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In vitro antiviral activity of arbidol against Chikungunya virus and characteristics of a selected resistant mutant

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ABSTRACT

Arbidol (ARB) is an antiviral drug originally licensed in Russia for use against influenza and other respiratory viral infections. Although a broad-spectrum antiviral activity has been reported for this drug, there is until now no data regarding its effects against alphavirus infection. Here, the *in vitro* antiviral effect of ARB on Chikungunya virus (CHIKV) replication was investigated and this compound was found to present potent inhibitory activity against the virus propagated onto immortalized Vero cells or primary human fibroblasts (MRC-5 lung cells) (IC_{50} < 10 μg/ml). A CHIKV resistant mutant was then selected and adapted to growth in the presence of 30 μg/ml ARB in MRC5 cells; its complete sequence analysis revealed a single amino acid substitution (G407R) localized in the E2 envelope protein. To confirm the G407R role in the molecular mechanism of ARB resistance, a CHIKV infectious clone harboring the same substitution was engineered, tested, and was found to display a similar level of resistance. Finally, our results demonstrated the effective *in vitro* antiviral activity of ARB against CHIKV and gave some tracks to understand the molecular basis of ARB activity.

1. Introduction

Chikungunya virus (CHIKV) is an arthropod-borne viral disease first described in Tanzania in 1952 (Robinson, 1955) which has reemerged since 2005 in Eastern Africa, the Indian Ocean, India and South-East Asia and even reached Europe in 2007 (Arankalle et al., 2007; Rezza et al., 2007). From 2005, this new variant has been responsible for millions of cases of CHIKV disease. The adaptation to its new vector, *Aedes albopictus* (Santhosh et al., 2008; Schuffenecker et al., 2006) rendered possible the spread of the virus in new territories in which *Aedes aegypti* was absent (e.g., Reunion Island, Mauritius, and the south of Europe). CHIKV infection is commonly an acute disease marked by febrile arthralgia and a frequent rash, but persisting arthralgia has been reported in a significant number of cases (Borgherini et al., 2008). Lethal infections are rare but severe cases have been described including neurological presentations and neonatal contaminations which were documented during the outbreak in Reunion Island (Economopoulou et al., 2009; Lemant et al., 2008). Current treatments of Chikungunya fever are for symptoms with no effective licensed vaccine nor specific antiviral drug available. The utilization of the antimalarial chloroquine proved to be poorly active *in vivo* despite it's *in cellulo* antiviral effect on CHIKV infection (de Lamballerie et al., 2008; Michault and Staikowsky, 2009; Thiboutot et al., 2010). Similarly, it has been shown that the combination of interferon-alpha and ribavirin is effective on CHIKV replication *in vitro* but these compounds have not been tested in animal models and/or clinical trials (Briolant et al., 2004; de Lamballerie et al., 2009).

The antiviral drug arbidol (ARB) (1-methyl-2-phenyl-thiomethyl-3-carbotoxy-4-dimetylaminomethyl-5-hydroxy-6-bromo-indolehydrochloride monohydrate) (Fig. 1) was originally developed at the Russian Research Chemical and Pharmaceutical Institute about 20 years ago (Panisheva et al., 1988) and since 1990 this drug has been used in Russia for prophylaxis and treatment of acute respiratory infections including influenza. Until now, it has been shown that ARB exhibits a wide range of activity against a number of RNA, DNA, enveloped and non-enveloped viruses (Boriskin et al., 2008). This suggests that ARB targets common critical step(s) in virus–cell interaction. Recent data showed that ARB incorporates into cellular membranes leading to perturbed membrane structures and inhibition of virus-mediated fusion (Villalain, 2010). In case of influenza viruses or hepatitis C virus (HCV), ARB blocks virus entry into target cells but exploits different modalities proving its effective broad-spectrum antiviral activity (Leneva et al., 2009; Pecheur et al., 2007).

In this study, we investigated the *in cellulo* antiviral ARB activity against CHIKV. Several cell lines were assayed (MRC-5 and Vero), in

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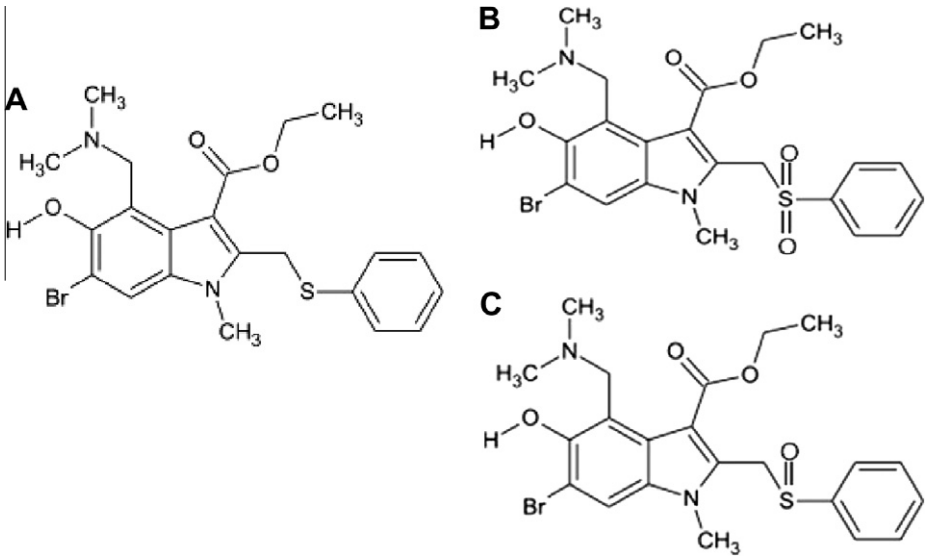


Fig. 1. Chemical structures of arbidol ARB (A), HZ1 (B) and HZ3 (C).

various conditions (pre and post-infection treatments) and using different ARB metabolites to demonstrated the *in vitro* inhibitory effects of ARB on CHIKV replication (IC_{50} < 10 μg/ml). To further characterize the mechanism of ARB action, we also selected ARB-resistant mutant of CHIKV, identified a single drug-resistant mutation in the E2 envelope viral protein (G407R) and confirmed its role in the virus resistance using infectious clones in *in vitro* assays.

