Supporting Information

Structure-Property Basis for Solving Transporter-Mediated Efflux and Pan-Genotypic Inhibition in HCV NS5B Inhibitors

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Table S1-1. Inhibitory Potency towards HCV Replicons (EC_{50}) and NS5B Enzyme (IC_{50}) Representing Genotypes 1 to 6 for Compound 12

Replicon EC_{50} (nM)[a]				**NS5B Enzyme (nM)**[a]			
1a	1b	1bC316N	2a	3a	4a	5a	6a
2.3±1.7	1.2±0.1	3.6±0.4	12±2.3	6.2[b]	1.2±0.7	8.4±2.9	2.5±0.2

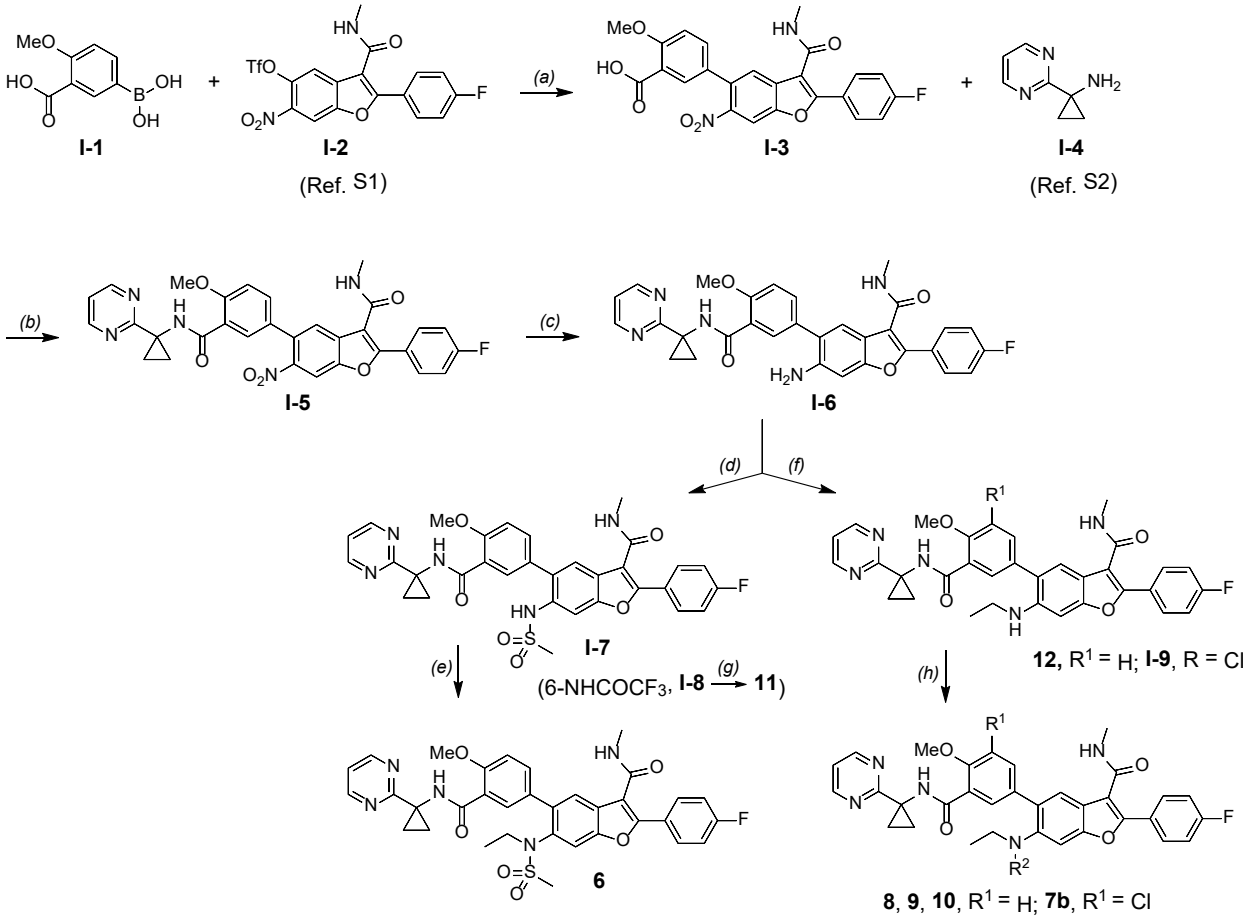
[a]Data are average values of n ≥2 independent experiments; [b]Data from a single experiment performed in duplicate

Compound Synthesis and Characterizations

The synthesis of compounds **2**, **3** and **4** can be found in ref. S1. The synthesis of compound **6** (and analogous for **5**) starting from intermediate **I-2** is shown in **Scheme S1**. Intermediates **I-2** and **I-4** were prepared according to refs. S1 and S2, respectively. The key 6-aminobenzofuran intermediate **I-6** was first sulfonylated to **I-7** and then alkylated to obtain compound **6**. Compound **11** was obtained from the trifluoroacetamide **I-8**, which was isolated as a side product if **I-7** was purified under reverse phase preparative HPLC using TFA as a buffer. Reductive amination of **I-6** provided compound **12**, which served as a penultimate intermediate for the synthesis of compounds **8**, **9** and **10**. Intermediate **I-9** was prepared in a similar manner to **12** and sulfonylated to provide **7b**, synthesis representative of those for **7a-d**. All final compounds were determined to

be ≥95% purity as determined by liquid chromatography methodologies. LC/MS and analytical HPLC conditions and purity are as indicated for each compound. R_t denotes retention time in minutes. Proton coupling constants for ^{1}H NMR spectra were reported in hertz (Hz) and chemical shifts δ in ppm. All reagents were purchased from commercial suppliers and used without purification. Anhydrous reactions were performed under a nitrogen atmosphere using anhydrous solvents from commercial sources.

Scheme S1. Synthesis of Compounds 6, 7b and 8-12



Reagents and conditions: (a) $Pd(PPh_3)_4$, Cs_2CO_3, 1:5 H_2O/1,4-dioxane, 90 °C, 4 hrs, 84%; (b) HATU, ^{i}Pr$_2$NEt, r.t., 3 hrs, 96%; (c) Zn, AcOH, MeOH, 50 °C then r.t., 91%; (d) (1) $MeSO_2Cl$,

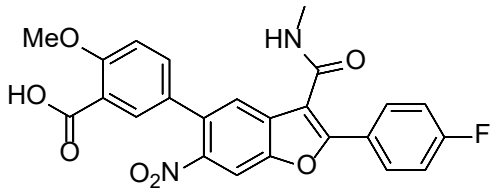
$^{i}Pr_2NEt$, $ClCH_2CH_2Cl$, overnight, r.t., (2) Cs_2CO_3, 1:5 H_2O/1,4-dioxane, 80 °C, 35 min., 38% (two steps); (e) EtI, Cs_2CO_3, DMF, r.t., 93%; (f) MeCHO, $NaCN(BH_3)$, 4Å ms, MeOH, r.t., 2 days, 46%; (g) EtI, Cs_2CO_3, DMF, r.t., 31%; (h) **8** (R^2 = MeO_2C): MeO_2CCl, Cs_2CO_3, DMF, 80 °C, overnight, 29%; **9** (R^2 = Me_2NCO): Me_2NCOCl, pyridine, r.t., 2 days, 42%; **10** (R^2 = MeCO): Ac_2O, pyridine, r.t., overnight, 55%; **7b** (R^2 = $MeSO_2$): $MeSO_2Cl$, DMAP, pyridine, r.t., 4 hrs, 24%.

References:

(S1) Yeung, K.-S.; Parcella, K. E.; Bender, J. A.; Beno, B. R.; Grant-Young, K. A.; Han, Y.; Hewawasam, P.; Kadow, J. F.; Nickel, A. Compounds for the treatment of hepatitis C. World Patent Application WO 2010/030592 A1, March 18, 2010.

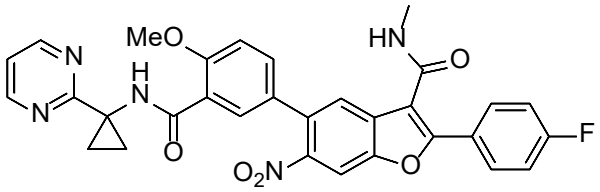
(S2) Corsi, M.; Faiferman, I.; Merlo-Pich, E.; Ratti, E.; Wren, P. B. 2-Amino-7,8-dihydropyrido[2,3-*d*] pyrimidin-7-one derivatives as CSBP/RK/p38 kinase inhibitors and their preparation, pharmaceutical compositions and use in the treatment of diseases. World Patent Application WO 2007/147103 A2, December 21, 2007.

