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Small molecule inhibitors of HIV RT Ribonuclease H

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ABSTRACT

Two classes of compounds, thiocarbamates **1** and triazoles **2**, have been identified as HIV RT RNase H inhibitors using a novel FRET-based HTS assay. The potent analogs in each series exhibited selectivity and were active in cell-based assays. In addition, saturable, 1:1 stoichiometric binding to target was established and time of addition studies were consistent with inhibition of RT-mediated HIV replication.

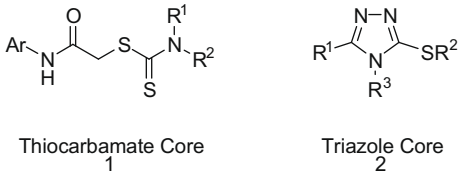
More than 20 years have elapsed since the discovery of the Human Immunodeficiency Virus (HIV) as the etiological agent for Acquired Immune Deficiency Syndrome (AIDS). During this time, progress in the treatment of this disease and its complications, ascribed largely to the 1996 introduction of combination therapy[1] to control viral load, has transformed AIDS into a chronic but manageable illness. While dramatic advances have slowed disease progression and reduced morbidity and mortality in patients, a complete cure in the form of drug therapy or a vaccine remains elusive. Drug resistance and persistent infection represent the major obstacles to disease control and consequently, there is still a need for new therapies with unique mechanisms of action.[2]

HIV reverse transcriptase (RT), one of three key encoded viral proteins, provides two critical functions for viral replication. Through RNA- and DNA-dependent DNA polymerization, it first oversees the conversion of single stranded RNA to double stranded DNA, followed by the degradation of the initial viral RNA strand.[3] The latter is the responsibility of HIV RT Ribonuclease H (RNase H), a non-specific, 15 kD endonuclease located at the C-terminal subunit of the p66 strand of the RT dimer.[4] To accomplish RNA hydrolysis, RNase H employs several amino acids. In particular, Asp443 and Asp498, in conjunction with Gly444, are believed to coordinate a divalent cation (Mg^{+2} or Mn^{+2}),[5] which in turn positions another water molecule to serve as a general base. In addition, Asp549 forms a critical hydrogen bond with a water molecule which, via interaction with His539, serves as the source of the nucleophile that ultimately cleaves the scissile phosphate bond.

That HIV RT RNase H is a viable drug target has been demonstrated, as point mutations within this domain have shown the endonuclease activities of this enzyme are required for viral infectivity.[6] Despite this and the numerous reports in the literature describing RNase H inhibitors,[7] to date there are no approved drugs with this mechanism of action.[8]

Several years ago, we embarked on a program to identify novel HIV RT RNase H inhibitors and we now disclose the preliminary details of this research. The two structural classes of inhibitors to be discussed, based on our initial HTS hits, are shown below (Fig. 1).

The thiocarbamates[9] **1** were synthesized by the convergent route shown in Scheme 1. First anilines **3** were treated with bromoacetyl bromide to give acetanilides **4**, which upon treatment



Thiocarbamate Core
1

Triazole Core
2

Figure 1. Two cores identified through HTS.

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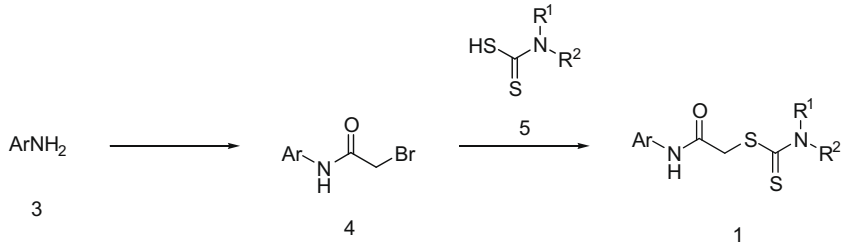
with various carbamodithioic acids, **5**, yielded the final products **1** in 25–55% overall yield. Intermediates **5** were prepared from commercially available amines and carbon disulfide.

As shown in Scheme 2, the triazoles[9] **2**, were prepared by first treating aryl carbohydrazides **6** with various isothiocyanates in hot acetonitrile to give crystalline hydrazine thiocarbamides **7**. Stirring compounds **7** with cesium carbonate in hot ethanol overnight induced cyclization to the corresponding thiones **8**, which were then S-alkylated with an alkylating agent in the presence of a suspension of sodium carbonate and sodium iodide in DMF at room temperature overnight. The desired products **2** were afforded in 30–65% yield, after reverse phase HPLC purification.

