Rapid and convenient assays to assess potential inhibitory activity on in vitro hepatitis A replication

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Shorten title: Antiviral assays for hepatitis A virus

Abstract

Three different antiviral assays were developed for the *in vitro* screening of inhibitors of the hepatitis A virus (HAV) of which (i) a cytopathic effect reduction assay suitable for medium-to-high-throughput screening and (ii) two virus yield reduction assays (based on quantification of viral RNA) for genotypes IB and IIIA. The assays were validated for antiviral studies with interferon-alpha (IFN α) and amantadine HCl, two known inhibitors of HAV replication. IFN α effectively inhibited HAV replication, whereas the activity of amantadine HCl appeared to be strain-dependent. Employing these assays, we assessed the effect of the known enterovirus inhibitors pleconaril, rupintrivir and enviroxime on HAV replication. Pleconaril exhibited some very moderate activity, the effect of rupintrivir proved to be strain-dependent. Enviroxime did not inhibit HAV replication, suggesting that phosphatidylinositol-4-kinase III β is not crucial in the HAV life cycle.

Keywords: Hepatitis A virus; Antiviral assay; Interferon; Amantadine; Enterovirus inhibitor

1. Introduction

Hepatitis A virus (HAV) is an atypical member of the *Picornaviridae* and among the leading causes of enterically transmitted hepatitis worldwide (Wasley *et al.*, 2006). Infections are often asymptomatic, especially in children, but result occasionally in jaundice, fever, fatigue and malaise. It is estimated that about 1.5 million clinical cases of hepatitis A occur annually (Wasley *et al.*, 2006). Full recovery is the rule for most symptomatic HAV infections, although some patients display relapsing infection or fulminant hepatitis, especially the elderly. This results in mortality rates up to 5.4% in those aged over 50 (WHO, 2011). Superinfections with HAV in patients chronically infected with hepatitis B or C are believed to increase morbidity and mortality (Keeffe *et al.*, 2000, Vento *et al.*, 1998), although these findings are still subject of debate (Shouval *et al.*, 2012). HAV is transmitted through the faeco-oral route and consequently, epidemics most often occur in regions with poor hygienic and sanitary conditions. Although efficacious vaccines are commercially available against the single HAV serotype, outbreaks are still reported rather frequently in developed countries (Donnan *et al.*, 2012, Bordi *et al.*, 2012). The current treatment is merely supportive: rest, adequate hydration and proper nutritional balance are advised (Jeong *et al.*, 2010). For severe infections and for the purpose of containment of epidemics, but also to shorten the period of illness (several weeks), a safe and potent antiviral molecule would be much appreciated. A recent report warned for the potential emergence of new serotypes (Pérez-Sautu *et al.*, 2011). Rapid intervention with an antiviral would be a useful tool in halting the spread of such vaccine-escape variants.

A limited number of molecules has been reported to inhibit the *in vitro* replication of HAV. Antiviral activities have been described for amantadine, ribavirin, glycyrrhizin, pyrazofurin, amphotericin B, atropine and protamine by using various methodologies such as solid-phase radioimmunoassays, antigen stainings and RNA hybridization assays (Widell *et*

61 *al.*, 1986, Superti *et al.*, 1987, Crance *et al.*, 1990, van Cuyck-Gandré *et al.*, 1995, Biziagos *et*
62 *al.*, 1990). Limited antiviral activity has been described for chlorpromazine and chloroquine
63 (Bishop *et al.*, 1998). However, clinical use of these compounds is hampered by poor
64 selectivity or toxic side effects. Interferon-alpha (IFN α) is active *in vitro* against HAV and has
65 shown some effect in the treatment of severe HAV infections (Crance *et al.*, 1995, Yoshida *et*
66 *al.*, 1994), but clinical use is associated with severe side effects.

67 Since the arrival of vaccines, research interest in HAV has decreased substantially (Martin
68 *et al.*, 2006). To the best of our knowledge, no cell-based screening assays against HAV have
69 been reported in the last decade. Moreover, the antiviral assays that were reported earlier are
70 labor-intensive and allow only to evaluate a small number of molecules. Here we report a
71 cytopathic effect (CPE) reduction assay amenable for high-throughput screening purposes. In
72 addition, we present virus yield reduction assays (based on reverse transcription quantitative
73 PCR (RT-qPCR)) that can be used as a secondary assay to confirm and validate the activities
74 of hits identified in the CPE-based assay. We employed these assays to assess the potential
75 inhibitory activity of a small panel of known enterovirus inhibitors (pleconaril, rupintrivir and
76 enviroxime) against HAV.

