4-Amino-1-hydroxy-2-oxo-1,8-naphthyridine-Containing Compounds Having High Potency against Raltegravir-Resistant Integrase Mutants of HIV-1

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ABSTRACT: There are currently three HIV-1 integrase (IN) strand transfer inhibitors (INSTIs) approved by the FDA for the treatment of AIDS. However, the emergence of drug-resistant mutants emphasizes the need to develop additional agents that have improved efficacies against the existent resistant mutants. As reported herein, we modified our recently disclosed 1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamides IN inhibitors to develop compounds that have improved efficacies against recombinant IN in biochemical assays. These new compounds show single-digit nanomolar antiviral potencies against HIV vectors that carry wild-type (WT) IN in a single round replication assay and have improved potency against vectors harboring the major forms of drug resistant IN mutants. These compounds also have low toxicity for cultured cells, which in several cases, results in selectivity indices (CC50/EC50) of greater than 10000. The compounds have the potential, with additional structural modifications, to yield clinical agents that are effective against the known strains of resistant viruses.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is an infectious disease caused by the human immunodeficiency virus (HIV). Reverse transcriptase (RT), protease (PR), and integrase (IN) are the three viral enzymes that are required for viral replication and all three have been targeted by anti-AIDS therapeutics. IN catalyzes the insertion of viral DNA into the host genome in two sequential steps, termed “3′-processing” (3′-P) and “strand transfer” (ST). The 3′-P reaction cleaves two nucleotides from the 3′ end of the viral DNA, exposing a deoxycytosine residue that is used in a nucleophilic attack on the host DNA in the ST reaction. Both of these reactions involve two Mg2+ ions held in place by three acidic residues Asp64, Asp116, and Glu152 that collectively constitute the “DDE” motif.1 IN inhibitors are the most recently developed class of anti-AIDS drugs. Merck’s raltegravir (RAL, 1) (October 2007)2 and Gilead’s elvitegravir (EVG) (August 2012)3 were the first two IN inhibitors to be approved by the FDA. The approved IN inhibitors selectively block the ST step, and members of this class of drugs are called “IN strand transfer inhibitors” (INSTIs) because of their ability to preferentially block the enzyme’s ST reaction relative to the 3′-P reaction.4 All of the known INSTIs share important structural features, which include a coplanar arrangement of three heteroatoms that chelate the two catalytic Mg2+ ions, and a halobenzyl ring that binds to the penultimate base (a deoxycytidine) adjacent to the deoxyadenosine that lies at the 3′ end of the viral DNA after the 3′-P reaction. Binding of INSTIs blocks the ST reaction by displacing the viral 3′-terminal deoxyadenosine from the catalytic Mg2+ ions. Treatment with 1 and EVG selects for resistant forms of HIV, and there is considerable cross-resistance to these two drugs. GlaxoSmithKline’s dolutegravir (DTG, 2)5,6 is a recently FDA-approved second-
generation INSTI (August 2013), which shows improved efficacies against RAL and EVG-resistant strains of HIV.\textsuperscript{7,8} However, 2 also selects for resistant strains of HIV.\textsuperscript{7} This emphasizes the need to develop agents that can overcome resistant strains of IN, including the emerging strains resistant to 2. We recently reported that 1-hydroxy-1,8-naphthyridin-2H-one-3-carboxamides, which include both 4-unsubstituted and 4-hydroxyl-containing analogues (3 and 4, respectively, Figure 1), potently inhibit wild-type (WT) IN in biochemical assays and show good antiviral efficacies in single-round infection assays of HIV-1 infectivity (Figure 1).\textsuperscript{9} Importantly, members of this series retain good antiviral potency against a set of mutants resistant to 1 in these latter assays. Here we describe structural variation at the 4-position of compound 3, which yielded agents of type 5 (Figure 1) that enhance their efficacy against additional mutant forms of IN that are resistant to 1.

**RESULTS AND DISCUSSION**

**Inhibitor Design.** IN is a member of the polynucleotidyl transferase class of enzymes that share similar catalytic mechanisms.\textsuperscript{10} There is also a large body of data that describes the known INSTIs, their efficacy against WT and drug-resistant forms of HIV, and their interactions with prototype foamy virus (PFV) IN. Accordingly, the structures of previously described inhibitors can be used to aid the design of new anti-IN compounds. This is exemplified by the development of 1 by Merck, which can be traced to dihydroxypyrimidine carboxamide inhibitors of the hepatitis C virus NS5b RNA polymerase (RNAP).\textsuperscript{10,11} Inhibitors of HIV-1 ribonuclease H (RNase H) have been reported, which, like INSTIs, inhibit their target enzyme by chelating two Mg\textsuperscript{2+} ions in the enzyme active site. In RNase H, the Mg\textsuperscript{2+} ions are held in place by a “DEDD motif” (D443, E478, D498, and D549).\textsuperscript{12–14} Compounds that inhibit both IN and RNase H have been reported.\textsuperscript{15,16} By analogy to the RNAP example, the known RNase H inhibitors might provide insights that can be used to design improved INSTI inhibitors. Accordingly, the design of our bicyclic 1-hydroxy-1,8-naphthyridin-2H-one INSTIs (3 and 4)\textsuperscript{9} was guided by the report that compounds such as the biaryl-containing 6 (Figure 2), which have submicromolar inhibitory potency against RNase H (IC\textsubscript{50} = 0.64 μM), also have low micromolar inhibitory potency against HIV-1 IN (ST IC\textsubscript{50} = 2.4 μM).\textsuperscript{14} In the case of 6, the reported EC\textsubscript{50} value (half-maximal concentration providing protection against viral-induced cell death) in a HIV-1 HXB2 single-cycle viral replication assay in HeLa P4-2 cells was 34 μM.\textsuperscript{14}

Metal chelation by the 1-hydroxy-1,8-naphthyridin-2H-one nucleus, which is common to 3, 4, and 6, can theoretically be achieved via the heteroatom triad formed by the N-hydroxyl group, the 2-oxo group, and the 8-naphthyridine nitrogen. However, an important component of 3 and 4, not found in 6, is a halobenzyl group, which is known to be important for binding to IN by interacting with the penultimate deoxy-cytosine in the 3’-end of enzyme-bound viral DNA.\textsuperscript{3} In the case of 3 and 4, this binding function is served by a 2′,4′-difluorobenzyl carboxamide group, which is appended at the 3-position of the bicyclic nucleus. Previous work has shown that the nature and pattern of halogen phenyl substitution can significantly affect the potency of INSTIs.\textsuperscript{17} In developing 3 and 4, we found that a 2′,4′-difluorobenzyl moiety, which is present in 2, was superior to the other halobenzyl rings we tested.\textsuperscript{9} An important feature of 3 and 4 is that the carbonyl oxygen of the 2′,4′-difluorobenzyl amide group may not be an obligatory component of the metal-chelating triad. As a consequence, there may be greater flexibility in this region of the molecule than is found with inhibitors, such as 1, where the halobenzyl amide carbonyl participates in Mg\textsuperscript{2+} chelation. This flexibility is reminiscent of what is seen with 2, where the flexibility of the haloamide component is thought to contribute to the ability of 2 to maintain efficacy against certain forms of IN that are resistant to 1.\textsuperscript{18–20}

