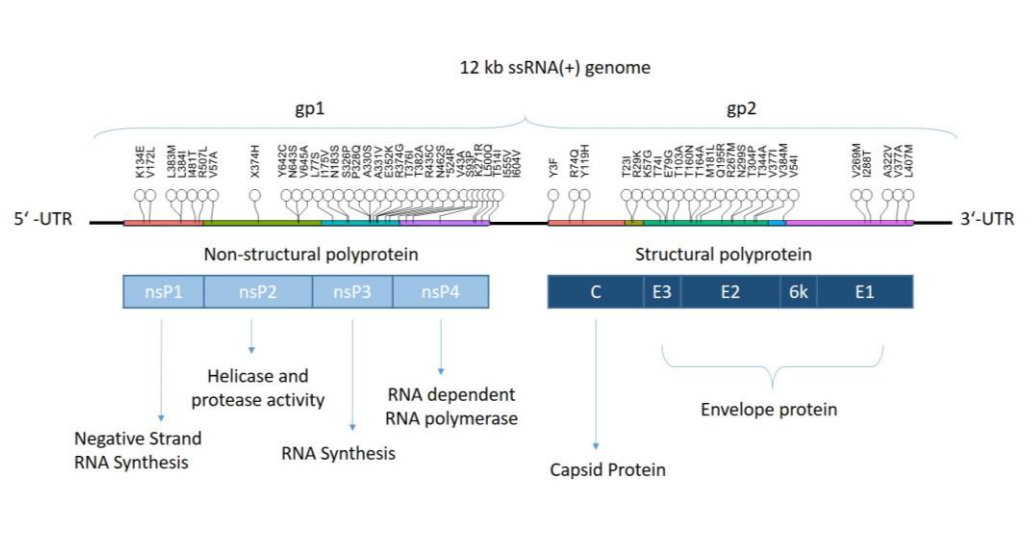
protocol. Paired-end sSequencing of the generated libraries was performed on a Illumina MiSeq platform using a Miseq Reagent Kit V2 500 cycles chemistry. De novo assemblies were generated for the two samples using the tool SPAdes version: 3.14.1. Pairwise alignments of the two generated whole genomes were generated using the ClustalW algorithm.



### 3 Results

#### 3.1 Genome differences between the two virus strains

The CHIKV virus strains CHIKV<sup>Ross</sup> and CHIKV<sup>Brazil</sup> belong to the ECSA genotype. Genome analysis revealed 57 amino acid differences in the structural and non-structural polyproteins between our Brazilian field isolate and the Ross strain as shown in Figure 1. For complete genome sequences of both virus strains see GenBank accession numbers Banklt2561907 Chikungunya\_Ross\_NH177 ON009843 and Banklt2561907 Chikungunya\_Brazil\_4497 ON009842.



**Figure 1. Genome structure and amino acid differences between CHIKV<sup>Brazil</sup> and CHIKV<sup>Ross</sup>**

Differences in amino acids (single letter code) as Brazil-position-Ross.

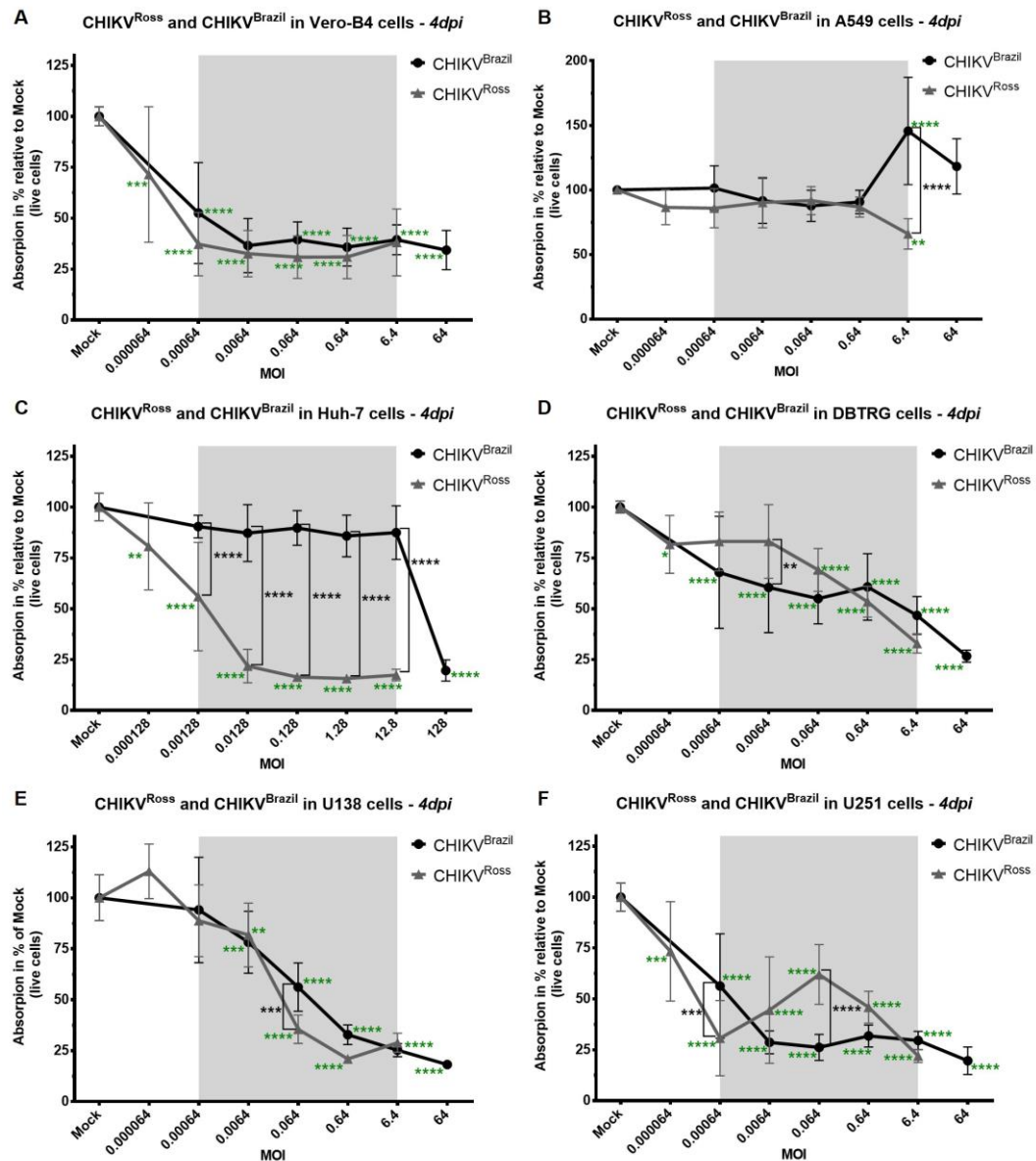
#### 3.2 Kill curve experiments

Vero-B4 cells are very sensitive to CHIKV infection. Even at an MOI of 0.000645 CHIKV Ross still killed more than 60% of Vero-B4 four days post infection (4dpi) in the MTS cell viability test. In a one-way ANOVA comparison of both CHIKV strains, no statistically significant difference could be detected with regard to cell infectivity and cell death between CHIKV<sup>Brazil</sup> and CHIKV<sup>Ross</sup> in Vero-B4 cells (Figure 2A).

