Hepatitis C virus *in vitro* replication is efficiently inhibited by acridone Fac4

Guilherme Rodrigues Fernandes Campos,[1] Cíntia Bittar,[1] Ana Carolina Gomes Jardim,[2] Jacqueline Farinha Shimizu,[1] Mariana Nogueira Batista,[1] Eder Ramos Paganini,[1] Letícia Ribeiro de Assis,[1] Christopher Bartlett,[3] Mark Harris,[3] Vanderlan da Silva Bolzani,[4] Luis Octavio Regasini[1,4] and Paula Rahal[1,*]

Abstract

Hepatitis C virus (HCV) affects about 170 million people worldwide. The current treatment has a high cost and variable response rates according to the virus genotype. Acridones, a group of compounds extracted from natural sources, showed potential antiviral actions against HCV. Thus, this study aimed to evaluate the effect of a panel of 14 synthetic acridones on the HCV life cycle. The compounds were screened using an Huh7.5 cell line stably harbouring the HCV genotype 2a subgenomic replicon SGR-Feo-JFH-1. Cells were incubated in the presence or absence of compounds for 72 h and cell viability and replication levels were assessed by MTT and luciferase assays, respectively. At a concentration of 5 µM the acridone Fac4 exhibited a >90 % inhibition of HCV replication with no effect on cell viability. The effects of Fac4 on virus replication, entry and release steps were evaluated in Huh7.5 cells infected with the JFH-1 isolate of HCV (HCVcc). Fac4 inhibited JFH-1 replication to approximately 70 %, while no effect was observed on virus entry. The antiviral activity of Fac4 was also observed on viral release, with almost 80 % of inhibition. No inhibitory effect was observed against genotype 3 replication. Fac4 was able to intercalate into dsRNA, however did not inhibit NS5B polymerase activity or translation driven by the HCV IRES. Although its mode of action is partly understood, Fac4 presents significant inhibition of HCV replication and can therefore be considered as a candidate for the development of a future anti-HCV treatment.

INTRODUCTION

Hepatitis C virus (HCV) is a global health problem, widely distributed, that affects approximately 170 million people around the world [1, 2]. HCV is a single-stranded RNA-positive genome virus that belongs to the *Flaviviridae* family and is classified as a group IV virus, according to Baltimore classification [3–5]. With a genome of 9.6 kb, flanked by 3′ and 5′ untranslated regions, the open reading frame codes for a polyprotein of about 3000 amino acids [6]. Viral and host proteases cleave this polyprotein, producing three structural proteins (Core, E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [4, 7, 8].

Due to the high genetic variability, mainly derived from the lack of proof-reading activity of RNA-dependent RNA polymerase NS5B and high replication rate during infection [9], HCV is divided into genotypes (1 to 7) and subtypes (classified by lowercase letters – a, b, c) [10–12]. Furthermore, in an infected individual it circulates as a pool of genetically related variants, named quasispecies, which provide a favourable environment for the emergence of mutations resulting in drug resistance [13–16]. Therefore, the quasispecies nature of HCV has a direct impact on the effectiveness of treatment with usual medications, as well as the development of new antivirals [17].

With the current development of direct-acting antivirals (DAAs) such as protease, polymerase and NS5A inhibitors, the most effective treatment is the administration of DAAs with or without PEG-IFN-α and ribavirin [18–20]. Treatment strategy is designed based on virological, clinical and liver pathology aspects. The sustained virological response (SVR) is variable and dependent on virus genotype and the stage of liver disease. Jacobson *et al.* evaluated two groups of patients during 12 weeks of treatment with sofosbuvir and ribavirin. The group infected with HCV genotype 2 presented around 90 % of SVR, while patients infected with genotype 3 showed only 61 % of SVR [21]. This reduced efficacy in genotype 3, coupled with potential side effects such as anaemia, autoimmune disorders, diarrhoea, rash, retinopathy and weight loss, as well as the elevated cost, means that additional therapeutic options are still required [22].

Alkaloids are a central class of natural products, which have been extensively used as modern drug prototypes and drugs [23]. Among these, acridones are planar compounds isolated from Rutaceae plants, which exhibit several bioactivities, including antimicrobial, cytotoxic, algicidal, moluscicidal, anti-allergic and antidiabetic [24]. Also, synthetic compounds containing the acridone framework have demonstrated correlated bioactivities to their natural analogues [25]. The antiviral action of acridones is well known in the literature against HSV-2 and CMV replication and inhibiting HIV-1 transcription [26–28]. Recent studies revealed the potential anti-HCV effect of acridone derivatives as NS3 helicase inhibitors and dsRNA intercalants, inhibiting viral replication [29, 30].

Considering the high cost, several side effects and the emergence of resistance mutations which decrease the response to treatment, the development of new drugs against the virus remains an important subject of research. The aim of this study was to investigate the effects of the synthetic acridone Fac4 on the HCV life cycle by the use of *in vitro* approaches.

RESULTS

Inhibitory activity of Fac4 on JFH-1 replication

We performed screening of a panel of 14 synthetic acridones to select those with potential antiviral activity on HCV replication. Huh7.5 cells stably harbouring SGR-Feo-JFH-1 were treated with compounds at 50, 10, 2 and 0.4 µM. After 72 h incubation, luciferase and MTT assays were performed in parallel to evaluate the replication inhibition and cell viability under treatment with the compounds, respectively. Amongst the tested acridones (Fig. S1, available in the online Supplementary Material), Fac4 presented a potential activity against HCV replication. At 10 µM Fac4 presented a cell viability of 85 % with inhibition of viral replication by approximately 93 %. Fac4 inhibited replication in a concentration-dependent manner (data not shown) with EC_{50} of 1.33 µM and a selectivity index (SI) (CC_{50}/EC_{50}) of 42.14. To determine a useful SI (favourable ratio of

cytotoxicity to antiviral potency), screening was performed with Fac4 at concentrations from 1 to 10 µM. We observed that Fac4 at 5 µM inhibited 92 % of HCV replication (Fig. 1). Therefore, this concentration was selected for further experiments.

