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

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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 harboring the HCV genotype 2a subgenomic replicon SGR-JFH1-FEO. Cells were incubated in the presence or absence of compounds for 72 hours and cell viability and replication levels were assessed by MTT and luciferase assays, respectively. The acridone Fac4 at 5 µM inhibited approximately 90 % of HCV replication with 100 % of 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 approximately 70 % of JFH-1 replication, while no effect was observed on virus entry. The antiviral activity of Fac4 was also observed on the viral release, with almost 80% of inhibition. No inhibitory effect was observed against genotype 3 replication. Fac4 demonstrated 40% of intercalation into dsRNA, however did not inhibit T7 polymerase activity, as well as translation by IRES interaction. Although its mode of action is partly understood, the Fac4 presents significant inhibition of Hepatitis C virus replication and can therefore be considered as a candidate for the development of a future anti-HCV treatment.

Keywords: acridones; antivirals; HCV, inhibition of viral replication; treatment.

Introduction

Hepatitis C virus (HCV) is a global health problem, widely distributed, that affects approximately 170 million people around the world (Alter & Seeff, 2000; Houghton, 2009). HCV, the causative agent of this disease, is a single stranded RNA positive genome virus that belongs to *Flaviviridae* family and is classified as a group IV virus, according to Baltimore classification (Baltimore, 1971; Penin *et al.*, 2004). 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 (Suzuki *et al.*, 2007). Viral and host proteases cleave this polyprotein, producing 3 structural proteins (Core, E1 and E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Dustin & Rice, 2007; Lindenbach & Rice, 2005; Penin *et al.*, 2004).

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 (Argentini *et al.*, 2009), HCV is divided into genotypes (1 to 7) and subtypes (classified by lowercase letters – a, b, c) (Murphy *et al.*, 2007; Simmonds *et al.*, 2005; Simmonds *et al.*, 1993). Furthermore, in an infected individual it circulates as a pool of variants genetically related, named quasispecies which provides a favorable environment for the emergence of mutations resulting in drugs resistance (Cristina *et al.*, 2007; Davis, 1999; Martell *et al.*, 1992; Pawlotsky, 2006). Therefore, the quasispecies nature of HCV has a direct impact in the effectiveness of treatment with usual medications, as well as the development of new antivirals (Le Guillou-Guillemette *et al.*, 2007).

With the current development of the Direct Acting Antivirals (DAAs) such as protease, polymerase and NS5A inhibitors, the most effective treatment is the administration of DAAs with or without pegIFN-α and ribavirin (Gao *et al.*, 2010; Lawitz *et al.*, 2013; Rosenquist *et al.*, 2014). Treatment strategy is designed based on virological, clinical and liver pathology aspects. The 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 (Jacobson *et al.*, 2013). This reduced efficacy in genotype 3, coupled with potential side effects such as anemia, autoimmune disorders, diarrhea, rash, retinopathy and weight loss, as well as the elevated cost, means that additional therapeutic options are still required (Munir *et al.*, 2010).

Alkaloids is a central class of natural products, which have been extensively used as modern drug prototypes and drugs (Newman & Cragg, 2016). Among these, acridones are planar compounds isolated from Rutaceae plants, and exhibited several bioactivities, including; antimicrobial, cytotoxic, algicidal, moluscicidal, anti-allergic and antidiabetic (Michael, 2008). Also, synthetic compounds containing acridone framework have demonstrated correlated bioactivities to their natural analogues (Alwan *et al.*, 2015). The antiviral action of acridones is well known in literature against HSV-2 and CMV replication and inhibiting HIV-1 transcription (Fujiwara *et al.*, 1999; Turpin *et al.*, 1998; Yamamoto *et al.*, 1989). Recent studies revealed the potential anti-HCV effect of acridone derivatives as NS3 helicase inhibitor and as dsRNA intercalant, inhibiting viral replication (Stankiewicz-Drogon *et al.*, 2010; Stankiewicz-Drogon *et al.*, 2008).

Considering the high cost and several side effects of current HCV 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 synthetic acridone Fac4 on HCV life cycle by the use of *in vitro* approaches.

Results

Inhibitory activity of Fac4 on JFH1 replication.

