Antiviral Research 98 (2013) 325–331

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

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

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ARTICLE INFO

Article history:
Received 29 November 2012
Revised 20 February 2013
Accepted 15 March 2013
Available online 23 March 2013

Keywords:
Hepatitis A virus
Antiviral assay
Interferon
Amantadine
Enterovirus inhibitor

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 (IFNα) and amantadine HCl, two known inhibitors of HAV replication. IFNα 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 IIIβ is not crucial in the HAV life cycle.

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 (World Health Organization, 2011). Superinfections with HAV in patients chronically infected with hepatitis B or C are believed to increase morbidity and mortality (Keeffe, 2000; Vento et al., 1998), although these findings are still subject of debate (Shouval, 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 hydratation and proper nutritional balance are advised (Jeong and Lee, 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 al., 1986; Superti et al., 1987; Crance et al., 1990; van Cuyck-Gandré et al., 1995; Biziagos et al., 1990). Limited antiviral activity has been described for chlorpromazine and chloroquine (Bishop, 1998). However, clinical use of these compounds is hampered by poor selectivity or toxic side effects. Interferon-alpha (IFNα) is active *in vitro* against HAV and has shown some effect in the treatment of severe HAV infections (Crance et al., 1995; Yoshiba et al., 1994), but clinical use is associated with severe side effects.

Since the arrival of vaccines, research interest in HAV has decreased substantially (Martin and Lemon, 2006). To the best of our knowledge, no cell-based screening assays against HAV have been reported in the last decade. Moreover, the antiviral assays that were reported earlier are labor-intensive and allow only to evaluate a small number of molecules. Here we report a cytopathic effect (CPE) reduction assay amenable for high-throughput screening purposes. In addition, we present virus yield reduction assays (based on reverse transcription quantitative PCR (RT-qPCR)) that

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http://dx.doi.org/10.1016/j.antiviral.2013.03.016

can be used as a secondary assay to confirm and validate the activities of hits identified in the CPE-based assay. We employed these assays to assess the potential inhibitory activity of a small panel of known enterovirus inhibitors (pleconaril, rupintrivir and enviroxime) against HAV.

