AAC Accepts, published online ahead of print on 7 April 2014
Antimicrob. Agents Chemother. doi:10.1128/AAC.00113-14

Identification of AP80978, a Novel Small Molecule Inhibitor of Hepatitis C Virus Replication that Targets NS4B

Jodi Dufner-Beattie[1,3], Andrew O'Guin[1,4], Stephanie O'Guin[1,5], Aaron Briley[1,6], Bin Wang[1,7], Jennifer Balsarotti[1,8], Robert Roth[1], Gale Starkey[1,9], Urszula Slomczynska[1,3], Amine Noueiry[1,10], Paul D. Olivo[1,11], Charles M. Rice[2#]

[1]Apath LLC, 760 Parkside Avenue, Suite 310, Brooklyn, NY 11226

[2]Laboratory of Virology and Infectious Disease, The Rockefeller University, 1270 York Avenue, New York, NY 10065

[3]Present address: Monsanto Company, Chesterfield, MO

[4]Present address: Fluidigm Corp., South San Francisco, CA

[5]Present address: Genome Technology Access Center, Washington University in St. Louis, St. Louis, MO

[6]Present address: American Type Culture Collection, Manassas, VA

[7]Present address: Dept. of Pathology and Immunology, Washington University in St. Louis, St. Louis, MO

[8]Present address: Dept. of Neurology, Washington University in St. Louis, St. Louis, MO

[9]Present address: Global Patent Group, St. Louis, MO

[10]Present address: Polsinelli PC, St. Louis, MO

[11]Present address: CPH, LLC., St. Louis, MO

#corresponding author: ricec@mail.rockefeller.edu

running title: A novel hepatitis C virus NS4B inhibitor

Abstract

A small molecule inhibitor of hepatitis C virus (HCV) designated AP89652 was identified by screening a compound library with an HCV genotype 1b subgenomic replicon assay. AP89652 contains two chiral centers, and testing of two *syn* enantiomers revealed that activity in the replicon assay resided with only one, AP80978, whose EC_{50} was 630 nM. AP80978 was inhibitory against HCV genotypes 1a and 1b but not genotype 2a. In a replicon clearance assay, potency and clearance rate of AP80978 were similar to telaprevir (VX950) and cyclosporine A (CsA). AP80978 was non-toxic when tested against a panel of human cell lines, and inhibitory activity was HCV-specific in that there was limited activity against negative-strand viruses, an alphavirus, and flaviviruses. By selection of resistant replicons and assessment of activity in 1b/2a intergenotypic replicons, the viral protein target of this compound was identified as NS4B. NS4B F98V/L substitutions were confirmed by site-directed mutagenesis as AP80978 resistance-associated mutations. When tested against HCV produced in cell culture, the compound was significantly more potent than other HCV inhibitors, including VX950, CsA, and 2'-C-methyl-adenosine (2'C-meA). In addition, AP80977, the enantiomer that was inactive in the replicon assay, had activity against the virus, although it was lower than AP80978. These results suggest that AP80978 has the potential to be optimized into an effective antiviral drug and is a useful tool to further study the role of NS4B in HCV replication.

Introduction

HCV is a positive-strand RNA virus belonging to the *Flaviviridae* family. Within the viral genome, the internal ribosome entry site (IRES) drives translation of a single polypeptide that is cleaved by both cellular peptidases and viral proteases to produce viral structural and non-structural proteins (1). The virus-encoded RNA-dependent RNA polymerase NS5B is exceptionally error-prone, resulting in significant genome sequence variability. Based on sequence differences, HCV can be categorized into seven distinct genotypes, which differ both in global distribution and response to therapy (2, 3).

Worldwide, over 170 million people are infected with this virus (4). HCV infection is a major cause of chronic liver disease such as cirrhosis and hepatocellular carcinoma, and is a leading cause of liver transplantation (5, 6). Until recently, the standard of care (SOC) was a combination of pegylated interferon and ribavirin, which is commonly associated with severe side effects and low sustained virological response rates for patients infected with genotype 1, the most prevalent genotype in North America and Europe (7, 8). Direct-acting antivirals (DAA) have been the focus of intensive drug discovery efforts, particularly the viral NS3-4A protease, the NS5A phosphoprotein, and the NS5B polymerase. A triple combination composed of the SOC with one of two protease inhibitors, VX950 or boceprevir enhances cure rates and is now approved for treatment of patients with chronic genotype 1 HCV infection (9, 10). However, resistance develops quickly to these as well as other antiviral compounds, and severe side effects and drug interactions complicate treatment (11). New protease and polymerase inhibitors have recently been approved but the development of

additional classes of antiviral compounds against novel viral targets will broaden treatment options and provide multiple options for interferon-free HCV therapy (1, 3, 12-14).

To this end, we carried out a high throughput, cell-based genotype 1b subgenomic replicon screen to identify novel compounds with antiviral activity against HCV. One compound that was selected for further study was a molecule with two chiral centers, designated AP89652. After separation of enantiomers, antiviral activity was found to be associated with AP80978, one of the two tested isomers. The active enantiomer was genotype 1-specific, non-cytotoxic, and inactive against numerous other virus replication systems, which included other flaviviruses. Two approaches were taken to study the molecular target of the compound, including selection of resistant replicons and generating intergenotypic 1b/2a replicons with differential susceptibility to the compound. Both approaches indicated a novel target of this compound, HCV NS4B. When tested against HCV produced in cell culture, the compound was significantly more potent than other HCV inhibitors, including VX950, CsA, and 2'C-meA.

Materials and Methods

Maintenance of Huh-7.5 cells

Huh-7.5 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Cells were subcultured by washing once

with phosphate buffered saline (PBS) (Invitrogen), followed by incubating for up to 5 min in 0.05% Trypsin-EDTA (Invitrogen) at 37°C until cells detached from the vessel. Upon detachment, complete medium was added to inactivate trypsin, and cells were counted and seeded at the desired density into T-flasks (TPP, Midwest Scientific, St Louis, MO). Cells were grown to 80% maximum confluence, and were seeded at a density no less than 13,000 cells/cm^2.

Preparation of CA32 replicon cells

The replicon utilized in the primary and secondary screens, CA32, was created at Apath, LLC, and is a transient genotype 1b subgenomic replicon generated from the Con1 strain. In this replicon, the HCV IRES within the 5' non-translated region (NTR) drives translation of the first 32 amino acids of the core protein fused to humanized *Renilla* luciferase (hRluc). The encephalomycarditis virus (EMCV) IRES lies 3' of the hRluc open reading frame (ORF) and drives translation of non-structural (NS) proteins NS3 through NS5B, which is flanked at its 3' end with the HCV 3' NTR (for schematic, see Figure 1a).

To prepare CA32 replicon cells, the replicon-encoding plasmid APP660 was linearized with the unique restriction endonuclease *ScaI* (NEB, Ipswitch, MA) at 37°C for 4 h. The reaction was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen) and once with chloroform:isoamyl alcohol (24:1). The linearized plasmid was precipitated with 1/10 volume 3M sodium acetate (pH 5.2) (Sigma-Aldrich, St. Louis, MO) and 2 volumes ethanol, and the pellet was washed twice with 70% ethanol. Linearized DNA (1 μg) was *in vitro* transcribed (IVT) using T7 polymerase from the

MEGAscript® T7 Kit (Invitrogen). RNA size and integrity were verified by resolving 1 μg IVT RNA on a standard formaldehyde-agarose gel and staining with ethidium bromide (Sigma-Aldrich). Approximately 24 h prior to electroporation, Huh-7.5 cells were seeded at a density of 5.5 x 10^6 cells per 150 cm^2 T-flask. On the day of electroporation, cells were washed once with PBS prior to incubation with trypsin-EDTA for up to 5 min at 37°C. After the cells detached, complete medium was added, and cells were pelleted via centrifugation at 200 x g for 5 min at 4°C. Cells were washed by resuspending in ice-cold PBS and pelleting at 200 x g for 5 min at 4°C, resuspended once more in ice-cold PBS, passed through a 70 μm cell strainer (VWR, Radnor, PA) and counted prior to a final centrifugation at 200 x g for 5 min at 4°C. After resuspension in ice-cold PBS at a density of 1.5 x 10^7 cells per ml, 6 x 10^6 cells were combined with 1 μg IVT RNA and electroporated in a 2 mm-gap electroporation cuvette (Bio-Rad, Hercules, CA) using the BTX Electrosquare Porator with the following settings: 820V, 99 μsec pulse length, 5 pulses, 1.1 sec intervals. Following electroporation, cells recovered at RT for 10 min prior to plating in complete medium. Replicon-containing cells were passaged as they reached 70-80% confluence and were seeded at a density no lower than 13,000 cells/cm^2.

