Supplementary Material

Synthesis of Compounds 16, 17

Scheme 1 depicts the sequence of reactions that led to the preparation of compounds 16, 17 using 2,6-difluorobenzonitrile (35) as the starting material. 2,6-difluorobenzonitrile (35) was reacted by refluxing with sodium 2-benzyloxy-ethanolate in DMSO, which afforded 2-(2-benzyl-oxoxyethoxy)-6-fluorobenzonitrile (36) or 2,6-bis-(2-benzyloxy-ethoxy)-benzonitrile (41). To synthesize compound 16, 36 was reacted by refluxing with sodium benzyloxide in DMSO to afford 2-benzyloxy-6-(2-benzyloxyethoxy)-benzonitrile (37), which was subsequently hydrolyzed to give 2-benzyloxy-6-(2-benzyloxyethoxy)-benzamide (38) [1]. The key intermediate 40 was synthesized by the reaction of 9H-fluoren-9-yl isocyanate (39) with 38 in refluxing toluene. Finally, the target compound 16 was obtained by reacting 40 in 50% THF/MeOH with Pd/C in an atmosphere of hydrogen at room temperature. To synthesize compound 17, 41 was hydrolyzed to 2,6-bis-(2-benzyloxyethoxy)benzamide (42) [1], and then 17 was obtained following the same route used in the synthesis of 16.

Reagents and conditions: (a) C₆H₅CH₂OCH₂CH₂ONa, DMSO, 120 °C; (b) sodium benzyloxide, DMSO, 120 °C; (c) KOH, trace water, 130 °C; (d) toluene, reflux; (e) H₂, Pd/C, THF, MeOH, r. t.

Scheme S1. The synthesis of compounds 16, 17.
2-(2-Benzoxylthoxy)-6-fluorobenzonitrile (36). To a solution of 2-benzoxylthanol (0.96 g) in dimethyl sulfoxide (DMSO, 5 mL), was added 60% sodium hydride (0.156 g) and the mixture was stirred in an atmosphere of nitrogen at room temperature for 3 h. To the mixture, 2,6-difluorobenzonitrile (35, 0.47 g) was added and then the temperature was increased to 110 °C. The reaction mixture was stirred for 10 h and, after cooling, poured into 100 mL water, and extracted with dichloromethane (DCM, 40mL) three times. The organic layers were combined, dried with anhydrous sodium sulfate, and then concentrated to dryness. The residue was purified by flash column chromatography on silica gel, eluted with a mixture of ethyl acetate (EA)/PE (1:5, v/v), to afford 36 (0.77 g, 84%) as a white solid: 1H-NMR (CDCl3) δ: 7.54–7.44 (m, 1H), 7.44–7.25 (m, 5H), 6.80 (dt, J = 8.4, 3.8 Hz, 2H), 4.68 (s, 2H), 4.33–4.25 (m, 2H), 3.94–3.87 (m, 2H).

2,6-Bis-(2-benzoyloxyethoxy)-benzonitrile (41). To a solution of 2-benzoxylthethanol (1.7 g) in DMSO (5 mL), was added 60% sodium hydride (0.45 g) and the mixture was stirred in an atmosphere of nitrogen at room temperature for 3 h. To the mixture, 2,6-difluorobenzonitrile (35, 0.47 g) was added and then the temperature was increased to 110 °C. The reaction mixture was stirred for 10 h and, after cooling it was poured into water (100 mL), and extracted with DCM (40mL) three times. The organic layers were combined, dried with anhydrous sodium sulfate, then concentrated to dryness. The residue was purified by flash column chromatography on silica gel, eluted with a mixture of EA/PE (1:5, v/v), to afford 41 (1.1 g, 81%) as a white solid: 1H-NMR (CDCl3) δ: 7.49–7.21 (m, 12H), 6.54 (d, J = 8.5 Hz, 1H), 4.67 (s, 2H), 4.56 (s, 2H), 4.23 (t, J = 4.8 Hz, 2H), 3.88 (t, J = 4.8 Hz, 2H), 3.81–3.71 (m, 2H), 3.66–3.53 (m, 2H).

