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Pharmacophore-based design of novel 3-hydroxypyrimidine-2,4-dione subtypes as inhibitors of HIV reverse transcriptase-associated RNase H: tolerance of a nonflexible linker

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Abstract

The pharmacophore of active site inhibitors of human immunodeficiency virus (HIV) reverse transcriptase (RT)-associated RNase H typically entails a flexible linker connecting the chelating core and the hydrophobic aromatics. We report herein that novel 3-hydroxypyrimidine-2,4-dione (HPD) subtypes with a nonflexible C-6 carbonyl linkage exhibited potent and selective biochemical inhibitory profiles with strong RNase H inhibition at low nM, weak to moderate integrase strand transfer (INST) inhibition at low μ M, and no to marginal RT polymerase (pol) inhibition up to 10 μ M. A few analogues also demonstrated significant antiviral activity without cytotoxicity. The overall inhibitory profile is comparable to or better than that of previous HPD subtypes with a flexible C-6 linker, suggesting that the nonflexible carbonyl linker can be tolerated in the design of novel HIV RNase H active site inhibitors.

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Appendix A. Supplementary Material

Synthesis and characterization data of intermediates **11a-11e**, **12e-k**, **13a-f**, **14a-l**. This material is available free of charge via the Internet at

Graphical abstract

Keywords

Human Immunodeficiency Virus (HIV); reverse transcriptase (RT); RNase H; inhibitors; 3-hydroxyisoxanthine-2,4-dione (HPD)

Introduction

HIV RT consists of two distinct domains[1]: a pol domain responsible for both RNA-dependent and DNA-dependent viral DNA polymerization, and an RNase H domain [1–2] which degrades the RNA strand from the RNA/DNA heteroduplex reverse transcription intermediate and processes primers for both RNA-dependent and DNA-dependent viral DNA polymerization[3]. RT pol is targeted by all currently known nucleoside RT inhibitors (NRTIs) [4] and non-nucleoside RT inhibitors (NNRTIs)[5], whereas inhibitors of RT-associated RNase H have yet to enter development pipeline. Nevertheless, the critical roles of RNase H for HIV replication are corroborated by the observed close correlation between attenuated RNase H enzymatic activity and reduced HIV replication in cell culture[6], suggesting that selectively inhibiting RNase H with small molecules could confer a similar antiviral phenotype.

Medicinal chemistry targeting HIV RNase H active site is well documented[7–9]. Reported inhibitor types, such as 2-hydroxyisoquinolinedione (HID)[10–11], β -thujaplicinol[12], dihydroxycoumarin[13], diketoacid (DKA)[14], HPD[15–17] and 3-hydroxy thienopyrimidine-2,4-dione [18], largely build on a chelating core capable of binding two divalent metal ions, a critical pharmacophore component also found in inhibitors of HIV INST[19–22], influenza PA endonuclease [23–27] and human cytomegalovirus (HCMV) terminase pUL89[28–30], all sharing a similar active site fold and a divalent metal requirement for catalytic activity[31]. Notably, structurally more elaborate inhibitor types, including pyrimidinol carboxylic acid **1**[32], hydroxynaphthyridine **2**[33], pyridopyrimidone **3**[34], hydroxypyridonecarboxylic acid (HPCA) **4**[35], redesigned HID **5**[36], and redesigned HPD **6**[37–39] (Figure 1), all contain an additional hydrophobic aromatic moiety (cyan) connected to the chelating core through a one-atom linker (blue), constituting an RNase H inhibitor pharmacophore reminiscent of that observed for canonical INSTIs[40–41]. Despite these medicinal chemistry efforts *bona fide* RNase H inhibitors remain elusive as reported ones generally do not confer significant antiviral activity in cell culture, or in a few cases where they do, direct evidence of RNase H as the antiviral target is lacking. This deficiency likely reflects the steep biochemical barrier of small molecules competing against much larger RNA/DNA substrates[34,42]. Biochemically, many reported RNase H inhibitors

also did not exhibit selective RNase H inhibition over the inhibition of RT pol and / or INST activities. These challenges highlight the need to continue identifying novel chemotypes with potent and selective biochemical RNase H inhibition that could confer antiviral activity. We have previously designed and synthesized a few variants of HPD subtype (**6**) which strictly conform to the afore-mentioned pharmacophore. Interestingly, while potent RNase H inhibition was observed with all variants, the flexible one-atom linker at C-6 appeared to profoundly influence the inhibitory activity both *in vitro* and *in cellulo*: the –O– (**6a**) and the –NH– (**6b**) linkers conferred submicromolar RNase H inhibition without significant antiviral activity, whereas analogues with a –S– (**6c**) or –CH₂– (**6d**) linker inhibited RNase H in low nanomolar range with significant antiviral activity (Figure 2). To further explore the linker effect, particularly the impact of altering the linker flexibility, we designed novel HPD subtypes **7–8** in which the flexible one-atom linker was replaced by a carbonyl linker which would exert reduced rotational freedom (Figure 2). We report herein the synthesis, biochemical and antiviral studies, and molecular modeling of the C-6 carbonyl HPD subtypes **7–8**.

Results and Discussion

Chemistry.

Many HPD subtypes are synthetically accessed from the 3-OBn-6-Clpyrimidine-2,4-dione **9** intermediate which can be easily prepared based on our reported procedures[16–17, 38–39]. To install the carbonyl moiety, the NH (1) of **9** was first protected with an ethoxymethyl group to yield **10**. The key C-C bond formation at C-6 was achieved by reacting **10** with various *in situ* generated ArCHNaCN to afford **11–12**. The cyanomethine was then converted into the desired keton functionality *via* an oxidative decyanation [43–44] to produce intermediates **13–14**. Benzyl deprotection *via* catalytic hydrogenation yielded HPD **15–16** which were converted into desired HPD subtypes **7–8** upon N-1 deprotection under acidic condition. From intermediates **11–12**, OBn deprotection also afforded C-6 cyanomethine HPD **17–18** (Scheme 1).

While the synthetic route depicted in Scheme 1 worked well for **7** where the Ar group is a mono phenyl ring derived from various readily available ArCH₂CN (Ar = phenyl), for subtype **8** some of the requisite ArCH₂CN (Ar = biphenyl) are not readily available. In these cases, we worked out an alternative synthesis for subtype **8** (Scheme 2). Key to this route was the synthesis of two bromo-substituted intermediates **13f–g** according to Scheme 1. The second phenyl ring was then introduced *via* a Suzuki coupling reaction to produce **14** which upon sequential debenzoylation and N-1 deprotection yielded **16** and **8**, respectively.

Biology.

All final compounds of subtypes **7** and **8** were first tested in biochemical assays measuring the catalytic activity of RT-associated RNase H, RT pol, and INST to establish their biochemical inhibitory profiles. They all were then subjected to further cell-based evaluation using a MAGI assay for antiviral activity against HIV-1 and a XTT colorimetric assay for cytotoxicity. Reported inhibitor **3** was used as the control for RT assays whereas two FDA-

approved INSTIs raltegravir (RAL) and dolutegravir (DTG) were used as controls for the INST assay and the MAGI antiviral assay.

C-6 Carbonyl HPD subtypes potently inhibited RT-associated RNase H.—In the biochemical assay against RNase H activity (Table 1), all analogues of **7** and **8** exhibited potent inhibition in the nanomolar range with IC_{50} values all <100 nM (15–93 nM), except for compound **8d** ($IC_{50} = 530$ nM). These observations clearly indicate that having a carbonyl linkage at the C-6 position is viable for RNase H inhibition. Interestingly, the level of inhibition observed herein is superior to the C-6 –O– (**6a**) and the C-6 –NH– (**6b**) HPD subtypes and slightly inferior to the C-6 –S– (**6c**) and the C-6 –CH₂– (**6d**) subtypes (Figure 2). When comparing the aromatic domain of these two series, the biphenyl moiety in **8** appeared to confer better inhibition than the mono phenyl ring in **7** (**8a–e** vs. **7a–e**, Table 1), a structure-activity-relationship (SAR) trend consistent with previous subtypes. Similar nanomolar potency was observed with additional analogues (**8f–i**, Table 1), which further substantiates subtype **8** as a potent inhibitor type of RT-associated RNase H. Interestingly, two synthetic intermediates **16f–g** bearing an N-1 substituent inhibited RNase H with substantially reduced potency (**16f**: $IC_{50} = 0.42$ μ M vs. **8f**: $IC_{50} = 0.022$ μ M; **16g**: $IC_{50} = 1.1$ μ M vs. **8g**: $IC_{50} = 0.017$ μ M), clearly indicating that the N-1 substituent disfavors target binding and that the position should be left unsubstituted for optimal inhibition, consistent with our previously report on HPDs[38]. Another interesting SAR was that synthetic intermediates **17e** and **18f** with a cyano methine linkage exhibited drastically reduced potency (**17e**: $IC_{50} = 3.7$ μ M vs. **7e**: $IC_{50} = 0.090$ μ M; **18f**: $IC_{50} = 0.76$ μ M vs. **8f**: $IC_{50} = 0.022$ μ M).