2. Materials and methods

2.1. Cells and viruses

The MRC-5 cells (ATCC number CCL-171) were grown in Basal Medium Eagle (BME) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, under 5% CO2. The Vero cell line (ATCC number CCL-81) was grown in minimal essential medium (MEM) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, under 5% CO2. HEK-293 cells (ATCC number CRL-1573) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/l of D-glucose, 1 mM of sodium pyruvate and 2 mM of L-glutamine, supplemented with 10% decomplemented fetal calf serum (FCS) and antibiotics.

The CHIKV strain used in this study for antiviral assays or the construction of infectious clone was LR2006 OPY1 (GenBank accession number DQ443544), isolated from a patient during the outbreak on Reunion Island in 2006 (Parola et al., 2006).

2.2. Compounds

Purified arbidol (HZ2 = ARB) and two derived metabolites, HZ1 (6-bromo-4-(dimethylaminomethyl)-5-hydroxy-1-methyl-2-(phenylsulphonylmethyl)-1H-indole-3-carboxylate) and HZ3 (6-bromo-4-(dimethylaminomethyl)-5-hydroxy-1-methyl-2-(methylphenylsulphoxyde)-1H-indole-3-carboxylate) (Fig. 1) were provided by Stragen Pharma SA (Geneva, Switzerland). HZ2, HZ1 and HZ3 powders were dissolved to completion in dimethyl sulfoxide (DMSO) at a final concentration of 10 mg/ml followed by dilution in sterile distilled water to prepare stocks at 1 mg/ml. After storage at −20 °C, these samples were used for preparation of required drug solutions in all experiments. The final 0.005% maximum DMSO concentration was also added to all mock control samples.

2.3. Cell viability assay

The ARB cytotoxicity in MRC5 and Vero cells was evaluated using neutral red (NR) dye uptake assays and microscopic observations (Repetto et al., 2008). Briefly, for NR dye uptake assays, 96-well tissue culture plates were seeded with cells then exposed at 90% confluence to varying ARB concentrations (0–100 μg/ml). Plates were then incubated at 37 °C, 5% CO2 for 18 or 48 h (respectively, for Vero or MRC5 cells), at which times medium containing neutral red (40 μg/ml) was added to each well. After 3 h of incubation, the dye was extracted with acidified ethanol solution and optical densities (OD) were read using a microplate spectrophotometer at 540 nm (TECAN Sunrise). Results were expressed as a percentage of OD value of treated cell cultures with respect to untreated ones and the 50% cytotoxic (CC_{50}) concentrations of ARB for MRC5 and Vero cells were determined by regression analysis.

2.4. Selection of an ARB-resistant mutant

MRC-5 cells grown in 12-well plates were infected with different dilutions of CHIKV (LR2006 OPY1 strain) at a multiplicity of infection (MOI) of 1–0.001 $TCID_{50}$/cell. Virus growth in the presence or absence of ARB was examined at each passage by direct microscopic observations of cytopathic effect (CPE) on MRC-5 cells. The clarified supernatant from the highest dilution providing CPE was used for subsequent passage. Initially, CHIKV did not grow in the presence of ARB at a concentration >10 μg/ml; its concentration was increased gradually from 4 to 30 μg/ml during successive virus passages using 4, 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 22, 24, 26, 28 and 30 μg/ml ARB. By the 17th passage, the virus appeared to have adapted to 30 μg/ml ARB in MRC-5 cell. This specific clarified supernatant was stored at −80 °C for further analysis.

2.5. Sequence analysis

Viral RNA was extracted from samples using the EZ1 virus mini kit on an EZ1 Biorobot workstation (Qiagen). Viral genomes were reverse-transcribed and amplified by use a one-step RT-PCR kit (Access RT-PCR core reagent kit, Promega) according to the manufacturer's instructions. PCR products were purified using the Qiagen PCR extraction kit and sequenced using the ABI PRISM

BigDye terminator cycle sequencing kit on an ABI Prism 31310X Genetic Analyser sequencer. The sequencing primers were those already used for the complete sequencing of the LR2006 OPY1 CHIK strain (Parola et al., 2006).

2.6. Construction of CHIKV infectious clones

The preparation of an infectious clone from the LR2006 CHIKV strain was reported elsewhere (Tsetsarkin et al., 2006). The original construct was modified by adding a CMV promoter and the Hepatitis D virus ribozyme at the 5′ and 3′ ends of the genome, respectively, in order to be able to transfect DNA and generate *in cellulo* infectious viral RNAs. The resulting construct (set-up in a pBR322 plasmid possessing an ampicilline resistance gene) was named "Tonile". The infectious CHIKV clone containing the mutation G407R called "Tonile-ARB-R" was produced by standard molecular biology techniques. The PCR fragments containing the mutation were cloned and inserted between the unique restriction sites AgeI and XhoI into the Tonile plasmid backbone (Vanlandingham et al., 2005) (Fig. 5). The Tonile and Tonile-ARB-R infectious clones were sequenced completely as described previously to validate the constructions.

