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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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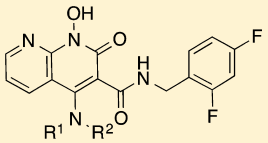
ABSTRACT: There are currently three HIV-1 integrase (IN) strand transfer inhibitors (INSTIs) approved by the FDA for the treatment of AIDS. However, the emergence of drug-resistant mutants emphasizes the need to develop additional agents that have improved efficacies against the existent resistant mutants. 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 (CC_{50}/EC_{50}) 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 Mg^{2+} 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**, Figure 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 Mg^{2+} 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 Mg^{2+} 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**, Figure 1)[5,6] is a recently FDA-approved second-

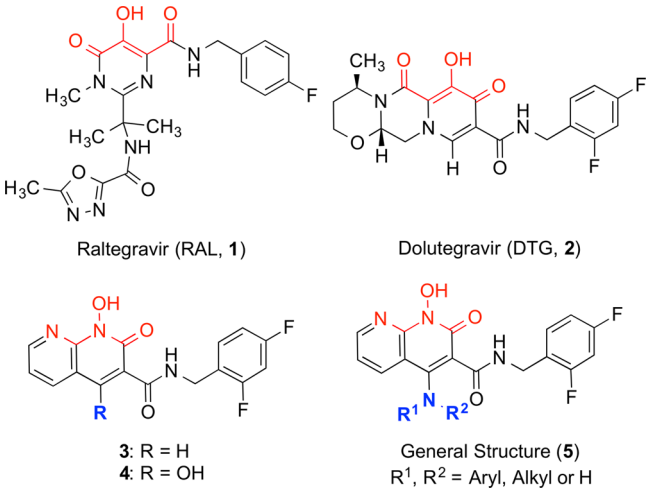


Figure 1. Structures of HIV-1 integrase inhibitors described in the text. Mg^{2+}-chelating heteroatoms are shown in red, with the 4-position of the 1-hydroxy-1,8-naphthyridin-2(1*H*)-one ring being indicated in blue.

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generation INSTI (August 2013), which shows improved efficacies against RAL and EVG-resistant strains of HIV.[7,8] However, **2** also selects for resistant strains of HIV.[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-2*H*-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).[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.[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).[10,11] Inhibitors of HIV-1 ribonuclease H (RNase H) have been reported, which, like INSTIs, inhibit their target enzyme by chelating two Mg^{2+} ions in the enzyme active site. In RNase H, the Mg^{2+} ions are held in place by a "DEDD motif" (D443, E478, D498, and D549).[12−14] Compounds that inhibit both IN and RNase H have been reported.[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-2*H*-one INSTIs (**3** and **4**)[9] was guided by the report that compounds such as the biaryl-containing **6** (Figure 2), which have submicromolar inhibitory potency against

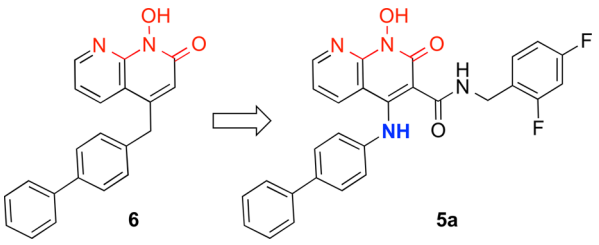

Figure 2. HIV-1 ribonuclease H (RNase H) inhibitors **6** and integrase inhibitor **5a** with the core metal-chelating 1-hydroxy-1,8-naphthyridin-2(1*H*)-one system shown in red and the key 4-amino group indicated in blue.

RNase H (IC_{50} = 0.64 μM), also have low micromolar inhibitory potency against HIV-1 IN (ST IC_{50} = 2.4 μM).[14] In the case of **6**, the reported EC_{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.[14]

Metal chelation by the 1-hydroxy-1,8-naphthyridin-2*H*-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 deoxycytosine in the 3′-end of enzyme-bound viral DNA.[1] 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.[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.[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^{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**.[18−20]

In our current work, we further modified the 1-hydroxy-1,8-naphthyridin-2*H*-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.[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)[9,21] using 2,4-difluorobenzylamine gave the known amide **8** in 70% yield (Scheme 1).[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_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.[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_{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.[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_{50} = 0.018 ± 0.006 μM, Table 1).

