

ORIGINAL ARTICLE

Assessment of flavaglines as potential chikungunya virus entry inhibitors

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ABSTRACT

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne alphavirus that recently caused large epidemics in islands in, and countries around, the Indian Ocean. There is currently no specific drug for therapeutic treatment or for use as a prophylactic agent against infection and no commercially available vaccine. Prohibitin has been identified as a receptor protein used by chikungunya virus to enter mammalian cells. Recently, synthetic sulfonyl amidines and flavaglines (FLs), a class of naturally occurring plant compounds with potent anti-cancer and cytoprotective and neuroprotective activities, have been shown to interact directly with prohibitin. This study therefore sought to determine whether three prohibitin ligands (sulfonyl amidine 1 m and the flavaglines FL3 and FL23) were able to inhibit CHIKV infection of mammalian Hek293T/17 cells. All three compounds inhibited infection and reduced virus production when cells were treated before infection but not when added after infection. Pretreatment of cells for only 15 minutes prior to infection followed by washing out of the compound resulted in significant inhibition of entry and virus production. These results suggest that further investigation of prohibitin ligands as potential Chikungunya virus entry inhibitors is warranted.

Key words Chikungunya virus, entry inhibitor, flavaglines, sulfonyl amidines.

Chikungunya virus, the cause of CHIKF, is transmitted to humans by the bites of infected *Ae* mosquitos, most commonly *Ae. aegypti* and *Ae. albopictus*; CHIKV-infected patients develop symptoms some 2–4 days (range 1 to 12 days) days after being bitten (1). Clinically, the disease is similar to dengue fever, patients present with a sudden febrile illness with rash, headache, edema of the extremities, gastrointestinal complaints, myalgia, and polyarthralgia (a hallmark of CHIKV infection) that frequently persists for two or more months (1).

Chikungunya virus belongs to the *Alphavirus* genus, family *Togaviridae*. The genome is an 11.8 kb positive sense single stranded RNA with a 5'-methylguanylate cap and a 3'-poly A tail (2). The genome possesses two open reading frames, which encode for four nsPs (nsP1–nsP4), three structural proteins (capsid, E1 and E2) and two proteins of ill-defined function (E3 and 6K). The CHIKV virion is approximately 70 nm in diameter and contains a nucleocapsid surrounded by a lipid bilayer envelope in which are embedded 80 trimeric spikes composed of 240 heterodimers of the E1 and E2

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List of Abbreviations: *Ae.*, Aedes; CC_{50} , 50% cytotoxicity concentration; CHIKF, Chikungunya fever; CHIKV, Chikungunya virus; DMEM, Dulbecco's modified Eagle's medium; E, envelope; ECSA, East Central and South Africa; FL, flavagline; IC_{50} , 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nsP, nonstructural protein(s); pfu, plaque forming unit; PHB, prohibitin.

glycoproteins (3). The 52 kDa E1 glycoprotein mediates fusion of virus with host cell membrane, whereas the 50 kDa E2 glycoprotein is responsible for receptor binding (2).

Chikungunya fever was first formally described after an outbreak in Tanzania in 1952 (4). The virus was first isolated from the same outbreak (5) and was subsequently shown to be present in several parts of Africa and South and Southeast Asia (6). An outbreak of CHIKF in Kenya in 2004 (7) spread to the Indian Ocean islands of Comoros and Seychelles in 2005 (8) and then to Mauritius and La Reunion (9). Some 266,000 cases of CHIKF were believed to have occurred in La Reunion (10, 11) and outbreaks were subsequently reported in India starting from 2005, and later in Singapore, Malaysia and Thailand and other parts of Southeast Asia (12–14). CHIKV has now been reported in many countries, and has caused particular concern through cases of autochthonous transmission in European countries (15).

A number of cell types, including epithelial, endothelial, fibroblast and monocyte-derived macrophage, are susceptible to CHIKV infection, whereas monocytes, lymphocytes, NK cells and monocyte-derived dendritic cells are reportedly not susceptible to CHIKV infection (16, 17). However, one study has shown CHIKV infection and replication in monocytes (18). The mechanism of entry of the virus remains somewhat unclear. Sourisseau and colleagues proposed that CHIKV entry (into HeLa cells) was via a clathrin-dependent pathway (16); however, Bernard and colleagues proposed that entry was via a clathrin-independent, Eps-15-dependent endocytic pathway (19). Both studies proposed that a cholesterol and pH dependent step is required for infection (19, 16).

In a recent study, PHB1 was identified as a receptor protein for CHIKV entry into mammalian cells (20). PHB1 and its homologue PHB2 are pleiotropic scaffold proteins that act as signaling hubs (21). Multiple heterodimers of PHB1 and PHB2 organize into large,

ring-like structures with diameters of 20–25 nm in the mitochondria (22–24), whereas in the cytoplasm, endoplasmic reticulum, nucleus and plasma membrane PHB forms heterodimers with signaling proteins to regulate many aspect of cell physiology, including mitochondrial biogenesis, survival, metabolism and cell division (25).

Cell surface-expressed PHB has been shown to act as an interacting molecule for the Vi polysaccharide of *Salmonella typhi* (26), whereas PHB expressed on the surfaces of insect cells has been shown to be a receptor for dengue virus (27) and an interacting protein for Cry4B, one of the major insecticidal toxins produced by *Bacillus thuringiensis* (28).

There is currently no specific antiviral treatment or commercially available vaccine to either protect against or treat CHIKV infections, although there are a number of vaccine development programs (29–32). Flavaglines are a family of plant natural products that have potent anticancer and neuro- and cardio-protective properties (33–35); studies have shown that prohibitins are directly interacting targets of this class of molecules (36). The synthetic sulfonyl amidine 1 m, which inhibits bone remodeling, also reportedly binds PHB1 (37). This study therefore sought to determine whether the synthetic flavaglines FL3 and FL23 and sulfonyl amidine 1 m are able to interfere with the CHIKV–prohibitin interaction that occurs at the receptor binding stage of CHIKV infection of mammalian cells.

