Title: Oligonucleotide-lipid conjugates forming G-quadruplex structures are potent and pangenotypic hepatitis C virus entry inhibitors *in vitro* and *ex vivo*

Short title: Lipoquads inhibit hepatitis C virus

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Abbreviations used in this manuscript:

HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men who have sex with men; DAA, direct-acting antiviral; GAGs, glycosaminoglycans; HSPG, heparan sulfate proteoglycans; ApoE, Apolipoprotein E; SDC, syndecan; L-SIGN , liver/lymph node-specific intercellular adhesion molecule 3-grabbing integrin; LDLR, low-density lipoprotein receptor; SR-B1, scavenger receptor class B type 1; CLDN1, claudin 1; OCLN, occludin; TfR1, transferrin receptor 1; EGFR, epidermal growth factor receptor; EphA2, ephrin receptor A2; NPC1L1, Niemann-Pick C1-like 1 cholesterol absorption receptor; HCVcc, HCV cell-culture; JFH1, Japanese fulminant hepatitis 1; IC_{50}, half maximal inhibitory concentration; CC_{50}, half maximal cytotoxic concentration; HCVpp, HCV pseudoparticles; VSV-G, vesicular stomatitis virus glycoprotein; VSV-Gpp, VSV-G pseudoparticles; EGFP, enhanced green fluorescence protein (EGFP); DMSO, dimethyl sulfoxide; SOF, sofosbuvir; LDV, ledipasvir

Keywords: Oligonucleotide-cholesterol conjugates; DNA G-quadruplex structures; antiviral treatment, HCV entry inhibitor; HCV cell-to-cell inhibitor; HCV pangenotypic inhibitor

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ABSTRACT

An HCV epidemic affecting HIV-infected men who have sex with men (MSM) is expanding worldwide. Albeit the improved cure rates obtained with the new direct-acting antiviral drug (DAA) combinations, the high rate of reinfection within this population calls urgently for novel preventive interventions. Here we show in cell culture and *ex vivo* experiments with human colorectal tissue that lipoquads, G-quadruplex DNA structures fused to cholesterol, are efficient HCV pangenotypic entry and cell-to-cell transmission inhibitors. Thus, lipoquads may be promising candidates for the development of rectally-applied gels to prevent HCV transmission.

INTRODUCTION

Hepatitis C Virus (HCV) infection of human immunodeficiency virus (HIV)-infected men who have sex with men (MSM) has emerged since the early 2000s as a growing epidemic worldwide (1). Although the introduction of interferon-free direct-acting antiviral (DAA) therapies improved significantly the sustained treatment responses, the rates of reinfection after treatment termination among HIV/HCV co-infected MSM are high (2, 3). Consequently, preventive interventions tailored to the MSM community are urgently needed. Given that this HCV epidemic is linked to high risk sexual behaviors that include unprotected anal sex, formulations of water-soluble molecules as rectally-applied gels that prevent HCV transmission would represent an ideal option. Currently, there are no prophylactic therapies for HCV in this setting.

G-Quadruplexes comprise a distinct category of nucleic acid secondary structures that are formed from G-rich DNA and RNA sequences (4). Polymorphisms in these structures can be observed in the number (from one to four) and orientation of the strands, the number of stacked G-tetrads, differences in the loop (length, type and/or location) and finally the dimension of the four grooves (4). The guanosine quartet AR177 (Zintevir™, Aronex Pharmaceuticals, Inc) is a 17-base oligonucleotide composed by deoxyguanosines and thymidines on a phosphodiester backbone supplemented by phosphorothioate internucleoside linkages at the 5′ and 3′ ends. AR177 is a potent inhibitor of laboratory strains and clinical isolates of HIV-1 with 50% effective concentrations (EC_{50}) ranging between 0.025 and 3 μM in cell culture tests (5, 6). The effect is an inhibition of viral entry by blocking a step before membrane fusion and viral resistant strains have shown mutations in the HIV gp120 gene (7). Similar G/T rich phosphorothioate oligonucleotides have been reported to have antiviral activity against Herpes Simplex Virus-2 (8). G-quadruplexes are polyanionic structures like sulfated polysaccharides and hence their inhibition mechanism may include mimetics of the glycosaminoglycans and other cell-surface attachment receptors involved in virus-cell attachment. Thus, these molecules compete with viral envelope glycoproteins during binding with their main receptors.

HCV cell-free virions enter into hepatocytes through a highly coordinated process which involves the two viral envelope glycoproteins E1 and E2 and multiple host cell factors. HCV firstly associates with its target cells through interactions of basic residues in its glycoproteins with glycosaminoglycans (GAGs), including heparan sulfate proteoglycans (HSPG) (9-11). Apolipoprotein E (ApoE), which associates with HCV virions, play also a role in the initial attachment through interaction with the HSPG

125 associated with syndecan 1 (SDC1) (12) and syndecan 4 (SDC4) (13). The liver/lymph
126 node-specific intercellular adhesion molecule 3-grabbing integrin (L-SIGN) (14, 15) and
127 the low-density lipoprotein receptor (LDLR) (16, 17) have been also implicated in the
128 preliminary attachment of cell-free viruses. Although the exact sequential order of
129 receptor engagement is still unclear, some evidence suggest that HCV viruses interact
130 with scavenger receptor class B type 1 (SR-B1), CD81, tight junction proteins claudin-1
131 (CLDN1), occludin (OCLN) and possibly other factors (18). Virions are later
132 internalized through clathrin-mediated endocytosis and fuse with the host membrane
133 following endosomal acidification (19). Finally, the transferrin receptor 1 (TfR1) (20),
134 epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) (21), and
135 Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1) (22) have also been
136 implicated in HCV entry. Cell-to-cell spread has been also presented as an important
137 route for HCV transmission within the infected liver. The requirements for this
138 alternative route suggest that SR-B1, CLDN1, OCLN (23), EGFR, EphA2 (24) and
139 NPC1L1 (25) are implicated for both cell-free and cell-to-cell spread.
140
141 In the present study, we characterized the anti-HCV inhibitory capacity of novel lipid-G-
142 Quadruplex conjugate structures, designated lipoquads (Fig. 1A). Firstly, we show that
143 the anti-HCV potency of lipoquads is correlated with the ability of the G-rich sequences
144 to form stable structures. Then, by using the HCV pseudoparticles, which is a well-
145 established system to investigate HCV entry and neutralization (26, 27) and cell-culture
146 produced viruses (HCVcc), we characterize the inhibition mechanism of lipoquads acting
147 at the early steps of HCV entry including the attachment phase, and demonstrate that this
148 inhibition is linked to basic amino acids in the hypervariable region 1 (HVR1) of the E2
149 glycoprotein and/or to the HVR1 itself. Moreover, lipoquads also inhibit cell-to-cell

HCV transmission and potently inhibit all major HCV genotypes. Finally, we provide evidence that lipoquads inhibit HCV infection *ex vivo* in a mucosal model based on colorectal tissue explants. Our results, pave the way for the use of lipoquads in the development of prevention strategies against HCV.

MATERIALS AND METHODS

Oligonucleotide synthesis and G-quadruplex preparation

Oligonucleotide sequences 5′-TTGGGGGGTACAGTGCA-3′-cholesterol and the A-rich control sequence 5′-TTGAAAGGTACAGTGCA-3′-cholesterol were assembled using an automatic oligonucleotide synthesizer (Applied Biosystems 3400, Foster City, CA). The solid support functionalized with cholesterol and the rest of the chemicals were from commercial sources (Link Technologies, Scotland, UK). After the assembly of the sequences, supports were treated with ammonia and the desired oligonucleotides were purified by reverse-phase HPLC. The oligonucleotides were next suspended at a concentration of 1.6 mM in an annealing buffer (lipoquad solvent, 20 mM Tris Acetate pH 7.0 and 50 mM Potassium Acetate), boiled for 5 minutes at 95°C, slowly cooled down to room temperature and incubated at 25°C for at least 14 days. G-quadruplex formation was followed by 10% non-denaturing polyacrylamide gel electrophoresis as described previously (28) and verified by circular dichroism. The compounds were stored at -20°C until use.

Cell culture

The human hepatocarcinoma cell lines Huh7/Scr , Huh7.5.1 Cl.2 (kindly provided by F. Chisari) and the human embryonic kidney cell line 293T (HEK293T cells, American Type Culture Collection, Manassas, VA, CRL-1573) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 10% non-essential amino acids, 100 units/ml penicillin and 100 units/ml streptomycin. Cells were grown in an incubator with 5% CO_2 at 37ºC.