In our current work, we further modified the 1-hydroxy-1,8-naphthyridin-2H-one nucleus by incorporating new functionalities at the 4-position. In undertaking these efforts, we noted that for RNase H inhibitors such as 6, an extended aryl functionality increased their inhibitory potency.\textsuperscript{14} Therefore, we began by preparing inhibitor 5a (Figure 2). In contrast to 6, where the aryl functionality is attached through a methylene unit, for reasons of synthetic simplicity, we employed a 4-amine group in 5a. Subsequently, we prepared a series of analogues (5a–5v) using an iterative process of design, synthesis, biological evaluation, and redesign.

**Synthesis.** Amidation of methyl ester 7 (obtained in three steps from commercially available methyl 2-fluoronicotinate)\textsuperscript{9,21} using 2,4-difluorobenzylamine gave the known amide 8 in 70% yield (Scheme 1).\textsuperscript{9,21} Subsequent reaction with toluenesulfonyl chloride produced the tosylated analogue 9 (93% yield), which was treated with a variety of amines to provide 10a–10v (Scheme 1). A subset of these amines (10a–10u) was converted to final products (5a–5u) by hydrogenolysis of the N-benzyloxy group (H\textsubscript{2}/10% Pd·C). In the case of final product 5p, acetylation of intermediate 10o to yield 10p was done prior to debenzylation. For final product 5v, treatment of intermediate 10v with TFA yielded the free amine 11 prior to debenzylation (Scheme 1).

**Biological Evaluation.** Compounds were evaluated in biochemical assays using radiolabeled oligonucleotides to measure their inhibitory potential in the 3’-P and ST reactions.\textsuperscript{9,22} The initial series of compounds was designed to examine the role of aromatic functionality at the 4-position. The IN ST inhibitory potency of 5a (IC\textsubscript{50} = 0.34 ± 0.08 μM, Table 1) was approximately 10-fold better than the value previously reported for 6, which contains a similar 1,4-phenyl group.\textsuperscript{14} The conformationally constrained biphenyl amine analogue 5b was slightly less potent than 5a. In contrast, introducing a 4′-nitrile or a 4′-amino group onto 5a (giving 5c and 5d, respectively) slightly increased the potency of the compound in the ST reaction relative to 5a. Importantly, shortening the 4-substituent by removal of one phenyl ring gave an approximate 19-fold enhancement in potency relative to 5a (5e, ST IC\textsubscript{50} = 0.018 ± 0.006 μM, Table 1).
Antiviral potencies were evaluated in a cell-based assay using lentiviral vectors carrying WT IN as well as mutant forms of IN that are resistant to Y143R, N155H, and the double mutant, G140S/Q148H. In these assays, amine 5a was approximately two orders-of-magnitude more potent against the WT enzyme (EC\textsubscript{50} = 372 ± 63 nM, Table 2) than what has been reported for 6 (32 \mu M). All members of the series (5a–5e) showed nanomolar ST inhibitory potencies against WT enzyme, with 5d and 5e being significantly more potent (EC\textsubscript{50} = 6.3 ± 2.4 and 14 ± 1.9 nM, respectively) than other members of the series (EC\textsubscript{50} values >100 nM). Because all compounds showed no cytotoxicity up to 250 \mu M, selectivity indices (SI = CC\textsubscript{50}/EC\textsubscript{50}) were from at least 500 to greater than approximately 40000 (Table 2). Of particular note, while compound 5e was only slightly less potent against the WT vector than what has been reported for 1 (EC\textsubscript{50} = 4 ± 2 nM), it was significantly less susceptible to loss of efficacy against the mutants: Y143R (2-fold loss versus a reported 54-fold loss for 1), N155H (8-fold loss versus a reported 39-fold loss for 1), and G140S/Q148H (32-fold versus a reported 425-fold loss for 1). The large loss of potency incurred by 1 against the Y143R mutant derives from a loss of \pi-\pi stacking of the inhibitor with the Y143 phenyl ring. The ability of compounds in the current series to retain good efficacy against the Y143R mutant indicates that they do not have a similar interaction as 1 with the aryl ring of Y143.

We prepared an additional series of analogues (5f–5v) in which several alkylamines were introduced at the 4-position (Table 3). In biochemical assays in vitro, most of these analogues exhibited low nanomolar inhibitory potencies in the ST reaction. However, compounds 5i and 5k, which contained cycloheptyl and n-butylphenyl substituents, respectively, had ST IC\textsubscript{50} values of 0.46 ± 0.18 and 0.28 ± 0.11 \mu M, respectively, which were markedly elevated relative to other members of the series. A third member of the series, having an (S)-ethyl N-prolinate group, was also significantly less potent [(S)-5u, ST IC\textsubscript{50} = 0.31 ± 0.04 \mu M] (Table 3).

Antiviral potencies were determined for 5f–5v in cells infected with viral vectors harboring WT and mutant forms of IN (Table 4). Most compounds of the series displayed EC\textsubscript{50} values in the low nanomolar range against the WT vector, with a majority of the compounds showing single-digit nanomolar potencies. These compounds also showed low cytotoxicity, which resulted in good SI values, with several compounds showing SI > 10000. Noteworthy exceptions were compounds 5g, 5h, and (S)-5u, which not only had significantly reduced...
antiviral potencies (EC_{50} values of 268 ± 8, 1200 ± 260, and 590 ± 72 nM, respectively) but also showed greater cytotoxicity (CC_{50} values of 13 ± 1.8, 8.4 ± 3.2, and 18 ± 7 µM, respectively) (Table 4). These latter compounds are the only members of the series having tertiary amines at the 4-position. As such, these analogues would not be able to form an intramolecular hydrogen bond between the 4-amino group and the 3-carboxamide carbonyl oxygen. In spite of their poor antiviral efficacies, the in vitro ST IC_{50} values for 5g and 5h (87 and 79 nM, respectively, Table 3) were only modestly elevated relative to most other members of the series (typically 30 nM or lower). In some cases, the in vitro IC_{50} values for 5g and 5h were better than compounds such as 5i and 5k (460 and 280 nM, respectively, Table 3), which paradoxically exhibited good EC_{50} values against the WT vector (12 ± 3 and 50 ± 13 nM, respectively, Table 4). These data could indicate that being able to form an intramolecular hydrogen bond between the 4-amino group and the 3-carboxamide carbonyl oxygen has a more important role for the activity of the compounds in an antiviral assay done in cultured cells than in the biochemical assay done in vitro.