MATERIAL AND METHODS

Compounds

The structures of flavaglines FL3 ($C_{25}H_{23}BrO_5$; MW: 483.36), FL23 ($C_{26}H_{24}BrNO_5$; MW 510.38) and sulfonyl amidine 1 m ($C_{20}H_{32}N_2O_2S$; MW 364.55) are shown in Figure 1. FL3 and sulfonyl amidine 1 m were synthesized according to described procedures (38, 21). The purity of these compounds was over 95% based on reversed-phase

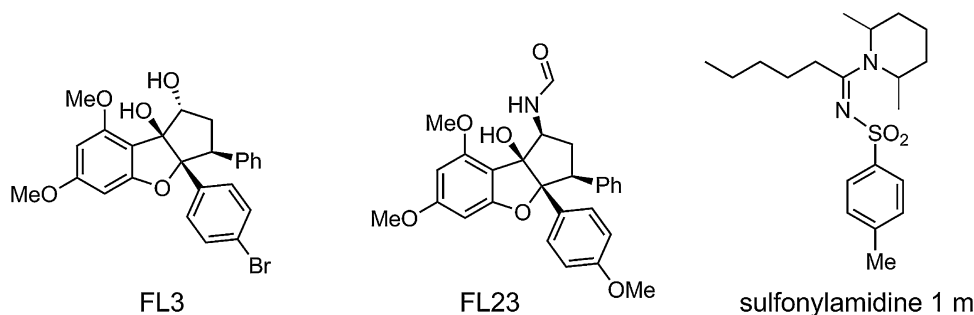


Fig. 1. Structures of flavagline FL3, FL23 and sulfonyl amidine 1 m.

HPLC analyses (Hypersil Gold column 30 1 mm, C18, Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: flow rate, 0.3 mL/min; buffer A, CH₃CN, buffer B, 0.01% aqueous trifluoroacetic acid; gradient, 98–10% buffer B over 8 min (detection, $\lambda = 220/254$ nm). The compounds were initially dissolved in DMSO as stock and serially diluted in DMEM for working solutions with a final DMSO concentration of less than 0.1%. Vehicle/DMEM used as a control consisted of DMSO diluted to 0.1% in DMEM.

Cell culture and virus propagation

The human embryonic kidney cell line Hek293T/17 (ATCC Cat No. CRL-11268) was cultured in DMEM supplemented with 10% heat-inactivated FBS and 100 units/mL of penicillin/streptomycin at 37 °C with 5% CO₂, whereas Vero (ATCC Cat No. CCL-81) was cultured with 5% FBS and 100 units/mL of penicillin/streptomycin.

The CHIKV used in this study was a Thai isolate of an ECSA strain with genotype E1:226V ECSA; it was propagated and stock virus produced as described previously (17). Stock titers were determined by standard plaque assays, undertaken essentially as described elsewhere (17, 20).

Immunofluorescence

Immunofluorescence was performed essentially as described previously (39) by incubating Hek293T/17 cells with 20 pfu/cell CHIKV for 1 hr at 4 °C in the presence or absence of various compounds at 100 nM. The cells were subsequently fixed by treatment with pre-cooled 1% paraformaldehyde in PBS for 20 min and then incubated with a 1:100 dilution of a mouse monoclonal anti-alphavirus (3581) antibody (STSC-58088, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a 1:200 dilution of a goat polyclonal anti-PHB-1 antibody (SC-18196, Santa Cruz Biotechnology) followed by incubation with a 1:150 dilution of an Alexa Fluor 488 labeled donkey anti-mouse IgG antibody (A11029, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and a 1:150 dilution of an Alexa Fluor 568 labeled donkey anti-goat IgG antibody (A11057, Invitrogen, Thermo Fisher Scientific). The cells were visualized under an Olympus FluoView 1000 confocal microscope (Olympus, Shinjuku-ku, Tokyo, Japan) equipped with Olympus FluoView Software v. 1.6. Image analysis and calculation of Pearson correlation coefficients and CIs were carried out as described previously (40).

Cell viability analysis

Cell viability was determined using a Vybrant MTT Cell proliferation assay kit (V13154, Invitrogen, Thermo

Fisher Scientific) according to the manufacturer's recommendations. The percentage cell viability was calculated from the average of eight sample measurements compared with negative control (vehicle/DMEM).

Flow cytometry

Flow cytometry to determine cellular infection with CHIKV was undertaken exactly as described elsewhere (17). The cells were analyzed on a BD FACalibur cytometer (Becton Dickinson, BD Biosciences, San Jose, CA, USA) and data analyzed using CELLQuest™ software.

Analysis of apoptosis

Analysis of induction of apoptosis in response to CHIKV infection was performed exactly as described elsewhere (17). As a positive control, Hek293T/17 cells were treated with 5% DMSO diluted in complete DMEM for 24 hrs. All experiments were undertaken independently in triplicate.

Assay of effect of compounds on CHIKV infection

Hek293T/17 cells were seeded in six well culture plates and grown under standard conditions until the cells reached approximately 90% confluence. The cells were then pre incubated with different concentrations of FL3, FL23 and sulfonyl amidine 1 m finally diluted in DMEM for the times stated, or incubated with the equivalent volume of vehicle/DMEM, after which they were washed and incubated with 10 pfu/cell of CHIKV ECSA genotype (E1:226V) in the absence of the drug. Following infection, cells were washed three times with DMEM, after which they were re-incubated in complete medium containing the compound or the equivalent of vehicle/DMEM. At 20 hrs the cell pellets were analyzed by flow cytometry and the supernatant by standard plaque assay to determine CHIKV titers. In some experiments, compounds were added at designated times after the virus infection step. All experiments were undertaken independently in triplicate, with duplicate plaque assays.

Assay of effect of pulse addition of compounds on CHIKV infection

Hek293T/17 cells were seeded in six wells culture plates and grown under standard conditions until the cells reached approximately 90% confluence. Cells were then pre incubated with different concentrations of FL3, FL23 and sulfonyl amidine 1 m finally diluted in DMEM or incubated with the equivalent volume of vehicle/DMEM for 15 minutes, after which they were washed twice with

DMEM and subsequently incubated with 10 pfu/cell of CHIKV ECSA genotype (E1:226V) in the absence of the drug. Following infection cells were washed three times with DMEM, after which they were re-incubated in complete medium. At 20 hrs the cell pellets were analyzed by flow cytometry and the supernatant analyzed by standard plaque assay to determine CHIKV titers. All experiments were performed independently in triplicate, with duplicate plaque assays.