2-Benzoxyl-6-(2-benzoxylthoxy)-benzonitrile (37). To a solution of sodium benzyloxide (0.48 g) in DMSO (10 mL), was added 2-(2-benzoxylthoxy)-6-fluorobenzonitrile (36, 0.45 g) with stirring and then the temperature was rapidly increased to 110 °C for 10 h. The reaction mixture was cooled and poured into water (100 mL), and extracted with DCM (40 mL) three times. The organic layers were combined, dried with anhydrous sodium sulfate, then concentrated to dryness. The residue was purified by flash column chromatography on silica gel, eluted with a mixture of EA/PE (1:5, v/v), to afford 37 (0.53 g, 89%) as a white solid: 1H-NMR (CDCl3) δ: 7.42–7.15 (m, 11H), 6.49 (t, J = 8.3 Hz, 2H), 5.12 (s, 2H), 4.59 (s, 2H), 4.16 (t, J = 4.8 Hz, 2H), 3.85–3.78 (m, 2H).

2-Benzoxyl-6-(2-benzoxylthoxy)-benzamide (38). A mixture of 37 (0.36 g), tetrabutylammonium bromide (0.25 g) and potassium hydroxide (1 g) in benzyl alcohol (3 mL) and water (0.25 mL) was heated to 140 °C for 24 h, after which the solvent was distilled off. The residue was treated with water (50 mL), and the resulting solid collected was purified by flash column chromatography on silica gel, eluted with a mixture of EA/PE (1:4, v/v), to afford 38 (166 mg, 44%) as a white solid: 1H-NMR (400 MHz, CDCl3) δ: 7.44 (d, J = 7.3 Hz, 2H), 7.39–7.27 (m, 8H), 7.23 (t, J = 8.4 Hz, 1H), 6.61 (dd, J = 14.2, 8.4 Hz, 2H), 5.14 (s, 2H), 4.61 (s, 2H), 4.21 (t, J = 4.8 Hz, 2H), 3.84 (t, J = 4.8 Hz, 2H); EI-MS m/z 377.2 (M+).

1-[2-Benzoxyl-6-(2-benzoxylthoxy)-benzoyl]-3-(9H-fluoren-9-yl)-urea (40). To a solution of 9H-fluoren-9-yl isocyanate (39, 0.21 g) was added 38 (0.32 g) with stirring at room temperature. The mixture was heated to 110 °C for 10 h. The solvent was evaporated under vacuum to give the crude
product that was purified by flash column chromatography on silica gel, eluted with a mixture of THF/DCM/PE (1:5:3, v/v/v), to afford 40 (347 mg, 70%) as a white solid: EI-MS m/z 584.2 (M⁺).

1-(9H-Fluoren-9-yl)-3-[2-hydroxy-6-(2-hydroxyethoxy)-benzoyl]-urea (16). A mixture of 40 (0.5 g), 10% palladium on charcoal (0.2 g), methanol (30 mL) and THF (30 mL) was stirred at 40 °C for 48 h in an atmosphere of hydrogen. The solvent was evaporated under vacuum and then the residue was purified by flash column chromatography on silica gel, eluted with a mixture of THF/DCM/PE (1:4:3, v/v/v), to afford 16 (246 mg, 71%) as a white solid: mp 208–213 °C; ¹H-NMR (acetone-d₆) δ: 12.16 (s, 1H), 10.84 (s, 1H), 8.62 (d, J = 7.8 Hz, 1H), 7.86 (d, J = 7.5 Hz, 2H), 7.71 (d, J = 7.4 Hz, 2H), 7.42 (dd, J = 30.3, 12.8, 7.3 Hz, 5H), 6.71 (d, J = 8.3 Hz, 1H), 6.58 (d, J = 8.4 Hz, 1H), 6.10 (d, J = 8.1 Hz, 1H), 4.43–4.36 (m, 2H), 4.29 (t, J = 5.0 Hz, 1H), 4.09 (dd, J = 9.4, 4.8 Hz, 2H); ESI-MS m/z 403.0 [M−H]⁻; HRMS (ESI) m/z calcd C₂₃H₂₀N₂O₅ [M−H]⁻ 403.1294, found 403.1299.