We performed a screening with a panel of 14 synthetic acridones to select those with potential antiviral activity on HCV replication. Huh 7.5 cells stably harboring 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 the treatment with the compounds, respectively. Among tested acridones, Fac4 presented a potential activity against HCV replication. This acridone at 10 µM presented cell viability of 85 % with inhibition of viral replication by approximately 89 %. Fac4 inhibited replication in a concentration-dependent manner (data not shown) with EC_{50} of 1.33uM and SI (CC_{50}/EC_{50}) of 42.14. To find the useful selectivity index (favorable ratio of cytotoxicity to antiviral potency), a 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. 2a)**. Therefore, this concentration was selected to the further experiments.

Thus, we evaluate the effects of Fac4 on the HCV replication in the context of full length virus. Huh 7.5 cells were infected with JFH1 HCVcc and after 4h, viral supernatant was removed and cells were treated with Fac4 for 72 hours. Cells were fixed, stained and titrated. Fac4 inhibited approximately 70 % of HCV replication **(Fig. 2b)**, 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. 2b)**.

Once Fac4 presented a potential inhibition of HCV genotype 2a JFH1 replication, we decided to test if these results are genotype-specific. For that, Huh 7.5 cells stably harboring the genotype 3

subgenomic replicon S52/SG-Feo were treated with Fac4 at 5μM and replication levels were analyzed by luciferase assay. No inhibition of genotype 3 replication was observed **(Fig. 2c)**.

Fac4 as a dsRNA intercalator.

To further investigate the antiviral mode of action of Fac4, we analyzed the capacity of this compound to intercalate into dsRNA. Using the 3'UTR region of JFH1 HCV as a template, we produced an amplicon flanked by T7 promoter that was used for *in vitro* transcription, synthesizing a dsRNA molecule of 273 bp. This dsRNA was incubated with Fac4 at 5μM or the controls (DMSO 0.1% and Doxorubicin at 100μM) and was analyzed by a migration retardation assay. Fac4 presented 40% of dsRNA intercalation when compared to the DMSO negative control, quantified by densitometry **(Fig. 3a)**. Notice that the sample treated with Doxorrubicin (positive control of intercalation) does not appear in the image, reasserting the observed result.

T7 RNA polymerase inhibition assay

In order to investigate if Fac4 has an inhibitory effect on the polymerase activity, we performed an *in vitro* transcription of HCV JFH-1 RNA in the presence of Fac4. For that, T7 RNA polymerase was used, which as stated earlier, is similar to NS5B viral polymerase(Stankiewicz-Drogon *et al.*, 2010; Stankiewicz-Drogon *et al.*, 2008). Fac4 did not present any activity on T7 enzymatic process since no difference was observed when compared to the control **(Fig. 3b)**. The results suggest it is unlikely that Fac4 has an effect on NS5B, however this possibility cannot be completely discarded.

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 the IRES-mediated translation. Cells electroporated with SGR-Feo-JFH-1 or SGR-luc-JFH1/GND were immediately incubated with Fac4 or controls and RNA replication was estimated after 4h by luciferase expression analysis. Fac4 did not influence the viral RNA translation, since luciferase values of both wild type replicon and the GND replication defective replicon at 4h were not reduced, demonstrating the input RNA was translated in to the cells **(Fig. 3c)**.

Fac4 does not block virus entry.

To evaluate whether Fac4 possess antiviral activity on HCV entry, Huh 7.5 cells were infected with JFH1 virus in the presence or absence of Fac4 for 4 h. Viral inoculum was replaced by fresh media and intracellular virus was quantified72 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 viral entry process, we decided to analyze the release step. We observed that intracellular RNA in Fac4-treated cells displayed similar values as the non-treated cells. However, a pronounced effect on virus release (extracellular RNA) is observed since there was 80% of difference in the amount of intra and extracellular HCV RNA **(Fig. 5)**.

Discussion

The antiviral effect of acridones has been described in 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) (Sepulveda *et al.*, 2013). 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 (Chansriniyom *et al.*, 2009; Itoigawa *et al.*, 2003; Takemura *et al.*, 1995; Yamamoto *et al.*, 1989). For RNA viruses, acridones presented activity against HIV-1, bovine viral diarrhea virus (BVDV), all serotypes of dengue virus (DENV) and HCV, the last three belonging to the *Flaviviridae* family (Fujiwara *et al.*, 1999; Houe, 2003; Mazzucco *et al.*, 2015; Raney *et al.*, 2010; Sepulveda *et al.*, 2008; Stankiewicz-Drogon *et al.*, 2010; Stankiewicz-Drogon *et al.*, 2008; Tabarrini *et al.*, 2006; Turpin *et al.*, 1998).