2. Materials and methods

2.1. Cells and viruses

FRhK-4 cells (ATCC CRL-1688) and BS-C-1 cells (ATCC CCL-26) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gent, Belgium) supplemented with 10% fetal bovine serum (FBS, Integro, Leuvenheim, the Netherlands). Huh7-A-I cells supporting stable HAV growth (Konduru and Kaplan, 2006) were grown in DMEM supplemented with 10% FBS and were subcultured twice a week at a 1:5 ratio. Huh7-A-I 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% CO_2 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 and Kaplan, 2006) was cultured in Huh7-A-I cells in DMEM with 10% FBS and blasticidin (Invitrogen, Carlsbad, CA, added 24 h post infection) at 2 μg/mL at 35 °C. Virus was harvested by 3 freeze–thaw cycles followed by centrifugation at 1000*g* for 10 min at 4 °C. Since a fungal contamination was reported for the PA21 strain by ATCC, the purchased stock was filtered through a 0.20 μm Millex filter (Millipore, Billerica, MA) and after infection cultured for 3 weeks in medium containing penicillin (100 U/mL, Gibco), streptomycin (100 μg/mL, Gibco) and nystatin (50 μg/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% CO_2 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 $2 \cdot 10^4$ cells per well in 100 μL of DMEM supplemented with 2% FBS and incubated at 37 °C. After 24 h, cells were confluent, medium was removed and 100 μL of a 1:10 virus dilution series in medium was added to each well. For HAV8Y-Bsd, Huh7-A-I cells were seeded at $5 \cdot 10^3$ cells per well in 100 μL of DMEM with 10% FBS and 100 μL of a 1:10 virus dilution series in medium was added to each well immediately. After 24 h, blasticidin was added to each well to a final concentration of 2 μg/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 ($TCID_{50}$) 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 and Muench, 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 $3 \cdot 10^5$ 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 $2 \cdot 10^4$ cells per well in 100 μL of DMEM supplemented with 2% FBS and incubated at 37 °C. After 24 h, cells were confluent, medium was removed and 100 μL of a 1:2 compound dilution series and 100 μL 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 4 h at 37 °C, the optical density (OD) at 498 nm was determined for each well. Antiviral activity was calculated with following formula: % antiviral activity = % CPE reduction = $(OD_{virus+compound} - OD_{VC})/(OD_{CC} - OD_{VC}) * 100$. Alternatively, quantification was done with the ATPlite luminescence assay system (PerkinElmer, Waltham, MA). The 50% effective concentration (EC_{50}) 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, 100 μL of plain medium was added. The 50% cytotoxic concentration (CC_{50}) 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 $2 \cdot 10^4$ cells per well in 100 μL of DMEM supplemented with 2% FBS and incubated at 37 °C. After 24 h, cells were confluent. Medium was removed and replaced with 20 μL of medium containing compound and HM175/18f virus at an m.o.i. of 0.2. Plates were incubated at 35 °C. The inoculum was removed after 1 h and cell layers were washed 3 times with 100 μL of PBS. To each well, 100 μL of compound dilution in medium was added. After incubation at 35 °C for 3 days, the viral load in 100 μL of culture medium was determined by RT-qPCR. For evaluation of compound toxicity, 100 μL of MTS/PMS dilution was added to each well after medium removal, plates were incubated at 37 °C for 4 h 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 μL of MEM rega 3 supplemented with 10% FBS, 1% L-glutamine and 1% sodium bicarbonate and incubated at 37 °C. After 24 h, medium was removed and replaced with 50 μL of medium containing compound and PA21 virus at 1.3×10^5 viral RNA copies per well. Plates were incubated at 35 °C. The inoculum was removed after 4 h and cell layers were washed twice with

100 μL of PBS. To each well, 100 μL of compound dilution in medium was added. After incubation at 35 °C for 7 days, the viral load in 100 μL of culture medium was determined by RT-qPCR. For toxicity evaluation, 100 μL of MTS/PMS dilution was added to each well after medium removal, plates were incubated at 37 °C for 1 h 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 $5 \cdot 10^3$ cells per well in 100 μL of DMEM supplemented with 10% FBS and incubated at 37 °C. After 24 h, medium was removed and replaced with 100 μL of medium containing compound and HAV8Y-Bsd virus at an m.o.i. of 0.002. Plates were incubated at 35 °C. The inoculum was removed after 1 h and cell layers were washed 2 times with 100 μL of PBS. To each well, 100 μL of compound dilution in medium was added. After incubation at 35 °C for 5 days, the viral load in 100 μL of culture medium was determined by RT-qPCR. For evaluation of compound toxicity, 100 μL of MTS/PMS dilution was added to each well after medium removal, plates were incubated at 37 °C for 1 h 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′-CACATCTGCCA AAGACAGAATGT-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-CAAGGTATTTTCCAGACTGTTGG-GAGTGGTCT-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 25 μL containing 3 μM of each primer, 67 nM of probe and 5 μL 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, Fig. 1). IFNα was used for validation of the antiviral assay (Fig. 2A and C). 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 (Fig. 2B and 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 (Fig. 2C and 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 μM, as assessed by the MTS/PMS-method (Fig. 2E) and corroborated by microscopical evaluation (Fig. 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 ± 11% at 25 μM and a calculated EC_{50} of 9 ± 3 μM (Fig. 2F). Neither rupintrivir nor enviroxime inhibited virus-induced CPE formation (Fig. 2G and 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 72 h 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 IFNα and 90 ± 23 μM for amantadine HCl (Fig. 3A and B). The moderate antiviral effect of pleconaril as detected in the CPE assay was confirmed (EC_{50} of 7 ± 4 μM, Fig. 3C). Neither rupintrivir nor enviroxime reduced viral RNA levels, thus confirming the results from the CPE reduction assay (Fig. 3D and 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 IFNα 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 ± 46 μM, Fig. 4A and