2.7. Transfection and production of CHIKV infectious clones

HEK-293 cells were seeded on 75 cm^2 flask in complete DMEM without antibiotics. The day after, cells were transfected with the CHIKV infectious clones (Tonile or Tonile-ARB-R) using Lipofectamine 2000 (Invitrogen) with a ratio of 1 μg of DNA per 1.5 μl of Lipofectamine. Four hours post-transfection, transfected cells were washed three times in Hanks balanced salt solution (HBSS) then incubated for 16 h in complete DMEM supplemented with antibiotics and 30 μg/ml ARB for cells transfected with Tonile-ARB-R. For each viral production, stocks were stored at −80 °C and a sample was used to perform complete genome sequencing and virus titration.

2.8. Virus titration

Virus titers were determined by the Tissue Culture Infectious $Dose_{50}$ ($TCID_{50}$) method in Vero cell cultures (Reed and Muench, 1938). Briefly, the $TCID_{50}$ assay was performed on Vero cells seeded in 96-well plates. When the cells reached 80% confluence, six replicates were infected with 150 μl of ten-fold serial dilutions of the virus sample, and then incubated 7 days before microscopic observations and positive CPE well counting. For each supernatant sample, the infectivity titer was expressed as $TCID_{50}$/ml using the Karber formulae.

2.9. In vitro antiviral activity of ARB

2.9.1. IC_{50} determination using Indirect Immunofluorescence Assays (IFA).

MRC-5 cells were grown in an 8-microchamber Lab-Tek II slide (Nalge Nunc international) to reach 80% confluence. Cells were then infected with different dilutions of CHIKV at MOIs from 1 to 0.0001 $TCID_{50}$/cell. One hour after infection, the viral inoculum was removed and cells were washed one time with PBS. Complete medium (250 μl) supplemented with 0.9% final concentration of Methocult (H4100, StemCell Technologies INC), containing different concentration of ARB (0.9; 1.8; 3.75; 7.5; 15 and 30 μg/ml) were added to each well and cells were grown for 48 h. After PBS washing, cells were fixed with acetone for 20 min at room temperature and viral antigens were detected by IFA using CHIKV-specific immune human serum (1:20) and fluorescein-conjugated anti-human IgG (1:400). The percentage of fluorescent cells in each well was determined. The IC_{50} value (i.e. the concentration of compound required to inhibit cell infection by 50%) was determined by plotting the percentage inhibition of cell infection as a function of ARB concentration after 4 independent experiments.

2.9.2. Virucidal activity

To evaluate a putative virucidal action of ARB on CHIKV, the same experiments were performed using CHIKV pre incubated during 0.5 or 1 h with ARB concentrations ranging from 1 to 30 μg/ml before MRC5 infection. Each assay was realized in duplicate, and 2 independent experiments were performed. The IC_{50} values were determined as described previously.

2.9.3. Kinetics of ARB cell treatment

To evaluate the influence of pre- and post-infection ARB treatment on virus replication, the same experiments were performed using MRC5 cells with the addition of ARB 1, 3, 5, 8, 24 h before and post infection. Each assay was realized in duplicate and 2 independent experiments were performed. The IC_{50} values were determined as described previously. The Mann–Whitney *U* statistical test was then used to analyze IC_{50} values obtained from different times in comparison with the chosen reference one (IC_{50} value corresponding to the time 1 h post infection).

2.9.4. IC_{50} determination using comparative quantitative RT-PCR analysis

Antiviral assays were carried out in Vero cells in 48-well plates in duplicate and two independent experiments were performed. Briefly, 1 day after seeding, cells were infected with 100 μl of the viral inoculum (at MOIs of either 0.1 or 0.01 $TCID_{50}$/cell) for 90 min. at 37 °C, 5% CO2. Following incubation, the viral inoculum was removed and cultures were washed once with HBSS after which 500 μL of fresh complete MEM medium supplemented with 0, 10, 20, 30, 40 or 50 μg/ml ARB (HZ2) were added to the wells. After 18 h p.i., supernatants were harvested and viral RNA was extracted from 100 μl of cell culture clarified supernatant using the NucleoSpin 96 virus kit according to the manufacturer's protocol (Macherey–Nagel, Duren, Germany) and an epMotion 5075 workstation (Eppendorf France SARL). One-step qRT-PCR was performed on the Applied Biosystems 7900HT Fast Real-Time PCR System using primers and probes already described (Pastorino et al., 2005). For comparative quantification, data were expressed as the percentage of untreated virus control, and log reduction values were calculated. The IC_{50} value (i.e. the concentration of compound required to inhibit viral RNA load by 50%) was determined by plotting the percentage inhibition of cell infection as a function of ARB concentration.

2.9.5. IC_{50} determination using virus titration assays

Supernatant used in antiviral assays and stored at −80 °C were titrated using the method described above. For each sample, the viral titer was represented as percentage of positive control (viral titer from infected cell supernatant sample without antiviral compound). The IC_{50} value (i.e. the concentration of compound required to inhibit infectious virus titer by 50%) was determined by plotting the percentage inhibition of cell infection as a function of ARB concentration.