Our results showed that Fac4 inhibited up to 92% of HCV replication in the context of either the subgenomic replicon or full length JFH1 HCVcc. Also, NS5A viral protein expression could not be detected after the 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 (Gentile *et al.*, 2014; Hayashi *et al.*, 2014; Rosenquist *et al.*, 2014; Summa *et al.*, 2012). Altogether, the hypothesis that Fac4 is interfering with NS3 protease is strengthened. However, to determine whether Fac4 is inhibiting NS3 protease activity further functional studies are needed.

Despite 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 (Adams, 2002; Stankiewicz-Drogon *et al.*, 2010;

174 Stankiewicz-Drogon *et al.*, 2008). For HCV, data presented by Stankiewicz-Drogon and co-
175 workers (Stankiewicz-Drogon *et al.*, 2010; Stankiewicz-Drogon *et al.*, 2008) reinforces this
176 assumption. Acridones showed inhibition of NS3 helicase, T7 RNA polymerase (topology and
177 function similar to HCV NS5B) and strong double-stranded RNA intercalation property. All
178 these elements are involved in the replication step.

179 It is not clear yet if there is a combination of the reported effects of acridones on the inhibition of
180 replication. Some acridones described in the literature present dsRNA intercalation property,
181 others show inhibition of NS3 helicase and NS5B polymerase, and some present both effects
182 (Manfroni *et al.*, 2009; Stankiewicz-Drogon *et al.*, 2010). However, all these studies performed
183 in isolated assays, evaluating inhibition of enzymatic activity or dsRNA intercalation
184 individually. According to our results, Fac4 presented the ability to partially intercalate in
185 dsRNA, however it does not inhibit T7 RNA polymerase. As reported before (Stankiewicz-
186 Drogon *et al.*, 2010; Stankiewicz-Drogon *et al.*, 2008), it is presumed that HCV replication
187 cannot be inhibited by dsRNA intercalation alone, and probably it is due to a combined effect
188 between different modes of action. Therefore, replication inhibition by Fac4 may be somewhat
189 related to dsRNA intercalation, which is a replication intermediate. However it is likely that
190 other mode of action, such as enzymatic inhibition is also involved (Stankiewicz-Drogon *et al.*,
191 2010; Stankiewicz-Drogon *et al.*, 2008; Tabarrini *et al.*, 2006). Another possible explanation for
192 the antiviral activity of Fac4 is the targeting of cellular components. Some acridone derivatives,
193 such as cycloferon (CMA), are described as compounds which can induce the interferon pathway
194 (Kovalenko *et al.*, 2000; Storch *et al.*, 1986). However, these assumptions remain to be
195 investigated.
196
197 The result observed in viral release assay reinforces the antiviral activity of Fac4. The compound
198 presented almost 80% of inhibition in HCV release step (extracellular RNA level). This assay is

199 performed 24h after treatment and to explain the lack of effect in the intracellular levels, we
200 performed a replication assay 24h after treatment, where luciferase levels were similar to the
201 control (data not shown). The inhibition of replication was observed 72h after treatment. These
202 results could indicate that, after 24h, Fac4 has not yet influenced HCV replication in a significant
203 way, however some interaction between the acridone and the viral RNA is occurring in a way
204 that prevents the release of new viral particles.

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

209

Materials and Methods

Synthesis and Identification of Fac4

The trihydroxylated acridone Fac4 was synthesized as the protocol previously described by Herath and co-authors (Herath *et al.*, 2004). A mixture of phloroglucinol (19 mmol), 2-amino-3-hydroxybenzoic acid (13 mmol), and *para*-toluenosulphonic acid (0.5 mmol) in 1-hexanol (65 mL) was refluxed for 8 h. The heterogenous mixture was stirred with cold hexane. The crude pale yellow product was recrystallized from mixture of ethanol and acetone (1:1), yielding 43% of Fac4. The structure of Fac4 was elucidated by analysis of Nuclear Magnetic Resonance (NMR) spectra, including NMR ^{1}H and ^{13}C NMR. Chemical shifts (δ) were expressed in ppm. Coupling constants (J) were expressed in Hz, and splitting patterns are described as follows; s = singlet, d = doublet and dd = double of doublets **(Fig. 1a)**.