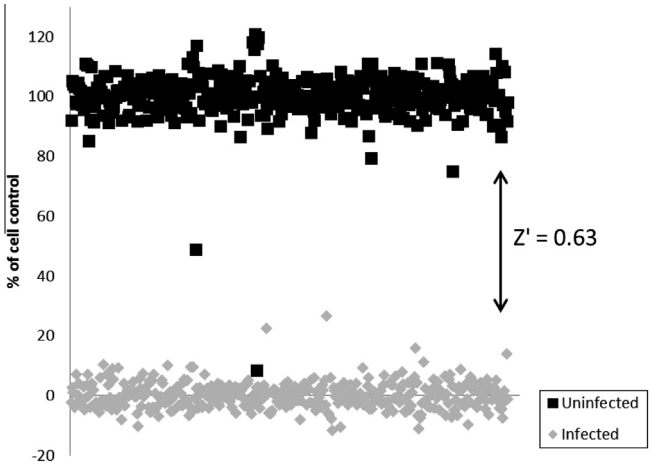

Fig. 1. Assay quality as assessed by calculation of Z' value. For both conditions, a total of 390 values was obtained over 3 independent experiments.

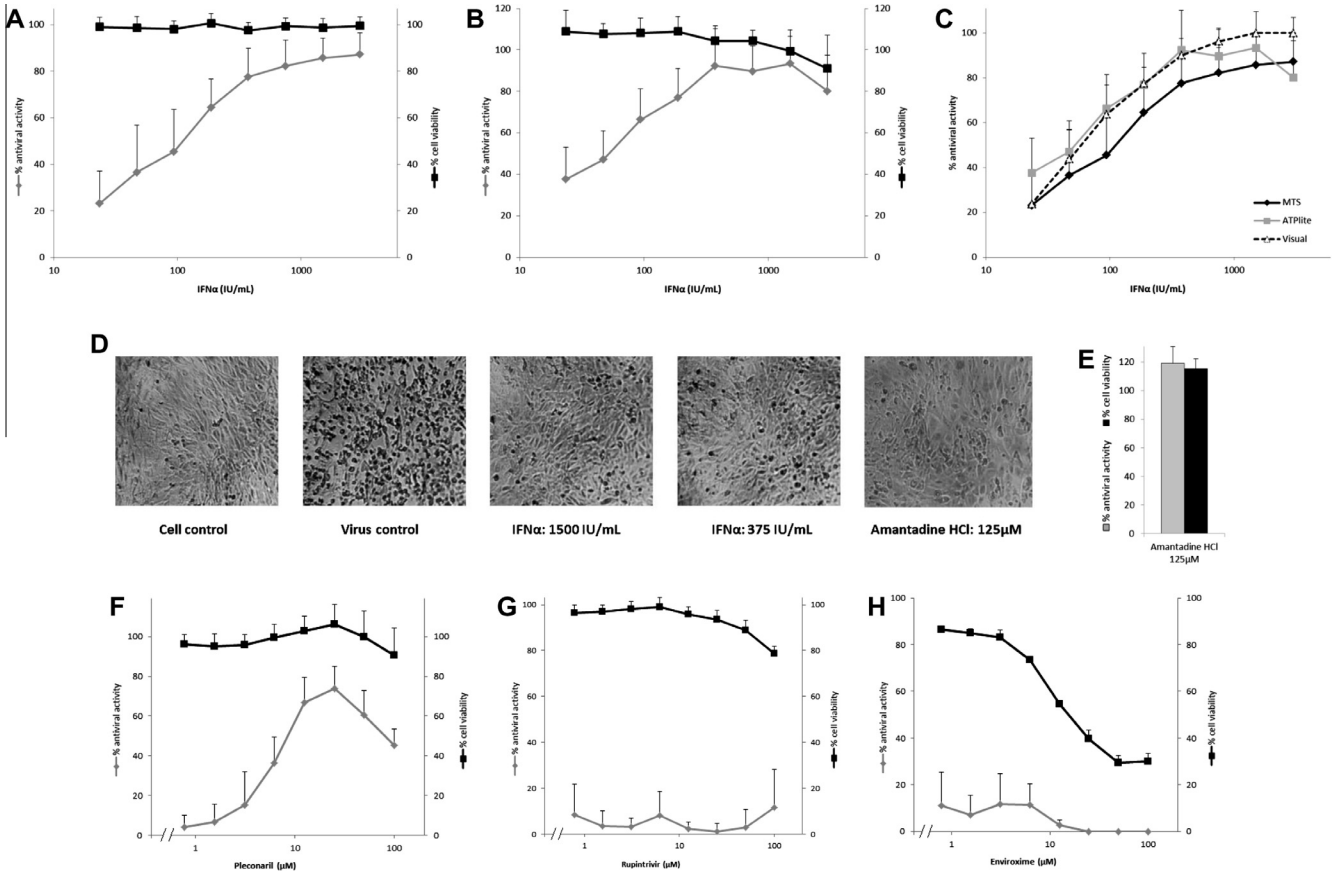

Fig. 2. CPE reduction assay. Inhibition of CPE formation by HM175/18f and cell viability after IFNα treatment were assessed by MTS (A) and ATPlite (B) read-out. Results are similar for both methods and correspond to values obtained by microscopic scoring (C). HM175/18f causes extensive CPE after 7 days,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

B). Pleconaril proved somewhat more toxic in Huh7-A-IS than in FRhK-4 cells, but also exhibited antiviral activity (EC_{50} 7 ± 3 μM, Fig. 4C). In contrast to the HM175/18f, some activity of the 3C inhibitor rupintrivir was observed (EC_{50} of 11 ± 4 μM, Fig. 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 (Fig. 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 and Kaplan, 2006). Huh7-A-I cells allow stable growth of this almost wild-type virus. Treatment with IFNα resulted in complete inhibition over a large concentration range (Supplementary Fig. 1A), (EC_{50} of 0.5 ± 0.2 IU/mL). EC_{50}'s obtained for amantadine HCl (170 ± 90 μM) and pleconaril (10 ± 10 μM) are in the same order of magnitude as those calculated in the above described assays (Supplementary Fig. 1B and C). Rupintrivir displayed a limited antiviral effect (EC_{50} of 51 ± 9 μM) while enviroxime did not inhibit HAV8Y-Bsd replication (Supplementary Fig. 1D and 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