Thus, we evaluate the effects of Fac4 on HCV replication in the context of full length virus. Huh7.5 cells were infected with JFH-1 HCVcc and after 4 h, viral supernatant was removed and cells were treated with Fac4 for 72 h. Cells were fixed, stained and titrated. Fac4 inhibited approximately 70 % of HCV replication (Fig. 1), corroborating the potential antiviral activity against HCV observed in the preliminary replicon assays. As expected, protein expression levels were also significantly reduced in the presence of Fac4 since NS5A was undetectable when cells were treated with Fac4 (Fig. 1).

Once Fac4 presented a potential inhibition of HCV genotype 2a JFH-1 replication, we decided to test if these results are genotype-specific. For that, Huh7.5 cells stably harbouring the genotype 3 subgenomic replicon SGR-Feo-S52 were treated with Fac4 at 5 µM and replication levels were analysed by luciferase assay. No inhibition of genotype 3 replication was observed (Fig. 1).

Fac4 as a dsRNA intercalator

To further investigate the antiviral mode of action of Fac4, we analysed the capacity of this compound to intercalate into dsRNA. Using the 3′UTR region of JFH-1 HCV as a template, we produced an amplicon flanked by a T7 promoter that was used for *in vitro* transcription, synthesizing a dsRNA molecule of 273 bp. This dsRNA was incubated with Fac4 at 5 µM or the controls (DMSO 0.1 % and doxorubicin at 100 µM) and was analysed by a migration retardation assay. Fac4 presented 40 % of dsRNA intercalation when compared to the DMSO negative control, quantified by densitometry (Fig. 2a). Notice that the sample treated with doxorubicin (positive control of intercalation) does not appear in the image, reasserting the observed result.

Fac4 and IRES-mediated translation

An IRES-mediated translation assay was carried out to investigate a possible interaction between Fac4 and IRES and therefore to evaluate if the inhibition of viral replication is related to IRES-mediated translation. Cells electroporated with SGR-Feo-JFH-1 or SGR-luc-JFH-1/GND were immediately incubated with Fac4 or controls and RNA replication was measured after 4 h by luciferase expression analysis. Fac4 did not influence viral RNA translation, since luciferase values of both wild-type replicon and the GND replication defective replicon at 4 h were not reduced, demonstrating that input RNA was translated in the cells (Fig. 2b).

NS5B activity is not inhibited by Fac4

To analyse if Fac4 interferes with the NS5B polymerase, we performed an NS5B RNA-dependent RNA polymerase *in vitro* activity assay. The obtained results showed that Fac4 is
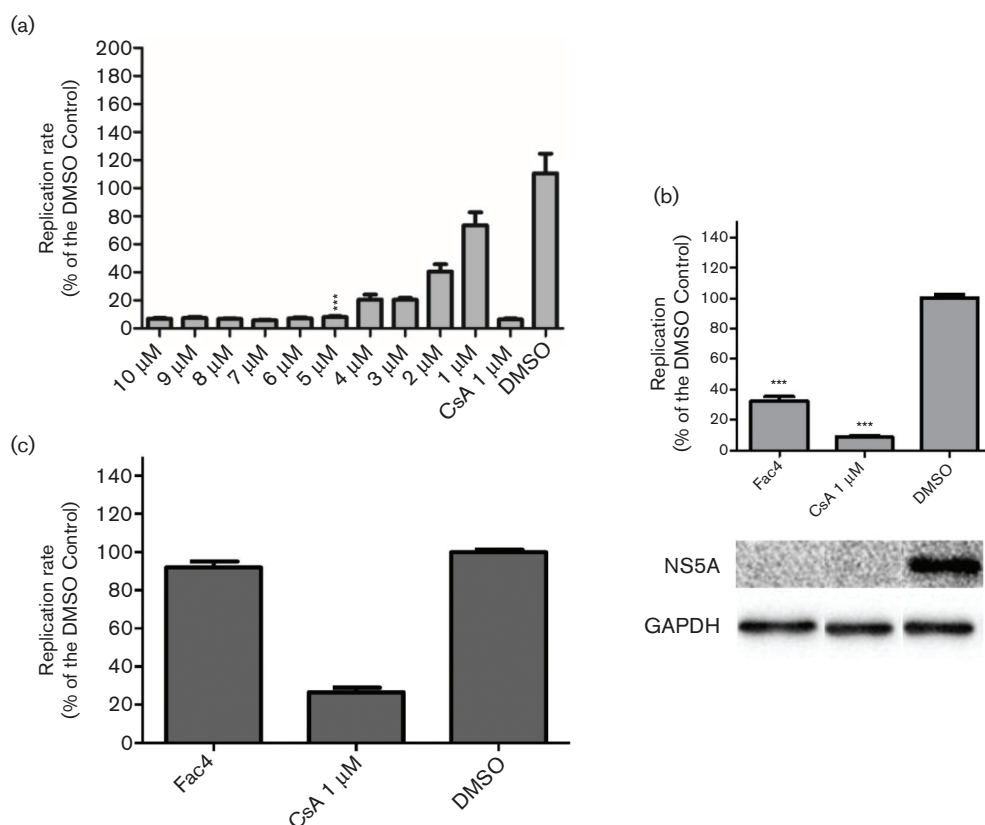
Fig. 1. Fac4 effect on HCV replication: (a) Fac4 concentration screening, determining 5 µM as the working concentration (the lower concentration with the best inhibitory effect and cell viability). (b) Replication assay in Huh7.5 infected with JFH-1 HCVcc and Western blot for NS5A viral protein detection. (c) Replication assay in Huh7.5 stably expressing genotype 3 subgenomic replicon SGR-Feo-S52. Cyclosporin (CsA) used as positive control. The three asterisks represent a significant difference between control group and treated group at $P<0001$.

not capable of inhibiting NS5B enzymatic activity, at any of the tested concentrations (50, 5 and 0.5 µM) (Fig. 3).

Fac4 does not block virus entry

To evaluate whether Fac4 possess antiviral activity on HCV entry, Huh7.5 cells were infected with JFH-1 virus in the presence or absence of Fac4 for 4 h. Viral inoculum was replaced by fresh media and intracellular virus was quantified 72 h post-infection (h p.i.). No blockage of viral entry was observed (Fig. 4).