1,3,5-trihydroxy-9(10*H*)-acridinone. ^{1}H NMR (11.7 T;DMSO-d_6): 5.98 (d; 2.0; H-2), 6.67 (d; 2.0; H-4), 7.14 (dd; 2.5 and 7.5; H-6), 7.05 (dd; 8.0 and 7.5; H-7), 7.61 (dd; 2.5 and 8.0; H-8), 11.1 (s; H-10); 14.3 (s; 1-OH), 10.3 (s; 3-OH), 10.6 (s; 5-OH). ^{13}C NMR (11.7 T; DMSO-d_6): 163.8 (C-1), 95.6 (C-2), 163.4 (C-3), 91.9 (C-4), 145.4 (C-5), 115.9 (C-6), 120.9 (C-7), 114.8 (C-8), 180.0 (C-9), 143.2 (C-4a), 131.4 (C-5a), 119.8 (C-8a), and 103.4 (C-9a).

Virus Constructs

The HCV subgenomic replicon SGR-Feo-JFH-1 was used in initial screening to evaluate the effect of the compounds on virus replication (Wyles *et al.*, 2009). This construct carries the phosphotransferase luciferase-neomycin fusion gene. To evaluate if the inhibitory effect was genotype-specific, the genotype 3 subgenomic replicon S52/SG-Feo was used (Saeed *et al.*,

2012). For replication, entry, release and for virus protein expression analysis, infection assays were carried out with full length HCV JFH-1 isolate (Wakita *et al.*, 2005) **(Fig. 1b)**.

Cell culture

Huh 7.5 cells and Huh 7.5 stably harboring subgenomic replicons SGR-Feo-JFH-1 and S52/SG-Feo were cultured in Dulbecco's modified Eagles 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 ºC 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 the assay. The final concentration of DMSO in all assays was 0.1 %. For each compound, cytotoxicity and replication assays were performed. Huh 7.5 cells harboring SGR-JFH1-FEO were seeded in 96 well plates at the density of 3×10^3 and incubated in the presence or absence of compounds for 72h. Cyclosporine A at 1 µM was used as a control for replication inhibition and DMSO 0.1% as 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 System (Promega) in a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Data was normalized by DMSO control.

Cytotoxicity assay

After 72h of treatment, the media was removed, cells were incubated at 37ºC with DMEM containing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich) at 1 mg mL^{-1}. After 30 minutes, 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 JFH1 RNA was electroporated into Huh 7.5 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.

JFH1 Replication assay

Huh 7.5 cells were seeded in 96 well plates the day before the assay. Cells were infected with JFH1 virus (MOI of 0.2) for 4 h at 37ºC 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 (Macdonald *et al.*, 2003) and Alexa Fluor anti-sheep 594 secondary antibody. Virus titers were obtained by focus formation unit analysis. Data was normalized by DMSO control and cyclosporine at 1µM was used as a control of inhibition of replication.

Viral entry

For virus entry experiments, infectious supernatant and Fac4 were added simultaneously to Huh-7.5 cells. Four hours post-infection (h.p.i), supernatant was removed, washed extensively and replaced with fresh medium. Cells were incubated for 48 hours. 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 analyze Fac4 effect on HCV secretion, 2×10^5 JFH-1 infected cells were seeded 48 h before treatment. Then, the medium was replaced by fresh medium supplemented with Fac4 was added at 5 µM or controls as previously described (Nahmias *et al.*, 2008). DMSO 0.1 % was used as non-treated control and naringenin (NR) at 400 µM was used as control of HCV secretion inhibition (Nahmias *et al.*, 2008). After 24 h of incubation, RNA was extracted from the supernatant and from the cells using TRIzol reagent (Life Technologies), and cDNA was synthesized with 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 samples quality and normalization of levels of expression were obtained by amplification of the endogenous gene GAPDH. JFH1 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 manufacturers protocol. Approximately 10 µg of protein was resolved in SDS-PAGE electrophoresis, transferred to a nitrocellulose membrane and blocked with nonfat milk 10% in TBS-T solution. The membrane was probed at 4ºC with sheep Anti-NS5A antibody overnight (Macdonald *et al.*, 2003) and then with secondary Anti-sheep IgG antibody conjugated with HRP (Sigma-Aldrich) at room temperature for 1h. 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, membrane was probed for 1h at room temperature with Anti-GAPDH antibody conjugated with HRP. After exposure to ECL, the blotting was analyzed in ChemiDoc.

dsRNA intercalation assay

To analyze the ability of Fac4 to intercalate in dsRNA, a migration retardation assay was performed based on the previously described protocol of Krawczyk *et al.*(Krawczyk *et al.*, 2009). The HCV JFH1 3' untranslated region (UTR) (accession no. AB047639) was amplified by PCR using primers flanked by a T7 promoter site (Forward:TAATACGACTCACTATAGGGGGCACACACTAGGTACA; Reverse: TAATACGACTCACTATAGGGACATGATCTGCAGAGAG; 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 (5µM) for 45 min, and analyzed 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 positive control of intercalation.