2,6-Bis-(2-benzyloxyethoxy)-benzamide (42). A mixture of 41 (0.81 g), tetrabutylammonium bromide (0.5 g) and potassium hydroxide (2 g) in benzyl alcohol (5 mL) and water (1 mL) was heated to 140 °C for 24 h, after which the solvent was distilled off. The residue was treated with water (50 mL), and the resulting solid collected was purified by flash column chromatography on silica gel, eluted with a mixture of EA/PE (1:4, v/v), to afford 42 (440 mg, 52%) as a white solid: ¹H-NMR (CDCl₃) δ: 7.36–7.19 (m, 11H), 6.57 (d, J = 8.4 Hz, 2H), 6.07 (s, 1H), 5.70 (s, 1H), 4.59 (s, 4H), 4.18 (t, J = 4.9 Hz, 4H), 3.82 (t, J = 4.9 Hz, 4H); EI-MS m/z 422.2 (M⁺).

1-[2,6-Bis-(2-benzyloxyethoxy)benzoyl]-3-(9H-fluoren-9-yl)-urea (43). To a solution of 9H-fluoren-9-yl isocyanate (39, 0.19 g) was added 42 (0.32 g) with stirring at room temperature. The mixture was heated to 110 °C for 10 h. The solvent was evaporated under vacuum to give the crude product that was purified by flash column chromatography on silica gel, eluted with a mixture of THF/DCM/PE (1:5:3, v/v/v), to afford 43 (340 mg, 71%) as a white solid: mp 143–145 °C; ¹H-NMR (acetone-d₆) δ: 9.77 (s, 1H), 8.94 (d, J = 6.8 Hz, 1H), 7.82 (d, J = 7.6 Hz, 2H), 7.58 (d, J = 7.5 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.37–7.19 (m, 13H), 6.74 (d, J = 8.4 Hz, 2H), 6.10 (d, J = 8.1 Hz, 1H), 4.62 (s, 4H), 4.28–4.19 (m, 4H), 3.87–3.77 (m, 4H); EI-MS m/z 628.2 (M⁺); 91.0 (100%); HRMS (EI) m/z calcd C₃₉H₃₆N₂O₆ (M⁺) 628.2573, found 628.2573.

1-[2,6-Bis-(2-hydroxyethoxy)benzoyl]-3-(9H-fluoren-9-yl)-urea (17). A mixture of 43 (0.5 g), 10% palladium on charcoal (0.2 g), methanol (30 mL) and THF (30 mL) was stirred at 40 °C for 48 h in an atmosphere of hydrogen. The solvent was evaporated under vacuum and then the residue was purified by flash column chromatography on silica gel, eluted with a mixture of THF/DCM/PE (1:4:3, v/v/v), to afford 17 (221 mg, 62%) as a white solid: mp 158–163 °C; ¹H-NMR (acetone-d₆) δ: 9.82 (s, 1H), 8.83 (d, J = 7.7 Hz, 1H), 7.87 (d, J = 7.5 Hz, 2H), 7.74 (d, J = 7.5 Hz, 2H), 7.47 (t, J = 7.3 Hz, 2H), 7.43–7.32 (m, 3H), 6.77 (d, J = 8.4 Hz, 2H), 6.12 (d, J = 8.0 Hz, 1H), 4.21–4.15 (m, 4H), 3.96 (t, J = 5.9 Hz, 2H), 3.82 (dd, J = 10.2, 5.4 Hz, 4H); EI-MS m/z 448.2 (M⁺); 180.1 (100%); HRMS (EI) m/z calcd C₂₅H₂₄N₂O₆ (M⁺) 448.1634, found 448.1635.
HPLC Analysis Data of Compounds 14–33

Table S1. HPLC analysis data of compounds 14–33. The purities of identified compounds were essential to the conclusions drawn in the text and were determined by the same instrumentation under several different conditions given in the following table. The peak purity was checked by UV spectroscopy.

