Project Title:

HTS Assays for Inhibitors of HIV RNase H

Grant Number:

MH077605

Screening Center Name:

University of Pittsburgh Molecular Screening Center

Principal Investigator of Screening Center:

John S. Lazo

Assay Submitter & Institution:

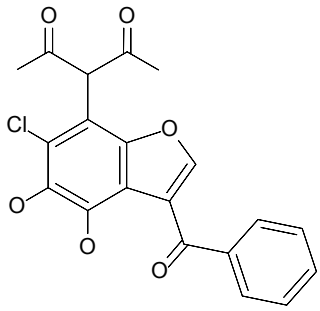
Michael Parniak
University of Pittsburgh

Assay or Pathway Target:

HIV-1 Reverse Transcriptase Associated Ribonuclease H (RT-RNH)

Probe PubChem Compound Identifier (CID):

PubChem-CID 665013 (PubChem-SID 863762)



Specific Aim: The conversion of HIV genomic RNA into DNA comprises multiple steps, each catalyzed by the essential viral enzyme, reverse transcriptase (RT). To catalyze this conversion, RT is multifunctional, possessing both DNA polymerase activity (RNA-dependent and DNA-dependent) and ribonuclease H (RNH) activity. Eleven of the therapeutics currently used for the clinical treatment of HIV infection target the viral DNA polymerase, reverse transcriptase (RT). Seemingly thousands of inhibitors of RT DNA polymerase activity have been identified, and all clinically used antivirals targeting RT are directed at this activity. Viral resistance to these drugs is an increasingly serious problem in the therapeutic management of HIV infection, and new drugs are needed, especially drugs that inhibit new targets of HIV. RT RNH is absolutely essential for HIV replication, and therefore represents a logical target for antiviral development. Unfortunately, very few inhibitors of HIV RT RNH have been described, and none are in preclinical development, let alone in clinical use. One of the reasons for the scarcity of RNH inhibitors has been the lack of an HTS assay to measure RNH activity. Traditional gel based RNH assays are cumbersome and time consuming, unsuitable for high throughput screening (HTS) of large libraries of compounds. We have developed a rapid and simple fluorescence based assay for RNH and have validated this assay in HTS screens using 96- and 384-well microtiter plates. We therefore believe that this assay is suitable for use in the Molecular Libraries Screening Center Network (MLSCN). To this end, we propose the following Specific Aims to be carried out by the MLSCN:

1. To transfer and implement our HTS assay technology in the MLSCN.
2. To validate hits obtained in the primary screen.
3. To evaluate the cytotoxicity of validated hits.
4. To conduct preliminary structural optimization of selected validated hits.

Significance: The rapid development of HIV-1 resistance to antiretroviral agents is a major clinical problem. This troubling phenomenon has been observed with each class of current clinically used anti-HIV agents. An increasingly serious clinical problem is the emergence of multidrug resistant variants of HIV-1 that show varying levels of resistance to many of the experimental drugs in the preclinical pipeline, since these analogs are directed at the same viral target as those agents to which the virus has developed resistance. There is an urgent need to identify and validate new HIV targets for the discovery and development of novel therapeutics. HIV-1 RT RNH represents one such possibility, but this target has not yet been validated for therapeutic utility at the level of cell based antiviral assays as the inhibitors so far described lack drug like characteristics. We hope that by using our HTS assay in the MLSCN system, suitable drug like leads may be identified, optimized and used to validate HIV RT-RNH as a target for antiretroviral drug development.