Plasmids

Plasmids used to produce HCVcc: the plasmid pFK-Jc1 has been previously described (29). It encodes a chimeric HCV consisting of codons 1 to 846 derived from J6/CF (genotype 2a, GenBank accession number AF177036) combined with codons 847 to 3033 of JFH1 (genotype 2a, GenBank accession number AB047639). The plasmid pFK-Luc-Jc1 (30) consists of a bicistronic construct where the HCV polyprotein-coding region is located in the second cistron and is expressed via an internal ribosome entry site (IRES) element derived from the encephalomyocarditis virus (EMCV) while the first cistron contains the *Firefly* luciferase reporter gene.. Plasmids encoding for the HVR1 mutants are also based on the Jc1 genome and have been described elsewhere (32). Briefly, these plasmids are pFK-Luc-Jc1 derivatives. The pFK-Luc-Jc1-ΔHVR1 plasmid contains a 27-amino acid deletion of the E2 HVR1 region while the pFK-Luc-Jc1-basic plasmid possesses an alanine substitution in all basic amino acids of the HVR1 ("basic-" mutant). HCV genotype 1-7 plasmids are JFH1 based reporter virus constructs (NS3-NS5B of JFH-1 origin, genotype 2a), carrying *Renilla* luciferase inserted at the NS5A gene and expressing core-nonstructural protein 2 (NS2) of genotype 1 to 7 prototype isolates (33). HCV Jc1FLAG(p7-nsGluc2A) is a cell culture

derived virus chimera of J6 and JFH-1(genotype 2a/genotype 2a chimera), which is fully-infectious and carries the *Gaussia* luciferase as a reporter gene (34).

Plasmid used to produce HCV subgenomic replicon: the subgenomic replicon plasmid carries a bicistronic construct in which a *Firefly* luciferase gene is expressed via HCV IRES and an EMCV IRES drives expression of JFH1 non-structural proteins (NS3 to NS5B) (31).

Plasmids used to produce HCVpp: the plasmid pTN7-Stopp (kindly provided by M. Dittmar) carries the HIV-1 genome with the following modifications: the *Renilla* luciferase reporter gene has replaced the *nef* gene and lacks a functional *env* gene (35). The plasmid, which encodes strain HC-J6CH E1E2 glycoproteins and is designated pcDNA3.1-ΔcE1E2-J6CH, has been described elsewhere (32). The plasmid pVPack-VSV-G, which encodes for the vesicular stomatitis virus glycoprotein (VSV-G) has been purchased by Agilent Technologies (Santa Clara, CA).

In vitro transcription, electroporation and preparation of virus stocks

Plasmids carrying Jc1 constructs were linearized with the *MluI* enzyme while plasmids carrying genotype 1-7/JFH1 chimeric viruses and the *Gaussia* reporter were linearized with the *XbaI* enzyme. Plasmid DNA was purified with the QIAquick PCR purification kit (Qiagen, Düsseldorf, Germany). Purified DNA was subjected to an *in vitro* transcription reaction with the MEGAscript® T7 kit (Applied Biosystems, Foster city, CA) according to the manufacturer's protocol. RNA from the *in vitro* transcription reaction was purified with the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany), RNA integrity was verified by formaldehyde agarose gel electrophoresis and the concentration was determined by measurement of the optical density at 260 nm. For RNA electroporations, single cell suspensions of Huh7.5.1 Cl.2 cells were prepared by

trypsinization of cell monolayers. Cells were washed with phosphate-buffered saline (PBS), counted, and resuspended at 1.5×10^7 cells per ml in cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4 pH=7.6, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$, final pH=7.6 adjusted with KOH) (36) containing 2mM ATP and 5mM glutathione. Ten µg of *in vitro* transcribed RNA was mixed with 400 µl of the cell suspension. Cells were then electroporated, immediately transferred to 10 ml of culture medium and seeded in a 10-cm dish. Electroporation conditions were 975 µF and 270 V by using a Gene Pulser Xcell™ system (Bio-Rad, Munich, Germany) and a cuvette with a gap width of 0.4 cm (Bio-Rad). Supernatants of the electroporated cells were harvested 72h post electroporation, cleared by passing them through 45-µm-pore-size filters and stored at -80ºC.

For the determination of viral titers Huh7/Scr cells were seeded at a concentration of 4×10^4 cells per well in a 96-well plate in a total volume of 200 µl. Twenty-four hours later, serial dilutions of virus containing supernatant were added (6 wells per dilution). Three days later, cells were washed with PBS and fixed for 20 min with ice-cold methanol at −20°C. After three washes with PBS, NS5A was detected with a 1:2000 dilution of the mouse anti-NS5A antibody 9E10 (kindly provided by C. Rice, The Rockefeller University, NY) in PBS supplemented with 5% BSA for 1h at room temperature. Cells were washed again three times with PBS, and the bound primary antibodies were detected by incubation in PBS + 5% BSA with goat anti-mouse IgG-peroxidase conjugated antibody (Sigma-Aldrich, St. Louis, MO) at 1:400 dilution. After 1h incubation at room temperature, cells were washed three times with PBS; the Vector NovaRED substrate kit (Linaris Biologische Produkte GmbH, Wertheim, Germany) was used for detection of peroxidase. Virus titers [50% tissue culture infective dose per ml

249 ($TCID_{50}$/ml)] were calculated based on the method described by Spearman and Kärber
250 (37, 38).
251
252 **Preparation of HCV and VSV-G pseudoparticles (HCVpp and VSV-Gpp)**
253 HIV-based pseudoparticles bearing HCV or VSV-G glycoproteins were generated by
254 calcium phosphate co-transfection of 293T cells. Briefly, 3.6 x 10^6 293T cells were
255 seeded in 10-cm dishes one day before transfection with equal amounts of pTN7-Stopp
256 plasmid and pcDNA3.1-ΔcE1E2-J6CH or pVPack-VSV-G (Agilent Technologies,
257 Santa Clara, CA) for HCVpp or VSV-Gpp, respectively. A total amount of 20 µg of
258 DNA was mixed with a 2M $CaCl_2$ solution and then 2X Hepes buffered Saline (HBS)
259 was added dropwise to form a precipitate which was added to the cells. The medium
260 was replaced the following day and supernatants containing the pseudoparticles were
261 harvested 48h later, cleared by passage through 0.45-µm-pore-size filters, and used for
262 luciferase infection assays.
263
264 **Luciferase infection assays**
265 For standard infection assays Huh7/Scr cells were seeded at a density of $4x10^4$
266 cells/well in 96-well plates. One day later, cells were pre-incubated for 1h at 37ºC with
267 the pertinent compounds and then inoculated with the virus and the compounds for 4h at
268 37ºC. HCVpp were left for 6 instead of 4h. Finally, virus-containing media was
269 replaced by a fresh media-compounds mix. Luciferase activity was assayed 72h post
270 infection. Cells were washed with PBS, lysed in 150µl of passive lysis buffer and
271 frozen. Upon thawing, lysates were resuspended by pipeting and 50µl were mixed with
272 25µl of a luciferin solution and measured in a luminometer for 2s. The luciferin solution
273 was LARII for *Firefly* luciferase assays and Stop&Glo® reagent (Promega) for *Renilla*

luciferase assays. The BioLux® Gaussia Luciferase Assay Kit (Promega) was used to assess *Gaussia* luciferase activity according to manufacturer´s instructions. Cytotoxicity (viability) was measured in all infection assays using the CytoTox-Glo cytotoxicity assay (Promega) as described by the manufacturer using a plate luminometer FLUOstar OPTIMA (BMG LABTECH) according to the manufacturer's instructions. The MOI used in the infections was 0.01-0.03 $TCID_{50}$/cell. Unless otherwise stated, results for both infectivity and viability are the means (± SEM; n=4) from two replicate infections measured in duplicates and expressed as relative RLU compared to the infection of control (mock) cells.