Compound screening with CA32 replicon cells

Replicon cells were seeded into 96-well plates at 20,000 cells/well in screening medium, consisting of DMEM with 10% FBS, 1X penicillin/streptomycin, 1X non-essential amino acids, and 100 ng/ml Fungizone (Invitrogen). After 4 h attachment, cells were treated with compound in 1% DMSO (Sigma-Aldrich) in a final volume of 200 μl for 24 to 48 h.

Pilot studies indicated that 1% DMSO caused no adverse effects on cell viability for this assay duration. For primary screening, compounds were added at a single dose (10 μM) in single replicate without toxicity evaluation. Compounds that caused a 50% reduction in *Renilla* luciferase levels were progressed to secondary screening for EC_{50} and CC_{50} determination. Identical culture conditions were used for EC_{50} and CC_{50} determination. For secondary screening, compound was added to wells using a five point 3-fold serial dilution series with four replicate treatments per dose. *Renilla* luciferase levels were assayed using the *Renilla* Luciferase Assay Kit (Promega, Madison, WI), and toxicity/cell viability was assayed using CellTiter-Glo Luminescent Cell Viability Assay (Promega). Both EC_{50} (the concentration at which a 50% reduction in *Renilla* luciferase levels was observed relative to an untreated control) and CC_{50} (the concentration resulting in a 50% decrease in cell viability) values were determined from the raw data using a proprietary software program based on a Hill Plot calculated from a four-parameter logistic model.

Compound synthesis and enantiomer separation

AP89652 [3-chloro-5-(furan-2-yl)-N-(thiophene-2-yl-methyl)—7-(trifluoromethyl)-4,5,6,7-tetrahydropyrazolo[1,5,a]pyrimidine-2-carboxamide] (Figure 1b) was synthesized in two steps using a procedure described by Dalinger et al. (15). The pyrazolo[1,5-a]pyrimidine ring was assembled by condensation of 5-amino-4-chloro-1H-pyrazole-3-carboxylic acid with 4,4,4-trifluoro-1-(furan-2-yl)-butane-1,3-dione and the pyrimidine ring was reduced stereoselectively with sodium borohydride in ethanol, yielding only one pair of diastereoisomers that corresponds to 2,4-*syn*-isomers. Separation of two enantiomers

(AP80977 and AP80978) was achieved by an HPLC chromatography method using a chiral column Chiralcel OD-H and heptane/2-propanol as a mobile phase. Absolute configuration of AP80978 was determined by x-ray diffraction (oXray Ltd.) and the active enantiomer was found to be *(5S,7R)* 3-chloro-5-(furan-2-yl)-N-(thiophene-2-yl-methyl)-7-(trifluoromethyl)-4,5,6,7-tetrahydropyrazolo [1,5,a]pyrimidine-2-carboxamide. The synthetic procedure for AP89652, its separation into two enantiomers, and determination of the absolute configuration for the active AP80978 enantiomer were described previously (16, 17).

Control inhibitors

Control inhibitors ribavirin, a viral RNA synthesis inhibitor (18), and CsA, an HCV replication inhibitor (19), were obtained from Sigma Aldrich (St. Louis, MO). VX950, an NS3-4A protease inhibitor (10), was synthesized by Stereochem Research Centre (Hyderabad, India). 2'C-meA, an NS5B polymerase inhibitor (20), was obtained from Carbosynth Limited (Berkshire, UK).

Replicon clearance assay

The genotype 1b replicon cells used in this assay harbor the replicon Con1/SG-Neo(I)hRLuc2aUb, which was created at Apath, LLC and is encoded by plasmid APP76. In this replicon, the HCV IRES within the 5' NTR drives translation of the first 15 amino acids of the core protein fused to Neo. The EMCV IRES is 3' of Neo and drives translation of an hRluc-ubiquitin (Ub)-foot-and-mouth disease virus 2a peptide (FMDV2a)-NS3-NS5B fusion protein (Figure 2A). The presence of FMDV2a and Ub

increases the likelihood of cleavage of the hRluc reporter from the C-terminal HCV non-structural proteins. Preparation of stable replicon cells was carried out as described for the genotype 2a replicon J6/JFHEMCVIRES2aRlucNeo.

Con1/SG-Neo((I)hRLuc2aUb replicon cells were seeded into 12-well cluster plates at a density of 5 x 10^4 cells/well in complete Huh-7.5 media lacking G418 (Invitrogen). After 4 h attachment, triplicate wells were harvested for RNA extraction as a 0 h control. Additional wells were treated in triplicate with either 1% DMSO (untreated control), 7.5 μM VX950, 2.5 μM CsA, or 3.75 μM or 15 μM AP80978 (replicon clearance phase). Cell density was monitored daily, and when the cells reached 80% confluence, a sample was collected for RNA extraction and a subset of the remaining cells were split at a 1:3 ratio into the appropriate compound-containing media. At the fourth passage, cells were collected and passaged into media lacking compound in the presence of 250 μg/ml G418 to initiate the rebound phase. Untreated cells surviving the treatment were collected as they reached 80% confluence. RNA from all time points and treatments was extracted using a 96-well RNeasy RNA Extraction Kit (Qiagen, Valencia, CA). RNA levels of HCV 3' NTR and the housekeeping gene GAPDH were quantified via RT-qPCR. GAPDH RT-qPCR was carried out using the following primers: forward, 5'-CCTGCACCACCAACTGCTTA -3'; reverse, 5'-GCAGTGATGGCATGGACTGT-3'; probe, 5'- Cy5-CTGGCCAAGGTCATCCATGACAACT-BHQ-2-3'. HCV RNA levels were normalized to those of GAPDH and were expressed as HCV $\log_{10}$ change relative to the HCV RNA level in untreated cells on day 0 using the comparative Ct method (Applied Biosystems). At each time point, normalization of HCV to GAPDH was determined as follows:

dCt=(Ct_{HCV})-(Ct_{GAPDH}). Calculation of ddCt value relative to t = 0 was determined as follows: ddCt=(dCt $_{time\ x}$)-(dCt $_{time\ 0}$). HCV RNA log change was calculated as log(2^((-1)*ddCt)).

Evaluation of AP80978 against genotype 1a and 2a replicons

The genotype 2a replicon, J6/JFHEMCVIRES2aRlucNeo, was created at Apath, LLC and is encoded by the plasmid APP40. It is a stable subgenomic replicon with elements from both the JFH and J6 strains. The JFH IRES within the 5' NTR drives translation of the first 19 amino acids of the JFH core protein linked to an hRluc-neomycin phosphotransferase (Neo) fusion protein. The EMCV IRES is 3' of the hRluc-Neo ORF and drives translation of JFH NS3-NS5B, which is flanked at its 3' end with the J6 3' NTR. The differences between the JFH and J6 3'NTRs are within the variable region.