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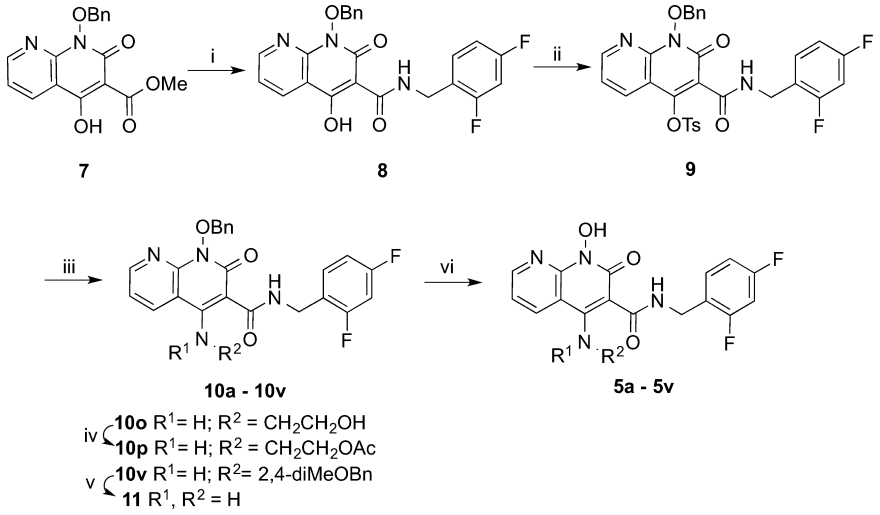
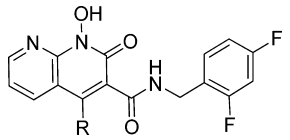
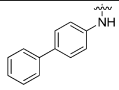
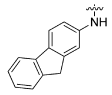
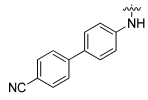
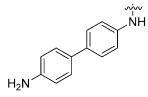
Scheme 1. Synthesis of 4-Amino-*N*-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamides 5a−5v[a]



[a]Reagents and conditions: (i) 2,4-diFBnNH$_2$; (ii) TsCl, TEA, MeCN; (iii) R^{1}R^{2}NH or R^{1}R^{2}NH-HCl, DIEA, DMF; (iv) Ac$_2$O, TEA, DCM; (v) TFA, DCM; (vi) H$_2$, 10% Pd·C, MeOH.

Table 1. Inhibitory Potencies of Carboxamides 5a−5e Obtained Using an in Vitro IN Assay[a]



Comp.	R	IC$_{50}$ (μM)	
		3′-Processing	Strand Transfer
5a		52 ± 5	0.34 ± 0.08
5b		46 ± 8	0.84 ± 0.21
5c		70 ± 7	0.13 ± 0.03
5d		80 ± 11	0.13 ± 0.02
5e		6.9 ± 1.1	0.018 ± 0.006

[a]Assays were performed using a gel-based protocol with Mg^{2+} cofactor as describe in ref 22.

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 **1**, Y143R, N155H, and the double mutant, G140S/Q148H.[23−25] In these assays, amine **5a** was approximately two orders-of-magnitude more potent against the WT enzyme (EC$_{50}$ = 372 ± 63 nM, Table 2) than what has been reported for **6** (32 μM).[14] All members of the series (**5a−5e**) showed nanomolar ST inhibitory potencies against WT enzyme, with **5d** and **5e** being significantly more potent (EC$_{50}$ = 6.3 ± 2.4 and 14 ± 1.9 nM, respectively) than other members of the series (EC$_{50}$ values >100 nM). Because all compounds showed no cytotoxicity up to 250 μM), selectivity indices (SI = CC$_{50}$/EC$_{50}$) 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$_{50}$ = 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**).[26] The large loss of potency incurred by **1** against the Y143R mutant derives from a loss of π−π 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$_{50}$ values of 0.46 ± 0.18 and 0.28 ± 0.11 μM, 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$_{50}$ = 0.31 ± 0.04 μM] (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$_{50}$ 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

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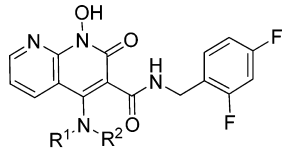
Table 2. Antiviral Potencies of Carboxamides 5a−5e in Cells Infected with HIV-1 Constructs Containing WT or Mutant IN

			EC_{50} (FC, IN mutants[c])			
compd	CC_{50} (μM)[a]	EC_{50} (nM, WT)[b]	Y143R	N155H	G140S/Q148H	SI[d]
5a	>250	372 ± 63	1×	N/A[e]	N/A[e]	>672
5b	>250	171 ± 57	3×	N/A[e]	N/A[e]	>1462
5c	>250	123 ± 21	3×	N/A[e]	N/A[e]	>2033
5d	>250	6.3 ± 2.4	16×	67×	N/A[e]	>39683
5e	>250	14 ± 1.9	2×	8×	32×	>17857