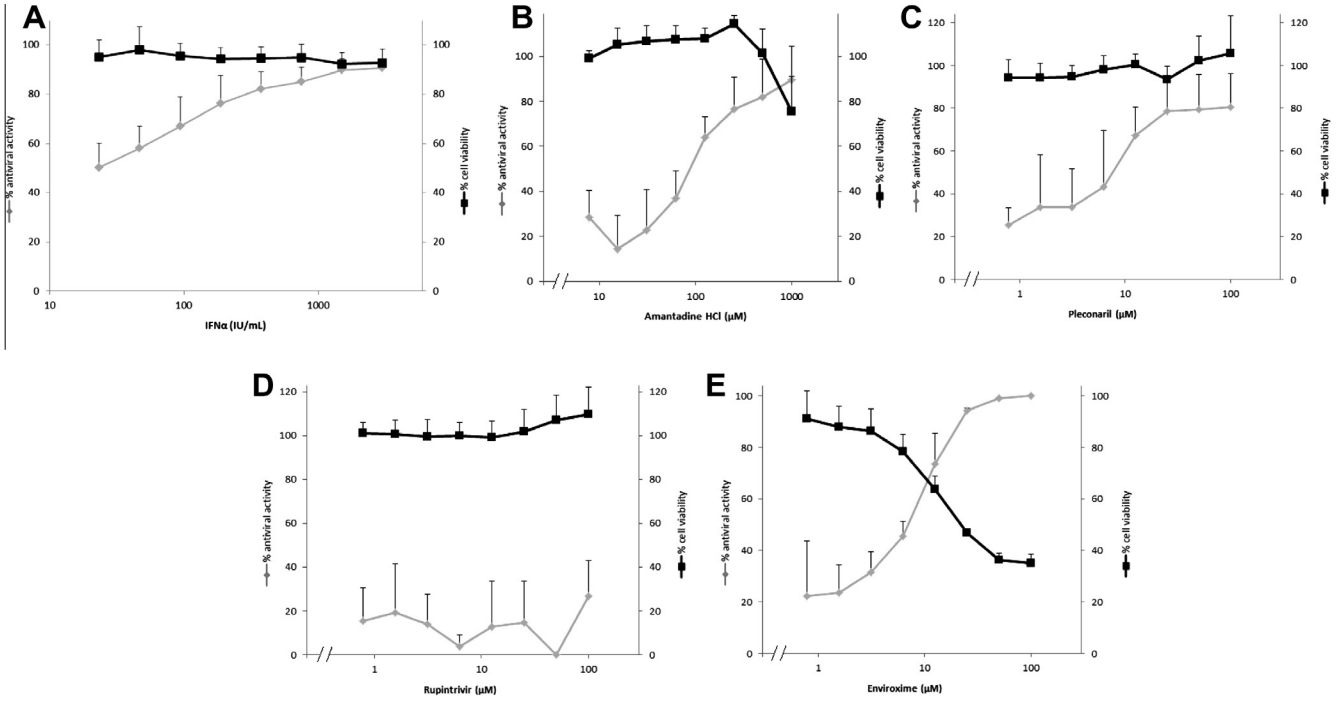

Fig. 3. Virus yield assay for HAV HM175/18f with RT-qPCR over 72 h. Extensive antiviral activity was recorded for IFNα (A). To a more limited extent, amantadine HCl (B) and pleconaril (C) proved to be active as well. No selective antiviral effects were observed for rupintrivir (D) and enviroxime (E).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