Preparation of stable genotype 2a replicon cells was performed as outlined above for CA32 cells except that 24 h after plating, medium was removed and replaced with selection media (i.e. complete media containing 1 g/L G418 (Invitrogen). Cells were monitored daily and passaged as needed to maintain a subconfluent culture. Selection was considered complete when cells electroporated in parallel with a polymerase-deficient replicon lacked any viable cells. After this observation, the concentration of G418 in the media was decreased to 250 μg/ml for culture maintenance. Screening with the resulting genotype 2a replicon cells (APC140) was as described for CA32 replicon cells except that seeding density was 12,000 cells/well and cells were assayed 48 h post-treatment in the absence of selection agent.

The genotype 1a replicon cells (Huh-7.5 containing the H/SG-Neo(L+I) replicon, APC89) was previously reported (21). Replicon cells were seeded into 12-well plates at

247 a density of 40,000 cells/well. After 4 h attachment, cells were treated in triplicate using
248 a five point 3-fold serial dilution series in 1% DMSO, which caused no adverse effects
249 on cell viability for this assay duration in pilot studies. After 72 h, RNA was extracted
250 using the RNeasy RNA Extraction Kit (Qiagen). Total RNA concentration was
251 determined using Ribogreen RNA Quantitation Reagent (Invitrogen), and were
252 normalized between samples. HCV RNA levels were quantified via quantitative RT-PCR
253 (RT-qPCR) using MultiCode-RTx HCV Viral Load Primer Mix and RNA Reagent Set
254 (EraGen Biosciences, Madison, WI) on an Applied Biosystems 7300 Real-Time PCR
255 System. EC_{50} values were determined as described above.
256
257 **Evaluation of AP80978 against other viruses**
258 BHK-S cells harboring a subgenomic, puromycin-selectable *Renilla* luciferase reporter
259 yellow fever virus (YFV) replicon (YF-hRUPac) (22) were seeded at 10,000 cells/well
260 into 96-well plates. The serotype 2 dengue virus replicon was constructed by removing
261 a structural protein-coding region, between capsid gene codon 28 and the last 26
262 codons at the 3' end of the envelope gene from an infectious clone DEN2 16681 (23).
263 This deletion was replaced with the humanized *Renilla* luciferase-ubiquitin-puromycin
264 acetyl transferase (hRUPac) cassette from the yellow fever replicon (22) to generate
265 pD2-hRUPac. BHK-S cells harboring the dengue virus replicon were seeded at 8,000
266 cells/well in 96-well plates. The respiratory syncytial virus (RSV) replicon is similar to
267 that reported by Malykhina et al. (24) except that it also contains a *Renilla* luciferase
268 reporter located between the GFP and NS1 coding sequence. Screening was carried
269 out by seeding 1,500 RSV BHK-SR19-T7 replicon cells and 8,500 BHK-S parental

cells/well in 96-well plates. For West Nile Virus (WNV), BHK-WNV-Rep replicon cells (22) were seeded at 10,000 cells/well into 96-well plates. The ebola virus (EBOV) mini-genome expresses a *Renilla* luciferase reporter-encoding plasmid that carries *cis*-acting elements for ebola virus replication. The EBOV minigenome was transfected into BHK-SINRep T7 cells, along with four expression plasmids that encode the replicase proteins NP, VP35, L, and VP30 (25). EBOV mini-genome cells were seeded at 20,000 cells/well into 96-well plates.

BHK-S cells harboring a subgenomic, puromycin-selectable firefly luciferase reporter Sindbis virus replicon (SINrep19.FLuc.Pac) (26) were seeded at 10,000 cells/well into 96-well plates. The influenza A virus (FluA) mini-genome expressing firefly luciferase reporter carries *cis*-acting elements for FluA replication. The FluA minigenome was transfected into 293T cells, along with four expression plasmids that encode the replicase proteins NP, PA, PB1, and PB2. FluA mini-genome cells (27) were seeded at 20,000 cells/well into 96-well plates.

For determination of anti-replicon activity, all cells were treated with compounds using a five point 3-fold serial dilution series with four replicate treatments per dose. A 24h incubation period was chosen to maximize screening throughput, following which, *Renilla* (dengue, YFV, RSV, WNV, EBOV) or firefly (Sindbis or FluA) luciferase levels were evaluated to determine an EC_{50} value. In parallel, cell toxicity/cell viability was assayed on parental cells using CellTiter-Glo Luminescent Cell Viability Assay.

Cytotoxicity evaluation in other cell types

HepG2 cells, Caco-2, MRC-5, and Jurkat cells were obtained from the ATCC (Manassas, VA). HepG2 and MRC-5 cells were maintained in Eagle's Minimal Essential Medium (Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Caco-2 cells were grown in the same media except with 20% FBS. Jurkat cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were incubated at 37°C in a humidified 5% CO_2 incubator. Cytotoxicity screening was carried out by incubating cells (20,000 cells/well for HepG2, Caco-2, and MRC-5 cell lines, and 40,000 cells/well for Jurkat cells) in 96-well plates in the presence of serial dilutions of compound for 24 hours, a time point chosen as a realistic interval to observe control inhibitor activity, and which also maximized assay throughput. At the end of the incubation period, cell viability was assessed by the CellTiter-Glo Luminescent Cell Viability Assay.

Generation and evaluation of AP80978-resistant replicons

Clone A cells (28) contain a genotype 1b (Con1) stable subgenomic replicon, and were used for resistant mutant selection. Cells were seeded at a density of 1 x 10^6 cells per 75 cm^2 T-flask. After 4 h attachment, cells were treated with complete medium supplemented with either 1% DMSO (untreated control) or 10 μM AP80978. Medium was changed every 3-4 d to replenish compound and G418, and cells were split as needed to maintain subconfluent cultures. After 12 d of culture in the presence of 10 μM AP80978, the concentration was increased to 20 μM, and cells were cultured for an additional 19 d. After this period, a sample of cells from both AP80978-treated and control cells were collected for evaluation of response to AP80978 as follows: cells

were seeded into 12-well plates at a density of 40,000 cells/well in the absence of G418. After 4 h attachment, cells were treated in triplicate with AP80978 using a five point, 3-fold serial dilution series in 1% DMSO. After 72 hours, total RNA was extracted using the RNeasy RNA Extraction Kit (Qiagen). Total RNA was quantified using the Ribogreen RNA Quantitation Reagent (Invitrogen), and concentrations were normalized between samples. HCV RNA levels were quantified via quantitative RT-PCR using MultiCode-RTx HCV Viral Load Primer Mix and RNA Reagent Set on an Applied Biosystems 7300 Real-Time PCR System. EC_{50} values were determined as described above.

Evaluation of cell-associated versus replicon-associated resistance

Total RNA (10 μg) extracted from control and AP80978-treated cells was electroporated into the Clone A parental cell line Huh7. Cells were selected with 1 mg/ml G418, and after selection was complete (determined by complete cell death in cells electroporated without RNA), cells were pooled and evaluated for response to AP80978 as described above.

Replicon sequence analysis

Total RNA was extracted from control and AP80978-resistant replicon cell lines using the RNeasy RNA Extraction Kit (Qiagen). RT-PCR was carried out with replicon-specific primers using SuperScript III reverse transcriptase (Invitrogen) and iProof high fidelity polymerase (BioRad). PCR amplicons were sequenced and compared against the wild-type reference sequence using Clone Manager software. The four mutations identified

within NS4B in the AP80978-resistant cells (G60 no change, F98V, F98L, S238Y) were introduced individually into the genotype 1b replicon-encoding plasmid APP76 using standard PCR-based techniques. The mutated plasmids were sequenced to verify that only the desired mutations had been introduced.