77 **2. Materials and methods**

78
79 **2.1. Cells and viruses** FRhK-4 cells (ATCC CRL-1688) and BS-C-1 cells (ATCC CCL-
80 26) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gent, Belgium)
81 supplemented with 10% fetal bovine serum (FBS, Integro, Leuvenheim, the Netherlands).
82 Huh7-A-I cells supporting stable HAV growth (Konduru *et al.*, 2006) were grown in DMEM
83 supplemented with 10% FBS and were subcultured twice a week at a 1:5 ratio. Huh7-A-I
84 slow (Huh7-A-IS) cells were obtained by subculturing Huh7-A-I once a week (1:5 split ratio)

for 20 passages in DMEM with 10% FBS and then for 10 passages in minimal essential medium (MEM) rega 3 (Gibco) supplemented with 10% FBS, 1% L-glutamine (Gibco) and 1% sodium bicarbonate (Gibco). All cell lines were grown in a humidified 5% CO₂ incubator at 37°C.

Cell culture-adapted cytopathic HAV strain HM175/18f (genotype IB, ATCC VR-1402) (Lemon *et al.*, 1991) and HAV strain PA21 (genotype IIIA, ATCC VR-1357) (Binn *et al.*, 1984) were grown in BS-C-1 cells in DMEM with 2% FBS at 35°C. Blasticidin resistance-conferring HAV8Y-Bsd virus (Konduru *et al.*, 2006) was cultured in Huh7-A-I cells in DMEM with 10% FBS and blasticidin (Invitrogen; Carlsbad, CA, added 24h post infection) at 2µg/mL at 35°C. Virus was harvested by 3 freeze-thaw cycles followed by centrifugation at 1000g for 10 minutes at 4°C. Since a fungal contamination was reported for the PA21 strain by ATCC, the purchased stock was filtered through a 0.20µm Millex filter (Millipore, Billerica, MA) and after infection cultured for 3 weeks in medium containing penicillin (100 U/mL, Gibco), streptomycin (100µg/mL, Gibco) and nystatin (50µg/mL, Sigma-Aldrich, St. Louis, MO) with weekly medium changes. Amphotericin B, although suggested by ATCC, was not used since a (limited) antiviral effect has been reported against HAV (Crance *et al.*, 1990, van Cuyck-Gandré *et al.*, 1995). All antiviral experiments were performed in a humidified 5% CO₂ incubator.

HM175/18f and HAV8Y-Bsd stocks were titrated by end-point dilution. For HM175/18f, FRhK-4 cells were seeded in 96-well plates (BD Falcon, Franklin Lakes, NJ) at 2.10⁴ cells per well in 100µL of DMEM supplemented with 2% FBS and incubated at 37°C. After 24h, cells were confluent, medium was removed and 100µL of a 1:10 virus dilution series in medium was added to each well. For HAV8Y-Bsd, Huh7-A-I cells were seeded at 5.10³ cells per well in 100µL of DMEM with 10% FBS and 100µL of a 1:10 virus dilution series in medium was added to each well immediately. After 24h, blasticidin was added to

each well to a final concentration of 2µg/mL. Each dilution was analyzed in 6-fold for both strains. Plates were incubated at 35°C for 7 days and subsequently scored by microscopy. The tissue culture infectious dose 50 (TCID₅₀) corresponds to a viral dose sufficient to induce CPE (HM175/18f) or protect from blasticidin toxicity (HAV8Y-Bsd) in half of the cells in a tissue culture and was calculated by the method of Reed and Muench (Reed *et al.*, 1938). Since the PA21 strain does not induce CPE, the number of RNA copies per mL as determined by RT-qPCR was used as a proxy for the infectious titer.

2.2. Compounds Interferon-alpha 2b (IFNα, Intron-A®) was purchased from Schering Plough (Kenilworth, NJ), diluted to 3.10⁵ international units (IU)/mL in phosphate-buffered saline (PBS, Lonza, Verviers, Belgium) supplemented with 10% glycerol and 0.1% bovine serum albumin, stored at -80°C and kept at 4°C after thawing. Amantadine HCl was from Sigma-Aldrich. Ribavirin [1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (Virazole®)] was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Pleconaril and enviroxime were synthesized as described before (Diana *et al.*, 1995, Paget *et al.*, 1978), rupintrivir (AG-7088) was a gift from Pfizer (New York, NY). Compounds were dissolved in DMSO and stored at 4°C.