Assay for virucidal activity

Stock CHIKV ECSA genotype (E1:226V) was incubated directly with different concentrations of FL3, FL23 and sulfonyl amidine 1 m as indicated for 1 hr. The titers of the virus were then directly determined by standard plaque assay. These preparations were also used to infect Hek293T/17 cells that had been cultured in six well plates until they had reached approximately 90% confluence.

Statistical analysis

Viral infection and viral production data were analyzed using the GraphPad Prism program (GrapPad Software). Statistical analysis of significance was undertaken by a paired sample *t*-tests using SPSS (SPSS, Chicago, IL, USA); $P < 0.05$ was considered significant. CC_{50} and IC_{50} values were calculated using freeware ED50plus (v1.0) software (<http://sciencegateway.org/protocols/cellbio/drug/data/ed50v10.xls>).

RESULTS

Colocalization between prohibitin and CHIKV E2 protein

In our previous study identifying PHB as a CHIKV receptor protein, the majority of the analysis was undertaken in CHME-5 (human microglial) cells (20). To investigate the effect of flavaglines on CHIKV entry in this study, we decided to use Hek293T/17 cells because these cells are more suitable for flow cytometry analysis. Our previous study showed that Hek293T/17 cells expressed PHB, which was capable of binding CHIKV (20). We therefore initially confirmed that CHIKV colocalized with PHB on the surfaces of Hek293T/17 cells, as previously shown for CHME-5 cells (20). As shown in Figure 2, we found cell surface expression of PHB and distinct colocalization between PHB and CHIKV.

Evaluation of cytotoxicity, cell death and virucidal activity

We incubated Hek293T/17 cells individually with various concentrations of each compound under

investigation for 24 hrs, after which we assessed cell viability using an MTT assay. We calculated the percentage cell viability from the average of eight replicates and compared it with negative (vehicle/DMEM) and positive (5% DMSO) controls. We found that after 24 hrs incubation, cell viability for FL3, FL23 and sulfonyl amidine 1 m at concentrations of 20 nM or less (0.1, 0.25, 0.5, 1, 5, 10 and 20 nM) was comparable to that of the negative control; however, we saw significant cytotoxicity at values above 20 nM (Supplementary Fig. S1). The compounds showed approximately equal CC_{50} values of 118.77 nM (FL3), 92.18 nM (FL23) and 138.53 nM (sulfonyl amidine 1 m) as calculated from dose response curves (see Supplementary Fig. S2).

To confirm the results of the MTT assay, we cultured Hek293T/17 cells and incubated them with various concentrations of FL3, FL23 and sulfonyl amidine 1 m at 37 °C or mock incubated them with vehicle/DMEM for 1 hr, after which we washed the cells and re-incubated them with the same concentration of drug for a further 24 hrs. We employed this two-step incubation to more closely mimic infection protocols as undertaken at later stages. We found approximately 80% apoptosis induction in cells treated with 5% DMSO as a positive control; however, amounts of apoptosis in cells treated with different concentrations (1, 5, 10 and 20 nM) of the compounds under investigation were comparable to that in negative control (mock) cells treated with vehicle/DMEM (Supplementary Fig. S3).

To evaluate potential virucidal activity, we incubated stock CHIKV directly with each compound separately at different concentrations for 1 hr or with vehicle/DMEM, after which we determined virus titers by standard plaque assays on Vero cells. We observed no direct effect of the compounds on the virus (Supplementary Fig. S4). We repeated the experiment using virus/compound mixtures to infect Hek293T/17 cells and found no significant effect on the ability of the virus to infect into Hek293T/17 cells. Moreover, plaque assay analysis of the highest concentration treatments showed no significant effect on virus production (Supplementary Fig. S4). Combined, these results show that the three compounds tested do not possess anti-CHIKV virucidal activity.

Effect of compounds on CHIKV infection

To determine the effects of the compounds on CHIKV infection, we cultured Hek293T/17 cells and then pre-incubated them with and without FL3, FL23 or sulfonyl amidine 1 m at various concentrations for 1 hr followed by infection with 10 pfu/cells of CHIKV or mock infected in serum-free medium without the compounds