Rationale: Very few inhibitors of RT-RNH have been described, and those identified to date lack the drug like characteristics needed for therapeutic utility. One of the problems with RT-RNH as a target for drug discovery has been the lack of a suitable high-throughput screening (HTS) assay. We have developed a fluorescence resonance energy transfer assay readily applicable to 96-well and 384-well microplate formats with robotic manipulation to enable high-throughput screening for inhibitors of HIV-1 RT-RNH. The substrate is an 18 nucleotide 3' - fluorescein labeled RNA annealed to a complementary 18 nucleotide 5' - DABCYL modified DNA. The intact duplex has an extremely low background fluorescent signal and provides up to 50-fold fluorescent signal enhancement following hydrolysis. The size and sequence of the duplex are such that RT-RNH cuts the RNA strand four nucleotides from the 3'-end. The labeled tetraribonucleotide readily dissociates from the complementary DNA at ambient temperature with immediate generation of a fluorescent signal. The assay is rapid, inexpensive and robust, providing Z' factors of 0.8 and coefficients of variation of about 5%. The assay requires only two addition steps with no washing and is thus suitable for robotic operation. We hope that by using our HTS assay in the MLSCN suitable drug like leads may be identified and optimized.

Assay Implementation and Screening

PubChem Bioassay Names:

1. Primary Screen: HIV-1 RT-RNH H MLSCN HTS MH077605 **AID 565**
2. Confirmation Assay: HIV-1 RT-RNH H MLSCN HTS MH077605 Confirmation Assay **AID 651**
3. Concentration Response Assay: HIV-1 RT-RNH H MLSCN MH077605 Probe Assessment: Dose response Assay **AID 652**

Primary Assay Description as defined in PubChem:

The HIV-1 RT-RNH H assay was submitted by Dr. Michael Parniak of the University of Pittsburgh, MLSCN XO1 MH077605, and the HTS was developed and screened at the University of Pittsburgh Molecular Library Screening Center (PMLSC). We have developed and implemented a 384-well format homogeneous fluorescence resonance energy transfer assay to screen for inhibitors of HIV-1 RT-RNH. The substrate is an 18 nucleotide 3' - fluorescein labeled RNA annealed to a complementary 18 nucleotide 5' - DABCYL modified DNA. The intact duplex has an extremely low background fluorescent signal and provides up to 50-fold fluorescent signal enhancement following hydrolysis. The size and sequence of the duplex are such that RT-RNH cuts the RNA strand four nucleotides from the 3'-end. The labeled tetraribonucleotide readily dissociates from the complementary DNA at ambient temperature with immediate generation of a fluorescent signal.

RT-RNH HTS Assay Conditions

Final Assay Buffer: 50 mM Tris pH 8.0, 60 mM KCl, 5 mM $MgCl_2$

1. Compounds: add 10 μL of 100 μM compounds to obtain 10μM and 1% DMSO final concentrations
2. RT-RNH Enzyme: add 40 μL containing 60 ng of RT-RNH
3. RNA/DNA duplex substrate: add 50 μL containing 70 nM RNA/DNA duplex
4. Incubate for 20 min at ambient temperature
5. Terminate reaction with 10 μL of 1M EDTA pH 9.0
6. Read the fluorescent intensity in the wells of the 384-well microtiter plates on the Spectromax M5; Excitation: 490 nm, Emission: 528 nm, Cutoff: 515 nm.

Center Summary of the Primary Screening Assays:

- Primary HTS - 65,239 compounds were screened at 10 μM, **AID 565**
  - 1190 (1.82%) compounds exhibited ≥ 50 % inhibition
  - A cherry pick order for 1042 compounds was submitted to Biofocus-DPI, compounds that exhibited ≥ 50 % inhibition and a Z-score < -3 in the primary screen
- Re-test of primary HTS actives at 10 μM in duplicate to confirm their activity in the RT-RNH assay, **AID 651**
  - 390 (37.4 %) compounds were confirmed active by exhibiting ≥ 50 % inhibition in both duplicate tests
- Compounds confirmed active in **AID 651** were counter screened against FITC to confirm that they do not simply quench the fluorescent signal
  - None of the compounds appear to quench FITC fluorescence significantly (≥ 20% quenching of fluorescence)
- 10-point concentration response confirmations, **AID 652**
  - 372 (95.4%) compounds exhibited IC_{50}s < 50 μM; 41 exhibited IC_{50}s < 1.0 μM, 257 exhibited IC_{50}s in the 1.0 to 10 μM range, and 74 exhibited IC_{50}s in the 10 to 50 μM range

- Compound structures that exhibited IC_{50}s < 50 μM were analyzed using the Leadscope Enterprise 2.4.6-1 software and subjected to structure based clustering and classification techniques based on recursive partitioning
  - 65 singletons and 64 clusters ranging from 2 to 24 structures per cluster

Summary Secondary Assays Performed by Assay Provider.

