Supporting Information

Discovery of Piperazinylquinoline Derivatives as Novel Respiratory Syncytial Virus Fusion Inhibitors

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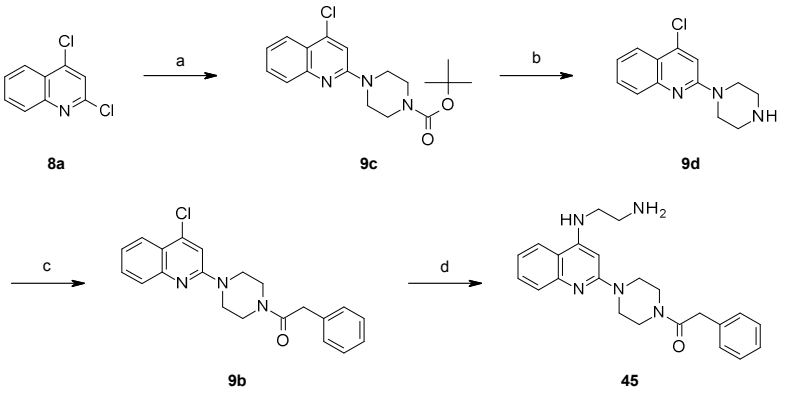
Virtual screening:

MOS(Maximal overlap set, 2D similarity) and ROCS(Rapid Overlay of Chemical Structures, 3D similarity) were used in parallel to screen the Roche Smart library (more than one million small molecules) for potential RSV fusion inhibitors. The ligands JNJ-2408068 (**1**), TMC-353121 (**2**) and BMS-433771 (**3**) were chosen as reference compounds for the virtual screening. Each method chose the best 1000 similar compounds to every reference compound in the library based on similarity scores, which resulted in totally 6000 similar compounds for 3 reference compounds. After removing the duplicated structures, less than 3,000 compounds were finally selected by the chemists for anti-RSV activity in reduction of cytopathic effect (CPE) assay based on chemical stability and drug-likeness.

Experimental details:

All starting materials were obtained commercially and were used without further purification. All reported yields are of isolated products and are not optimized. All the final compounds were purified by preparative HPLC on reversed phase column using X BridgeTM Perp C18 (OBDTM 30 × 100 mm) column or SunFireTM Perp C18 (5 m, OBDTM 30 × 100 mm) column. The high resolution mass spectra were run on an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. And NMR Spectra were obtained using Bruker Avance 400MHz. All the target compounds (7, 12-56) have purities of > 95% based upon LC/MS, and ^{1}H-NMR.

Synthesis of Piperazinylquinoline 45[a]



[a] Reagents and conditions: (a) *tert*-butyl piperazine-1-carboxylate, DIPEA, toluene, reflux; (b) HCl/EtOAc, 5 °C; (c) 2-phenylacetyl chloride, TEA, DCM, 0 °C to r.t.; (d) ethane-1,2-diamine, neat, microwave, 100 to 160 °C.

To a solution of **8a** (27 g, 0.14 mol) in 300 mL of toluene was added *tert*-butyl piperazine-1-carboxylate (50 g, 0.27 mol) and DIPEA (10 mL). The resulting mixture was heated with stirring at 110 °C for 16 h. The solvent was removed *in vacuo*, and the residue was suspended in 200 mL of water. The aqueous was extracted with DCM (200 mL × 3). The combined organic layer was washed with water (200 mL × 2) and dried with anhydrous sodium sulfate. The solvent was concentrated *in vacuo* and the residue was purified by column (eluting with 5% to 10% EtOAc in PE) to give the product **9c** (14 g, yield: 29%).

To a solution of **9c** (14 g, 0.04 mol) in 50 mL of EtOAc was added 4N of HCl(g)/EtOAc solution (200 mL) slowly at 0 to 5°C. After the addition, the resulting mixture was kept at this temperature for 14 h. The solvent was removed *in vacuo* to give the product **9d** as a white solid (10.2 g, HCl salt, yield: 90%).

To a solution of **9d** (500 mg, 2 mmol) in 20 mL of DCM was added 2-phenylacetyl chloride (500 mg, 3.2 mmol) and 3 drops of Et_3N slowly at 0 °C in an ice-water bath. After being stirred at room temperature overnight, the resulting mixture was poured into 40 mL of water and extracted with DCM (20 mL × 2). The combined organic layer was dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by preparative TLC (eluting with 30% of EtOAc in PE) to give the desired product **9b** (220 mg, yield: 30%).