2.10. Hemagglutination assay

Hemagglutination titration of the CHIKV strain LR2006 OPY1 was first performed using standard methods (Clarke and Casals, 1958): twofold serial dilution of virus samples (cell supernatant) on U-bottom microplates were carried out with 0.4% bovine albumin/borate saline pH 9.0 solution (final volume: 35 μl/well). Thirty-five microliters of pre-diluted goose red blood cells (1/150

using the final pH 6.0 adjusting diluents) were added, the mixture was homogenized, incubated 45 min at room temperature and then read using four scoring symbols: ++ for complete hemagglutination, + for partial hemagglutination, +/− for trace hemagglutination and − for negative hemagglutination. The titer was the reciprocal of the last dilution in which + was observed. To assess the effect of ARB on hemagglutination, we then performed a hemagglutination assay in the same condition with 3 amounts of virus (8, 4 and 2 UHA/well). Each concentration of virus was used in triplicate in presence of various ARB concentrations (0, 15, 30 and 60 μg/ml). Negative controls contained no virus and allowed us to observe the effect of ARB on goose red blood cells sedimentation.

3. Results

3.1. Effect of arbidol and its metabolites HZ1 and HZ3 on CHIKV strain LR2006 OPY1

The cytotoxic effect of ARB was evaluated using NR cytotoxicity assay and microscopic observations. Fig. 2C shows the 50% cytotoxic concentration values (CC_{50}) obtained for confluent Vero and MRC5 cells after, respectively, 18 or 48 h of ARB treatment.

Then, the effect of ARB and two sulfone and sulfoxide metabolites (HZ1 and HZ3) (Fig. 1) on CHIKV replication was determined using specific indirect immunofluorescent assays (IFA). As shown in Fig. 2A, ARB was found to inhibit CHIKV infection with IC_{50} values at 6.49 ± 1.17 μg/ml. This was further confirmed using infected Vero cells and qRT-PCR assays which also provided estimated IC_{50} values ⩽10 μg/ml (Fig. 2B). ARB selectivity indices (CC_{50}/IC_{50}) calculated using Vero and MRC5 cell line, provided values about 28 and 36, respectively (Fig. 2C). Moreover, subconfluent monolayers of Vero and MRC5 cells treated for 18 or 48 h with ARB at concentrations of 0–30 μg/ml did not show any microscopically visible changes in cell morphology or cell density.

In the case of arbidol HZ1 and HZ3 compounds, a weak antiviral activity was observed, with IC_{50} values reaching 30 μg/ml. Furthermore, pre-incubation of ARB for 12 h at 37 °C did not improve its antiviral effect on MRC5 cells (HZ2a, IC_{50} value 6.21 ± 0.73 μg/ml) (see Fig. 2A). These results suggested that the arbidol antiviral activity against CHIKV was due to the HZ2 molecule and was not extended to its metabolites or degradation products.

Finally, to investigate the direct inactivating effect of ARB, CHIKV was pre treated for 0.5 or 1 h with concentrations ranging from 1 to 30 μg/ml. The IC_{50} obtained (respectively, 12.3 ± 2.67 μg/ml and 16.85 ± 3.87 μg/ml) indicated that the antiviral activity of ARB on CHIKV infection was not due to a virucidal activity (Fig. 2A).

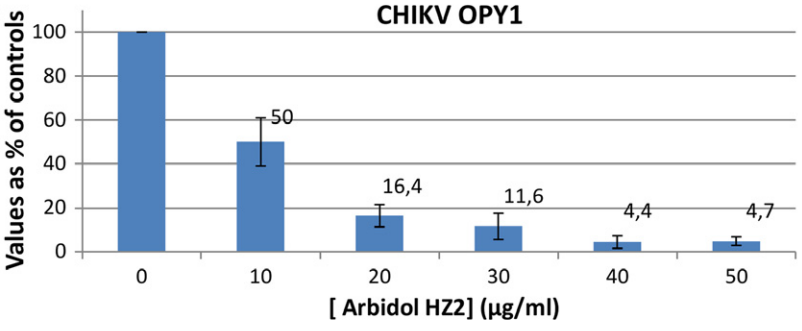
3.2. Time-of-drug-addition studies

To examine the mechanism of viral inhibition by ARB, a time-of-drug-addition experiment was carried out. Various concentrations of ARB HZ2 were added to CHIKV-MRC5 infected cells at several time points before or post infection. As shown in Fig. 3, a decrease in the IC_{50} values was observed from times 0 to 24 before infection and the IC_{50} value at 24 h before infection reached a statistically significant difference from IC_{50} observed 1 h post infection ($P < 0.05$). Moreover, antiviral activity was progressively reduced when ARB was added at post infection stages and the increased IC_{50} values at 3, 5 and 8 h post infection were statistically different

A

Compound	IC_{50} [a]	
	(μg/ml)	(μM)
HZ2	6.49 ± 1.17	12.2 ± 2.2
HZ2 + virus (30 min.)	12.3 ± 2.67	23.1 ± 5
HZ2 + virus (60 min.)	16.85 ± 3.87	31.7 ± 7.2
HZ2a	6.21 ± 0.73	11.7 ± 0.1
HZ1	> 30	> 56.4
HZ3	28.99 ± 3.09	54.5 ± 5.8

B



C

Cell line	CC_{50} [a]		SI [b]
	μg/ml	μM	
Vero	360	676.9	28
MRC5	200	376	36

Fig. 2. Antiviral activity of ARB against CHIKV strain LR2006 OPY1. (A) The IC_{50} values were determined using indirect Immunofluorescence Assay (IFA) and MRC5 cells. HZ2 + virus (30 or 60 min.) represented experiments where CHIKV was pre incubated during 30 or 60 min. with various ARB concentrations before cell infection. HZ2a represented experiments where ARB was pre-incubated for 12 h at 37 °C before cell addition. [a]Mean ± SD values are determined from four independent experiments. (B) Effect of ARB on CHIKV replication using Vero cells and comparative qRT-PCR assays. Data were expressed as the percentage of untreated virus control and each point represents the mean of two replicate in two independent experiments). (C) Cytotoxicity of ARB in our experimental conditions. [a]The 50% cytotoxic (CC_{50}) concentrations of ARB for MRC5 and Vero cells were determined using neutral red (NR) dye uptake assays after, respectively, 18 or 24 h ARB treatment. [b]Selectivity index (SI) is expressed as the ratio CC_{50}/IC_{50}.