Attachment assay and qRT-PCR

Huh7/Scr cells were seeded in 24-well plates at $1.5x10^5$ cells/well. The following day, cells were set on ice for 30 min to cool down and then incubated with pre-chilled Jc1 HCVcc virus (at an MOI ~10 $TCID_{50}$/cell) in the presence or absence of compounds for 2h at 4ºC. Cells were washed 3x with ice-cold PBS, lysed and RNA was extracted using the Nucleo Spin RNA II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. RNA concentration was determined by measurement of the optical density at 260 nm. Twenty-five µg of the total RNA sample was used for quantitative PCR analysis using a 7500 Real-Time PCR sequence detector system (Applied Biosystems, Waltham, MA). HCV-specific qRT-PCRs were conducted in duplicate for each sample with the OneStep RT-PCR kit (QIAGEN, Hilden, Germany) using the following 5′ NTR-specific probe: S-292, 5'-6-carboxyfluorescein-CCTGATAGGGTGCTTGCGAGTGCC -tetrachloro-6-carboxyfluorescein-3'; and primers: S-271, 5'- GCGAAAGGCCTTGTGGTACT-3'; and A-337, 5'-CACGGTCTACGAGACCTCCC -3' (Biomers, Ulm, Germany). Reactions were

299 performed in three stages by using the following conditions: stage 1, 60 min at 55°C
300 (reverse transcription); stage 2, 15 min at 95°C (heat inactivation of reverse
301 transcriptase and activation of Taq polymerase); and stage 3, 40 cycles of 15 s at 95°C
302 and 1 min 60°C (amplification). The total volume of the reaction mix was 15 µl, and it
303 contained the following components: 2.66 µM 6-carboxy-X-rhodamine (passive
304 reference), 4 mM $MgCl_2$, 0.66 mM deoxynucleoside triphosphates, 0.266 µM HCV
305 probe, 1 µM of each HCV primer, and 0.6 µl enzyme mix. The amount of HCV RNA
306 was calculated by comparison to serially diluted *in vitro* transcripts.
307
308 **Subgenomic replicon assay**
309 Huh7/Scr cells were seeded in 24-well plates at $1.5x10^5$ cells/well. The following day,
310 cells were pre-incubated for 1h at 37ºC with the pertinent compounds [lipoquads or
311 telaprevir (VX-950), the latter purchased by Selleck Chemicals, Houston, TX] and then
312 transfected with subgenomic replicon RNA using the Lipofectamine® 2000 reagent
313 (Invitrogen, Carlsbad, CA) according to manufacturer´s instructions. Four-hours post
314 transfection lipofectamine-RNA-containing media was replaced by a fresh media-
315 compounds mix and *Firefly* luciferase activity was measured 24h later as described
316 above.
317
318 **Cell-to-cell transmission assays**
319 The first day, Huh7/Scr cells were infected with Jc1 HCVcc virus at an MOI ~10
320 $TCID_{50}$/cell. These cells were then used as HCV donor cells while Huh7.5-EGFP-NLS-
321 IPS cells were used as acceptor cells. The latter cells stably express a chimeric protein
322 that encompasses the enhanced green fluorescent protein (EGFP) associated to the
323 SV40 nuclear localization sequence (NLS) followed by the mitochondrially tethered

interferon-β promoter stimulator protein 1 (IPS-1, all together EGFP-NLS-IPS), which upon HCV infection is redistributed from mitochondria to the nucleus (39, 40). The following day, a 1:1 ratio of donor: acceptor cells was used and a total of $2x10^5$ cells/well were plated in 24-well chambered cover glasses with medium containing lipoquads or dasatinib that inhibits HCV cell-to-cell spread (24), lipoquads buffer (0.1% v/v) or DMSO (0.1% v/v). Cells were covered with fresh medium containing 1% low-melting temperature agarose and compounds 4h after seeding and further cultured for 20h. Finally, cells were fixed with 4% paraformaldehyde, and stained with anti-NS5A antibodies. Cell-to-cell spread was analyzed in a Leica TCS-SP5 confocal microscope. Quantification of Huh7.5/EGFP-NLS-IPS cells was done in 3 independent wells by taking 3 independent pictures of different fields of each well that contained at least 200 cells in total. Data are expressed as percentage to lipoquads solvent (for lipoquads) or DMSO (for dasatinib) treated cells and represent mean values of the 3 independent fields of three biological replicates (±SEM).

Patients and tissue explants

Surgically-resected specimens of colorectal tissue were collected at St. Mary's Hospital, Imperial College London, UK. All tissues were collected after receiving signed informed consent from all patients and under protocols approved by the Local Research Ethics Committee. The tissue was obtained from patients undergoing rectocele repair and colectomy for colorectal cancer. Only healthy tissue that was 10 to 15 cm away from any tumor was used. All patients were HIV and HCV negative. On arrival in the laboratory, resected tissue was cut into 2-3 mm^3 explants comprising both epithelial and muscularis mucosae as described previously (41). Colorectal explants were maintained with DMEM containing 10% fetal calf serum, 2mM L-glutamine and antibiotics (100 U

of penicillin/ml, 100 µg of streptomycin/ml, 80 µg of gentamicin/ml) at 37ºC in an atmosphere containing 5% CO_2.

Tissue inhibition assays

Tissue explants were incubated with lipoquads or DAA [sofosbuvir (SOF) and ledipasvir (LDV), both at 1 µM final concentration, purchased by Selleck Chemicals, Houston, TX] for 1h prior to virus addition for 2h. Explants were then washed 4 times with PBS to remove unbound virus and drug, transferred on gel foam rafts (Welbeck Pharmaceuticals, UK) and cultured in complete medium in the presence (sustained) or absence (pulse) of drug for 48h at 37ºC. Viral levels were measured by *Gaussia* luciferase quantification (Promega, Madison, WI) in a Synergy HT Multi-Detection microplate reader (BioTek Instruments, Inc., Burlington, VT) as described above.

Statistical and mathematical analysis

The statistical comparison between two groups was made by an unpaired-t test. *p value < 0.05, **p value < 0.01 and ***p value < 0.001 were considered to indicate a significant difference while ns = non-significant. IC_{50} values were calculated from sigmoid curve fitting (GraphPad Prism, Graph Pad Software, La Jolla, CA) fulfilling the criterion of $R^2 > 0.7$.

RESULTS

Lipoquads inhibit HCVcc infection

To evaluate the inhibitory effects of lipoquads (Fig. 1A) on HCV infection we used the HCV cell-culture (HCVcc) system (30, 42, 43). Unless otherwise stated, we used the highly permissive Huh7/Scr cells (44) for HCV propagation *in vitro*. Furthermore, we used the highly infectious genotype 2a HCVcc virus (Jc1 chimera (29)). To simplify the quantification of infection, we used the bicistronic Jc1 luciferase reporter construct, designated Luc-Jc1 HCVcc (10). Cell viability was monitored in parallel by a commercial ATP assay (45). Briefly, cells were pre-incubated for 1h at 37ºC with lipoquads and then inoculated with the virus in the presence of lipoquads for 4h at 37ºC. After this time, virus-containing media were replaced by fresh media-lipoquads mix. Luciferase activity was assayed 72h post infection. As shown in Fig. 1B, Luc-Jc1 HCVcc virus infection of Huh7/Scr cells was completely inhibited by lipoquads at concentrations in the 5-10 μM range [Maximum Percent Inhibition (MPI)] without affecting cell viability. The half maximal inhibitory concentration (IC_{50}) for lipoquads was estimated to be ~0.8 μM with a half maximal cytotoxic concentration (CC_{50}) above 10 μM. Under the same infection conditions, dasatinib (46), an FDA-approved anti-cancer kinase inhibitor which has been shown to inhibit HCV entry, inhibited HCV with an IC_{50} of ~3.2 μM and a CC_{50} of 51.8 μM. Disruption of the G-quadruplex structure by exchanging three of the guanines with adenines resulted in a ~7.8-fold increase of the IC50 value (Figure 1C), indicating a certain specificity mediated by the G-quadruplex element.

Lipoquads inhibit the infection of HCV pseudoparticles

To examine if lipoquads inhibit HCV entry we used HCV pseudoparticles (HCVpp) that carried the same E1E2 glycoproteins as Luc-Jc1 HCVcc. HCVpp is a well-established system to investigate HCV entry and neutralization (26, 27, 47). HCVpp infection conditions with lipoquads were similar than those used with the Luc-Jc1 HCVcc virus, except that Huh7/Scr cells were inoculated with HCVpp for 6h. Dasatinib was used again as positive control. At 5 μM concentration, which has been shown previously to inhibit Luc-Jc1 HCVcc infection potently, lipoquads inhibited HCVpp entry by ~8-fold while pseudoparticles carrying the vesicular stomatitis virus glycoprotein (VSV-Gpp) were inhibited only by ~2-fold (Fig. 2A). Thus, lipoquads inhibit E1E2-mediated HCV entry into target cells with a preference over the VSV-G envelope.