5-(2-(4-Fluorophenyl)-3-(methylcarbamoyl)-6-nitrobenzofuran-5-yl)-2-methoxybenzoic acid (**I-3**)



To a sealed tube was added 2-(4-fluorophenyl)-3-(methylcarbamoyl)-6-nitrobenzofuran-5-yl trifluoromethanesulfonate **I-2** (4.23 g, 9.15 mmol) in 1,4-dioxane (120 mL) along with water (24 mL), cesium carbonate (8.94 g, 27.4 mmol), 5-borono-2-methoxybenzoic acid **I-1** (2.331 g, 11.89 mmol) and palladium tetrakis(triphenylphosphine) (1.057 g, 0.960 mmol). The resulting yellow solution was degassed and then heated at 90 °C for 4 hrs. The black mixture was diluted with 1N HCl (85 mL) ans then with then ice cold water (125 mL). The greenish black solid obtained were filtered, and washed with water (2 x 20 mL). The semi-solid was triturated with ether (4 x 40 mL), and dried to give intermediate **I-3** as a brown solid (3.58 g, 84% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.80 (s, 1 H), 8.62 (m, 1 H), 8.50 (s, 1 H), 8.06 (dd, J = 8.53, 5.40, 2 H), 7.71 (s, 1 H), 7.67 (d, J = 2.80, 1 H), 7.52 (dd, J = 8.53, 2.26, 1 H), 7.46 (t, J = 8.91, 2 H), 7.25 (d, J = 8.78, 1 H), 3.90 (s, 3 H), 2.86 (d, J = 4.52, 3 H). LC/MS method: Phenomenex Luna 3 μm C18, 2 x 50 mm column, with a gradient of 0-100%B (B = 90% MeCN/0.1% TFA/10% H_2O, A = 90% H_2O/0.1% TFA/10% MeCN), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) m/z = 465.2 $(M + H)^+$, calculated for $C_{24}H_{18}FN_2O_7$ = 465.1; R_t = 2.848 min. (UV at 220 nm).

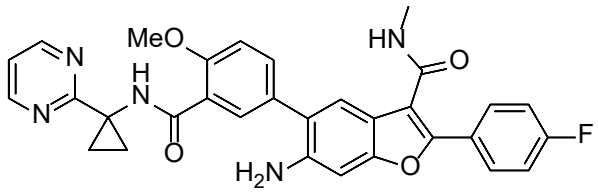
2-(4-Fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methyl-6-nitrobenzofuran-3-carboxamide (**I-5**)



To a 250 mL round bottom flask (RBF) was added 5-(2-(4-fluorophenyl)-3-(methylcarbamoyl)-6-nitrobenzofuran-5-yl)-2-methoxybenzoic acid **I-3** (3.58 g, 7.71 mmol), DMF (100 mL), *N*-ethyl-*N*-isopropylpropan-2-amine (5.37 mL, 30.8 mmol), 1-(pyrimidin-2-yl)cyclopropanamine.2HCl **I-**

4 (2.246 g, 10.79 mmol), and 2-(3*H*-[1,2,3]triazolo[4,5-b]pyridin-3-yl)-1,1,3,3-tetramethylisouroniumhexafluorophosphate(V) (HATU) (5.86 g, 15.42 mmol). The mixture was stirred at r.t. for 3 hrs. LCMS analysis showed completion of the reaction. The mixture was cooled to 0 °C, diluted with 1M HCl (100 mL) and water (200 mL). The yellow solid was collected, washed with water and ether, and dried to give intermediate **I-5** (4.29 g, 96% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 1 H), 8.71 (d, J = 4.77, 2 H), 8.65 (m, 1 H), 8.51 (s, 1 H), 8.06 (dd, J = 8.78, 5.52, 2 H), 7.88 (d, J = 2.26, 1 H), 7.70 (s, 1 H), 7.53 (dd, J = 8.53, 2.26, 1 H), 7.46 (t, J = 8.91, 2 H), 7.34-7.28 (m, 2 H), 4.02 (s, 3 H), 2.85 (d, J = 4.52, 3 H), 1.68-1.59 (m, 2 H), 1.50-1.42 (m, 2 H). LC/MS method: Phenomenex Luna 3 μm C18, 2 x 50 mm column, with a gradient of 0-100%B (B = 90% MeCN/0.1% TFA/10% H_2O, A = 90% H_2O/0.1% TFA/10% MeCN), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) *m/z* = 582.3 $(M + H)^+$, calculated for $C_{31}H_{25}FN_5O_6$ = 582.2; R_t = 2.991 min. (UV at 220 nm).

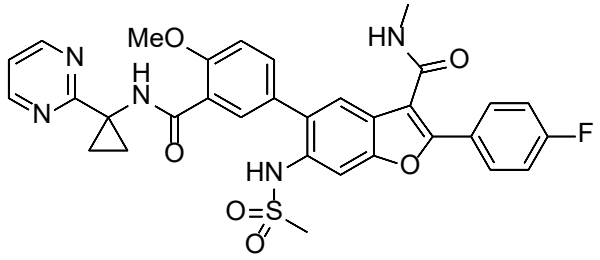
6-Amino-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methylbenzofuran-3-carboxamide (**I-6**)



To a RBF was added 2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methyl-6-nitrobenzofuran-3-carboxamide **I-5** (4.29 g, 7.38 mmol), MeOH (430 mL), zinc (2.89 g, 44.3 mmol) and acetic acid (3.38 mL, 59.0 mmol). The mixture was placed under nitrogen and stirred at 50 °C for 2 hrs, and then at r.t. for 2 hrs. Another quantities of zinc (1 g) and acetic acid (1.2 mL) were added to the mixture, which was stirred for

1 hr. LCMS analysis showed completion of the reaction. The mixture was filtred while warm, and the solid was washed with MeOH. The filtrate was evaporated, and the residue diluted with CH_2Cl_2 (300 mL). The organic solution was washed with water, K_3PO_4 solution, brine, and dried over $MgSO_4$. The mixture was filtered and the filtrate evaporated to dryness to give the product 6-amino-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methylbenzofuran-3-carboxamide **I-6** as yellow solid (3.69 g, 91% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.67 (d, J = 4.80, 2 H), 8.08 (d, J = 2.40, 1 H), 7.92-7.88 (m, 2 H), 7.65 (dd, J = 8.40, 2.40, 1 H), 7.32 (s, 1 H), 7.31 (d overlapping with s, 1 H), 7.25 - 7.20 (m, 3 H), 6.98 (s, 1 H), 4.08 (s, 3 H), 2.93 (s, 3 H), 1.82-1.79 (m, 2 H), 1.54-1.51 (m, 2 H). LC/MS method: Phenomenex Luna 3 μm C18, 2 x 50 mm column, with a gradient of 0-100%B (B = 90% MeCN/0.1% TFA/10% H_2O, A = 90% H_2O/0.1% TFA/10% MeCN), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) m/z = 552.3 $(M + H)^+$, calculated for $C_{31}H_{27}FN_5O_4$ = 552.2; R_t = 2.227 min. (UV at 220 nm).

2-(4-Fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methyl-6-(methylsulfonamido)benzofuran-3-carboxamide (**I-7**)



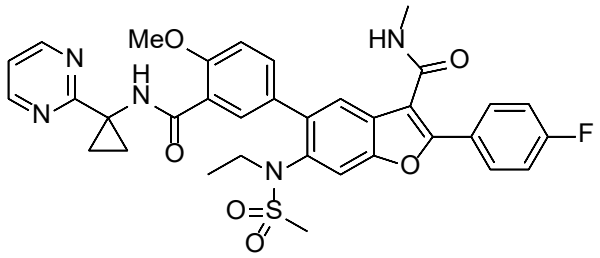
Step 1: To a RBF, under a nitrogen atmosphere, was added 6-amino-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methylbenzofuran-3-carboxamide **I-6** (36.1 mg, 0.065 mmol), 1,2-dichloroethane (3 mL), and *N*-ethyl-*N*-

isopropylpropan-2-amine (0.046 mL, 0.262 mmol). The mixture was stirred for 30 minutes at r.t. To the resulting mixture was added methanesulfonyl chloride (0.020 mL, 0.262 mmol). The tan mixture was stirred overnight at room temperature. The mixture was diluted with about 30 mL of 5% MeOH/CH_2Cl_2, washed with sat. aq. sodium bicarbonate, brine, dried over sodium sulfate, filtered, evaporated and dried under vacuum for 2 hours. LCMS analysis of the crude product showed a ~1:1 mixture of mono-sulfonylated **I-7** and its bis-sulfonylated side product. (Note if the reaction mixture was purified by reverse phase preparative HPLC using TFA as a buffer, trifluoroacetamide **I-8** was also isloated as a side product.)