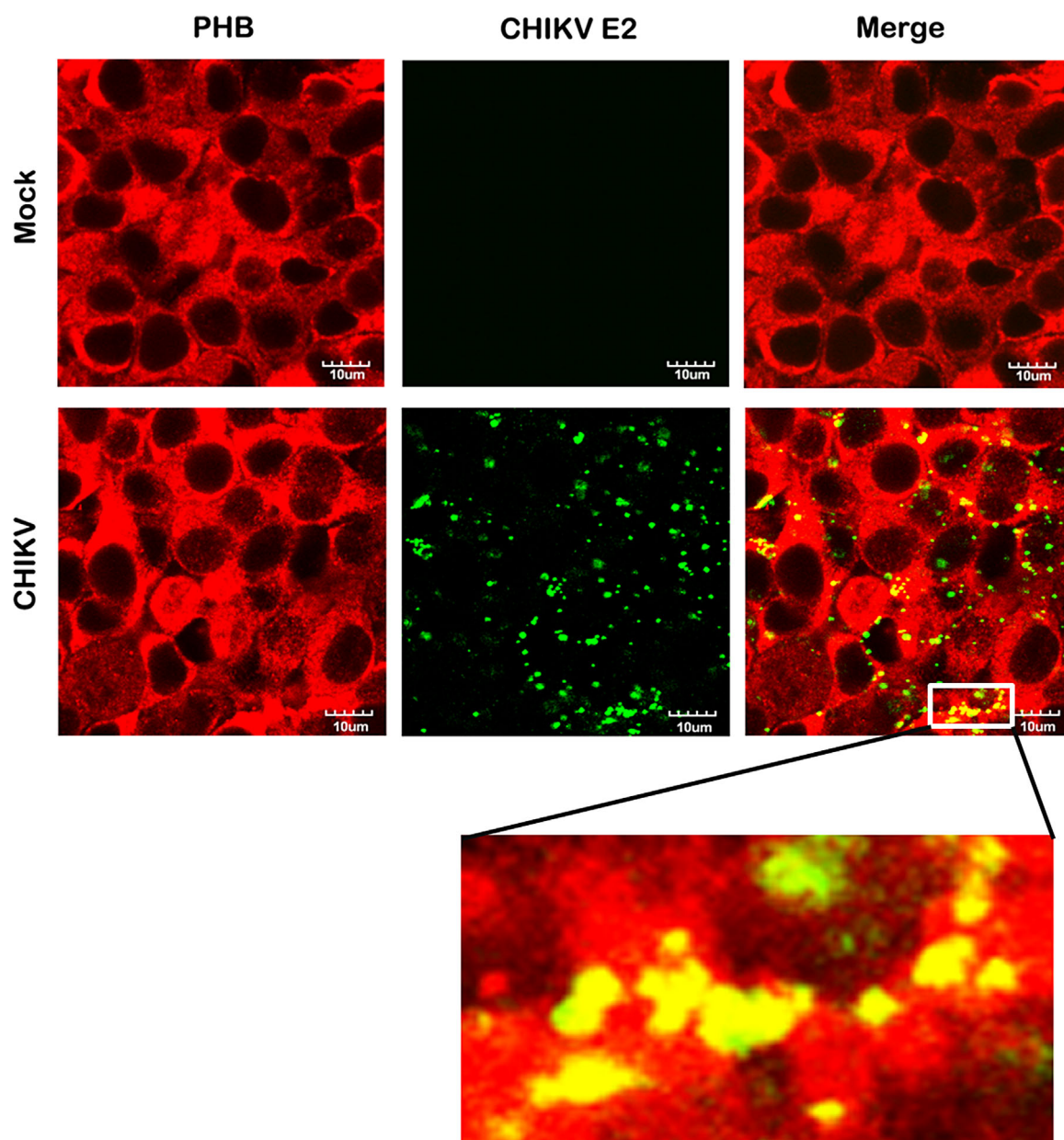


Fig. 2. Colocalization of CHIKV E2 protein with PHB. (a) Hek293T/17 cells were grown on glass slides and incubated with CHIKV or mock incubated and examined for the cell surface colocalization of PHB (red) and CHIKV E2 protein (green). Fluorescent signals were observed using an Olympus Fluoview 1000 confocal microscope. Scale bar for magnification is shown. Representative, non-contrast adjusted, unmerged and merged images are shown.

under investigation. After 2 hr, we removed extracellular virus by washing three times and subsequently incubated the cells in complete medium under standard conditions in the presence of the compounds for 20 hrs, after which we harvested the cells for flow cytometry and collected the supernatant for standard plaque assay. We found a reduction in the percentage of infected cells for all three compounds at concentrations of 10 and 20 nM (Fig. 3a,c,

e), whereas FL3 additionally showed a significant reduction in the percentage of cells infected with CHIKV at 5 nM (Fig. 3a). Similarly, we observed a significant reduction in viral production in cells incubated with 10 and 20 nM of the three compounds (Fig. 3b,d,f and Supplementary Fig. S5), whereas FL23 additionally showed a significant effect on virus production at 1 and 5 nM (Fig. 3d and Supplementary Fig. S5). The