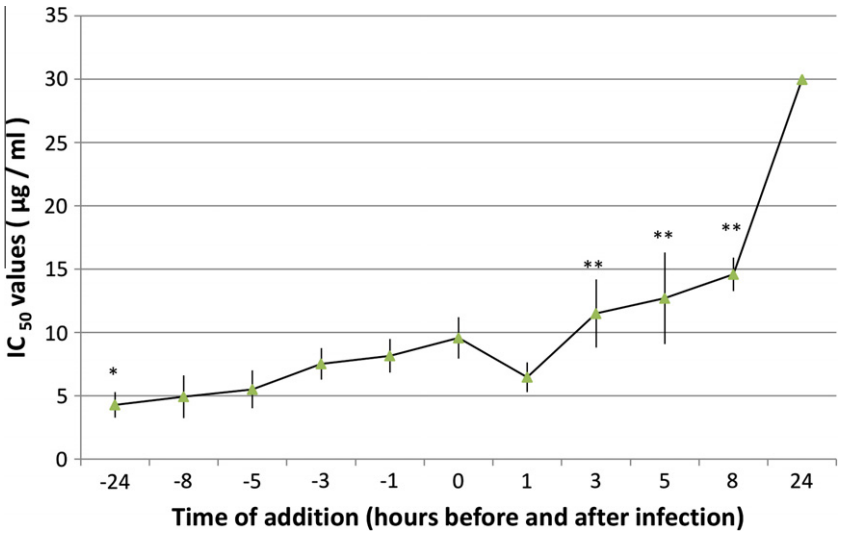


Fig. 3. Time-of-addition study. Experiments were performed using MRC5 cells with the addition of ARB 1, 3, 5, 8, 24 h before and post infection. The IC_{50} values were determined using IFA as described previously. Each point represents the mean of two replicate in two independent experiments. $^{*}P < 0.05$ vs. reference value at 1 h post infection; $^{**}P < 0.01$ vs. reference value at 1 h post infection.

from IC_{50} observed 1 h post infection ($P < 0.01$). These results suggested that ARB interferes with the earliest stages of the viral replication cycle (i.e., virus attachment/entry).

3.3. Effect of ARB on CHIKV hemagglutination

The ability of viruses to agglutinate erythrocyte is a potentially simple model for the study of virus attachment to cellular receptors. Therefore, to make precise the mechanism of ARB action, hemagglutination assays were performed and analyzed. As shown in Fig. 4, ARB concentration equal or higher than 15 µg/ml inhibited the hemagglutination of CHIKV onto goose red blood cells depending on the amount of virus. With 2 UHA/well and 30 µg/ml ARB, the hemagglutination-inhibition reached around 50%. When 60 µg/ml ARB was added in the assay, no CHIKV hemagglutination was detected. Clearly, these results showed that ARB prevents the interaction of CHIKV with goose red blood which could suggest by analogy that the drug blocks the CHIKV replication cycle at the cell adsorption step.

3.4. Selection and characteristics of an ARB-resistant mutant

CHIKV LR2006 OPY1 strain was used to select an ARB-resistant variant following passage on MRC5 cells with increasing concentrations of the drug (from 4 to 30 µg/ml). Efficient virus propagation was ultimately obtained at the 17th passage after 4 months of culture. The resistant mutant (called CHIKV-R) was purified by end-point dilution in the presence of 30 µg/ml ARB and a virus stock was prepared under the same concentration of

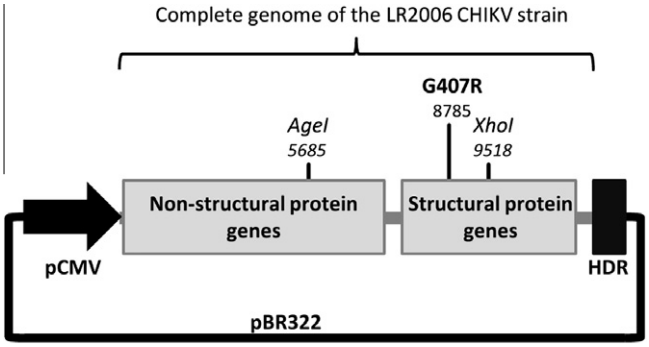


Fig. 5. Schematic representation of the infectious clone Tonile-ARB-R containing the G407R mutation. Tonile-ARB-R derives from the Tonile Infectious clone which contains the whole genome of the LR2006 CHIKV strain. The promoter CMV (pCMV) and the Hepatitis Delta virus ribozyme (HDR) allow to generate *in cellulo* infectious viral RNAs. All of them are inserted into a pBR322 plasmid possessing an ampicilline resistance gene. The unique restriction sites AgeI and XhoI were used to add the G407R mutation. Positions indicated in this figure are based on the complete genome sequence.