Fac4 inhibits HCV release

Since Fac4 has antiviral activity against HCV replication but does not act during the viral entry process, we decided to analyse the release step. We observed that intracellular RNA in Fac4-treated cells displayed similar values as the non-treated cells (Fig. 5), and this is corroborated by the result obtained in the replication assay performed 24 h after treatment, where luciferase levels were similar to the control (Fig. S2). Despite this fact, a pronounced effect on virus release (extracellular RNA) is observed, since there was a difference of 80% in the amount of intra and extracellular HCV RNA (Fig. 5).

DISCUSSION

The antiviral effect of acridones has been described in the literature and the activity spectrum of this class of alkaloids varies depending on the type of viral genome (double-stranded DNA genome or RNA viruses) [31]. Some acridones from Rutaceae plants showed great antiviral activity against viruses with DNA genomes like herpes simplex virus serotypes 1 and 2 (HSV-1 and HSV-2), human cytomegalovirus (HCMV) and Epstein–Barr virus [28, 32–34]. For RNA viruses, acridones presented activity against HIV-1, bovine viral diarrhoea virus (BVDV), all serotypes of dengue virus (DENV) and HCV, the last three belonging to the *Flaviviridae* family [26, 27, 29, 30, 35–39].

Our results showed that Fac4 inhibited up to 92 % of HCV replication in the context of either the subgenomic replicon or full length JFH-1 HCVcc. Also, NS5A viral protein expression could not be detected after treatment with this compound. Despite its considerable effect on HCV genotype 2, inhibition was not observed on HCV genotype 3 replication. So far, all NS3 protease inhibitors available have also no effect on HCV genotype 3 [20, 40–42]. Altogether, the hypothesis that Fac4 may be interfering with NS3
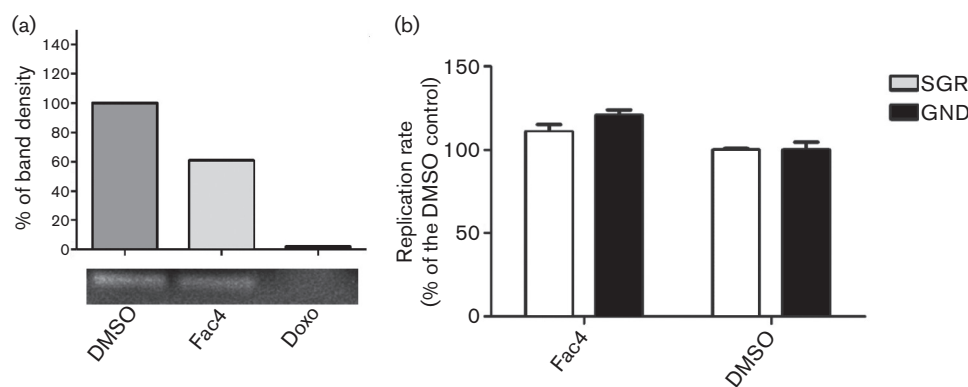
Fig. 2. Fac4 possible mechanisms of action: (a) intercalation assay, evaluating Fac4 dsRNA intercalation property. Doxorrubicin (Doxo) was used as a positive control. (b) IRES interaction assay in Huh7.5 cells. Replication rate 4 h after electroporations. Subgenomic replicon SGR-Feo-JFH-1 (SGR); defective mutated subgenomic replicon SGR-luc-JFH-1/GND (GND).

protease is strengthened. However, to determine whether Fac4 is inhibiting NS3 protease activity, further functional studies are needed.

Regardless of the observed inhibition of replication, Fac4 had no activity on HCV viral entry. This could be explained by the way acridones usually act against virus infection. Some authors argue that their nucleic acid intercalation ability and interaction with viral enzymes are the main mechanisms by which these compounds act [29, 30, 43]. For HCV, data presented by Stankiewicz-Drogon *et al.* reinforces this assumption. Acridones showed inhibition of NS3 helicase, T7 RNA polymerase (topology and function similar to HCV NS5B) and strong double-stranded RNA intercalation property. All these elements are involved in the replication step [29, 30].

It is not clear yet if there is a combination of the reported effects of acridones on the inhibition of replication. Some acridones described in the literature present dsRNA intercalation property, others show inhibition of NS3 helicase and NS5B polymerase, and some present both effects [29, 44]. However, all these studies were performed in isolated assays, evaluating inhibition of enzymatic activity or dsRNA intercalation individually. According to our results, Fac4 presented the ability to partially intercalate into dsRNA; however, it did not inhibit RNA polymerase activity, as we observed *de novo* transcript production in the NS5B activity assay. As reported before [29, 30], it is presumed that HCV replication cannot be inhibited by dsRNA intercalation alone, and probably it is due to a combined effect between different modes of action. Therefore, replication inhibition by Fac4 may be somewhat related to dsRNA intercalation, which is a replication intermediate. However, it is likely that another mode of action is also involved [29, 30, 39]. Another possible explanation for the antiviral activity of Fac4 is the targeting of cellular components. Some acridone derivatives, such as cycloferon (CMA), are described as compounds which can induce the interferon pathway [45,

46]. However, these assumptions for Fac4 remain to be investigated.

The result observed in viral release assay reinforces the antiviral activity of Fac4. The compound presented almost 80 % of inhibition in HCV release (extracellular RNA level). This assay is performed 24 h after treatment and to explain the lack of effect in the intracellular levels, the replication assay performed 24 h after treatment shows that Fac4 does not inhibit viral replication at this early time point, where luciferase levels were similar to the control. The inhibition of replication was observed 72 h after treatment. These results could indicate that, after 24 h, Fac4 has not yet influenced HCV replication significantly. However, some interaction between the acridone and the viral RNA is occurring in a way that prevents the release of new viral particles.