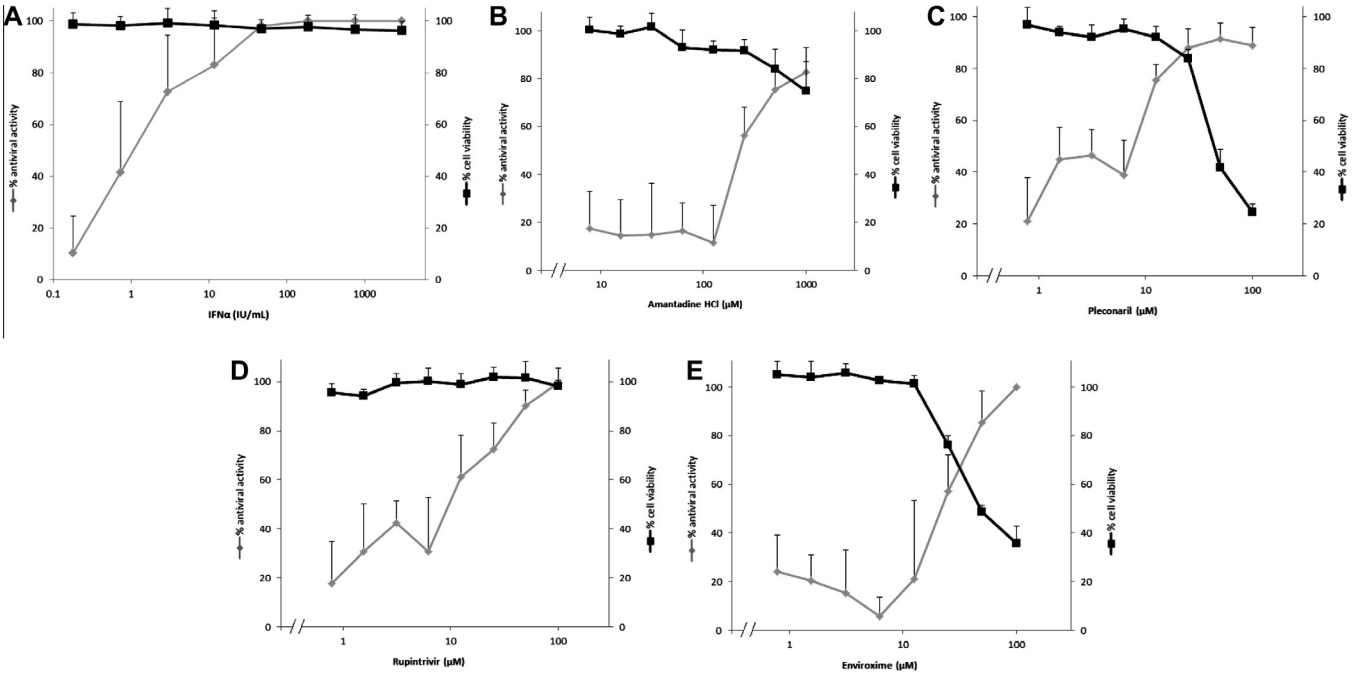

Fig. 4. Virus yield assay for HAV PA21 with RT-qPCR over 7 days. Extensive antiviral activity was found for IFNα (A). Amantadine HCl and pleconaril exhibit only small selectivity windows (B and C). Toxicity is more pronounced for pleconaril in Huh7-A-IS compared to FRhK-4 (Fig. 3C). Contrary to results for HM175/18f, rupintrivir displays an antiviral effect against PA21 (D). No selective inhibition was observed for enviroxime (E). A and B: 8 measurements, 3 independent experiments. C and D: 5 measurements, 3 independent experiments. E: 4 measurements, 2 independent experiments. Values represent mean ± SD.

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 and Kaplan, 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 IFNα, both *in vitro* and *in vivo* (Crance et al., 1995; Yoshiba et al., 1994). We here report EC_{50} 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 (EC_{50} = 58 μM) (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 EC_{50}'s in the range of 0.002–1 μM (Pevear et al., 1999)), here we report EC_{50}'s between 7 and 10 μM 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 and Lemon, 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 PI4KIIIβ 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 PI4KIIIβ 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).

Acknowledgments

We would like to thank Stijn Delmotte for excellent technical assistance. We are also grateful to Jolien Nelissen for critical editing of the manuscript and to Kai Dallmeier, Suzanne Kaptein and Hendrik Thibaut for input on experiments and figures. Yannick Debing is a fellow of the Research Foundation – Flanders (FWO). This work is supported by KU Leuven, geconcerteerde onderzoeksactie (GOA/10/014) and EU FP7 project SILVER (260644).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.03.016.

References

Bergmann, E.M., Mosimann, S.C., Chernaia, M.M., Malcolm, B.A., James, M.N., 1997. The refined crystal structure of the 3C gene product from hepatitis A virus: specific proteinase activity and RNA recognition. J. Virol. 71, 2436–2448.

Binn, L.N., Lemon, S.M., Marchwicki, R.H., Redfield, R.R., Gates, N.L., Bancroft, W.H., 1984. Primary isolation and serial passage of hepatitis A virus strains in primate cell cultures. J. Clin. Microbiol. 20, 28–33.

Bishop, N.E., 1998. Examination of potential inhibitors of hepatitis A virus uncoating. Intervirology 41, 261–271.

Biziagos, E., Crance, J.M., Passagot, J., Deloince, R., 1990. Inhibitory effect of atropine, protamine, and their combination on hepatitis A virus replication in PLC/PRF/5 cells. Antimicrob. Agents Chemother. 34, 1112–1117.