2.3. CPE reduction assay FRhK-4 cells were seeded in 96-well plates at 2.10⁴ cells per well in 100µL of DMEM supplemented with 2% FBS and incubated at 37°C. After 24h, cells were confluent, medium was removed and 100µL of a 1:2 compound dilution series and 100µL of HM175/18f dilution in medium, corresponding to a multiplicity of infection (m.o.i.) of 0.02, were added to each well. Virus controls (VC) and cell controls (CC) were included in each plate. Plates were incubated at 35°C. Seven days post infection, medium was removed and replaced with 100µL of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazinemethosulfate (MTS/PMS, Promega, Leiden, the

Netherlands) solution, as described (Jochmans *et al.*, 2012). After an incubation period of 4h at 37°C, the optical density (OD) at 498nm was determined for each well. Antiviral activity was calculated with following formula: % antiviral activity = % CPE reduction = $(OD_{\text{virus+compound}} - OD_{\text{VC}}) / (OD_{\text{CC}} - OD_{\text{VC}}) * 100$. Alternatively, quantification was done with the ATPlite luminescence assay system (PerkinElmer, Waltham, MA). The 50% effective concentration (EC₅₀) is the concentration of compound that causes 50% protection of cells from virus-induced CPE and was calculated through logarithmic interpolation. For toxicity evaluation, plates were prepared in parallel with antiviral assays, but instead of virus dilution, 100µL of plain medium was added. The 50% cytotoxic concentration (CC₅₀) is the concentration of compound that causes cell death in 50% of cultured cells and was estimated through logarithmic interpolation as well. Z'-values were calculated as described (Zhang *et al.*, 1999).

2.4. HM175/18f virus yield assay FRhK-4 cells were seeded in 96-well plates at 2.10^4 cells per well in 100µL of DMEM supplemented with 2% FBS and incubated at 37°C. After 24h, cells were confluent. Medium was removed and replaced with 20µL of medium containing compound (1:2 dilution series) and HM175/18f virus at an m.o.i. of 0.2. Plates were incubated at 35°C. The inoculum was removed after 1h and cell layers were washed 3 times with 100µL of PBS. To each well, 100µL of compound dilution in medium was added. After incubation at 35°C for 3 days, the viral load in 100µL of culture medium was determined by RT-qPCR. For evaluation of compound toxicity, 100µL of MTS/PMS dilution was added to each well after medium removal, plates were incubated at 37°C for 4h and OD's were determined as described.

2.5. PA21 virus yield assay

Huh7-A-IS cells were seeded in 96-well plates at 10^4 cells per well in 100 μ L of MEM rega 3 supplemented with 10% FBS, 1% L-glutamine and 1% sodium bicarbonate and incubated at 37°C. After 24h, medium was removed and replaced with 50 μ L of medium containing compound (1:2 dilution series) and PA21 virus at 1.3×10^5 viral RNA copies per well. Plates were incubated at 35°C. The inoculum was removed after 4h and cell layers were washed twice with 100 μ L of PBS. To each well, 100 μ L of compound dilution in medium was added. After incubation at 35°C for 7 days, the viral load in 100 μ L of culture medium was determined by RT-qPCR. For toxicity evaluation, 100 μ L of MTS/PMS dilution was added to each well after medium removal, plates were incubated at 37°C for 1h and OD's were determined as described.

2.6. HAV8Y-Bsd virus yield assay

Huh7-A-I cells were seeded in 96-well plates at 5.10^3 cells per well in 100 μ L of DMEM supplemented with 10% FBS and incubated at 37°C. After 24h, medium was removed and replaced with 100 μ L of medium containing compound (1:2 dilution series) and HAV8Y-Bsd virus at an m.o.i. of 0.002. Plates were incubated at 35°C. The inoculum was removed after 1h and cell layers were washed 2 times with 100 μ L of PBS. To each well, 100 μ L of compound dilution in medium was added. After incubation at 35°C for 5 days, the viral load in 100 μ L of culture medium was determined by RT-qPCR. For evaluation of compound toxicity, 100 μ L of MTS/PMS dilution was added to each well after medium removal, plates were incubated at 37°C for 1h and OD's were determined as described.