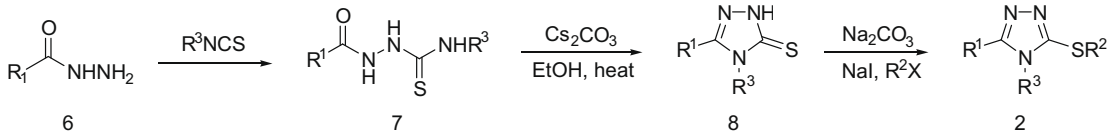
The thiocarbamate and triazole analogs **1a** and **2c** were identified in an HTS using a novel FRET-based assay (Fig. 2).[10] In summary, an appropriate probe oligonucleotide substrate labeled with a fluorophore at the 3′ terminus and a quencher at the 5′ end was annealed to complementary substrate RNA, thus minimizing the interaction between the two labels. In the absence of drug, fully functional RNase H digests the RNA strand of the hybrid oligonucleotide, releasing the tagged DNA strand. Upon liberation, the designed DNA strand snaps into a hairpin loop, bringing the 5′ and 3′ ends in close proximity, and allowing the quencher and fluorophore to interact. The resulting reduction in the fluorescent signal is proportional to RNase H activity.[11] The advantages of this assay are that it is high throughput (readily amenable to 96-, 384- and 1536-well formats), is extremely sensitive, and allows for direct determination of RNase H activity.

Compounds of interest from the HTS were required to have IC_{50} $\leqslant$10 μM in the primary assay, with at least 15-fold selectivity over HIV RT (polymerase), *Escherichia coli* RNase H, and several human DNA polymerases.[12] Compounds meeting these criteria were next screened for cell-based activity utilizing a cytopathic effect (CPE) reduction assay in MT4 cells, where an IC_{50} $\leqslant$10 μM and a therapeutic index of at least 20 was sought. Additionally, potential lead compounds were further evaluated in a binding assay that utilizes changes in the intrinsic fluorescence of the enzyme to confirm saturable, stoichiometric binding to target.[13] To further characterize these compounds, some were assayed in time of addition studies. A total of 500,000 compounds were screened from the Wyeth corporate database and some 1500 hits were identified that then underwent confirmation and secondary screening to yield putative lead series for a discovery program.

The HTS hit **1a** for the thiocarbamate series (Table 1) had modest potency but exhibited good selectivity over the polymerase activity of RT[14] as well as the related *E. coli* RNase H, and screened human polymerases.[15] Newly prepared analogs displayed definable, albeit limited, SAR. For instance, replacing one of the benzyl groups on the thiocarbamate nitrogen (i.e., R^1 or R^2) of **1b** or **1c** with a phenyl moiety resulted in compounds with decreased potency against RNase H (**1b** vs **1g** and



Scheme 1. The synthetic route for the preparation of thiocarbamates **1**.



Scheme 2. The synthetic route for the preparation of triazoles **2**.

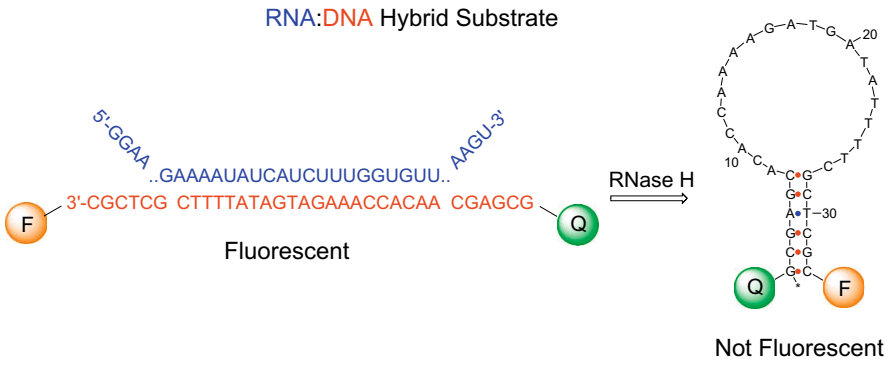
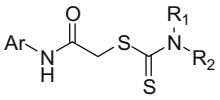


Figure 2. A schematic representation of the high-throughput FRET-based RNase H assay.