T7 RNA polymerase inhibition assay

The T7 polymerase presents similar topology and function to the HCV RdRp NS5B (Stankiewicz-Drogon *et al.*, 2010; Stankiewicz-Drogon *et al.*, 2008). In order to evaluate whether Fac4 interacts with T7 consequently inhibiting viral replication, we tested the effects of FAC4 on *in vitro* RNA transcription. Five *in vitro* transcription reactions for JFH1 RNA were performed, using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega). The compound was tested in three different concentrations (0.5 µM, 5µM and 50µM). The experiment was set up according to manufacturer instructions apart to the addition of acridone Fac4. For controls, we performed a standard reaction for JFH1 RNA synthesis and a reaction adding only DMSO, the solvent of the compounds. Synthesized RNA was quantified and analyzed in a RNA denaturant 1 % agarose gel.

IRES interaction assay

Huh7.5 cells were electroporated with SGR-Feo-JFH-1or SGR-luc-JFH1/GND. Immediately after electroporation, cells were seeded in 96 well plates and incubated with Fac4 (5µM) or DMSO. Cells were harvested by lysis with PLB (Promega) 4h post-electroporation and HCV RNA replication/translation was quantified by measuring luciferase activity using the Luciferase Assay System (Promega).

Data analysis

Cytotoxicity, subgenomic replicon and complete viral genome (JFH1) 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 5.0 software. All data was normalized by the non-treated control and multiplied by a hundred to obtain values in percentage.

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Figure legends

Figure 1. Structure of Fac4 and constructs: A) Structure of acridone Fac4; **B)** Subgenomic Replicon SGR-JFH1-FEO, which express the genotype 2 non-structural viral proteins and the phosphotransferase fusion protein luciferase-neomycin, and JFH-1 replicon, which express all viral proteins. Subgenômic Replicon S52/SG-Feo express the genotype 3 non-structural viral proteins.

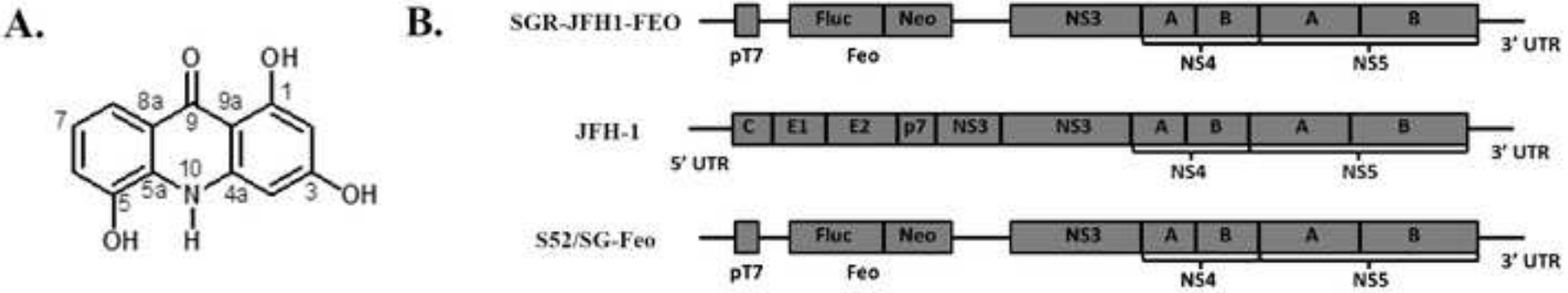
Figure 2. Fac4 effect on HCV replication: A) Fac4 concentration screening, determining 5μM as working concentration (the lower concentration with best inhibitory effect and cell viability). **B)** Replication assay in Huh 7.5 infected with JFH1 HCVcc and Western Blot for NS5A viral protein detection. **C)** Replication assay in Huh 7.5 stably expressing genotype 3 subgenomic replicon S52/SG-Feo. Cyclosporin (CsA) used as positive control. The three asterisks represent a significant difference between control group and treated group at $p < 0{,}001$.