The mixture of compound **9b** (220 mg, 0.6 mmol) and ethane-1,2-diamine (150 mg, 2.5 mmol) was heated in microwave at 130 °C for 1 h. The crude product was separated by HPLC to give **45** (105 mg, yield: 45%). 97% purity; HRMS m/z: $[M+H]^+$ calcd for C25H31N5O, 418.2601; found, 418.2602; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 1.60 (s, 6 H), 3.37 - 4.05 (m, 12 H), 6.00 (s, 1 H), 7.26 - 7.36 (m, 3 H), 7.39 - 7.46 (m, 2 H), 7.50 (ddd, J = 8.34, 6.71, 1.51 Hz, 1 H), 7.70 - 7.82 (m, 2 H), 8.11 (d, J = 8.28 Hz, 1 H).

Analog **7**: 95% purity; HRMS m/z: $[M+H]^+$ calcd for C23H28N4O, 377.2336; found, 377.2332; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 1.75 - 1.95 (m, 2 H), 2.05 (d, J = 11.47 Hz, 2 H), 2.97 (dd, J = 12.79, 9.70 Hz, 2 H), 3.20 (dd, J = 12.79, 2.87 Hz, 1 H), 3.32 - 3.45 (m, 2 H), 3.54 - 3.67 (m, 2 H), 4.13 - 4.24 (m, 1 H), 4.42 (d, J = 13.45 Hz, 2 H), 6.19 (s, 1 H), 7.16 - 7.22 (m, 1 H), 7.24 - 7.36 (m, 4 H), 7.46 (ddd, J = 8.38, 5.84, 2.32 Hz, 1 H), 7.66 - 7.80 (m, 2 H), 8.10 (d, J = 8.16 Hz, 1 H).

Analog **22**: 97% purity; HRMS m/z: $[M+H]^+$ calcd for C23H27N5O, 390.2288; found, 390.2283; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 3.25 (s, 2 H), 3.68 - 3.91 (m, 12 H), 6.05 (s, 1 H), 7.17 - 7.35 (m, 5 H), 7.46 (d, J = 5.07 Hz, 1 H), 7.74 (br. s., 2 H), 8.11 (d, J = 8.38 Hz, 1 H).

Analog **27**: 98% purity; HRMS m/z: $[M+H]^+$ calcd for C23H26ClN5O, 424.1899; found, 424.1898; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 3.31 - 3.34 (m, 2 H), 3.76 - 3.96 (m, 12 H), 6.06 (s, 1 H), 7.16 - 7.34 (m, 4 H), 7.47 (ddd, J = 8.38, 5.18, 3.20 Hz, 1 H), 7.72 - 7.77 (m, 2 H), 8.11 (d, J = 8.38 Hz, 1 H).

Analog **30**: 96% purity; HRMS m/z: $[M+H]^+$ calcd for C23H26FN5O, 408.2194; found, 408.2194; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 3.34 - 3.38 (m, 2 H), 3.71 - 4.07 (m, 12 H), 6.10 (s, 1 H), 6.93 - 7.20 (m, 3 H), 7.38 (td, J = 7.97, 6.15 Hz, 1 H), 7.51 (ddd, J = 8.41, 5.02, 3.39 Hz, 1 H), 7.72 - 7.83 (m, 2 H), 8.14 (d, J = 8.28 Hz, 1 H).

Analog **33**: 96% purity; HRMS m/z: $[M+H]^+$ calcd for C24H29N5O, 404.2445; found, 404.2441; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 2.29 (s, 3 H), 3.28 (s, 1 H), 3.32 - 3.37 (m, 1 H), 3.63 - 3.89 (m, 12 H), 6.05 (s, 1 H), 7.00 - 7.11 (m, 3 H), 7.15 - 7.25 (m, 1 H), 7.46 (ddd, J = 8.32, 6.45, 1.98 Hz, 1 H), 7.71 - 7.78 (m, 2 H), 8.06 - 8.19 (m, 1 H).

Analog **36**: 97% purity; HRMS m/z: $[M+H]^+$ calcd for C24H29N5O2, 420.2394; found, 420.2395; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 3.34 (d, J = 6.17 Hz, 2 H), 3.68 - 3.90 (m, 12 H), 3.76 (s, 3 H), 6.06 (s, 1 H), 6.75 - 6.93 (m, 3 H), 7.23 - 7.29 (m, 1 H), 7.45 - 7.57 (m, 1 H), 7.75 (d, J = 3.53 Hz, 2 H), 8.06 - 8.18 (m, 1 H).