A549 did not show any cytopathogenic effects (CPE) when infected with either CHIKV strain (Figure 2B). Only at the highest MOI (6.45) with CHIKV<sup>Ross</sup>, limited cell death could be observed (65.96% ±11.74% viable cells). Infection with a MOI of 63.5 and 6.35 of wt CHIKV even indicated proliferating cells (>100% viable cells).

The human hepatoma cell line Huh-7 only showed cell death when infected with wt CHIKV<sup>Brazil</sup> at a very high MOI of 127 (Figure 2C). Infection with MOI of 12.7 and lower did not result in a statistically different cell viability than non-infected Huh-7 cells. Infection with CHIKV<sup>Ross</sup> resulted in extensive cell death 4dpi when a MOI was between 0.0129 and 12.9 (80% dead Huh-7 cells). CHIKV<sup>Ross</sup> infection at a

MOI 0.00129 still killed 45% of Huh-7 cells 4dpi. The comparison of the two CHIKV strains at corresponding MOI displayed a highly significant difference with  $p < 0.0001$  between 0.0129 and 12.9 (Figure 2C).



**Figure 2. Effect of CHIKV<sup>Ross</sup> and CHIKV<sup>Brazil</sup> on different cell lines**

Comparison of the infectivity/cell damage caused by two CHIKV strains CHIKV<sup>Brazil/Ross</sup> at increasing MOI. Cell viability was measured in a colorimetric assay (MTS cell viability test) 4dpi. Data are means  $\pm$ SD of at least three independent experiments with three technical replicates, with 100% corresponding to non-infected cells (Mock). Asterisks indicating the p-values generated in a one-way ANOVA test comparison of non-infected cells with infected cells (green asterisks), and of the different virus strains at the same MOI (grey area and black asterisks). p-values are indicated as follows: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

(A) Vero-B4 cells ( $1 \times 10^4$  cells/well); (B) A549 cells ( $1 \times 10^4$  cells/well); (C) Huh-7 cells ( $5 \times 10^3$  cells/well); (D) DBTRG cells ( $1 \times 10^4$  cells/well); (E) U138 cells ( $1 \times 10^4$  cells/well); (F) U251 cells ( $1 \times 10^4$  cells/well).

The brain derived cell line DBTRG was susceptible to CHIKV infection in a dose dependent manner. At  $MOI \geq 0.064$ , both virus strains showed diminished cell viability that was statistically significant ( $p < 0.0001$ ) from non-infected cells

(Figure 2D). The wt CHIKV<sup>Brazil</sup> had similar significance at MOI 0.00064. High MOI ( $\geq 6.4$ ) of both virus strains were needed to achieve extensive cell death  $> 50\%$ . The U138 cell line was susceptible to CHIKV infection and the cells showed extensive CPE 4dpi with either CHIKV strain. CHIKV<sup>Ross</sup> showed significantly more dead cells at a MOI 0.064 than CHIKV<sup>Brazil</sup> (35.5% vs. 56.2% surviving cells;  $p < 0.001$ ). Likewise at a MOI of 0.64, 32% of the U138 cells survived CHIKV<sup>Brazil</sup> while 21% survived CHIKV<sup>Ross</sup> (Figure 2E).

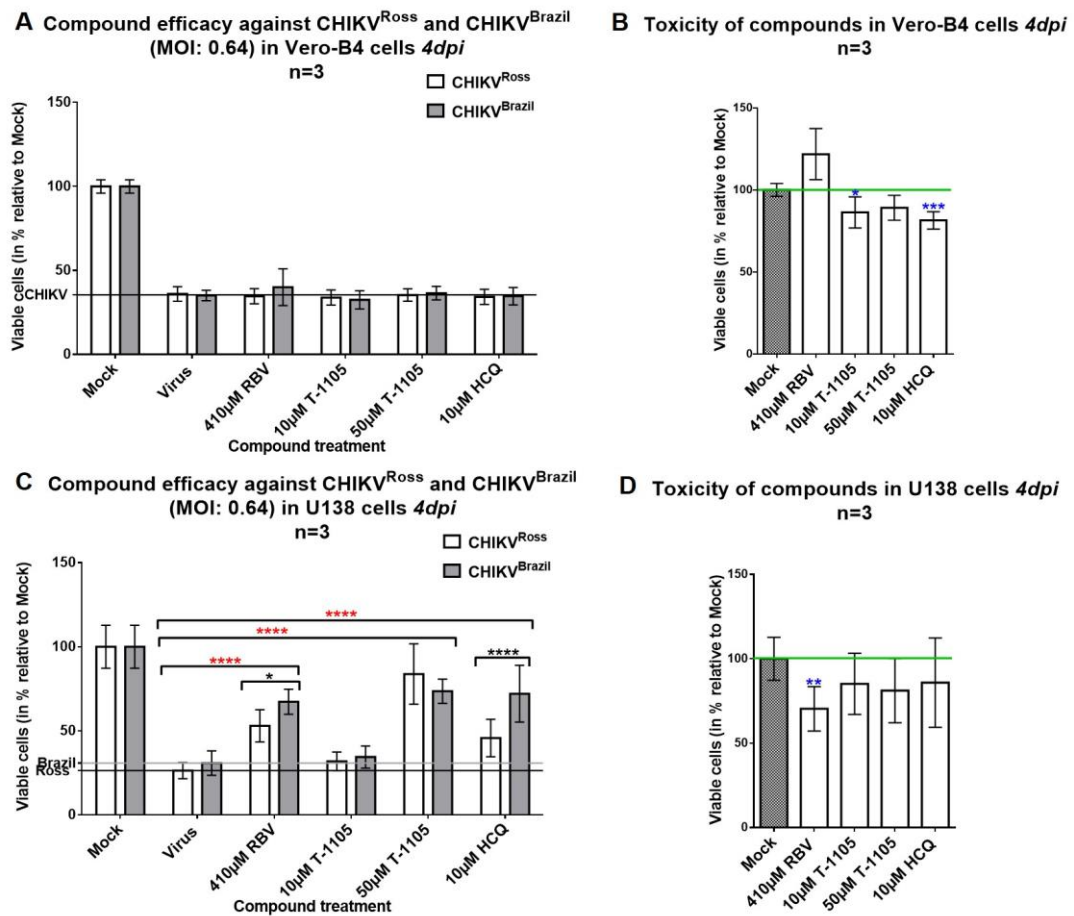
U138 did not show plaques when infected with CHIKV<sup>Brazil</sup>, although the plaque assays with U138 were conducted the same way as with Vero-B4.

U251 cells were more sensitive to CHIKV infection than U138 cells. At a MOI of 0.00064 of CHIKV Ross only  $30.69 \pm 18.46\%$  of U251 cells survived after 4 days. There is however, no MOI dependent linear progression of the curve but rather an undulated one as far as CHIKV<sup>Ross</sup> on U251 is concerned (Figure 2F). Four days after infection of U251 cells with CHIKV<sup>Brazil</sup> at MOI 0.00064,  $56.32 \pm 25.64\%$  of the cells had survived. CHIKV<sup>Brazil</sup> at MOI  $\geq 0.0064$  kills  $> 65\text{--}70\%$  of the U251 cells.

