Supporting Information

Imidazo[1,2-*a*]pyridines That Directly Interact with Hepatitis C Nonstructural Protein 4B: Preclinical Characterization

Methods Described:

I. **References Chemical Syntheses and Purities of Final Compounds**
II. **Chemical Syntheses of Novel Compounds**
III. **Chemical Syntheses of Radioligands**
IV. **NS4B Binding Assay & NS4B Protein Purification**
V. **Replicon Assay**
VI. **In vivo/in vitro DMPK Assays**
VII. **Supplementary Potency/SAR Data Tables**

I. Chemical Synthesis and purities of Final Compounds 1a, 1b and 5-21

Syntheses of **1b**, and **5**-**21**, including all novel amines, have been previously described. References to the specific patent describing synthetic protocols and analytical characterization at the time of synthesis (LCMS, NMR, HPLC Purity) are included in Table SI-1 below and are consistent with the structures as presented with purities > 90%. Protocols for syntheses of novel compounds **1a**, **1c**, and **1d** are described in Section II and III.

Table SI-1

Cmpd.	Ref.	Cmpd.	Ref.	Cmpd.	Ref.
1b	WO2010091411	**10**	WO2010091411	**16**	WO2011041713
5	WO2009023179	**11**	WO2010091411	**17**	WO2009023179
6	WO2009023179	**12**	WO2010091411	**18**	WO2011041713
7	WO2009023179	**13**	WO2010091411	**19**	WO2011041713
8	WO2009023179	**14**	WO2010091411	**20**	WO2011041713
9	WO20100204265	**15**	WO2011041713	**21**	WO2011041713

(6a) Banka, Anna; Catalano, John G.; Chong, Pek Yoke; Fang, Jing; Garrido, Dulce Maria; Peat, Andrew James; Price, Daniel J.; Shotwell, John Brad; Tai, Vincent; Zhang, Huichang . Preparation of piperazinyl antiviral agents. WO 2011041713 (2011). (6b) Baskaran, Subramanian; Maung, Jack; Neitzel, Martin Leon; Rai, Roopa; Slododov, Irina; Tai, Vincent W-F. Preparation of imidazopyridine derivatives for treating viral infections. US 20100204265 (2010). (6c) Baskaran, Subramanian; Maung, Jack; Neitzel, Martin; Rai, Roopa; Slobodov, Irina; Tai, Vincent. Preparation of imidazopyridine derivatives for treating viral infections . WO 2010091409 (2010). (6d) Banka, Anna; Baskaran, Subramanian; Catalano, John; Chong, Pek; Dickson, Hamilton; Fang, Jing; Maung, Jack; Neitzel, Martin Leon; Peat, Andy; Price, Daniel; et. al. Preparation of piperidinyl cyclic amido compounds as HCV antiviral agents . WO 2010091411 (2010). (6e) Schmitz, Franz Ulrich; Tai, Vincent; Rai, Roopa; Roberts, Christopher; Abadi, Ali Dehghani Mohammad; Baskaran, Subramanian; Slobodov, Irina; Maung, Jack; Neitzel, Martin Leon. Preparation of substituted imidazopyridine derivatives and analogs for use as antiviral agents. WO 2009023179 (2009).

Immediate Processing Quality Control Protocol (IPQC)

All compounds underwent IPQC to confirm identity and determine relative purity immediately before processing in the replicon or NS4B binding assays (described below). Analysis is by UPLC-MS with UV diode array detection to determine purity and MS used to confirm molecular weight. A Waters Acquity UPLC system comprising Binary Solvent manager, Sample Manager, PDA Detector, Waters ZQ or SQD mass spectrometer, Waters Acquity Evaporative Light Scattering Detector or Polymer Laboratories

Evaporative Light Scattering Detector were employed. Mobile phases: acetonitrile + 0.1% formic acid; water + 0.1% formic acid. Wash solutions: strong wash 100% acetonitrile + 0.1% formic acid; weak wash 50:50 acetonitrile:water + 0.1% formic acid.