Analog **43**: 96% purity; HRMS m/z: $[M+H]^+$ calcd for C24H29N5O, 404.2445; found, 404.2447; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 1.45 (d, J = 6.78 Hz, 3 H), 3.21 - 3.29 (m, 1 H), 3.34 - 3.36 (m, 1 H), 3.62 - 4.04 (m, 10 H), 4.16 (q, J = 6.86 Hz, 1 H), 6.03 (s, 1 H), 7.18 - 7.42 (m, 5 H), 7.51 (ddd, J = 8.34, 6.34, 1.88 Hz, 1 H), 7.71 - 7.83 (m, 2 H), 8.12 (d, J = 8.28 Hz, 1 H).

Analog **48**: 95% purity; HRMS m/z: $[M+H]^+$ calcd for C26H31N5O, 430.2601; found, 430.2605; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 1.87 - 2.02 (m, 1 H), 2.04 - 2.17 (m, 1 H), 2.43 - 2.57 (m, 2 H), 2.82 - 2.97 (m, 2 H), 3.27 - 3.31 (m, 2 H), 3.35 - 3.42 (m, 3 H), 3.69 - 3.99 (m, 7 H), 6.01 (s, 1 H), 7.25 - 7.34 (m, 1 H), 7.39 - 7.55 (m, 5 H), 7.72 - 7.81 (m, 2 H), 8.11 (d, J = 8.28 Hz, 1 H).

Analog **50**: 98% purity; HRMS m/z: $[M+H]^+$ calcd for C26H33N5O, 432.2758; found, 432.2759; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 1.58 (s, 6 H), 2.49 (s, 3 H), 2.88 - 3.99 (m, 12 H), 5.96 (s, 1 H), 7.19 (t, J = 7.04 Hz, 1 H), 7.26 - 7.37 (m, 3 H), 7.38 - 7.44 (m, 2 H), 7.45 - 7.56 (m, 2 H), 7.87 (d, J = 8.03 Hz, 1 H).

Analog **53**: 98% purity; HRMS m/z: $[M+H]^+$ calcd for C25H30N4O2, 419.2442; found, 419.2443; ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm: 1.60 (s, 6 H), 3.36 - 3.98 (m, 12 H), 6.00 (s, 1 H), 7.25 - 7.36 (m, 3 H), 7.39 - 7.53 (m, 3 H), 7.67 - 7.78 (m, 2 H), 8.13 (d, J = 8.28 Hz, 1 H).

Biological assays and results:

Viral cytopathic effect (CPE) assay: To measure anti-RSV activity of compounds, 96-well plates are seeded with 6x10^3 HEp-2 cells per well in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells are infected the next day with sufficient RSV Long strain (ATCC) to produce an approximately 80-90% cytopathic effect after 6 days, in the presence of serial half-log diluted compound in a total volume of 200 µL

per well. The viability of cells is assessed after 6 days using Cell Counting kit-8 (Dojindo Molecular Technologies). The absorbance at 450 nm and referenced at 630 nm is measured to determine 50% effective concentration (EC_{50}).

Cytotoxicity assay: The procedure is similar with CPE assay. For cytotoxicity evaluation, RSV viruses were not added and 50% cytotoxicity concentration (CC_{50}) was determined by CCK8.

RSV fusion inhibition assay: F gene of RSV Long strain was codon-optimized and synthesized by GenScript. Then the full length of F gene was clone into pcDNA5 TOPO TA vector (Invitrogen). Then RSV-F stable cell lines were generated by co-transfection of the RSV-F-pcDNA5 and pOG44 plasmid into the Flp-In T-Rex 293 cell line (Invitrogen) and 100 μg/ml hygromycin B was used to select stable transfectants. RSV-F expression was induced by 1μg/ml tetracycline. Compounds were serial diluted and added into the RSV-F expressing cells. The cell-cell fusion was observed by microscopy at three days after compound addition.