Herein, we reported the acridone Fac4 as a potent inhibitor of *in vitro* HCV genotype 2 in two steps of viral life cycle, replication and release. This inhibition was correlated to dsRNA intercalation and possibly associated with other mechanisms. Although the mode of action of this compound is partly understood, this drug is a candidate for further studies as a future anti-HCV agent.

METHODS

Synthesis and identification of Fac4

The trihydroxylated acridone Fac4 was synthesized by the group of Dr Luis Octavio Regasini at the Institute of Bioscience, Language and Exact Science (IBILCE) of São Paulo State University (UNESP) in São José do Rio Preto, Brazil, using the protocol previously described [47]. Details of synthesis and NMR analysis are available upon request (Fig. 6a).

Virus and subgenomic replicon constructs

The HCV subgenomic replicon SGR-Feo-JFH-1 was used in initial screening to evaluate the effect of the compounds on
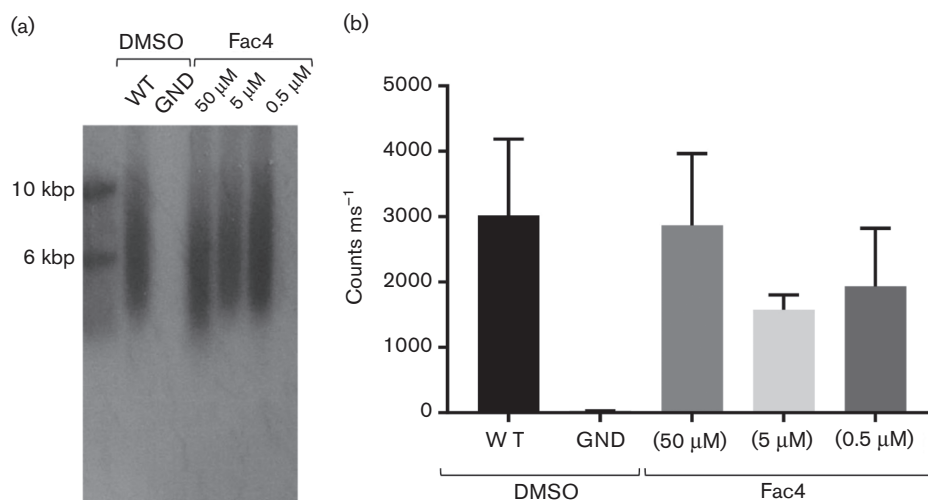
Fig. 3. Activity of JFH-1 NS5B in the presence of Fac4: (a) reaction products generated from a JFH-1 genomic RNA template. Purified wild-type or inactive point mutant (GND) NS5B (250 nM) was incubated with *in vitro* transcribed JFH-1 [GND] genomic RNA in the presence of $[\alpha^{32}P]$-CTP. Reactions were supplemented with either DMSO vehicle control or Fac4 at the indicated concentrations. Purified reaction products were separated by denaturing formaldehyde-agarose gel electrophoresis, the gel dried and imaged by autoradiography. Radiolabelled marker RNAs are indicated on the left. (b) Activity of NS5B in the presence of Fac4. Purified reaction products were subjected to liquid scintillation counting to quantify the amount of $[\alpha^{32}P]$–CTP incorporation into newly synthesized RNA. Data represent the mean ± standard error of the mean from three independent experiments conducted in duplicate.

virus replication [48]. This construct carries the phosphotransferase luciferase-neomycin fusion gene. To evaluate if the inhibitory effect was genotype-specific, the genotype 3 subgenomic replicon SGR-Feo-S52 was used [49]. For replication, entry, release and for virus protein expression

analysis, infection assays were carried out with full length HCV JFH-1 isolate [50] (Fig. 6b).

Cell culture

Huh7.5 cells and Huh7.5 stably harbouring subgenomic replicons SGR-Feo-JFH-1 and S52/SG-Feo were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1}, 0.5 mg mL^{-1} of geneticin (G418), 10 % fetal calf serum and incubated at 37 °C and 5 % CO$_2$.

Experimental delineation for initial screening

An initial screening was performed to test a panel of acridones for their antiviral activity on HCV replication. Compounds were dissolved in DMSO (dimethyl sulfoxide-Sigma Aldrich) and diluted in media immediately prior to the assay. The final concentration of DMSO in all assays was 0.1 %. For each compound, cytotoxicity and replication assays were performed. Huh7.5 cells harbouring SGR-Feo-JFH-1 were seeded in 96 well plates at a density of 3×10^3 and incubated in the presence or absence of compounds for 72 h. Cyclosporine A at 1 µM was used as a control for replication inhibition and DMSO 0.1 % as a non-treated control. Assays were performed in triplicates, and a minimum of three times. Four concentrations were tested (50, 10, 2 and 0.4 µM).

Replication assay for subgenomic replicons

After treatment, cells were harvested with Passive Lysis Buffer (PLB) (Promega). Replication levels were quantified by measuring luciferase activity with the Luciferase Assay
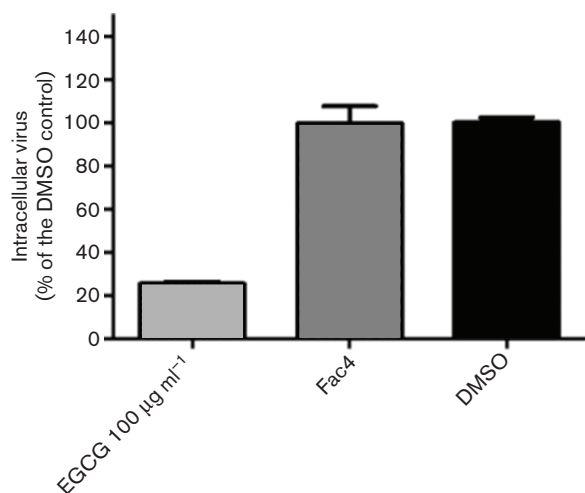