Step 2: To a small RBF was added the above mixture of sulfonylated products (24.77 mg, assumed 0.035 mmol), dioxane (4 mL), water (0.800 mL) and cesium carbonate (45.6 mg, 0.140 mmol). The mixture was heated to 80 °C for 35 minutes. The mixture was cooled, diluted with CH_2Cl_2, washed with sat. aq. sodium bicarbonate, brine, dried over sodium sulfate, filtered, evaporated and dried under vacuum. The crude reaction mixture was taken up in 6 mL of acetonitrile and 1mL of DMSO and purified using a Shimadzu preparative HPLC employing acetonitrile/water/ammonium acetate where solvent A was 5% acetonitrile / 95% H_2O / 10 mM ammonium acetate and solvent B was 5% H_2O / 95% acetonitrile / 10 mM ammonium acetate with a Phenomenex-Luna 10 μm C18 30x100mm column at a gradient of 40-100% B and a flow rate of 30 mL/min. over 10 minutes with a 10 minute hold. The desired compound eluted at 5.27 min. and he corresponding fractions were combined and concentrated overnight under nitrogen to obtain **I-7** as an yellow oil (15.6 mg, 38% yield two steps). ^{1}H NMR (400 MHz, THF-d_8) δ 8.86 (s, 1 H), 8.58 (d, J = 4.52, 2 H), 8.17 (d, J = 2.4, 1 H), 8.16-8.07 (m, 2 H), 7.85 (s, 1 H), 7.82 (br. s, 1 H), 7.60 (s, 1 H), 7.57 (dd, J = 8.28, 2.51, 1 H), 7.49 (br. s, 1 H), 7.25 (d, J = 8.4, 1 H), 7.20 (t, J = 8.80, 2 H), 7.10 (t, J = 4.77, 1 H), 4.07 (s, 3 H), 2.89 (s, 3 H), 2.87 (d, J = 4.8, 3 H), 1.69-1.62 (m, 2 H), 1.62-1.55 (m, 2 H). The

LC/MS data was obtained on a Shimadzu analytical LC /Micromass Platform LC (ESI+) at 220 nm using the following set of conditions: Phenomenex Luna 3 μm C18, 2 x 50 mm column, with a gradient of 0-100%B (B = 90% HPLC grade acetonitrile/0.1% trifluoroacetic acid/10% HPLC grade water), (A = 90% HPLC grade water/0.1% trifluoroacetic acid/10% HPLC grade acetonitrile), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) *m/z* = 630.4 $(M + H)^+$, calculated for $C_{32}H_{29}FN_5O6_S$ = 630.2; R_t = 2.875 min.

6-(N-ethylmethylsulfonamido)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methylbenzofuran-3-carboxamide (**6**)



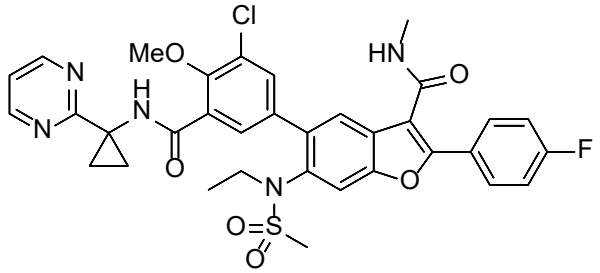
To a round bottom flask was added 2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methyl-6-(methylsulfonamido)benzofuran-3-carboxamide **I-7** (68.6 mg, 0.109 mmol), DMF (3.89 mL), cesium carbonate (53.2 mg, 0.163 mmol) and iodoethane (13.21 μL, 0.163 mmol) under nitrogen. The mixture was stirred at r.t. overnight. The crude reaction mixture was then evacuated to near dryness, taken up in 8 mL of methanol and purified using a Shimadzu preparative HPLC employing methanol/water/TFA where solvent A was 10% MeOH / 90% H_2O / 0.1% trifluoroacetic acid and solvent B was 10% H_2O / 90% MeOH / 0.1% trifluoroacetic acid with a Phenomenex-Luna 10u C18 30x100mm column at a gradient of 30-100% B and a flow rate of 40 mL/min. over 15 minutes with a 10 minute hold. Desired material eluted at 12.8 minutes (77% methanol). Tubes with the desired fractions were combined and

solvent removed on rotovap to a white slurry, which then placed under a stream of nitrogen overnight. The material was rinsed three times with diethyl ether, evaporated on rotovap to obtain an oil. The oil was taken up in 50 mL of EtOAc, washed with 15 mL of 0.5 N NaOH, brine, dried over sodium sulfate, filtered and evaporated on the rotovap, and then dried under high vacuum overnight to obtain 6-(N-ethylmethylsulfonamido)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methylbenzofuran-3-carboxamide (**6**) as a light yellow powder (68 mg, 93% yield).

HRMS (ES+) *m/z* found 658.2106 $(M+H)^+$; calculated for $C_{34}H_{33}FN_5O_6S$, 658.2136. ^{1}H NMR (400 MHz, THF-d_8) δ 8.87 (s, 1 H), 8.58 (d, J = 4.77, 2 H), 8.23-8.12 (m, 3 H), 7.74 (m, J = 8.41, 2.38, 1 H), 7.68 (d, J = 6.02, 2 H), 7.54 (m, 1 H), 7.27-7.15 (m, 3 H), 7.09 (t, J = 4.77, 1 H), 4.07 (s, 3 H), 2.95 (s, 3 H), 2.89 (d, J = 4.77, 3 H), 1.66-1.58 (m, 4 H), 1.29 (m, 2 H), 0.98 (t, J = 7.03, 3 H). ^{13}C NMR (101 MHz, THF-d_8) δ 171.86, 165.95, 164.35, 163.56, 158.60, 157.97, 155.68, 153.80, 139.12, 136.36, 135.04, 134.49, 133.89, 131.24, 131.17, 128.99, 127.56, 123.89, 123.52, 119.16, 116.78, 116.57, 115.29, 113.73, 112.70, 56.92, 47.21, 40.29, 39.04, 26.85, 20.02, 14.10. ^{19}F NMR (376.46 MHz, THF-d_8): δ -112.08. LC/MS method: Phenomenex Luna 3 μm C18, 2 x 50 mm column, with a gradient of 0-100%B (B = 90% MeCN/0.1% TFA/10% H_2O, A = 90% H_2O/0.1% TFA/10% MeCN), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) *m/z* = 658.5 $(M + H)^+$; R_t = 3.146 min. (UV at 220 nm). Analytical HPLC method: (a) Waters Sunfire C18 3.5 μm 4.6 x 150 mm column, with a gradient of 10-100% B, (B = 95% MeCN/0.1% TFA/5% H_2O, A = 95% H_2O/0.1% TFA/5% MeCN), in 10 minutes with a 10 minute hold at a rate of 1 mL/minute; 98.1% purity, R_t = 8.286 min. (b) Phenomenex Gemini C18 3.0 μm 4.6 x 150 mm column, with a gradient of 10-100% B (B = 95% MeOH /10 mM NH_4HCO_3/5% H_2O, A =

95% H_2O/10 mM NH_4HCO_3/5% MeOH), in 10 minutes with a 10 minute hold at a rate of 1 mL/minute; 98.9% purity, R_t = 10.604 min. (UV at 254 nm and 256 nm).

5-(3-Chloro-4-methoxy-5-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-6-(N-ethylmethylsulfonamido)-2-(4-fluorophenyl)-N-methylbenzofuran-3-carboxamide (**7b**)

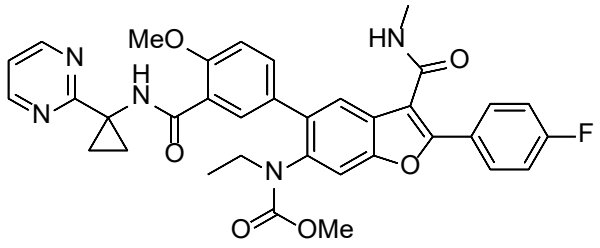


A mixture of 5-(3-chloro-4-methoxy-5-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-6-(ethylamino)-2-(4-fluorophenyl)-*N*-methylbenzofuran-3-carboxamide **I-9** (0.1 g, 0.163 mmol) in pyridine (1 mL) in a sealed tube was cooled to 0° C. To the mixture was added DMAP (1.989 mg, 0.016 mmol) followed by the addition of methanesulfonyl chloride (0.015 mL, 0.195 mmol). The reaction mixture was then stirred at room temperatue for about 4 hrs. The mixture was added water and extracted with ethylacetate. The organic layer was dried and concentrated. The crude product was purified by reverse phase preparative HPLC to give **7b** (27 mg, 24% yield).