Colony formation assay

IVT RNA corresponding to wild-type replicons or replicons with F98V or F98L resistance mutations were electroporated into Huh-7.5 cells as described for the genotype 2a replicon. Electroporated cells (225, 450, 900, or 1800) were seeded in triplicate into 6-well cluster plates along with cells electroporated with a polymerase-defective construct to achieve a total cell number of 90,000 cells/well. After 24 h attachment, cells were treated with either 1 mg/mL G418 alone or G418 in the presence of 20 μM AP80978. Media was changed every 3-4 days to replenish compound and remove dead cells. After 2-3 wks, media were removed, cells were fixed with 7% formaldehyde and stained with 1% crystal violet (Sigma-Aldrich) in 50% ethanol, and colonies were counted.

Generation and testing of intergenotypic 2a/1b replicons

Standard PCR-based techniques were used to produce a series of genotype 2a intergenotypic replicons (encoded by plasmid APP40, description above) in which the genotype 2a NS4B-encoded amino acids were replaced with the corresponding sequence from genotype 1b: NS4B(Con1 $_{7\text{-}254}$), NS4B(Con1 $_{7\text{-}52}$), NS4B(Con1 $_{53\text{-}254}$), NS4B(Con1 $_{219\text{-}254}$), NS4B(Con1 $_{7\text{-}52,\ 219\text{-}254}$), NS4B(Con1 $_{53\text{-}219}$). Polymerase-defective versions of each construct were prepared in parallel to evaluate replication of each

362 intergenotypic replicon. The resulting plasmids were sequenced to verify that the
363 desired constructs had been produced. Preparation of intergenotypic replicon cells and
364 compound screening was performed as outlined above for genotype 2a replicon cells.
365 The genotype 2a replicon containing the NS4B configuration NS4B(Con1 $_{7-254}$) did not
366 replicate by comparison with the polymerase-defective counterpart.
367
368 **Generation and testing of AP80978-sensitive virus**
369 To construct an AP80978-sensitive hepatitis C virus, a 3355 bp *SpeI-RsrII* (NEB) DNA
370 fragment from the genotype 2a replicon, encoding genotype 1b NS4B amino acids 53
371 through 219 (i.e. NS4B(Con1 $_{53-219}$)) was excised and inserted in the same restriction
372 sites in the genotype 2a/2a J6/JFH Jc1 (29) HCV plasmid construct (see Figure 6A).
373 This HCVcc construct is referred to as J6/JFH Jc1/Con 1 NS4B $_{53-218}$.
374 The resulting plasmid was linearized with *XbaI* (NEB), and DNA was prepared for
375 *in vitro* transcription and electroporation as described above for the replicons. Following
376 electroporation, cell culture supernatants were collected and concentrated using Amicon
377 Centrifugal Filter Units with a 100,000 molecular weight cut-off (EMD Millipore
378 Corporation, Billerica, MA). The virus titer was determined by infecting replicate wells
379 containing Huh-7.5 cells with serially-diluted virus, staining 4 d later with NS5A
380 monoclonal antibody, and calculating the $TCID_{50}$/ml from the number of positively-
381 stained replicates for each dilution.
382 To evaluate the effect of AP80978 on J6/JFH Jc1/Con1 NS4B $_{53-218}$ virus, Huh-
383 7.5 cells were seeded into 96-well plates at a density of 20,000 cells/well. After 24 h
384 attachment, cells were treated in quadruplicate with serially diluted compound in 1%

DMSO in the presence of 2000 $TCID_{50}$ units/well virus (MOI=0.1). Following 48 h incubation, cells were washed and RNA was extracted using RNeasy RNA Extraction kit (Qiagen). RNA levels of HCV 3' NTR were quantified via RT-qPCR and were expressed as HCV log change relative to untreated control cells using the comparative Ct method.

Results

Identification of AP89652 in a genotype 1b replicon assay

In order to identify new classes of antiviral compounds that inhibit HCV replication, a high throughput cell-based screen was carried out using a transient genotype 1b subgenomic *Renilla* luciferase replicon (Figure 1A). A primary screen of 93,000 commercially available compounds against these replicon cells identified 209 compounds that decreased *Renilla* luciferase levels by 50%. As the primary screen did not employ a method to identify cytotoxic compounds, these 209 compounds were further evaluated in a secondary assay to calculate efficacy (EC_{50}) and toxicity (CC_{50}). Evaluation of these compounds in the secondary screen identified 38 compounds with EC_{50} values less than 10 μM and CC_{50} greater than 100 μM. The 38 compounds belonged to 13 distinct chemical classes, with 15 compounds categorized as orphan compounds. One compound, AP89652 (Figure 1B), that belongs to a tetrahydropyrazole-[1,5,a]-pyrimidine chemical series was selected for further study based on its favorable physico-chemical properties, novelty, and amenability of this chemical series to SAR (structure activity relation) studies.

AP89652 contains two chiral centers (Figure 1B), yielding four potential isomers. However, based on the literature describing the synthesis of tetrahydropyrazolo-[1,5a]-pyrimidine chemical series (15) and our results, it was found that AP89652 is a racemic mixture of two *syn*-enantiomers, as shown in Figure 1C. To determine which enantiomer of AP89652 was responsible for HCV replication inhibitory activity, the optically pure enantiomers were isolated via chiral chromatography and evaluated against the replicon cell line. Screening against CA32 genotype 1b subgenomic replicon cells revealed that only one of the two enantiomers, AP80978, had replicon-inhibitory activity, with an EC_{50} of 0.63 μM, while the other, AP80977, lacked activity (Table 1). Similar results were obtained when replicon levels were quantified using RT-qPCR (data not shown).

HCV Replicon Clearance by AP80978

The HCV replicon clearance assay served as a cell culture model for virus clearance. At the onset of the assay, cells harboring a stable HCV replicon were cultured under non-selection conditions in the presence or absence of compound. During this "replicon clearance phase", cells were able to proliferate independently of HCV replicon replication. The "rebound phase" was initiated by inhibitor removal and resumption of G418 selection, allowing growth of only HCV replicon-harboring survivor cells.

In this assay, the cells harboring the stable genotype 1b subgenomic replicon Con1/SG-Neo(I)hRluc2aUb (Figure 2A) were treated with either 15 μM or 3.75 μM AP80978 in the absence of neomycin for 17 d. To compare efficacy of AP80978 with other antiviral compounds, parallel cultures were treated with 2.5 μM CsA or 7.5 μM VX950. Compound-treated cells were passaged as needed to maintain a subconfluent

431 culture, replenishing compound and collecting an aliquot of cells at each passage. On
432 d17, cells were seeded in G418-containing selection media in the absence of
433 compound, and cells were cultured for an additional 7 d, during which untreated cells
434 approached confluence. Massive cell death was observed in cultures that had been
435 treated with compounds. At each passage, HCV RNA levels were quantified and
436 normalized relative to GAPDH via RT-qPCR for treated and control samples. HCV RNA
437 levels remained similar over the course of the assay in control cells grown in the
438 absence of inhibitors, while cells treated with both doses of AP80978 exhibited a
439 progressive decrease in HCV RNA levels during the replicon clearance phase, with a
440 reduction of 1.5-1.75 $\log_{10}$ RNA copies by d17 of the assay (Figure 2B). Similar to
441 results with AP80978, treatment with CsA and VX950 was associated with a maximal
442 1.5 to 2 $\log_{10}$ decrease in HCV RNA levels. During the rebound phase, HCV RNA levels
443 in all inhibitor-treated cells, including AP80978 and control CsA and VX950, were
444 undetectable in the presence of G418, indicating the absence of functional replicon
445 RNA within these cells (Figure 2B). In control untreated cells, replicon RNA was
446 slightly lower than baseline, but within the variation typically observed in RT-qPCR in
447 the clearance assay.
448
449 **Genotype- and Virus-specificity of AP80978**
450 To evaluate the genotype specificity of AP80978, the compound was screened against
451 cells harboring the genotype 1a replicon H/SG-Neo(L+I) (21) and the genotype 2a
452 replicon J6/JFHEMCVIRES2aRlucNeo. AP80978 inhibited replication of the genotype
453 1a replicon, with an EC_{50} value (0.62 μM) similar to that for the genotype 1b replicon

(Table 2). However, the compound was completely inactive against the genotype 2a replicon (EC_{50} >25 μM). Screening against genotype 1a, 1b, and 2a replicons by two external laboratories confirmed similar EC_{50} values for genotypes 1a and 1b, and inactivity against genotype 2a (data not shown).