### 3.3 Comparison Antiviral Compounds vs Virus/Cell line

In Vero-B4 cells none of the administered compounds displayed any efficacy against either CHIKV strain (MOI: 0.64) at the administered concentrations (Figure 3A). In U138 cells, RBV (410  $\mu\text{M}$ ), T-1105 (50  $\mu\text{M}$ ), and HCQ (10  $\mu\text{M}$ ) showed statistically significant efficacy against both CHIKV strains ( $p < 0.0001$ ) (Figure 3C). RBV and HCQ protected U138 cells significantly better from CPE caused by wt CHIKV<sup>Brazil</sup> than from the lab strain CHIKV<sup>Ross</sup> ( $p < 0.001$  and  $< 0.0001$  respectively).

In the toxicity testing Vero-B4 cells treated with T-1105, and HCQ showed a low toxic effect of the compound with 80 – 90% ( $\pm 5.31$  – 9.45%) of the cells surviving (Figure 3C). HCQ showed a highly significant difference to untreated cells with a cell survival of  $81.44 \pm 5.31\%$  and  $p < 0.0001$ . RBV treatment resulted in more viable Vero-B4 cells than the untreated control ( $121.82 \pm 15.57\%$  viable cells), while in U138, RBV lead to statistically significant toxicity ( $70.43 \pm 13.14\%$  viable cells) (Figure 3B and D). The difference in RBV toxicity between the two cell lines was statistically significant with  $p < 0.0001$ . Neither T-1105 nor HCQ led to significant cell damage in U138 cells (Figure 3D).



**Figure 3. Comparison of compound efficacy and toxicity against CHIKV<sup>Ross</sup> and CHIKV<sup>Brazil</sup> in the cell lines Vero-B4 and U138**

Cells were treated with certain concentrations of HCQ, RBV or T-1105 and were either infected with CHIKV (efficacy test (A) and (C)) or not (toxicity test (B) and (D)). Four days after infection/treatment, cell survival was determined with MTS. Values are given as percentages in relation to Mock control and are means of three independent experiments each with at least three technical replicates. (A) Vero-B4 and (C) U138 cells were infected with CHIKV<sup>Ross</sup> (white columns) or CHIKV<sup>Brazil</sup> (grey columns). Statistically significant differences of the compound efficacies between the different virus strains Ross and wt Brazil in the same cell line were evaluated in a one-way ANOVA test and are indicated by black asterisks. Red asterisks indicate significant (positive) differences between the positive control (black and grey line) and treated, infected cells (same corresponding virus strain and cell line). (B) & (D) Compound toxicity in Vero-B4 (B) and U138 (D) cells. Statistically significant (negative) differences between Mock control (grey bar and green line) and the treated cells (white bars), are indicated by blue asterisks. The number of asterisks indicate p-values as follows: \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.0001.

### 3.4 IC<sub>50</sub>/CC<sub>50</sub> evaluation of RDV, HCQ and T-1105 in Vero-B4 and U138 cells

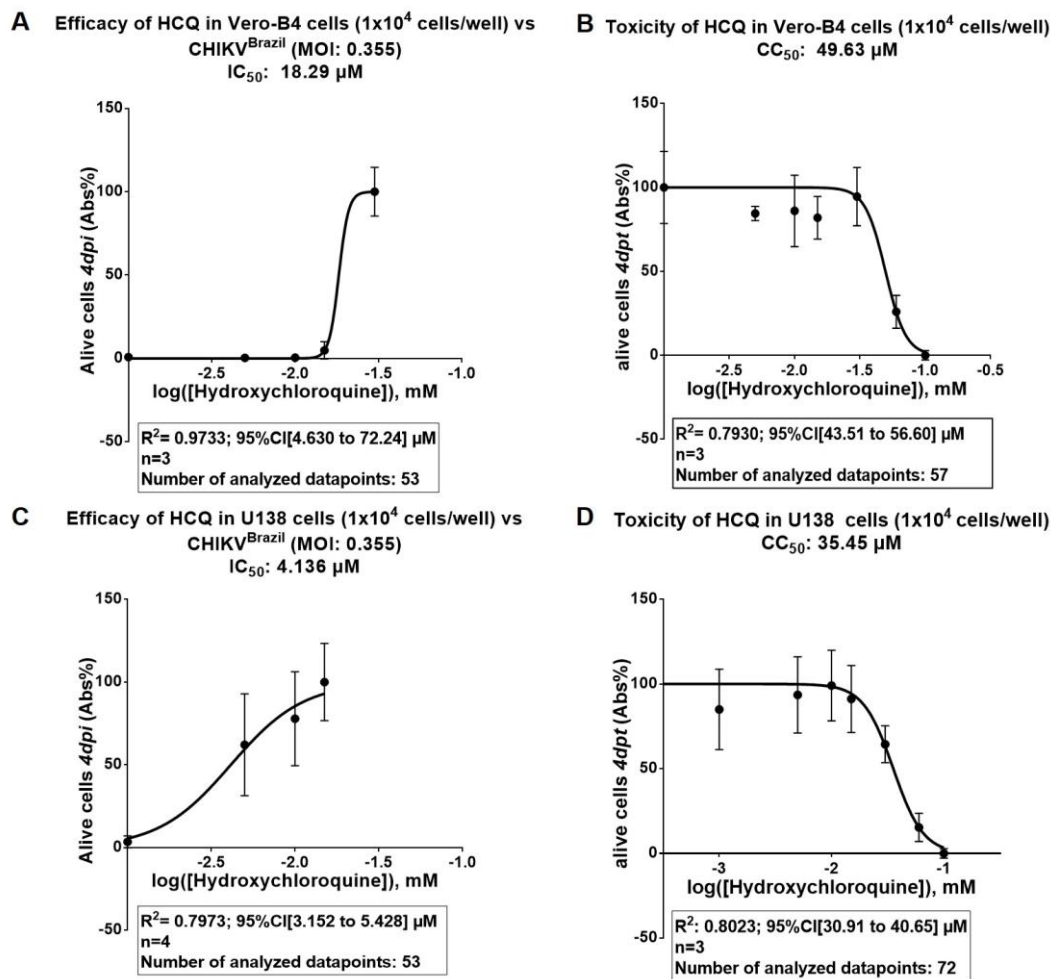
A dose-dependent inhibition of CPE in both cell lines could be observed with HCQ and RBV. However, only in U138 cells was a dose-dependent effect for T-1105 detectable. Efficacies of HCQ and RBV differed considerably in the two cell lines (Table 1).