^{1}H NMR (400 MHz , DMSO-d_6): δ 9.03 (s, 1 H), 8.70 (d, J = 4.8, 2 H), 8.54-8.52 (m, 1 H), 8.04-8.01 (m, 2 H), 7.98 (s, 1 H), 7.79 (d, J = 2.4, 1 H), 7.66 (s, 1 H), 7.68 (d, J = 2.4, 1 H), 7.46-7.41 (overlapping m, 2 H), 7.30 (t, J = 4.8, 1 H), 3.98 (s, 3 H), 3.40-3.30 (broad m, 2H), 3.15 (s, 3 H), 2.84 (d, J = 4.8, 3 H), 1.62-1.60 (m, 2 H), 1.41-1.39 (m, 2 H), 0.93 (t, J = 7.2, 3 H). ^{19}F NMR (376.57 MHz, DMSO-d_6): δ -110.24. LC/MS method: Solvent A = 2% MeCN-98% H_2O-10 mM NH_4CHO_2, Solvent B = 98% MeCN-2% H_2O-10 mM NH_4CHO_2, Time(min)/%B = 0/0, 1.4/100, 3/100, Flow Rate = 1.0 ml/min, Column: Ascentis Express C18, 50 x 2.1 mm, 2.7 μm; (ES+) *m/z*

= 692.8 $(M+H)^+$, calculated for $C_{34}H_{32}ClFN_5O_6S$ = 692.2; R_t = 1.892 min. (UV at 220 nm). Analytical HPLC method: Buffer : 0.05% TFA in water pH 2.5, Mobile Phase A: Buffer:MeCN (95:5), Mobile Phase B: MeCN:Buffer (95:5), Time(min)/%B = 0/10, 25/100, 30/100, Flow Rate = 1 ml/min, Column: Sunfire C18, 3.5 µm, 4.6 x 150 mm, 99.9% purity, R_t = 17.613 min; Xbridge Phenyl 3.5 µm, 4.6 x 150 mm, 99.9% purity, R_t = 16.509 min. (UV at 220 nm and 254 nm).

Methyl ethyl(2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-3-(methylcarbamoyl)benzofuran-6-yl)carbamate (**8**)

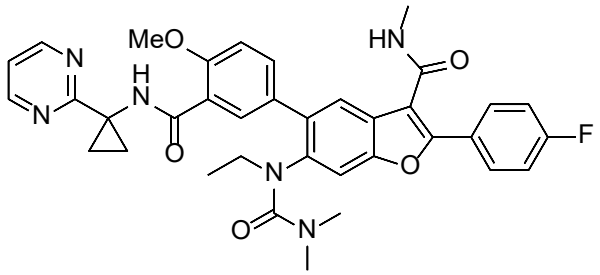


To a mixture of 6-(ethylamino)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methylbenzofuran-3-carboxamide **12** (40 mg, 0.069 mmol) in DMF (2.5 mL) at room temperature was added cesium carbonate (67.5 mg, 0.207 mmol) followed by methyl chloroformate (65.2 mg, 0.690 mmol) at r.t. The mixture was stirred at 80 °C overnight. The mixture was then diluted with water, extracted with EtOAc, and the organic layer washed with brine, dried over Na_2SO_4 and concentrated. The crude product was purified by reverse phase preparative HPLC using the conditions: column: Atlontis dc18(19x250mm)10µm; mobile phase: A:10 mM NH_4OAc in water, B:MeCN flow: 15 ml/min; gradient: Time = 0/10 min, %B = 20/80; runtime = 20 min; product retention time: 9.18 min. to give **8** (13 mg, 29%).

^{1}H NMR (400 MHz , DMSO-d_6): δ 8.90 (s, 1 H), 8.70 (d, J = 4.8, 2 H), 8.55-8.50 (m, 1 H), 8.03-7.99 (m, 2 H), 7.86 (broad s, 1 H), 7.70 (s, 1 H), 7.54 (s, 1 H), 7.44-7.39 (overlapping m, 3 H), 7.30-7.26 (overlapping m, 2 H), 4.00 (s, 3 H), 3.52 (s, 3 H), 3.70-355 (m, 2 H), 2.83 (d, J = 4.8, 3

H), 1.63-1.60 (m, 2 H), 1.45-1.43 (m, 2 H), 0.97 (t, J = 6.8, 3 H). ^{19}F NMR (376.57 MHz, DMSO-d_6): δ -110.53. LC/MS method: Solvent A = 10% MeCN-90% H_2O-20 mM NH_4OAc, Solvent B = 90% MeCN-10% H_2O-20 mM NH_4OAc, Time(min)/%B = 0/0, 2/100, 2.5/100, 3/0, Flow Rate = 2.5 ml/min, Column: Purospher STAR RP-18 (4 x 55) mm, 3 µm; (ES-) m/z = 636.2 $(M-H)^-$, calculated for $C_{35}H_{31}FN_5O_6$ = 636.2; R_t = 1.795 min. (UV at 220 nm). Analytical HPLC method: Buffer : 0.05% TFA in water pH 2.5, Mobile Phase A: Buffer:MeCN (95:5), Mobile Phase B: MeCN:Buffer (95:5), Time(min)/%B = 0/10, 25/100, 30/100, Flow Rate = 1 ml/min, Column: Sunfire C18, 3.5 µm, 4.6 x 150 mm, 98.6% purity, R_t = 17.519 min; Xbridge Phenyl 3.5 µm, 4.6 x 150 mm, 99.2% purity, R_t = 16.138 min. (UV at 220 nm and 254 nm).

6-(1-Ethyl-3,3-dimethylureido)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methylbenzofuran-3-carboxamide (**9**)

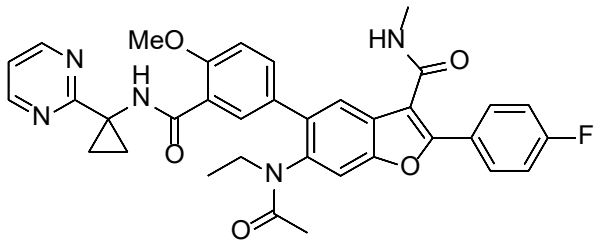


To a mixture of 6-(ethylamino)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methylbenzofuran-3-carboxamide **12** (50 mg, 0.086 mmol) and pyridine (1.5 mL) at room temperature was added dimethylcarbamic chloride (46.4 mg, 0.431 mmol) at r.t. The reaction mixture was stirred at r.t. for 2 days. The mixture was then diluted with water and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The crude product was purified by reverse phase preparative HPLC using the conditions: column**:** sunfire C18 (150 x19) 5 µm; mobile phase: A:10 mM NH_4OAc in water,

B:MeCN flow: 15 ml/min; gradient: Time = 0/11/11.3 min, %B = 25/70/100; runtime = 20 min; product retention time: 10.30 min. to give **9** (23.46 mg, 42%).

^{1}H NMR (400 MHz , DMSO-*d*$_6$): δ 8.85 (s, 1 H), 8.69 (d, *J* = 4.8, 2 H), 8.50-8.46 (m, 1 H), 8.00-7.97 (m, 2 H), 7.91 (d, *J* = 2.8, 1 H), 7.61 (s, 1 H), 7.51 (dd, *J* = 8.4, 2.4, 1 H), 7.48 (s, 1 H), 7.43-7.38 (m, 2 H), 7.29 (t, *J* = 4.8, 1 H), 7.24 (d, *J* = 8.8, 1 H), 3.99 (s, 3 H), 3.54-3.49 (m, 2 H), 2.82 (d, *J* = 4.8, 3 H), 2.38 (s, 6 H), 1.63-1.60 (m, 2 H), 1.46-1.43 (m, 2 H), 1.07 (t, *J* = 7.2, 3 H). ^{19}F NMR (376.57 MHz, DMSO-*d*$_6$): δ -110.76. LC/MS method: Solvent A = 10% MeCN-90% H_2O-20 mM NH_4OAc, Solvent B = 90% MeCN-10% H_2O-20 mM NH_4OAc, Time(min)/%B = 0/0, 2/100, 2.5/100, 3/0, Flow Rate = 2.5 ml/min, Column: Purospher STAR RP-18 (4 x 55) mm, 3 µm; (ES+) *m/z* = 651.2 $(M+H)^+$, calculated for $C_{36}H_{36}FN_6O_5$ = 651.3; R_t = 1.678 min. (UV at 254). Analytical HPLC method: Buffer : 0.05% TFA in water pH 2.5, Mobile Phase A: Buffer:MeCN (95:5), Mobile Phase B: MeCN:Buffer (95:5), Time(min)/%B = 0/10, 12/100, 15/100, Flow Rate = 1 ml/min, Column: Sunfire C18, 3.5 µm, 4.6 x 150 mm, 99.1% purity, R_t = 9.826 min; Xbridge Phenyl 3.5 µm, 4.6 x 150 mm, 99.1% purity, R_t = 8.600 min. (UV at 220 nm and 254 nm).