Some C-6 carbonyl HPD subtypes weakly inhibited RT pol and INST.—In stark contrast to the exceptional potency inhibiting RT-associated RNase H, analogues of subtypes **7–8** were generally inactive against RT pol at concentrations up to 10 μ M, with the exception of compounds **8h–k** which moderately inhibited RT pol in low micromolar range ($IC_{50} = 5.9$ – 9.6 μ M, Table 1). In the INST assay, all analogues of subtype **7** and most analogues of subtype **8** demonstrated inhibitory activities in low μ M range (**7a–e**, **8a**, **8c–g**, **8k**: $IC_{50} = 2.1$ – 13 μ M, Table 1). Three analogues of subtype **8** inhibited INST at submicromolar concentrations (**8i**, **8j**, **8l**: $IC_{50} = 0.33$ – 0.53 μ M) and two (**8b** and **8h**) did not inhibit at concentrations up to 100 μ M. It is noteworthy that in our assays compound **3**, which is among the best RNase H inhibitors known, inhibited RNase H with much lower potency ($IC_{50} = 0.70$ μ M) and essentially no selectivity over RT pol inhibition ($IC_{50} = 0.85$ μ M) when compared to **7** and **8**, whereas both RAL and DTG inhibited INST in the nanomolar range without inhibiting RNase H at concentrations up to 10 μ M. Taken together, these biochemical assays characterize the carbonyl HPD subtypes **7** and **8** as viable scaffolds for potent and selective inhibitors of RT-associated RNase H.

Antiviral and cytotoxicity evaluation.—In the MAGI antiviral assay, analogues of subtype **7** were mostly inactive at concentrations up to 20 μ M (**7a–d**, Table 2). One **7** analogue (**7e**: $EC_{50} = 9.5$ μ M, $CC_{50} = 21$ μ M) and one **8** analogue (**7j**: $EC_{50} = 6.7$ μ M, $CC_{50} = 8.4$ μ M) did show antiviral activity in low micromolar range, though the apparent antiviral EC_{50} values largely reflect the observed cytotoxicity. Interestingly, two analogues of **8**, **8a**

(EC₅₀ = 4.7 μ M, CC₅₀ = 22 μ M) and **8b** (EC₅₀ = 5.3 μ M, CC₅₀ = 17 μ M), inhibited HIV-1 with a moderate selectivity (CC₅₀ / EC₅₀). The best results were observed with **8c** (EC₅₀ = 9.0 μ M, CC₅₀ >100 μ M), **8f** (EC₅₀ = 15 μ M, CC₅₀ >100 μ M) and **8l** (EC₅₀ = 18 μ M, CC₅₀ > 100 μ M), all inhibiting HIV-1 without cytotoxicity at 100 μ M. These results indicate that the C-6 carbonyl HPD subtype **8** could represent a viable scaffold for identifying antivirals targeting RNase H.

Molecular modeling.

Docking analysis was performed with Glide XP (version 6.4)[45–46] using two metal sites as a constraint within the active site of RNase H. The predicted binding mode of compound **7a** within the active site of RNase H (PDB code: 5J1E[35]) suggests potential chelating interactions between the HPD core (the 2-C=O-3-OH-4-C=O chelating triad) and the two metal cofactors (Mg²⁺) which are coordinated to the conserved active site acidic residues D443, E478, D498, and D549. The molecular model also reveals a potential interaction between the keto group at the 4-position (4-C=O) of **7a** and the imidazole ring of the highly conserved H539, which could help to stabilize the inhibitor at the active site. The carbonyl group at 6-position of HPD core restricts the free rotation of the aryl group within **7a**. The predicted binding mode of **8a** within the active site of RNase H is similar to that of **7a**, with exception that the biaryl moiety of **8a** is positioned to potentially reach and interact with other protein residues or nucleic acid substrate, which may explain the observed increased binding affinity compared to **7a**. These predicted binding modes conform to the general pharmacophore of active site-directed RNase H inhibitors, hence corroborating the active site inhibition mechanism (Figure 3).

Conclusions

New HPD subtypes **7–8** featuring a C-6 carbonyl linkage were synthesized as inhibitors of HIV RT-associated RNase H. In biochemical assays, all analogues inhibited RNase H in nanomolar range without significantly inhibiting RT pol at 10 μ M. All analogues of **7** and some of **8** also inhibited INST, though with much reduced potency comparing to RNase H inhibition. Significant antiviral activity against HIV-1 was observed with five analogues of **8** in low μ M range with modest or no cytotoxicity. Molecular modeling predicted a binding model consistent with RT RNase H active site binding. The overall inhibitory profiles of HPD subtypes **7–8** are comparable to or better than the previous subtypes **6a–d**, suggesting that carbonyl can be a viable linker in HIV RNase H inhibitor design.

EXPERIMENTAL

Chemistry

General Procedures. All commercial chemicals were used as supplied unless otherwise indicated. Flash chromatography was performed on a Teledyne CombiFlash RF-200 with RediSep columns (silica) and indicated mobile phase. All moisture sensitive reactions were performed under an inert atmosphere of ultra-pure argon with oven-dried glassware. ¹H and ¹³C NMR spectra were recorded on a Varian 600 MHz spectrometer. Mass data were acquired using an Agilent 6230 TOF LC/MS spectrometer. Melting points were determined

in open glass capillaries using a MEL-TEMP melting-point apparatus. Analysis of sample purity was performed on a Varian Prepstar SD-1 HPLC system with a Phenomenex Gemini, 5 μ m C18 column (250mm $\times$ 4.6 mm). HPLC conditions: solvent A: H₂O with 0.1% TFA; solvent B: MeCN; flow rate 1.0 mL/min; compounds were eluted with a gradient of 20% MeCN/H₂O with 0.1% TFA to 100% MeCN for 20 min. All tested compounds have a purity 97%.

General procedure for the synthesis of 7 and 8.—To a solution of **13** or **14** (0.1 mmol, 1 equiv.) in 1 mL of MeOH was added Pd 10 wt. % on activated carbon (10% equiv.) in one portion. The mixture was subjected to hydrogen atmosphere for 20 minutes at room temperature (the cleavage of the N-OH bond was observed under longer reaction time). Upon completion of the hydrogenation reaction, the mixture was filtered through celite and concentrated under vacuum to give the desired compound **15** or **16** which can be used directly for the next step.

A solution of **15** or **16** (0.08 mmol) in 1 mL of TFA was irradiated under microwave at 120 °C for 20 minutes. Upon completion of the reaction as monitored with LC-MS, TFA was removed under vacuum and the crude mixture was purified via trituration to give the desired pure product.

3-Hydroxy-6-(4-methylbenzoyl)pyrimidine-2,4(1H,3H)-dione (7a).—Yield 54%. Mp 149–150 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 11.52 (s, 1H), 11.28 (s, 1H), 7.91 (d, *J* = 8.4 Hz, 2H), 7.14 (d, *J* = 7.8 Hz, 2H), 5.74 (s, 1H), 2.49 (s, 3H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 188.8, 164.2, 151.5, 148.4, 146.0, 132.0, 130.5, 130.0, 103.4, 21.8; HRMS (ESI-) *m/z* calculated for C₁₂H₁₀N₂O₄ [M-H][−] 245.0641, found 245.0641.