The main objective of the current study was to derive minimally cytotoxic inhibitors having good antiviral potency against cells infected with WT virus, which also retained their efficacy against viruses harboring mutant forms of IN that are resistant to 1. As shown in Table 4, relative to their potencies against WT, almost all members of the current series maintained complete or nearly complete efficacy against virus having the Y143R mutant. In addition, most members of the series showed good retention of efficacy against virus having the N155H and G140S/Q148H mutants (with a few exceptions, 10-fold or less loss of potency) (Table 4). Members of the series also commonly exhibited high SI values, in the range of four orders-of-magnitude.

On the basis of these data, we examined selected members of the series (5o–5q and 5v) against a more extensive panel of INSTI-resistant mutants that included R263 K and G118R mutants, which have recently been identified through in vitro selection studies with second-generation INSTIs. For reference, we also included 1 and 2 as well as the parent 1-hydroxy-1,8-naphthyridin-2H-ones (3 and 4), which formed the starting points for the current series. Although 1 is potent against viral vectors that carry WT IN (EC_{50} = 4 ± 2 nM), it shows extensive loss of antiviral efficacy against the mutants, Y143R (EC_{50} = 162 ± 16 nM; 41-fold loss), N155H (EC_{50} = 154 ± 33 nM; 38-fold loss), and G140S/Q148H (EC_{50} = 1900 ± 300 nM; 475-fold loss). Compound 1 is more tolerant of the G118R mutant (EC_{50} = 36 ± 5 nM; 9-fold loss) and even less affected by the R263 K mutant (EC_{50} = 9 ± 4 nM; 2-fold loss) (Table 5). In contrast, the recently FDA-approved second-generation inhibitor 2, while showing similar potency to 1 against WT vector (EC_{50} = 1.6 ± 0.9 nM), exhibits a significantly smaller loss of potency against vectors having the mutants Y143R (EC_{50} = 4.3 ± 1.2 nM; 3-fold loss), N155H (EC_{50} = 3.6 ± 1.3 nM; 2-fold loss), and G140S/Q148H (EC_{50} = 5.8 ± 0.5 nM; 4-fold loss) (Table 5). However, as expected, 2 shows some loss of potency against virus having the R263 K (EC_{50} = 11 ± 3 nM; 7-fold loss) and G118R (EC_{50} = 13 ± 5 nM; 8-fold loss) mutants. Among the current series, the antiviral potencies of 5o–5q are approximately equivalent to 1 against WT enzyme, with 5v

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**Table 2. Antiviral Potencies of Carboxamides 5a–5e in Cells Infected with HIV-1 Constructs Containing WT or Mutant IN**

| compd | EC_{50} (µM) | IC_{50} (µM) |
|-------|-------------|-------------|
| 5a    | >250        | 372 ± 63    |
| 5b    | >250        | 171 ± 57    |
| 5c    | >250        | 123 ± 21    |
| 5d    | >250        | 6.3 ± 2.4   |
| 5e    | >250        | 14 ± 1.9    |

**Table 3. Inhibitory Potencies of Carboxamides 5f–5v Obtained Using an in Vitro IN Assay**

| compd | R¹     | R²     | IC_{50} (µM) |
|-------|--------|--------|-------------|
| 5f    | −CH₃   | −H     | 3.7 ± 0.4   |
| 5g    | −CH₅   | −CH₃   | 21 ± 2      |
| 5h    | −morpholinino | −H | 77 ± 12 |
| 5i    | −cycloheptyl | −H | 13 ± 1.1 |
| 5j    | −CH₂CH₂Ph | −H | 8.0 ± 1.5 |
| 5k    | −CH₂(CH₂)₃Ph | −H | 12 ± 2.0 |
| 5l    | −CH₂(CH₂)₂CH₃ | −H | 8.2 ± 1.6 |
| 5m    | −CH₂CH₂ | −H | 1.8 ± 0.2 |
| 5n    | −CH₂CH₂NH₂ | −H | 4.5 ± 0.2 |
| 5o    | −CH₂CO₂H | −H | 0.55 ± 0.07 |
| 5p    | −CH₂CH₂OAc | −H | 5.3 ± 0.5 |
| 5q    | −CH₂CO₂CH₃ | −H | 0.71 ± 0.10 |
| (S)-5r | −NHCH(CH₂) | −H | 7.4 ± 0.8 |
| (R)-5r | −NHCH(CH₂) | −H | 5.8 ± 0.6 |
| (S)-5s | −NHCH(CH₂) | −H | 16.7 ± 1.4 |
| (R)-5s | −NHCH(CH₂) | −H | 13.5 ± 1.0 |
| (S)-5t | −NHCH(CH₂) | −H | 5.8 ± 0.5 |
| (R)-5t | −NHCH(CH₂) | −H | 4.4 ± 0.5 |
| 5u    | −Pro-OEt | −H | 8.6 ± 0.6 |
| 5v    | −H     | −H     | 2.5 ± 0.3   |