Table 1
In vitro IC_{50} data for thiocarbamates **1**



Compound	HIV RT RNase H IC_{50} (μM)	*E. coli* RNase H IC_{50} (μM)	HIV RT IC_{50} (μM)	Ar	R^1	R^2
1a	1.9	>80	>80	2,4-$(Me)_2$–Ph	$PhCH_2$	$PhCH_2$
1b	3.3	>80	>80	2,3,4-Cl_3–Ph	$PhCH_2$	$PhCH_2$
1c	4.7	>80	>80	3-CN–Ph	$PhCH_2$	$PhCH_2$
1d	5.8	>168	>168.3	2,4-Cl_2–Ph	$PhCH_2$	$PhCH_2$
1e	8.3	>80	>80	2-OMe-5-Me–Ph	$PhCH_2$	Ph
1f	11.4	>80	>80	3-CN–Ph	$PhCH_2$	Ph
1g	26.6	>80	>80	2,3,4-Cl_3–Ph	$PhCH_2$	Ph

Table 2
EC_{50} data for selected thiocarbamates **1** in the CPE reduction assay

Compound	HIV RT RNase H IC_{50} (μM)	MTS IC_{50} (μM)	MT4 cells EC_{50} (μM)	MT4 cells TC_{50} (μM)	TI
1a	1.9	>115	3.7	115.1	31.3
1b	3.3	>100	24.8	>98.2	4
1c	4.7	>100	6.5	>116	18
1d	5.8	>120	1.3	105.2	80.9

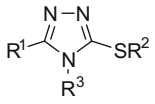
1c vs **1f**). Additionally, increasing the bulk on the amide aryl group (**1a** vs **1d** vs **1g**) or incorporation of electron donating groups was not well tolerated, and the thiocarbamate linkage was required (data not shown).

Despite these limitations, several compounds had profiles that matched our selection criteria and were studied further in a series of cell-based assays. As shown in Table 2, compounds **1a**–**d** were non-cytotoxic in an MTS assay[16] in Vero cells (entries 1–4). In a CPE reduction assay in MT4 cells, **1a**, **1c**, and **1d** had TIs of >18 in MT4 cells and EC_{50}s in the 1.3–6 μM range.[17] Encouraged by these results, we sought to confirm binding to target via a fluorescent binding assay using holoenzyme.[13] Thus, when RT was treated with increasing concentrations of compound **1a**, the data indicated a 1:1 binding stoichiometry and a K_d of 1 μM.

The HTS hit **2c** from the triazole series (Table 3)[18] was a potent inhibitor of RNase H that was more than 100-fold selective over both *E. coli* RNase H and HIV RT and was essentially devoid of activity in human polymerase assays.[15] Modification of this core at R^1–R^3 allowed for a diverse set of analogs that maintained a similar selectivity profile to **2c** (with a few exceptions) and had better defined SAR than the thiocarbamate series. From these data, several general trends were obvious. Thus, the preferred R^3 group on the triazole nitrogen was unsubstituted benzyl, and with rare exceptions (i.e., **2n**), compounds that carried strongly electron donating, electron withdrawing or essentially electron neutral groups showed diminished activity relative to a simple benzyl group. For R^1, 2-thiophene appeared to be optimal, though in some cases, this preference was marginal (compare **2a** and **2h**; **2b** and **2e**–**g**; **2c** and **2i**). Finally, aside from not tolerating strongly electron withdrawing groups (**2l** is an exception), RNase H was very tolerant of substitution at R^2 on sulfur.[19]

Drilling down further, for the subseries where R^3 is benzyl and R^1 is 2-thiophene, the most potent analogs prepared had either a benzyl or picolyl group at R^2 (**2a** and **2b**). In all other cases, incorporation of substituents on R^2 that either affected electron density and/or lipophilicity diminished activity (compare **2a** or **2b** to entries **2c** or **2m**). In some instances, lipophilicity had a greater negative impact on activity relative to increasing electron density (compare **2d** or **2e**–**2o**).[19] Finally, some compounds such as **2d**,