2.7. RT-qPCR

Viral RNA was extracted from culture medium with the NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Primers and probes for TaqMan-based quantification of HAV RNA were based on published sequences (Silberstein *et al.*, 2003). As a forward primer 5'-GGCATTTAGGTTTTTCCTCATTCTTA-3' was used for HM175/18f and HAV8Y-Bsd and

5'-GGCATTTAGGTTTTTCCTCATCAATA-3' for PA21, reverse primers were 5'-
AATGTCTGCCAAAGACAGGATGT-3' for HM175/18f and HAV8Y-Bsd and 5'-
CACATCTGCCAAAGACAGAATGT-3' for PA21. Probes were labeled with 6-
carboxyfluorescein (FAM) at the 5' end and with a minor groove binder (MGB) at the 3' end
(5'-6FAM-CAAGGTATTTTCCAGACTGTTGGGAGTGGTCT-MGBNFQ-3' for
HM175/18f and HAV8Y-Bsd,
5'-6FAM-CAAGGTATTTTCCAGACTGTTGGGAGTGGCCT-3'-MGBNFQ for PA21).
Reactions were performed with One-Step qRT-PCR mix (Eurogentec, Seraing, Belgium) in a
final volume of 25µL containing 3µM of each primer, 67nM of probe and 5µL of RNA
sample. PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied
Biosystems, Foster City, CA) under following conditions: 30 min at 48°C and 10 min at
95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed with ABI
PRISM 7500 SDS software (version 1.3.1, Applied Biosystems). For absolute quantification,
standard curves were generated using 10-fold dilutions of template preparations of known
concentrations.

3. Results

A CPE reduction assay was developed to allow the rapid, convenient and reproducible
evaluation of the potential anti-HAV activity of compound libraries. The cell culture-adapted,
cytopathic HM175/18f strain was used to this end since it induces a rapid and complete CPE
in FRhK-4 cells (Lemon *et al.*, 1991). Initial optimization experiments indicated that infection
at an m.o.i. of 0.02 on confluent FRhK-4 cells with subsequent incubation for 7 days at 35°C
results in sufficient CPE for detection with the MTS/PMS method. The Z'-value of the assay
was calculated to be 0.63. This calculation was based on 390 wells of both uninfected and
infected cultures (collected from 3 independent experiments, figure 1). IFNα was used for

validation of the antiviral assay (figure 2A, 2C). An EC_{50} of 110 ± 70 IU/mL was calculated and no cytotoxicity was detected at concentrations up to 3000 IU/mL. When CPE reduction was quantified by measuring intracellular ATP levels, an EC_{50} of 60 ± 20 IU/mL was calculated (figure 2B-C). Dose response curves generated by both methods were comparable. Microscopic scoring confirmed these data obtained by the MTS/PMS and the ATP detection methods (figure 2C-D). Amantadine HCl, a known inhibitor of HAV replication (Kanda *et al.*, 2005), resulted in complete inhibition of virus-induced CPE at a concentration of $125 \mu\text{M}$, as assessed by the MTS/PMS-method (figure 2E) and corroborated by microscopical evaluation (figure 2D).

The potential antiviral activity of 3 known inhibitors of *in vitro* enterovirus replication, i.e. pleconaril, rupintrivir (also known as AG7088) and enviroxime, was evaluated in the CPE reduction assay. Pleconaril exhibited moderate antiviral activity with a maximal inhibition of $70 \pm 11\%$ at $25 \mu\text{M}$ and a calculated EC_{50} of $9 \pm 3 \mu\text{M}$ (figure 2F). Neither rupintrivir nor enviroxime inhibited virus-induced CPE formation (figure 2G-H).

To allow confirmation of the antiviral activity of hits identified in (future) antiviral screens in the CPE-based assay, we developed an RT-qPCR-based virus yield assay using the HM175/18f strain and FRhK-4 cells. From a pilot study, we observed that infection at an m.o.i. of 0.2 and an incubation period of 72h at 35°C yielded sufficient levels of viral RNA for the efficient detection of potential antiviral effects. Using this assay, an EC_{50} of 20 ± 22 IU/mL was calculated for IFN α and $90 \pm 23 \mu\text{M}$ for amantadine HCl (figure 3A-B). The moderate antiviral effect of pleconaril as detected in the CPE assay was confirmed (EC_{50} of $7 \pm 4 \mu\text{M}$, figure 3C). Neither rupintrivir nor enviroxime reduced viral RNA levels, thus confirming the results from the CPE reduction assay (figure 3D-E).