| Equipment | Agilent 1200 with quaternary pump and diode-array detector (DAD) |
|-----------|---------------------------------------------------------------|
| Column    | BECKMAN ULTRASPHERE C18 ODS column (4.6 × 250 mm, 5 μm particle size) |
| System Condition | CH₃OH/H₂O, 80% (v/v) of CH₃OH gradient, flow rate: 0.5 mL·min⁻¹; the relative purity of each compound was calculated at 254 nM |

| Compound | Retention Time (min) | Relative Purity (%) | Compound | Retention Time (min) | Relative Purity (%) |
|----------|----------------------|---------------------|----------|----------------------|---------------------|
| 14       | 8.67 a               | 99.0                | 15       | 7.87                 | 95.3                |
| 16       | 9.22                 | 97.3                | 17       | 7.52                 | 98.3                |
| 18       | 8.59                 | 95.2                | 26       | 8.15                 | 98.4                |
| 19       | 7.36                 | 95.0                | 27       | 9.82                 | 99.4                |
| 20       | 5.26                 | 98.1                | 28       | 11.09                | 96.9                |
| 21       | 7.79                 | 98.6                | 29       | 10.48                | 96.4                |
| 22       | 8.53                 | 95.2                | 30       | 12.56                | 95.3                |
| 23       | 8.46                 | 95.0                | 31       | 7.88                 | 98.0                |
| 24       | 8.60                 | 95.1                | 32       | 7.94                 | 96.0                |
| 25       | 9.36                 | 95.4                | 33       | 8.08                 | 96.7                |

a CH₃OH/H₂O, 90% (v/v) of CH₃OH gradient, flow rate: 0.5 mL·min⁻¹; the relative purity was calculated at 254 nM.

Establishment of HCV Virus and Subgenomic Replicon Cell Lines

The HCV virus assay was constructed by using the method developed as previously described with a small modification [2,3]. In brief, the pRLuc-JFH-1 plasmid was constructed as follows: based on the plasmid of pJFH-1, a gift from Apath, LLC (New York, NY, USA), a humanized Renilla luciferase reporter gene was introduced into the C-terminus of NS5A in the JFH-1 genome. The plasmid pRLuc-JFH-1 was made through digestion with XbaI restriction enzyme, and used as a template for RNA transcription. The virus transcripts were prepared in vitro by using the Ambion MEGAscript Kit, and then 10 μg RNA was mixed with 400 mL of Huh7.5.1 cells, which were a kind gift of Jin Zhong (Institute Pasteur of Shanghai, Chinese Academy of Science, Shanghai, China), at a concentration of 1 × 10⁷ cells·mL⁻¹. After electroporation, the Huh7.5.1 cells containing virus transcripts were seeded in a 10 cm dish. After cells were cultured for 4 days, the supernatant was collected and filtered to obtain the stock solution of virus JFH-1. To obtain virus titer, the virus stocks were diluted at a gradient of 1:10, and incubated the Huh7.5.1 cells for 48 h at 37 °C. Then the cells were harvested and the luminescence was detected as manufacturer’s protocol of Renilla-Glo™ Luciferase Assay System (Promega, Beijing, China).

The plasmids containing HCV replicon genotype 1b (Con-1), 1a (1a H77) containing a luciferase reporter gene was a gift from Apath, LLC. Creation of the plasmid of genotype 2a (2a JFH-1) subgenomic replicon has been reported previously [4]. All subgenomic HCV replicons encode an HCV internal
ribosome entry site (IRES)-driven neomycin cassette and encephalomyocarditis virus-controlled coding region containing a humanized Renilla luciferase reporter gene and the nonstructural NS3, NS4A, NS4B, NS5A, and NS5B polypeptide sequences, separated by a FMDV cleavage site. All stable a linearized bicistronic construct as previously. All Huh7.5.1-based replicon cell lines were grown subgenomic replicon cells were created by electroporating Huh7.5.1 cells with RNA transcribed from in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Twenty-four hours after electroporation, replicon-containing cell clones were selected using 0.5 mg·mL⁻¹ G418 (Gibco; Invitrogen, Shanghai, China). Stable replicon cell lines were selected and maintained in medium containing 0.5 mg·mL⁻¹ G418.