Plaque reduction assay: Hep-2 cell monolayers were infected with 1 ml of 400 PFU/ml of RSV Long strain per well of 6-well plate with or without the presence of serial diluted compounds. After two hours, cells were overlaid with DMEM/F12 medium containing 2% FBS and 0.55% agarose. Plates were incubated for three days and the cells were then fixed with 4% paraformaldehyde for six hours. The agarose plugs were removed, and viral plaques were visualized by immunostaining. Cells were blocked with 1×TBS buffer with 1% BSA and 0.5% Triton X-100. Plates were then incubated in the presence of a mouse anti-RSV monoclonal antibody (NCL-RSV3; Novocastra) at 1:500 dilution followed by a rabbit anti-mouse horseradish peroxidase-labeled secondary antibody. The plaque staining was developed with 4-chloro-1-naphthol in the presence of hydrogen peroxide.

Single dose PK study: To evaluate PK parameters of compounds in male Wistar rats, **45** and **50** were dissolved in 6% Solutol solution (Solutol:Ethanol, 1:1, v/v) and 94% 0.9% saline to yield a final concentration of 2 mg/mL for intravenous administration (pH = 7) and in 0.445% microcrystalline cellulose, 0.055% carboxymethyl cellulose sodium and 1.6% lactose water solution to yield a final concentration of 2 mg/mL for oral administration (pH = 5-6). The

plasma samples were collected after dosing, and the drug concentrations in plasma were determined by LC-MS/MS.

Table SI-1. SDPK study of 45 and 50

Compds	Cl (mL/min/kg)[a]	$T_{1/2}$	V_{ss} (L/kg)	$AUC_{(0-t)}$ (ng*h/mL)[b]	F (%)
45	142.6	3 h	36.9	401.10	57
50	222	7.3 h	67.9	463.43	41

[a] Single dose i.v. administration of 2 mg/kg to male Wistar rats.

[b] Single dose p.o. administration of 10 mg/kg to male Wistar rats.

Figure SI-1. Plaque reduction assay of 45 and 50

Compound **45** and **50** can potently inhibit RSV replication in plaque reduction assay. The viral plaques were visualized by immuno-staining. The plaque reduction assay was repeated three times, and representative images are presented. Plaques were detected in Elispot reader (Autoimmun Diagnostika GmbH Germany) and counted using the AID EliSpot Software 'algorithm C' with emphasis settings were set on “tiny”. The EC_{50} was analyzed by non-linear regression using GraphPad™ Prism™ software. The EC_{50} of Compound **45** was 39.8 nM (35.6-44.3 nM, 95% CI); EC_{50} of Compound **50** was 122.6 nM (94.9-158.4 nM, 95% CI).

Fig. SI-1, A: # cpd 45, repeat-1 (raw-data)

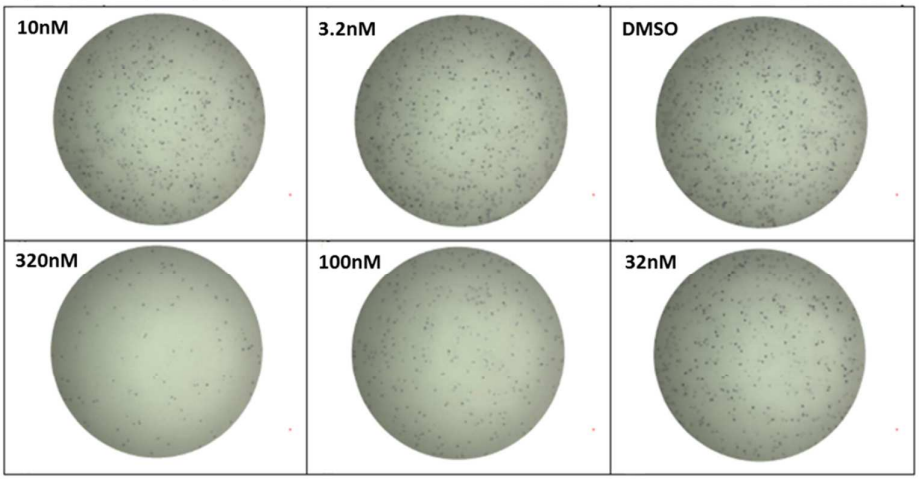


Fig. SI-1, B: # cpd 50, repeat-1 (raw-data)