A second RNA virus yield assay was developed using the PA21 strain and Huh7-A-IS cells. This strain is only weakly adapted to cell culture (Binn *et al.*, 1984), hence sufficiently high viral RNA titers were only reached at 7 days post infection. A critical point for this assay is the requirement for 10% FBS in the medium over the entire assay period, initial tests with 2% FBS (as is standard in most antiviral assays) revealed that Huh7-A-IS (but also regular Huh7-A-I) become apoptotic after 3-4 days of culturing, in particular when confluent (data not shown). Since Huh7-A-IS divide markedly slower than the parent Huh7-A-I, they are well suited for extended incubation. As shown, the PA21 strain in these cells is considerably more sensitive to IFN α than for instance the HM175/18f strain in FRhK-4 cells. In fact, complete inhibition of viral replication was noted over a wide concentration range (EC_{50} of 2 ± 3 IU/mL). The PA21 virus in Huh7-A-IS was however less sensitive to amantadine HCl than HM175/18f on FRhK-4 (EC_{50} of $230\pm 46\mu M$, figure 4A-B). Pleconaril proved somewhat more toxic in Huh7-A-IS than in FRhK-4 cells, but also exhibited antiviral activity (EC_{50} $7\pm 3\mu M$, figure 4C). In contrast to the HM175/18f, some activity of the 3C inhibitor rupintrivir was observed (EC_{50} of $11\pm 4\mu M$, figure 4D). These observed differences may possibly be explained by the use of the particular virus strains, the cellular background or a combination of both. Also in this assay enviroxime proved not effective (figure 4E).

Finally the effect of the 5 molecules studied was assessed against an almost wild-type strain, HAV8Y-Bsd. Apart from the blasticidin resistance gene, it contains only one adaptive mutation in the 2B-coding region (A216V) that does not seem to affect virulence (Konduru *et al.*, 2006). Huh7-A-I cells allow stable growth of this almost wild-type virus. Treatment with IFN α resulted in complete inhibition over a large concentration range (supplementary figure 1A), (EC_{50} of 0.5 ± 0.2 IU/mL). EC_{50} 's obtained for amantadine HCl ($170\pm 90\mu M$) and pleconaril ($10\pm 10\mu M$) are in the same order of magnitude as those calculated in the above described assays (supplementary figure 1B-C). Rupintrivir displayed a limited antiviral effect

(EC₅₀ of 51±9µM) while enviroxime did not inhibit HAV8Y-Bsd replication (supplementary figure 1D-E).

4. Discussion

We established three antiviral assays to identify inhibitors of the HAV. The HM175/18f-based CPE reduction assay is suitable for medium-to-high-throughput screening purposes. Two virus RNA yield assays were developed that allow to assess the potential antiviral activity against genotypes IB and IIIA. Since the latter assays are rather costly and labor-intensive, they would be less suitable for large scale screening campaigns, but very useful for further confirmation of hits identified in the CPE reduction assay. The systems were validated for antiviral studies with IFNα and amantadine HCl, two known inhibitors of *in vitro* HAV replication. Moreover, the potential antiviral effect of a selection of three enterovirus inhibitors, i.e. the capsid binder pleconaril, the 3C^{pro} inhibitor rupintrivir and the 3A/phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ)-targeting compound enviroxime, was assessed. The effect of pleconaril and rupintrivir appeared to be cell type and/or virus strain-specific. Enviroxime, a direct PI4KIIIβ inhibitor with broad spectrum anti-enterovirus activity (van der Schaar *et al.*, 2012), was devoid of any activity against HAV. In addition results were confirmed with an almost wild-type HAV strain (HAV8Y-Bsd).

The HM175/18f strain is a highly passaged HAV strain and adapted to growth in African green monkey kidney cell lines such as BS-C-1 and FRhK-4. When compared to wild-type HM175, 44 mutations were noted (Lemon *et al.*, 1991). Although the physiological relevance of this highly adapted strain could be questioned, the mutations concern < 1% of the entire 7.5kb HAV genome, which is far less than for instance the 15% sequence divergence between different genotypes (Costa-Mattioli *et al.*, 2003). Another concern could be the use of the non-hepatic FRhK-4 cell line, while wild-type HAV mainly replicates in the liver. We

propose therefore to assess the potential antiviral effect of hits from a screen using HM175/18f in the PA21 virus yield assay using the hepatocyte cell line Huh7-A-IS. The PA21 virus has only been passaged a limited number of times (Binn *et al.*, 1984). A potential issue for the PA21 virus yield system is the limited dynamic range (Ct values of 27-28 were obtained in the infected cultures at 7 days post infection). This however is virtually inevitable for a slow growing virus such as HAV and is probably not a significant issue in the initial assessment of an antiviral effect.