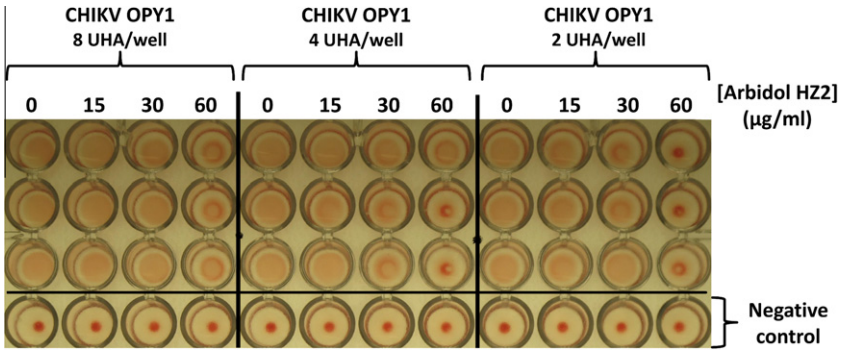


Fig. 4. Hemagglutination assay. Three amounts of virus (8, 4 and 2 UHA/well) were used in triplicate and in presence of various ARB concentrations (0, 15, 30 and 60 µg/ml) to observe modification on goose red blood cells hemagglutination. Negative controls represented wells without virus.

drug. Complete nucleotide sequence of CHIKV-R revealed a single amino acid substitution G407R localized in the E2 viral envelope protein (Fig. 5). CHIKV-R was further characterized for ARB resistance and replication fitness. For both studies, CHIKV replication was determined on Vero cells using comparative qRT-PCR. As described in Fig. 6, CHIKV-R was highly resistant to ARB, with no significant difference in virus replication after 18 h post infection when exposed to 10 from 50 μg/ml ARB. The fitness of CHIK OPY1 and CHIKV-R was compared in infection assays using the same MOIs (0.1 and 0.01 $TCID_{50}$/cell) for both viruses. Viral RNA load was quantified every eight hours for four days in the absence of any antiviral drug using specific one-step qRT-PCR detection (Pastorino et al., 2005) (Fig. 7). Despite slight initial difference in the numbers of viral RNA copies, the growth curves exhibited the same pattern with a similar exponential growth phase until reaching a plateau at the end of the second day and CPE apparition at day 3. The rate of viral growth was identical between hours 8 and 56 for both viruses with no significant difference in viral RNA copies (Fig. 7).

However, in the absence of ARB selection pressure, CHIKV-R seemed to be unstable as the reversion G407R G407 was rapidly detected: when CHIKV-R was propagated on Vero cells without ARB in the medium culture, sequence analysis of viral production revealed the emergence of the G407 revertant as soon as 3 days post infection. This suggests that the wild-type virus is much better adapted to replication than the G407R variant in our experimental conditions.

3.5. Effect of arbidol on Tonile-ARB-R, an infectious clone of CHIKV containing the mutation G407R

Tonile and Tonile-ARB-R CHIKV infectious clones were used to infect Vero cells at the same MOIs (0.1 and 0.01 $TCID_{50}$/cell) in the presence of various ARB concentrations (0–50 μg/ml). Eighteen

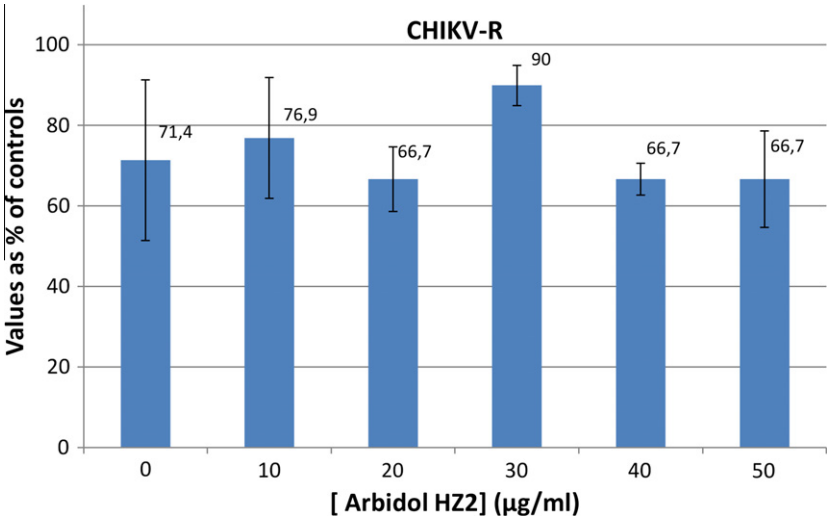


Fig. 6. Effect of ARB on CHIKV-R mutant replication. The antiviral activity of ARB on CHIKV-R mutant replication was evaluated using Vero cells and comparative qRT-PCR assays as described previously. Data were expressed as the percentage of untreated virus control and each point represents the mean of two replicate in two independent experiments.