Table SI-2: IPQC Purities for Final Compounds 1b and 5-21 at time of assay

Cmpd.	Purity (%)	Cmpd.	Purity (%)	Cmpd.	Purity (%)
1b	100	**10**	96	**16**	100
5	100	**11**	85	**17**	100
6	100	**12**	98-100*	**18**	100
7	99	**13**	96	**19**	100
8	99	**14**	100	**20**	100
9	91	**15**	100	**21**	99

*Data for compound **12** represents average of 8 distinct lots of compound, all with purity between 98-100%

II. Chemical Syntheses of Novel Compound 1a

Compound 1a. (3-bromo-6-(1H-pyrazol-4-yl)-8-(trifluoromethyl)imidazo[1,2-a]pyridin-2-yl)(3-(thiophen-2-yl)pyrrolidin-1-yl)methanone. To a suspension of 3-bromo-6-(1H-pyrazol-4-yl)-8-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxylic acid (281 mg, 0.75 mmol, see reference 6e, WO2009023179), 3-(2-thienyl)pyrrolidine (231 mg, 0.864 mmol), N,N-diisopropylethylamine (655 µl, 3.75 mmol) in N,N-Dimethylformamide (DMF) (3750 µl) was added PyBrop (367 mg, 0.788 mmol) at RT. After 1 hour, the mixture was diluted with EtOAc (100 mL) and washed with saturated aqueous $NaHCO_3$ (40 mL), then brine (40 mL), dried (Na2SO4), filtered and concentrated to give 577 mg of crude material. The crude residue was purified by reverse phase hplc, concentrated, suspended in ACN/water and lyophilized to give 3-bromo-6-(1H-pyrazol-4-yl)-2-{[3-(2-thienyl)-1-pyrrolidinyl]carbonyl}-8-(trifluoromethyl)imidazo[1,2-a]pyridine (130 mg, 0.255 mmol, 34.0 % yield) as a white solid. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 1.90 - 2.15 (m, 1 H), 2.26 - 2.43 (m, 1 H), 3.45 - 4.13 (m, 4.5 H), 4.13 - 4.31 (m, 0.5 H), 6.89 - 7.11 (m, 2 H), 7.40 (ddd, *J*=12.2, 4.8, 1.4 Hz, 1 H), 8.12 - 8.71 (m, 3 H), 8.76 (d, *J*=5.5 Hz, 1 H), 13.17 (br. s., 1 H). MS(ESI): 509.99 (MH+).

III. Chemical Syntheses of Radioligands 1c and 1d.

Compound 1c: [^{3}H]-[3-Bromo-6-(1H-pyrazol-4-yl)-8-trifluoromethyl-imidazo[1,2-a]pyridin-2-yl]-(3-thiophen-2-yl-pyrrolidin-1-yl)-methanone

Step 1: [^{3}H]-3-Thiophen-2-yl-pyrrolidine (1c). *Synthesis of tritium-labeled **1c** was carried out at ViTrax, Inc., Placentia, CA.* 10% Palladium on carbon (3.5 mg) was added to a Tritium reaction vessel followed by a solution of 3-thiophen-2-yl-2,5-dihydro-pyrrole-1-carboxylic acid tert-butyl ester (3.5 mg, 0.014 mmol) in DMF (0.2 mL). The vessel was attached to the Tritium line and pressurized to 0.5 atm with Tritium gas. The solution was stirred overnight at room temperature. The crude material was then filtered through Celite and washed with MeOH (4 x 5 mL). The solvent was concentrated followed by co-evaporation with MeOH (3 x 20 mL). The crude material was purified by preparative reverse-phase HPLC to yield 105 mCi of [^{3}H]-3-thiophen-2-yl-2,5-dihydro-pyrrole-1-carboxylic acid tert-butyl ester with >99% purity. To this material was added dichloromethane (1.4 mL) and trifluoroacetic acid (0.6 mL) in a 10 mL conical flask. The solution was stirred at room temperature for 30 min. The solvent was removed and co-evaporated with toluene (2 x 2 mL) to give [^{3}H]-3-thiophen-2-yl-pyrrolidine as the TFA salt which was used in the next step without further purification.

Step 2: [^{3}H]-[3-Bromo-6-(1H-pyrazol-4-yl)-8-trifluoromethyl-imidazo[1,2-a]pyridin-2-yl]-(3-thiophen-2-yl-pyrrolidin-1-yl)-methanone. [^{3}H]-3-thiophen-2-yl-pyrrolidine TFA salt (105 mCi) was concentrated to dryness in a 10 mL conical flask, then DMF (0.3 mL), 3-bromo-6-(1H-pyrazol-4-yl)-8-trifluoromethyl-imidazo[1,2-a]pyridine-2-carboxylic acid (from step 4, 4 mg, 0.011 mmol), N,N-diisopropylethylamine (0.01 mL, 0.057 mmol), and HATU (4 mg, 0.011 mmol) were added. The solution was stirred at room temperature for 1 hour. The mixture was diluted with EtOAc (20 mL) and washed with saturated aqueous $NaHCO_3$ (10 mL), then brine (10 mL). The organic phase was dried (Na_2SO_4), filtered and concentrated. The crude material was purified by reverse phase HPLC to yield 14 mCi of [^{3}H]-[3-bromo-6-(1H-pyrazol-4-yl)-8-trifluoromethyl-imidazo[1,2-a]pyridin-2-yl]-(3-thiophen-2-yl-pyrrolidin-1-yl)-methanone with >99% purity.