**Notes:**
- CC_{50} values result in 50% reduction in the level of ATP in human osteosarcoma (HOS) cells.
- In vitro IC_{50} values for 5f–5v were obtained using an in vitro IN assay.
- EC_{50} values for Y143R, N155H, G140S/Q148H, and SI values were obtained using an in vitro assay.
- Assays were performed using a gel-based protocol with Mg^{2+} cofactor as described in refs 9 and 22.
Table 4. Antiviral Potencies of Carboxamides 5f–5v in Cells Infected with HIV-1 Constructs Containing WT or Mutant IN

| compd | CC50 (μM)a | EC50 (nM, WT)b | EC50 (nM, IN mutants)c | Sifd |
|-------|------------|----------------|-----------------------|------|
| 5f    | >250       | 3.1 ± 0.6      | 6.1 ± 2.5 (2x)        | 87 ± 11 (28x) | >80645 |
| 5g    | 13 ± 1.8   | 268 ± 8        | 273 ± 52 (1x)         | 2300 ± 700 (9x) | 49 |
| 5h    | 8.4 ± 3.2  | 1200 ± 260     | 1800 ± 870 (1.5x)     | 3730 ± 920 (3x) | 6920 ± 2000 (6x) | 7 |
| 5i    | 68 ± 8.7   | 12 ± 3         | 6.6 ± 1.7 (0.55x)     | 14 ± 1 (1x)   | 35 ± 12 (3x)     | 5667 |
| 5j    | >250       | 14 ± 8         | 18 ± 2.6 (1x)         | 79 ± 5.2 (6x) | 119 ± 3.4 (9x)   | >17857 |
| 5k    | 102 ± 8    | 50 ± 13        | 40 ± 15 (0.80x)       | 116 ± 22 (2x) | 243 ± 39 (5x)    | 2040 |
| 5l    | 94 ± 24    | 7.4 ± 1.4      | 8.8 ± 2.7 (1x)        | 12 ± 4.5 (2x) | 71 ± 0.14 (10x)  | 12703 |
| 5m    | >250       | 7.2 ± 3.0      | 7.4 ± 0.5 (1x)        | 44 ± 6.7 (6x) | 154 ± 16 (21x)   | >34722 |
| 5n    | 9.6 ± 3.7  | 35 ± 11        | 57 ± 13 (2x)          | N/A  | N/A  | 277 |
| 5o    | 24 ± 3     | 5.2 ± 0.6      | 4.6 ± 1.8 (0.88x)     | 25 ± 4 (5x)  | 43 ± 15 (8x)     | 4615 |
| 5p    | >250       | 4.5 ± 1.5      | 4.8 ± 2.9 (1x)        | 3.1 ± 0.3 (0.69x) | 35 ± 14 (8x) | >55556 |
| 5q    | >250       | 3.8 ± 1.2      | 4.6 ± 2.2 (1x)        | 19 ± 5 (7x)  | 36 ± 16 (9x)     | >65789 |
| (S)-5r| >250       | 4.2 ± 1.6      | 4.8 ± 1.4 (1x)        | 15.3 ± 3.3 (4x) | 141 ± 20 (33x) | >59524 |
| (R)-5r| >250       | 11.3 ± 4.7     | 8.1 ± 1.7 (0.7x)      | 27 ± 9.9 (2x) | 89 ± 26 (8x)     | >22124 |
| (S)-5s| >250       | 9.2 ± 4.4      | 8.5 ± 1.9 (0.9x)      | 17.6 ± 6.4 (2x) | 55.6 ± 6.2 (6x) | >27778 |
| (R)-5s| >250       | 7.4 ± 3.3      | 8.2 ± 1.8 (1 x)       | 22 ± 4 (3 x ) | 48 ± 12 (6 x)    | >33784 |
| (S)-5t| >250       | 9.7 ± 3        | 11 ± 4 (1 x )         | 29 ± 8 (3 x) | 122 ± 33 (13 x ) | >25773 |
| (R)-5t| >250       | 14.2 ± 3.7     | 10.2 ± 1.4 (0.7 x)    | 71 ± 5 (5 x) | 284 ± 120 (20 x) | >17606 |
| (S)-5u| 18 ± 7     | 590 ± 72       | 1660 ± 920 (3 x )     | N/A  | N/A  | 31 |
| 5v    | >250       | 1.1 ± 0.7      | 2.5 ± 0.6 (2 x )      | 5.3 ± 2.3 (5 x) | 35 ± 9 (32 x) | >227273 |

Notes:
- aCytotoxicity concentration resulting in 50% reduction in the level of ATP in human osteosarcoma (HOS) cells.
- bValues obtained from cells infected with lentiviral vector carrying WT IN.
- cCells were infected with viral constructs carrying IN mutations and indicated values correspond to the fold-change (FC) in EC50 relative to WT.
- dSelectivity index (SI) calculated as the ratio of CC50 to EC50. Not available.

Table 5. Antiviral Potencies of 5o–5q and 5v Compared with 1, 2, and Previously Reported Carboxamides 3 and 4 in Cells Infected with HIV-1 Constructs Containing WT or Mutant IN

| compd | EC50 (nM, WT)d | EC50 (nM/[FC], IN mutants)c |
|-------|----------------|-----------------------------|
| 1     | 4 ± 2          | 162 ± 16 (41x)              |
| 2     | 1.6 ± 0.9      | 4.3 ± 1.2 (3x)              |
| 3     | 5.1 ± 1.9      | 4.9 ± 0.8 (1x)              |
| 4     | 6.2 ± 2.9      | 11 ± 2 (2x)                 |
| 5o    | 5.2 ± 0.6      | 4.6 ± 1.8 (0.88x)           |
| 5p    | 4.5 ± 1.5      | 4.8 ± 2.9 (1x)              |
| 5q    | 3.8 ± 1.2      | 4.6 ± 2.2 (1x)              |
| 5v    | 1.1 ± 0.66     | 2.5 ± 0.6 (2x)              |

Notes:
- aValues obtained from cells infected with lentiviral vector harboring WT IN.
- bCells were infected with viral constructs carrying IN mutations and indicated values correspond to the fold-change (FC) in EC50 relative to WT.
- cNot available.
control in the polymerase inhibition assays. The polymerase inhibition assays show that all three compounds are able to inhibit the DNA-dependent DNA polymerase activity of HIV-1 RT (Figure 3A). However, the data also show that the compounds differ in their potency. All of the compounds are less potent than nevirapine in the polymerase assay, Compound 5v the most potent, followed by 5p, then 5q. It is not clear at this point whether the compounds are binding the Mg\(^{2+}\) ions at the polymerase active site or binding within the NNRTI-binding pocket near the polymerase active site, similar to nevirapine. It is also possible that they may be binding at some other site in HIV-1 RT.