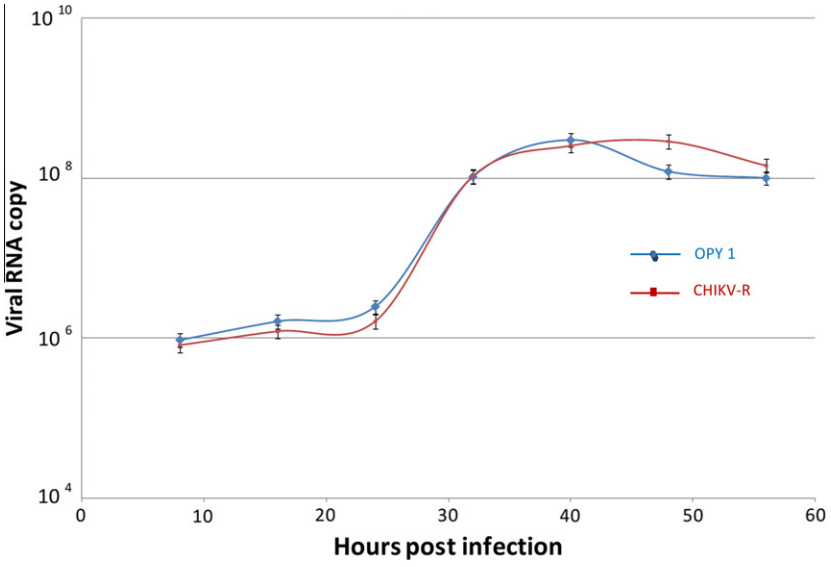


Fig. 7. Replication kinetics of CHIKV OPY1 and CHIKV-R mutant. Vero cells were infected with CHIKV OPY1 or CHIKV-R strains at the same MOIs (0.1 and 0.01 $TCID_{50}$/cell). The viral growth in the absence of antiviral compound was followed by measuring viral RNA copies every 8 h for 4 days using specific real-time RT-PCR assay.

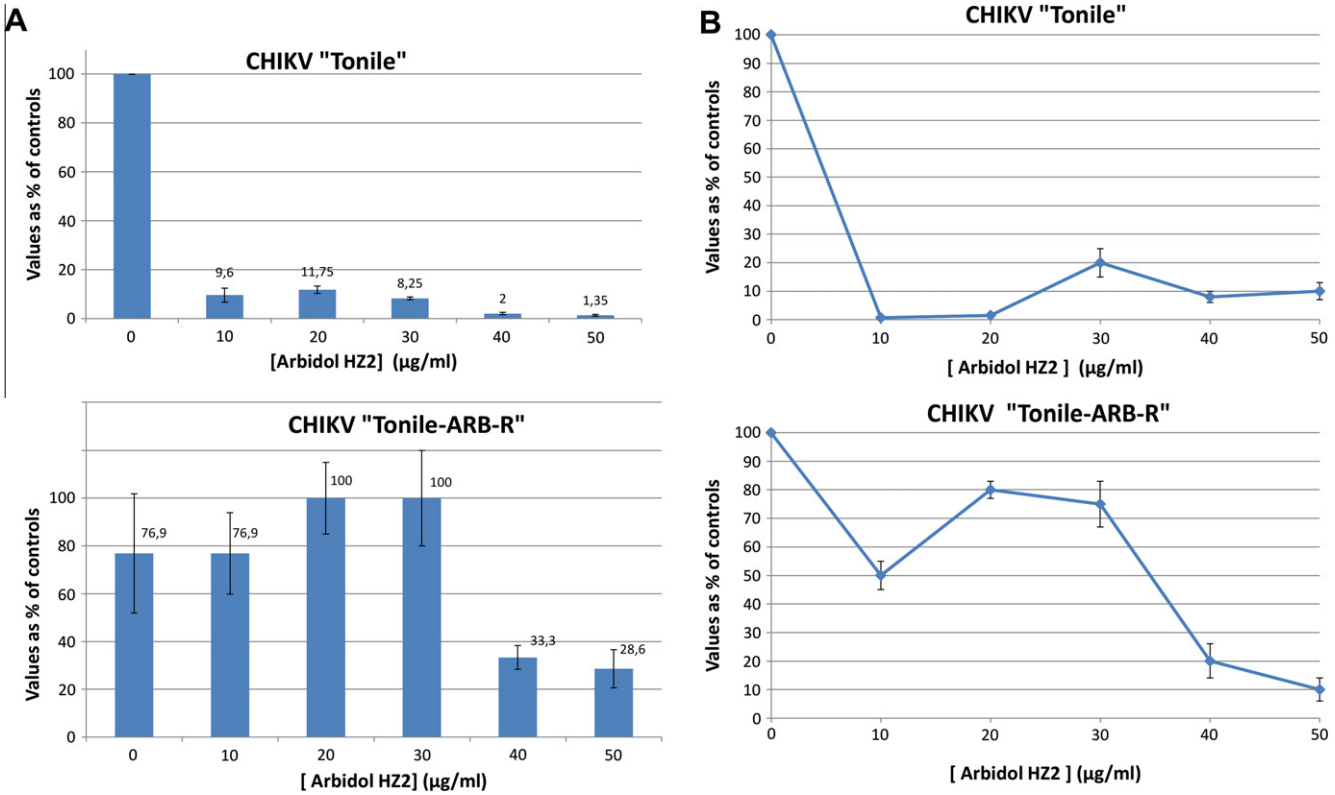


Fig. 8. Effect of ARB on CHIKV infectious clones replication. (A) The effect of ARB on Tonile and Tonile-ARB-R replication was assayed using Vero cells and comparative qRT-PCR assays as already described. Data were expressed as the percentage of untreated virus control and each point represents the mean of two replicate in two independent experiments. (B) The effect of ARB on Tonile and Tonile-ARB-R infectivity were determined using Vero cells and titration of cell culture supernatant. Data were expressed as the percentage of untreated virus control and each point represents the mean of two replicates in two independent experiments.

hours post infection, infected cell supernatants were harvested and compared using viral RNA quantification and virus titration assays. As expected (see Fig. 8A), a strong viral load decrease was observed for Tonile CHIKV when arbidol was added to the culture medium. A 90% reduction of viral RNA was observed after addition of 10 µg/ml ARB in the culture medium. The same experiment using Tonile-ARB-R CHIKV infectious clone resulted in no significant difference for viral load when 0 to 30 µg/ml of arbidol was added to the culture medium. A 70% reduction of viral RNA was only observed when the medium was supplemented with 40 or 50 µg/ml of the drug. Virus titration assays confirmed the results obtained for viral RNA quantification (Fig. 8B) with a complete suppression of Tonile CHIKV production using 10 µg/ml ARB whereas the infectivity of Tonile-ARB-R was only significantly reduced with a 40 µg/ml ARB concentration.