Compound 1d: [^{3}H]-3-(1-(3-chloro-6-isopropyl-8-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carbonyl)piperidin-4-yl)oxazolidin-2-one

*Tritium-labeled **1d** was synthesized at RC TRITEC AG, Switzerland using similar protocol as above*. Stock solution of phenyl sulfide was prepared as 10 μL phenyl sulfide dissolved in 1 mL MeOH.

A suspension of 3-(1-{[3-chloro-6-(1-methylethenyl)-8-(trifluoromethyl)imidazo[1,2-a]pyridin-2-yl]carbonyl}-4-piperidinyl)-1,3-oxazolidin-2-one (20 mg, 0.044 mmol, Compound 106 in WO2010091411A1), 10% palladium on carbon (5 mg, 4.70 µmol, Engelhard code C3645 from Aldrich cat#520888), phenyl sulfide (72.9 µL, 4.38 µmol) was stirred under hydrogen (1 atm) in methanol (2 mL). After 2.25 hours, the catalyst was filtered and solvent removed under reduced pressure. This was loaded on preparative TLC plate and eluted with EtOAc to give 3-(1-{[3-chloro-6-(1-methylethyl)-8-(trifluoromethyl)imidazo[1,2-a]pyridin-2-yl]carbonyl}-4-piperidinyl)-1,3-oxazolidin-2-one (18.5 mg, 0.040 mmol, 92 % yield) as a white powder (after lyophilization). ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 1.29 (d, J=6.9 Hz, 6 H), 1.54 - 1.72 (m, 3 H), 1.78 (br. s., 1 H), 2.89 (td, J=12.8, 2.3 Hz, 1 H), 3.11 - 3.25 (m, 2 H), 3.44 - 3.61 (m, 2 H), 3.72 - 3.89 (m, 1 H), 4.12 - 4.22 (m, 1 H), 4.25 (t, J=8.0 Hz, 2 H), 4.58 (br. s., 1 H), 7.90 (s, 1 H), 8.42 (s, 1 H); MS(ESI) m/z: 459.4 (MH^+)

IV. NS4B Binding Assay & NS4B Protein Purification

HCV1b NS4B (1-261) (in pFastBac1) was expressed as a C-terminal 6xHis fusion in baculovirus infected SF9 (super9)cells. Purification was accomplished by IMAC (immobilized metal affinity chromatography) followed by ion exchange and size exclusion chromatography (SEC).

Briefly, frozen cells were re-suspended (~5-10ml per gram of cells) in lysis buffer (25mM Hepes pH=7.5, 200mM NaCl and 1% DDM (n-Dodecyl-β-D maltopyranoside) and lysed via two passes through a Rannie APV homogenizer (7-8 K PSI). The lysate was subjected to centrifugation (20,000G x 45 min) and the resultant supernatant filtered with cheese cloth and 0.2 micron filter (GE Healthcare). The imidazole concentration of the sample was adjusted to ~40mM before incubation in batch mode with Ni sepharose 6 Fast Flow resin (GE Healthcare) equilibrated with 25 mM Hepes pH=7.5, 200mM NaCl, 40 mM Imidazole, 0.03% DDM. Following incubation, the sample was loaded into a XK-26 column, washed with the same buffer and eluted with a two column volume (CV) linear gradient (40-500mM imidazole). The peak fractions were pooled, diluted 8 fold with 25mM Hepes 7.5, .03% DDM, 2mM DTT and

subjected to ion exchange over Poros HS and Poros HQ resins (Life Technologies) (XK26-6cm columns) connected in tandem and equilibrated with 25mM Hepes pH=7.5, .03% DDM, 25mM NaCl and 2mM DTT. The flow through containing NS4B was then loaded onto 2 x 5ml HisTrap columns (GE Healthcare) equilibrated with affinity buffer. NS4B was eluted with a 2 CV imidazole gradient(40-500mm Imidazole), pooled and concentrated with a Centriprep 10 concentrator to a volume of ~6mls. The sample was subjected to SEC by applying to a pre-equilibrated (25mM Hepes pH= 8.0, 200mM NaCl, .03% DDM, 5mM DTT) XK-26, 60 cm column packed with Superdex 75 resin (GE Healthcare). Peak fractions were pooled for use in subsequent assays.