Bordi, L., Rozera, G., Scognamiglio, P., Minosse, C., Loffredo, M., Anitori, A., Narciso, P., Ippolito, G., Girardi, E., Capobianchi, M.R., GEAS group, 2012. Monophyletic outbreak of hepatitis A involving HIV-infected men who have sex with men, Rome, Italy 2008–2009. J. Clin. Virol. 54, 26–29.

Costa-Mattioli, M., Di Napoli, A., Ferré, V., Billaudel, S., Perez-bercoff, R., Cristina, J., 2003. Genetic variability of hepatitis A virus. J. Gen. Virol. 84, 3191–3201.

Crance, J.-M., Biziagos, E., Passagot, J., van Cuyck-Gandré, H., Deloince, R., 1990. Inhibition of hepatitis A virus replication *in vitro* by antiviral compounds. J. Med. Virol. 31, 155–160.

Crance, J.-M., Lévêque, F., Chousterman, S., Jouan, A., Trépo, C., Deloince, R., 1995. Antiviral activity of recombinant interferon-α on hepatitis A virus replication in human liver cells. Antiviral Res. 28, 69–80.

Diana, G.D., Rudewicz, P., Pevear, D.C., Nitz, T.J., Aldous, S.C., Aldous, D.J., Robinson, D.T., Draper, T., Dutko, F.J., Aldi, C., et al., 1995. Picornavirus inhibitors: trifluoromethyl substitution provides a global protective effect against hepatic metabolism. J. Med. Chem. 14, 1355–1371.

Donnan, E.J., Fielding, J.E., Gregory, J.E., Lalor, K., Rowe, S., Goldsmith, P., Antoniou, M., Fullerton, K.E., Knope, K., Copland, J.G., Bowden, D.S., Tracy, S.L., Hogg, G.G., Tan, A., Adamopoulos, J., Gaston, J., Vally, H., 2012. A multistate outbreak of hepatitis A associated with semidried tomatoes in Australia, 2009. Clin. Infect. Dis., 775–781.

Jeong, S.-H., Lee, H.-S., 2010. Hepatitis A: clinical manifestations and management. Intervirology 53, 15–19.

Jochmans, D., Leyssen, P., Neyts, J., 2012. A novel method for high-throughput screening to quantify antiviral activity against viruses that induce limited CPE. J. Virol. Methods 183, 176–179.

Kanda, T., Imazeki, F., Nakamoto, S., Okitsu, K., Fujiwara, K., Yokosuka, O., 2010. Internal ribosome entry-site activities of clinical isolate-derived hepatitis A virus and inhibitory effects of amantadine. Hepatol. Res. 40, 415–423.

Kanda, T., Yokosuka, O., Imazeki, F., Fujiwara, K., Nagao, K., Saisho, H., 2005. Amantadine inhibits hepatitis A virus internal ribosomal entry site-mediated translation in human hepatoma cells. Biochem. Biophys. Res. Commun. 331, 621–629.

Keeffe, E., 2000. Hepatitis A in patients with chronic liver disease – severity of illness and prevention with vaccination. J. Viral. Hepat. 7 (Suppl. 1), 15–17.

Konduru, K., Kaplan, G.G., 2006. Stable growth of wild-type hepatitis A virus in cell culture. J. Virol. 80, 1352–1360.

Lemon, S.M., Murphy, P.C., Shields, P.A., Ping, L.-H., Feinstone, S.M., Cromeans, T., Jansen, R.W., 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. J. Virol. 65, 2056–2065.

Martin, A., Lemon, S.M., 2006. Hepatitis A virus: from discovery to vaccines. Hepatology 43, S164–S172.

Nainan, O.V., Xia, G., Vaughan, G., Margolis, H.S., 2006. Diagnosis of hepatitis A virus infection: a molecular approach. Clin. Microbiol. Rev. 19, 63–79.

Paget, C.J., Chamberlin, J.W., Wikel, J.H., 1978. Carbonyl-substituted 1-sulfonylbenzimidazoles. US Patent #4118742.