**Table 1. IC<sub>50</sub> and CC<sub>50</sub> values of different compounds against wt CHIKV<sup>Brazil</sup> (MOI: 0.355) in Vero-B4 and U138 cells**

Compound	IC <sub>50</sub> (μM)		CC <sub>50</sub> (μM)		SI	
	Vero-B4	U138	Vero-B4	U138	Vero-B4	U138
<b>Ribavirin</b>	n.d.	165.8	> 1000	> 500	> 1.5	> 3
<b>Hydroxychloroquine</b>	18.29	4.136	49.63	35.45	2.7	8.57
<b>T-1105</b>	n.d.	34.21	> 100	> 100	n.d.	> 3

Abbreviations: CC<sub>50</sub>, half maximal cytotoxic concentration; IC<sub>50</sub>, half maximal inhibitory concentration; n.d., not determined; SI, selectivity index;

Of the four tested potential CHIKV antiviral substances (RBV, HCQ, and T-1105) only HCQ and RBV showed dose dependent efficacies in Vero-B4 cells. However, even at 1000 μM concentration of RBV, only 37.55 ± 6.15% (at MOI 0.325) surviving cells were detectable and thus no IC<sub>50</sub> value could be generated. (data not shown). Efficacy of HCQ was observable between the concentrations of 1 μM and 30 μM. At concentrations > 30 μM HCQ was considerably toxic. An IC<sub>50</sub> value of 18.29 μM and a CC<sub>50</sub> of 49.63 μM could be generated for HCQ in Vero-B4 cells at MOI 0.355 (Figure 4A and B) leading to an SI of 2.7. For T-1105 no dose dependent efficacy against CHIKV in Vero-B4 could be observed (concentration range: 5-100 μM).



**Figure 4.  $IC_{50}$  and  $CC_{50}$  of HCQ in Vero-B4 and U138 cells**

Hydroxychloroquine inhibits CHIKV<sup>Brazil</sup>-induced cell death in Vero-B4 (A) and U138 (C) cells in a dose-dependent manner. Cells ( $1 \times 10^4$  cells/well) were infected at an MOI of 0.355 and treated with a serial dilution of HCQ. After 4 days, cell death was determined via a colorimetric cell viability assay (MTS). Toxicity assays in Vero-B4 (B) and U138 (D) cells were performed similarly without infection of the cells. The data represent means  $\pm$  SD of raw data from at least 3 independent experiments performed with three technical replicates. Normalized fit of dose-response curves was calculated with GraphPad Prism 6 Software.

All tested compounds showed a dose dependent antiviral effect against wt CHIKV<sup>Brazil</sup> in U138 cells. Efficacy of HCQ in U138 was observed using the compound at concentrations between 1 and 15  $\mu$ M, as concentrations above 15  $\mu$ M were toxic to the cells. An  $IC_{50}$  value of 4.136  $\mu$ M and a  $CC_{50}$  of 35.45  $\mu$ M was observed (Figure 4C and D), leading to an SI of 8.57 for HCQ in U138.

RBV was effective against CHIKV<sup>Brazil</sup> with an  $IC_{50}$  of 165.8  $\mu$ M (See A and B in Supplemental materials). No maximal toxic effect was observable at the highest concentration of 500  $\mu$ M (data not shown). Consequently it was not possible to generate an exact  $CC_{50}$  value. As  $CC_{50}$  is  $> 500$   $\mu$ M, the SI would therefore be  $> 3$ . The compound T-1105 (the defluorinated analog of favipiravir) was effective against wt CHIKV in U138 cells with an  $IC_{50}$  of 34.21  $\mu$ M (see supplemental materials Supp. Fig. 3). At the highest concentration (100  $\mu$ M), no significant CPE was observable. The SI can thus be assumed to be  $> 3$ .

Taken together, these data demonstrate that HCQ, RBV, and T-1105 inhibit CHIKV induced cell death of U138 cells in a dose-dependent manner. With the exception of HCQ the compounds had no significant toxic effect on this particular cell line at the tested concentrations.

## 4 Discussion

### 4.1. Kill curve experiments

CHIKV *in vitro* experiments are usually conducted in Vero cells as they propagate the virus well and show extensive CPE [23]. However, Vero cells originate from the kidney of an African green monkey and do not represent the usual site of infection in humans. As the latest CHIKV outbreaks reported an increase in neurological complications following CHIKV, it was one of our objectives to find a human derived neurological (immortalised) cell line to establish an *in vitro* model for neurogenic CHIKV (and possibly other neurogenetic alphavirus) infection.

There is a report of another glioblastoma cell line (U-87 MG (ATCC HTB-14)) being tested in CHIKV experiments [24, 25]. This study evaluated the glioblastoma cell line (U87-MG) with wt CHIKV isolate (RGCB355/KL08 CHIKV strain) with regard to susceptibility to infection, visible CPE, autophagy, apoptosis and innate immune response. However, there are indications that this cell line is not the original cell line published by Ponten in 1968 [26, 27]. The DNA profile of the U87MG is different from that of the original and thus the origin of this cell line is unknown [25].

For these reasons, we tested different human glioblastoma cell lines (DBTRG, U138 and U251) for the susceptibility of infection with CHIKV and their suitability for cell viability assays with this virus. Furthermore, Huh-7 and A549, for which controversial data with regard to CHIKV infectivity have been published, were evaluated with the same objectives. As these differences might be due to the fact that different CHIKV strains have been used in the aforementioned studies, we compared the lab adapted CHIKV strain Ross and the field isolate from Brazil in *in vitro* cell cultures and by full genome analysis.

In our study, all tested glioblastoma cell lines were susceptible to CHIKV infection. However, extensive CPE with > 50% nonviable cells could only be achieved in DBTRG cells at  $\text{MOI} \geq 6.4$ . U138 and U251 cells showed extensive CPE 4dpi with either CHIKV strain (Figure 2E). U251 cells were more sensitive to wt CHIKV<sup>Brazil</sup> infection than U138 cells. However, the kill curve of U251 infected with CHIKV<sup>Ross</sup> strain was not a strictly dose-dependent linear progression but rather an undulated one (Figure 2F). Furthermore, SD in U251 was also rather high (sometimes > 25%). One important observation was that cell viability assay with MTS in U251 is not working reliably when the experiment duration exceeds 3 days and the initial cell concentration is  $\geq 1 \times 10^4$  cells/well. The reason might be that the cells double in 23 hours and are sensitive to overgrowing [26, 28]. Too many cells will cause U251 to stop proliferating, curb their metabolic rates and reach a state of stasis. In this state, U251 cells no longer reduce MTS into its formazan product. Consequently, the absorbance of the plate appears to be the same as in dead cells although there are a multitude of alive U251 cells. This results in false interpretation of test results. Seeding too few cells, on the other hand, results in badly proliferating cells, since it was our observation that both U251 (and U138) cells need close cell-to-cell contacts



in order to form a stable layer. For these reasons U251 were not used in the other experiments, since those experiments were designed to run for 4 days. Still, U251 cells might be a useful cell line for CHIKV studies if the experimental parameters are adapted accordingly.