Figure 3. Fac4 possible mechanisms of action: A) Intercalation assay, evaluating Fac4 dsRNA intercalation property. Doxorrubicin (Doxo) was used as positive control. **B)** Evaluation of Fac4 effect over T7 RNA polymerase activity during *in vitro* transcription. Above the RNA bands are the quantification values, in μg/μl. **C) :** IRES interaction assay in Huh 7.5 cells. Replication rate 4h after electroporations. Subgenomic replicon SGR-JFH1-FEO (SGR); Defective mutated subgenomic replicon SGR-luc-JFH1/GND (GND);

Figure 4. Fac4 effect on HCV entry step: Entry assay in Huh 7.5 infected with JFH1 HCVcc. EGCG ((-)-epigallocatechin gallate – positive control).

Figure 5. Fac4 effect on HCV release step: Viral release assay based on HCV 5'UTR qPCR. Cells Huh 7.5 JFH1-infected were treated with FAC-4 along 24h. DMSO was used as negative control and Naringerin 400 μM (NR) used like positive control for release inhibition. The bars present the triplicate of two independent assays. The three asterisks represent a significant difference between control group and treated group at $p < 0{,}001$.

Figure



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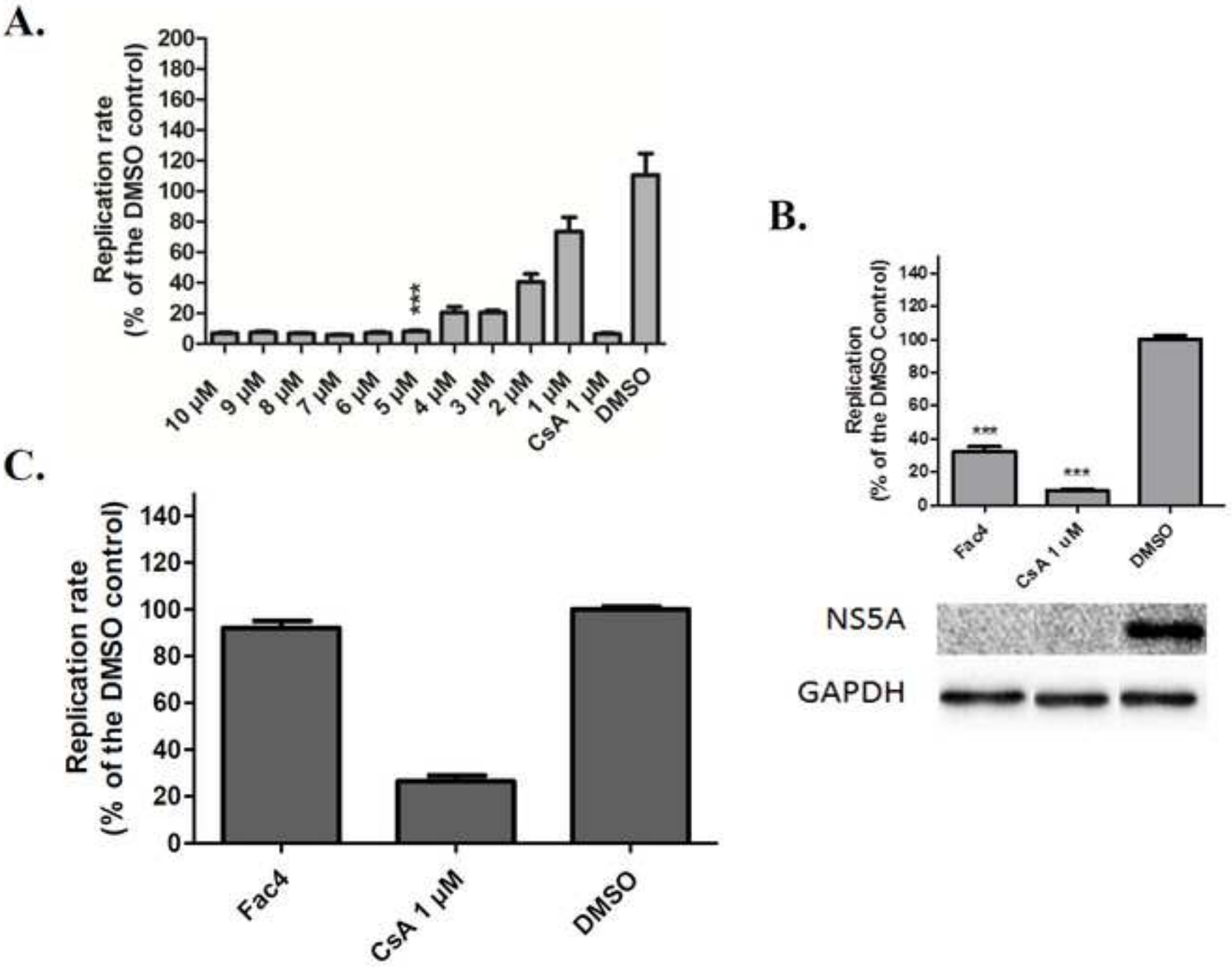