6-(N-ethylacetamido)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methylbenzofuran-3-carboxamide (**10**)

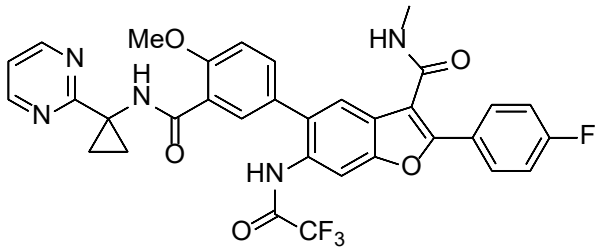


To a mixture of 6-(ethylamino)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methylbenzofuran-3-carboxamide **12** (50 mg, 0.086 mmol) and pyridine (2.0 mL) at room temperature was added Ac_2O (8.14 µl, 0.086 mmol), and the mixture

stirred at r.t. overnight. The mixture was then diluted with water and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The crude product was purified by reverse phase preparative HPLC using the conditions: column**:** X-Terra RP 18 (150x10x5µm); mobile phase: A:10 mM NH_4OAc in water, B:MeCN flow: 12 ml/min; gradient: Time = 0/6/8.4/8.5/10.5/10.6/12.5 min, %B = 10/40/40/100/100/10/10; runtime = 13 min; product retention time: 7.50 min. to give **10** (30 mg, 55% yield).

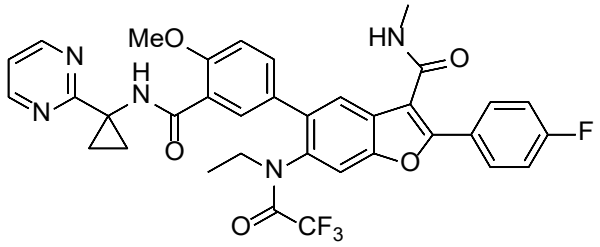
^{1}H NMR (400 MHz , DMSO-*d*$_6$): δ 8.91 (s, 1 H), 8.70 (d, J = 4.8, 2 H), 8.55-8.50 (m, 1 H), 8.02-7.99 (m, 2 H), 7.88 (d, J = 2.8, 1 H), 7.80 (s, 1 H), 7.62 (s, 1 H), 7.48 (dd, J = 8.8, 2.4, 1 H), 7.44-7.40 (m, 2 H), 7.31-7.27 (overlapping m, 2 H), 4.00 (s, 3 H), 3.90-3.83 (m, 1 H), 2.83 (d, J = 4.8, 3 H), 2.62-2.55 (m, 1 H), 1.85 (s, 3 H), 1.62-1.60 (m, 2 H), 1.45-1.44 (m, 2 H), 0.97 (t, J = 7.2, 3 H). ^{19}F NMR (376.57 MHz, DMSO-*d*$_6$): δ -110.38. LC/MS method: Solvent A = 10% MeCN-90% H_2O-20 mM NH_4OAc, Solvent B = 90% MeCN-10% H_2O-20 mM NH_4OAc, Time(min)/%B = 0/0, 2/100, 2.5/100, 3/0, Flow Rate = 2.5 ml/min, Column: Purospher STAR RP-18 (4 x 55) mm, 3 µm; (ES+) *m/z* = 622.2 $(M+H)^+$, calculated for $C_{35}H_{33}FN_5O_5$ = 622.2; R_t = 1.624 min. (UV at 220). Analytical HPLC method: Buffer : 0.05% TFA in water pH 2.5, Mobile Phase A: Buffer:MeCN (95:5), Mobile Phase B: MeCN:Buffer (95:5), Time(min)/%B = 0/10, 12/100, 15/100, Flow Rate = 1 ml/min, Column: Sunfire C18, 3.5 µm, 4.6 x 150 mm, 98.8% purity, R_t = 9.177 min; Xbridge Phenyl 3.5 µm, 4.6 x 150 mm, 99.1% purity, R_t = 8.691 min. (UV at 220 nm and 254 nm).

2-(4-Fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methyl-6-(2,2,2-trifluoroacetamido)benzofuran-3-carboxamide (**I-8**)



Intermedaite **I-8** was isolated in the first step for the preparation of **I-7** if the reaction crude product was purified by using the following method: Shimadzu preparative HPLC employing methanol/water/TFA where solvent A was 10% MeOH / 90% H_2O / 0.1% trifluoroacetic acid and solvent B was 10% H_2O / 90% MeOH / 0.1% trifluoroacetic acid with a Phenomenex-Luna 10 μm C18 30x100 mm column at a gradient of 40-100% B and a flow rate of 40 mL/min. over 10 minutes with a 10 minute hold. The desired fractions were combined and evaporated. The product was diluted with CH_2Cl_2, washed with sat. aq. sodium bicarbonate solution and brine, dried over sodium sulfate, filtered and evaporated to give **I-8**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.15 (s, 1 H), 8.90 (s, 1 H), 8.70 (d, J = 4.77, 2 H), 8.53 (m, 1 H), 8.0-7.98 (m, 2 H), 7.93 (d, J = 2.26, 1 H), 7.81 (s, 1 H), 7.61 (s, 1 H), 7.52 (dd, J = 8.53, 2.51, 1 H), 7.42 (t, J = 8.78, 2 H), 7.33-7.22 (m, 2 H), 4.01 (s, 3 H), 2.84 (d, J = 4.77, 3 H), 1.70-1.57 (m, 2 H), 1.49-1.38 (m, 2 H). The LC/MS data was obtained on a Shimadzu analytical LC/Micromass Platform LC (ESI+) at 220 nm using the following set of conditions: Phenomenex Luna 3 μm C18, 2 x 50 mm column, with a gradient of 0-100% B (B = 90% HPLC grade acetonitrile/ 0.1% trifluoroacetic acid/ 10% HPLC grade water), (A = 90% HPLC grade water / 0.1% trifluoroacetic acid/ 10% HPLC grade acetonitrile), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) m/z = 648.3 $(M + H)^+$, calculated for $C_{33}H_{26}F_4N_5O_5$ = 648.2; R_t = 3.026 min.

6-(N-ethyl-2,2,2-trifluoroacetamido)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methylbenzofuran-3-carboxamide (**11**)

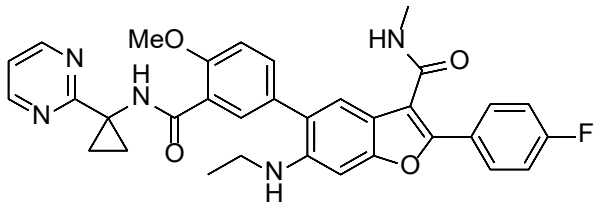


To a scintillation vial was added 2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methyl-6-(2,2,2-trifluoroacetamido)benzofuran-3-carboxamide **I-8** (39.5 mg, 0.052 mmol, assumed one TFA), DMF (2 mL), cesium carbonate (67.6 mg, 0.207 mmol) and finally iodoethane (8.30 µl, 0.104 mmol). The vial was capped and the heterogeneous mixture stirred overnight at r.t. The crude reaction mixture was further diluted with methanol (2 mL) and purified using a Shimadzu preparative HPLC employing methanol/water/TFA where solvent A was 10% MeOH / 90% H_2O / 0.1% trifluoroacetic acid and solvent B was 10% H_2O / 90% MeOH / 0.1% trifluoroacetic acid with a Phenomenex-Luna 10u C18 30x100mm column at a gradient of 30-100% B and a flow rate of 40 mL/min. over 15 minutes with a 10 minute hold. The desired product eluted at 12.1-13.2 minutes. The desired fractions were combined and volatiles removed under a stream of nitrogen overnight, and then further dried under high vacuum for 3 hours to obtain **11** as a tan powder (13 mg, 31%).

^{1}H NMR (400 MHz, THF-d_8) δ 8.91 (s, 1 H), 8.59 (d, J = 4.77 Hz, 2 H), 8.22-8.12 (m, 3 H), 7.77 (s, 1 H), 7.57 (m, 2 H), 7.43 (dd, J = 8.53, 2.51 Hz, 1 H), 7.28-7.18 (m, 3 H), 7.10 (t, J = 4.89 Hz, 1 H), 4.07 (s, 3 H), 3.88 (m, 2 H), 2.89 (d, J = 4.77 Hz, 3 H), 1.68-1.58 (m, 4 H), 1.14-0.98 (t, J = 1.06 Hz, 3 H). LC/MS method: Phenomenex Luna 3 µm C18, 2 x 50 mm column, with a gradient of 0-100%B (B = 90% MeCN/0.1% TFA/10% H_2O, A = 90% H_2O/0.1% TFA/10% HPLC MeCN), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) m/z = 676.4 $(M + H)^+$,

calculated for $C_{35}H_{30}F_4N_5O_5$ = 676.2; R_t = 3.145 min. (UV at 220 nm). Analytical HPLC method: (a) Waters Sunfire C18 3.5 μm 4.6 x 150 mm column, with a gradient of 10-100% B, (B = 95% MeCN/0.1% TFA/5% H_2O, A = 95% H_2O/0.1% TFA/5% MeCN), in 10 minutes with a 10 minute hold at a rate of 1 mL/minute; 95.3% purity, R_t = 10.183 min. (b) Phenomenex Gemini C18 3.0 μm 4.6 x 150 mm column, with a gradient of 10-100% B (B = 95% MeOH/10 mM NH_4HCO_3/5% H_2O, A = 95% H_2O/10 mM NH_4HCO_3/5% MeOH), in 10 minutes with a 10 minute hold at a rate of 1 mL/minute; 99.1% purity, R_t = 11.794 min. (UV at 254 nm and 256 nm).

