Supporting Information

2,4,5-Trisubstituted Pyrimidines as Potent HIV-1 NNRTIs: Rational Design, Synthesis, Activity Evaluation, and Crystallographic Studies

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Table S1. Data collection and refinement statistics

	RT52A-16c (PDB ID 7KWU)
Wavelength (Å)	1.03326
Number of merged data sets	3
Resolution range	29.4 - 2.02 (2.06 - 2.02)
Space group	C2
Unit cell (Å / deg)	163.379 72.622 109.723 / 90 100.205 90
Unique reflections	83,044 (4,512)
Multiplicity	17.8 (13.7)
Completeness (%)	99.95 (100.00)
Mean I/sigma(I)	9.6 (1.3)
Wilson B-factor (Å^2)	32.778
R-merge	0.503 (12.136)
R-meas	0.518 (12.606)
R-pim	0.124 (3.396)
CC1/2	0.995 (0.383)
R-work	0.2116 (0.3129)
R-free	0.2588 (0.3655)
Number of non-hydrogen	8,826
macromolecules	8,039
ligands	91
solvent	696
Protein residues	970
RMS bonds (Å)	0.003
RMS angles (deg)	0.66
Ramachandran favored (%)	97.20
Ramachandran allowed (%)	2.70
Ramachandran outliers (%)	0.10
Rotamer outliers (%)	0.11
Clashscore	4.79
Average B-factor (Å^2)	51.41
macromolecules	51.28
ligands	69.31
solvent	50.61