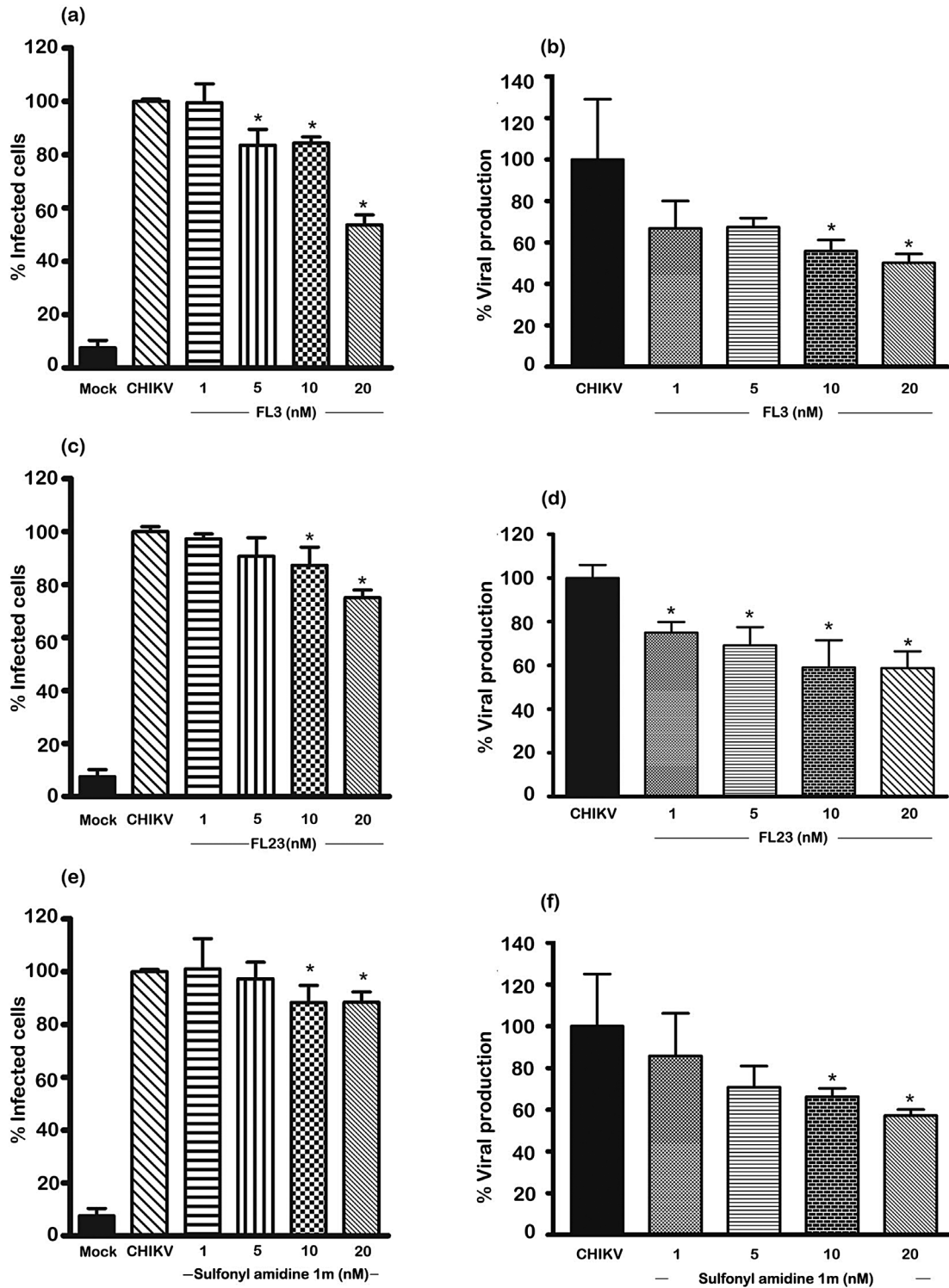


Fig. 3. Effects of various compounds on CHIKV infection of Hek293T/17 cells. Hek293T/17 cells were pre-incubated with vehicle/DMEM (mock and CHIKV) or pre-incubated with (a, b) FL3, (c,d) FL23 or (e,f) sulfonyl amidine 1m for 1 hr before being infected with 10 pfu/cell of CHIKV or being mock infected (Mock). After infection, cells were incubated under standard conditions in the presence or absence of the appropriate drug for 20 hrs before (a, c, e) determination of degree of infection of cells by flow cytometry or (b, d, f) assay of the supernatant for virus titer by standard plaque assay. All experiments were undertaken independently in triplicate with duplicate plaque assays; error bars show SD. *, $P < 0.05$. Mock, pre-incubation with vehicle/DMEM and mock (CHIKV) infection; CHIKV (incubation with vehicle/DMEM and standard infection).