Table 3
In vitro IC_{50} data for triazoles **2**



2

Compound	HIV RT RNase H IC_{50} (μM)	*E. coli* RNase H IC_{50} (μM)	HIV RT IC_{50} (μM)	R^{1*}	R^2	R^3
2a	0.2	>99.8	11.6	2-Thio	4-$PyrCH_2$	$PhCH_2$
2b	<0.21	105	27.3	2-Thio	$PhCH_2$	$PhCH_2$
2c	1	>102.8	103.2	2-Thio	4-Cl-$PhCH_2$	$PhCH_2$
2d	1	>103.6	4.5	Ph	4-MeO–$PhCH_2$	$PhCH_2$
2e	1	>105.6	46.2	Ph	$PhCH_2$	$PhCH_2$
2f	1.2	>106.4	12.4	2-Fur	$PhCH_2$	$PhCH_2$
2g	1.7	>80	30.4	2-Pyr	$PhCH_2$	$PhCH_2$
2h	2.6	>64.4	21.9	2-Pyr	4-$PyrCH_2$	$PhCH_2$
2i	3.8	>104	56.8	2-Fur	4-Cl–$PhCH_2$	$PhCH_2$
2j	3.8	>104	57.0	2-Fur	4-MeO–$PhCH_2$	$PhCH_2$
2k	4	>80	25.2	2-Fur	4-$PyrCH_2$	$PhCH_2$
2l	4.7	>80	80.0	2-Fur	4-CF_3–$PhCH_2$	Ph
2m	5.9	>103.2	>103.2	2-Thio	4-MeO–$PhCH_2$	$PhCH_2$
2n	6.5	>100.4	100.4	2-Fur	4-CF_3–$PhCH_2$	4-MeO–$PhCH_2$
2o	6.0	>103.2	>103.2	Ph	4-Cl–$PhCH_2$	$PhCH_2$

* 2-Thio = 2-thiophene; 2-Fur = 2-furan; 2-Pyr = 2-pyridine.

Table 4
EC_{50} data for selected triazoles **2** in the CPE reduction assay

Compound	HIV RT RNase H IC_{50} (μM)	MTS IC_{50} (μM)	MT4 cells EC_{50} (μM)	MT4 cells TC_{50} (μM)	TI
2a	0.2	109.7	4.0	>104.6	26.0
2b	<0.21	41	4.4	>131.5	30.0
2c	1	16.9	1.4	27.6	19.9
2d	1	33.9	1.1	7.9	7.3
2e	1	28.9	5.8	3.1	0.6
2f	1.2	46	18.5	59.5	3.2
2g	1.7	34	3.6	32.0	8.8
2h	2.6	>80	3.9	>85.2	22.1
2i	3.8	29.4	3.3	15.5	4.7
2j	3.8	35	5.9	1.0	0.2
2k	4	78.6	4.7	<0.005	–
2l	4.7	33.8	18.7	16.9	0.9
2m	5.9	24.6	4.3	2.5	0.6

2h, and **2k** had IC_{50}s against HIV RT (polymerase) that were within 10-fold of their corresponding RNase H IC_{50}s. It is possible that these select compounds bind to different or multiple sites on RT.[20]

As with the thiocarbamate series, the best triazoles analogs were screened further in our cell-based assays and the results are shown in Table 4. In general, this class appeared to have slightly higher inherent cellular toxicity (i.e., MTS values <50 μM) relative to the thiocarbamate class.[21] Still, several compounds that were essentially inactive in the MTS assay were potent in our CPE reduction assay and had TI >20 (see **2a–c** and **2h**). Of the analogs that failed to meet our selection criterion of TI >20-fold, those that had a 2-furan at R^1 failed with the greatest frequency (**2f** and **2i–2l**), perhaps at least in part due to the lability of this group. And whereas the thiocarbamates displayed a reasonable correlation between MTS and TC_{50} data, for some of the triazoles, especially for compounds with TI <10, the correlation was poor. Moreover, these compounds exhibited greater cellular toxicity in MT4 cells than Vero.

For compounds that met our requirements, we further assayed them in our fluorescent binding assay, where we were able to confirm, for instance, saturable, 1:1 stoichiometric binding (K_d = 2 μM) for compound **2c**.[13]

In order to confirm these compounds were operating in cell at the level of RT-associated HIV replication, time of addition studies with compounds **1a** and **2c** were conducted.[22] The data, shown in Figure 3, is consistent with this hypothesis.