Statistics for the highest-resolution shell are shown in parentheses

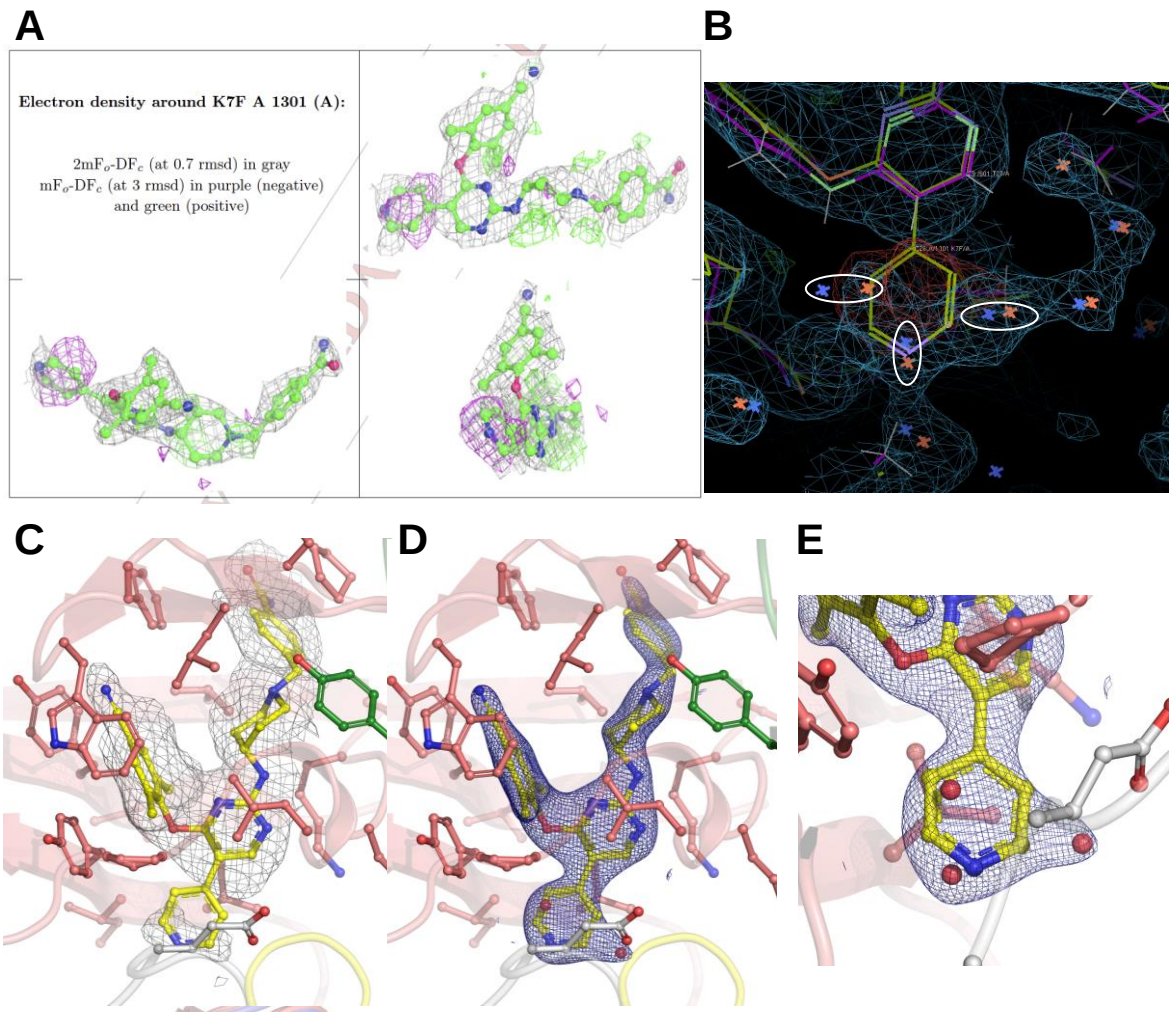

Figure S1. A) Electron density of compound **16c** (legend included), as appearing in the eposition validation report of the RT52A-**16c** structure. Negative density is observed in the 4-pyridyl substituent location, while positive density is observed beside the piperidine moiety. The first may be due to displacement of water molecules present in the RT NNIBP pocket before ligand binding and have been modeled in an alternative conformation to **16c**. The second might reflect the flexibility of the piperidine moiety, likely present in more than one conformation (but not possible to model reliably). B) Zoomed-in of the 4-pyridyl substituent binding to the RT NNIBP of the RT52A-**16c** structure (yellow sticks and red water molecules) superposed to the RT-RPV structure (PDB ID 4G1Q, violet sticks and blue water molecules), with the 2Fo – Fc map (blue mesh, 0.5σ) of the first. The snapshot shows that there are three water molecules (conserved in the RT-RPV structure and circled) displaced by the substituent. C) Electron density (in grey) of compound **16c** in the structure (calculated through a Polder omit map at 3.5σ contour, reference for Polder omit map: PMID 28177311), with color code as in the main manuscript. D) Electron density (in blue) of compound **16c** in the structure (calculated through a maximum entropy map (MEM) at 1σ contour, aimed at improving the phases, reference for MEM map: PMID 24419387), with color code as in the main manuscript. E) Zoomed-in of D) in the region of the 4-pyridyl substituent, showing the improved electron density for the substituent and the displaced water molecules. F) Superposition of the the RT52A-**16c** structure with related RT-piperidine-substituted NNRTI structures.

***In vitro* assay of anti-HIV activities in MT-4 cells**

The anti-HIV activity and cytotoxicity of the newly synthesized derivatives were evaluated with wild-type HIV-1 strain (HIV-IIIB), single mutant strains (L100I, K103N, Y181C, Y188L, and E138K), double mutant strains (F227L+V106A and K103N+Y181C), and HIV-2 (strain ROD) in MT-4 cell cultures using the MTT method as described previously.[1, 2] Stock solutions (10×final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells in order to allow simultaneous evaluation of the effects on mock- and HIV-infected cells. Using a Biomek 3000 robot (Beckman Instruments, Fullerton, CA), serial five-fold dilutions of the test compounds (final 200 μL volume per well) were made directly in flat-bottomed 96-well microtiter trays, including untreated control HIV-1 and mock-infected cell samples for each sample. HIV-1 strains stock (50 μL at 100-300 $CCID_{50}$) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of compounds on uninfected cells to assess cytotoxicity. Exponentially growing MT-4 cells were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6×105 cells/mL, and 50 μL aliquots were transferred to the microtiter tray wells. At five days after infection, the viability of mock- and HIV-infected cells was determined spectrophotometrically with MTT assay.

The MTT assay is based on the reduction of yellow-colored MTT (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to form a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer at the wavelengths of 540 and 690 nm. All data were calculated using the median optical density (OD) value of three wells. The EC_{50} was defined as the concentration of the test compound affording 50% protection from viral cytopathogenicity.

The CC_{50} was defined as the compound concentration that reduced the absorbance of mock-infected cells by 50%.