The compounds were also tested for their ability to inhibit the RNase H activity of RT. When RT binds an RNA−DNA template/primer (T/P), the 3′ end of the DNA primer is preferentially located at the polymerase active site; the RNase H active site contacts the RNA template approximately 17 to 18 nucleotides (NT) from the polymerase active site.\(^{27}\) The initial RNase H cleavage occurs approximately 17 NT from the polymerase active site. These cleavages have been designated as the −17 family of cleavages. The RT then alters its interactions with the T/P, so that RNase H can make additional secondary cleavages approximately 8 NT from the 3′ end of the primer (−8 cleavages). The full length RNA is 60 NT in length. We found that 5p, 5q, and 5v varied in their abilities to inhibit RNase H activity (Figure 3B−D). However, the ranking of the potencies of the compounds in the RNase H assay is different from the polymerase assay (Figure 3A), which suggests that the compounds are interacting with the RT in different places in the two assays. The data show that 5q is the most potent inhibitor of RNase H (it was the least potent compound in the polymerase assay). Compound 5q did not completely block RNase H activity, even at the highest concentration; a large amount of full length RNA was present in the reaction that contained 5q at the 10 μM concentration. It is also apparent that more of the product of the −17 cleavages remained after 10 and 15 min as compared to the “no compound” controls. This results from partial blocking the −8 cleavages.

Figure 3. Polymerase and RNase H inhibition assays (A): The effects of 5p, 5q, and 5v on the DNA-dependent DNA polymerase activity of RT are shown. Reactions were performed with WT RT in the presence of various concentrations of compounds (0, 0.02, 0.1, 0.5, 1, or 10 μM). Samples were precipitated with EtOH and then fractionated on a 15% polyacrylamide sequencing gel. After electrophoresis, the gel was exposed to X-ray film. RNase H inhibition assay (B,C,D): The effects of 5p, 5q, and 5v on the RNase H activity of RT are shown. The reactions were incubated for the amount of time indicated (1, 5, 10, and 15 min) in the presence of the indicated concentration of the individual compounds (0, 0.1, 0.5, 1, and 10 μM). The size of intact RNA (full length) is 60 NT, as shown in the “No-RT” lane. The RNA fragments derived from the −17 and −8 families of cleavages are shown.
results were obtained with 5v, while 5p had very little effect on RNase H activity. At the highest concentration, there were subtle effects on the ~8 cleavages, but the compound is obviously less potent, in the RNase H assay, than the other two. It is quite difficult to use the results of the in vitro assays to estimate IC₅₀ values. In the polymerase assay, it is clear that all of the compounds are less potent than nevirapine. We did not have a potent RNase H inhibitor that would allow us to make a similar comparison in the RNase H inhibitor assay. Although it is clear that the current compounds are primarily IN inhibitors, because these compounds can inhibit both the RNase H and polymerase activity of HIV-1 RT, they could serve as the starting point for the synthesis of additional compounds that would be specifically designed to inhibit these alternate targets. In that regard, because there are no potent RNase H inhibitors, and because many of the known RNase H inhibitors are relatively toxic, using these compounds as leads to develop new RNase H inhibitors is a potentially attractive option.

## CONCLUSION

Our current study examines the effects of introducing an amine functionality at the 4-position of our previously reported 1-hydroxy-1,8-naphthyridin-2H-ones (3 and 4). The focus of the work was to enhance antiviral potency, with particular emphasis on retaining efficacy against viruses harboring mutant forms of IN that have been shown to be resistant to the first-generation INSTIs. For reference we employed 2, which is a recently FDA-approved second-generation INSTI with improved performance against the known resistant mutants. Most members of the series of new inhibitors (5) show single-digit nanomolar antiviral potency against WT enzyme, with SI values greater than 10000. As a whole, the family of new inhibitors exhibited a smaller fold-change than 1 in antiviral assays that employed IN mutants Y143R, N155H, and the double mutant G140S/Q148H. Among the new inhibitors, compound 5p showed a profile against the panel of mutants that was comparable to 2, with 5v exhibiting the best overall performance among the new inhibitors, approximately 5- to 10-fold enhancement relative to the starting compound 4. Although 2 is more effective than first-generation INSTIs in its ability to retain efficacy against resistant forms of IN, it has been shown that 2 can select for resistant forms of the enzyme. Therefore, there is a continuing need for the development of new agents as potential alternatives to the currently approved panel of three FDA-approved INSTIs. The structural class of agents presented herein may represent an attractive platform for developing such next-generation INSTIs.

## EXPERIMENTAL SECTION

### General Synthetic.

1H and 13C NMR data were obtained on a Varian 400 MHz spectrometer or a Varian 500 MHz spectrometer and are reported in ppm relative to TMS and referenced to the solvent in which the spectra were collected. Solvent was removed by rotary evaporation under reduced pressure, and anhydrous solvents were obtained commercially and used without further drying. Purification by silica gel chromatography was performed using Combiflash Rf 200 with EtOAc–hexanes solvent systems. Preparative high pressure liquid chromatography (HPLC) was conducted using a Waters Prep LC4000 system having photodiode array detection and Phenomenex 1.8 μm columns (catalogue no. 00G-4436-P0-AX, 250 mm × 21.2 mm, 10 μm particle size, 110 Å pore) at a flow rate of 10 mL/min. Binary solvent systems consisting of A = 0.1% aqueous TFA and B = 0.1% TFA in acetonitrile were employed with gradients as indicated. Products were obtained as amorphous solids following lyophilization.

Electrospray ionization–mass spectra (ESI-MS) were acquired with an Agilent LC/MSD system equipped with a multimode ion source. Purities of samples subjected to biological testing were assessed using this system and shown to be ≥95%. High-resolution mass spectra (HRMS) were acquired by LC/MS/ESI using an LTQ-Orbitrap-XL at 30k resolution.

### General Procedure A.

Preparation of 1-(Benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (10a–10v). A solution of 1-(benzyloxy)-4-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylic acid (8) in 0.1% aqueous TFA (150 μL) and ethyl acetate (150 μL) (6.0 mmol) was heated at 50 °C (1 h). The crude mixture was purified by Combiflash silica gel chromatography (hexanes and ethyl acetate) to provide amides (10a–10v).

### General Procedure B.

Preparation of N-(2,4-Difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamides (5a–5v). Amides (10a–10u or 11; 0.2 mmol) were dissolved in MeOH (15 mL) and EtOH (5 mL) and the solution degassed and stirred at room temperature under H₂ over Pd-C (10%, 0.2 mmol) (1 h). The mixture was filtered and the filtrate was concentrated, and the resulting residue was purified by HPLC to provide amides (5a–5v).
1-(4-(Amino-[1,1-biphenyl]-4-ylamino)-1-benzoyloxy)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)ethyl acetate (10a). Treatment of 9 with dimethylamine hydrochloride as outlined in general procedure A, provided 10a as a colorless solid in 96% yield. 1H NMR (400 MHz, CDCl3) δ 11.99 (t, J = 4.6 Hz, 1H), 1.079 (t, J = 5.7 Hz, 1H), 6.88–8.66 (m, 1H), 8.31 (dd, J = 8.2, 1.5 Hz, 1H), 7.68 (dd, J = 7.5, 1.5 Hz, 2H), 7.39–7.33 (m, 3H), 7.26 (dd, J = 10.9, 3.9 Hz, 3H), 7.18–7.12 (m, 4H), 6.83–6.78 (m, 2H), 5.24 (s, 2H), 4.62 (d, J = 5.7 Hz, 2H), 3.68 (q, J = 6.3 Hz, 2H), 1.26 (t, J = 7.0 Hz, 2H), 1.81 (qd, J = 6.9, 3.5 Hz, 4H). ESI-MS m/z: 569.2 (M+ H+).