4. Discussion

Arbidol is a small indole-derivatives molecule that was first marketed in Russia in 1993 and in China in 2006 for prophylaxis and treatment of infections by influenza A and B viruses (Boriskin et al., 2008; Brooks et al., 2004). This drug proved to be efficient to reduce the duration of illness and to prevent the development of post-influenza complications (Leneva et al., 2009). Moreover, despite a clinical use for more than 15 years, no ARB-resistant viruses have been isolated so far and clinical trials revealed that the drug was well tolerated with minor side effects (Liu et al., 2009). More than 17 tonnes of ARB are used yearly in Russia for the treatment of acute respiratory infectious syndromes. In addition to possible immune-modulatory effects, ARB demonstrated a broad-spectrum antiviral activity against a number of enveloped and non-enveloped viruses which could in part be due to its membranotropism (Shi et al., 2007). Indeed, it has been shown that ARB was an entry inhibitor of influenza virus infection by stabilizing the influenza HA and preventing the endosomal membrane fusion (Leneva et al., 2009). Similarly, ARB proved to be active *in vitro* against HCV infection and the antiviral mechanism was related to inhibition of the HCV glycoprotein conformational changes needed for the membrane fusion process (Boriskin et al., 2008; Pecheur et al., 2007; Teissier et al., 2011).

Since 2004, CHIKV has been identified as a reemerging arbovirus with a high epidemic potential in relation with its possible dissemination by rapid long-distance travels and its transmissibility by urban mosquito vectors (Chevillon et al., 2008). Originally confined to the developing nations, the virus began to encroach into the boundaries of the developing world but there is currently no vaccine available or effective treatment for CHIKV infection. In this context, the development of prophylactic and therapeutic strategies for CHIKV infection is important (Couderc et al., 2009; Thiboutot et al., 2010).

Until now, our understanding of the interactions of CHIKV with human cells remains limited. However, CHIKV is capable of replication *in vitro* in a variety of mammalian cells with the production of characteristic CPE. Among these cells, continuous Vero and MRC5 ones are highly permissive to CHIKV infection. Primary human fibroblasts as MRC5 are cells of specific interest since human fibroblasts are known targets of CHIKV infection (Couderc et al., 2008; Sourisseau et al., 2007).

In the current study, we used these two cell lines to demonstrate that ARB was able to inhibit CHIKV replication when added before infection. The IC_{50} values obtained $\leqslant$10 µg/ml indicated that

ARB was active at concentrations which were significantly lower than its cytotoxic concentrations ($CC_{50} \geqslant 200$ μg/ml), and similar to those observed for the influenza A virus. The high CC_{50} values measured could be explained by the short time of drug exposition (18 or 48 h) in our experiments. This choice was justified on the one hand by the high rate of CHIKV growth and on the other hand by the low half-life of ARB in cultured cells (about 18 h). Moreover, in relation with its ester prodrug nature, it is known that ARB antiviral activity may be produced by one or several hydrolyzed metabolites. Among them oxidized sulfone and sulfoxide forms of ARB are strongly represented (Anisimova et al., 1995; Boriskin et al., 2008). However, our results did not confirm this hypothesis in the case of CHIKV, with no effect of two derived synthetic HZ2 products (HZ1, HZ3) on CHIKV replication. In addition, no virucidal effect of ARB HZ2 for CHIKV was observed, in opposition with previous studies on influenza A virus, respiratory syncitial virus, human rhinovirus type 14 and coxsackie virus B3 (Shi et al., 2007) but in agreement with data reported for Hantaan virus (Deng et al., 2009). This result combined with those obtained in our time-of-drug-addition experiment indicated that ARB blocks the earliest stages of the CHIKV replication cycle (i.e., virus attachment and/or virus entry) as previously demonstrated for other enveloped viruses (Boriskin et al., 2008). To further characterize the mechanism of ARB action, we selected a CHIKV mutant and identified a single crucial amino acid substitution G407R localized in the E2 viral envelope protein. Sequence alignments revealed that this residue G407 was not conserved throughout the alphavirus genus. Based on recent structural analysis of the CHIKV glycoprotein, this mutation was shown to be localized in the domain A of E2 and more precisely in the "wings" insertion which could be involved in alphavirus interactions with cell receptors (Voss et al., 2010). Accordingly, the mechanism or arbidol antiviral activity may be related to cell adsorption. This hypothesis was reinforced by the observed inhibition of CHIKV hemagglutination by ARB. However, structural studies have also demonstrated that CHIKV E2 protein was tightly associated with E1, a viral protein mostly implicated in membrane fusion (Kielian, 2010; Li et al., 2010). Although the E2 domain B has been identified as the covered E1 fusion loop, it cannot be excluded that residue 407 may be indirectly involved in the fusion process. Altogether, while a precise mechanism remains to be fully elucidated, our results strongly suggested that arbidol interferes with the early stages of Chikungunya virus infection (virus attachment or entry) by targeting the cellular membrane. In conclusion, arbidol, a molecule which has been extensively used previously for the treatment of viral respiratory infections in humans, was found to be a potent inhibitor against *in vitro* CHIKV infection. In addition to the elucidation of its cellular mode of action, this drug should be further evaluated for the prevention of CHIKV infection and for the management of severe presentations.

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