[a]Cytotoxic concentration resulting in 50% reduction in the level of ATP in human osteosarcoma (HOS) cells. [b]Values obtained from cells infected with lentiviral vector harboring WT IN. [c]Cells were infected with viral constructs carrying IN mutations and indicated values correspond to the fold-change (FC) in EC_{50} relative to WT. [d]Selectivity index calculated as the ratio of CC_{50} to EC_{50}. [e]Not available.

Table 3. Inhibitory Potencies of Carboxamides 5f−5v Obtained Using an in Vitro IN Assay[a]



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			IC_{50} (μM)	
compd	R^1	R^2	3′-processing	strand transfer
5f	$-CH_3$	−H	3.7 ± 0.4	0.027 ± 0.004
5g	$-CH_3$	$-CH_3$	21 ± 2	0.087 ± 0.012
5h	−morpholino		77 ± 12	0.079 ± 0.013
5i	−cycloheptyl	−H	13 ± 1.1	0.46 ± 0.18
5j	$-CH_2CH_2Ph$	−H	8.0 ± 1.5	0.050 ± 0.012
5k	$-CH_2(CH_2)_3Ph$	−H	12 ± 2.0	0.28 ± 0.11
5l	$-CH_2(CH_2)_3CH_3$	−H	8.2 ± 1.6	0.024 ± 0.009
5m	$-CH(CH_3)_2$	−H	1.8 ± 0.2	0.016 ± 0.004
5n	$-CH_2CH_2NH_2$	−H	4.5 ± 0.2	0.039 ± 0.006
5o	$-CH_2CH_2OH$	−H	0.55 ± 0.07	0.010 ± 0.009
5p	$-CH_2CH_2OAc$	−H	5.3 ± 0.5	0.027 ± 0.006
5q	$-CH_2CO_2CH_3$	−H	0.71 ± 0.10	0.021 ± 0.011
(*S*)-**5r**	$-NHCH(CH_3)CO_2CH_3$	−H	7.4 ± 0.8	0.017 ± 0.011
(*R*)-**5r**	$-NHCH(CH_3)CO_2CH_3$	−H	5.8 ± 0.6	0.027 ± 0.005
(*S*)-**5s**	$-NHCH(Ph)CO_2CH_3$	−H	16.7 ± 1.4	0.010 ± 0.002
(*R*)-**5s**	$-NHCH(Ph)CO_2CH_3$	−H	13.5 ± 1.0	0.0082 ± 0.0015
(*S*)-**5t**	$-NHCH(CH_2OH)CO_2CH_3$	−H	5.8 ± 0.5	0.0084 ± 0.0032
(*R*)-**5t**	$-NHCH(CH_2OH)CO_2CH_3$	−H	4.4 ± 0.5	0.013 ± 0.04
(*S*)-**5u**	−Pro-OEt		86 ± 6	0.31 ± 0.04
5v	−H	−H	2.5 ± 0.3	0.019 ± 0.002

[a]Assays were performed using a gel-based protocol with Mg^{2+} cofactor as describe in refs 9 and 22.

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 internal hydrogen bonds between their 4-amino groups 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.[5] For reference, we also included **1** and **2** as well as the parent 1-hydroxy-1,8-naphthyridin-2*H*-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 4. Antiviral Potencies of Carboxamides 5f−5v in Cells Infected with HIV-1 Constructs Containing WT or Mutant IN