We have identified two classes of compounds, thiocarbamates **1** and triazoles **2** as HIV RT RNase H inhibitors using a novel FRET-based HTS assay and demonstrated preliminary SAR for both series. The potent analogs in each series exhibited selectivity against the polymerase activity of HIV RT as well as several human polymerases and the related *E. coli* RNase H. Moreover, several analogs were active and relatively non-cytotoxic in cell-based assays. In addition, saturable, stoichiometric binding to target was established and time of addition studies suggest these inhibitors are operating at the level of RT-mediated HIV replication. Further results expanding on these preliminary data will be reported in due course.

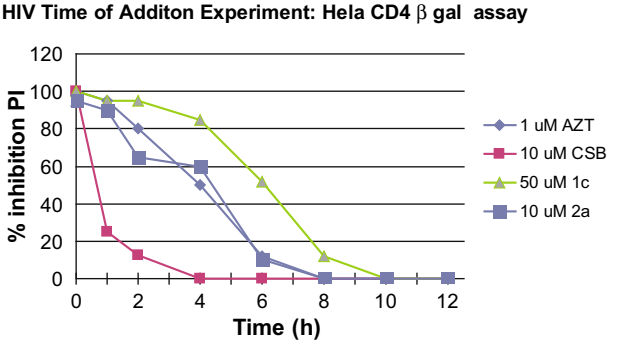


Figure 3. Time of addition analysis of HIV for compounds **1a** and **2c**.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.043.

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9. The routes to the final compounds were optimized using a test case (i.e., a single experiment) and all intermediates were characterized by NMR and MS at a minimum. Once the chemistry was established, subsequent compounds were prepared in parallel, evaluated by LC/MS, and were at least >85% pure based on HPLC data. Compounds of interest were subsequently resynthesized and fully characterized by HPLC, NMR and MS. (a) For a detailed description of the preparation of the reported thiocarbamates, please see: Olson, M. W.; Di Grandi, M.; Prashad, A. U.S. Pat. Appl. Publ. US 2005203176 A1; (b) for a detailed description of the preparation of the reported triazoles, please see: Olson, M.; Di Grandi, M. US Patent 7,563,905, July 21, 2009.
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11. All compounds were evaluated under initial-rate conditions at or near the empirically determined K_M values for all substrates. DMSO concentration was 2% due to the addition of compound. *HIV RT RNase H:* Assays were carried out at 25 °C in a final volume of 25 μL in reaction buffer (50 mM HEPES (pH 8) with 2 mM MgOAc, 200 mM KOAc, 3% glycerol, 1 mM DTT, 0.02% NP-40 and 50 mg/ml BSA), 100 nM RNA/DNA substrate and 2 nM HIV RT (K_M for this substrate was 33 nM). *E. coli RNase H assay: E. coli* RNase H (Ambion) assays were carried using the same buffer and substrate conditions as HIV RT. Enzyme

concentration was 150 pM (K_M for this substrate was 150 nM). *HIV RT assay*: assays were carried out at 25 °C in a final volume of 25 μL in reaction buffer (50 mM HEPES (pH 8.5) with 6 mM $MgCl_2$, 80 mM KCl, 3% glycerol, 1.5 mM DTT, 0.05% TX100 and 50 mg/ml BSA), 40 μM poly rA-oligodT16 (Pharmacia/GE BioTech) 20 mM TTP (Pharmacia/GE BioTech) 10 nM a-33P-TTP (0.03 μCi, Perkin Elmer) and 1.6 nM HIV RT. Substrate hydrolysis was monitored as a function of time using Wallac Victor II fluorescence microplate reader (Perkin Elmer Life Sciences, Inc., Boston MA). For the FRET-based assays, excitation and emission wavelengths were set at 585 and 615 nm, respectively, (10 nm band pass). HIV RT was either purchased from Replidine, Inc. (Denver Colorado) or expressed and purified in house. Instrument data collection was monitored with a personal computer compatible with 32-bit Windows Workstation software, designed to utilize the full capabilities of Windows 95/98/NT. Fluorescent measurements were taken every 30 s.