Fig. 4. Fac4 effect on HCV entry step: entry assay with JFH-1 HCVcc. Infectious supernatant and Fac4 were added simultaneously to Huh7.5 cells. Supernatant was removed 4 h p.i. and cells were incubated in fresh medium for 48 h. EGCG [(–)-epigallocatechin gallate] was used as a positive control and DMSO as a negative control.
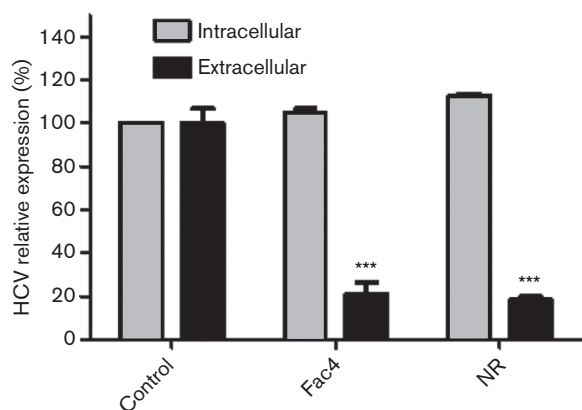
Fig. 5. Fac4 effect on HCV release step: viral release assay based on HCV 5′ UTR qPCR. JFH-1-infected Huh7.5 cells were treated with FAC-4 for 24 h. DMSO was used as a negative control and Naringerin 400 µM (NR) used as a positive control for release inhibition. The bars present the triplicate of two independent assays. The three asterisks represent a significant difference between the control group and treated group at *P*<0001.

System (Promega) in a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Data was normalized by DMSO control.

Cytotoxicity assay

After 72 h of treatment, the media were removed, cells were incubated at 37 °C with DMEM containing MTT (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich) at 1 mg ml^{-1}. After 30 min, MTT was removed and 100 µl of DMSO was added to solubilize formazan crystals. Cell viability was determined by measuring optical density in the microplate reader. Compounds were classified as non-toxic, when cells presented viability over 80 %.

Effective concentration 50 % (EC$_{50}$)

The effective concentration 50 % (EC$_{50}$) was calculated using Prism (GraphPad) and cytotoxicity assays were carried out in parallel to determine the cytotoxic concentration 50 % (CC$_{50}$) using a MTT-based system as described in cytotoxic assay section. The values of CC$_{50}$ and EC$_{50}$ were

used to calculate the selectivity index (SI = CC$_{50}$/EC$_{50}$), which suggests the potential antiviral activity of the compounds.

Viral production

HCV JFH-1 RNA was electroporated into Huh7.5 cells at 270 V, 950 µF and ∞ resistance, using a 4 mm cuvette in the Gene PulserXcell Electroporation System (Bio-Rad, Philadelphia, PA, USA). Fifteen days after electroporation supernatant was collected, concentrated with PEG 8000 (polyethylene glycol) (Sigma-Aldrich) and titrated by focus formation unit assay.

JFH-1 replication assay

Huh7.5 cells were seeded in 96 well plates the day before the assay. Cells were infected with JFH-1 virus (m.o.i. of 0.2) for 4 h at 37 °C and 5 % CO$_2$, washed extensively to remove virus and subsequently treated with Fac4 (5 µM). After 72 h, cells were fixed with 4 % PFA (Synth) and stained for NS5A using sheep anti-NS5A [51] and Alexa Fluor anti-sheep 594 secondary antibody. Virus titres were obtained by focus formation unit analysis. Data was normalized by DMSO control and cyclosporine at 1 µM was used as a replication inhibition control.

Viral entry

For virus entry experiments, infectious supernatant and Fac4 were added simultaneously to Huh7.5 cells. Then, 4 h p.i., supernatant was removed, washed extensively and replaced with a fresh medium. Cells were incubated for 48 h. DMSO and (−)-epigallocatechin gallate (EGCG, Sigma-Aldrich) were used as negative and positive controls, respectively. Cells were fixed and intra cellular virus was titrated.

Viral release analysis

To analyse Fac4 effect on HCV release, 2×10^5 JFH-1 infected cells were seeded 48 h before treatment. Then, the medium was replaced by a fresh medium supplemented with Fac4 at 5 µM or controls as previously described [52]. DMSO 0.1 % was used as a non-treated control and naringenin (NR) at 400 µM was used as a control of HCV secretion inhibition [52]. After 24 h of incubation, RNA was extracted from the supernatant and from the cells using TRIzol reagent (Life Technologies), and cDNA was synthesized
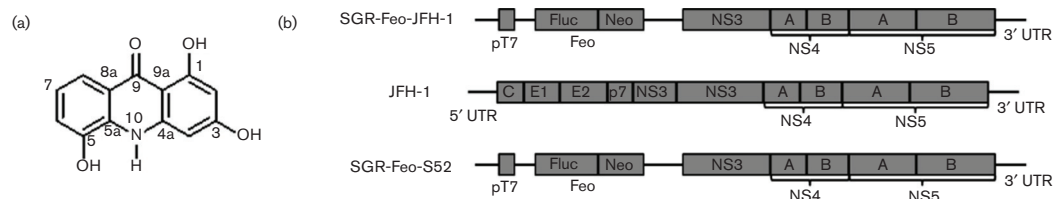

Fig. 6. Structure of Fac4 and constructs: (a) structure of acridone Fac4; (b) subgenomic replicon SGR-Feo-JFH-1, which expresses the genotype 2 non-structural viral proteins and the phosphotransferase fusion protein luciferase-neomycin, and JFH-1 virus, which expresses all viral proteins. Subgenomic replicon SGR-Feo-S52 expresses the genotype 3 non-structural viral proteins.

with the High-Capacity cDNA Archive (Applied Biosystems). HCV expression analysis was performed by TaqMan Universal PCR Master Mix no AmpErase UNG (Applied Biosystems) detecting the amplification of the HCV 5′ UTR region (forward: CGGGAGAGCCATAGTGG; reverse: AGTACCAACAAGGCCTTTCG). The sample quality and normalization of expression levels were obtained by amplification of the endogenous gene GAPDH. JFH-1 release inhibition was calculated as a percentage of negative control.