			EC_{50} (nM, IN mutants[c])			
compd	CC_{50} (μM)[a]	EC_{50} (nM, WT)[b]	Y143R	N155H	G140S/Q148H	SI[d]
5f	>250	3.1 ± 0.6	6.1 ± 2.5 (2×)	18 ± 5.2 (6×)	87 ± 11 (28×)	>80645
5g	13 ± 1.8	268 ± 8	273 ± 52 (1×)	2300 ± 700 (9×)	6800 ± 400 (25×)	49
5h	8.4 ± 3.2	1200 ± 260	1800 ± 870 (1.5×)	3730 ± 920 (3×)	6920 ± 2000 (6×)	7
5i	68 ± 8.7	12 ± 3	6.6 ± 1.7 (0.55×)	14 ± 4 (1×)	35 ± 12 (3×)	5667
5j	>250	14 ± 8	18 ± 2.6 (1×)	79 ± 5.2 (6×)	119 ± 3.4 (9×)	>17857
5k	102 ± 8	50 ± 13	40 ± 15 (0.80×)	116 ± 22 (2×)	243 ± 39 (5×)	2040
5l	94 ± 24	7.4 ± 1.4	8.8 ± 2.7 (1×)	12 ± 4.5 (2×)	71 ± 0.14 (10×)	12703
5m	>250	7.2 ± 3.0	7.4 ± 0.5 (1×)	44 ± 6.7 (6×)	154 ± 16 (21×)	>34722
5n	9.6 ± 3.7	35 ± 11	57 ± 13 (2×)	N/A[e]	N/A[e]	277
5o	24 ± 3	5.2 ± 0.6	4.6 ± 1.8 (0.88×)	25 ± 4 (5×)	43 ± 15 (8×)	4615
5p	>250	4.5 ± 1.5	4.8 ± 2.9 (1×)	3.1 ± 0.3 (0.69×)	35 ± 14 (8×)	>55556
5q	>250	3.8 ± 1.2	4.6 ± 2.2 (1×)	19 ± 7 (5×)	36 ± 16 (9×)	>65789
(*S*)-**5r**	>250	4.2 ± 1.6	4.8 ± 1.4 (1×)	15.3 ± 3.3 (4×)	141 ± 20 (33×)	>59 524
(*R*)-**5r**	>250	11.3 ± 4.7	8.1 ± 1.7 (0.7×)	27 ± 9.9 (2×)	89 ± 26 (8×)	>22 124
(*S*)-**5s**	>250	9 ± 2.4	8.5 ± 1.9 (0.9×)	17.6 ± 6.4 (2×)	55.6 ± 6.2 (6×)	>27 778
(*R*)-**5s**	>250	7.4 ± 3.3	8.2 ± 1.8 (1 ×)	22 ± 4 (3 ×)	48 ± 12 (6 ×)	>33 784
(*S*)-**5t**	>250	9.7 ± 3	11 ± 4 (1 ×)	29 ± 8 (3 ×)	122 ± 33 (13 ×)	>25 773
(*R*)-**5t**	>250	14.2 ± 3.7	10.2 ± 1.4 (0.7 ×)	71 ± 5 (5 ×)	284 ± 120 (20 ×)	>17 606
(*S*)-**5u**	18 ± 7	590 ± 72	1660 ± 920 (3 ×)	N/A[e]	N/A[e]	31
5v	>250	1.1 ± 0.7	2.5 ± 0.6 (2 ×)	5.3 ± 2.3 (5 ×)	35 ± 9 (32 ×)	>227 273

[a]Cytotoxic concentration resulting in 50% reduction in the level of ATP in human osteosarcoma (HOS) cells. [b]Values obtained from cells infected with lentiviral vector carrying WT IN. [c]Cells were infected with viral constructs carrying IN mutations and indicated values correspond to the fold-change (FC) in EC_{50} relative to WT. [d]Selectivity index (SI) calculated as the ratio of CC_{50} to EC_{50}. [e]Not available.

Table 5. Antiviral Potencies of 5o−5q and 5v Compared with 1, 2, and Previously Reported Carboxamides 3 and 4[9] in Cells Infected with HIV-1 Constructs Containing WT or Mutant IN

		EC_{50} [nM/(FC), IN mutants[b]]				
compd	EC_{50} (nM, WT)[a]	Y143R	N155H	R263 K	G118R	G140S/Q148H
1	4 ± 2	162 ± 16 (41×)	154 ± 33 (38×)	9 ± 4 (2×)	36 ± 5 (9×)	1900 ± 300 (475×)
2	1.6 ± 0.9	4.3 ± 1.2 (3×)	3.6 ± 1.3 (2×)	11 ± 3 (7×)	13 ± 5 (8×)	5.8 ± 0.5 (4×)
3	5.1 ± 1.9	4.9 ± 0.8 (1×)	134 ± 23 (26×)	N/A[c]	N/A[c]	438 ± 121 (86×)
4	6.2 ± 2.9	11 ± 2 (2×)	31 ± 8 (5×)	36 ± 8 (6×)	107 ± 8 (17×)	308 ± 125 (50×)
5o	5.2 ± 0.6	4.6 ± 1.8 (0.88×)	25 ± 4 (5×)	26 (5×)	27 ± 1 (5×)	43 ± 15 (8×)
5p	4.5 ± 1.5	4.8 ± 2.9 (1×)	3.1 ± 0.3 (0.69×)	16 ± 5 (4×)	44 ± 11 (10×)	35 ± 14 (8×)
5q	3.8 ± 1.2	4.6 ± 2.2 (1×)	19 ± 7 (5×)	26 ± 8 (7×)	41 ± 18 (11×)	36 ± 16 (9×)
5v	1.1 ± 0.66	2.5 ± 0.6 (2×)	5.3 ± 2.3 (5×)	6.4 ± 2.3 (6×)	16 ± 5 (15×)	35 ± 9 (32×)