1-(Benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-4-(pentylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (10b). Treatment of 9 with pentan-1-amine as outlined in general procedure A, provided 10b as a colorless oil in 95% yield. 1H NMR (400 MHz, CDCl3) δ 11.99 (t, J = 4.6 Hz, 1H), 10.79 (t, J = 5.7 Hz, 1H), 6.88–8.60 (m, 1H), 8.31 (dd, J = 8.2, 1.5 Hz, 1H), 7.68 (dd, J = 7.5, 1.5 Hz, 2H), 7.39–7.32 (m, 3H), 7.26 (dd, J = 10.9, 3.9 Hz, 3H), 7.18–7.12 (m, 4H), 6.83–6.78 (m, 2H), 5.24 (s, 2H), 4.62 (d, J = 5.7 Hz, 2H), 3.68 (q, J = 6.3 Hz, 2H), 1.26 (t, J = 7.0 Hz, 2H), 1.81 (qd, J = 6.9, 3.5 Hz, 4H). ESI-MS m/z: 569.2 (M+ H+).
Methyl (1-Benzoyl)-3-(2,4-difluorobenzoyl)carbamoyl-2-oxo-1,2-dihydro-1,8-naphthyridine-4(9H)-yl]acetonitrile (10a)

Treatment of methyl (2,4-difluorobenzoyl)carbamoyl chloride as outlined in general procedure A, provided (10a) as a white solid in 95% yield. 1H NMR (400 MHz, CDCl3) 8.55 (J ≈ 7.8 Hz, 1H), 8.46 (J ≈ 8.1 Hz, 1H), 8.38 (J ≈ 8.2 Hz, 1H), 7.63 (J ≈ 8.1 Hz, 1H), 7.56 (J ≈ 8.1 Hz, 1H), 7.32 (m, 3H), 7.20 (m, 3H), 7.13 (J ≈ 8.5 Hz, 2H), 7.03 (J ≈ 8.0 Hz, 2H), 4.66 (J ≈ 8.1 Hz, 2H), 1.35 (s, 3H). EI-MS m/z: 504.1 (MH+).

Methyl (1-Benzoyl)-3-(2,4-difluorobenzoyl)carbamoyl-2-oxo-1,2-dihydro-1,8-naphthyridine-4(9H)-yl]acetate (10b)

Treatment of methyl (2,4-difluorobenzoyl)carbamoyl chloride as outlined in general procedure A, provided (10b) as a white solid in 95% yield. 1H NMR (400 MHz, CDCl3) 8.55 (J ≈ 7.8 Hz, 1H), 8.46 (J ≈ 8.1 Hz, 1H), 8.38 (J ≈ 8.2 Hz, 1H), 7.63 (J ≈ 8.1 Hz, 1H), 7.56 (J ≈ 8.1 Hz, 1H), 7.32 (m, 3H), 7.20 (m, 3H), 7.03 (J ≈ 8.0 Hz, 2H), 4.66 (J ≈ 8.1 Hz, 2H), 1.35 (s, 3H). EI-MS m/z: 504.1 (MH+).

Methyl (1-Benzoyl)-3-(2,4-difluorobenzoyl)carbamoyl-2-oxo-1,2-dihydro-1,8-naphthyridine-4(9H)-yl]acetonitrile (10c)

Treatment of methyl (2,4-difluorobenzoyl)carbamoyl chloride as outlined in general procedure A, provided (10c) as a white solid in 95% yield. 1H NMR (400 MHz, CDCl3) 8.55 (J ≈ 7.8 Hz, 1H), 8.46 (J ≈ 8.1 Hz, 1H), 8.38 (J ≈ 8.2 Hz, 1H), 7.63 (J ≈ 8.1 Hz, 1H), 7.56 (J ≈ 8.1 Hz, 1H), 7.32 (m, 3H), 7.20 (m, 3H), 7.03 (J ≈ 8.0 Hz, 2H), 4.66 (J ≈ 8.1 Hz, 2H), 1.35 (s, 3H). EI-MS m/z: 504.1 (MH+).
N-(2,4-Difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5f).

Treatment of 10f as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 80% B over 30 min; retention time = 23.7 min) provided 5f as a yellow solid in 72% yield. 1H NMR (400 MHz, DMSO-d6) δ 10.47 (s, 1H), 8.75 (dd, J = 8.1, 5.7 Hz, 1H), 7.74 (dd, J = 8.2, 4.6 Hz, 1H), 7.19 (dd, J = 10.5, 4.6 Hz, 1H), 7.04–7.69 (m, 2H), 4.46 (dd, J = 8.5, 2.8 Hz, 2H), 3.60–3.57 (m, 2H), 1.57 (dd, J = 14.0, 7.0 Hz, 2H), 1.29–1.21 (m, 4H), 0.81 (t, J = 7.1 Hz, 3H). ESI-MS m/z: 471.1 (MH+). HRMS calcld C24H21F2N4O3 [MH+], 479.1889; found, 479.1894.

N-(2,4-Difluorobenzyl)-1-hydroxy-2-oxo-4-(pentylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5g).

Treatment of 10g as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 80% B over 30 min; retention time = 21.6 min) provided 5g as a yellow solid in 89% yield. 1H NMR (400 MHz, DMSO-d6) δ 8.79 (t, J = 5.9 Hz, 1H), 8.57 (dd, J = 4.5, 1.5 Hz, 1H), 8.19 (dd, J = 8.0, 1.6 Hz, 1H), 7.70 (dd, J = 15.5, 8.7 Hz, 1H), 7.25 (dd, J = 8.0, 4.6 Hz, 1H), 7.18–7.13 (m, 1H), 7.02 (td, J = 8.5, 2.6 Hz, 1H), 4.38 (d, J = 5.8 Hz, 2H), 2.76 (s, 6H). ESI-MS m/z: 361.1 (MH+). HRMS calcld C17H17F2N4O2 [MH+], 361.1107; found, 361.1105.