6-(Ethylamino)-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-N-methylbenzofuran-3-carboxamide (**12**)



Acetaldehyde (0.163 mL, 2.90 mmol) was added to a suspension of 6-amino-2-(4-fluorophenyl)-5-(4-methoxy-3-((1-(pyrimidin-2-yl)cyclopropyl)carbamoyl)phenyl)-*N*-methylbenzofuran-3-carboxamide **I-6** (200 mg, 0.363 mmol) and molecular sieves (4Å, 400 mg) in MeOH (35 mL) at r.t. The mixture was stirred at r.t for 1 hr followed by addition of sodium cyanoborohydride (182 mg, 2.90 mmol), and then stirred for one day. Another quantities of acetaldehyde (0.1 mL) and sodium cyanoborohydride (110 mg) were added to the mixture. The reaction mixture were stirred at r.t. for 24 hrs, and then diluted with MeOH (200 mL) and filtered. The filtrate was evaporated, and the residue dissolved in CH_2Cl_2 (200 mL). The mixture was washed with water and brine, dried over $MgSO_4$, and concentrated to give crude product as a brown solid. The crude product was purified by preparative HPLC to give 135 mg target compound as an off white solid using the

conditions: XTERRA 30x100 mm S5 column(4), with moblie phase A = 10% MeCN-90% water-0.1%TFA, B = 90% MeCN-10% water-0.1%TFA; Gradient time 10 min, Start %B: 15, final %B: 90; Flow rate 40 mL/min; Stop time 25 min. The material was recrystallized from EtOH by dissolving in EtOH (24 mL) and then concentrated to 8 mL to give **12** (96 mg, 46% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.66 (d, J = 4.8, 2 H), 8.04 (d, J = 2.4, 1 H), 7.92-7.89 (m, 2 H), 7.62-7.60 (m, 1 H), 7.32-7.29 (m, 2 H), 7.25-7.20 (m, 3 H), 6.86 (s, 1 H), 4.08 (s, 3 H), 3.22 (dd, J = 14.4, 7.2, 2 H), 2.93 (s, 3 H), 1.82-1.79 (m, 2 H), 1.54-1.51 (m, 2 H), 1.22 (t, J = 7.2, 3 H). ^{19}F NMR (376.46 MHz, CD_3OD): δ -114.23. LC/MS method: Phenomenex Luna 3 μm C18, 2 x 50 mm column, with a gradient of 0-100%B (B = 90% MeCN/0.1% TFA/10% H_2O, A = 90% H_2O/0.1% TFA/10% MeCN), in 4 minutes with a 1 minute hold at a rate of 0.8 mL/minute. (ES+) m/z = 580.4 $(M + H)^+$, calculated for $C_{33}H_{31}FN_5O_4$ = 580.2; R_t = 2.606 min. (UV at 220 nm); 98.7% purity. Analytical HPLC method: (a) Waters Sunfire C18 3.5 μm 4.6 x 150 mm column, with a gradient of 10-100% B, (B = 95% MeCN/0.1% TFA/5% H_2O, A = 95% H_2O/0.1% TFA/5% MeCN), in 15 minutes with a 5 minute hold at a rate of 1 mL/minute; 99.5% purity, R_t = 10.22 min. (UV at 220 nm and 254 nm).

Biological Methods

HCV NS5B RdRp enzyme assay. A 384-well on-bead solid phase homogeneous assay was used to assess NS5B inhibitors (Wang Y-K, Rigat K, Roberts S, and Gao M (2006) Anal Biochem, 359: 106-111). Specifically, the biotinylated oligo dT12 primer was captured on streptavidin-coupled imaging beads (GE, RPNQ0261) by mixing primer and beads in 1x assay buffer and incubating at room temperature for three hours. Unbound primer was removed after centrifugation. The primer-

bound beads were resuspended in 3x reaction mix (20 mM Hepes buffer, pH 7.5, dT primer coupled beads, poly A template, ^{3}H-UTP, and RNAse inhibitor (Promega N2515). Compounds were serially diluted 1:3 in DMSO and aliquoted into assay plates. Equal volumes (10 μL) of water, 3X reaction mix, and enzyme in 3x assay buffer (60 mM Hepes buffer, pH 7.5, 7.5 mM $MgCl_2$, 7.5 mM KCl, 3 mM DTT, 0.03 mg/ml BSA and 6% glycerol were added to the diluted compound on the assay plate. Final concentration of components in 384-well assay: 0.36 nM template, 15 nM primer, 0.29 μM ^{3}H-UTP (0.3 μCi), 1.6 U/μL RNAse inhibitor, 7 nM NS5B enzyme, 0.010 mg/mL BSA, 1 mM DTT, and 0.33 μg/μL beads, 20 mM Hepes buffer, pH 7.5, 2.5 mM $MgCl_2$, 2.5 mM KCl, 0.1% DMSO. Reactions were allowed to proceed for 4 or 24 hours at 30º C. For the 24 hour reactions, enzyme and inhibitors were pre-incubated for 24 hours before adding template and primer. Reactions were then terminated by the addition of 50 mM EDTA (10 μL). After incubating for at least 15 minutes, plates were read on an Amersham LEADseeker multimodality imaging system

IC_{50} values for compounds were determined using ten different [I]. IC_{50} values were calculated from the inhibition using the four-parameter logistic formula y = A+((B-A)/(1+((C/x)^D))), where A and B denote minimal and maximal % inhibition, respectively, C is the IC50, D is hill slope and x represent compound concentration.

Cell lines. The cell lines used to evaluate compounds consist of a human hepatocyte derived cell line (Huh-7) that constitutively expresses HCV replicon containing a Renilla luciferase reporter gene. These cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 100 U/mL penicillin/streptomycin and 1.0 mg/mL G418.

HCV replicon luciferase assay. To evaluate compound efficacy, titrated compounds were transferred to sterile 384-well tissue culture treated plates, and the plates were seeded with HCV replicon cells (50 μL at a density of 2.4 x 10^3 cells/well) in DMEM containing 4 % FCS (final DMSO concentration at 0.5 %). After 3 days incubation at 37°C, cells were analyzed for Renilla Luciferase activity using the EnduRen substrate (Promega cat #E6485) according to the manufacturer's directions. Briefly, the EnduRen substrate was diluted in DMEM and then added to the plates to a final concentration of 7.5 μM. The plates were incubated for at least 1 h at 37ºC then read on a Viewlux Imager (PerkinElmer) using a luminescence program. The 50% effective concentration (EC_{50}) was calculated using the four-parameter logistic formula noted above.

To assess cytotoxicity of compounds, Cell Titer-Blue (Promega) was added to the EnduRen-containing plates and incubated for at least 4 hrs at 37°C. The fluorescence signal from each well was read using a Viewlux Imager. All CC_{50} values were calculated using the four-parameter logistic formula.

Table S1-2. **Cytotoxicity in HCV gt-1a Replicon (CC_{50}) and Inhibitory Potency towards gt-2a Replicon (EC_{50}) for Compounds 2-12**

Compd	gt-1a Replicon[a]		gt-2a Replicon[c]	
	CC_{50} (μM)	SD (±)	EC_{50} (nM)	SD (±)
2	8.5	5.5	515[b]	0.0
3	56.7	0.9	22	9.2
4	6.5	2.6	8.6	2.6
5	>100	0.0	5.4	2.9
6	>100	0.0	13	3.7
7a	37.5	21.6	8.8	4.9

7b	>100	0.0	6.6	1.6
7c	66.5	40.9	16	3.3
7d	70.4[b]	0.0	9.0	5.4
8	39.0	12.0	15	10
9	>100[b]	0.0	165[b]	0.0
10	71.7	1.7	35	21
11	>100	0.0	20	8.6
12	>100	0.0	12	2.3

[a]Data are average values of n ≥2 independent experiments; [b]Data from a single experiment performed in duplicate; [c]EC_{50} values are the same as in **Tables 1**, **2** and **4**.