All NS4B binding and ligand displacement experiments were performed in low volume, white 384-well plates containing either 0.1 µL of test compound in DMSO or DMSO only in a final assay volume of 10 µL. The assay buffer consisted of 25 mM MOPS pH 7.5, 0.1 mg/mL BSA, 50 mM KCl, 1 mM CHAPS, and 50 µM n-Dodecyl β-D-Maltopyranoside (DDM). Unless noted, final assay concentrations were 125 nM purified C-His NS4B, 8 nM biotinylated anti-His antibody, 20 nM **1d** (RC TRITEC Ltd., Teufen, Switzerland), and 0.5 mg/mL streptavidin coupled polystyrene LEADseeker imaging beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). All plates were read on a ViewLux ultraHTS Microplate Imager (PerkinElmer, Inc., Waltham, MA).

K_d determination

C-His NS4B was serial diluted in assay buffer and added to a low volume, white 384-well plate containing either 0.1 µL DMSO or 0.1 µL of 1 mM **1b** to determine the background signal. A streptavidin coupled polystyrene LEADseeker imaging bead, biotinylated anti-HIS antibody and **1d** mixture was added to the plate containing the serial diluted C-His NS4B. The plate was allowed to equilibrate overnight and was then read on a ViewLux ultraHTS Microplate Imager. Background signal was subtracted and data were fit to equation 1 by nonlinear regression using SigmaPlot 2002 (Systat Software, Inc., San Jose, CA).

$$[AR] = \frac{B_{max} \times [A]}{[A] + K_d} \qquad \text{(equation 1)}$$

[A] equals the concentration of **1d**, [R] equals the concentration of C-His NS4B, [AR] equals the concentration of the **1d** – C-His NS4B complex, B_{max} equals the concentration of complex AR at saturating concentration of A, and K_d equals the dissociation constant of complex AR.

pIC_{50} determination

In preparation for the assay, test compounds were serially diluted in DMSO from a starting concentration of 5 mM and plated (0.1 µL) in low volume, white 384-well plates. Positive (100% inhibition) control wells contained 0.1 µL of 5 mM **1a** in DMSO, and negative (0% inhibition) control wells contained 0.1 µL of DMSO only. 10 µL of assay buffer containing 125 nM purified C-His NS4B, 8 nM biotinylated anti-His antibody, 20 nM **1d**, and 0.5 mg/mL Streptavidin coupled polystyrene LEADseeker imaging beads were dispensed to each well. The plates were allowed to equilibrate overnight before being read on the ViewLux ultraHTS Microplate Imager.

The data for dose responses were plotted as % Inhibition versus compound concentration following normalization using the formula 100-100*(U-C2)/(C1-C2), where U was the unknown value, C1 was the average of the negative (0% Inhibition) control wells and C2 was the average of the positive (100%

Inhibition) control wells. Curve fitting was performed with ActivityBase XE (**ID Business Solutions Ltd., Guildford, UK) using** equation 2.

$$y = A + \left(\frac{B-A}{1+\left(\frac{10^{X}}{10^{C}}\right)^{D}}\right) \qquad \text{(equation 2)}$$

A was the minimum response, B was the maximum response, C was the log(IC_{50}) and D was the Hill slope. The results for each test compound were recorded as pIC_{50} values (-C in the above equation) from which IC_{50} values were calculated.

V. HCV Replicon Assay

50µL of a 5mM stock solution in DMSO of each test compound was transferred into in the first or thirteenth column of a 384 well, V-bottom microplate, to give 200 times the top concentration of the required dilution series. Aliquots of 16.67 µL were added to each well of the remaining rows containing 33.33 µL of DMSO giving a 1:3 dilution series over ten points. Columns 11 and 12 contained DMSO only for the positive and negative control, respectively. 10µL of each well were transferred into the assay plate.