N-(2,4-Difluorobenzyl)-4-(dimethylamino)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5h).

Treatment of 10h as described under general procedure B and purification by preparative HPLC (linear gradient of 20% B to 45% B over 30 min; retention time = 20.5 min) provided 5h as a yellow solid in 82% yield. 1H NMR (400 MHz, DMSO-d6) δ 8.91 (t, J = 5.9 Hz, 1H), 8.62–8.61 (m, 1H), 8.23 (dd, J = 8.0, 1.6 Hz, 1H), 7.70 (dd, J = 15.4, 8.7 Hz, 1H), 7.30 (dd, J = 8.0, 4.6 Hz, 1H), 7.21–7.16 (m, 1H), 7.05 (dd, J = 8.5, 2.2 Hz, 1H), 4.41 (d, J = 5.8 Hz, 2H), 3.67–3.65 (m, 4H), 2.98–2.96 (m, 4H). ESI-MS m/z: 417.1 (MH+). HRMS calcld C17H17F2N4O2 [MH+], 417.1369; found, 417.1364.

N-(2,4-Difluorobenzyl)-1-hydroxy-4-morpholino-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5i).

Treatment of 10i as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 80% B over 30 min; retention time = 25.8 min) provided 5i as a yellow solid in 87% yield. 1H NMR (400 MHz, DMSO-d6) δ 8.96 (bs, 1H), 9.4 (t, J = 5.7 Hz, 1H), 8.62 (dd, J = 4.5, 1.5 Hz, 1H), 8.35 (dd, J = 8.3, 1.5 Hz, 1H), 7.45–7.39 (m, 1H), 7.26 (dd, J = 8.2, 4.6 Hz, 1H), 7.17 (ddd, J = 10.5, 9.4, 2.6 Hz, 1H), 7.03–6.98 (m, 1H), 4.44 (d, J = 5.7 Hz, 2H), 4.02 (bs, 1H), 1.93–1.88 (m, 2H), 1.58–1.50 (m, 4H), 1.47–1.45 (m, 4H), 1.31–1.38 (s, 2H). ESI-MS m/z: 443.1 (MH+). HRMS calcld C17H17F2N4O2 [MH+], 443.1897; found, 443.1897.

N-(2,4-Difluorobenzyl)-1-hydroxy-2-oxo-4-(phenethylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5j).

Treatment of 10j as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 80% B over 30 min; retention time = 22.0 min) provided 5j as a yellow solid in 93% yield. 1H NMR (400 MHz, DMSO-d6) δ 10.80 (bs, 1H), 10.36 (t, J = 5.8 Hz, 1H), 8.59 (dd, J = 4.5, 1.5 Hz, 1H), 8.48 (dd, J = 8.3, 1.6 Hz, 1H), 7.42 (dd, J = 15.3, 8.6 Hz, 1H), 7.22–7.18 (m, 6H), 7.18–7.14 (m, 1H), 7.03–6.98 (m, 1H), 4.43 (d, J = 5.8 Hz, 2H), 3.82–3.78 (m, 2H), 2.87 (t, J = 7.3 Hz, 2H). ESI-MS m/z: 451.1 (MH+). HRMS calcld C23H22F2N4O2 [MH+], 451.1576; found, 451.1584.

N-(2,4-Difluorobenzyl)-1-hydroxy-2-oxo-4-((4-phenylbutyl)amino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (5k).

Treatment of 10k as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 80% B over 30 min; retention time = 26.7 min) provided 5k as a yellow solid in 78% yield. 1H NMR (400 MHz, DMSO-d6) δ 10.96 (bs, 1H), 10.48 (t, J = 5.5 Hz, 1H), 8.62 (dd, J = 4.6, 1.5 Hz, 1H), 8.51 (dd, J = 8.3, 1.6 Hz, 1H), 7.42 (dd, J = 15.4, 8.7 Hz, 1H), 7.25–7.16 (m, 4H), 7.12–7.07 (m, 3H), 7.07 (d, J = 7.0 Hz, 1H), 7.01–6.97 (m, 1H), 4.46 (d, J = 5.7 Hz, 2H), 3.61 (bs, 2H), 2.54 (t, J = 6.9 Hz, 2H), 1.61–1.60 (m, 4H). ESI-MS m/z: 479.2 (MH+). HRMS calcld C26H25F2N4O2 [MH+], 479.1889; found, 479.1894.
Methyl (R)-2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-3-hydroxypropionate ([S]-5r). Treatment of (S)-10r as described under general procedure B and purification by preparative HPLC (linear gradient of 30% B to 50% B over 30 min; retention time = 26.4 min) provided (S)-5r as a white solid in 48% yield. 1H NMR (400 MHz, DMSO-d6) δ 7.12 (m, 1H), 7.00 (td, J = 8.7, 1.4 Hz, 1H), 6.98 (m, 1H), 7.10 (dd, J = 8.2, 2.4 Hz, 1H), 4.42 (s, 2H), 4.41 (s, 2H), 3.60 (s, 3H). ESI-MS m/z: 419.1 (MH+). HRMS calc C22H19F2N4O5 [MH+], 419.1162; found, 419.1163.

Ethyl (S)-1-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)propanoate ([S]-5u). Treatment of (S)-10u as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 70% B over 30 min; retention time = 12.5 min) provided (S)-5u as a yellow solid in 48% yield. 1H NMR (400 MHz, DMSO-d6) δ 8.91 (t, J = 5.9 Hz, 1H), 8.65 (dd, J = 4.6, 1.5 Hz, 1H), 8.39 (dd, J = 8.0, 1.6 Hz, 1H), 7.73 (dd, J = 15.4, 8.7 Hz, 1H), 7.34 (dd, J = 8.0, 4.6 Hz, 1H), 7.26−7.20 (m, 1H), 7.09 (td, J = 8.6, 2.4 Hz, 1H), 4.51 (s, J = 15.1, 6.3 Hz, 1H), 4.38−4.30 (m, 2H), 3.95 (q, J = 7.1 Hz, 2H), 3.64 (J = 15.2, 6.4 Hz, 1H), 3.18 (J = 12.8, 8.1 Hz, 1H), 2.11−1.93 (m, 1H, 1H), 1.92−1.87 (m, 2H), 1.80−1.76 (m, 1H), 1.03 (q, J = 7.0 Hz, 3H). ESI-MS/m/z: 473.2 (MH+). HRMS calc C22H19F2N4O5 [MH+], 473.1631; found, 473.1618.