3-Hydroxy-6-(4-(trifluoromethyl)benzoyl)pyrimidine-2,4(1H,3H)-dione (7b).—Yield 58%. Mp 197–198 °C; ¹H NMR (600 MHz, CD₃OD) δ 7.82 (d, *J* = 8.4 Hz, 2H), 7.26 (d, *J* = 8.4 Hz, 2H), 5.74 (s, 1H); HRMS (ESI-) *m/z* calculated for C₁₂H₇F₃N₂O₄ [M-H][−] 299.0358, found 299.0358.

6-(4-Fluorobenzoyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (7c).—Yield 63%. Mp 224–225 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 11.44 (s, 1H), 11.28 (s, 1H), 8.23 (t, *J* = 8.6, 2H), 7.50 (t, *J* = 8.6 Hz, 2H), 5.79 (s, 1H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 187.9, 167.0, 165.4, 164.2, 151.4, 147.9, 133.5, 131.3, 116.7, 116.6, 103.9; HRMS (ESI-) *m/z* calculated for C₁₁H₇FN₂O₄ [M-H][−] 249.0391 ([M]-H), found 249.0390.

Methyl 4-(1-hydroxy-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbonyl)benzoate (7d).—Yield 52%. Mp 245–246 °C; ¹H NMR (600 MHz, CD₃OD) δ 8.11 (d, *J* = 8.3 Hz, 2H), 7.88 (d, *J* = 8.3 Hz, 2H), 6.01 (s, 1H), 3.88 (s, 3H); HRMS (ESI-) *m/z* calculated for C₁₃H₁₀N₂O₆ [M-H][−] 289.0539, found 289.0539.

6-(3,5-Dimethylbenzoyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (7e).—Yield 43%. Mp 130–131 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 11.43 (s, 1H), 11.26 (s, 1H), 7.59 (s, 2H), 7.46 (s, 1H), 5.73 (s, 1H), 2.42 (s, 6H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 188.8,

164.2, 151.4, 148.4, 146.0, 132.0, 130.5, 130.0, 103.4, 21.8; HRMS (ESI-) m/z calculated for $C_{13}H_{12}N_2O_4$ $[M-H]^-$ 259.0797, found 259.0798.

3-Hydroxy-6-(4'-methyl-[1,1'-biphenyl]-4-carbonyl)pyrimidine-2,4(1H,3H)-dione (8a).—Yield 46%. Mp 243–244°C; 1H NMR (600 MHz, DMSO- d_6) δ 11.65 (s, 1H), 10.62 (s, 1H), 7.97 (d, J = 8.0 Hz, 2H), 7.85 (d, J = 8.0 Hz, 2H), 7.66 (d, J = 7.7 Hz, 2H), 7.31 (d, J = 7.6 Hz, 2H), 5.91 (s, 1H), 2.34 (s, 3H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 188.4, 160.27, 149.8, 146.3, 145.2, 138.8, 136.0, 133.0, 131.2, 130.2, 127.4, 127.2, 103.3, 21.2; HRMS (ESI-) m/z calculated for $C_{18}H_{14}N_2O_4$ $[M-H]^-$ 321.0881, found 321.0897.

3-Hydroxy-6-(4'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbonyl)pyrimidine-2,4(1H,3H)-dione (8b).—Mp 230–231°C; Yield 56%. 1H NMR (600 MHz, DMSO- d_6) δ 11.66 (s, 1H), 10.64 (s, 1H), 8.02 (d, J = 7.9 Hz, 2H), 7.98 (d, J = 7.9 Hz, 2H), 7.94 (d, J = 8.0 Hz, 2H), 7.86 (d, J = 8.0 Hz, 2H), 5.93 (s, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 188.5, 160.3, 149.5, 144.8, 144.5, 143.0, 134.2, 131.2, 128.5, 128.0, 126.4, 126.4 (dd, J_{C-F} = 7.3, 3.7 Hz), 103.7; HRMS (ESI-) m/z calculated for $C_{18}H_{11}F_3N_2O_4$ $[M-H]^-$ 375.0596, found 375.0606.

6-(4'-Fluoro-[1,1'-biphenyl]-4-carbonyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (8c).—Yield 46%. Mp 223–224°C; 1H NMR (600 MHz, DMSO- d_6) δ 11.65 (s, 1H), 10.63 (s, 2H), 7.98 (d, J = 8.3 Hz, 2H), 7.86 (d, J = 8.3 Hz, 2H), 7.82 (dd, J = 8.5, 5.5 Hz, 2H), 7.33 (t, J = 8.8 Hz, 2H), 5.91 (d, J = 1.9 Hz, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 188.4, 163.8, 162.2, 160.3, 149.8, 145.1 (d, J_{C-F} = 30.6 Hz), 135.43 (d, J_{C-F} = 3.0 Hz), 133.3, 131.2, 129.8, 129.7, 127.5, 116.5 (d, J_{C-F} = 21.5 Hz), 103.5; HRMS (ESI-) m/z calculated for $C_{17}H_{11}FN_2O_4$ $[M-H]^-$ 325.0630, found 325.0647.

Methyl 4'-(1-hydroxy-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbonyl)-[1,1'-biphenyl]-4-carboxylate (8d).—Yield 69%. Mp >250°C; 1H NMR (600 MHz, DMSO- d_6) δ 11.66 (s, 1H), 10.64 (s, 1H), 8.06 (d, J = 8.3 Hz, 2H), 8.02 (d, J = 8.3 Hz, 2H), 7.94 (d, J = 8.4 Hz, 2H), 7.92 (d, J = 8.3 Hz, 2H), 5.93 (s, 1H), 3.86 (s, 3H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 188.4, 166.3, 160.3, 149.7, 144.9, 144.8, 143.4, 134.1, 131.2, 130.4, 130.0, 128.0, 127.9, 103.7, 52.7; HRMS (ESI-) m/z calculated for $C_{19}H_{14}N_2O_6$ $[M-H]^-$ 365.0779, found 365.0795.

6-(3',5'-Dimethyl-[1,1'-biphenyl]-4-carbonyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (8e).—Yield 52%. Mp 169–170°C; 1H NMR (600 MHz, DMSO- D_6) δ 11.46 (s, 1H), 11.31 (s, 1H), 8.06 (d, J = 8.4 Hz, 2H), 7.94 (d, J = 8.4 Hz, 2H), 7.45 (s, 2H), 7.16 (s, 1H), 5.82 (s, 1H); ^{13}C NMR (151 MHz, DMSO- D_6) δ 188.8, 164.3, 151.5, 148.3, 146.6, 138.9, 138.7, 133.2, 131.0, 130.6, 127.5, 125.3, 103.6; HRMS (ESI-) m/z calculated for $C_{19}H_{16}N_2O_4$ $[M-H]^-$ 335.1110, found 335.1110.

6-([1,1'-Biphenyl]-4-carbonyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (8f).—Yield 70%. Mp >250°C; 1H NMR (600 MHz, DMSO- d_6) δ 11.66 (s, 1H), 10.61 (s, 1H), 7.99 (d, J = 8.2 Hz, 2H), 7.88 (d, J = 8.3 Hz, 2H), 7.76 (d, J = 7.5 Hz, 2H), 7.50 (t, J = 7.5 Hz, 2H), 7.43 (t, J = 7.2 Hz, 1H), 5.91 (s, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 188.4,

160.3, 149.8, 146.3, 145.1, 139.0, 133.3, 131.2, 129.6, 129.2, 127.6, 127.5, 103.4; HRMS (ESI-) m/z calculated for $C_{17}H_{12}N_2O_4$ $[M-H]^-$ 307.0724, found 307.0726.

6-([1,1'-Biphenyl]-3-carbonyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (8g).—Yield 87%. 1H NMR (600 MHz, DMSO-*d*6) δ 11.66 (s, 1H), 10.62 (s, 1H), 8.12 (s, 1H), 8.03 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.71 (d, J = 7.6 Hz, 2H), 7.67 (t, J = 7.7 Hz, 1H), 7.48 (t, J = 7.5 Hz, 2H), 7.40 (t, J = 7.2 Hz, 1H), 5.93 (s, 1H); ^{13}C NMR (151 MHz, DMSO-*d*6) δ 189.0, 160.3, 149.8, 144.8, 141.2, 139.2, 135.3, 133.1, 130.1, 129.6, 129.4, 128.6, 128.3, 127.4, 104.0; HRMS (ESI-) m/z calculated for $C_{17}H_{12}N_2O_4$ $[M-H]^-$ 307.0736, found 307.0734.