Lipoquads interact with HCV and inhibit early entry steps of HCV life-cycle

To identify which HCV entry step was inhibited, we carried out time-of-addition experiments using Luc-Jc1 HCVcc viruses. To this end, we carried out 4 different incubation protocols (Fig. 3A): i) Huh7/Scr cells were pre-incubated with lipoquads for 1h prior to inoculation ii) Luc-Jc1 HCVcc viruses were pre-incubated for 1h with lipoquads prior to inoculation and then viruses containing lipoquads were added to the cells iii) lipoquads were present only during infection for 4h or iv) lipoquads were added to cells post inoculation (from 4h until the time of luciferase assays). As shown in Fig. 3B, lipoquads inhibited Luc-Jc1 HCVcc infection only when they were pre-incubated with Luc-Jc1 HCVcc viruses or added simultaneously to the cell-virus mix indicating that lipoquads act on the viral particles and/or initial steps of HCV entry. Further, in order to test if lipoquads inhibits HCV attachment on the surface of target cells, Huh7/Scr cells were incubated with Jc1 HCVcc virus without reporters in the

presence or absence of inhibitors for 2h at 4°C. Under these conditions, virus attaches to the cells but does not efficiently enter. Heparin sodium salt, which is known to inhibit HCV entry at the attachment step, was used as a positive control (10). After 2h, viruses were removed, cells were washed extensively to remove the unbound virus and the bound HCV was quantified with qRT-PCR. As shown in Fig. 4, lipoquads efficiently inhibited HCV attachment indicating a role at least in early entry steps.

Lipoquads neither inhibit HCV RNA translation nor HCV RNA replication

To test whether lipoquads exert an additional effect in HCV translation and/or replication, we transfected Huh7/Scr cells with a subgenomic JFH1 luciferase replicon (SGR-JFH1). Lipoquads were present for a pre-incubation period of 1h, during transfection (4h) and post transfection, as described previously for Luc-Jc1 HCVcc infections. RNA replication was monitored 24h post transfection by luciferase assays. As control, we incubated the cells with increasing doses of the NS3-4A protease inhibitor telaprevir (VX-950). As shown in Fig.5, lipoquads did not affect luciferase expression, while the NS3-4A inhibitor telaprevir showed a sharp reduction in luciferase expression, indicating that lipoquads do not inhibit HCV RNA translation and/or replication.

Basic residues of hypervariable region 1 (HVR1) and HVR1 itself play a role in lipoquads-mediated HCV neutralization.

The hypervariable region 1 (HVR1) of the HCV E2 glycoprotein is known to facilitate virus-host cell interactions (48). To investigate whether this region is involved in the lipoquad-mediated HCV inhibition, we performed inhibition experiments with HCV mutants carrying altered HVR1 regions (Fig. 6). To this end, we used Luc-Jc1 WT

449 HCVcc viruses, Luc-Jc1 HCVcc viruses which harbor a total deletion of the HVR1
450 region [ΔHVR1 (49)] or a Luc-Jc1 HCVcc virus mutant that possesses alanines instead
451 of the basic amino acids in the HVR1 [basic- (21)]. In order to observe subtle
452 differences in lipoquads inhibition, we used a 1 μM final concentration in this setting,
453 which according to Fig.1 is higher than the IC_{50} but below 100% inhibition. As shown
454 in Fig. 6, WT virus was inhibited up to ~80%, while both mutants (ΔHVR1 and basic-)
455 were not affected. This suggests a direct role of the basic amino acids of the HVR1
456 and/or of the HVR1 region itself in lipoquads antiviral activity.
457
458 **Lipoquads inhibit HCV cell-to-cell transmission**
459 To assess whether lipoquads can inhibit HCV cell-to-cell transmission, we used an
460 agarose overlay-based assay which inhibits cell-free virus, using a previously described
461 infection reporter system (40). In this infection reporter system, Jc1 HCVcc-infected
462 Huh7/Scr cells act as virus donor cells and uninfected Huh7.5/ EGFP-NLS-IPS as
463 acceptor cells (40, 50). The latter cells stably express a chimeric EGFP protein, which
464 upon HCV infection is redistributed from mitochondria to the nucleus (a complete
465 description of these cells is available in the *Materials and Methods* section). Moreover,
466 HCV infection was evaluated by anti-NS5A immunofluorescence. For contingency to
467 the previous experiments, the lipoquads incubation period was similar to that used for
468 cell-free infections. As shown in Fig. 7A & B, lipoquads and the control dasatinib
469 efficiently inhibited HCV cell-to-cell transmission, as deduced by the number of NS5A
470 positive acceptor cells and EGFP redistribution to the nucleus.
471
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473

Lipoquads inhibit entry of all major HCV genotypes

To determine the antiviral efficiency of lipoquads against all major HCV genotypes, we utilized JFH1-based reporter virus constructs (JFH1 NS3-NS5B proteins), carrying *Renilla* luciferase inserted at the NS5A gene and core to NS2 proteins from all major HCV genotypes: 1a (isolate TN), 1b (isolate J4), 2b (isolate J8), 3a (isolate S52), 4a (isolate ED43), 5a (isolate SA13), 6a (isolate HK6a) and 7a (isolate QC69) (51). Strikingly, lipoquads showed antiviral activity against all major HCV genotypes (Fig. 8). The estimated IC_{50} for each HCV genotype (Table 1) was comparable to the IC_{50} estimated in the first set of experiments for the genotype 2a Luc-Jc1 HCVcc.

Lipoquads inhibit HCV *ex vivo* in a mucosal model

To assess lipoquads activity *ex vivo*, we tested lipoquads in a mucosal model based on *ex vivo* viral challenge of colorectal tissue explants (52, 53). This model allowed us to evaluate the potency of lipoquads as an HCV entry inhibitor against a reporter HCV, Jc1FLAG(p7-nsGluc2A), by measurement of *Gaussia* luciferase expressed and secreted upon viral entry, translation and/or replication of the HCV genome (34). Pulse exposure of explants to drug for 3h resulted in a dose-dependent reduction of viral levels (Fig. 9A; IC_{50} of 11.88 ± 7.35 µM). Interestingly, the level of inhibition reached with the highest drug concentration tested was similar to that obtained with a combination of SOF/LDV (both at 1 µM final concentration). With sustained exposure to lipoquads (compound maintained throughout explant exposure to virus and culture), a decrease in the IC_{50} value to 1.08 ± 0.13 µM and an increase in Jc1FLAG(p7-nsGluc2A) infection inhibition up to 93% were reached within the concentration range tested (Fig. 9B). Altogether, these data demonstrate the potential of lipoquads as candidate drugs for topical HCV prevention strategies.

DISCUSSION

The introduction of DAA therapies in the Standard-of-Care (SoC) treatment of HCV has increased significantly the sustained virological response (SVR) rates accompanied by manageable adverse effects (54). However, these compounds target one of the non-structural proteins of HCV (NS3-4A, NS5A and/or NS5B) and thus, cannot prevent HCV infection. In this study, we report a class of novel oligonucleotide-lipid conjugates forming G-quadruplex structures, designated lipoquads, as potent and pangenotypic HCV entry inhibitors *in vitro*. Moreover, we were able to show anti-HCV activity *ex vivo* in an intestinal mucosa explant model, using genotype 2a HCVcc. Notably, lipoquads exhibited comparable IC_{50} values to known FDA-approved drugs like dasatinib and telaprevir (VX-950). Besides, lipoquads were able to achieve a MPI at ~5 – 10 µM. These data for lipoquads as antiviral compounds are important if breakthrough and/or resistance need to be tested. Along with their absence of toxicity in the concentrations tested and their high water solubility, lipoquads present attractive candidates for the development of HCV prevention strategies.