Suspensions were prepared from cultures of Huh-7 cells stably transfected with sub-genomic HCV NS3-NS5B replicons of either genotype 1b (the ET subline described by Pietschmann,T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D. & Bartenschlager, R., *Journal of Virology*, 2002, **76**, 4008-4021), genotype 1a (subline 1.19 constructed in-house) or genotype 1b H94N (constructed in-house), or genotype 1b V105M (constructed in-house) linked to a firefly luciferase reporter gene. Monolayers nearing confluency were stripped from growth flasks with versene-trypsin solution and the cells re-suspended in assay medium comprising DMEM. 50µL of suspension containing either 15,000 cells (genotype 1b luciferase replicon and genotype 1a luciferase replicon) were added to all wells of a 384 well plate (NUNC, #164610), except medium controls in column 12 of the assay plate. The cell suspension added to 0.5 µL of compound and the plate was incubated for 48 hours at 37°C in a 5% CO_2 atmosphere.

For toxicity the cells in one plate were treated with Cell Titer Glo (Promega, #G7573). A solution of Cell Titer Glo was prepared according to the manufacturer's instructions, and 10 µL added to each well. The plate was then read for luminescence on a ViewLux.

For potency a solution of Steady Glo (Promega, #E2550) was prepared according to the manufacturer's instructions and 10-20 µL added to each well. After a ten minute incubation the plate was then read for luminescence on a ViewLux.

Certain mutations including NS3/A156T, NS5A/L31V, and NS5B/M414T were analyzed by transient transfection of HCV replicon RNA. For this work, replicon-bearing DNA plasmids were digested with the restriction enzymes AseI/ScaI and purified with a QiaQuick PCR purification kit (Qiagen, #28104). RNA was prepared by in vitro transcription (Promega, #P1320) and purified with spin columns (Qiagen, #74104). Cells were electroporated with 5 ug of RNA using a GenePulser system (Bio-Rad), plated in the presence of compound, and incubated for 72 hours at 37°C in a 5% CO_2 atmosphere. Potency was determined by the addition of 25-50 µL Bright glo Reagent (Promega, #E2650). A solution of Bright Glo was prepared according to the manufacturer's instructions, and 100 µL added to each well. The plate was then read for luminescence on an Envision Plate Reader (Perkin Elmer).

Data Analysis

Toxicity: The luminescence values from duplicate wells were averaged and expressed as a percentage of the mean absorbance of compound free control wells to determine comparative cell viability. Compound cytotoxicity was expressed either as the lowest concentration at which a significant reduction in viability was observed or a 50% toxic concentration ($CCID_{50}$) was determined by plotting percentage cytotoxicity against compound concentration using ActivityBase (IDBS Software) or Bioassay with curve fitting done using XE runner.

Potency: The luminescence values from all compound-free wells containing cells were averaged to obtain a positive control value. The mean luminescence value from the compound-free wells that had received no cells was used to provide the negative (background) control value. The readings from the wells at each compound concentration were taken and after the subtraction of the mean background from all values, were expressed as a percentage of the positive control signal. The quantifiable and specific reduction of luciferase signal in the presence of a drug is a direct measure of replicon inhibition. ActivityBase (IDBS Software) with XE Runner for curve fitting was used to plot the curve of percentage inhibition against compound concentration and derive the 50% inhibitory concentration (IC_{50}) for the compound.

EC_{50} Calculation (used for NS4B binding and HCV Replicon assays)

For each assay, an analysis of variance (ANOVA) model was fit to link the PXC_{50} values to the compounds. Compounds with PXC_{50} values < 4.3 or > 9.07 were excluded from the ANOVA modeling. The ANOVA model was used to estimate a pooled standard deviation. From this, 95% confidence intervals were computed for each compound's PXC_{50} mean. For compounds with PXC_{50} values < 4.3, the estimated mean and confidence interval have a "$<$" modifier. For compounds with PXC_{50} values > 9.07, the estimated mean and confidence interval have a "$>$" modifier. XC_{50} means and confidence intervals were computed by exponentiating those created for PXC_{50}s.

VI. In Vivo/In Vitro DMPK Profiling

All studies were conducted after review by the Institutional Animal Care and Use Committee at GSK (or at the institution where the work was performed, if not at GSK) and in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals.

Pharmacokinetics

Male Sprague-Dawley rats (Charles River Labs, Raleigh, NC) were dosed intravenously at 1 mg/kg and orally at doses ranging from 5 mg/kg to 1000 mg/kg for pharmacokinetic evaluations. Male Beagle Dogs were dosed intravenously at 1 mg/kg and orally at 5 mg/kg for pharmacokinetic evaluations. Blood samples were collected at various time points into EDTA tubes, and centrifuged to form plasma. Plasma samples were stored at -70°C until sample analysis. Samples were extracted by protein precipitation and analyzed by LC/MS/MS.