3-Hydroxy-6-(4'-methoxy-[1,1'-biphenyl]-4-carbonyl)pyrimidine-2,4(1H,3H)-dione (8h).—Yield 61%. Mp 249–250°C; 1H NMR (600 MHz, DMSO-*d*6) δ 11.74 (s, 1H), 10.72 (s, 1H), 8.04 (d, J = 8.3 Hz, 2H), 7.85 (d, J = 8.3 Hz, 2H), 7.76 (d, J = 8.6 Hz, 1H), 7.56 (s, 1H), 7.24 (d, J = 8.7 Hz, 2H), 6.00 (s, 1H), 3.96 (s, 3H); ^{13}C NMR (151 MHz, DMSO-*d*6) δ 188.2, 160.3, 158.4, 149.8, 146.3, 145.2, 132.5, 131.3, 130.8, 129.1, 126.9, 111.9, 103.2, 56.1; HRMS (ESI-) m/z calculated for $C_{18}H_{14}N_2O_5$ $[M-H]^-$ 337.0903, found 337.0903.

4'-(1-Hydroxy-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-carbonyl)-[1,1'-biphenyl]-4-carbonitrile (8i).—Yield 55%. Mp 220–221°C; 1H NMR (600 MHz, DMSO-*d*6) δ 11.38 (s, 1H), 11.23 (s, 1H), 8.21 – 7.99 (m, 3H), 8.01 – 7.89 (m, 5H), 5.75 (s, 1H); ^{13}C NMR (151 MHz, DMSO-*d*6) δ 188.8, 164.2, 151.4, 147.9, 144.1, 143.5, 134.4, 133.5, 131.1, 128.5, 128.1, 111.6, 104.1; HRMS (ESI-) m/z calculated for $C_{18}H_{11}N_3O_4$ $[M-H]^-$ 332.0750, found 332.0750.

6-(3',4'-Dimethoxy-[1,1'-biphenyl]-4-carbonyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (8j).—Yield 58%. Mp 202–204°C; 1H NMR (600 MHz, CD₃OD) δ 8.08 (d, J = 8.4 Hz, 1H), 7.88 (s, 1H), 7.76 (s, 1H), 6.00 (s, 1H), 3.88 (s, 3H), 3.86 (s, 3H); HRMS (ESI-) m/z calculated for $C_{19}H_{16}N_2O_6$ $[M-H]^-$ 367.1008, found 367.1008.

6-(4'-Fluoro-3'-methyl-[1,1'-biphenyl]-4-carbonyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (8k).—Yield 59%. Mp 209–210°C; 1H NMR (600 MHz, CD₃OD-*d*4) δ 7.91 – 7.80 (m, 2H), 7.77 – 7.55 (m, 2H), 7.49 (d, J = 7.3 Hz, 1H), 7.44 (s, 1H), 7.05 (t, J = 9.0 Hz, 1H), 6.03 (s, 1H), 2.26 (s, 3H); HRMS (ESI-) m/z calculated for $C_{18}H_{13}FN_2O_4$ $[M-H]^-$ 339.0859, found 339.0859.

6-(4'-Chloro-3'-fluoro-[1,1'-biphenyl]-4-carbonyl)-3-hydroxypyrimidine-2,4(1H,3H)-dione (8l).—Yield 51%. Mp 210–211°C; 1H NMR (600 MHz, DMSO-*d*6) δ 11.37 (s, 1H), 11.22 (s, 1H), 8.02 – 7.96 (m, 2H), 7.97 – 7.91 (m, 2H), 7.89 (dd, J = 10.7, 1.8 Hz, 1H), 7.71 (dd, J = 7.8, 4.0 Hz, 1H), 7.67 (dd, J = 8.3, 1.8 Hz, 1H), 5.89 (m, 1H); ^{13}C NMR (151 MHz, DMSO-*d*6) δ 188.8, 164.3, 158.9, 151.4, 148.0, 143.6, 140.1, 134.1, 131.7, 131.1, 127.7, 124.8, 120.4, 116.1, 115.9, 104.0; HRMS (ESI-) m/z calculated for $C_{17}H_{10}ClFN_2O_4$ $[M-H]^-$ 359.0313, found 359.0313.

Synthesis of 16f and 16g—To a solution of **14f** or **14g** (0.1 mmol, 1 equiv.) in 1 mL of MeOH was added Pd 10 wt. % on activated carbon (10% equiv.) in one portion. The mixture was subjected to hydrogen atmosphere for 20 minutes at room temperature (the cleavage of the N-OH bond was observed under longer reaction time). Upon completion of the hydrogenation reaction, the mixture was filtered through celite, concentrated under vacuum and recrystallized in MeOH to afford compound **16f** or **16g**.

6-([1,1'-Biphenyl]-4-carbonyl)-1-(ethoxymethyl)-3-hydroxypyrimidine-2,4(1H, 3H)-dione (16f).—Yield 95%. Mp 133–134°C; ¹H NMR (600 MHz, CD₃OD-*d*₄) δ 8.07 (d, *J* = 7.7 Hz, 2H), 7.85 (d, *J* = 7.7 Hz, 2H), 7.72 (d, *J* = 8.0 Hz, 2H), 7.49 (t, *J* = 7.5 Hz, 2H), 7.43 (t, *J* = 7.0 Hz, 1H), 5.96 (s, 1H), 5.41 (s, 2H), 3.46 (q, *J* = 6.8 Hz, 2H), 0.88 (t, *J* = 6.7 Hz, 3H); ¹³C NMR (151 MHz, CD₃OD) δ 187.8, 159.4, 150.3, 147.5, 147.1, 139.1, 133.0, 130.6, 128.7, 128.4, 127.0, 126.9, 102.7, 73.5, 64.1, 13.3; HRMS (ESI-) *m/z* calculated for C₂₀H₁₈N₂O₅ [M-H][−] 365.1143, found 365.1136.

6-([1,1'-Biphenyl]-3-carbonyl)-1-(ethoxymethyl)-3-hydroxypyrimidine-2,4(1H, 3H)-dione (16g).—Yield 97%. ¹H NMR (600 MHz, CD₃OD) δ 8.08 (s, 1H), 7.84 (dd, *J* = 17.9, 7.6 Hz, 2H), 7.56 – 7.46 (m, 3H), 7.34 (t, *J* = 7.5 Hz, 2H), 7.26 (t, *J* = 7.2 Hz, 1H), 5.83 (s, 1H), 5.28 (s, 2H), 3.33 (dd, *J* = 13.9, 6.9 Hz, 2H), 0.71 (t, *J* = 6.9 Hz, 3H); ¹³C NMR (151 MHz, CD₃OD) δ 188.3, 159.4, 150.3, 146.8, 141.9, 139.3, 134.9, 133.1, 129.2, 129.0, 128.7, 127.9, 127.8, 126.7, 102.9, 73.5, 64.1, 13.3; HRMS (ESI-) *m/z* calculated for C₂₀H₁₈N₂O₅ [M-H][−] 365.1136, found 365.1149.

Synthesis of 17e and 18f.—**17e** and **18f** were synthesized from **11e** and **12f** (0.1 mmol, 1 equiv.) respectively following the same hydrogenation procedure as described for the synthesis of **16f** and **16g**.

2-(3,5-Dimethylphenyl)-2-(3-(ethoxymethyl)-1-hydroxy-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)acetonitrile (17e).—Yield 83%. Mp 111–112°C; ¹H NMR (600 MHz, CDCl₃) δ 7.60 (s, 1H), 7.26 (s, 2H), 6.52 (s, 1H), 5.91 (d, *J* = 11.7 Hz, 1H), 5.84 (s, 1H), 5.41 (d, *J* = 11.5 Hz, 1H), 4.08 (q, *J* = 7.0 Hz, 2H), 2.68 (s, 6H), 1.59 (t, *J* = 7.0 Hz, 3H); HRMS (ESI-) *m/z* calculated for C₁₇H₁₉N₃O₄ [M-H][−] 328.1376, found 328.1375.