The human lung derived cell line A549 proved unsuitable experiments testing cell viability since it displayed no CPE after infection with wt CHIKV<sup>Brazil</sup> and only limited cell death at the highest MOI (6.45) of CHIKV<sup>Ross</sup> at 4dpi (Figure 2B). The kill curve in MTS assays of wt CHIKV<sup>Brazil</sup> on A549 cells even indicated more viable cells at the highest MOI (63.5 and 6.35) than in the non-infected control. This phenomenon might be explained with some of the cells dying at such high MOI (possibly due to apoptosis), which then leaves more space and substrate for the surviving cells. At lower MOI and in the control, the A549 cells were overconfluent and might have curbed down their metabolism, leading to a lower MTS reduction (which leads to lower OD values). Apart from a bad or unreliable CPE, the amount of virus needed to conduct viability experiments on A549 is very high. The A549 cell line has been described in CHIKV experiments before, but reports are contradictory. Sourisseau, Schilte [29] state that wt CHIKV virions bind to A549 cells without replicating within the cell, and Solignat, Gay [30] did not observe any CPE on wt CHIKV infected A549. Other studies do not recommend this cell line claiming that CHIKV does not reproduce in A549 [31]. Franco, Rodriquez [32] however, used this cell line to test RBV and favipiravir against an attenuated CHIKV strain (vaccine strain 181/clone25) at MOI 0.1 in a yield assay, looking at virus in the supernatant. This would indicate this particular CHIKV strain does replicate in A549 cells and is secreted into the supernatant. It is possible, that the laboratory-generated, attenuated vaccine strain (181/clone25) has some affinity to this cell line, however, for cell viability experiments with our clinical isolate of CHIKV<sup>Brazil</sup> and the Ross strain, A549 cell cannot be recommended.

The Huh-7 human hepatocarcinoma cell line is often used to evaluate hepatocellular toxicity of compounds *in vitro* [33]. Huh-7 cells only showed cell death after infection with wt CHIKV<sup>Brazil</sup> at a very high MOI of 127. Data showed that an increased initial MOI of CHIKV promotes the effect of CHIKV-induced cellular transcriptional shutoff in cells and thus leads to apoptosis [34]. This effect could be observed in cells infected with higher MOI [34], and it could explain the CPE in A549 and Huh-7 at very high MOI. We observed the biggest difference in CPE between the two virus strains in Huh-7 cells. While the wt CHIKV was not able to significantly damage Huh-7 cells at MOI  $\leq 12.7$ , the Ross strain showed a dose-dependent CPE (Figure 2C).

Solignat, Gay [30] has successfully used Huh-7 cells in CHIKV experiments before. In his work, Huh-7 cells were infected at higher MOI with the West African CHIKV strain 5'CHIKV-EGFP that encodes a GFP protein. According to the study, there was detectable viral replication and CPE [30]. Antiviral efficacy assays measuring virus yield were conducted using Huh-7 cells by Franco, Rodriquez [32] (vaccine strain of CHIKV (181/clone 25)) and Ferreira, Reis [35] (CHIKV (Asian strain), not

further specified). Additionally, a study from Roberts, Zothner [36] evaluated a variety of cell lines for their use in experiments with a sub-genomic replicon (SGR) system CHIKV SGR (CHIKV-D-Luc-SGR), derived from the ECSA strain (ICRES). To test infectious virus, the group used a full-length infectious cDNA clone of CHIKV-LR2006 OPY1. According to the group, Huh-7 cells could be infected by said CHIKV construct and did yield infectious virus in moderate amounts. A549 cells on the other hand were less suited. No observations were done in regard of CPE in the two cell lines in this particular study. Thus, the results of the research cannot be transferred to cell viability assays with wt CHIKV.

The fact that other studies have successfully used the Huh-7 cell line in CHIKV cell viability assays might be due to the use of different, lab adapted or modified CHIKV strains. Interestingly, the field isolate tested in this study showed no CPE on Huh-7 cells while the Ross strain displayed extended cell kill. This might be due to cell culture adaption of CHIKV<sup>Ross</sup>. Genome analysis of both strains revealed that both CHIKV strains (CHIKV<sup>Ross</sup> and CHIKV<sup>Brazil</sup>) belong to the ESCA clade.

For other arboviruses like Dengue Virus (DENV) or Zika Virus (ZIKV), A549 and Huh-7 are very useful cell lines, as these viruses replicate well and show CPE [31, 37-39]. Since coinfections of CHIKV, DENV and ZIKV occur due to geographical overlapping in tropical regions, cell lines in which all these viruses may be propagated might have been one objective as to why A549 and Huh-7 cells have repeatedly been tried in CHIKV experiments. Especially DENV and CHIKV cause similar fever-like symptoms, and are difficult to diagnose [31].

To our knowledge a comparison of lab-adapted CHIKV strain with wt CHIKV isolates with regard to cell affinity in different cell lines has only been done by Wikan, Sakoonwatanyoo [40]. The group tested a panel of cell lines with different CHIKV strains (two field isolates and the original Ross strain). However, their cell line panel did not encompass Huh-7 and A549 cells.

The reasons for the different CPE of CHIKV<sup>Brazil</sup> and CHIKV<sup>Ross</sup> on various cell lines are currently unknown. One possible explanation might be the presence or absence of specific cell surface receptors and/or host proteins which are necessary for an efficient infection, replication and virus production with cell lysis. Even if certain cell lines have already been described as susceptible, different CHIKV strains might still not work.

Various studies observed strain differences in CHIKV tropism and virulence [41]. The cell culture adapted CHIKV strain 181/25, which had been investigated as a possible vaccine strain after being passaged various times *in vitro*, displays increased glycosaminoglycan (GAG) binding due to a specific mutation in the E2 glycoprotein (G82R) [41-45]. GAGs serve as attachment factors for many pathogenic viruses and are among the central factors which trigger CHIKV attachment [44]. The viral spike glycoproteins E2 and E1 play an important role for the infection of target cells. While the E2 protein is thought to be responsible for receptor binding, the E1 protein contains a hydrophobic fusion peptide and is necessary for viral and cellular membrane fusion [46].

da Silva and colleagues could demonstrate by reciprocal amino acid substitutions at residue 82 of the E2 glycoprotein that the exchange G82R resulted in a phenotype switch in CHIKV [44]. Their data suggest that an Arginine at position 82 of E2 increases the affinity of the glycoprotein for GAGs [44]. These findings also support the hypothesis that the G82R substitution in E2 of CHIKV strain 181/25 contributes to attenuation of the vaccine strain due to GAG binding [45]. Further research *in vitro* and *in vivo* suggested that an arginine at residue 82 lead to a greater dependence on GAGs for infection of mammalian cells [41]. These results suggest that GAG utilisation plays a role in regulating CHIKV tropism and host responses that contribute to arthritis, a cardinal symptom of CHIKV disease [41].

Other point mutations in the E2 protein (e.g. E79K, E266K and E166K) affecting GAG binding were observed in cell-culture adapted CHIKV strains [43, 47, 48]. These strains were more dependent on GAGs for infection and showed reduced *in vivo* replication. By increasing the positive charge in domain A of the E2 protein, these point mutations affected the binding affinity of the virus. The positive charge acquisition is a phenomenon commonly observed in cell culture adapted alphaviruses and often correlates with an attenuated phenotype *in vivo* [45, 49, 50]. Mutation at critical points of the envelope surface proteins may introduce changes in charge and hydrophobicity of the CHIKV the E1 and E2 glycoprotein [51]. Such changes in the E1/E2 proteins can influence pH sensitivity and dramatically affect virus structure and production [52, 53]. Furthermore, mutations in specific regions of the E2 protein may directly influence interactions with a specific cell surface receptor thus influencing virulence and adaption [48, 54].