Reactive metabolite

Test compounds (10 μM) were incubated for 30 minutes with rat and human liver microsomes in the presence of glutathione as a trapping agent to assess the potential for reactive metabolites to occur. Reactions were quenched with the addition of 2% trifluoroacetic acid (TFA) and cleaned up for sample

analysis by solid phase extraction (SPE). The presence of a glutathione adduct was measured by LC/MS/MS as a neutral loss of 129 mass units from glutathione.

P450 inhibition

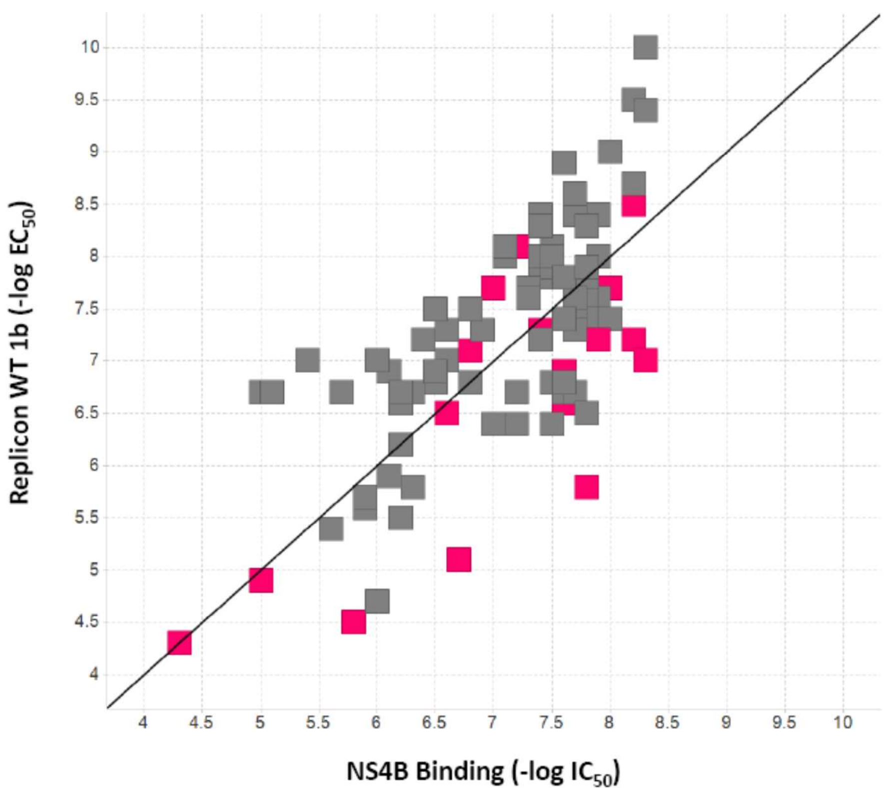
Test compounds were incubated with pooled, mixed-gender human liver microsomes to assess direct inhibition of CYP1A2, 2C9, 2C19, 2D6, and 3A4. In a 96 well plate, a 10 mM DMSO stock of test compound was diluted 1:3 for 7 concentrations ranging from 33 μM to 33 nM. Two microsomal incubations containing 0.1 mg/ml microsomal protein were incubated the probe cocktails (either 3 CYP3A4 substrates or substrates of CYP1A2, 2C9, 2C19, and 2D6) along with dilutions of test compound for 7 minutes (CYP3A4 cocktail) or 10 minutes (CYP1A2, 2C9, 2C19, and 2D6 cocktail). Reactions were quenched with an equal volume of acetonitrile:methanol. The amount of the metabolites formed from each substrate in the probe cocktail was quantified by LC/MS/MS analysis. Enzyme activity in the presence of test compound was normalized for the enzyme activity in the absence of test compounds and expressed as percent control activity. Percent control activity versus concentrations plots were fitted and IC_{50} curves were generated using XLFit software (IDBS, Guildford, UK).

VII. Supplementary Potency/SAR Data Tables

Table SI-3. Replicon and NS4B Binding for 21 and Related Isomers

	EC_{50} (nM)		IC_{50} (nM)
Analog	**WT 1a**	**WT 1b**	**4B Binding**
21	0.81	5.4	60
SI-1	79	79	320
SI-2	6.3	1.3	7.9

Table SI-4: Replicon vs. NS4B binding Correlation*



[*]Compounds described in text (pink) in addition to compounds for which full curve data exists described in patents from Reference 6 (grey), ceiling for NS4B binding assay is pIC_{50} = 8.4. NOTE: pIC_{50} = - log IC_{50}