2-([1,1'-Biphenyl]-4-yl)-2-(3-(ethoxymethyl)-1-hydroxy-2,6-dioxo-1,2,3,6-tetrahydro pyrimidin-4-yl)acetonitrile (18f).—Yield 80%. Mp 129–130°C; ¹H NMR (600 MHz, CDCl₃) δ 7.60 (d, *J* = 8.2 Hz, 2H), 7.50 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.6 Hz, 2H), 7.34 (dd, *J* = 13.4, 7.8 Hz, 3H), 6.10 (s, 1H), 5.57 (s, 1H), 5.52 (d, *J* = 11.6 Hz, 1H), 5.06 (d, *J* = 11.7 Hz, 1H), 3.61 (q, *J* = 7.0 Hz, 2H), 1.18 (t, *J* = 7.0 Hz, 3H); HRMS (ESI-) *m/z* calculated for C₂₁H₁₉N₃O₄ [M-H][−] 376.1376, found 376.1375.

Biology

Reagents

Biologicals. Recombinant HIV-1 reverse transcriptase (RT) was expressed and purified as previously described[47]. P4R5 HIV infection indicator cells were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (p4R5.MAGI from Dr. Nathaniel

Landau). These cells express CD4, CXCR4 and CCR5 as well as a β -galactosidase reporter gene under the control of an HIV LTR promoter.

Chemicals.: DNA and RNA oligonucleotides for the preparation of RNA/DNA duplexes for assay of RNase H activity were purchased from Trilink (San Diego, CA).

RNase H assay—RNase H activity was measured essentially as previously described[48]. RNA/DNA duplex substrate HTS-1 (RNA 5'-gaucugagccugggagcu -3'-fluorescein annealed to DNA 3'-CTAGACTCGGACCCTCGA -5'-Dabcyl) is a high sensitivity duplex that assesses non-specific internal cleavage.

RT polymerase assay—RT pol assays [49–52] were carried out in 96-well plates by measuring the extension of an 18 nucleotide DNA primer (5'-GTCAGTGTTCGAGCACCA-3') annealed to a 100 nucleotide DNA template (5'-ATGTGTGTGCCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTCAGACCC TTTTAGTCAGTGTGGAATATCTCATAGCTTGGTGCTCGAACAGTGAC-3'). Reactions containing 20 nM RT, 40 nM template/primer, and 10 μ M deoxynucleotide triphosphates (dNTPs) in a buffer containing 50 mM Tris (pH 7.8) and 50 mM NaCl were initiated by the addition of 6 mM $MgCl_2$. Reactions contained 1% DMSO and increasing concentrations of compounds. DNA synthesis was carried out for 30 min at 37°C, and reactions were arrested by the addition of 100 mM EDTA. The QuantiFluor dsDNA System (Promega) was used to quantify the amount of formed double-stranded DNA. Reactions were read at ex/em 504/531 nm in a PerkinElmer EnSpire Multilabel plate reader. Results were analyzed using Prism software (GraphPad Software, San Diego, CA) for nonlinear regression to fit dose-response data to logistic curve models.

HIV IN assay—HIV integrase was expressed and purified as previously reported[53]. Inhibition assays were performed using a modified protocol of our reported method[53]. Briefly, 2.1 μ L of compound suspended in DMSO was placed in duplicate into a Black 96 well non-binding plate (Corning 3991). Compounds were plated in duplicate to a final concentration of 0.13 – 100 μ M. To each well of the plate 186.9 μ L of reaction mixture without DNA substrate was added (10 mM HEPES pH 7.5, 10 % glycerol w/v, 10 mM $MnCl_2$, 1 mM DTT, 1 μ M integrase). The enzyme was incubated with inhibitor for 10 min at 25 °C after which the reaction was initiated by the addition of 21 μ L of 500 nM oligo (5' biotin ATGTGGAAAATCTCTAGCA annealed with ACTGCTAGAGATTTTCCACAT 3' Cy5). Reactions were incubated at 37 °C for 30 min and then quenched by the addition of 5.2 μ L 500 mM EDTA. Each reaction was moved (200 μ L) to a MultiScreen HTS PCR plate (Millipore MSSLBPC10) containing 20 μ L streptavidin agarose beads (Life Technologies S951) and incubated with shaking for 30 min. A vacuum manifold was used to remove the reaction mixture and the beads were similarly washed 3 times with wash buffer (0.05% SDS, 1 mM EDTA in PBS). The plates were further washed 3 times with 200 μ L 50 mM NaOH to denature DNA not covalently linked to the biotin modification. For each denaturation step the plate was incubated with shaking at 25 °C for 5 min and the NaOH was removed by centrifugation at 1000 g for 1 min. The reaction products were eluted from the beads by the

addition of 150 μ L formamide. The plate was incubated at 25 °C for 10 min and read directly at 635/675 in a SpectraMax i3 plate reader (Molecular Devices).

Antiviral assays—Antiviral MAGI assays were carried out using P4R5 indicator cells essentially as previously described[54]. P4R5 cells were cultured in 96-well microplates (4×10^3 cells per well and maintained in DMEM/10% FBS supplemented with puromycin (1 μ g/ml). Cells were incubated with either 1% DMSO or varying concentrations of the compounds for 24 h and then exposed to HIV-1 (MOI of 1.25) followed by an additional incubation period of 48 h. The extent of infection was assessed using a fluorescence-based β -galactosidase detection assay, as previously described with minor modifications [55]. After the 48 h incubation period, cells were lysed and 4-methylumbelliferyl galactoside (MUG) substrate was added. The β -galactosidase produced during infection acts on the MUG substrate and yields a fluorescent product, 4-methylumbelliferone (4-MU), that could be detected fluorimetrically with excitation wavelength 365 nm and emission wavelength 446 nm.

Cytotoxicity—For cytotoxicity assays, P4R5 cells were cultured in 96-well microplates with 4×10^3 cells per well and maintained in DMEM/10% FBS supplemented with puromycin (1 μ g/mL). Cells were incubated with either 1% DMSO or varying concentrations of compounds for 72 h and then assessed with the Cell Proliferation Kit II (XTT) (Roche) according to the manufacturer's instructions.

Molecular docking and modeling analysis—Molecular modeling was performed using the Schrödinger small molecule drug discovery suite 2014–3. The crystal structure of a hydroxypyridone carboxylic acid active-site RNase H inhibitor in complex with HIV-1 RT was extracted from the Protein Data Bank (PDB code 5J1E) as reported by Kankanala *et al.* [35]. This model was subjected to Protein Preparation Wizard (Schrödinger Inc.) [56–57] in which missing hydrogens atoms were added and zero-order bonds to metals were created followed by the generation of metal binding states. The protein structure was minimized using OPLS 2005 force field [58] to optimize hydrogen bonding networks and converge heavy atoms to an rmsd of 0.3 Å. The receptor grid generation tool in Maestro (Schrödinger Inc.) was used to define an active site around the native ligand to cover all the residues within 14 Å of the compound with both the metal cofactors (Mg^{2+}) as constraints to identify the chelating triad during docking.