To evaluate whether AP80978 was active against other viruses, the compound was tested against virus-free mini-genome and replicon systems that have been developed for high-throughput screening to identify replication inhibitors of negative-strand and other positive-strand viruses. AP80978 was inactive against infection-free negative-strand RNA virus systems for ebola virus, influenza A virus, and respiratory syncytial virus, although the controls (ribavirin, CsA, and 2'C-meA) for each of these systems behaved as expected (Table 3). Similarly, the compound was inactive against the alphavirus Sindbis replicon, as well as other positive-strand flavivirus replicons for dengue virus, yellow fever virus, and West Nile virus, indicating that AP80978 is an HCV-specific antiviral compound. To confirm that the compound that was used in these studies was active, AP80978 was tested in parallel against HCV replicon cells, and showed efficacy.

Cytotoxicity of AP80978 in Different Cell Types

To determine the effect of AP80978 on other cell types, cytotoxicity was evaluated against a panel of cell lines from an array of different tissues. Similar to observations using the Huh-7.5 cell line that supports HCV replication in cell culture, AP80978 was non-toxic against another human liver cell line, HepG2, with a CC_{50} value >100 μM

(Table 4). It was also non-toxic in human cell lines derived from intestinal epithelium (Caco-2), lung fibroblasts (MRC-5), and T-lymphocytes (Jurkat).

Mechanism of Action of AP80978

Selection and characterization of AP80978 resistance mutations

Preliminary biochemical studies showed that AP80978 had no activity against the HCV NS3-4A serine protease and NS5B polymerase (not shown), suggesting a novel viral or host target. Due to the error-prone nature of the HCV RNA polymerase, culture of HCV replicon cells in the presence of replication inhibitors can result in the selection of inhibitor-resistant replicons. In an effort to gain insight into the molecular target of AP80978, Clone A cells resistant to AP80978 were selected by sequential passaging in the presence of compound. Clone A cells are a human hepatoma cell line that contains a stable genotype 1b (Con1 strain) subgenomic replicon (28). Cells were cultured under neomycin-containing conditions for 12 d in medium supplemented with 10 μM AP80978, followed by culture for an additional 19 d in 20 μM AP80978. Control Clone A cells grown in the presence of diluent only were cultured in parallel. After this culture period, cells were evaluated for response to AP80978 by culturing treated and control cells in the presence of serial dilutions of AP80978 for 72 h and subsequently quantifying HCV RNA levels via RT-qPCR to determine the EC_{50}. Clone A cells that had undergone selection exhibited greater than 10-fold decreased sensitivity to AP80978 (EC_{50} >20 μM) relative to the control cells (EC_{50}=1.8 μM).

To determine whether resistance was encoded by the replicon, total RNA was extracted from both AP80978-resistant and control cells, and RNA was re-introduced

via electroporation into naïve Huh7 cells, the parental cell line of Clone A cells. Electroporated cells that harbored actively replicating replicons were selected with G418, pooled, and assayed for sensitivity to AP80978 as described above. Cells electroporated with RNA from control cells were sensitive to AP80978 (EC_{50}=0.91 μM), while those electroporated with RNA from AP80978-resistant cells maintained resistance to the compound (EC_{50} >20 μ□), indicating that the resistance was associated with the replicon.

To identify nucleotide changes that conferred resistance to AP80978, RNA extracted from AP80978-resistant and control Clone A cells was reverse transcribed and amplified using replicon-specific primers. Sequence analysis of the amplified replicons revealed four point mutations present within the sequence encoding NS4B from the AP80978-resistant cells that were absent from the control or wild-type replicon sequence. Three of the four mutations resulted in amino acid changes corresponding to F98L, F98V, and S238Y within NS4B. One silent mutation at codon 60 was also observed.

Each mutation was introduced independently into a stable genotype 1b subgenomic reporter replicon plasmid (Figure 3A) via site-directed mutagenesis. Sequencing of the resulting plasmids revealed that they were void of additional mutations. IVT RNA generated from each mutant and parental replicon plasmid was introduced into Huh-7.5 cells, a highly permissive human hepatoma cell line. Resulting replicon cell lines were evaluated for response to AP80978 and the positive control compounds CsA and VX950. A differential response was observed between cells electroporated with different replicon RNAs. Cells containing replicons with F98V and

522 F98L amino acid substitutions were resistant to AP80978 (EC_{50} >20 μM), while those
523 containing parental wild-type replicon (EC_{50} = 0.29 μM), the S238Y substitution (EC_{50} =
524 0.41 μM) or the silent mutation (G60nc, EC_{50} =0.24 μM) remained sensitive to AP80978
525 (Table 5). These data suggest that NS4B is the target of AP80978 and that changing
526 the amino acid at residue 98 of NS4B to either valine or leucine was sufficient to confer
527 resistance to the compound. Cells electroporated with IVT RNA from each construct
528 were responsive to CsA (EC_{50} values ranged from 0.1 to 0.16 μM) and VX950
529 (replicons cleared from all cell lines, data not shown), indicating that resistance to
530 AP80978 did not confer cross-resistance to either of these compounds.

531 Alignment of the NS4B amino acid sequences from genotypes 1b and 2a revealed
532 a leucine at codon 98 of NS4B in genotype 2a (Figure 3B). Since mutation that
533 changed phenylalanine to leucine at this position in the genotype 1b replicon conferred
534 resistance to AP80978, mutagenesis was carried out to determine whether a
535 phenylalanine at this position in the genotype 2a replicon was sufficient to confer
536 sensitivity to the compound. The resulting genotype 2a replicon with the L98F
537 substitution remained resistant to the compound (EC_{50} > 20 μM), indicating that a
538 mutation resulting in amino acid substitution in this residue alone was insufficient to
539 confer AP80978 sensitivity to an HCV genotype 2a subgenomic replicon.