To characterize the lipoquads inhibition mechanism we used several molecular tools in diverse infection assays. Firstly, we used the HCVpp system in order to isolate the entry process from other HCV functions i.e. replication/translation and assemble/release. Because lipoquads inhibited HCVpp and had no effect on the subgenomic replicon, we conclude that lipoquads target viral entry. HCV entry is a complex process that requires several entry molecules (see (55, 56) for reviews). It comprises the steps from particle binding to the host cell up to the delivery of the viral genome to the replication site within the cell. Initial binding is mediated by interactions between HCV E1E2 envelope

524 glycoproteins and glycosaminoglycans (GAGs) (11). Also low density lipoprotein
525 receptors (LDLR) on host cells may function as initial attachment factors due to the
526 association of HCV with (V)LDL (57, 58). Following this initial engagement,
527 tetraspanin CD81 and SR-B1 together with the tight junction proteins CLDN1 and
528 OCLN are the main receptors contributing to HCV uptake (59-62). Our results show
529 that lipoquads inhibits HCV infection by negatively affecting virus-cell binding. This
530 effect is presumably mediated via direct interaction of the compound with the virus
531 since pre-exposure of the cells to lipoquads did not result in decreased infectivity.
532 Lipoquads are therefore responsible for blocking the interaction of the virus with the
533 cellular receptors.
534
535 To shed more light into the mechanism of interaction between lipoquads and HCV, we
536 used two HCV mutants for the E2 HVR1, one in which all basic aminoacids of the
537 region were replaced by alanines (basic-), and other that lacked the region itself
538 (ΔHVR1). Importantly, none of them was inhibited by lipoquads. Although HVR1 is
539 not essential for HCV productive infection, viruses lacking this domain are less
540 infectious, both *in vitro* (63) and *in vivo* (64). HVR1 displays high genetic variability
541 between HCV isolates, which is likely contributing to immune evasion of HCV.
542 Previous experiments performed in HVR1-deleted mutants suggest that HVR1 may be
543 acting as immunological decoy since it shields conserved neutralizing epitopes (63).
544 Indeed, ΔHVR1 mutants show increased neutralization susceptibility to HCV patient
545 sera (63, 65). It has been proposed that a complex interplay between several regions of
546 E2 is responsible for modulating receptor binding, possibly through intramolecular
547 interactions (66). As deduced by our results, lipoquads may affect this interplay.
548 Particularly, lipoquads display negative charges so that a positive-negative electrostatic

549 interaction is likely to occur. Furthermore, alignment of the HVR1 domains of the
550 different HCV genotypes reveals a conserved presence of positively charged residues
551 (data not shown). Thus, HCV pangenotypic activity of lipoquads can also be explained
552 by the interaction with basic aminoacids in the E2 glycoprotein. Overall, although
553 conclusive evidence for the target of lipoquads is lacking, we hypothesize that lipoquads
554 use a common mechanism that involves electrostatic interactions with positively-
555 charged residues in viral entry proteins.
556

557 HCV entry into target hepatocytes, as cell-free virus, has been proven to be a well-
558 orchestrated process with spatio-temporal requirements. Notably, in addition to
559 infection by cell-free virus, direct cell-to-cell transmission also occurs in the liver (67,
560 68) and in cell-cultured hepatocytes (69-73). This route of viral spread may provide a
561 way to avoid neutralization, resulting in viral persistence and hampering viral
562 eradication (73). DAA-resistant variants have been shown to use cell-to-cell
563 transmission as the main route of viral spread in cell culture (74). Hence, lipoquads
564 possess a great additional value owing to their cell-to-cell virus transmission inhibition
565 capacity. Most host entry factors are shared between the two routes. These include SR-
566 B1, CLDN1, OCLN, NPC1L1 as well as EGFR and its signal transducer HRas (21, 25,
567 75-77). The dependence on CD81 in cell-to-cell transmission remains controversial (23,
568 78). While the role of these factors during HCV cell-free transmission has been
569 extensively studied, mechanistic insights about their spatio-temporal role during HCV
570 cell-to-cell spread are still far from being satisfactory. Thus, although lipoquads appear
571 to inhibit initial HCV cell-free attachment and therefore interfere with the interaction of
572 HCV with attachment molecules/receptors, the exact mechanism of HCV cell-to-cell

573 inhibition by lipoquads needs further investigation. Importantly, the majority of
574 monoclonal antibodies targeting the viral envelope has been shown to fail to inhibit cell-
575 to-cell transmission. In contrast, several host-targeting entry inhibitors (HTEIs) have
576 succeeded in blocking this route of viral spread (21, 75). Since lipoquads target directly
577 the viral particles and do not interfere with cellular processes, they present a significant
578 advantage against HTEIs.
579
580 Antivirals formulated for topical applications are known as microbicides and viral entry
581 into the target cell represent an important point where microbicides can inhibit mucosal
582 transmission. In more details, the intestinal mucosa is composed of intestinal epithelial
583 cells (enterocytes, secretory cells and intestinal epithelial stem cells), stromal cells and
584 myofibroblasts. Colorectal tissue also contains T cells, B cells, dendritic cells,
585 macrophages, and innate lymphoid cells in the lamina propia (79, 80). Furthermore, the
586 expression of certain HCV entry receptors in the intestinal mucosa has been described
587 previously (81-85). These cells could be the primary target cells for virus infection
588 through mucosal transmission. In the field of human immunodeficiency virus (HIV)
589 prevention, proof of concept that microbicides can block HIV transmission was
590 obtained in a clinical efficacy trial (86); although, ultimately, the efficacy of any
591 microbicide product will depend upon adherence as well as appropriate drug delivery
592 (87). Microbicides are topical antivirals and therefore their inhibitory activity needs to
593 be considered within the context of mucosal transmission. Mucosal tissue explants
594 models are becoming an essential tool for pre-clinical screening of candidates for oral-
595 and topical-pre-exposure prophylaxis against HIV (53) and are increasingly being used
596 in HIV-prevention clinical trials (88-91). In this study we expanded the usage of this
597 mucosal model to assess the inhibitory potency of lipoquads against HCV. Our results

598 are important because they clearly show HCV transmission through the mucosa,
599 although HCV primarily infects and replicates in hepatocytes. Nevertheless, we were
600 able to inhibit HCV intestinal mucosa infection by DAA, suggesting that HCV
601 replicates in this *ex vivo* model as well. Furthermore, pulse exposure of the tissue to
602 lipoquads showed limited potency reaching a maximum of 55% of inhibition; however,
603 sustained exposure showed a significant increase of activity with a decrease in the IC_{50}
604 and an increase in the level of inhibition reached at the highest concentration tested.
605 Because microbicides are topically applied, higher local drug concentrations can be
606 delivered to mucosal surfaces without significant systemic exposure, thereby reducing
607 the risk of long-term toxicity in healthy but at-risk individuals.
608
609 In conclusion, permucosal HCV transmission has been confirmed as the most likely
610 mode of HCV infection in MSM and became a significant source of new HCV
611 infections. In essence, HCV sexual transmission concerns the general population,
612 independently of sexual preferences. This calls for new antiviral drugs that permit an
613 efficient topical pre-exposure prophylaxis. Our work here now demonstrates that
614 lipoquads are efficient pangenotypic HCV entry inhibitors with interesting properties
615 that make them candidates for further studies on drug formulations and dosing
616 evaluations. Providing safe and acceptable microbicides to the community at risk to
617 sexually acquire HCV will be an important step in HCV transmission control. Lipoquad
618 presents itself as a candidate compound for development in this direction.
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623 **Table 1**

Antiviral activity (IC_{50}) of lipoquads across all major HCV genotypes	
HCV genotype	IC_{50} [μM]
1a (TN)	0.9
1b (J4)	0.2
2b (J8)	0.3
3a (S52)	0.3
4a (ED43)	0.9
5a (SA13)	1.2
6a (HK6a)	1.5
7a (QC69)	0.5