[a]Values obtained from cells infected with lentiviral vector harboring WT IN. [b]Cells were infected with viral constructs carrying IN mutations and indicated values correspond to the fold-change (FC) in EC_{50} relative to WT. [c]Not available.

being slightly more potent (equivalent to **2**) (Table 5). Compounds **5p**, **5q**, and **5v** also show fold-loss of potencies that are approximately equivalent to **2** against the panel of mutants, with the exception of **5v**, which shows greater fold-loss of potency against the mutant G140S/Q138H (32-fold). However, because **5v** has greater potency against the WT virus than either **5p** or **5q**, the effective antiviral potencies against virus having the G140S/Q138H mutant are approximately equal for the three compounds (EC_{50} = 35 nM). A summary analysis of Table 5 shows that **5p** exhibits a profile against the panel of mutants that is similar to **2**. Because of its slightly better potency against WT IN, compound **5v** exhibits the best overall performance among the new inhibitors, with both absolute potencies and fold-loss of potencies against the various mutant vectors that are similar to **2**. The sole exception is the G140S/Q148H mutant, where **5v** suffers an approximate 7-fold loss of potency relative to **2**. Importantly, **5v** shows from 5- to 10-fold enhanced performance across the entire panel relative to the starting compound **4**. The standard assays are performed in the presence of 5% fetal bovine serum (FBS). To examine the potential effects of serum protein binding on their antiviral potencies, compounds **5p**, **5q**, and **5v** were evaluated against the WT vector in the presence of increasing amounts FBS (5%, 10%, and 15%). These experiments showed that the antiviral potencies of the compounds were essentially unchanged relative to values shown in Tables 4 and 5, which were obtained in the presence of 5% fetal bovine serum protein (data not shown).

The title compounds were originally designed to chelate the two Mg^{2+} ions at the IN active site. However, HIV-1 reverse transcriptase (RT) also contains two active sites, the polymerase active site and the RNase H active site, each of which has two bound Mg^{2+} ions. To examine whether representative compounds of the series could also bind Mg^{2+} ions at one or both of the active sites in RT, we tested **5p**, **5q**, and **5v**, to see whether they inhibited either the RNase H or the polymerase activity of RT. Nevirapine, a well-known nonnucleoside reverse transcriptase inhibitor (NNRTI), was included as a positive

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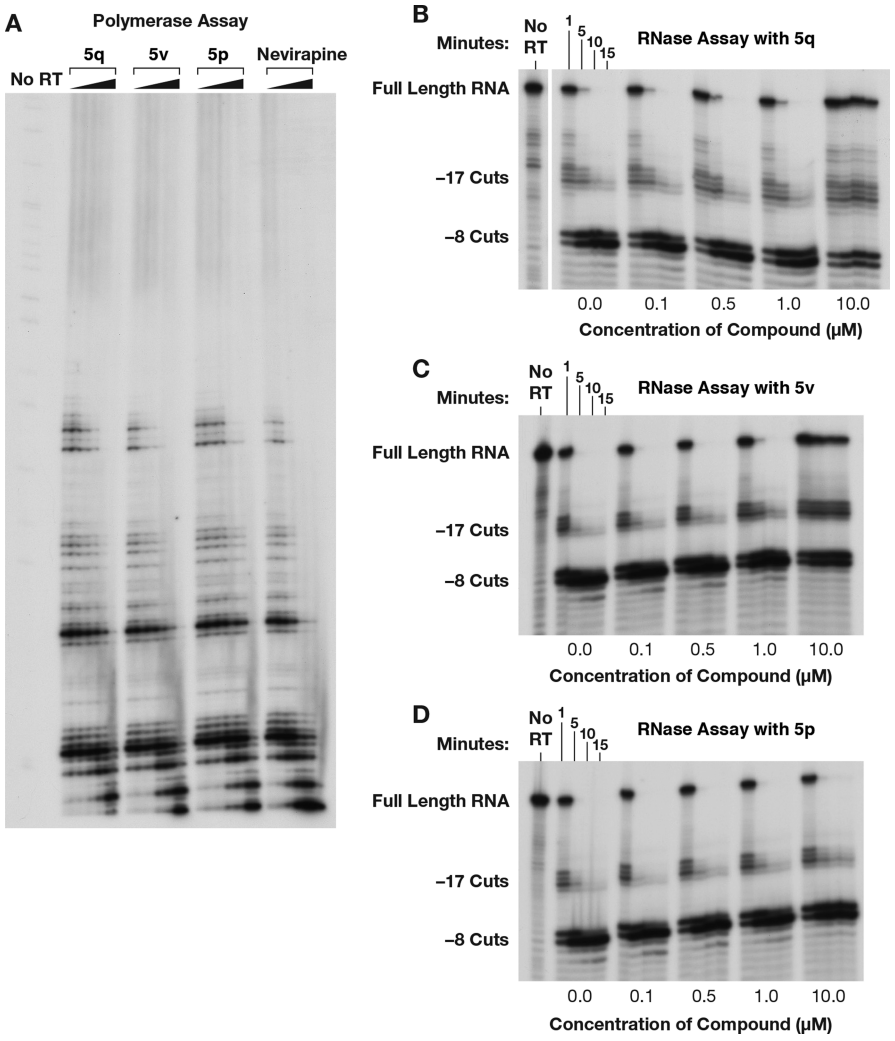