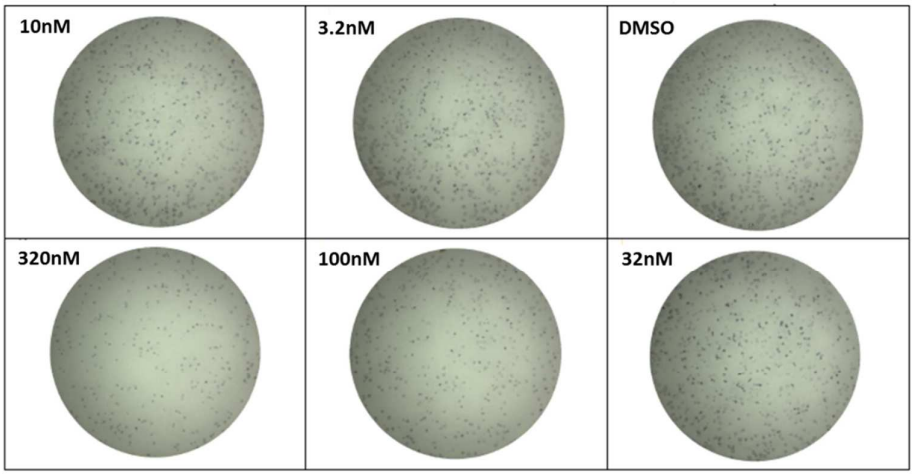


Fig. SI-1, C: # cpd 45, repeat-1 (PFUs were counted with Elispot reader, AID)

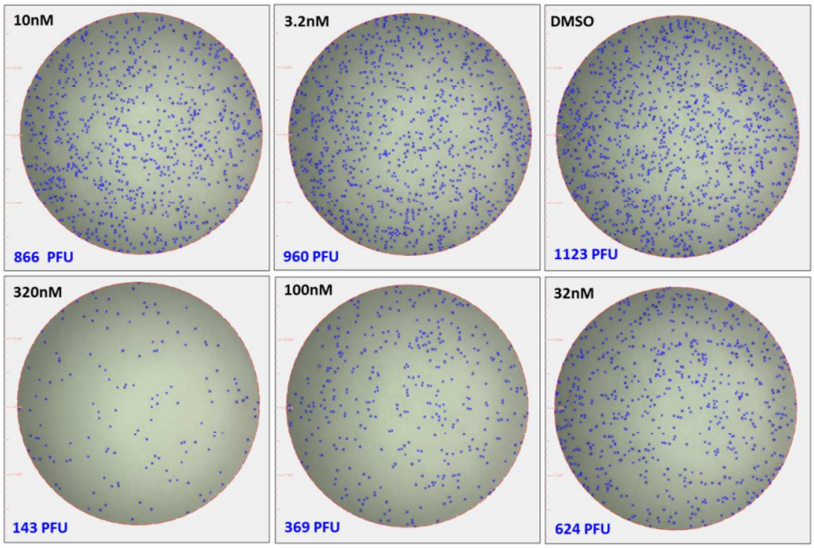


Fig. SI-1, D: # cpd 50, repeat-1 (PFUs were counted with Elispot reader, AID)