Compounds **7a** and **8a** were drawn using Maestro and subjected to Lig Prep [59] to generate conformers, possible protonation at pH of 7 ± 3 , and metal binding states that serve as an input for the docking process. All docking experiments were performed using Glide XP (Glide, version 6.4) [45–46] with both the Mg^{2+} metal cofactors as a constraint. The van der Waals radii of nonpolar atoms for each of the ligands were scaled by a factor of 0.8. The predicted binding mode of compounds **7a** and **8a** features the critical interaction between the chelating triad and to the divalent metal cofactors. The ligands within the active site of RNase H was further refined post docking by minimizing under implicit solvent to account for the local protein flexibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIV	human immunodeficiency virus
RT	reverse transcriptase
HPD	3-hydroxypyrimidine-2,4-dione
INST	integrase strand transfer
Pol	polymerase
NRTIs	nucleoside RT inhibitors
NNRTIs	nonnucleoside RT inhibitors
HID	2-hydroxyisoquinolinedione
DKA	diketoacid
HCMV	human cytomegalovirus
HPCA	hydroxypyridonecarboxylic acid
RAL	raltegravir
DTG	dolutegravir
SAR	structure-activity relationship

References

1. Sarafianos SG; Marchand B; Das K; Himmel DM; Parniak MA; Hughes SH; Arnold E, Structure and Function of HIV-1 Reverse Transcriptase: Molecular Mechanisms of Polymerization and Inhibition. *J. Mol. Biol* 385 (2009) 693–713. [PubMed: 19022262]
2. Beilhartz GL; Gotte M, HIV-1 Ribonuclease H: Structure, Catalytic Mechanism and Inhibitors. *Viruses* 2 (2010) 900–26. [PubMed: 21994660]
3. Huber HE; Richardson CC, Processing of the primer for plus strand DNA synthesis by human immunodeficiency virus 1 reverse transcriptase. *J Biol Chem* 265 (1990) 10565–73. [PubMed: 1693920]
4. Cihlar T; Ray AS, Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antiviral Res.* 85 (2010) 39–58. [PubMed: 19887088]
5. de Bethune MP, Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: A review of the last 20 years (1989–2009). *Antiviral Res.* 85 (2010) 75–90. [PubMed: 19781578]
6. Parniak MA, Unpublished data.

7. Wang XS; Gao P; Menendez-Arias L; Liu XY; Zhan P, Update on Recent Developments in Small Molecular HIV-1 RNase H Inhibitors (2013–2016): Opportunities and Challenges. *Curr. Med. Chem* 25 (2018) 1682–1702. [PubMed: 28088905]
8. Cao LL; Song WG; De Clercq E; Zhan P; Liu XY, Recent Progress in the Research of Small Molecule HIV-1 RNase H Inhibitors. *Curr. Med. Chem* 21 (2014) 1956–1967. [PubMed: 24438523]
9. Tramontano E; Di Santo R, HIV-1 RT-Associated RNase H Function Inhibitors: Recent Advances in Drug Development. *Curr. Med. Chem* 17 (2010) 2837–2853. [PubMed: 20858167]
10. Klumpp K; Hang JQ; Rajendran S; Yang YL; Derosier A; In PWK; Overton H; Parkes KEB; Cammack N; Martin JA, Two-metal ion mechanism of RNA cleavage by HIV RNase H and mechanism-based design of selective HIV RNase H inhibitors. *Nucleic Acids Res.* 31 (2003) 6852–6859. [PubMed: 14627818]
11. Tang J; Vernekar SKV; Chen Y-L; Miller L; Huber AD; Myshakina N; Sarafianos SG; Parniak MA; Wang Z, Synthesis, biological evaluation and molecular modeling of 2-Hydroxyisoquinoline-1,3-dione analogues as inhibitors of HIV reverse transcriptase associated ribonuclease H and polymerase. *Eur. J. Med. Chem* 133 (2017) 85–96. [PubMed: 28384548]
12. Himmel DM; Maegley KA; Pauly TA; Bauman JD; Das K; Dharia C; Clark AD Jr.; Ryan K; Hickey MJ; Love RA; Hughes SH; Bergqvist S; Arnold E, Structure of HIV-1 reverse transcriptase with the inhibitor beta-Thujaplicinol bound at the RNase H active site. *Structure* 17 (2009) 1625–1635. [PubMed: 20004166]
13. Himmel DM; Myshakina NS; Ilina T; Van Ry A; Ho WC; Parniak MA; Arnold E, Structure of a dihydroxycoumarin active-site inhibitor in complex with the RNase H domain of HIV-1 reverse transcriptase and structure-activity analysis of inhibitor analogs. *J. Mol. Biol* 426 (2014) 2617–31. [PubMed: 24840303]
14. Shaw-Reid CA; Munshi V; Graham P; Wolfe A; Witmer M; Danzeisen R; Olsen DB; Carroll SS; Embrey M; Wai JS; Miller MD; Cole JL; Hazuda DJ, Inhibition of HIV-1 ribonuclease H by a novel diketo acid, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid. *J. Biol. Chem* 278 (2003) 2777–80. [PubMed: 12480948]
15. Tang J; Kirby KA; Huber AD; Casey MC; Ji J; Wilson DJ; Sarafianos SG; Wang Z, 6-Cyclohexylmethyl-3-hydroxypyrimidine-2,4-dione as an inhibitor scaffold of HIV reverse transcriptase: Impacts of the 3-OH on inhibiting RNase H and polymerase. *Eur. J. Med. Chem* 128 (2017) 168–179. [PubMed: 28182989]
16. Wu BL; Tang J; Wilson DJ; Huber AD; Casey MC; Ji J; Kankanala J; Xie JS; Sarafianos SG; Wang ZQ, 3-Hydroxypyrimidine-2,4-dione-5-N-benzylcarboxamides Potently Inhibit HIV-1 Integrase and RNase H. *J. Med. Chem* 59 (2016) 6136–6148. [PubMed: 27283261]
17. Vernekar SKV; Tang J; Wu BL; Huber AD; Casey MC; Myshakina N; Wilson DJ; Kankanala J; Kirby KA; Parniak MA; Sarafianos SG; Wang ZQ, Double-Winged 3-Hydroxypyrimidine-2,4-diones: Potent and Selective Inhibition against HIV-1 RNase H with Significant Antiviral Activity. *J. Med. Chem* 60 (2017) 5045–5056. [PubMed: 28525279]
18. Kankanala J; Kirby KA; Huber AD; Casey MC; Wilson DJ; Sarafianos SG; Wang ZQ, Design, synthesis and biological evaluations of N-Hydroxy thienopyrimidine-2,4-diones as inhibitors of HIV reverse transcriptase-associated RNase H. *Eur. J. Med. Chem* 141 (2017) 149–161. [PubMed: 29031062]
19. Dayam R; Sanchez T; Neamati N, Diketo acid pharmacophore. 2. Discovery of structurally diverse inhibitors of HIV-1 integrase. *J Med Chem* 48 (2005) 8009–15. [PubMed: 16335925]
20. Dayam R; Sanchez T; Clement O; Shoemaker R; Sei S; Neamati N, Beta-diketo acid pharmacophore hypothesis. 1. Discovery of a novel class of HIV-1 integrase inhibitors. *J Med Chem* 48 (2005) 111–20. [PubMed: 15634005]
21. Zhao XZ; Smith SJ; Maskell DP; Metifiot M; Pye VE; Fesen K; Marchand C; Pommier Y; Cherepanov P; Hughes SH; Burke TR, Structure-Guided Optimization of HIV Integrase Strand Transfer Inhibitors. *J. Med. Chem* 60 (2017) 7315–7332. [PubMed: 28737946]
22. Zhao XZ; Smith SJ; Metifiot M; Johnson BC; Marchand C; Pommier Y; Hughes SH; Burke TR, Bicyclic 1-Hydroxy-2-oxo-1,2-dihydropyridine-3-carboxamide-Containing HIV-1 Integrase Inhibitors Having High Antiviral Potency against Cells Harboring Raltegravir-Resistant Integrase Mutants. *J. Med. Chem* 57 (2014) 1573–1582. [PubMed: 24471816]