Figure 3. Polymerase and RNase H inhibition assays (A): The effects of **5p**, **5q**, and **5v** on the DNA-dependent DNA polymerase activity of RT are shown. Reactions were performed with WT RT in the presence of various concentrations of compounds (0, 0.02, 0.1, 0.5, 1, or 10 μM). Samples were precipitated 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.

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. Similar

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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 IC_{50} 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-2*H*-ones (**3** and **4**).[9] 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 INSTI, **1**. 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 absolute 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 ^{13}C 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 R_f 200i 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 C_{18} 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 *p*-methylbenzenesulfonate (**9**) (1.0 mmol), *N*-ethyl-*N*-isopropylpropan-2-amine (10 mmol) and amines R^1R^2NH or $R^1R^2NH{\cdot}HCl$ (5.0 mmol) in DMF (2.0 mL) was heated to 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 EtOAc (5 mL) and the solution degassed and stirred at room temperature under H_2 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-(Benzyloxy)-N-(2,4-difluorobenzyl)-4-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**8**).* A solution of methyl 1-(benzyloxy)-4-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylate (**7**)[9] (1.0 g, 3.17 mmol) and (2,4-difluorophenyl)methanamine (2.0 mL, 15.86 mmol) in DMF (3.0 mL) was subjected to microwave irradiation with stirring (140 °C; 2 h). The resulting product mixture was purified by CombiFlash silica gel chromatography to provide **8** as a white solid (964 mg, 70% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 10.35 (t, J = 5.5 Hz, 1H), 8.80 (dd, J = 4.7, 1.8 Hz, 1H), 8.49 (dd, J = 7.9, 1.8 Hz, 1H), 7.67 (dd, J = 7.6, 1.8 Hz, 2H), 7.42−7.36 (m, 4H), 7.31 (dd, J = 7.9, 4.7 Hz, 1H), 6.89−6.82 (m, 2H), 5.28 (s, 2H), 4.66 (d, J = 6.0 Hz, 2H). ESI-MS m/z: 438.1 (MH^+).

*1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl 4-Methylbenzenesulfonate (**9**).* To a suspension of **8** (967 mg, 2.21 mmol) in CH_3CN (10 mL) and CH_2Cl_2 (2.0 mL) were added *N*-ethyl-*N*-isobutyl-2-methylpropan-1-amine (2.81 mL, 13.27 mmol) and 4-methylbenzene-1-sulfonyl chloride (1.27 g, 6.63 mmol), and the mixture was stirred at room temperature (overnight). The resulting mixture was purified by CombiFlash silica gel chromatography using solid loading to provide **9** as a colorless oil (1.21 g, 93% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.76 (dd, J = 4.7, 1.7 Hz, 1H), 8.28 (t, J = 5.8 Hz, 1H), 8.23 (dd, J = 8.1, 1.7 Hz, 1H), 7.90−7.88 (m, 2H), 7.66−7.64 (m, 2H), 7.47−7.45 (m, 1H), 7.40−7.35 (m, 5H), 7.9−7.26 (m, 1H), 6.86−6.78 (m, 2H), 5.30 (s, 2H), 4.50 (d, J = 5.9 Hz, 2H). ESI-MS m/z: 592.1 (MH^+).