**Whole genome sequencing** revealed 5 differences in the E1 glycoprotein (Figure 1). One difference was at the E1 protein position 322. While Ross has a valine in this position, Brazil has an alanine. Studies showed that membrane fusion of endosomes containing CHIKV is triggered by E1 glycoproteins and that this process is pH dependent. Mutations in the E1 protein at position 226 can lead to phenotypes which require lower pH compared to the parent strains to trigger fusion [55, 56]. Differences in the E1 protein between the two strains may be responsible for the differences in HCQ response, as HCQ (and the more toxic base substance chloroquine (CQ)) is known to raise the endosomal pH and thus intervene with CHIKV membrane fusion [57]. It is thus possible that some of these changes have an impact on the acid pH-triggered conformational changes in alphavirus E1 during membrane fusion [58].

Furthermore, whole genome sequencing of the strains used in this study revealed differences at four positions in the nsP2, a protein known to be connected with cytopathogenicity especially of old-world alphaviruses. Apart from other functions, the nsP2 inhibits host transcription which eventually induces cell death [59].

Whether the discovered genome differences between CHIKV<sup>Ross</sup> and CHIKV<sup>Brazil</sup> are responsible for the differences in cell affinity (especially concerning Huh-7 cells) needs to be further investigated using mutagenesis of the respective sites and observation on the effects on cell tropism in reverse genetics experiments.

## 4.2. Comparison of Compound efficacy in different cell lines against two different CHIKV strains

Despite being treated with compounds that should potentially confer some protection at the concentrations used, Vero-B4 cells showed no significant cell survival after 4 days of CHIKV challenge. A possible reason of the inefficacy of the compounds might be the higher MOI of 0.64 with which the cells were infected (compared to an MOI of 0.355 in the IC<sub>50</sub>/CC<sub>50</sub> experiments and considerably lower MOIs of 0.005 – 0.01 in previous studies with the same setup [21].

Previously published data states that RBV was efficient against wt CHIKV (MOI: 0.005) with an IC<sub>50</sub> of 423.6 µM and a CC<sub>50</sub> > 500 µM [21]. The same study states CQ's IC<sub>50</sub> as 5 – 10.6 µM with a CC<sub>50</sub> of > 36 µM. Delang, Segura Guerrero [22] however tested CQ against CHIKV Indian Ocean strain 899 (lab) at MOI 0.01 in Vero cells and generated IC<sub>50</sub> values of 11 and 28 µM. Delang also tested T-1105 against this lab CHIKV strain at MOI 0.01 and IC<sub>50</sub> values were 7 – 47 µM, with a CC<sub>50</sub> value of 571 µM [22]. HCQ is a less toxic derivative of CQ and its efficacy is comparable to CQ.

U138 cells on the other hand benefited considerably from RBV, T-1105 (50 µM), and HCQ treatment, despite the higher MOI. The reason for the difference in compound efficacy between the two cell lines might be due to the different ability of the respective cells to process the compounds into their active analogues.

Furthermore, there was a significant difference in RBV toxicity between the two cell lines. While RBV lead to an increase of the MTS signal in Vero-B4 cells, U138 cells showed diminished signals which can be interpreted as fewer viable cells. There might be different reasons for this observation:

- (i) The compounds kill some cells, leave space for the remaining cells which then have spare room and medium and become highly metabolically active, hence they are able to reduce MTS into the signal yielding formazan product more effectively.
- (ii) Vero-B4 have a higher proliferation rate (doubling time 24 hours) than U138 cells (doubling time 47-72 hours). Although the absorption values are set in relation with an untreated and uninfected control of the same cell line,
- (iii) it is still possible, that RBV actually causes cell proliferation or an activation of metabolism in Vero-B4 cells, while U138 cells actually are hampered/damaged by the compound. This is possibly due to the cell proliferation rate of the different cell lines. While Vero-B4 double every 24 hours, U138 have a doubling time of > 46 hours [28, 60]. It is thus possible that Vero-B4 cells have a higher metabolism.

When comparing efficacies of the compounds between the two strains, RBV and HCQ protected U138 cells significantly better from wt CHIKV than from CHIKV Ross.

CQ/HCQ are effective at early stages of viral infection [61]. The drugs seem to impair cell-virus surface interactions. Pre-treatment of Vero cells with CQ impairs terminal glycosylation of ACE2, a cell surface receptor used by severe acute respiratory syndrome corona virus (SARS-CoV) for cell attachment [62]. Khan *et al.* suggested a similar mechanism to be responsible for the inhibition of CHIKV

infection by CQ *in vitro* [61]. In the case of other Alphaviruses like Sindbis virus (SINV) and Semliki Forest virus (SFV), viral fusion with the host cell membrane is achieved via conformational changes in the viral envelope glycoprotein that are triggered by clathrin-mediated endocytosis by the target cell and the low pH of the endosomal compartment [63]. This low endosomal pH is said to be required for CHIKV entry into cells as well [29]. Bernard and colleagues could demonstrate that the base CQ raises the endosomal pH by interfering with the protonation of the endocytic vesicles. This prevents the E1 fusion step needed for the release of CHIKV RNA into the cell cytoplasm [64].

In our comparative experiments, HCQ showed a statistically significant higher efficacy against the wt CHIKV<sup>Brazil</sup> than against the CHIKV<sup>Ross</sup> strain. The CHIKV<sup>Brazil</sup> strain may rely on a lower pH to grant membrane fusion (possibly due to mutations in the E1 glycoprotein as mentioned above), or the strain CHIKV<sup>Ross</sup> has gained a more efficient way to grant fusion with the host cell membrane during its repeated passage in Vero cells (possibly due to mutations in the E2 protein). It should be mentioned that HCQ is only used as a control for measuring efficacy *in vitro*, as patients do not benefit from HCQ treatment during acute CHIKV disease and the drug has no suppressive effect on peripheral viral load in patients [65].

### 4.3. Differences of IC<sub>50</sub>/CC<sub>50</sub> values in different cell lines

Both, IC<sub>50</sub> and CC<sub>50</sub> of HCQ observed in this study are higher than previously published data of chloroquine in Vero cells. This might be due to a different MOI. RBV did show a dose dependent efficacy, however, the maximal protection of Vero-B4 cells at the highest drug concentrations did not outnumber 37.55 ± 6.15% (at MOI 0.325) surviving cells and thus no IC<sub>50</sub> value could be generated. Published data from comparable experiments give IC<sub>50</sub> values for RBV of 423.6 – 765.8 µM in Vero-E6 cells [6, 21]. One possible explanation for not exceeding 37.55% surviving Vero-B4 cells might be the fact that the aforementioned publication used different CHIKV strains, VeroE6 cells, and infected with a lower MOI (0.005). At the highest concentration (1000 µM) RBV showed no toxic effect on Vero-B4 cells. The other compounds neither displayed a positive effect against CHIKV<sup>Brazil</sup> nor negative effects on Vero-B4 cells at the used concentrations. Altogether, the experiments showed that HCQ and RBV inhibit CHIKV<sup>Brazil</sup>-induced cell death of Vero-B4 cells in a dose-dependent manner and that HCQ was considerably more effective in preventing CHIKV-related CPE in Vero-B4 than RBV (Table 1).