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Figure 1

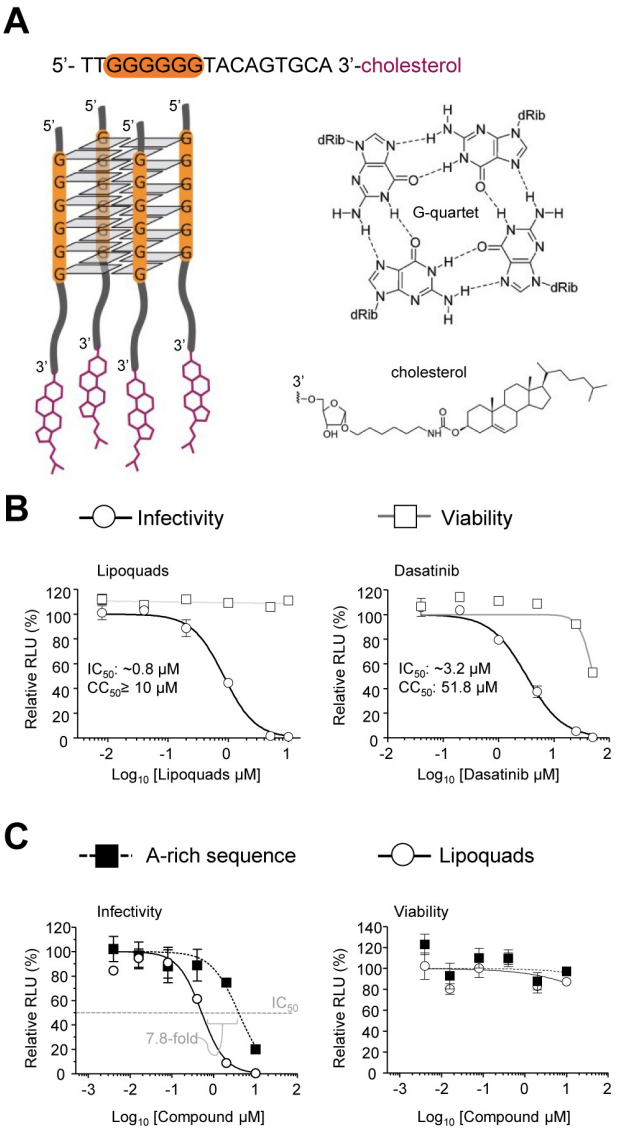

Figure 1. Quadruplex DNA structures (lipoquads) are potent HCV inhibitors. (A) Sequence and schematic structure of the parallel tetramolecular lipoquads of the present study, formed by the association of four 17-mer oligonucleotides containing the GGGGGG (orange) repeat. The structure is held by six stacked G-quartets. The 3' extremities of the individual strands are conjugated with a cholesteryl function (magenta), forming a lipophilic tail. (B) Anti-HCV activity of lipoquads and viability of Huh7/Scr cells infected with Luc-Jc1 HCVcc viruses (at an MOI 0.01-0.03 $TCID_{50}$/cell) and treated with increasing concentrations of lipoquads or dasatinib (positive control). (C) Anti-HCV activity and viability on Huh7/Scr cells infected with Luc-Jc1 HCVcc viruses and treated with increasing concentrations of lipoquads or A-rich control sequence. Infectivity and viability were determined 72h post infection by luciferase assays. Results are the means (± SEM) from two replicate infections measured in duplicates and expressed as relative RLU compared to the infection of control (mock) cells. The data presented are from a single experiment and are representative of three independent experiments.

Figure 2

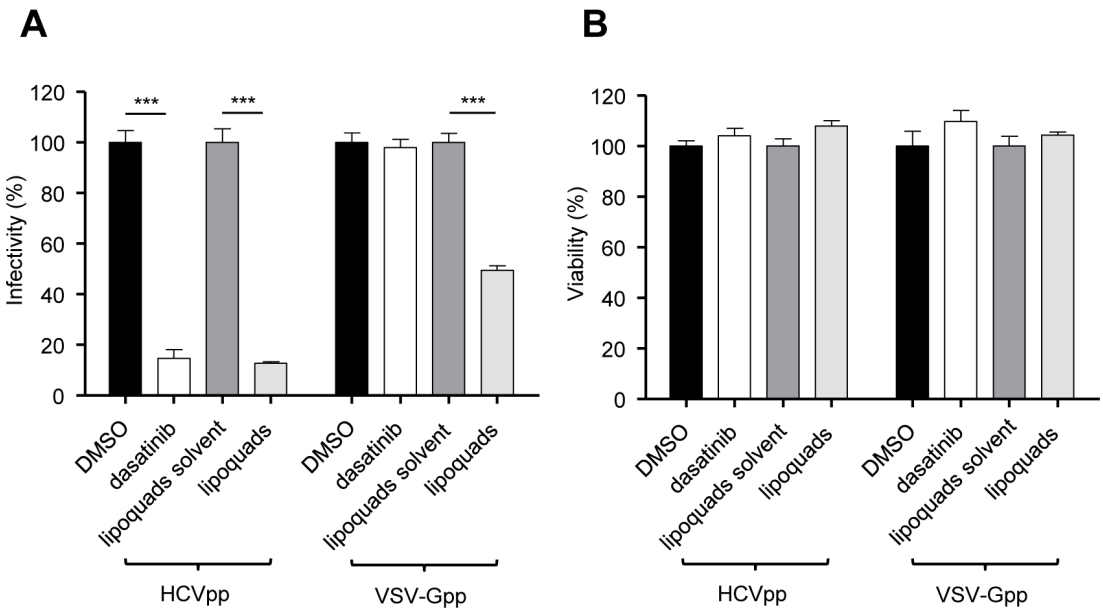

Figure 2. Lipoquads preferentially inhibit HCV pseudoparticles (HCVpp). Huh7/Scr cells were pretreated for 1h with 5 µM of lipoquads or dasatinib. Then, cells were inoculated with HCVpp or VSV-Gpp in the presence of compounds. Six hours later medium was replaced with fresh media-compounds mix and cells. Infectivity and viability were determined 72h post infection by *Renilla* luciferase for infectivity (A) or *Firefly* luciferase assays for cell viability (B). Results are the means (± SEM) from two replicate infections measured in duplicates and expressed as relative RLU compared to the infection of control (lipoquads solvent for lipoquads or DMSO for dasatinib) cells for both infectivity and viability. The data presented are from a single experiment and are representative of three independent experiments.

Figure 3

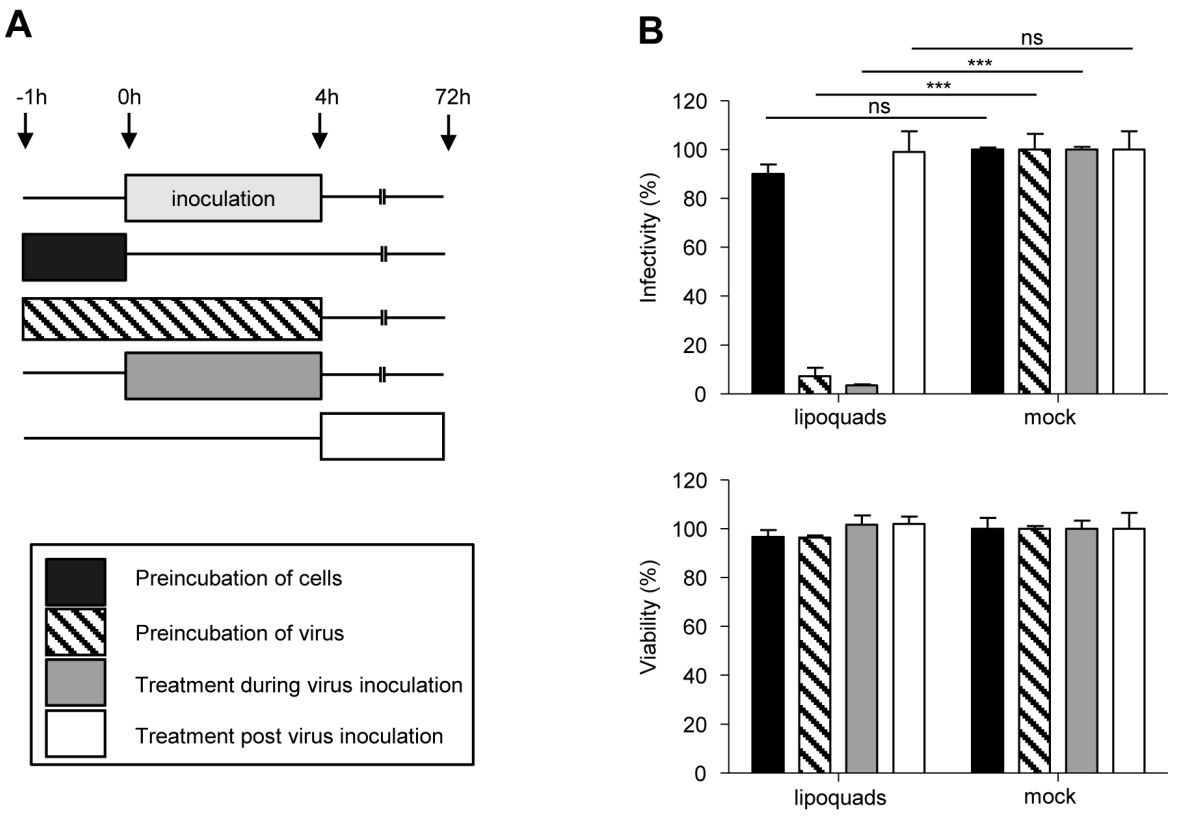

Figure 3. Lipoquads interact with HCV particles and selectively inhibit early HCV entry events. Huh7/Scr cells were inoculated with Luc-Jc1 HCVcc viruses (at an MOI 0.01-0.03 $TCID_{50}$/cell) prepared in the absence of drugs. Lipoquads (final concentration 1 µM) were added to the cells only before inoculation for 1h (black), pre-incubated with the viruses (stripes), only during inoculation (grey) or selectively after infection (white), as schematically depicted in (A). Infectivity and viability (B) were determined 72h later by *Firefly* luciferase assays. Results are the means (± SEM) from two replicate infections measured in duplicates and expressed as relative RLU compared to the infection of control (mock) cells. The data presented are from a single experiment and are representative of three independent experiments.