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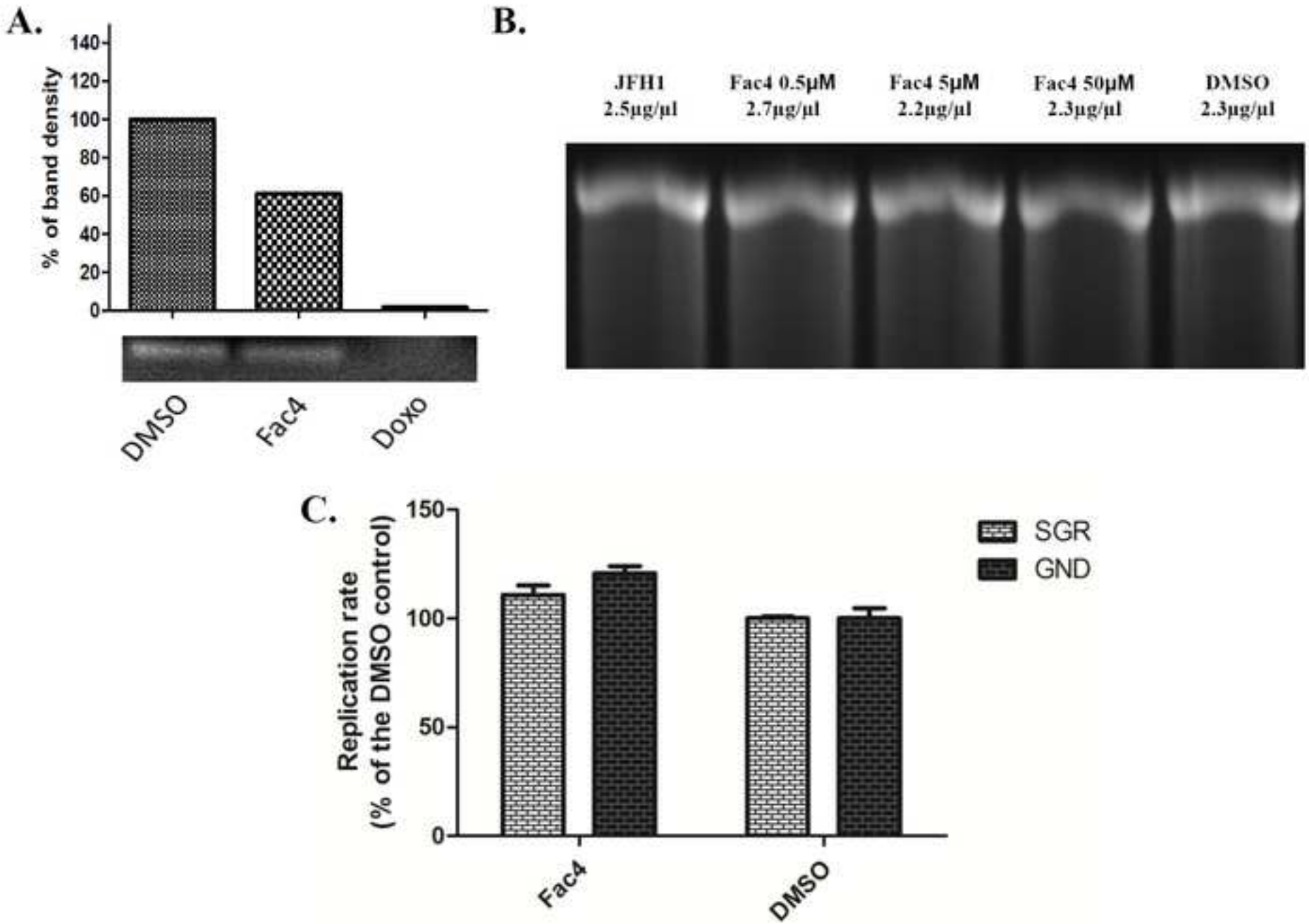