23. Ju H; Zhang J; Huang B; Kang D; Huang B; Liu X; Zhan P, Inhibitors of Influenza Virus Polymerase Acidic (PA) Endonuclease: Contemporary Developments and Perspectives. *J Med Chem* 60 (2017) 3533–3551. [PubMed: 28118010]
24. Jones JC; Marathe BM; Lerner C; Kreis L; Gasser R; Pascua PN; Najera I; Govorkova EA, A Novel Endonuclease Inhibitor Exhibits Broad-Spectrum Anti-Influenza Virus Activity In Vitro. *Antimicrob Agents Chemother* 60 (2016) 5504–14. [PubMed: 27381402]
25. Credille CV; Chen Y; Cohen SM, Fragment-Based Identification of Influenza Endonuclease Inhibitors. *J Med Chem* 59 (2016) 6444–54. [PubMed: 27291165]
26. Sagong HY; Bauman JD; Patel D; Das K; Arnold E; LaVoie EJ, Phenyl substituted 4-hydroxypyridazin-3(2H)-ones and 5-hydroxypyrimidin-4(3H)-ones: inhibitors of influenza A endonuclease. *J Med Chem* 57 (2014) 8086–98. [PubMed: 25225968]
27. Credille CV; Dick BL; Morrison CN; Stokes RW; Adamek RN; Wu NC; Wilson IA; Cohen SM, Structure-Activity Relationships in Metal-Binding Pharmacophores for Influenza Endonuclease. *J. Med. Chem* 61 (2018) 10206–10217. [PubMed: 30351002]
28. Wang Y; Tang J; Wang Z; Geraghty RJ, Metal-chelating 3-hydroxypyrimidine-2,4-diones inhibit human cytomegalovirus pUL89 endonuclease activity and virus replication. *Antiviral Res* 152 (2018) 10–17. [PubMed: 29425830]
29. Kankanala J; Wang Y; Geraghty RJ; Wang Z, Hydroxypyridonecarboxylic Acids as Inhibitors of Human Cytomegalovirus pUL89 Endonuclease. *ChemMedChem* 13 (2018) 1658–1663. [PubMed: 29968426]
30. Wang Y; Mao L; Kankanala J; Wang Z; Geraghty RJ, Inhibition of Human Cytomegalovirus pUL89 Terminase Subunit Blocks Virus Replication and Genome Cleavage. *J Virol* 91 (2017).
31. Nowotny M, Retroviral integrase superfamily: the structural perspective. *EMBO reports* 10 (2009) 144–51. [PubMed: 19165139]
32. Kirschberg TA; Balakrishnan M; Squires NH; Barnes T; Brendza KM; Chen X; Eisenberg EJ; Jin W; Kutty N; Leavitt S; Licican A; Liu Q; Liu X; Mak J; Perry JK; Wang M; Watkins WJ; Lansdon EB, RNase H active site inhibitors of human immunodeficiency virus type 1 reverse transcriptase: design, biochemical activity, and structural information. *J. Med. Chem* 52 (2009) 5781–4. [PubMed: 19791799]
33. Williams PD; Staas DD; Venkatraman S; Loughran HM; Ruzek RD; Booth TM; Lyle TA; Wai JS; Vacca JP; Feuston BP; Ecto LT; Flynn JA; DiStefano DJ; Hazuda DJ; Bahnck CM; Himmelberger AL; Dornadula G; Hrin RC; Stillmock KA; Witmer MV; Miller MD; Grobler JA, Potent and selective HIV-1 ribonuclease H inhibitors based on a 1-hydroxy-1,8-naphthyridin-2(1H)-one scaffold. *Bioorg. Med. Chem. Lett* 20 (2010) 6754–6757. [PubMed: 20869872]
34. Beilhartz GL; Ngure M; Johns BA; Deanda F; Gerondelis P; Gotte M, Inhibition of the Ribonuclease H Activity of HIV-1 Reverse Transcriptase by GSK5750 Correlates with Slow Enzyme-Inhibitor Dissociation. *J. Biol. Chem* (2014).
35. Kankanala J; Kirby KA; Liu F; Miller L; Nagy E; Wilson DJ; Parniak MA; Sarafianos SG; Wang ZQ, Design, Synthesis, and Biological Evaluations of Hydroxypyridonecarboxylic Acids as Inhibitors of HIV Reverse Transcriptase Associated RNase H. *J. Med. Chem* 59 (2016) 5051–5062. [PubMed: 27094954]
36. Vernekar SKV; Liu Z; Nagy E; Miller L; Kirby KA; Wilson DJ; Kankanala J; Sarafianos SG; Parniak MA; Wang ZQ, Design, Synthesis, Biochemical, and Antiviral Evaluations of C6 Benzyl and C6 Biaryl methyl Substituted 2-Hydroxylisoquinoline-1,3-diones: Dual Inhibition against HIV Reverse Transcriptase-Associated RNase H and Polymerase with Antiviral Activities. *J. Med. Chem* 58 (2015) 651–664. [PubMed: 25522204]
37. Wang L; Tang J; Huber AD; Casey MC; Kirby KA; Wilson DJ; Kankanala J; Parniak MA; Sarafianos SG; Wang Z, 6-Biphenylmethyl-3-hydroxypyrimidine-2,4-diones potently and selectively inhibited HIV reverse transcriptase-associated RNase H. *Eur. J. Med. Chem* 156 (2018) 680–691. [PubMed: 30031978]
38. Tang J; Liu F; Nagy E; Miller L; Kirby KA; Wilson DJ; Wu BL; Sarafianos SG; Parniak MA; Wang Z, 3-Hydroxypyrimidine-2,4-diones as Selective Active Site Inhibitors of HIV Reverse Transcriptase-Associated RNase H: Design, Synthesis, and Biochemical Evaluations. *J. Med. Chem* 59 (2016) 2648–2659. [PubMed: 26927866]

39. Wang L; Tang J; Huber AD; Casey MC; Kirby KA; Wilson DJ; Kankanala J; Xie J; Parniak MA; Sarafianos SG; Wang Z, 6-Arylthio-3-hydroxypyrimidine-2,4-diones potently inhibited HIV reverse transcriptase-associated RNase H with antiviral activity. *Eur. J. Med. Chem* 156 (2018) 652–665. [PubMed: 30031976]
40. Li Y; Xuan S; Feng Y; Yan A, Targeting HIV-1 integrase with strand transfer inhibitors. *Drug Discov. Today* 20 (2015) 435–449. [PubMed: 25486307]
41. McColl DJ; Chen X, Strand transfer inhibitors of HIV-1 integrase: bringing IN a new era of antiretroviral therapy. *Antiviral Res.* 85 (2010) 101–118. [PubMed: 19925830]
42. Schwaib AG; Cornella-Taracido I, Causes and Significance of Increased Compound Potency in Cellular or Physiological Contexts. *J. Med. Chem* 61 (2018) 1767–1773. [PubMed: 28820267]
43. Selikson SJ; Watt DS, The oxidative decyanation of arylacetonitriles via α -thiophenoxy arylacetonitriles. *Tetrahedron Letters* 15 (1974) 3029–3032.
44. Freerksen RW; Selikson SJ; Wroble RR; Kyler KS; Watt DS, Oxidative decyanation of secondary nitriles to ketones. *The Journal of Organic Chemistry* 48 (1983) 4087–4096.
45. Schrödinger Small-Molecule Drug Discovery Suite 2014–3: Glide, version 6.4, Schrödinger, LLC, New York, NY, 2014, 2014.
46. Friesner RA; Murphy RB; Repasky MP; Frye LL; Greenwood JR; Halgren TA; Sanschagrin PC; Mainz DT, Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem* 49 (2006) 6177–96. [PubMed: 17034125]
47. Fletcher RS; Holleschak G; Nagy E; Arion D; Borkow G; Gu Z; Wainberg MA; Parniak MA, Single-step purification of recombinant wild-type and mutant HIV-1 reverse transcriptase. *Protein Expres. Purif* 7 (1996) 27–32.
48. Parniak MA; Min KL; Budihas SR; Le Grice SF; Beutler JA, A fluorescence-based high-throughput screening assay for inhibitors of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H activity. *Anal. Biochem* 322 (2003) 33–9. [PubMed: 14705777]
49. Singh B. M. Kamalendra, Rai Devendra K., Sharma Bechan, Michailidis Eleftherios., Ryan K. B. M. Emily M., Leslie Maxwell D., Hagedorn Ariel N., Li Zhe, Norden Pieter R., Hachiya M. A. P. Atsuko, Xu Hong-Tao, Wainberg Mark A., and Sarafianos Stefan G., Biochemical Mechanism of HIV-1 Resistance to Rilpivirine. *The Journal of biological chemistry* 287 (2012) 38110–38123. [PubMed: 22955279]
50. Kankanala J; Kirby KA; Huber AD; Casey MC; Wilson DJ; Sarafianos SG; Wang Z, Design, synthesis and biological evaluations of N-Hydroxy thienopyrimidine-2,4-diones as inhibitors of HIV reverse transcriptase-associated RNase H. *European journal of medicinal chemistry* 141 (2017) 149–161. [PubMed: 29031062]
51. Tang J; Kirby KA; Huber AD; Casey MC; Ji J; Wilson DJ; Sarafianos SG; Wang Z, 6-Cyclohexylmethyl-3-hydroxypyrimidine-2,4-dione as an inhibitor scaffold of HIV reverse transcriptase: Impacts of the 3-OH on inhibiting RNase H and polymerase. *European journal of medicinal chemistry* 128 (2017) 168–179. [PubMed: 28182989]
52. Vernekar SKV; Tang J; Wu B; Huber AD; Casey MC; Myshakina N; Wilson DJ; Kankanala J; Kirby KA; Parniak MA; Sarafianos SG; Wang Z, Double-Winged 3-Hydroxypyrimidine-2,4-diones: Potent and Selective Inhibition against HIV-1 RNase H with Significant Antiviral Activity. *Journal of medicinal chemistry* 60 (2017) 5045–5056. [PubMed: 28525279]
53. Wang Z; Bennett EM; Wilson DJ; Salomon C; Vince R, Rationally designed dual inhibitors of HIV reverse transcriptase and integrase. *J. Med. Chem* 50 (2007) 3416–3419. [PubMed: 17608468]
54. Sirivolu VR; Vernekar SKV; Ilina T; Myshakina NS; Parniak MA; Wang ZQ, Clicking 3'-azidothymidine into novel potent inhibitors of human immunodeficiency virus. *J. Med. Chem* 56 (2013) 8765–8780. [PubMed: 24102161]
55. Abram ME; Parniak MA, Virion instability of human immunodeficiency virus type 1 reverse transcriptase (RT) mutated in the protease cleavage site between RT p51 and the RT RNase H domain. *J. Virol* 79 (2005) 11952–11961. [PubMed: 16140771]
56. Sastry GM; Adzhigirey M; Day T; Annabhimoju R; Sherman W, Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. *J Comput Aided Mol Des* 27 (2013) 221–34. [PubMed: 23579614]