(A) HIV-1 RT RNA-dependent DNA polymerase activity.

1. Assay protocol: HIV-1 RT RNA-dependent DNA polymerase activity in the absence or the presence of 10 μM compound was determined using 0.2 units/ml of poly(rA)-oligo$(dT)_{12-18}$, 2 nM recombinant RT p66/p51 heterodimer, 5 μM [^{3}H] TTP in a final volume of 50 μl of 50 mM Tris-HCl, pH 8.0 containing 60 mM KCl and 10 mM $MgCl_2$. Samples were incubated for 20 min at 37 ºC and then quenched by adding 200 μL of ice-cold 10% trichloroacetic acid containing 20 mM sodium pyrophosphate. After 20 minutes incubation on ice, samples were filtered through 1.2 μm glass fiber type C filter in 96-well microplates (Millipore, Bedford MA). The filters were washed sequentially with 10% TCA and ethanol, and the extent of [^{3}H]-TMP incorporation into the precipitated nucleic acid was quantified by liquid scintillation spectrometry.

2. Results: 238 compounds were assayed at 10 μM final concentration. Assays were carried out twice (2 separate experiments). Three compounds showed > 50% inhibition in both assays, 68 compounds showed 25 – 50% inhibition in both assays, and the rest were < 25% inhibition in both assays or showed substantial variability between assays, with at least one value less than 25% inhibition. These inhibition potencies are substantially weaker than those of therapeutically used HIV-1 RT RNA-dependent DNA polymerase inhibitors such as efavirenz (in vitro $IC_{50} \approx 1 – 5$ nM), thus we considered that IC_{50} determinations on the MLSCN compounds was not warranted. Data are summarized in Appendix Table 2.

(B) HIV-1 replication assay.

1. Assay protocol: Antiviral assays were carried out by evaluating single-cycle viral infectivity of P4R5 HIV-1 infection indicator cells cultured in 96-well microplates ($5x10^3$ cells per well). Cells were obtained from Dr. John Mellors, University of Pittsburgh, and maintained in DMEM/10% FBS supplemented with puromycin (0.5 μg/mL). P4R5 cells express CD4, CXCR4 and CCR5 as well as a β-galactosidase reporter gene under the control of an HIV LTR promoter (Munk et al., 2002; Parikh et al, 2005). The cells were incubated with varying concentrations of compound and inoculated with HIV-1. The extent of infection was assessed 48h post-infection using a fluorescence-based β-galactosidase detection assay using 4-methylumbelliferyl-β-D-galactopyranoside (4-MUG). Briefly, infected cells were washed, then incubated with 100 μL lysis buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 (pH 7.2), 1 mM $MgSO_4$, 100 mM β-mercaptoethanol, 2% [v/v] Triton X-100) for 1 h at 37°C. β-galactosidase activity was assessed by addition of 50 μL 4-MUG to a final concentration of 0.5 mM, followed by incubation for 1 h at 37°C, and quenching with 150 μL 0.2 M Na_2CO_3, pH 11.2. Fluorescence intensity was assessed with a SPECTRAmax GEMINI XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 355 nm and an emission wavelength of 480 nm, with cutoff filter set to 475 nm.

Munk, C., Brandt, S. M., Lucero, G., Landau, N. R., 2002. A dominant block to HIV-1 replication at reverse transcription in simian cells. Proc.Natl.Acad.Sci. U.S.A 99, 13843-13848.

Parikh, U. M., Koontz, D. L., Chu, C. K., Schinazi, R. F., Mellors, J. W. 2005. In vitro activity of structurally diverse nucleoside analogs against human immunodeficiency virus type 1 with the K65R mutation in reverse transcriptase. Antimicrob. Agents Chemother. **49:**1139-1144.