Western blotting

Cells were lysed using Cell Lytic (Sigma-Aldrich) and protein was quantified with Pierce BCA Protein Assay Kit (Thermo Scientific), following the manufacturer's protocol. Approximately 10 µg of protein was resolved in SDS-PAGE electrophoresis, transferred to a nitrocellulose membrane and blocked with non-fat milk 10 % in TBS-T solution. The membrane was probed at 4 °C with sheep anti-NS5A antibody overnight [51] and then with secondary Anti-sheep IgG antibody conjugated with HRP (Sigma-Aldrich) at room temperature for 1 h. The membrane was washed in TBS-T, exposed to ECL (enhanced chemiluminescent) and chemiluminescence was captured by ChemiDoc equipment (Bio-Rad, Philadelphia, PA, USA). After stripping, the membrane was probed for 1 h at room temperature with Anti-GAPDH antibody conjugated with HRP. After exposure to ECL, the blotting was analysed in ChemiDoc.

dsRNA intercalation assay

To analyse the ability of Fac4 to intercalate in dsRNA, a migration retardation assay was performed based on the previously described protocol of Krawczyk *et al.* [53]. The HCV JFH-1 3′ untranslated region (UTR) (accession no. AB047639) was amplified by PCR using primers flanked by a T7 promoter site (forward: TAATACGACTCACTA-TAGGGGGCACACACTAGGTACA; reverse: TAATAC-GACTCACTATAGGGACATGATCTGCAGAGAG; T7 promoter regions are underlined). The reaction product (273 bp) was purified by Zymoclean Gel DNA recovery Kit (Zymo Research) and used for *in vitro* transcription by the T7 Ribomax Express kit (large scale RNA production system) (Promega). The dsRNA molecule was obtained by complementary annealing and incubated at 15 nM with Fac4 (5 µM) for 45 min, and analysed in 1 % agarose 1X TAE gel stained with ethidium bromide. Since an intercalating compound competes with ethidium bromide, the intercalation of dsRNA is confirmed when the band of the treated sample is not visualized in the gel. Doxorubicin (100 µM) was used as a positive control of intercalation.

IRES interaction assay

Huh7.5 cells were electroporated with SGR-Feo-JFH-1or SGR-luc-JFH-1/GND. Immediately after electroporation, cells were seeded in 96 well plates and incubated with Fac4 (5 µM) or DMSO. Cells were harvested by lysis with PLB (Promega) 4 h post-electroporation and HCV RNA replication/translation was quantified by measuring luciferase activity using the Luciferase Assay System (Promega).

Expression and purification of JFH-1 NS5B from *E. coli*

Single colonies of BL21 (DE3) cells carrying the JFH-1 NS5B wild-type or inactive point mutant (GND) were grown in 10 ml of LB media (10 g l^{-1} tryptone, 10 g l^{-1} NaCl, 5 g l^{-1} yeast extract) containing 100 µg ml^{-1} ampicillin for 16 h at 37 °C on a rotary shaking incubator at 200 rpm. Overnight cultures were pelleted at 4000 g for 15 min, re-suspended in 10 ml LB media and inoculated 1 : 1000 into 500 ml LB containing ampicillin. Bacterial cultures were grown to an OD^{600} of 0.6–0.8 before induction of protein expression using 0.1 mM IPTG at 25 °C for 4 h. Final cultures were centrifuged at 4000 g for 15 min and stored at −20 °C.

Bacterial cell pellets were lysed in NS5B lysis buffer I (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM MgCl2, 2 % (v/v) Triton X-100, 2 mg ml^{-1} lysozyme, 1 Unit ml-1 DNase) for 30 min on ice before centrifugation at 20 000 g for 50 min at 4 °C. The resultant pellet was re-suspended in NS5B lysis buffer II [20 mM sodium phosphate (pH 7.5), 500 mM NaCl, 0.1 % (v/v) β-octylglucopyranoside, 20 mM imidazole, 50 % (v/v) glycerol] and sonicated on ice 10 times with 20 s on/off pulses using a Sanyo Soniprep 150 sonicator with a microtip at an amplitude of 6 microns. The sonicated suspension was centrifuged at 20 000 g for 20 min at 4 °C and the resultant supernatant applied to Ni-NTA-agarose (resin) for 16 h at 4 °C with end-over-end rotation. Resin was centrifuged at 500 g for 2 min, washed in NS5B lysis buffer II supplemented with 50 mM imidazole 10 times before protein elution in NS5B lysis buffer containing 250 mM imidazole. Eluted protein was quantified by measured absorbance at 280 nm and stored at −80 °C.

RNA-dependent RNA polymerase *in vitro* activity assay

Purified NS5B (250 nM) was incubated in RdRp reaction buffer (20 mM Tris-HCl [pH 7.6], 5 mM MgCl$_2$, 1 mM DTT, 25 mM KCl, 1 mM EDTA, 10 Units RNasin) with 0.5 µg *in vitro* transcribed JFH-1 (GND) genomic RNA template. *In vitro* transcribed RNA was re-folded before addition to RdRp reaction buffer by denaturation at 95 °C for 2 min, cooling on ice for 2 min and incubation in re-folding buffer [100 mM HEPES (pH 8.0), 100 mM NaCl, 6.6 mM MgCl$_2$] for 30 min at 37 °C. RdRp reactions were supplemented with DMSO vehicle control or Fac4, with a final DMSO concentration of 0.1 %. Reactions were started by the addition of NTP mix (5 µCi [α^{32}P]-CTP, 10 µM cold CTP, 1 mM ATP, 1 mM UTP, 5 mM GTP) and were incubated at 25 °C for 90 min before terminating the reaction with 1 vol 0.5 M EDTA. Products from RdRp *in vitro* transcription were purified using RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol.

For denaturing formaldehyde-agarose electrophoresis, 5 µl of purified reactions were loaded onto a 1 % (w/v) agarose MOPS gel (10 mM MOPS, 1.5 mM sodium acetate, 0.6 mM EDTA) containing 4.7 % (v/v) formaldehyde, and run at 70

V for 60 min. The gel was fixed (10 % (v/v) isopropanol, 10 % (v/v) acetic acid) for 60 min at room temperature with three buffer exchanges before drying at 60 °C under vacuum for 2 h. Radioactive transcripts were visualized by exposure of photographic film to the dried gel in the dark at −80 °C for 16 h. Liquid scintillation counting was conducted on a Tri-Card 2100 TR liquid scintillation counter using 1 µl purified *in vitro* transcribed reaction product mixed in a 1 ml Emulsifier-Safe liquid scintillation cocktail.