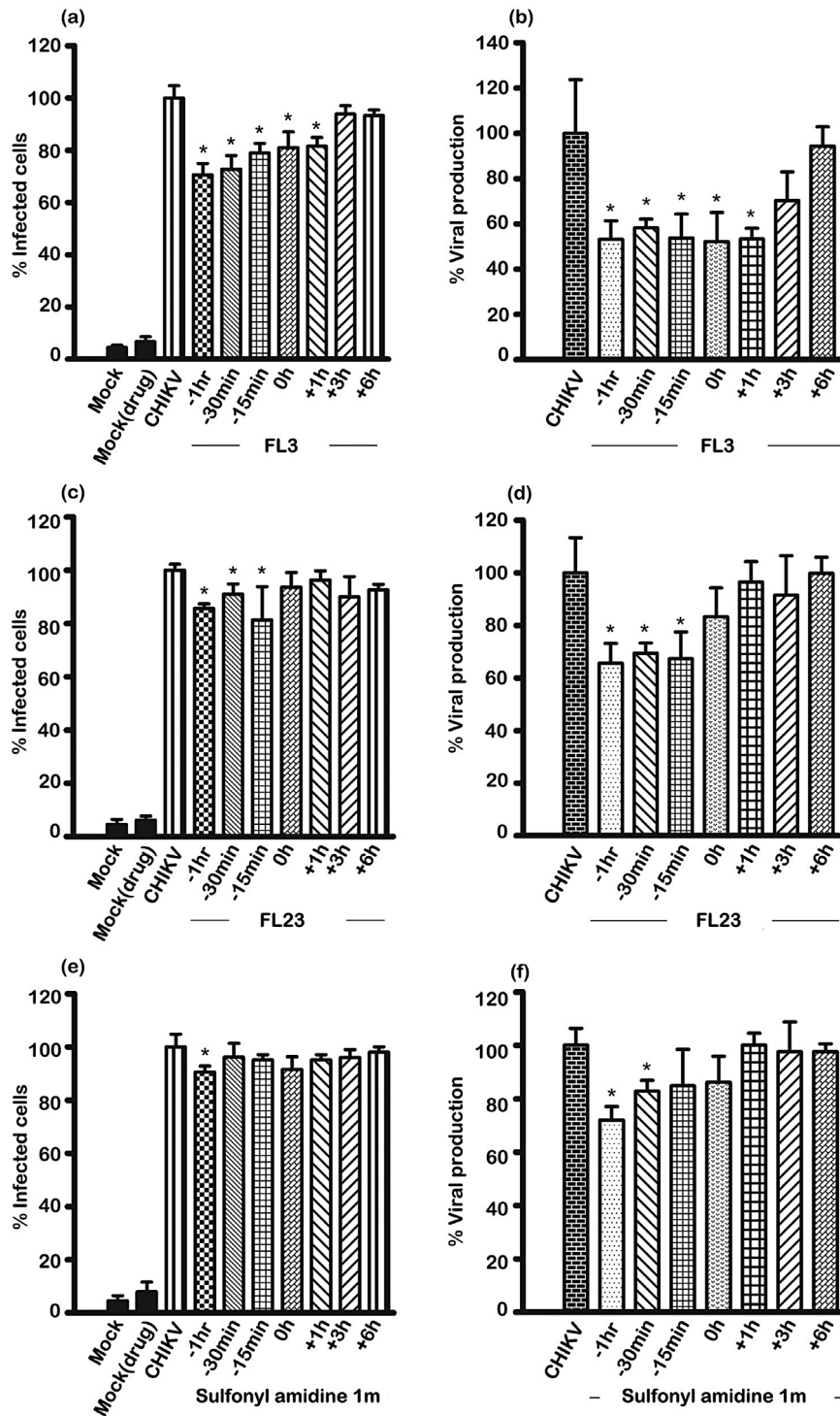


Fig. 4. Time course of effect of flavaglines on CHIKV infection of Hek293T/17 cells. Hek293T/17 cells were mock pre-incubated with vehicle/DMEM or pre-incubated with (a, b) FL3, (c, d) FL23 or (e, f) sulfonyl amidine 1m for the indicated times before being infected with 10 pfu/cell of CHIKV or being mock (Mock) infected, or the compounds were added at the indicated times post-infection. After infection, cells were incubated under standard conditions in the presence or absence of the drug as appropriate for 20 hrs before (a, c, e) determination of degree of infection of cells by flow cytometry or (b, d, f) the supernatant assayed for virus titer by standard plaque assay. Mock, incubation with vehicle/DMEM and mock infection; Mock (drug), incubation with compound and mock infection; CHIKV, incubation with vehicle/DMEM and standard CHIKV infection. All experiments were undertaken independently in triplicate with duplicate plaque assays; error bars show SD. *, $P < 0.05$.

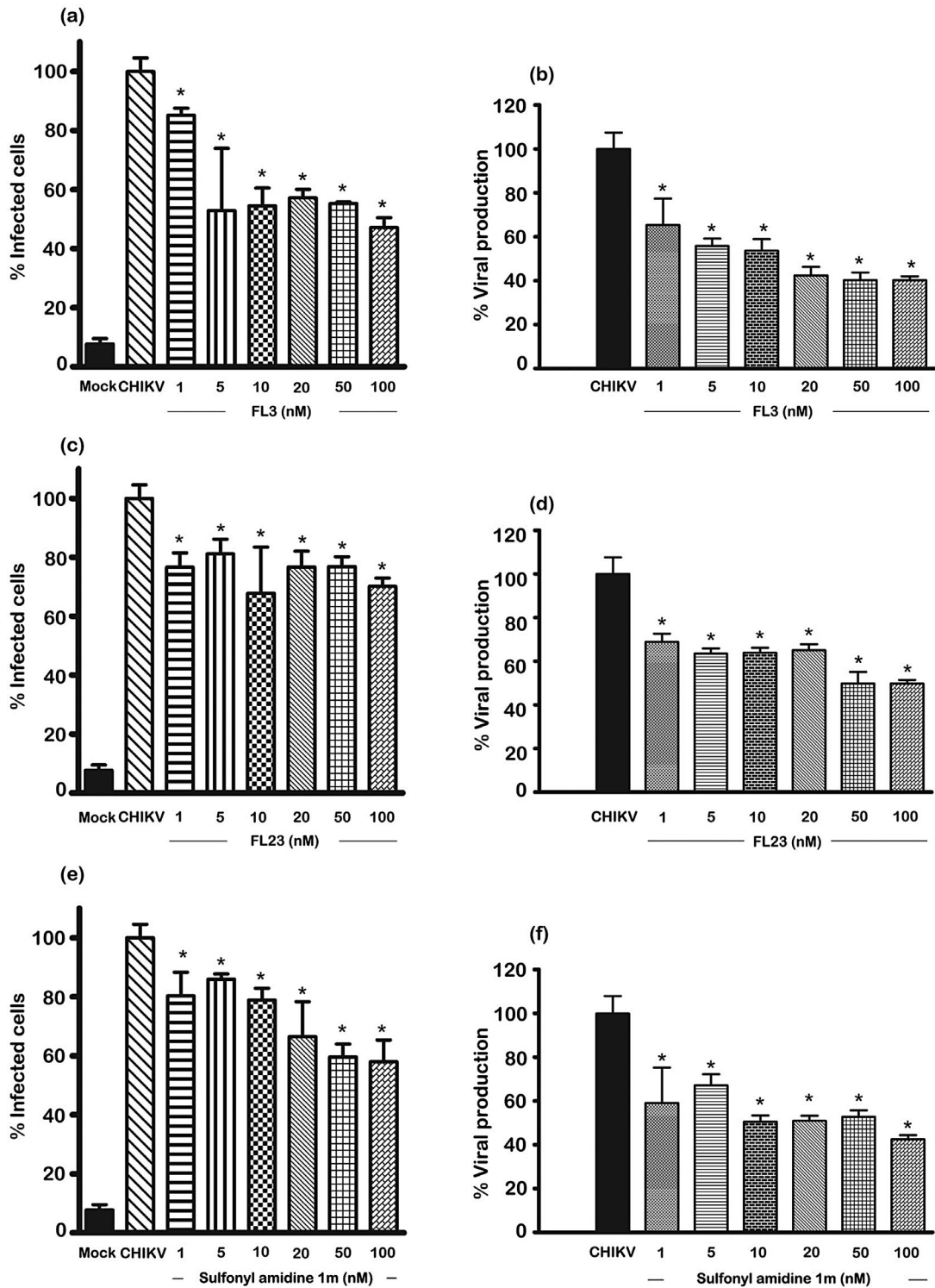


Fig. 5. Effect of pulse pre-treatment of compounds on CHIKV infection of Hek293T/17 cells. Hek293T/17 cells were mock pre-incubated with vehicle/DMEM or pre-incubated with (a, b) FL3, (c,d) FL23 or (e,f) sulfonyl amidine 1m for 15 minutes after which cells were washed twice before being infected with 10 pfu/cell of CHIKV or being mock infected. After infection, cells were incubated under standard conditions for 20 hrs before (a, c, e) determination of degree of infection of cells by flow cytometry or (b, d, f) assay of the supernatant for virus titer by standard plaque assay. All experiments were undertaken independently in triplicate with duplicate plaque assays; error bars show SD. *, $P < 0.05$. Mock, pre-incubation with vehicle/DMEM and mock (CHIKV) infection; CHIKV, (incubation with vehicle/DMEM and standard infection).

maximum effect observed was with 20 nM FL3, which reduced both infection and virus production by nearly 50%, the IC₅₀ for this compound was 22.4 nM.

Time course analysis of the effect of compounds on CHIKV infection

To determine whether the compounds were having an effect at the entry step of CHIKV infection, we incubated cells with the highest non-cytotoxic concentrations of the compounds (20 nM) for increasingly shorter times pre-infection, or added them only after infection. We analyzed cells and supernatant at 20 hrs post-infection as in previous experiments.