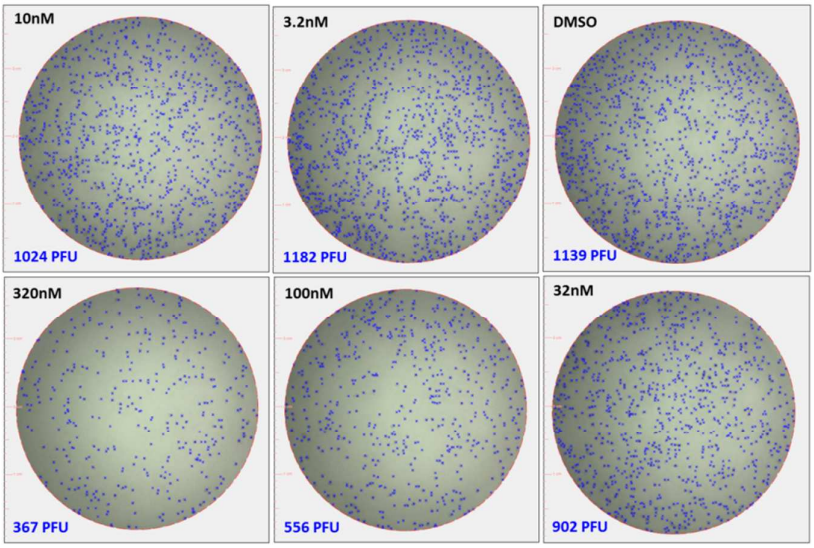
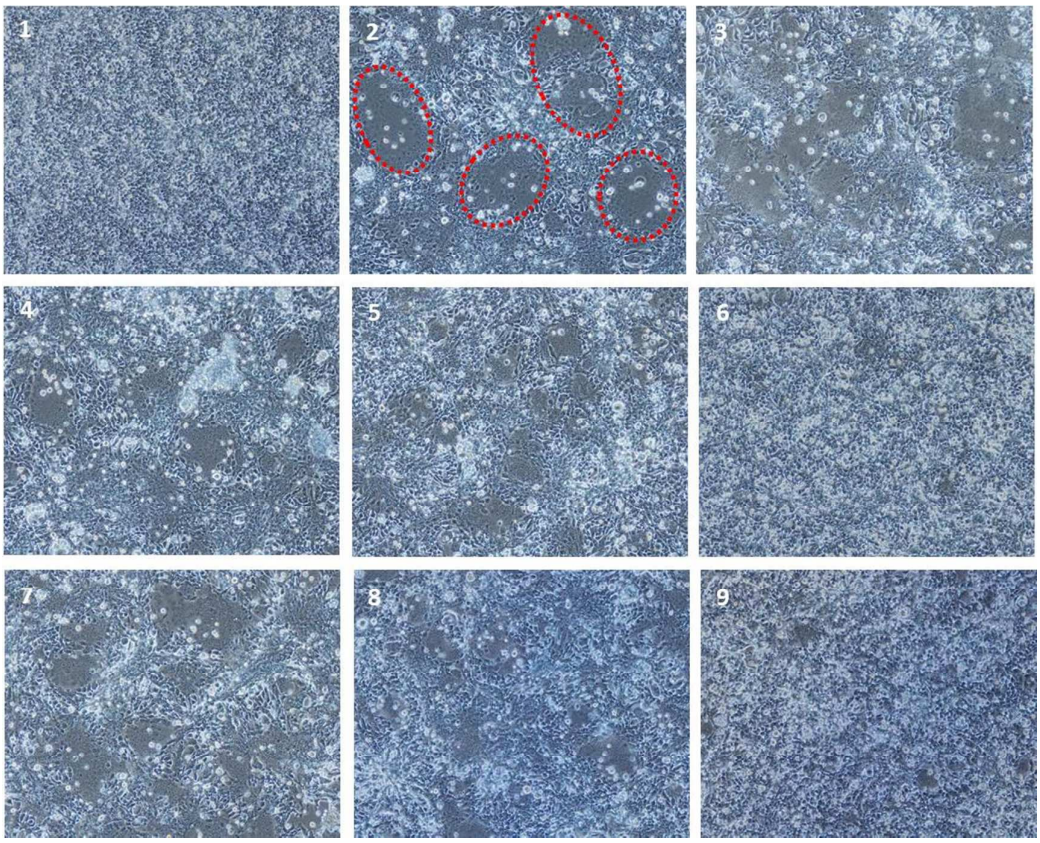


Figure SI-2. Fusion inhibition assay of 45 and 50

Compound **45** and **50** can potently inhibit cell-cell fusion in RSV-F expressing cells. The cell fusion induced by RSV Fusion protein was marked with red circles.

1. Negative control, without tetracycline;
2. Positive control, 1 μg/ml tetracycline;
3. 0.1 nM compound **45**;
4. 1 nM compound **45**;
5. 10 nM compound **45**;
6. 100 nM compound **45**;
7. 1 nM compound **50**;
8. 10 nM compound **50**;
9. 100 nM compound **50**.



Docking procedure:

Compound **7** and **45** were docked into the binding pocket (reported by McLellan, J. S.[23, 24]) by Gold (V5.3) with default parameters. 10 best docking poses were generated for each molecule. After the analysis of these 10 docking poses of each molecule, the binding pose of the molecule with a reasonable conformation and a best-fit to the binding pocket (judged by the modeler) was picked.