Vero-B4 cells could not be protected from CHIKV infection with T-1105 at the concentrations used. This was unexpected, since Delang reported IC<sub>50</sub> values of 7 – 47 µM for T-1105 in Vero cells in his study [22]. The concentrations used in the experiments for T-1105 in this study ranged from 5 to 100 µM, well in the range to detect an efficacy of the compound against CHIKV<sup>Brazil</sup>. However, Delang used VeroA cells, different CHIKV strains and infected the cells with an MOI of 0.1. It is possible that the difference in CHIKV strain, cell line, and MOI contributed to the

discrepancy between our results and previously published data. Since the compound did show efficacy against CHIKV<sup>Brazil</sup> in U138 cells, issues related to the compound itself (*e.g.*, degradation due to repeated thaw-freeze-cycles) can be ruled out.

Both RBV and T-1105 are antivirals that interfere with the viral genome replication by inhibiting the nsP4 polymerase. Both are synthetic purine nucleoside analogues [6], and act as broad-spectrum antivirals, with multiple mechanisms of action ascribed to them. Both might either block the RNA-dependent RNA polymerase (RdRp) function of the nsP4 by binding at certain domains of the enzyme and/or they might be incorporated into the viral genome and thus lead to lethal mutagenesis [32]. Others suggest that RBV interferes with the nsP1 guanylyl transferase and/or methyltransferase activity and thus leads to a production of untranslatable mRNAs [66]. RBV and T-1105 (as well as the fluorinated form favipiravir T-705) have to be phosphorylated by host cell kinases into their mono-, di- and triphosphate metabolites. The triphosphate form is the active metabolite which is eventually incorporated into the viral genome, thus leading to error catastrophe [67].

Resistance against RBV and favipiravir (T-705) has been reported and is explained by mutations in nsP4. RBV resistance was put down to a mutation from K291R in nsP4 while favipiravir resistance was explained by a C483Y mutation [22, 66]. Whole genome sequencing of our strains revealed that neither CHIKV Ross nor Brazil have these mutations. Our experiments confirmed the findings of Franco and colleagues, that compound efficacy varies between host cell lines. While Vero-B4 cells were refractory to the treatment of RDV, T-1105, and to a lesser extent HCQ, U138 cells could be protected by all three compounds considerably better. A study demonstrated that the accumulation of RBV is host-cell dependent due to the presence or absence of specific nucleoside transporters [68]. This could also hold true for other nucleoside analogues like T-1105. Furthermore, pro-drugs like RBV and T-1105 depend on host kinases for phosphorylation into their active metabolite. The resistance of some cell types to RBV may thus depend on the intracellular RBV metabolism [69]. A study on the cell line-dependent activation and antiviral activity of T-1105 revealed that T-1105 activation in Vero cells was hindered by inefficient conversion of the ribonucleoside monophosphate to the ribonucleoside diphosphate en route to forming the active triphosphate [70]. This might be one reason, why T-1105 is less potent in Vero-B4 than in U138 cells. It is likely that the distribution of host cell kinases differs between species and tissues and thus lead to a varying intracellular concentration of the triphosphate forms of RBV and possibly T-1105 [32].

## 5 Conclusion

Two glioblastoma cell lines (U138 and U251) were identified as potentially useful *in vitro* cell culture models for CHIKV infection and evaluation of antiviral activity. To our knowledge, this is the first time these two cell lines have been described in connection with CHIKV antiviral tests. Furthermore, A549 and Huh-7 cells cannot be recommended for cell viability assays with wt CHIKV, as these cell lines do not

show CPE. Furthermore, our experiments proved that there are differences in cytopathological effects and antiviral efficacies between wt and laboratory-adapted CHIKV strains.

## 6 Abbreviations

95% CI	95% confidence interval
Abs	absorption
CC <sub>50</sub>	half maximal cytotoxic concentration
CHIKF	Chikungunya fever
CHIKV	Chikungunya virus
Cp	capsid protein
CPE	cytopathic effect
CQ	chloroquine
DENV	Dengue virus
dpi	days post infection
ECSA	East-Central-South African
FBS	foetal bovine serum
FDA	U.S. Food and Drug Administration
GAG	glycosaminoglycans
HCQ	hydroxychloroquine
HG	“high glucose”; medium supplemented with 4.5 g/L of D-glucose
IC <sub>50</sub>	half maximal inhibitory concentration
IOL	Indian Ocean Lineage
LG	“low glucose”; medium supplemented with 1 g/L of D-glucose
MOI	multiplicity of infection
n	number of independent repetitions
NC	nucleocapsid
nsp	non-structural protein
RBV	ribavirin
RdRp	RNA-dependent RNA polymerase
SD	standard deviation
SFV	Semliki Forest virus
SI	selectivity index
SINV	Sindbis virus
WA	West African
wt	wild type
ZIKV	Zika virus

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## **8 Conflict of Interest:**

The authors declare no conflict of interest.

The authors declare that there is no financial or personal relationship with other people or organisations that could inappropriately influence the work. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by Bundeswehr Joint Medical Service or any other governmental institutions.

## **9 Compliance with Ethical Standards:**

All human cell lines used in this study are commercially available and their origin have been stated in the Materials and Methods section. A wt CHIKV was isolated from a diagnostic sample. No patient metadata was collected. No studies with human participants were conducted. Patient consent was obtained according to the national rules for the collection and prior to performing the diagnostic investigation.

## **10 Author contributions:**

JJB and FH conceived the layout of the project. FH performed all experiments with CHIKV. MB-W was responsible for all work connected with sequencing and genome analysis. FH, MB-W and PZ performed statistical analysis and generated the figures and tables. FH and MB-W wrote the first draft of the manuscript. MB-W, MB, AB, and JJB contributed providing additional information as well as reviewing the manuscript. JJB supervised and funded the project as well as oversaw data analysis, manuscript drafting and revision.

## **11 Supplemental material:**

### **11.1 IC<sub>50</sub>/CC<sub>50</sub> of DMSO on Vero-B4 and U138 cells**

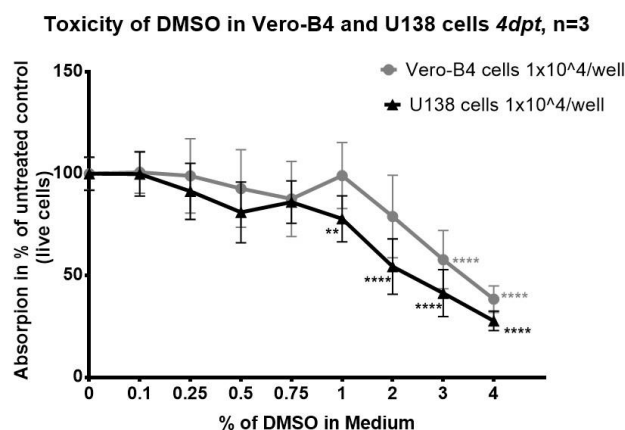
DMSO has cytotoxic properties at higher concentrations. It depends to a great deal on the cell line, at which concentration DMSO cubs cell proliferation. As no published data could be found for U138 cells, experiments were run to generate CC<sub>50</sub> (and possible IC<sub>50</sub>) values to rule out any cytotoxic or antiviral effect of DMSO in the actual experiment at the DMSO concentration used. Serial dilutions of DMSO (final concentration of compound in the wells were 4%, 3%, 2%, 1%, 0.75%, 0.5%, 0.25%, 0.1%) were prepared in assay medium (DMEM).