2. Results: 238 compounds were assayed at 10 μM and 50 μM final concentrations. Two compounds showed ≥ 50% inhibition of HIV-1 replication at 10 μM final concentration; these compounds were further evaluated by establishing IC_{50} values for inhibition of HIV-1 replication. Data are summarized in Appendix Table 2.

(C) Cytotoxicity. Formal cytotoxicity measurements were not carried out. Rather, cytotoxicity was evaluated qualitatively by microscopic assessment of P4R5 cell number and cell morphology 48h post exposure. Data are summarized in Appendix Table 2.

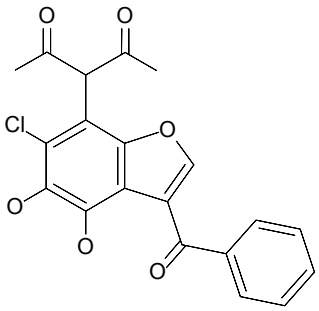
Probe Optimization
Chemical name of probe compound:

3-[3-(benzoyl)-6-chloro-4,5-dihydroxy-1-benzofuran-7-yl]pentane-2,4-dione

Molecular Formula: $C_{20}H_{15}ClO_6$

MW: 386.782

Draw probe chemical structure and show stereochemistry if known:

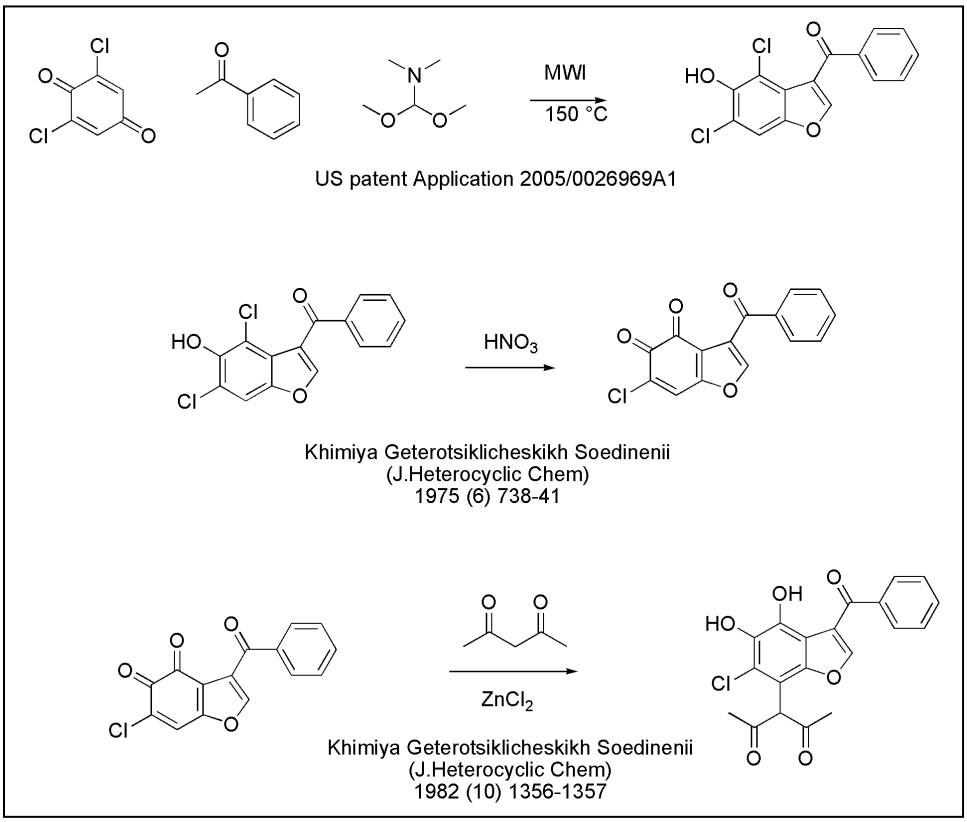


PubChem-CID 665013 Average IC_{50} of 30.7 ± 23.8 nM (n=5)

Describe mode of action for biological activity of probe:
No Mechanism of action Enzyme Kinetics data presently available

Has this compound been provided to the MLSMR:
Compound 665013 was obtained directly from MLSMR
MLSMR_SAMPLE_SUPPLIER : InterBioScreen
MLSMR_SUPPLIER_STRUCTURE_ID : STOCK4S-82292
MLSMR_COMPOUND_CLASS : DC
Center comments on chemistry strategy leading to probe identification:
Compound 665013 was obtained directly from MLSMR and is commercially available.