Data analysis

Cytotoxicity, subgenomic replicon and complete viral genome (JFH-1) assays were performed in triplicate and a minimum of three times. All data originated from these assays were evaluated using software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Average and standard deviation were represented in each graph. Statistical analyses were done using ANOVA test and Dunnett's multiple comparison test, considering $P<0.05$ as significant. The statistical analyses from the release assay were performed by two-way ANOVA with Bonferroni's post test using GraphPad Prism 5 software. All data was normalized by the non-treated control and multiplied by a hundred to obtain values in percentage.

Conflicts of interest
We declare that there are no conflicts of interest in this manuscript, including financial, political, religious or intellectual.

References

1. **Alter HJ, Seeff LB.** Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin Liver Dis* 2000;20:17–35.

2. **Houghton M.** The long and winding road leading to the identification of the hepatitis C virus. *J Hepatol* 2009;51:939–948.

3. **Baltimore D.** Expression of animal virus genomes. *Bacteriol Rev* 1971;35:235–241.

4. **Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM.** Structural biology of hepatitis C virus. *Hepatology* 2004;39:5–19.

5. **Simmonds P, Becher P, Bukh J, Gould EA, Meyers G et al.** ICTV virus taxonomy profile: *Flaviviridae. J Gen Virol* 2017;98:2–3.

6. **Suzuki T, Ishii K, Aizaki H, Wakita T.** Hepatitis C viral life cycle. *Adv Drug Deliv Rev* 2007;59:1200–1212.

7. **Dustin LB, Rice CM.** Flying under the radar: the immunobiology of hepatitis C. *Annu Rev Immunol* 2007;25:71–99.

8. **Lindenbach BD, Rice CM.** Unravelling hepatitis C virus replication from genome to function. *Nature* 2005;436:933–938.

9. **Argentini C, Genovese D, Dettori S, Rapicetta M.** HCV genetic variability: from quasispecies evolution to genotype classification. *Future Microbiol* 2009;4:359–373.

10. **Murphy D, Chamberland J, Dandavino R, Sablon E.** A new genotype of hepatitis C virus orginating from central Africa. *Hepatology* 2007;46:623.

11. **Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N et al.** Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005;42:962–973.

12. **Simmonds P, Holmes EC, Cha TA, Chan SW, Mcomish F et al.** Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* 1993;74:2391–2399.

13. **Cristina J, del Pilar Moreno M, Moratorio G.** Hepatitis C virus genetic variability in patients undergoing antiviral therapy. *Virus Res* 2007;127:185–194.

14. **Davis GL.** Hepatitis C virus genotypes and quasispecies. *Am J Med* 1999;107:21–26.

15. **Martell M, Esteban JI, Genescà J, Weiner A, Weiner A et al.** Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* 1992;66:3225–3229.

16. **Pawlotsky JM.** Hepatitis C virus population dynamics during infection. *Curr Top Microbiol Immunol* 2006;299:261–284.

17. **Le Guillou-Guillemette H, Vallet S, Gaudy-Graffin C, Payan C, Pivert A et al.** Genetic diversity of the hepatitis C virus: impact and issues in the antiviral therapy. *World J Gastroenterol* 2007;13: 2416–2426.

18. **Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN et al.** Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* 2010;465:96–100.

19. **Lawitz E, Lalezari JP, Hassanein T, Kowdley KV, Poordad FF et al.** Sofosbuvir in combination with peginterferon alfa-2a and ribavirin for non-cirrhotic, treatment-naive patients with genotypes 1, 2, and 3 hepatitis C infection: a randomised, double-blind, phase 2 trial. *Lancet Infect Dis* 2013;13:401–408.

20. **Rosenquist Å, Samuelsson B, Johansson PO, Cummings MD, Lenz O et al.** Discovery and development of simeprevir (TMC435), a HCV NS3/4A protease inhibitor. *J Med Chem* 2014;57:1673–1693.

21. **Jacobson IM, Gordon SC, Kowdley KV, Yoshida EM, Rodriguez-Torres M et al.** Sofosbuvir for hepatitis C genotype 2 or 3 in patients without treatment options. *N Engl J Med* 2013;368:1867–1877.

22. **Munir S, Saleem S, Idrees M, Tariq A, Butt S et al.** Hepatitis C treatment: current and future perspectives. *Virol J* 2010;7:296.

23. **Newman DJ, Cragg GM.** Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod* 2016;79:629–661.

24. **Michael JP.** Quinoline, quinazoline and acridone alkaloids. *Nat Prod Rep* 2008;25:166–187.

25. **Alwan WS, Mahajan AA, Rane RA, Amritkar AA, Naphade SS et al.** Acridone-based antitumor agents: a mini-review. *Anticancer Agents Med Chem* 2015;15:1012–1025.

26. **Fujiwara M, Okamoto M, Okamoto M, Watanabe M, Machida H et al.** Acridone derivatives are selective inhibitors of HIV-1 replication in chronically infected cells. *Antiviral Res* 1999;43:189–199.

27. **Turpin JA, Buckheit RW, Derse D, Hollingshead M, Williamson K et al.** Inhibition of acute-, latent-, and chronic-phase human immunodeficiency virus type 1 (HIV-1) replication by a bistriazoloacridone analog that selectively inhibits HIV-1 transcription. *Antimicrob Agents Chemother* 1998;42:487–494.

28. **Yamamoto N, Furukawa H, Ito Y, Yoshida S, Maeno K et al.** Anti-herpesvirus activity of citrusinine-l, a new acridone alkaloid, and related compounds. *Antiviral Res* 1989;12:21–36.

29. **Stankiewicz-Drogoń A, Dörner B, Erker T, Boguszewska-Chachulska AM.** Synthesis of new acridone derivatives, inhibitors of NS3 helicase, which efficiently and specifically inhibit subgenomic HCV replication. *J Med Chem* 2010;53:3117–3126.