540 The effect of AP80978 resistance mutations was evaluated in a long-term colony
541 formation assay. IVT RNA from either wild-type or resistant replicon constructs was
542 electroporated into naïve Huh-7.5 cells and plated at varying densities in the presence
543 of cells that had been electroporated with polymerase-defective constructs in order to
544 allow transient growth of cells in the presence of G418. Cells that had been

545 electroporated with all constructs grew in the presence of G418, with similar numbers of
546 colonies. However, only cells that had been electroporated with IVT RNA encoding
547 resistance mutations grew in the presence of AP80978 (Figure 4). In cells
548 electroporated with RNA encoding the F98L resistance-associated amino acid change,
549 there were fewer and smaller colonies in the presence of AP80978, suggesting that
550 maintaining resistance to this compound may be associated with a fitness cost.
551 However, colony number and size were similar in the absence and presence of
552 AP80978 for cells electroporated with the construct encoding the F98V substitution.
553
554 ***Evaluation of AP80978 against intergenotypic genotype 2a/1b replicons***
555 To confirm NS4B as the molecular target of AP80978, an alternate approach was taken
556 that took advantage of the differential response of genotypes 1b and 2a to AP80978.
557 Using the plasmid encoding J6/JFHEMCVIRES2aRlucNeo (a genotype 2a replicon that
558 is insensitive to AP80978) as a template, six intergenotypic constructs were made, in
559 which regions encoding the following amino acids of NS4B were replaced with the
560 corresponding amino acids from genotype 1b NS4B (sensitive to AP80978): (i) 7-254,
561 (ii) 7-52, (iii) 53-254, (iv) 219-254, (v) 7-52 and 219-254, and (vi) 53 to 218 (Figure 5).
562 Testing the resulting intergenotypic constructs would show which portions of genotype
563 1b NS4B protein confer AP80978 response. IVT RNA generated from each of the six
564 intergenotypic replicon cDNAs as well as the genotype 2a parental replicon was
565 electroporated into Huh-7.5 cells and evaluated for response to AP80978 and the
566 control compound CsA (Figure 5). The intergenotypic replicon that contained the nearly
567 full-length NS4B from genotype 1b (amino acids 7-254) did not replicate, similar to the

568 replication-defective control replicon, while the remaining intergenotypic replicons
569 replicated to varying degrees based on reporter levels. Compound screening was
570 initially carried out in unselected cells 96 h post-electroporation, and again following
571 selection with G418, with similar results. Two intergenotypic replicons that encoded
572 amino acid sequences from genotype 1b within the central region of NS4B (amino acids
573 53-254 and amino acids 53 to 218) were sensitive to AP80978, with EC_{50} values less
574 than 1 μM, while all other intergenotypic replicons, as well as the genotype 2a parental
575 replicon, did not respond to AP80978 treatment. Thus, the molecular target of AP80978
576 was located between amino acids 53 and 218 within NS4B of genotype 1b. All of the
577 intergenotypic and wild-type control constructs were responsive to CsA (not shown).
578
579 **Evaluation of AP80978 activity against cell culture-produced HCV**
580 To evaluate the effect of AP80978 on virus replication in the context of the fully-
581 infectious replication cycle, an AP80978-sensitive virus construct was produced by
582 cloning residues 53-218 from genotype 1b into a J6/JFH1 Jc1 construct, to produce
583 J6/JFH Jc1 (Con 1 NS4B $_{53\text{-}218}$) (Figure 6A). IVT RNA produced from this construct was
584 electroporated into Huh-7.5 cells, and intergenotypic virus was collected from the cell
585 culture medium. Titers were low, suggesting that the virus replicated poorly. Huh-7.5
586 cells were infected with either the intergenotypic or wild-type virus in the presence of
587 either AP80978, AP80977 (the second *syn* enantiomer that was inactive in the replicon
588 assay), AP89652 (the racemic mixture), or AP80935 (an analog of AP80978 with an
589 aromatic six-membered ring). Parallel cultures were treated with the HCV inhibitors
590 2'C-meA, VX950, and CsA. After 48 h, RNA was extracted and HCV RNA levels were

591 quantified. As expected, in cells infected with the J6/JFH Jc1 virus, HCV RNA levels did
592 not change appreciably in cells treated with 0.06-5 μM AP80978, AP80977, AP89652,
593 or AP80935 (Figure 6B), although high concentrations of the control inhibitors 2'C-meA
594 and VX950 were associated with a 2-3 $\log_{10}$ reduction (Figure 6C). Lower inhibitory
595 potency was observed for CsA for J6/JFH Jc1 HCV RNA (Figure 6C). In cells infected
596 with intergenotypic J6/JFH Jc1 (NS4B $_{53\text{-}218}$), HCV RNA levels decreased >1 $\log_{10}$ at 70
597 nM AP80978, and ~0.7 $\log_{10}$ at 20 nM (Figure 6D). Unexpectedly, AP80977, the
598 enantiomer that was inactive in the genotype 1b replicon assay, had inhibitory activity
599 against the intergenotypic virus, with a reduction of ~0.7 $\log_{10}$ HCV RNA at 0.67 μM.
600 Treatment with AP89652 and AP80935 also resulted in decreases in HCV RNA levels,
601 but potency was less than AP80978. However, AP80935 had similar potency as
602 AP80978 in a 24h genotype 1b replicon assay (not shown). One key finding is that
603 AP80978 maximally decreased HCV RNA by 1.5 $\log_{10}$ at 220 nM, which was lower than
604 the replicon assay EC_{50} and suggestive of enhanced activity in the context of replicating
605 virus. At a comparable concentration (190 nM), inhibitory efficacy was decreasing or
606 lost for the HCV inhibitors 2'C-meA, VX950, and CsA (Figure 6E).
607

608 **Discussion**

609 The purpose of this study was to identify new classes of anti-HCV compounds,
610 particularly ones with novel viral targets. Such compounds may be critical in patients
611 failing existing therapies, and/or who develop drug-resistant viruses. We identified a
612 novel small-molecule inhibitor, AP80978, with activity against genotype 1a and 1b, but
613 not 2a HCV, which was not toxic in multiple cell lines. AP80978 was similar to VX950,

614 an approved therapeutic for chronic HCV infection, in potency and clearance rate in the
615 replicon clearance assay and against replicating virus. Preliminary studies indicated that
616 AP80978 activity was not directed against HCV protease and polymerase, suggesting a
617 mechanism of action distinct from many compounds currently being evaluated in clinical
618 studies. Through two complementary approaches, resistant mutant generation and
619 evaluating the differential response of intergenotypic genomes to the compound, we
620 demonstrated that AP80978 acts against a novel target, NS4B.

621 Experimental data from numerous studies suggest several molecular functions for
622 the NS4B protein, including (i) formation of a membranous web, the modified membrane
623 structure located in the endoplasmic reticulum that is the proposed site for HCV
624 replication (30), (ii) modulation of NS5B RNA-dependent RNA polymerase activity (31),
625 (iii) modulation of HCV and host cellular translation (32-34), (iv) nucleotide binding and
626 GTPase activity (35), (v) modulation of NS5A hyperphosphorylation (36, 37) (vi) HCV
627 RNA binding (38). In theory, antiviral compounds could target any of these functions of
628 NS4B.

629 Other labs have recently identified compounds that inhibit HCV replication by
630 targeting NS4B, attesting to its potential as an effective drug target against HCV
631 (reviewed in (39)). Clemizole was identified after screening a compound library for
632 inhibitors of NS4B-HCV RNA binding, by using *in vitro* protein expression coupled with a
633 microfluidic affinity analysis (38). This compound inhibited HCV replication in cell
634 culture, and mutations conferring resistance to this compound displayed increased
635 affinity for the viral RNA. Viropharma discovered several classes of compounds that
636 interacted with NS4B using a biochemical binding assay, and demonstrated anti-

637 replicon activity, presumably by apoptosis induction of NS4B-expressing cells (40). A
638 new class of NS4B inhibitors was identified at Stanford University, by screening for
639 inhibitors of vesicle aggregation, which is mediated by the NS4B AH2 domain, an
640 amphipathic helix between amino acids 42-66 that is involved in membrane
641 rearrangement (41). Of particular interest is anguizole, an NS4B antagonist that targets
642 the AH2 domain and changes its subcellular distribution (42). Anguizole and AP80978
643 share two-dimensional structural similarity in their pyrazolopyrimidine core and
644 substitution nature and pattern, and it would be interesting to determine whether they
645 have similar mechanistic activity. A novel NS4B inhibitor with activity against genotype
646 1a and 1b replicons is PTC725, a small molecule whose inhibitory activity is additive to
647 synergistic with α-interferon and HCV protease and polymerase inhibitors, although
648 activity against replicating HCV was not assessed (43). Similar to our data, F98 was
649 implicated in the response to PTC725, with mutants encoding F98L/C amino acid
650 substitutions reduced in both inhibitor susceptibility and replicon fitness compared to
651 wild-type (43). In that study, immunofluorescence experiments of replicon cells showed
652 no changes in intracellular distribution of NS4B in the presence of the inhibitor or with
653 replicons expressing F98C-substituted NS4B (43). As AP80978 and PTC725 are
654 dissimilar in chemical structure, it would be informative to compare their three
655 dimensional models to determine if they share similar electrostatic, van der Waals
656 and/or hydrophobic interactions with the target, since both compounds select for
657 mutations encoding resistance at the same amino acid position. In this context, it will be
658 worthwhile to further understand the mechanistic properties of AP80978, particularly
659 whether it antagonizes NS4B by affecting interactions with HCV RNA and/or membrane

660 rearrangements. Finally, the fact that AP80978-resistant replicons remained sensitive
661 to CsA and VX950 suggests that drug combinations may be very effective. In this
662 regard, it would also be important to know whether AP80978 is potentially additive or
663 synergistic when combined with other inhibitors.