12. Though human RNase H enzymes were not screened, current literature suggests selectivity over these enzymes should be attainable, see Ref. 5a.
13. These experiments were used to confirm the binding of compounds to the target enzyme HIV RT. No inference as to the location of this binding with respect to the RNase H domain is implied. Moreover in several instances, that 1:1 binding stoichiometry was demonstrated suggests binding is related to enzyme inhibition. The intrinsic fluorescence of the enzyme was monitored at an excitation wavelength of 295 nm and emission of 340 nm in the absence and in the presence of increasing concentrations of the inhibitor. The progressive decrease in the fluorescence of the enzyme upon interaction with the compound was used to determine the binding affinity using a quadratic equation; see: Jamieson, E. R.; Jacobson, M. P.; Barnes, C. M.; Chow, C. S.; Lippard, S. J. *J. Biol. Chem.* **1999**, *274*, 12346.
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15. We screened against H DNA Pol α, H DNA Pol β, H DNA Pol γ, and H RNA Pol 2; IC_{50}s greater than 80 μM were observed; data not shown.
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17. Primary screening for anti-HIV activity was performed in a human T-cell line CEM-X174 infected with HIV-1 89.6 virus in the absence or presence of increasing concentrations of the test compound as described previously (vide infra). The toxicity of the compounds was determined simultaneously on the same plate in uninfected CEM-X174; see: (a) Watson, K. M.; Buckheit, C. E.; Buckheit, R. W., Jr. *Antimicrob. Agents Chemother.* **2008**, *52*, 2787; (b) Stephen, A. G.; Worthy, K. M.; Towler, E.; Mikovits, J. A.; Sei, S.; Roberts, P.; Yang, Q.; Akee, R. K.; Klausmeyer, P.; McCloud, T. G.; Henderson, L.; Rein, A.; Covell, D. G.; Currens, M.; Shoemaker, R. H.; Fisher, R. J. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 1228; (c) Weislow, O. S.; Kiser, R.; Fine, D. L.; Bader, J.; Shoemaker, R. H.; Boyd, M. R. *J. Natl. Cancer Inst.* **1989**, *81*, 577. In a typical CPE reduction assay, the EC_{50} is defined as the concentration of drug that reduced cell death in HIV-infected MT4 cells by 50%; the TC_{50} is the concentration of drug that induced 50% cell death in uninfected MT4 cells; TI = TC_{50}/EC_{50}.
18. Recently, Gilead scientists disclosed a series of triazoles based on Ref. [9b] as HIV-1 RT inhibitors; see: Kirschberg, T. A.; Balakrishnan, M.; Huang, W.; Hluhanich, R.; Kutty, N.; Liclican, A. C.; McColl, D. J.; Squires, N. H.; Lansdon, E. B. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1131. Compounds **2a–2d** in this Letter were also prepared by the Gilead group and for at least compounds **2a–2c**, our data suggests these compounds to be selective RNase H inhibitors.
19. In addition to the biological data presented in Table 3, these conclusions are based on data not shown.
20. Binding studies to determine stoichiometry were not conducted with these compounds. However, in other series, not reported in this Letter, occasionally, we observed greater than 1:1 binding stoichiometry. Alternatively, these compounds could be weak polymerase inhibitors with residual RNase H activity (see Ref. 14).
21. This result could reflect a higher inherent cytotoxicity for this series or could be a reflection of the small number of thiocarbamates screened in this assay.
22. Yang, Q.; Stephen, A. G.; Adelsberger, J. W.; Roberts, P. E.; Zhu, W.; Currens, M. J.; Feng, Y.; Crise, B. J.; Gorelick, R. J.; Rein, A. R.; Fisher, R. J.; Shoemaker, R. H.; Sei, S. *J. Virol.* **2005**, *79*, 6122. In order to examine possible steps of viral replication inhibited by the compounds, the addition of test compounds was delayed for 0, 1, 2, 4, 6, 8, 10, and 12 h after MAGI cells were inoculated with HIV-1NL4-3 (MAGI time-of-addition assay). In this time-of-addition assay format, compounds were added at the following concentrations 10 μM Chicago sky-blue 60, (CSB), 1 μM AZT, 10 μM **2c** 50 μM **1a**.; See: Kimpton, J.; Emerman, M. *J. Virol.* **1992**, *66*, 2232; Sei, S.; Yang, Q. E.; O'Neill, D.; Yoshimura, K.; Nagashima, K.; Mitsuya, H. *J. Virol.* **2000**, *74*, 4621.