30. **Stankiewicz-Drogon A, Palchykovska LG, Kostina VG, Alexeeva IV, Shved AD et al.** New acridone-4-carboxylic acid derivatives as

potential inhibitors of hepatitis C virus infection. *Bioorg Med Chem* 2008;16:8846–8852.

31. **Sepúlveda CS, Fascio ML, García CC, D'Accorso NB, Damonte EB.** Acridones as antiviral agents: synthesis, chemical and biological properties. *Curr Med Chem* 2013;20:2402–2414.

32. **Chansriniyom C, Ruangrungsi N, Lipipun V, Kumamoto T, Ishikawa T.** Isolation of acridone alkaloids and N-[(4-monoterpenyloxy)phenylethyl]-substituted sulfur-containing propanamide derivatives from *Glycosmis parva* and their anti-herpes simplex virus activity. *Chem Pharm Bull* 2009;57:1246–1250.

33. **Itoigawa M, Ito C, Wu TS, Enjo F, Tokuda H *et al.*** Cancer chemopreventive activity of acridone alkaloids on Epstein-Barr virus activation and two-stage mouse skin carcinogenesis. *Cancer Lett* 2003;193:133–138.

34. **Takemura Y, Ju-Ichi M, Ito C, Furukawa H, Tokuda H.** Studies on the inhibitory effects of some acridone alkaloids on Epstein-Barr virus activation. *Planta Med* 1995;61:366–368.

35. **Houe H.** Economic impact of BVDV infection in dairies. *Biologicals* 2003;31:137–143.

36. **Mazzucco MB, Talarico LB, Vatansever S, Carro AC, Fascio ML *et al.*** Antiviral activity of an N-allyl acridone against dengue virus. *J Biomed Sci* 2015;22:29.

37. **Raney KD, Sharma SD, Moustafa IM, Cameron CE.** Hepatitis C virus non-structural protein 3 (HCV NS3): a multifunctional antiviral target. *J Biol Chem* 2010;285:22725–22731.

38. **Sepúlveda CS, Fascio ML, Mazzucco MB, Palacios ML, Pellón RF *et al.*** Synthesis and evaluation of N-substituted acridones as antiviral agents against haemorrhagic fever viruses. *Antivir Chem Chemother* 2008;19:41–47.

39. **Tabarrini O, Manfroni G, Fravolini A, Cecchetti V, Sabatini S *et al.*** Synthesis and anti-BVDV activity of acridones as new potential antiviral agents. *J Med Chem* 2006;49:2621–2627.

40. **Gentile I, Buonomo AR, Borgia F, Zappulo E, Castaldo G *et al.*** MK-5172 : a second-generation protease inhibitor for the treatment of hepatitis C virus infection. *Expert Opin Investig Drugs* 2014;23:719–728.

41. **Hayashi N, Izumi N, Kumada H, Okanoue T, Tsubouchi H *et al.*** Simeprevir with peginterferon/ribavirin for treatment-naïve hepatitis C genotype 1 patients in Japan: CONCERTO-1, a phase III trial. *J Hepatol* 2014;61:219–227.

42. **Summa V, Ludmerer SW, McCauley JA, Fandozzi C, Burlein C *et al.*** MK-5172, a selective inhibitor of hepatitis C virus NS3/4a protease with broad activity across genotypes and resistant variants. *Antimicrob Agents Chemother* 2012;56:4161–4167.

43. **Adams A.** Crystal structures of acridines complexed with nucleic acids. *Curr Med Chem* 2002;9:1667–1675.

44. **Manfroni G, Paeshuyse J, Massari S, Zanoli S, Gatto B *et al.*** Inhibition of subgenomic hepatitis C virus RNA replication by acridone derivatives: identification of an NS3 helicase inhibitor. *J Med Chem* 2009;52:3354–3365.

45. **Kovalenko AL, Kazakov VI, Slita AV, Zarubaev VV, Sukhinin VP.** Intracellular localization of cycloferon, its binding with DNA and stimulation of cytokines expression after exposure to cycloferon. *Tsitologiia* 2000;42:659–664.

46. **Storch E, Kirchner H, Brehm G, Hüller K, Marcucci F.** Production of interferon-β by murine T-cell lines induced by 10-carboxymethyl-9-acridanone. *Scand J Immunol* 1986;23:195–199.

47. **Herath HMTB, Muller K, Diyabalanage HVK.** Synthesis of acrimarins from 1,3,5-trioxygenated-9-acridone derivatives. *J Heterocycl Chem* 2004;41:23–28.

48. **Wyles DL, Kaihara KA, Korba BE, Schooley RT, Beadle JR *et al.*** The octadecyloxyethyl ester of (S)-9-[3-hydroxy-2-(phosphonomethoxy) propyl]adenine is a potent and selective inhibitor of hepatitis C virus replication in genotype 1A, 1B, and 2A replicons. *Antimicrob Agents Chemother* 2009;53:2660–2662.

49. **Saeed M, Scheel TK, Gottwein JM, Marukian S, Dustin LB *et al.*** Efficient replication of genotype 3a and 4a hepatitis C virus replicons in human hepatoma cells. *Antimicrob Agents Chemother* 2012;56:5365–5373.

50. **Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M *et al.*** Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11:791–796.

51. **Macdonald A, Crowder K, Street A, McCormick C, Saksela K *et al.*** The hepatitis C virus non-structural NS5A protein inhibits activating protein-1 function by perturbing ras-ERK pathway signaling. *J Biol Chem* 2003;278:17775–17784.

52. **Nahmias Y, Goldwasser J, Casali M, van Poll D, Wakita T *et al.*** Apolipoprotein B-dependent hepatitis C virus secretion is inhibited by the grapefruit flavonoid naringenin. *Hepatology* 2008;47:1437–1445.

53. **Krawczyk M, Wasowska-Lukawska M, Oszczapowicz I, Boguszewska-Chachulska AM.** Amidinoanthracyclines – a new group of potential anti-hepatitis C virus compounds. *Biol Chem* 2009;390:351–360.