Table S1-3. **Inhibitory Potency towards HCV gt-1b, gt-1a and gt-1bC316N Replicons (EC_{50}) for Compounds 2-5**

Compd	**Replicon EC_{50} (nM)[a]**		
	gt-1b	**gt-1a**	**gt-1bC316N**
2	3.8±3.0	36±13	75±2.7
3	52±19	35±7.1	385±3.3
4	6.2±1.5	5.7±1.8	64±45
5	8.8±1.2	7.7±2.4	44±13

[a]Data are average values of n ≥2 independent experiments; EC_{50} are the same as in **Table 1**.

In Vivo Rat Pharmacokinetic Studies.

All animal studies were performed under the approval of the Bristol-Myers Squibb Animal Care and Use Committee and in accordance with the American Association for Accreditation of Laboratory Animal Care (AAALAC). Male Sprague−Dawley rats (300−350 g) with dual indwelling cannulae implanted in the jugular or intraportal veins were used in the pharmacokinetic studies. After dosing, serial blood samples (0.3 mL) were obtained from the appropriate cannula of each rat by collection into EDTA-containing tubes (Becton Dickinson, Franklin Lakes, NJ) and

centrifuged to separate plasma. Plasma was frozen until analysis. For IV studies, compound **6** or **12** was dosed (2 mg/kg) in a vehicle of PEG-400/ethanol (90/10, v/v) as a 10 min constant rate infusion into the jugular vein, with serial blood samples collected before dosing and at 10, 15, 30, 45, 60 min and 1, 2, 3, 5, 7, and 24 hr after dosing (n = 3 rats/dose group). For PO dosing, compound was administered (6 mg/kg) by gastric gavage as an aqueous solution (PEG-400/ethanol/TPGS (90/5/5, v/v) for **6** and PEG-400/ethanol (90/10, v/v) for **12**). Serial blood samples were collected before dosing and at 15, 30, 45, 60 min and 1, 2, 3, 5, 7, and 24 hr after dosing (n = 3 rats/dose group). Prior to all PO dosing, the rats were fasted overnight with free access to water. To assess liver exposure, livers were removed from rats at 24 hr after IV dosing, and 5 (using two satellite animals) and 24 hr after PO dosing. The tissues were rinsed, blotted dry, weighed, and stored frozen until analysis. Processing included addition of 2 volumes of 80% acetonitrile in Hanks balanced salt solution (HBSS) buffer per gram of tissue, homogenization with a T25 basic S1 generator (IKA Works, Wilminton, NC) using a S25N-8G dispersing tool and centrifugation (IEC Centra-8R at 3,000 rpm for 10 min). Aliquots of supernatant were removed and stored frozen until analysis by LC/MS/MS.

The pharmacokinetics parameters were obtained by noncompartmental analysis of plasma concentration versus time data (KINETICA software, version 2.4, InnaPhase Corporation, Philadelphia, PA). The peak concentration (Cmax) and time for Cmax (Tmax) were recorded directly from experimental observations. The area under the curve from time zero to the last sampling time (AUC_{0-T}) and the area under the curve from time zero to infinity (AUC_{INF}) were calculated using a combination of linear and log trapezoidal summations. The whole body plasma clearance (CL), steady-state volume of distribution (Vss), apparent terminal $t_{1/2}$, and mean residence time (MRT) were estimated following intravenous administration. The absolute oral

bioavailability (F) was estimated as the ratio of dose-normalized AUC values following PO and IV doses.

Permeability and P-Glycoprotein and BCRP Efflux Transporter Substrate Assay in Caco-2 Cells

Materials: Caco-2 (Caucasian colon adenocarcinoma) cells were obtained from the American Type Culture Collection (Manassas, VA), N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid (HEPES) buffer was purchased from GIBCO/Invitrogen (Carlsbad, CA), Transwell plates with 24 wells (surface area: 0.33 cm^2) with a 0.4-μm pore size polycarbonate membrane, were purchased from Corning (Corning, NY). Modified Hank's Balanced Salt Solution (MHBSS) was prepared by adjusting Hank's Balanced Salt Solution (HBSS) with HEPES to pH 7.4. HBSS and bovine serum albumin (BSA) were purchased from Sigma (Saint Louis, Missouri). All solvents were analytical grade.

Caco-2 cells were seeded onto a collagen coated polycarbonate filter membranes in 24-well transwell plates at a density of 1.45 x 10^5 cells/cm^2. Bi-directional permeability studies were conducted with the monolayers obtained from P-glycoprotein expressing Caco-2 cells cultured for approximately 13-27 days. The transport medium was modified Hank's balanced salt solution (MHBSS) containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4 for both apical and basolateral sides) for compounds **6**, **8**, **10**, **11** and **12** while assays evaluating compound **9**, 0.5 % BSA was added to the transport media. Stock solutions were prepared at 10 mM in DMSO (final concentration 1% DMSO) and were diluted to a final concentration of 3 μM (bi-directional studies).

The bi-directional permeability studies were initiated by addition of buffer containing compound to either the apical or basolateral side of the monolayer. To evaluate apical to basolateral transport (or A to B) an addition of 200 µL buffer containing compound was placed on the apical side of the monolayers with buffer alone (600 µL) in the basolateral compartment. To evaluate basolateral to apical transport (or B to A) 600 µLof buffer containing compound was placed on the basolateral side of the monolayer with buffer alone (200 µL) in the apical compartment. The monolayers were then placed in an incubator for 2 h at 37˚C. Samples were taken from both the apical and basolateral compartment at the end of the 2 h period and the concentration of test compound was analyzed by an LC-MS/MS method. Permeability coefficient (Pc) was calculated according to the following equation: $Pc = C_{At} \times V_a/(S \times C_{D0} \times t)$, where C_{At} is the concentration of the test compound in the acceptor well after time t, V_A is the volume in acceptor well, S is the surface area of the membrane (0.33 cm^2), and C_{D0} is the initial concentration (3 µM) in the donor well and t is the incubation time. The Efflux Ratio is calculated as $Pc_{(B->A)}/Pc_{(A->B)}$. The Pc values are expressed as nm/sec. Digoxin a substrate of P-glycoprotein efflux pump, and Sulfasalazine a substrate of BCRP transporter were used as standard controls.

Compound **6** were tested in the P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) efflux transporter substrate assay in Caco-2 cells under 3 conditions: in the absence of any inhibitors; in the presence of KO143 (1 µM), a known selective inhibitor of BCRP efflux transporter; and in the presence of zosuquidar (5 µM), a known selective inhibitor of P-gp efflux transporter. Caco-2 cells were seeded and the permeability measured the same as described in the

bi-directional experiments. Assay buffer was MHBSS without 0.5 % BSA. The Pc and efflux ratio were also calculated as described above.

Production of the Recombinant Protein:

Expression and Purification of HCV-NS5B-2a-JFH(1-574)-L30S

A pET21b-based plasmid containing the cDNA for HCV-NS5B-2a-JFH(1-574)-L30S was used to express the protein in *E. coli* Rosetta2 (DE3) cells. Following transformation of competent Rosetta2 (DE3) cells, a starter culture was grown overnight at 37 °C in 200 mL LB media with 15 µg/mL chloramphenicol and 30 µg/mL carbenacillin. The following morning, this culture was used to inoculate larger culture volumes (1:100 volume, or 10 mL per 1 L) in Overnight Express Instant TB Medium. The cultures were grown at 37 °C for 5.5 h, when the OD_{600} > 1.0 au. The temperature was then dropped to 20 ºC, and growth continued for another 21 h. The cells were harvested by centrifugation (5000 x g, 20 min, 4 ºC) and the pellets were frozen at -80 ºC until needed for purification. A total of 12 L of culture was grown.

For purification, a basic HBS buffer was used throughout the process, with various additions or omissions depending on the step. The basic HBS buffer was composed of 25 mM Hepes, pH 7.3, 400 mM NaCl, 5 mM DTT, 10% v/v glycerol and 0.1% (v/v) β-Octyl glucoside. For lysis, the cell paste (267 g, 6 L original culture volume) was resuspended in 475 mL of HBS buffer supplemented with 1 mM $MgSO_4$, protease inhibitor tablets (4 x Roche Complete tablets), and 25 mL of 10X BugBuster. Finally, Benzonase (10 units/mL, Sigma) and rLysozyme (EMD Biosciences, 100 µL) were added, and the suspended pellet stirred at rt for 60 min. Lysis was achieved by sonication, and the lysate clarified by centrifugation at 10,800 x g for 30 min at 4 ºC.