57. Schrödinger Release 2014–3: Schrödinger Suite 2014–3. (a) Protein Preparation Wizard. (b) Epik version 2.9; Schrödinger, LLC: New York, NY, 2014. (c) Impact version 6.4; Schrödinger, LLC: New York, NY, 2014, 2014.
58. Jorgensen WL; Maxwell DS; Tirado-Rives J, Development and Testing of the OPLS All-Atom Force Field on Conformational Energetics and Properties of Organic Liquids. *J. Am. Chem. Soc* 118 (1996) 11225–11236.
59. Schrödinger Release 2014–3: LigPrep, version 3.1, Schrödinger, LLC, New York, NY, 2014, 2014.

Highlights

- Novel and highly potent inhibitors of HIV RT-associated RNase H.
- Inhibitors uniquely feature a nonflexible carbonyl linkage.
- Low nM RNase H inhibition, moderate INST inhibition and marginal RT pol inhibition.
- A few analogues exhibited significant antiviral activity with no cytotoxicity.
- Molecular modeling study corroborated the RNase H active site binding mode.

Figure 1. Pharmacophore model of active site-directed HIV RNase H inhibitors. Best inhibitor types reported by others (**1–3**) and our own group (**4–6**) all contain a chelating triad (magenta) capable of binding two divalent metal ions, a hydrophobic aryl or biaryl moiety (cyan), and a one-atom flexible linker (blue).

Figure 2.
Impact of the C-6 linker (blue) for HPD subtype **6** and the design of novel HPD subtypes **7–8** featuring a less flexible carbonyl linker.

Figure 3.

Predicted binding mode of compound **7a** (A) and compound **8a** (B) within the active site of RNase H (PDB code: 5J1E[35]). The p66 and p51 subunits of RT are shown in orange and gray cartoon, respectively. Active site residues are shown as yellow sticks with metal ions (Mg^{2+}) as green spheres. Chelating and H-bond interactions are depicted as black dashed lines.

Scheme 1. Synthesis of HPD subtype 7–8^a

^a Reagents and conditions: a) chloromethyl ethyl ether, BSA, TBAI, DCM, rt, 81%; b) ArCH₂CN, NaH, DMF, 0°C to rt, 76%–92%; c) K₂CO₃, DMSO, O₂, rt, 37%–80%; d) Pd/C, MeOH, rt; e) TFA, MW, 120, 20 min, 43%–85%.

Scheme 2. Alternative synthesis of HPD subtype 8^a

^aReagents and conditions: a) R-PhB(OH)₂, Pd(PPh₃)₄, K₂CO₃, MeOH, 80 °C, 1 h, 37%–80%; b) Pd/C, MeOH, rt; c) TFA, MW, 120, 20 min, 46%–87%.

Table 1.

Biochemical inhibition of C-6 carbonyl HPD subtypes **7–8** against HIV-1 RNase H and pol activities of RT, and the INST activity of HIV-1 IN.

Compd	Ar	RT RNase H IC ₅₀ ^a (μM)	RT pol IC ₅₀ (μM)	INST IC ₅₀ (INST IC ₅₀ (μM))
7a		0.093 ± 0.014	>10	7.2 ± 2.1
7b		0.081 ± 0.008	>10	2.1 ± 0.5
7c		0.085 ± 0.010	>10	4.0 ± 1.0
7d		0.031 ± 0.017	>10	13 ± 2.6
7e		0.090 ± 0.017	>10	4.9 ± 0.9
8a		0.021 ± 0.002	>10	10 ± 2.7
8b		0.026 ± 0.006	>10	>100
8c		0.027 ± 0.005	>10	6.7 ± 0.8
8d		0.015 ± 0.001	>10	6.7 ± 1.5
8e		0.063 ± 0.009	>10	3.5 ± 1.0

Compd	Ar	RT RNase H IC ₅₀ ^a (μM)	RT pol IC ₅₀ (μM)	INST IC ₅₀ (INST IC ₅₀ (μM))
8f		0.022 ± 0.003	>10	2.5 ± 0.5
8g		0.017 ± 0.001	>10	7.1 ± 1.4
8h		0.53 ± 0.31	5.9 ± 0.8	>100
8i		0.024 ± 0.001	7.7 ± 2.3	0.53 ± 0.1
8j		0.074 ± 0.02	9.6 ± 3.7	0.33 ± 0.06
8k		0.019 ± 0.001	8.3 ± 2.2	4.3 ± 1.3
8l		0.023 ± 0.003	>10	0.37 ± 0.07
16f		0.42 ± 0.12	>10	>100
16g		1.1 ± 0.07	>10	>100
17e		3.7 ± 0.6	>10	>100
18f		0.76 ± 0.04	>10	>100

Compd	Ar	RT RNase H IC ₅₀ ^a (μM)	RT pol IC ₅₀ (μM)	INST IC ₅₀ (INST IC ₅₀ (μM))
3	--	0.70	0.85	NT
RAL	--	>10	NT	0.65 ± 0.1
DTG	--	>10	NT	0.068 ± 0.01

Concentration of compound inhibiting the enzymatic function by 50%, expressed as the mean ± standard deviation from at least two independent experiments.

Table 2.Antiviral activity of subtypes **7** and **8** analogues against HIV-1.

Compd	Antiviral Profile	
	EC ₅₀ ^a (μM)	CC ₅₀ ^b (μM)
7a	>20	48 ± 3.9
7b	>20	>100
7c	>20	>100
7d	>20	>100
7e	9.5 ± 1.8	21 ± 7.5
8a	4.7 ± 0.02	22 ± 0.8
8b	5.3 ± 0.06	17 ± 0.5
8c	9.0 ± 1.4	>100
8d	>20	>100
8e	>20	>100
8f	15 ± 0.7	>100
8g	>20	>100
8h	>20	>100
8i	>20	>100
8j	6.7 ± 0.9	8.4 ± 0.4
8k	>20	>100
8l	18 ± 0.2	>100
16f	>20	>100
16g	12 ± 1.2	73 ± 0.7
17e	>20	>100
18f	>20	>100
RAL	0.03	--
DTG	0.02	--

^aConcentration of compound inhibiting HIV-1 replication by 50%, expressed as the mean ± standard deviation from at least two independent experiments.

^bConcentration of compound causing 50% cytotoxicity, expressed as the mean ± standard deviation from at least two independent experiments.