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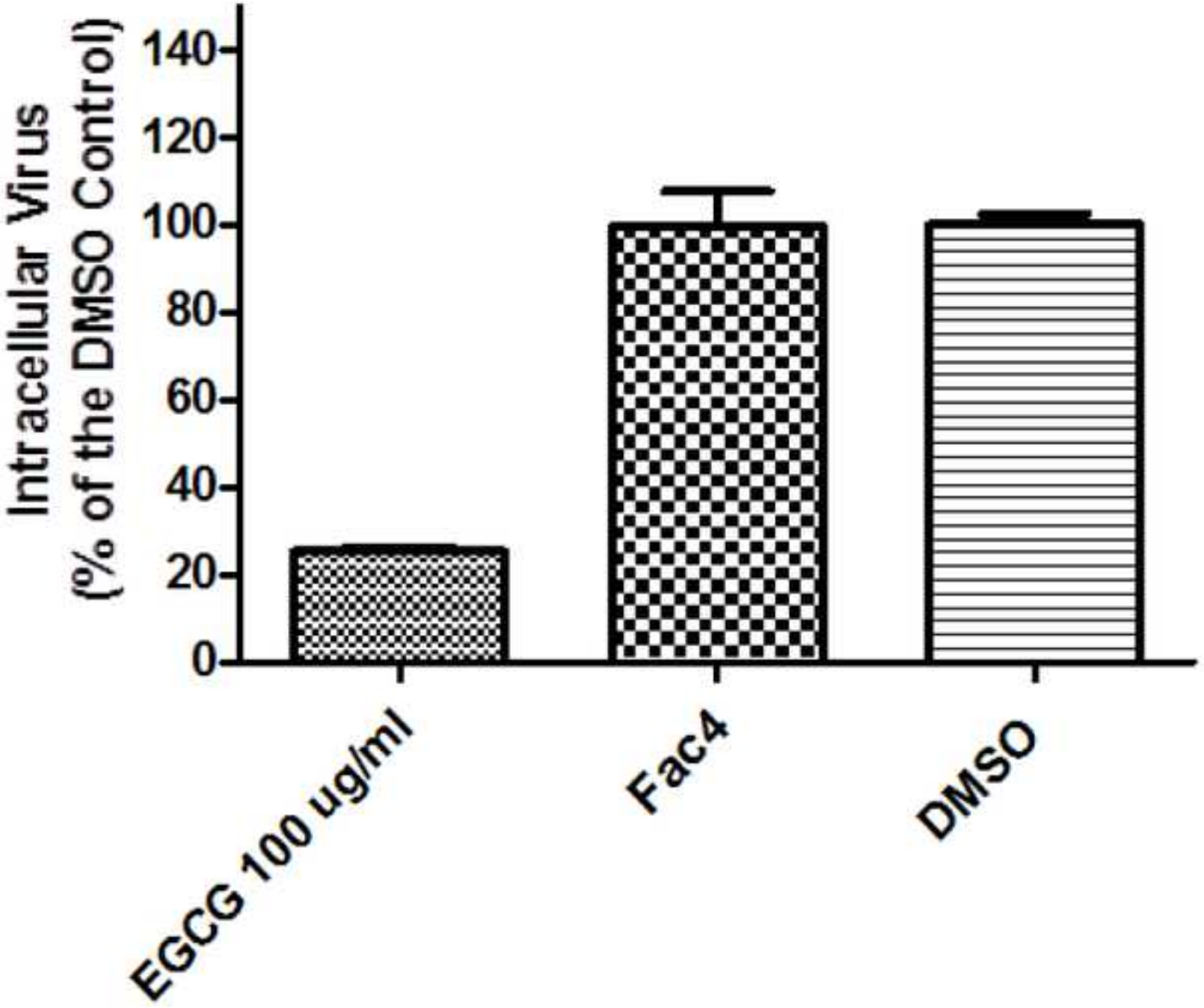

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