*4-([1,1′-Biphenyl]-4-ylamino)-1-(benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10a**).* Treatment of **9** with [1,1′-biphenyl]-4-amine as outlined in general procedure A, provided **10a** as a colorless oil in 97% yield. ^1H NMR (400 MHz, $CDCl_3$) δ 13.23 (s, 1H), 10.86 (t, J = 5.5 Hz, 1H), 8.62 (dd, J = 4.5, 1.5 Hz, 1H), 7.91 (dd, J = 8.2, 1.5 Hz, 1H), 7.71−7.69 (m, 2H), 7.58−7.34 (m, 7H), 7.28−7.23 (m, 5H), 7.09 (d, J = 8.5 Hz, 2H), 6.88 (dd, J = 8.3, 4.5 Hz, 1H), 6.84 (d, J = 8.8 Hz, 1H), 5.32 (s, 2H), 4.65 (d, J = 5.8 Hz, 2H). ESI-MS m/z: 589.2 (MH^+).

*4-((9H-Fluoren-2-yl)amino)-1-(benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10b**).* Treatment of **9** with 9*H*-fluoren-2-amine as outlined in general procedure A, provided **10b** as a colorless oil in 99% yield. ^1H NMR (400 MHz, $CDCl_3$) δ 13.31 (s, 1H), 10.87 (t, J = 5.7 Hz, 1H), 8.59 (ddd, J = 4.6, 1.7, 0.7 Hz, 1H), 7.85 (ddd, J = 8.3, 1.6, 0.7 Hz, 1H), 7.76−7.15 (m, 8H), 7.05 (dd, J = 8.1, 2.0 Hz, 1H), 6.88−6.84 (m, 3H), 6.83−6.78 (m, 2H), 6.72−6.66 (m, 2H), 5.33 (s, 2H), 4.65 (d, J = 5.8 Hz, 2H), 3.84 (s, 2H). ESI-MS m/z: 601.2 (MH^+).

*1-(Benzyloxy)-4-((4′-cyano-[1,1′-biphenyl]-4-yl)amino)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10c**).* Treatment of **9** with 4′-amino-[1,1′-biphenyl]-4-carbonitrile as outlined in general procedure A provided **10c** as a yellow solid in 98% yield. ^1H NMR (400 MHz, $CDCl_3$) δ 13.21 (s, 1H), 10.84 (t, J = 5.7 Hz, 1H), 8.66 (dd, J = 4.5, 1.6 Hz, 1H), 7.91 (dd, J = 8.2, 1.7 Hz, 1H), 7.74−7.60 (m, 4H), 7.55−7.53 (m, 2H), 7.44−7.38 (m, 4H), 7.13 (d, J

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= 8.5 Hz, 2H), 6.94 (dd, *J* = 8.3, 4.6 Hz, 1H), 6.88−6.80 (m, 2H), 6.78−6.75 (m, 2H), 5.33 (s, 2H), 4.65 (d, *J* = 5.8 Hz, 2H). ESI-MS *m*/*z*: 614.2 (MH^+).