We found that FL23 and sulfonyl amidine 1 m only had effects on the number of infected cells and the amount of virus production when the cells were pre-incubated with the compounds (Fig. 4). We saw no effect when we added these two compounds post-infection (Fig. 4c–f and Supplemental Fig. S6). FL23 showed some effect when the pre-incubation was as short as 15 minutes pre-infection, whereas sulfonyl amidine 1 m showed an effect on number of cells infected only with pre-incubation for 1 hr pre-infection.

FL3 showed both the largest effect and the greatest range of time points that had an effect. Even incubation with FL3 at 1 hr post-infection had a significant effect on both number of cells infected and amount of virus produced. This may mean that continued inhibition of virus entry was not completely prevented by the washing step and suggests that FL3 may remain associated with PHB for a longer time than either FL23 or sulfonyl amidine 1 m. However, addition of FL3 at 3 hrs post-infection showed no significant effect.

Effect of pulse treatment of compounds on CHIKV infection

The results of the time course experiment are consistent with the action of the compounds occurring at the attachment/entry step of CHIKV infection. To further verify this, we undertook experiments with a short (15 minutes) pulse pre-treatment with wash out of the compound before infection. Because the cell treatment was for a short period, it was possible that we could use higher concentrations of the compounds. To verify this, we treated cells for 15 minutes with different concentrations of the compounds (up to 100 nM), after which we washed the cells twice with DMEM before incubating them under standard conditions for 24 hrs and assessing cell viability as undertaken previously. We found no evident loss of cell viability, even at the highest concentration used (100 nM; Supplemental Fig. 7). We therefore repeated the experiment and infected the cells after washing with

CHIKV and assayed for degree of infection and virus production at 20 hrs post-infection as before. We found a significant reduction in both infection and virus production under all conditions tested (Fig. 5 and Supplemental Fig. S8). Virus output for both FL3 and sulfonyl amidine 1 m was reduced by nearly 60% by pulse treatment at 100 nM as compared with untreated cells.

Effect of compounds on CHIKV receptor binding

Finally, to confirm that the compounds acted by interfering with receptor binding, we repeated the initial colocalization experiment between PHB and CHIKV, this time in the presence or absence of each of the compounds. We found a marked reduction in colocalization between CHIKV and PHB when we incubated the virus with cells in the presence of the compounds at 100 nM, as compared to incubation with no compound (Fig. 6). The high degree of colocalization between CHIKV and PHB (Pearson correlation coefficient 0.85, 95% CI 0.82–0.88) was reduced to a statistically significantly degree in the presence of FL3 (Pearson correlation coefficient 0.22; 95% CI 0.09–0.34; $P < 0.05$) and FL23 (Pearson correlation coefficient 0.63; 95% CI 0.53–0.73; $P < 0.05$) and reduced, although not significantly, in the presence of sulfonyl amidine 1 m (Pearson correlation coefficient 0.72; 95% CI 0.34–1.09) These results confirm that the ligands interfere with the receptor binding of CHIKV.

DISCUSSION

Prohibitin is located in a number of cell compartments, primarily the mitochondria; cell surface expression has also been demonstrated in a number of studies (41, 42, 26, 20, 43, 44). PHB has been shown to interact with a number of different pathogens or pathogen proteins including CHIKV (20), dengue (27), severe acute respiratory syndrome coronavirus (45), HIV (46) and foot-and-mouth disease virus (47).

Prohibitins have been shown to be specific targets for flavaglines (36). Flavaglines are a family of natural products found in plants of the genus *Aglaia*, family *Meliaceae* (mahogany), of which the first identified molecule was rocaglamide (48). Rocaglamide was first identified based on its potent anti-leukemic activity; since then both natural and synthetic flavaglines have been shown to have potent anti-cancer properties, often in the low nanomolar range (34, 35). Recent studies have suggested that flavaglines, including rocaglamide and silvestrol, are promising candidates for the treatment of leukemia (49). Studies have shown that the anti-cancer