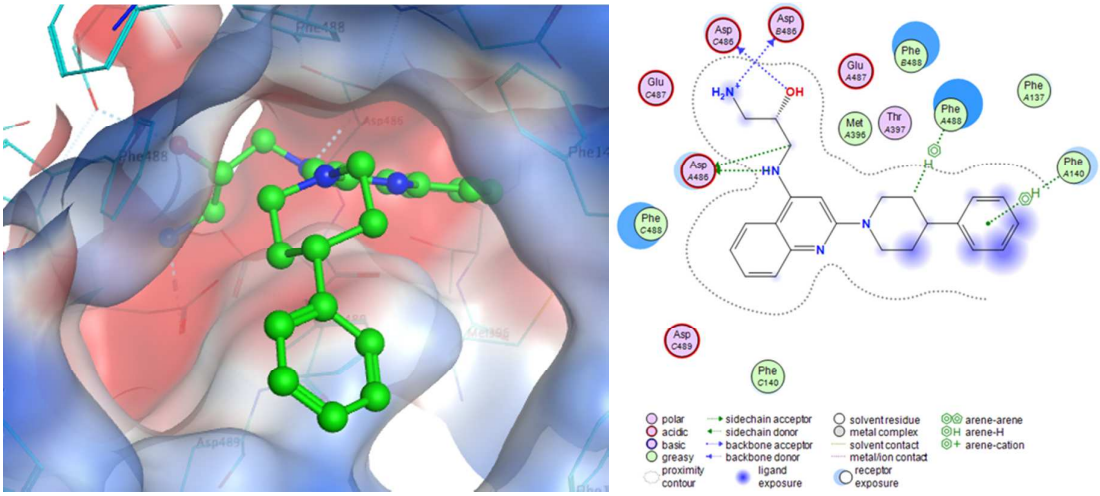


Figure SI-3. Docking model of 7 to RSV pre-fusion protein: Top view (left) of the compound **7** in the binding pocket of RSV F. The binding pocket is shown as the molecular surface with negative-charged area in red and positive-charged area in blue. Compound **7** is shown as ball-and-stick, with carbon atoms colored in green, nitrogen atoms in blue, oxygen atoms in red. 2D ligand-interaction diagram (*right*) was generated in MOE. The interactions of compound **7** with RSV F main-chain and side-chain atoms are shown as blue and green dotted lines respectively. And the arrowhead points to the acceptor

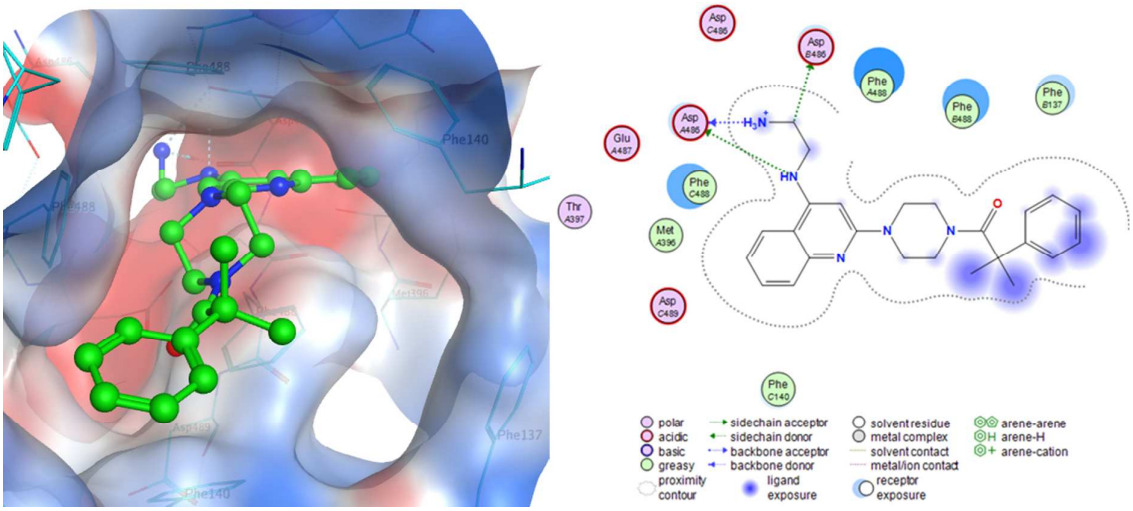


Figure SI-4. Docking model of 45 to RSV pre-fusion protein: Top view (left) of the compound **45** in the binding pocket of RSV F. The binding pocket is shown as the molecular surface with negative-charged area in red and positive-charged area in blue. Compound **45** is shown as ball-and-stick, with carbon atoms colored in green, nitrogen atoms in blue, oxygen atoms in red. 2D ligand-interaction diagram (*right*) was generated in MOE. The interactions of compound 45 with RSV F main-chain and side-chain atoms are shown as blue and green dotted lines respectively. And the arrowhead points to the acceptor.