The systems described here have several strengths and advantages. First, at the inoculum used in the CPE reduction assay, multiple cycles of viral replication are required to obtain a sufficient level of CPE for MTS or ATPlite read-out. This allows for detection of antiviral activity at any step of the replication cycle and evaluation of cell viability after 7 days allows for a stringent assessment of potential cytotoxicity. Second, the RT-qPCR-based virus yield assay is highly sensitive. By using only a 3-day incubation for the HM175/18f strain, it is possible to study the antiviral effect in just one or a few replication cycles. A higher inoculum is required for such assays than in the CPE reduction assay to provide a sufficient dynamic range. On the other hand, the PA21 virus yield assay employs a limitedly adapted genotype IIIA virus in a hepatocyte cell line and thus offers a set of more physiological conditions. To our knowledge no cytopathic variants of HAV have been described other than those belonging to genotype IA or IB. Also, genotypes I and III are the most common genotypes isolated (Nainan *et al.*, 2006). Third, compared to the assays described in the late 1980's – early 1990's, our systems do not require radioactivity or evaluation by immunostaining and the CPE reduction assay can be employed in medium-to-high-throughput screening campaigns. The methodologies reported here are convenient, robust, reproducible and reliable. We also implemented an antiviral screening assay based on the blasticidin resistance-carrying strain HAV8Y-Bsd in Huh7-A-I cells (Konduru *et al.*,

2006). A negative selection assay with addition of blasticidin to culture medium 2 days post infection and MTS read-out after 7 days was successfully developed, but yielded several false positive hits in an antiviral screen (unpublished results). This may be explained by a direct inhibition of the blasticidin S deaminase instead of blockage of viral replication.

It has since long been established that HAV is susceptible to the antiviral activity of IFN α , both *in vitro* and *in vivo* (Crance *et al.*, 1995, Yoshida *et al.*, 1994). We here report EC₅₀ ranging from 0.5 to 110 IU/mL depending on the assay, which is in line with values reported by Crance and colleagues (<10 to 90 IU/mL, m.o.i.-dependent) (Crance *et al.*, 1995). Differences between assays may be explained by differences in the cell types and virus genotypes employed, the extent of cell culture adaptation and the required time of incubation. The activity of amantadine has been reported as well with only a limited selectivity (EC₅₀ = 58 μ M) (Crance *et al.*, 1990). Although initially thought to act as an inhibitor of intravesicular acidification and thus HAV uncoating, amantadine was later reported to block IRES-mediated translation of the viral genome (Kanda *et al.*, 2005). The results presented here corroborate the antiviral effect, even though the selectivity is very limited and the activity seems to be strain-dependent, as was found for different naturally occurring IRES's as well (Kanda *et al.*, 2010).

Pleconaril is an enterovirus inhibitor that targets receptor attachment and uncoating through binding into a hydrophobic pocket underneath the canyon present in the enterovirus capsid (Thibaut *et al.*, 2012). The molecule is rather potent against most enteroviruses (with EC₅₀'s in the range of 0.002-1 μ M (Pevsner *et al.*, 1999)), here we report EC₅₀'s between 7 and 10 μ M for HAV. It is however rather surprising to observe activity of this molecule on HAV since the virion structure has no canyons comparable to those in enteroviruses (Martin *et al.*, 2006). Further experiments are required to unravel by which mechanism pleconaril inhibits HAV. The lack of activity found for rupintrivir in HM175/18f experiments can be explained