Pérez-Sautu, U., Costafreda, M.I., Caylà, J., Tortajada, C., Lite, J., Bosch, A., Pintó, R.M., 2011. Hepatitis A virus vaccine escape variants and potential new serotype emergence. Emerg. Infect. Dis. 17, 734–737.

Pevear, D.C., Tull, T.M., Seipel, M.E., Groarke, J.M., 1999. Activity of pleconaril against enteroviruses. Antimicrob. Agents Chemother. 43, 2109–2115.

Reed, L.J., Muench, H., 1938. A simple method for estimating fifty per cent endpoints. Am. J. Epidemiol. 27, 193–197.

Shouval, D., 2012. Hepatitis: new doubts about preventing HAV superinfection in chronic HCV. Nat. Rev. Gastroenterol. Hepatol. 29, 367–368.

Silberstein, E., Xing, L., van de Beek, W., Lu, J., Cheng, H., Kaplan, G.G., 2003. Alteration of hepatitis A virus (HAV) particles by a soluble form of HAV cellular receptor 1 containing the immunoglobin- and mucin-like regions. J. Virol. 77, 8765–8774.

Superti, F., Seganti, L., Orsi, N., Divizia, M., Gabrieli, R., Panà, A., 1987. The effect of lipophilic amines on the growth of hepatitis A virus in Frp/3 cells. Arch. Virol. 96, 289–296.

Tan, J., George, S., Kusov, Y., Perbandt, M., Anemüller, S., Mesters, J.R., Norder, H., Coutard, B., Lacroix, C., Leyssen, P., Neyts, J., Hilgenfeld, R., 2013. 3C protease of enterovirus 68: structure-based design of Michael acceptor inhibitors and their broad-spectrum antiviral effects against picornaviruses. J. Virol. 87, 4339–4351.

Thibaut, H.J., De Palma, A.M., Neyts, J., 2012. Combating enterovirus replication: state-of-the-art on antiviral research. Biochem. Pharmacol. 83, 185–192.

van Cuyck-Gandré, H., Job, A., Burckhart, M.F., Girond, S., Crance, J.M., 1995. Use of digoxigenin-labeled RNA probe to test hepatitis A virus antiviral drugs. Pathol. Biol. (Paris) 43, 411–415.

van der Schaar, H.M., van der Linden, L., Lanke, K.H., Strating, J.R., Pürstinger, G., de Vries, E., Neyts, J., van Kuppeveld, F.J., 2012. Coxsackievirus mutants that can bypass host factor PI4KIIIβ and the need for high levels of PI4P lipids for replication. Cell Res. 22, 1576–1592.

Vento, S., Garofano, T., Renzini, C., Cainelli, F., Casali, F., Ghironzi, G., Ferraro, T., Concia, E., 1998. Fulminant hepatitis associated with hepatitis A virus superinfection in patients with chronic hepatitis C. N. Engl. J. Med. 29, 286–290.

Wasley, A., Fiore, A., Bell, B.P., 2006. Hepatitis A in the era of vaccination. Epidemiol. Rev. 28, 101–111.

Widell, A., Hansson, B.G., Oberg, B., Nordenfelt, E., 1986. Influence of twenty potentially antiviral substances on *in vitro* multiplication of hepatitis A virus. Antiviral Res. 6, 103–112.

World Health Organization, 2011. The Immunological Basis for Immunization Series – Module 18: Hepatitis A. WHO Press, Geneva, Switzerland.

Yang, Y., Liang, Y., Qu, L., Chen, Z., Yi, M., Li, K., Lemon, S.M., 2007. Disruption of innate immunity due to mitochondrial targeting of a picornaviral protease precursor. Proc. Natl. Acad. Sci. USA 104, 7253–7258.

Yoshiba, M., Inoue, K., Sekiyama, K., 1994. Interferon for hepatitis A. Lancet 343, 288–289.

Zhang, J.-H., Chung, T.D.Y., Oldenburg, K.R., 1999. A simple statistic parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. 4, 67–73.