4-Amino-N-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide ([S]-5v). Treatment of 10v as described under general procedure B and purification by preparative HPLC (linear gradient of 30% B to 50% B over 30 min; retention time = 19.5 min) provided 5v as a white solid in 47% yield. 1H NMR (400 MHz, DMSO-d6) δ 10.59 (t, J = 5.8 Hz, 1H), 8.66 (dd, J = 4.5, 1.4 Hz, 1H), 8.61 (dd, J = 8.1, 1.6 Hz, 1H), 7.36 (d, J = 6.6 Hz, 1H), 7.30 (dd, J = 8.1, 4.6 Hz, 1H), 7.18 (d, J = 9.2 Hz, 1H), 7.00 (d, J = 2.4 Hz, 2H), 4.46 (J = 5.8 Hz, 2H). ESI-MS/m/z: 347.1 (MH+). HRMS calc C16H13F2N4O3 [MH+], 347.0950; found, 347.0953.

Integrate Biochemical Assays. Determination of IN 3’-P and ST inhibitory values using an in vitro assay IN reactions were carried out using [γ-32P]-labeled DNA as previously described.6,22

Cellular Cytotoxicity Assays. Cytotoxicity was measured using the human osteosarcoma cell line, HOS (Dr. Richard Schwartz, Michigan State University, East Lansing, MI), by monitoring ATP levels using a luciferase reporter assay as previously reported.29

Single-Round HIV-1 Infectivity Assays. As previously described,3 the human embryonic kidney cell line 293T was transfected with the pNLNoGIVR ΔLUC vector, which was made from pNLNoGIVR ΔEnv.HSA by removing the HSA reporter gene and replacing it with a luciferase reporter gene between the NotI and XhoI restriction sites.28 VSV-ΔΔV pseudotyped HIV was produced transfecting 293T cells as described previously.3 On the day prior to transfection, 293T cells were plated on 100 mm diameter dishes at a density of 1.5 × 105 cells per plate. 293T cells were transfected with 16 μg of pNLNoGIVR ΔLUC and 4 μg of PHCMV-Δ (obtained from Dr. Jane Burns, University of California, San Diego) using the calcium phosphate method. At approximately 6 h after the calcium phosphate precipitate was added, the 293T cells were washed twice with phosphate-buffered saline (PBS) and incubated with fresh media (48 h). The virus-containing supernatants were then harvested, clarified by low-speed centrifugation, filtered, and diluted for preparation in infection assays. On the day prior to the screen, HOS cells were seeded in a 96-well luminescence cell culture plate at a density of 4000 cells in 10 μL per well. On the day of the screen, cells were treated with the compounds from a concentration range of 10 μM to 0.0005 μM using 11 serial dilutions and then incubated at 37 °C (3 h). After this incubation, 100 μL of virus stock diluted to achieve a maximum luciferase signal between 0.2 and 1.5 RLUs was added to each well and the plates were incubated at 37 °C (48 h). Infectivity was measured by using the Steady-lite plus luminescence reporter gene assay system (PerkinElmer, Waltham, MA). Luciferase activity was measured by adding 100 μL of Steady-lite plus buffer (PerkinElmer) to the cells, incubating at room temperature (20 min), and measuring luminescence using a microplate reader. Activity was normalized to infectivity in the absence of target compounds. Kaleidagraph (Synergy
Software, Reading, PA) was used to perform regression analysis on the data. EC50 values were determined from the fit model.

**Vector Constructs.** pNLNoGIVR ΔEnv.LUC has been described previously.29 The IN coding region was removed from pNLNoGIVR ΔEnv.LUC (between Kpnl and SalI sites) and inserted between the Kpnl and SalI sites of pBluescript II KS+. Using this construct as the wild-type template, the following IN-resistant mutants were prepared via the QuikChange II XL (Stratagene, La Jolla, CA) site-directed mutagenesis protocol: G118R, Y143R, Q148K, Y155H, G140S + Q148H, G140A + Q48K, and E138 K + Q48 K. The samples were precipitated by the addition of EDTA. The compounds were added to give concentrations of 0, 0.02, 0.1, 0.5, 1, or 10 μM dCTP, dGTP, and TTP. The compounds were added to give the reaction volume was 12 μL. The reactions were initiated by the addition of 50 nM of RT and were incubated at 37 °C. Aliquots were removed at the indicated time points, and the reactions halted by addition of 2X gel loading buffer (Ambion). The reaction products were fractionated on a 15% polyacrylamide sequencing gel. Products were visualized by exposure to X-ray film.

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**Notes**
The authors declare no competing financial interest.

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**ABBREVIATIONS USED**
HIV-1, human immunodeficiency virus type 1; AIDS, acquired immune deficiency syndrome; FDA, Food and Drug Administration; IN, integrase; RNase H, ribonuclease H; RT, reverse transcriptase; NNRTI, nonnucleoside reverse transcriptase inhibitor; NT, nucleotides; RAL, raltegravir; EVG, elvitegravir; DTG, dolutegravir; 3′-P, 3′-processing; ST, strand transfer; INSTIs, integrase strand transfer inhibitors; DNA, deoxyribonucleic acid; ICS50, half-maximal inhibitory concentration; EC50, half maximal effective concentration; WT, wild-type; DMF, dimethylformamide; HPLC, high-pressure liquid chromatography; HRMS, high-resolution mass spectrometry

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**RnaseH Assays.** This procedure has been previously described.30 Briefly, RNA oligonucleotide (5′-GGGGCCACUUUUUAAAAGAAGGGGACUGAGGCGUAAUACUCAC-3′) was obtained from Dharmacon Research, Inc. The RNA oligonucleotide was 5′-end labeled and was then annealed to a DNA oligonucleotide (5′-GAGTGAATTAGCCCTTGGCGTCCC-3′) by heating and slow cooling. A 0.2 μM concentration of the RNA/DNA hybrid was suspended in 25 mM Tris (pH 8.0), 50 mM NaCl, 5.0 mM MgCl2, 100 μg of bovine serum albumin/mL, 10 nM CHAPS, and 1 U of Superscript (Ambion). The compounds were added to the reactions to give the following final concentrations (0.01, 0.05, 0.1, and 10 μM). The reaction volume was 12 μL. The reactions were initiated by the addition of 50.0 ng of RT and were incubated at 37 °C. Aliquots were removed at the indicated time points, and the reactions halted by addition of 2X gel loading buffer (Ambion). The reaction products were fractionated on a 15% polyacrylamide sequencing gel. Products were visualized by exposure to X-ray film.
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