The mutational JFH-1 recombinant virus was constructed based on the phRluc-JFH-1 plasmid. Site-directed mutagenesis was performed using a Quick Change Lightning Site-Directed Mutagenesis kit (Stratagene, Shanghai, China). The mutagenic primers were design as follows: NS5BS282T-Sense: 5′-CAGACGTTCGCGCCACCCGCTAACCACTAG-3′, NS5BS282T-Antisense: 5′-CTAGTG GTTAGCACCACCGGTGGCGCGCAACGTCTTCTG-3′. The constructs were confirmed by sequencing.

**Solution Stability Assay**

The most potent compound 25 was selected as a candidate for further testing by the HPLC method [5]. To a solution of 25 (1 mg) in DMSO (6 mL) was added PBS buffer (48 mg NaCl, 1.2 mg KCl, 22 mg Na₂HPO₄·12H₂O, 1.4 mg KH₂PO₄, pH 7.4) at 0 °C. Then the samples were incubated at 37 °C. At defined times the solution was analyzed and samples were run on a BECKMAN ULTRASPHERE C₁₈ ODS column (4.6 × 250 mm, 5 μm particle size) at 25 °C with a flow rate of 0.5 mL·min⁻¹ of an isocratic eluent composed of methanol/water (70/30, v/v), and the eluted analytes were detected at 254 nm.

| Time (h) | Peak Area of 25 | 25 Content a (%) | Time (h) | Peak Area of 25 | 25 Content a (%) |
|---------|----------------|------------------|---------|----------------|------------------|
| 0       | 83.59          | 100              | 5.0     | 83.77          | 100.21           |
| 0.5     | 93.42          | 99.79            | 6.0     | 83.80          | 100.24           |
| 1.0     | 83.70          | 100.13           | 7.0     | 83.88          | 100.34           |
| 1.5     | 83.40          | 99.76            | 8.0     | 83.84          | 100.30           |
| 2.0     | 84.20          | 100.72           | 9.0     | 83.00          | 99.29            |
| 2.5     | 84.47          | 100.05           | 10.0    | 82.66          | 98.88            |
| 3.0     | 84.16          | 100.67           | 11.0    | 82.28          | 98.43            |
| 3.5     | 84.37          | 100.93           | 12.0    | 82.46          | 98.65            |
| 4.0     | 84.21          | 100.74           |         |                |                  |

*25 content (%) was calculated using the formula: peak area of 25 at each timepoint/the peak area of 25 at 0 h × 100%.

**Pharmacokinetic Studies**

The pharmacokinetic studies of compound 25 were carried out in SD rats (male SD rats (200 ± 20 g) × 3, provided by the Sun Yat-sen University Animals Center). Before administration, to a solution of 25 (4 mg) in N,N-dimethylacetamide (DMA, 100 μL) was added castor oil (900 μL), and then the solution was diluted with normal saline to 10 mL. The rats were administered at a dose of 5 mL·kg⁻¹ intravenously.
After intravenous administration, blood samples (0.2 mL) were collected at designated time points (0, 5, 15, 30, 45 min, 1, 2, 3, 5, 8, 12 and 24 h) from the eye venous sinus of the rats. The blood samples were centrifuged at 14800 rpm for 10 min to obtain the plasma fraction. The plasma samples were kept frozen (−20 °C) until analysis by a liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Table S3. Pharmacokinetic parameters after intravenous administration of 25 to SD rats at 5 mL·kg⁻¹.

| Pharmacokinetic Parameters | 25 (Intravenous Injection of 2 mg 25/kg) | 1 (Intravenous injection of 2mg 25/kg) |
|----------------------------|-------------------------------------|-------------------------------------|
|                           | Rat 1  | Rat 2  | Rat 3  | Mean ± SD | Rat 1  | Rat 2  | Rat 3  | Mean ± SD |
| b C max (ng·mL⁻¹)         | 13.40  | N g g  | 27.54  | 20.47 ± 9.99 | 698.12 | 52.28  | 1110.90 | 904.5 ± 291.9 |
| d AUC₀-∞ (ng·h·mL⁻¹)     | 5.66   | N g g  | 5.30   | 5.48 ± 0.25  | 335.12 | 78.78  | 323.69  | 329.4 ± 8.08  |
| f t₁/₂ (h)               | 5.40   | N g g  | 0.12   | 2.76 ± 3.73  | 1.84   | 1.10   | 1.21    | 1.53 ± 0.45   |
| e MRT (h)                | 2.42   | N g g  | 0.21   | 1.3 ± 1.6    | 0.94   | 1.98   | 0.55    | 0.75 ± 0.28   |