Purification was achieved in two chromatographic steps. First, cation exchange chromatography was employed (40 mL self-packed column with SP-FF Sepharose resin, GE Healthcare), using the HBS buffer containing protease inhibitors. The NS5B-2a protein was eluted with a 400-1000 mM NaCl gradient in basic HBS buffer. Fractions containing the target protein were combined and the conductivity reduced with the HBS buffer lacking NaCl. The protein was concentrated to ~12 mg/mL, and polished by size exclusion chromatography (prepacked HiLoad 16/60 Superdex 75 column, GE Healthcare) in the basic HBS buffer. The final protein was ≥90% pure, judged by SDS-PAGE with Coomassie staining. LC/MS was used to demonstrate the protein was of expected mass for des-Met form (i.e. 63,751.9 Da observed vs. 63,751.7 Da predicted), and the concentration was determined by OD_{280} (ε = 83,610 M^{-1} cm^{-1}). The final protein was stored in aliquots at 3.67 mg/mL, flash frozen in LN2, and stored at -80 °C, until delivered for crystallization. From 6 L of original culture volume, a total of 119 mg of final purified protein was obtained.

Crystallization of HCV-NS5B-2A-JFH(1-574)-L30S

Crystals of HCV-NS5B-2a-JFH(1-574)-L30S were prepared by sitting drop vapor diffusion method. The protein stock solution consisted of 10.8 mg/ml (0.169 mM based on the calculated MW of 63,847 Da) in 400 mM NaCl, 10% Glycerol (v/v), 0.1% B-OG, 5 mM DTT, 25mM Hepes at pH 7.3. The protein was complexed with 0.676 mM (4.0 molar excess) of compound **5** or **13** on ice for 4 h. The mixture was clarified by centrifugation. Crystallization screens were prepared using a Mosquito LCP (TTP Labtech, Melbourn, UK) on a 96-well, 2-drop MRC tray. The optimized conditions consisted of a reservoir solution of 25 % (w/v) PEG4K, 0.2 M $(NH_4)_2SO_4$, 0.1 M sodium acetate, pH 4.6. Drops were formed from 0.4 µl of the protein solution and 0.4 µl

of the reservoir solution (total initial volume of 0.8 µl), mixed, and placed at 20º C to equilibrate. Crystals appeared within 14 days.

The crystals of HCV-NS5B-2a-JFH(1-574)-L30S were transferred to a cryo-protectant drop prepared by mixing 40% PEG 400 (v/v) and 40% glycerol (v/v) diluted with water. 2.5 µl of the 40:40 mixture was combined with 7.5 µl of crystallization growth condition. The cryo-solution was added serially to crystallization drop. Once crystals have been equilibrated in the cryo-solution crystals were flash frozen into liquid nitrogen.

Structure Determination

Data for HCV NS5B 2a L30S in complex with compound **5** was collected at the Canadian Light Source (Grochulski et al. 2011) using a Rayonix MX-300 detector and data for the complex with compound **13** was collected at the Advanced Photon Source on beamline 17-ID using a Dectris Pilatus 6M detector. The data were processed using autoPROC (GlobalPhasing, Ltd.), which used XDS for integration (Kabsch, 2010a,b) and SCALA (Evans, 2005) for scaling. Both structures were refined with BUSTER/TNT (GlobalPhasing, Ltd.) and used restraint dictionaries generated with GRADE (GlobalPhasing, Ltd.). The models were manipulated in real space with COOT (Emsley et al., 2010).

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Table S2. Crystallographic Statistics for Complexes of HCV NS5B 2a L30S with Compounds 5 and 13

Protein	**5**	**13**
Space Group	$P2_12_12_1$	$P2_12_12$
Unit Cell Axes (Å)	62.3, 90.0, 124.1	93.4, 116,6, 64.7
Resolution range (Å)	39.49-2.08 (2.10-2.08)	21.32-2.17 (2.23-2.17)
Completeness (%)	96.4 (81.1)	99.7 (99.3)
σ cutoff	0	0
No. of reflections, working set	39015 (784)	36064 (2777)
No. of reflections, test set	2066 (38)	1898 (151)
Final Rcryst	0.173 (0.188)	0.177 (0.188)
Final Rfree	0.210 (0.218)	0.210 (0.220)
No. of non-H atoms		
Protein	4273	4203
Ion (SO4)	-	55
Multi-atom solvent	67 (SCN GOL 2PE)	57 (GOL PG4)
Ligand	45	44
Water	458	322
R.m.s. deviations		
Bonds (Å)	0.010	0.010

Angles (°)	1.0	1.0
Average B factors (Å^2)		
Protein	27.2	28.4
Ion (SO4)		54.6
Multi-atom solvent	40.0 (SCN GOL 2PE)	54.3 (GOL PG4)
Ligand	20.6	23.6
Water	38.1	39.3
Ramachandran plot*		
Most favoured	93.0	92.9
Allowed	6.6	7.1

Numbers in parentheses represent the highest resolution shell.
*as defined in Laskowski, R.A.; MacArthur, M.W.; Moss, D.S.; Thornton, J.M. *PROCHECK*: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr*. **1993**, *26*, 283–291.

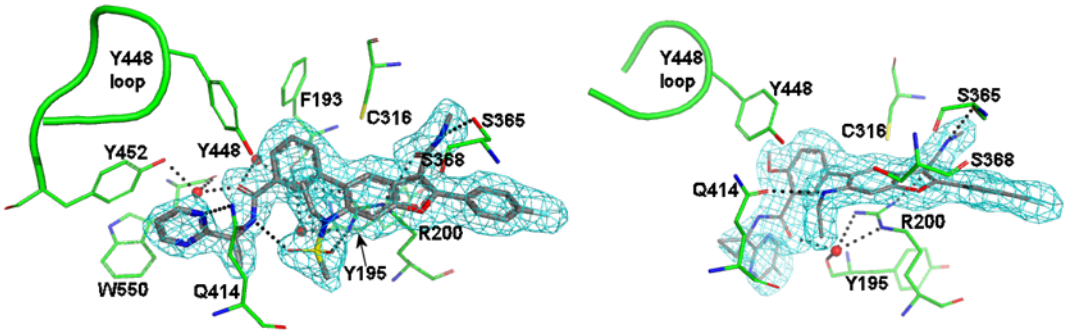

Figure S1. The X-ray co-crystal structures of the complexes of gt-2a NS5B L30S with **5** (left) and **13** (right). The omit electron density for the ligands is contoured at 3.0 r.m.s.d. in cyan mesh. Images were generated with The PyMol Molecular Graphics System (v. 2.0, Schrödinger, LLC). (Note: While compound **6** is structurally more similar to **13** than is **5**, an X-ray structure of the complex of gt-2a NS5B L30S with **6** obtained was of poor resolution (3.50 Å) and details of protein-ligand interactions could not be confidently discerned. Thus, the higher resolution

structure of the gt-2a NS5B L30S/**5** complex was selected for comparison with the gt-2a NS5B L30S/**13** complex in Figure 2 and the related parts of the discussion.)

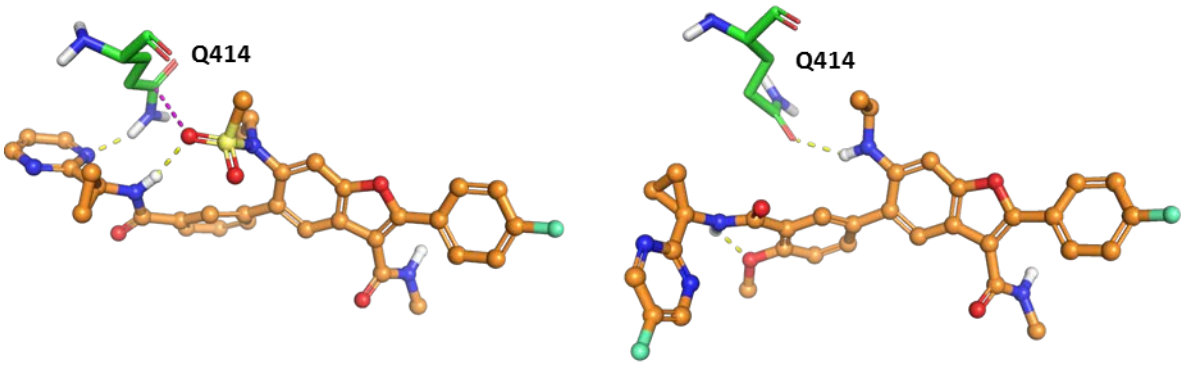

Figure S2. Close-up view of the interactions between ligand and Gln414 in the X-ray co-crystal structures of the complexes of gt-2a NS5B L30S with **5** (left) and **13** (right). Hydrogen bonds are denoted with dashed yellow lines and possible $n \rightarrow \pi^*$ interaction is shown as dashed purple line. Image generated with The PyMOL Molecular Graphics System (v. 2.0, Schrödinger, LLC).