Figure 4

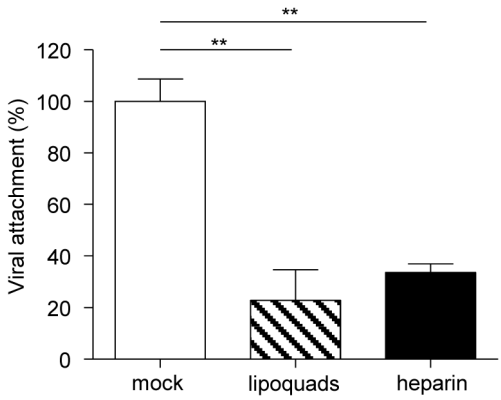

Figure 4. Lipoquads block virus attachment to the cell. Huh7/Scr cells were placed on ice for 30 min to cool down. Cells were then incubated for 2h with pre-chilled Jc1 HCVcc virus (MOI ~10 $TCID_{50}$/cell) at 4ºC. Lipoquads (5 µM) or heparin (100 µg/ml) or buffer (mock) were added to the cells simultaneously to virus inoculation. Cell monolayers were washed 3 times with cold PBS and then lysed and subjected to RNA extraction. HCV RNA was quantified by qRT-PCR in 25 ng of total RNA. Results are the means (± SEM) from two replicate infections measured in triplicates and expressed as relative RNA compared to the viral attachment of control (mock) cells. The data presented are from a single experiment and are representative of three independent experiments.

Figure 5

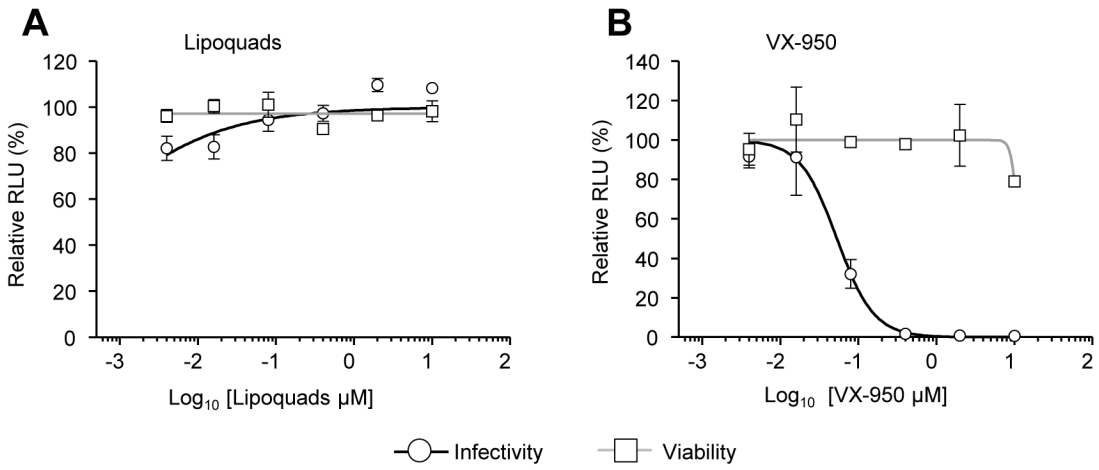

Figure 5. Lipoquads do not inhibit HCV subgenomic replicons. Huh7/Scr cells were pre-treated with lipoquads or telaprevir (VX-950) at increasing concentrations (0.004, 0.016, 0.08, 0.4, 2 and 10 µM) for 1h, transfected with SGR RNA (compounds present) and seeded in 96-well plates. Four-hours post transfection lipofectamine-RNA-containing media was replaced by a fresh media-compounds mix and *Firefly* luciferase activity was assayed 24h later. In both A and B results are the means (± SEM) from two replicate transfections measured in duplicates and expressed as relative RLU compared to the transfection of control (mock) cells for both infectivity and viability. The data presented are from a single experiment and are representative of three independent experiments.

Figure 6

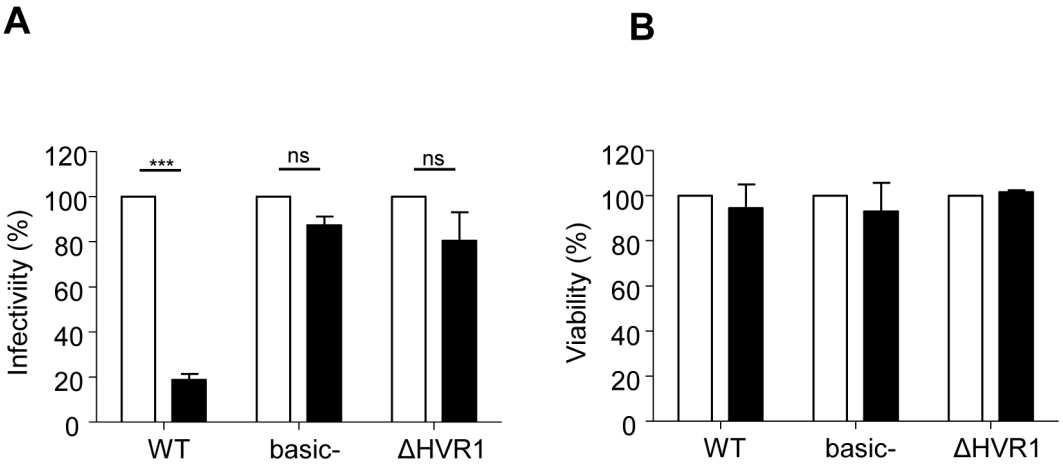

Figure 6. Basic aminoacis in E2 HVR1 and HVR1 itself play a role in lipoquad-mediated HCV inhibition. Huh7/Scr cells were inoculated with Luc-Jc1 WT HCVcc viruses or with the indicated mutants (at an MOI 0.01-0.03 $TCID_{50}$/cell). Lipoquads (final concentration 1 μM) were added to the cells simultaneously to inoculation. HCVcc-compounds mix were replaced 4h post infection with fresh media-compounds mix and 72h after infection cells were assayed for *Firefly* luciferase activity. Results are the means (± SEM) from two replicate infections measured in duplicates (black bars) and expressed as relative RLU compared to the infection of control (mock) cells (white bars) for both infectivity (A) and viability (B). The data presented are from a single experiment and are representative of three independent experiments.