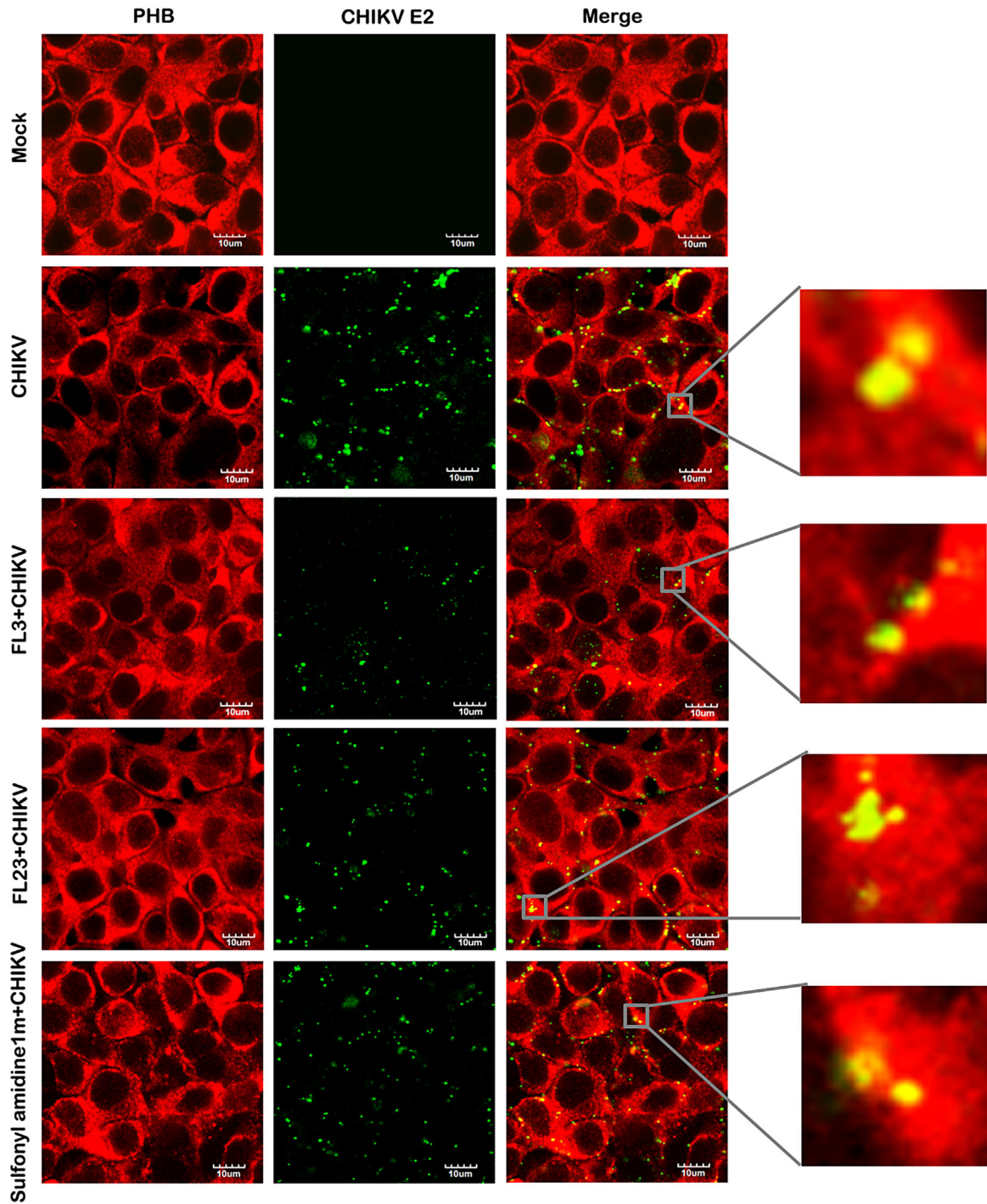


Fig. 6. Colocalization of CHIKV E2 protein with PHB in the presence and absence of test compounds. Hek293T/17 cells were grown on glass slides and incubated with CHIKV or mock incubated in the presence and absence of test compounds at 100 nM and examined for cell surface colocalization of PHB (red) and CHIKV E2 protein (green). Fluorescent signals were observed using an Olympus FluoView 1000 confocal microscope. Scale bar for magnification is shown. Representative, non-contrast adjusted, unmerged and merged images are shown.

action of flavaglines derives, at least in part, from a specific interaction between PHB and flavaglines (36). This study was undertaken to determine whether the interaction between flavaglines and PHB was sufficient to disrupt the interaction between PHB and CHIKV. All of our results are consistent with the compounds acting at the entry stage, possibly by physically interfering with the binding of CHIKV to the PHB receptor. The maximum inhibition observed was approximately 50% of viral entry (seen with pulse treatment of cells with FL3), a degree of inhibition consistent with that seen in previous antibody inhibition experiments (20). The compounds tested showed little effect when added post-entry; while this may suggest that flavaglines have only limited potential utility as prophylactic agents, because natural infections are not synchronous, administration during the course of the disease could have therapeutic effects by preventing new cells from becoming infected. The fact that both antibody inhibition experiments (20) and the compounds tested here showed a maximum effect on entry of about 50% may indicate that CHIKV uses multiple receptor protein to enter susceptible cells.

The compounds tested showed significant cytotoxicity to Hek293T/17 cells under conditions of continuous culture, consistent with the compounds known anticancer activities (34, 35). However, no cytotoxicity was observed after exposure to dramatically higher doses when the compounds were administered as short (15 minute) pulses. Importantly, these compounds show no toxicity to normal cells and do not display any evidence of toxicity *in vivo* (33), suggesting that these compounds, or indeed other ligands of PHB, could form the basis of a prophylactic or therapeutic regime in CHIKV outbreaks.

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DISCLOSURE

The authors have no conflict of interest to disclose.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1: Determination of cell viability. Hek293T/17 cells were incubated with different concentrations of (a) DMSO, (b) FL3, (c) FL23 or (d) sulfonyl amidine 1 m for 24 hours before determination of cell viability using the MTT assay. Data is derived from eight replicates; error bars show SD. *, $P < 0.05$; negative, vehicle/DMEM, positive, 5% DMSO.

Figure S2: Dose response curves generated by the ED50plus (v1.0) software used for CC_{50} calculations.

Figure S3: Analysis of apoptosis in response to treatment with flavaglines. Hek293T/17 cells were incubated with different concentrations of (a) FL3, (b) FL23 or (c) sulfonyl amidine 1 m for 24 hours before determination of the amount of apoptosis by annexin V/propidium iodide staining and analysis by flow cytometry. Experiments were undertaken independently in triplicate; error bars show SD. Mock, vehicle/DMEM.

Figure S4: Analysis of virucidal activity of flavaglines. Stock CHIKV was incubated for 1 hr in the presence or absence of various concentrations of FL3, FL23 and

sulfonyl amidine 1 m after which the virus was either (a) directly assayed by standard plaque assay or (b) used to infect Hek293T/17, with the percentage infection being determined at 20 hours by flow cytometry. (c) Virus titers in the supernatants from 20 nM treatments in (b) were assayed by standard plaque assay. Experiments were undertaken independently in triplicate with duplicate plaque assays; error bars show SD. Mock, mock virus incubated with vehicle/DMEM; CHIKV, CHIKV incubated with vehicle/DMEM.

Figure S5: Virus production data from Figure 3 plotted as pfu/mL.

Figure S6: Virus production data from Figure 4 plotted as pfu/mL.

Figure S7: Determination of cell viability after pulse treatment. Hek293T/17 cells were incubated with different concentrations of (a) FL3, (b) FL23 or (c) sulfonyl amidine 1 m for 15 minutes, after which cells were washed and incubated under standard conditions for a further 24 hrs before determination of cell viability using the MTT assay. Data is derived from eight replicates. Negative (vehicle/DMEM) and positive (5% DMSO) controls were run in parallel. Error bar show SD. *, $P < 0.05$.

Figure S8: Virus production data from Figure 5 plotted as pfu/mL.