by limited sequence identity and significant variations in specificity-determining amino acid residues between HAV and enterovirus 3C^{pro} (see e.g. Tan *et al.*, 2013). These authors reported several broad-spectrum picornaviral 3C^{pro} inhibitors that were inactive against a HAV subgenomic replicon (Tan *et al.*, 2013). The fact that we observed some activity against PA21 and limited inhibition of HAV8Y-Bsd may be attributable to sequence differences between virus strains. The full PA21 sequence is currently not available through GenBank, sequencing of the PA21 3C^{pro} region and comparison with HM175/18f may shed light on these differences. HAV8Y-Bsd and HM175/18f differ in 3C^{pro} by one amino acid (Q101E) (Lemon *et al.*, 1991), but this residue is not located near the catalytic side (Bergmann *et al.*, 1997). Alternative explanations may be found in the different cell types or m.o.i.'s employed in the experiments. Although enviroxime selects for drug-resistance mutations in 3A, it functions by directly inhibiting PI4KIII β which is an essential host factor that is hijacked by enteroviruses for replication complex formation (van der Schaar *et al.*, 2012). Most picornaviral 3A proteins have a transmembrane region that targets to the endoplasmatic reticulum (ER) from which the replication complexes are derived. Lack of activity of enviroxime against HAV is not unexpected since sequence homology between HAV and enteroviral 3A is very limited and more importantly, since HAV 3A is thought to target to the outer mitochondrial membrane, instead of to the ER, for derivation of replication complexes (Yang *et al.*, 2007). The lack of activity for enviroxime and the mitochondrial origin of replication complexes suggest that PI4KIII β does not play a role in HAV replication.

The assays described in the present report should allow for the first time medium-to-high-throughput screening campaigns against HAV. Despite the fact that it would be important to have (a) drug(s) at hand for the treatment of severe and life-threatening cases of HAV infection, the investment cost to develop such drug from a new molecular entity (NME) may possibly seem too high in light of the potential market. Therefore screening of approved

drug libraries may be an interesting option. Inhibitors (or combinations thereof) thus identified may be used off-label for the treatment of severe cases, to contain outbreaks and to combat the potential rise of vaccine-escape mutants (Pérez-Sautu *et al.*, 2011).

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Figure legends

Figure 1: Assay quality as assessed by calculation of Z' value. For both conditions, a total of 390 values was obtained over 3 independent experiments.

Figure 2: CPE reduction assay. Inhibition of CPE formation by HM175/18f and cell viability after IFN α treatment were assessed by MTS (A) and ATPlite (B) read-out. Results are similar for both methods and correspond to values obtained by microscopic scoring (C). HM175/18f causes extensive CPE after 7 days, which is efficiently inhibited by IFN α at 1500 and 375 IU/mL and by amantadine HCl at 125 μ M (D, E). Pleconaril exhibits moderate anti-HAV activity in the CPE reduction assay (F), no antiviral activity could be detected for rupintrivir or enviroxime (G-H). A-G: Results represent 7 to 10 measurements from at least 3 independent experiments. H: 4 to 5 measurements, 2 independent experiments. Values represent mean $\pm$ SD.

Figure 3: Virus yield assay for HAV HM175/18f with RT-qPCR over 72h. Extensive antiviral activity was recorded for IFN α (A). To a more limited extent, amantadine HCl (B) and pleconaril (C) proved to be active as well. No selective antiviral effects were observed for rupintrivir (D) and enviroxime (E). A-B: 8 measurements, 3 independent experiments. C-D: 5 measurements, 3 independent experiments. E: 4 measurements, 2 independent experiments. Values represent mean $\pm$ SD.

Figure 4: Virus yield assay for HAV PA21 with RT-qPCR over 7 days. Full antiviral activity was found for IFN α (A). Amantadine HCl and pleconaril exhibit only small selectivity windows (B-C). Toxicity is more pronounced for pleconaril in Huh7-A-IS compared to FRhK-4 (figure 3C). Contrary to results for HM175/18f, rupintrivir displays an antiviral effect against PA21 (D). No selective inhibition was observed for enviroxime (E). A-B: 8

525 measurements, 3 independent experiments. C-D: 5 measurements, 3 independent experiments.
526 E: 4 measurements, 2 independent experiments. Values represent mean $\pm$ SD.

527 **Supplementary figure 1:** Virus yield assay for HAV8Y-Bsd with RT-qPCR over 5 days.
528 Extensive antiviral activity was recorded for IFN α (A). To a more limited extent, amantadine
529 HCl (B), pleconaril (C) and rupintrivir (D) proved to be active. No selective antiviral effect
530 was observed for enviroxime (E). A-B: 8 measurements, 3 independent experiments. C-D: 5
531 measurements, 3 independent experiments. E: 4 measurements, 2 independent experiments.
532 Values represent mean $\pm$ SD.

A **B** **C** **D** **E**