Figure 7

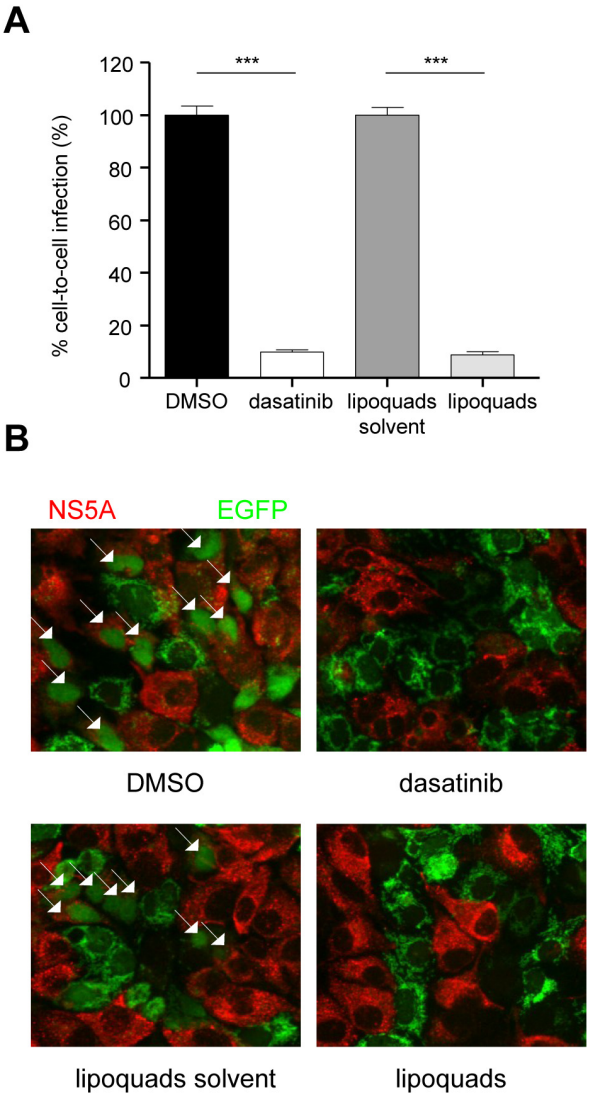

Figure 7. Lipoquads inhibit HCV cell-to-cell transmission. Huh7/Scr cells were infected with Jc1 HCVcc virus at an MOI ~10 $TCID_{50}$/cell and 20h later seeded on 24 well plates. Simultaneously, Huh7.5/EGFP-NLS-IPS cells were seeded on the same 24 well plates (co-culture, at a ratio 1:1 with Huh7/Scr cells) and lipoquads (10 µM) or dasatinib (10 µM) were added to the wells. The addition of the compounds solvent (lipoquads solvent or DMSO) served as negative control. Four hours later, medium was removed and cells were overlaid with 1% agarose with fresh compounds. Twenty four hours later, the HCV infection was analysed by immunofluoresce of NS5A staining. (A) Results are the means of quantification of Huh7.5/EGFP-NLS-IPS cells in 3 independent wells by taking 3 independent pictures of different fields of each well that contained at least 200 cells in total. Cell-to-cell inhibition is expressed as the percentage of acceptor cells with the EGFP-NLS + NS5A signal with respect to the total number of EGFP + NS5A cells in the DMSO (for dasatinib) or lipoquads buffer (for lipoquads) treated wells. (B) Representative images of co-culture cells stained with anti-NS5A antibodies. Huh7.5/EGFP-NLS-IPS cells with EGFP signal redestributed to the nucleus and cytoplasmatic NS5A signal are delineated with arrows. Magnification 63x.

Figure 8

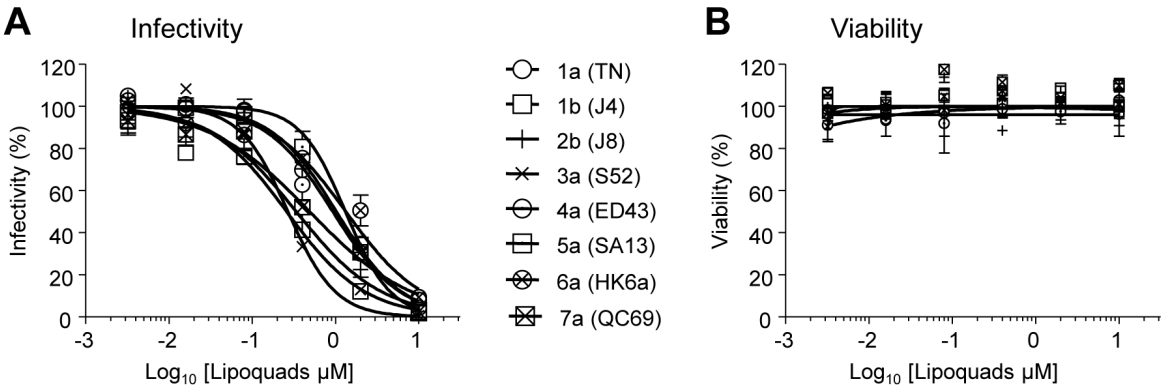

Figure 8. Lipoquads inhibits HCV of different genotypes. Huh7/Scr cells were inoculated with viruses of the indicated genotypes and isolates (at an MOI ~0.01-0.03 $TCID_{50}$/cell). Lipoquads in the indicated concentrations were added to the cells simultaneously to inoculation. HCVcc-compounds mix were replaced 4h post infection with fresh media-compound mix. Seventy two hours post infection cells were assayed for *Renilla* luciferase activity for infectivity (A) or *Firefly* luciferase for cell viability (B). Results are the means (± SEM) from two replicate infections measured in duplicates and expressed as relative RLU compared to the infection of control (mock) cells. The data presented are from a single experiment and are representative of three independent experiments.

Figure 9

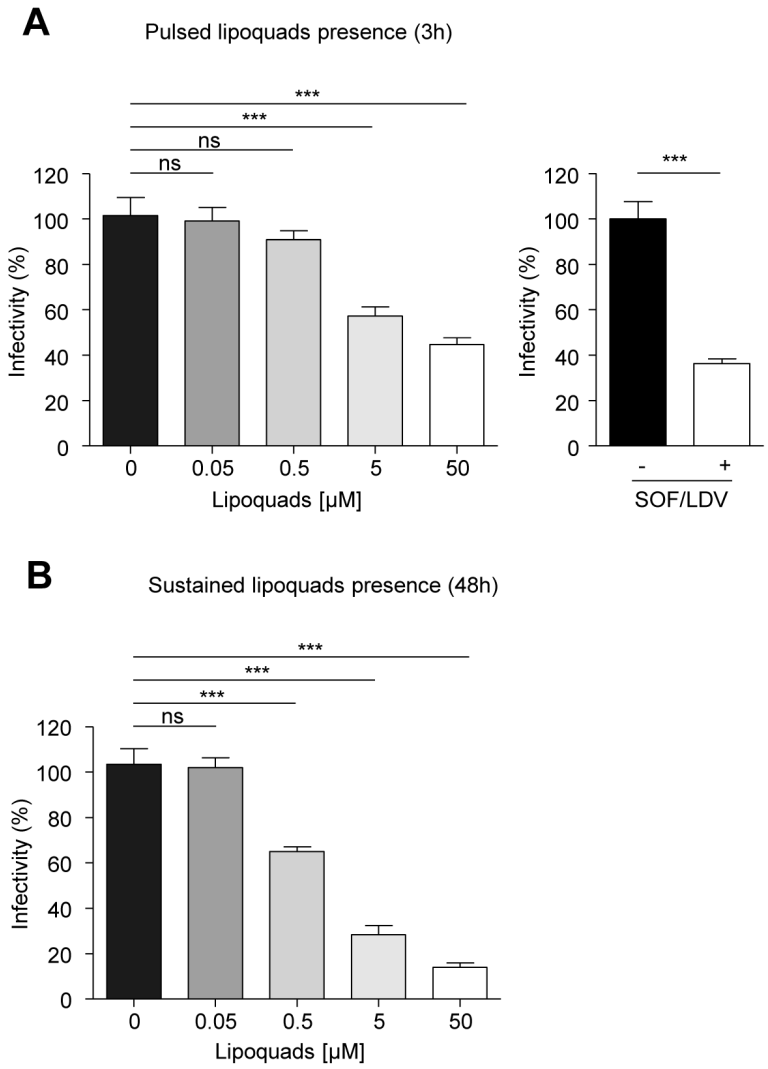

Figure 9. Inhibitory activity of lipoquads in colorectal tissue explants against HCVcc Jc1FLAG(p7-nsGluc2A). Mucosal explants were treated for 1h in the presence or absence of lipoquads (at the indicated concentrations) or SOF/LDV (both at 1 µM final concentration) prior to viral exposure for 2h (total compounds presence 3h). Explants were then washed four times with PBS and transferred to gel foam rafts. Tissue explants were kept in culture for 48h in the absence (pulse) (A) or in the sustained compounds presence (B). Culture supernatants were harvested for detection of *Gaussia* luciferase activity and the extent of infection was plotted as percentage relative to the relative light units (RLU) obtained for explants infected with virus in the absence of compound (100% infectivity). Data represent the means of three independent experiments performed in triplicates (±SEM).