664 One unexpected finding from our study was the increased potency of both
665 AP80978 and AP80977 in the virus assay relative to the replicon assay. In the replicon
666 assay, AP80977 was inactive, while in the infection assay using cell culture-produced
667 intergenotypic J6/JFH Jc1 (Con 1 NS4B $_{53\text{-}218}$) virus, its activity was similar to that of the
668 control HCV inhibitors. Similarly, AP80978, which was already active in the replicon
669 assay, had enhanced potency in the infection assay. The greater inhibitory effect on
670 infectious virus vs. replicon is unsurprising, because in addition to creating RNA
671 genome replication foci, NS4B also interacts with other replicase proteins as well as
672 itself during infectious virion production (44-46). Therefore, an inhibitor targeting NS4B
673 may block numerous steps in the context of fully infectious virus replication. Whether
674 this assay or replicon-based assays more closely reflect inhibitor performance in
675 patients with chronic HCV infection is unknown. It is also not known whether the lower
676 potency of the racemate in the replicon assay was due to dilution of the activity of
677 AP80978 by AP80977, or because of an inhibitory effect of AP80977 on the activity of
678 AP80978. Overall, however, the potency of AP80978 against fully-replicating HCV does
679 provide promise for *in vivo* efficacy and future mechanistic studies.

680 Additional studies to determine how this compound affects NS4B function, as well
681 as structure-activity relationship studies to further improve potency and genotype
682 coverage are warranted. Since genotype 1 is the most prominent HCV in North America

683 and Europe, and is also the most difficult to treat, AP80978 provides a scaffold for
684 development of newer inhibitors with pan-genotypic activity. Additionally, we believe that
685 AP80978 will be a useful tool to help dissect the multiple roles of NS4B in the HCV
686 replication cycle, and aid the design of efficacious antiviral therapeutics targeting non-
687 enzymatic viral replicase components.
688

689 **Acknowledgements**

690 This work was funded in part by NIH SBIR awards R43AI049604 and R44AI049604,
691 and is dedicated to the memory of Laura Janet Milton. The authors thank Mayla Hsu for
692 helpful comments and insightful editing.
693

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847 **Figure legends**

848

849 Figure 1. (A) Schematic of genotype 1b replicon (CA32) used in compound screening.

850 (B) Structure of AP89652, with two chiral centers indicated with asterisks. (C) Structure

851 of two *syn* enantiomers present within the AP89652 racemic mixture.

852

853 Figure 2. Clearance of HCV RNA from replicon cells by treatment with AP80978. (A)

854 Schematic of the genotype 1b replicon (Con1/SG-Neo(I)hRluc2aUb) used in the

855 replicon clearance assay. (B) Replicon cells were untreated (●) or treated with

856 AP80978 (15 μM (▼) or 3.75 μM (▲)) or control compounds (2.5 μM CsA (□), 7.5 μM

857 VX950 (O)) in the absence of G418 selection for four passages. Cells were then

858 cultured in selection media in the absence of compound to determine when replicon is

859 present and to allow "rebound". HCV RNA levels from each treatment and time point

860 were quantified and are expressed as $\log_{10}$ change compared with HCV RNA levels in

861 untreated samples at day 0, with all values normalized relative to GAPDH RNA. No

862 rebound was observed in replicon cells that had been cultured in the presence of CsA,

863 VX950 or AP80978 during the clearance phase of the assay. The mean and standard

864 deviation from three replicates are shown.

865

866 Figure 3. Localization of AP80978-selected amino acid substitutions within NS4B. (A)
867 Schematic of the genotype 1b replicon present within Clone A cells that was used to
868 select AP80978-resistant replicons. Location of amino acid substitutions within NS4B
869 (G60 no change (G60nc), F98V, F98L, and S238Y) is indicated. (B) Alignment of
870 NS4B from JFH1 (genotype 2a) and Con1 (genotype 1b), revealing the presence of a
871 leucine residue at amino acid 98 in JFH1 (boxed). Mutation to encode leucine at this
872 position in genotype 1b replicons confers resistance to AP80978.
873
874 Figure 4. Colony formation of wild-type and AP80978-resistant replicons in the
875 presence of AP80978. The top panels show images of a colony formation assay in
876 which cells were electroporated with wild-type or AP80978-resistant (F98V or F98L)
877 replicon RNA, plated in triplicate (1800 cells/well) in the absence (G418 only) or
878 presence (G418 + AP80978) of AP80978 under selection conditions, and surviving
879 colonies stained after 3 weeks. The bottom panels show graphic representations of
880 colony formation assays in which 225, 450, 900, and 1800 electroporated cells were
881 plated and cultured in the absence (G418 only) or presence (G418 + AP80978) of
882 AP80978. The mean and standard deviation from three replicates are shown.
883
884 Figure 5. Screening AP80978 against genotype (GT) 2a replicons containing
885 intergenotypic GT2a/1b NS4B. Schematics of intergenotypic NS4B regions within the
886 genotype 2a replicon are illustrated, with dark gray boxes representing genotype 1b and
887 gray boxes representing genotype 2a regions. The AP80978 EC_{50} values are indicated

888 to the right of each replicon construct. The replicon containing Con1 amino acids 7
889 through 254 (i.e. NS4B Con1 $_{7\text{-}254}$) failed to produce cells that survived G418 selection.
890
891 Figure 6. Effect of AP80978 and control compounds on virus replication. (A) Schematic
892 of the AP80978-sensitive virus J6/JFH Jc1 (Con 1 NS4B $_{53\text{-}218}$). (B) Huh-7.5 cells were
893 infected with J6/JFH Jc1 in the presence of the indicated concentrations of AP80978 or
894 its structural analogs. After 48 hours, HCV RNA levels from each treatment were
895 quantified by RT-qPCR and are expressed as $\log_{10}$ change compared with HCV RNA
896 levels in untreated samples. (C) Huh-7.5 cells were infected with J6/JFH Jc1 in the
897 presence of the indicated concentrations of the control compounds 2'C-meA, VX950, or
898 CsA. Experimental procedure and data analysis is the same as in (B). (D) Huh-7.5 cells
899 were infected with J6/JFH Jc1 (Con 1 NS4B $_{53\text{-}218}$) in the presence of the indicated
900 concentrations of AP80978 or its structural analogs. Experimental procedure and data
901 analysis are the same as in (B). (E) Huh-7.5 cells were infected with J6/JFH Jc1 (Con 1
902 NS4B $_{53\text{-}218}$) in the presence of the indicated concentrations of control compounds 2'C-
903 meA, VX950, or CsA. Experimental procedure and data analysis is the same as in (B).
904
905 **Tables**
906
907 Table 1. Activity of AP89652 isomers in replicon assays