Recombinant HIV-1 reverse transcriptase (RT) inhibitory assays

The HIV-1 RT inhibition assay was performed with an RT assay kit (Roche). The procedure was conducted as described in the kit protocol.[2] Firstly, The mixture of HIV-1 RT enzyme, reconstituted template and viral nucleotides [digoxigenin (DIG)-dUTP, biotin-dUTP and dTTP] in the incubation buffer with or without inhibitors was incubated for 2 h at 37ºC. Then, the mixture was transferred to a streptavidin-coated microtitre plate (MTP) and incubated for another 1 h at 37ºC. The biotin-labeled dNTPs that were incorporated into the cDNA chain in the presence of RT were bound to streptavidin. The unbound dNTPs were washed with washing buffer, and anti-DIG-POD was added to the MTPs.

After incubation for 1 h at 37ºC, the DIG-labeled dNTPs incorporated in cDNA were bound to the anti-DIG-POD antibody. The unbound anti-DIG-PODs were washed out and the peroxide substrate (ABTS) solution was added to the MTPs. The absorbance of the sample was determined at OD_{405} nm using a microtiter plate ELISA reader. The IC_{50} values correspond to the concentrations of the inhibitors required to inhibit biotin-dUTP incorporation by 50%.

Pharmacokinetics assays

Six male Sprague Dawley rats (200-220 g) were randomly divided into two groups to receive intravenous (2 $mg.kg^{-1}$) or oral administration (20 $mg.kg^{-1}$). A solution of **16c** was prepared by dissolving in a mixture of polyethylene glycol (peg) 400/normal saline (70/30, V/V). Blood samples of the intravenous group were collected from the sinus jugular is into heparinized centrifugation tubes at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6h and 10 h after dosing, and blood samples of the oral administration group were collected at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 10h,

and 12 h after dosing. Blood was taken at 100 μL each time. All the samples were centrifuged at 8000 rpm for 8 min to separate plasma. LC-MS/MS analysis was used to determine the concentration of **16c** in plasma. Briefly, 50 μL of plasma was added to 50 μL of internal standard and 300 μL of methanol in a 5 mL centrifugation tube, which was centrifuged at 3000g for 10 min. The supernatant layer was collected and a 20 μL aliquot was injected for LC-MS/MS analysis. Standard curves for **16c** in blood were generated by the addition of various concentrations of **16c** together with internal standard to blank plasma. Then all samples were quantified with an Agilent 1200 LC/MSD (Agilent, USA). The mobile phase was methanol/1.5% glacial acetic acid (50:50, V/V) at a flow rate of 1.0 mL/min, the test wavelength was 225 nm.

Acute toxicity experiment

Kunming mice (18-20 g) were purchased from the animal experimental center of Shandong University. Compounds **16c** was suspended in PEG-400 and normal saline at concentrations of 100 mg•mL^{-1}, and administered intragastrically by gavage after the mice had been fasted for 12 hours. Dosage of 2000 mg•kg^{-1} was administered to 10 mice per group (5 males, 5 females).

Assay procedures for hERG activity[3]

HEK293 cells with stably transfected with hERG cDNA was selected to test the inhibitory activity to the hERG potassium channel. HEK239 cells expressing hERG were plated in 35 mm dishes for 24 hours and maintained at 37 °C under 5% CO_2. A micropipette was drawn out from borosilicate glass to give a tip resistance between 3 ~ 5 MΩ. For each trial, a single dish of cells was removed from the incubator, washed two times with ECS and placed on the microscope stage. The whole-cell recordings were conducted with a commercially available patch clamp amplifier.

Tail currents were evoked once every 30 s by a 3 s, -50 mV repolarizing pulse following a 2 s, +50 mV depolarizing pulse with a hold voltage of -80 mV. A 50 ms depolarizing pulse to -50

mV at the beginning of the voltage protocol served as a baseline for calculating the amplitude of the peak tail current. Only stable cells with recording parameters exceed threshold were used for experiments. The hERG currents were allowed to stabilize over a 3-minute period under the condition that vehicle alone prior to test compound application. The cells were kept in the test solution until the peak tail current was stable (< 5% change) for ~5 sweeps. Peak tail amplitudes were then plotted as a function of the sweep number. Five peak tail current measurements at the steady state before the test compound application were averaged and used as the control current amplitude. Four or five peak tail current measurements at the steady state after test compound application were averaged and used as the remaining current amplitude after inhibition by the test compound.

References

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