The experiments were run and evaluated as described in chapter 2.4.1. Each experiment was repeated at least 3 times independently with three technical replicates.

### Results:

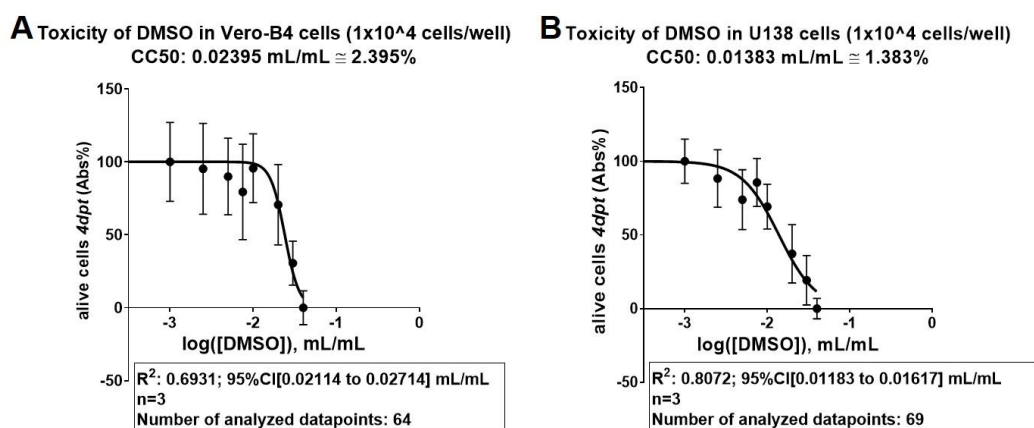
DMSO has no antiviral effect on CHIKV in Vero-B4 or U138 cells. The cytotoxic effect of DMSO was significant in Vero-B4 cells at concentrations  $\geq 3\%$  (Supp. Fig. 1). The  $CC_{50}$  in Vero-B4 cells calculated as 2.395% (Supp. Fig. 2A).



### Supp. Fig. 1. Cytotoxic effect of DMSO on Vero-B4 and U138 cells

Vero-B4 (grey graph) and U138 (black graph) cells were treated with different concentrations of DMSO (0.1-4%) for four days (4dpt). Cell survival was determined with a colorimetric cell viability endpoint assay (MTS). Statistically significant differences between the untreated Mock control (=100% viable cells) and the treated cells were evaluated in a one-way ANOVA test (GraphPad Prism6) and are indicated by asterisks. The number of asterisks indicate p-values as follows: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ . In Vero-B4 cells (grey graph and round symbols), DMSO showed a statistically significant cytotoxic effect at concentrations  $\geq 3\%$ . In U138 cells (black graph and triangle symbol), DMSO concentrations  $\geq 1\%$  showed statistically significant cytotoxicity. Abbreviations: dpt, days post treatment; DMSO, dimethyl sulfoxide; n, number of independent repetitions.

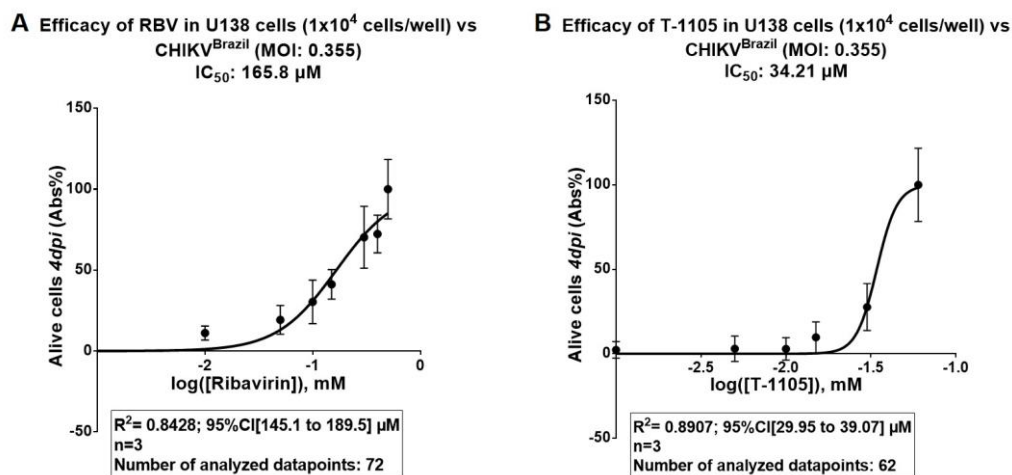
In U138 cells, DMSO concentrations  $\geq 1\%$  showed significant cytotoxicity (Supp. Fig. 1) and the  $CC_{50}$  calculated to 1.383% (Supp. Fig. 2B). Thus concentrations  $\leq 0.6\%$  DMSO during cell assay experiments were considered as acceptable to Vero-B4 and U138 cells.



**Supp. Fig. 2. CC<sub>50</sub> values of DMSO in Vero-B4 (A) and U138 (B) cells**

Vero-B4 (A) and U138 (B) cells ( $1 \times 10^4$  cells/well) were treated with different concentrations of DMSO for 4 days. Cell death was then determined via a colorimetric cell viability assay (MTS). The data represent means  $\pm$  SD of raw data from 3 independent experiments performed with three technical replicates. Normalized fit of dose-response curve was done with GraphPad Prism 6 Software. Abbreviations: 95% CI, 95% confidence interval; Abs, absorption; CC<sub>50</sub>, half maximal cytotoxic concentration; dpi, days post treatment; DMSO, dimethyl sulfoxide; n, number of independent repetitions.

**11.1.1 IC<sub>50</sub> of RBV and T-1105 in U138**



**Supp. Fig. 3. IC<sub>50</sub> of Ribavirin and T-1105 in U138 glioblastoma cells**

Ribavirin (A) and T-1105 (B) inhibit CHIKV-induced cell death in U138 cells in a dose-dependent manner. U138 ( $1 \times 10^4$  cells/well) were infected at an MOI of 0.355 and treated with RBV or T-1105 at the indicated concentrations. After 4 days, cell death was determined via a colorimetric cell viability assay (MTS). Toxicity assays were performed similarly without infection of the cells. The highest concentration of Ribavirin ( $500 \mu\text{M}$ ) used on U138 cells did not result in a maximal toxic effect on the cells, thus no CC<sub>50</sub> could be calculated with (data not shown). T-1105 had no significant toxic effect at the highest concentration ( $100 \mu\text{M}$ ; data not shown). Data represent means  $\pm$  SD of raw data from 3 independent experiments performed with three technical replicates. Normalized fit of dose-response curve was done with GraphPad Prism 6 Software. Abbreviations: 95% CI, 95% confidence interval; Abs, absorption; CC<sub>50</sub>, half maximal cytotoxic concentration; IC<sub>50</sub>, half maximal inhibitory concentration; n, number of independent repetitions; RBV, ribavirin; wt, wild type.

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