Detailed synthetic pathway for making probe:



Center Summary of Probe Properties

PubChem Listed Properties:
Molecular Weight: 386.7825 g/mol, Molecular Formula: $C_{20}H_{12}ClO_6$, Hydrogen Bond Donor Count: 2, Hydrogen Bond Acceptor Count: 6, Rotatable Bond Count: 5, Tautomer Count: 52, Topological Polar Surface Area: 105. XLogP: 3.7, Exact Mass: 386.055716, MonoIsotopic Mass: 386.055716, Heavy Atom Count: 27, Charge: 0, Complexity: 587, Isotope Atom Count: 0, Defined Atom StereoCenter Count: 0, Undefined Atom StereoCenter Count: 0, Defined Bond StereoCenter Count: 0' Undefined Bond StereoCenter Count: 0, Covalently-Bonded Unit Count: 1

LeadScope Calculated Properties:

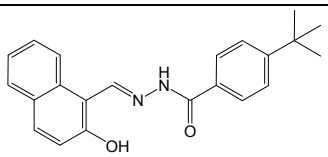
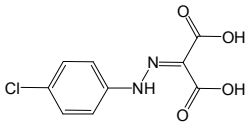
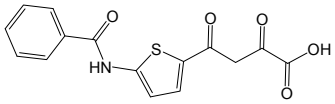
PubChem_SID	Hydrogen Bond Donors	Parent Molecular Weight	Hydrogen Bond Acceptors	Lipinski Score	Molecular Weight	Polar Surface Area	ALogP	Rotatable Bonds	Parent Atom Count
863762	2	386.8	5	0	386.8	104.8	3.99	5	27

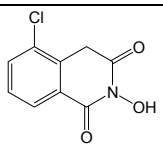
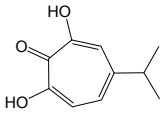
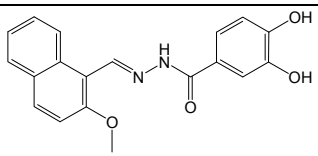
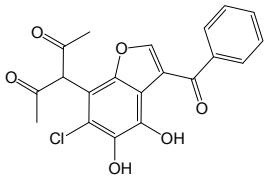
Appendices:

I. Comparative data on probe, similar compound structures and prior probes

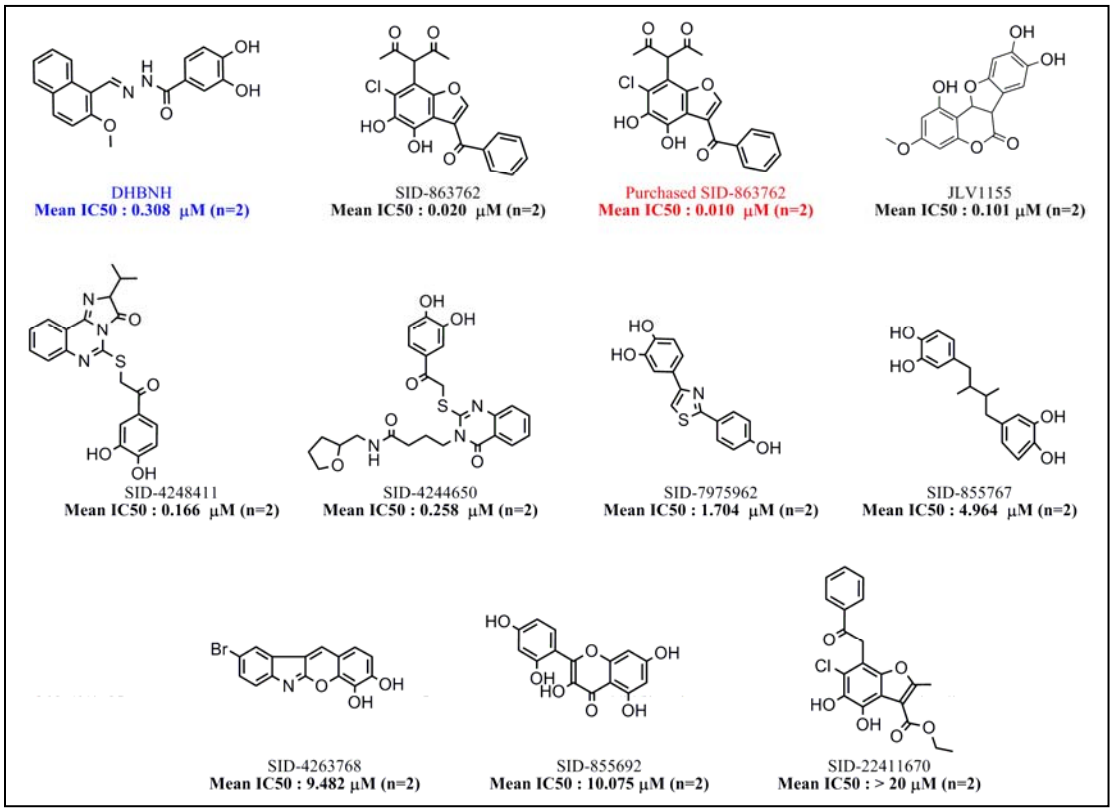
While many inhibitors of HIV RT DNA polymerase activity have been discovered and include eleven drugs in current clinical use, very few potent inhibitors of HIV RT-associated RNH have been described. The 'state-of – the-art' for RNHI is summarized in Table 1. Parniak's group was the first to describe an RNHI with reasonable potency[1]. The acylhydrazone N-(4-tert-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH; ***1***) inhibits HIV-1 RT RNH ($IC_{50} \approx 3$ μM) as well as Moloney murine leukemia virus RT RNH and *E. coli* RNH. Subsequently, Peliska's group identified the aryl hydrazone 4-chlorophenylhydrazone mesoxalate[2] (CPHM; ***2***). While BBNH and CPHM share some structural similarity, they show significant differences in inhibitory mechanism. BBNH is in fact a bifunctional inhibitor of RT, inhibiting both the DNA polymerase and RNH activities of the enzyme[1]. Various lines of evidence suggest that this bifunctional inhibition arises from binding to two sites on RT, one of which is in the RNH domain. In contrast, CPHM is specific for RNH and does not inhibit RT DNA polymerase activity and does not inhibit HIV-1 RT RNH primary cuts but rather acts at the level of secondary RNH cleavage events[2]. In contrast, acylhydrazones such as BBNH inhibit both primary and secondary RNH cleavages (Parniak, unpublished data). The Merck group has described a diketo acid RNHI[3] (***3***) discovered as an offshoot of their integrase inhibitor discovery program. Recently, two new classes of RNHIs have been described, N-hydroxyimides[4] (***4***) and hydroxytropolones[5] (***5***). None of the preceding compounds is a therapeutic candidate since they have poor 'drug-like' characteristics (e.g., charged functionality that prevents cell uptake), potency far too low to be of therapeutic value, significant cytotoxicity, or some combination of these problems. Parniak's group in collaboration with the Arnold group at Rutgers recently described DHBNH, an acylhydrazone analog of BBNH with substantially reduced cytotoxicity and reasonable antiviral potency[6]. DHBNH is the only published RNHI with these properties.