a Rat 2: parts of the sample of 25 was injected into the subcutaneous tissues which lead to the uncertainty of the plasma concentration, so this rat was not incorporated into the calculation of pharmacokinetic parameters;  
b C max: maximum plasma concentration taken directly from measured values; c T max: time to reach C max;  
d AUC₀-∞: integrated area under plasma concentration vs. time curve from time 0 to time infinity; e MRT: mean residence time; f t₁/₂: half-life. g N: parts of the data below the LLOQ, that result in the uncertainty of the pharmacokinetic parameters.

hERG Cardiac Toxicity Assay of Compound 25

Cell Culture and Cell Requirements

A CHO cell line stably transfected with hERG cDNA and expressing hERG channels was used for the study. The cells were cultured in medium containing the following components: Ham’s F12, 10% (v/v) heat inactivated FBS, 100 µg·mL⁻¹ hygromycin B and 100 µg·mL⁻¹ geneticin. The cells used in QPatch study must meet following criteria: under microscopy examination, the majority of cells in suspension should be single and isolated; their viability should be greater than 95%, with only a few debris and cell clumps (which may clog the holes in QPlate during whole-cell clamp recording); cell density should be ranged within 3–8 × 10⁶ cells·mL⁻¹ in the final suspension before applying to the QPatch stir chamber. After leaving CO₂ incubator, cells are maintained in serum-free medium buffered HEPES. Cells in such condition can be used for recording only for four hours after harvesting.

Recording System

Whole-cell recordings were performed using automated QPatch (Sophion, Ballerup, Denmark). The cells were voltage clamped at a holding potential of −80 mV. The hERG current was activated by
depolarizing at +20 mV for 5 s, after which the current was taken back to −50 mV for 5 s to remove the inactivation and observe the deactivating tail current. The maximum amount of tail current size was used to determine hERG current amplitude. The composition of the solutions used for the electrophysiological recordings were described in Table S4.

**Table S4.** Composition of internal and external solutions used in hERG QPatch studies a.

| Reagents  | External Solution (mM) | Internal Solution (mM) |
|-----------|------------------------|------------------------|
| CaCl₂     | 2                      | 5.374                  |
| MgCl₂     | 1                      | 1.75                   |
| KCl       | 4                      | 120                    |
| NaCl      | 145                    | -                      |
| Glucose   | 10                     | -                      |
| HEPES     | 10                     | 10                     |
| EGTA      | -                      | 5                      |
| Na-ATP    | -                      | 4                      |
| pH        | 7.4 (adjusted with NaOH), osmolarity ~305 mOsm | 7.25 (adjusted with KOH), osmolarity ~290 mOsm |

a solutions recommended by Sophion.

**Automated QPatch Procedures**

After achieving break-in (whole-cell) configuration, the cells were recorded for 120 s to assess current stability. The voltage protocol described above was then applied to the cells every 20 s throughout the whole procedure. Only stable cells with recording parameters above threshold were allowed to enter the drug addition procedure.

External solution containing 0.2% DMSO (vehicle) was applied to the cells to establish the baseline. After allowing the current to stabilize for 3 min, compound 25 (20 mM stocked in DMSO) was applied. The solution of 25 (40, 8, 1.6, 0.32, 0.064 and 0.0128 µM, first diluted in DMSO, and then serial-diluted in external solution to the final µM ranges before QPatch test. Final DMSO concentration was 0.2%) was added and the cells were kept in the test solution until the compound’s effect reached a steady state or for a maximum of 3 min. Washout with external solution might be performed until the recovery of the current reached a steady state. Positive control cisapride (dosing started at 3 µM) is used in the experiments to test the same batch of cells used for test compounds to ensure the normal response and the good quality of the cells. The method described above was performed according to [6–8].

**Supporting References**

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