Compound No.	Description	EC_{50} (μM*)	CC_{50} (μM)	SI
AP89652	Racemate	1.78	>100	>56
AP80977	Enantiomer 1	>25	>100	NA
AP80978	Enantiomer 2	0.63	>100	>158

908
909
910
911 Table 2. Activity of AP80978 against genotype 1a, 1b, and 2a replicons

Replicon	EC_{50} (μM)
Genotype 1a	0.62
Genotype 1b	1.76

Genotype 2a	>25

Table 3. Activity of AP80978 against other viruses

	Replicon EC_{50} (μM)					Mini-genome EC_{50} (μM)	
	Dengue	YFV	Sindbis	RSV	WNV	Ebola	FluA
AP80978	>20	>20	>20	>20	>20	>20	>20
Ribavirin	2.0	3.7	2.1	3.7	Nd	10.1	20.0
CsA	12.0	8.6	>20	2.7	5.5	5.4	10.2
2'C-meA	4.5	2.9	5.3	>20	4.0	>20	>20

YFV=yellow fever virus, RSV=respiratory syncytial virus, WNV=West Nile virus, FluA=influenza A virus

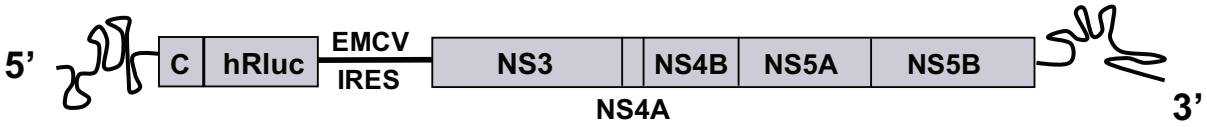
Table 4. Cytotoxicity of AP80978 in different cell types

Cell line	Tissue of origin	CC_{50} (μM)
HepG2	Liver	>100
Caco-2	Colorectal	>100
MRC-5	Lung	>100
Jurkat	T-lymphocyte	>100

Table 5. Activity of AP80978 against replicons containing amino acid substitutions. G60nc indicates a silent mutation at the G60 codon.

Replicon	EC_{50} (μM)
Wild-type	0.29
G60nc	0.24
F98V	>20
F98L	>20
S238Y	0.41

A



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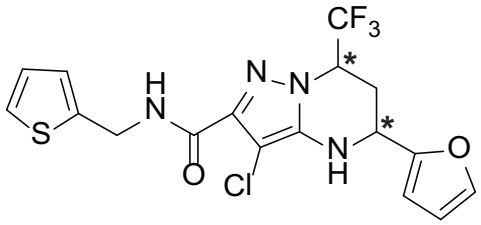

AP89652

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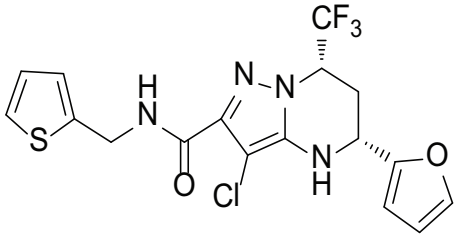

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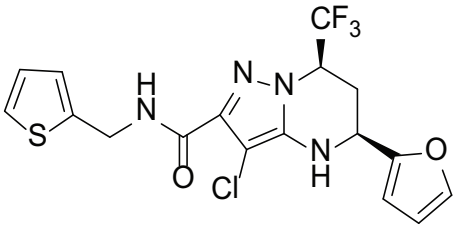

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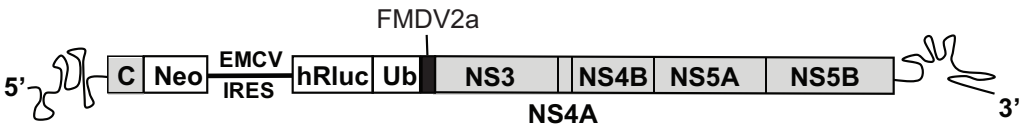

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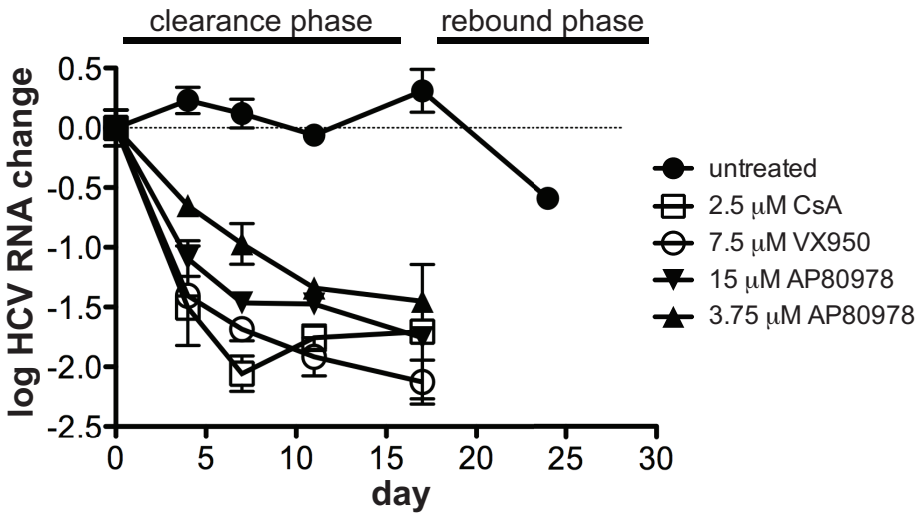

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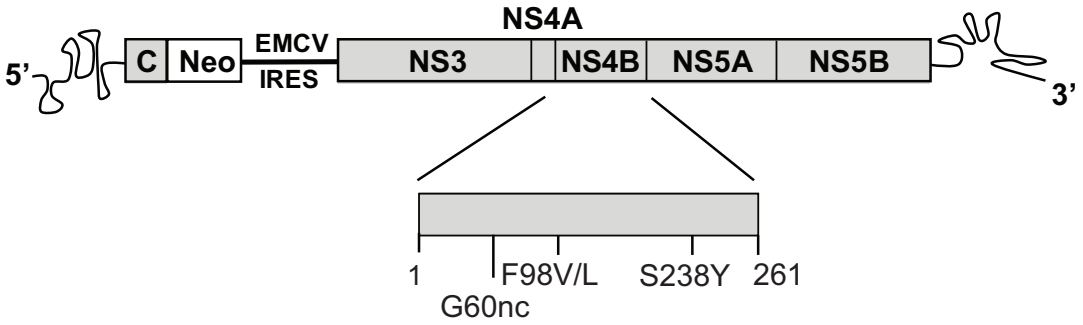

B

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Con1 ASHLPYIEQGMQLAEQFKQKAIGLLQTATKQAEAAAPVVESKWRTLEAFWAKHMWNFISGIQYLAGLSTL
JFH1 --RAAL--E-QRI--ML-S-IQ----Q-S---QDIQ-AMQAS-PKV-Q---R------------------

Con1 PGNPAIASLMAFTASITSPLTTQHTLLFNILGGWVAAQLAPPSAASAFVGAGIAGAAVGSIGLGKVLVDI
JFH1 -----V--M---S-AL----S-ST---L--M---L-S-I---AG-TG--VS-LV----------------

Con1 LAGYGAGVAGALVAFKVMSGEMPSTEDLVNLLPAILSPGALVVGVVCAAILRRHVGPGEGAVQWMNRLIA
JFH1 -------IS-------I----K--M--VI----G-----------I-----------------------

Con1 FASRGNHVSPTHYVPESDAAARVTQILSSLTITQLLKRLHQWINEDCSTPC
JFH1 --------A-----T----SQ----L-G-----S--R---N--T---PI--
```