Table 1. Structures and inhibitory properties of published RNHI in comparison to probe [SID]863762

COMPOUND #	STRUCTURE	RT RNH IC_{50} (μM)	RT RDDP IC_{50} (μM)	HIV-1 REPLICATION IC_{50} (μM)	COMMENTS
1		1.5 - 3	3	≈ 5	Cytotoxicity above 10 μM
2		3	> 50	Not reported	Shaw-Reid et al[3] report RDDP inhibition at 2.3 μM; we do not find any inhibition
3		3.2 ± 1.7	> 50	Not reported	

4		0.3	83	Not reported	
5		0.2	> 50	Not reported	
6		0.5 ± 0.2	> 25	5.5 ± 1.7	No cytotoxicity at 100 μM
[SID]863762		0.031 ± 0.024	63% inhibition at 10 μM IC_{50} n.d.	10 μM	Cytotoxicity noted at 25 μM

II. Structure Activity Data on the Inhibition of RT-RNH by Related Catechol Compounds.
Compounds were tested in two independent experiments in 10-pt 3-fold dilution series concentration response curves run in triplicate starting at a maximum concentration of 20 μM. A series of catechol compounds identified in the HTS were compared to DHBNH, and an additional compound (JLV1155) provided by the PMLSC chemistry core.



III. Cross Target Query of PubChem Database:

PubChem-SID	PubChem-CID	# Assays	Active Flags	Primary HTS Actives	Dose response Actives
863762	665013	122	12	10	2

AID	Description	Format
565	HIV-1 RT-RNH H MLSCN HTS MH077605	HTS
651	HIV-1 RT-RNH H MLSCN HTS MH077605 Confirmation Assay	HTS
652	HIV-1 RT-RNH H MLSCN MH077605 Probe Assessment: Dose response Assay	IC50
596	qHTS Assay for Tau Filament Binding	AC50
430	Fluorescent HTS Cytotoxicity/Cell Viability Assay HPDE-C7 Cells	HTS
431	Fluorescent HTS Cytotoxicity/Cell Viability Assay HPDE-C7K Cells	HTS
463	Cell Proliferation & Viability (Cytotoxicity) Primary Assay Jurkat Cells	HTS
598	Human H69AR Lung Tumor Cell Growth Inhibition Assay - 86K Screen	HTS
633	HTS for Estrogen Receptor-beta Coactivator Binding inhibitors	HTS
719	Human Lung Fibroblast Proliferation Assay	HTS
775	Screen for Chemicals that Extend Yeast Lifespan	HTS
777	CYP2C9 Assay	HTS

Bibliography

[1]Borkow G, Fletcher RS, Barnard J, Arion D, Motakis D, Dmitrienko GI, Parniak MA (1997) Inhibition of the ribonuclease H and DNA polymerase activities of HIV-1 reverse transcriptase by N-(4-t-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone. Biochemistry 36, 3179-3185.

[2]Davis WR, Tomsho J, Nikam S, Cook EM, Somand D, Peliska JA (2000) Inhibition of HIV-1 reverse transcriptase-catalyzed DNA strand transfer reactions by 4-chlorophenylhydrazone of mesoxalic acid. Biochemistry 39, 14279-14291.

[3]Shaw-Reid CA, Munshi V, Graham P, Wolfe A, Witmer M, Danzeiesen R, Olsen DB, Carroll SS, Embrey M, Wai JS, Miller MD, Cole JL, Hazuda DJ (2003) Inhibition of HIV-1 ribonuclease H by a novel diketoacid, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid. J Biol Chem 278, 2777-2780.

[4]Hang JQ, Rajendran S, Yang Y, Li Y, In PWK, Overton H, Parkes KEB, Klumpp K (2004) Activity of the isolated HIV RNase H domain and specific inhibition by N-hydroxyimides. Biochem Biophys Res Commun 317, 321-329.

[5]Budihas SR, Gorshkova I, Gaidamakov S, Wamiru A, Bona MK, Parniak MA, Crouch RJ, McMahon JB, Beutler JA, Le Grice SFJ. Selective inhibition of HIV-1 reverse transcriptase-associated ribonuclease H activity by hydroxylated tropolones. Nucl Acids Res 2005; 33, 1249-1256.

[6]Himmel DM, Sarafianos SG, Dhamasena S, Hossain MM, McCoy-Simandle K, Ilina T, Clark, AD Jr., Knight JL, Julias JG, Clark PK, Krogh-Jespersen K, Levy RM, Hughes SH, Parniak MA, Arnold E. 2006. HIV-1 reverse transcriptase structure with RNase H inhibitor DHBNH bound at a novel site. ACS Chem Biol 1, 702-712.