*4-((4′-Amino-[1,1′-biphenyl]-4-yl)amino)-1-(benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10d**).* Treatment of **9** with benzidine as outlined in general procedure A, provided **10d** as a colorless oil in 97% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.20 (s, 1H), 10.83 (t, *J* = 5.8 Hz, 1H), 8.66−8.50 (m, 1H), 7.88 (dd, *J* = 8.4, 1.5 Hz, 1H), 7.69−7.67 (m, 2H), 7.44 (d, *J* = 8.4 Hz, 2H), 7.39−7.34 (m, 3H), 7.32−7.287 (m, 4H), 7.02 (d, *J* = 8.6 Hz, 2H), 6.86−6.80 (m, 2H), 6.72−6.67 (m, 2H), 5.29 (s, 2H), 4.62 (d, *J* = 5.8 Hz, 2H). ESI-MS *m*/*z*: 604.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-4-(phenylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10e**).* Treatment of **9** with aniline as outlined in general procedure A, provided **10e** as a yellow solid in 65% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.19 (s, 1H), 10.85 (t, *J* = 5.7 Hz, 1H), 8.61 (dd, *J* = 4.5, 1.7 Hz, 1H), 7.81 (dd, *J* = 8.3, 1.6 Hz, 1H), 7.69 (dd, *J* = 7.8, 1.6 Hz, 2H), 7.43−7.36 (m, 4H), 7.31 (dd, *J* = 10.7, 4.9 Hz, 2H), 7.18 (t, *J* = 7.4 Hz, 1H), 7.06−7.041 (m, 2H), 6.87−6.79 (m, 3H), 5.32 (s, 2H), 4.64 (d, *J* = 5.8 Hz, 2H). ESI-MS *m*/*z*: 513.1 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-4-(methylamino)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10f**).* Treatment of **9** with methanamine (2.0 M in THF) as outlined in general procedure A, provided **10f** as a white solid in 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.09 (d, *J* = 4.9 Hz, 1H), 10.83 (t, *J* = 5.6 Hz, 1H), 8.70 (dd, *J* = 4.6, 1.5 Hz, 1H), 8.47 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.69 (dd, *J* = 7.7, 1.6 Hz, 2H), 7.41−7.35 (m, 4H), 7.20−7.17 (m, 1H), 6.86−6.78 (m, 2H), 5.26 (s, 2H), 4.61 (d, *J* = 5.8 Hz, 2H), 3.41 (d, *J* = 5.6 Hz, 3H). ESI-MS *m*/*z*: 451.1 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-4-(dimethylamino)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10g**).* Treatment of **9** with dimethylamine hydrochloride as outlined in general procedure A, provided **10g** as a white solid in 91% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (dd, *J* = 4.6, 1.7 Hz, 1H), 8.20 (dd, *J* = 8.1, 1.7 Hz, 1H), 8.05 (t, *J* = 5.9 Hz, 1H), 7.67 (dd, *J* = 7.5, 1.8 Hz, 2H), 7.55 (dd, *J* = 15.1, 8.5 Hz, 1H), 7.41−7.36 (m, 3H), 7.19 (dd, *J* = 8.1, 4.6 Hz, 1H), 6.88−6.78 (m, 2H), 5.22 (s, 2H), 4.66 (d, *J* = 6.0 Hz, 2H), 2.95 (s, 6H). ESI-MS *m*/*z*: 465.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-4-morpholino-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10h**).* Treatment of **9** with morpholine as outlined in general procedure A, provided **10h** as a colorless oil in 99% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (dd, *J* = 4.6, 1.6 Hz, 1H), 8.07−8.03 (m, 2H), 7.68−7.60 (m, 3H), 7.39 (dd, *J* = 4.2, 2.2 Hz, 3H), 7.13 (dd, *J* = 8.0, 4.6 Hz, 1H), 6.89−6.80 (m, 2H), 5.08 (s, 2H), 4.64 (d, *J* = 5.9 Hz, 2H), 3.61−3.59 (m, 4H), 3.05−3.03 (m, 4H). ESI-MS *m*/*z*: 507.1 (MH^+).

*1-(Benzyloxy)-4-(cycloheptylamino)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10i**).* Treatment of **9** with cycloheptanamine as outlined in general procedure A, provided **10i** as a colorless oil in 96% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.77 (d, *J* = 9.1 Hz, 1H), 10.77 (t, *J* = 5.7 Hz, 1H), 8.69 (dd, *J* = 4.6, 1.6 Hz, 1H), 8.24 (dd, *J* = 8.2, 1.5 Hz, 1H), 7.68 (dd, *J* = 7.8, 1.6 Hz, 2H), 7.41−7.34 (m, 4H), 7.19 (dd, *J* = 8.2, 4.5 Hz, 1H), 6.86−6.78 (m, 2H), 5.26 (s, 2H), 4.63 (d, *J* = 5.7 Hz, 2H), 4.01 (dq, *J* = 13.2, 4.4 Hz, 1H), 2.13−2.05 (m, 2H), 1.82−1.74 (m, 4H), 1.62−1.60 (m, 4H), 1.52−1.42 (m, 2H). ESI-MS *m*/*z*: 533.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-4-(phenethylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10j**).* Treatment of **9** with 2-phenylethanamine as outlined in general procedure A, provided **10j** as a colorless oil in 78% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.06 (t, *J* = 4.7 Hz, 1H), 10.77 (t, *J* = 5.7 Hz, 1H), 8.67−8.66 (m, 1H), 8.30 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.69−7.66 (m, 2H), 7.38−7.36 (m, 4H), 7.29−7.21 (m, 6H), 7.12 (dd, *J* = 8.2, 4.5 Hz, 1H), 6.88−6.79 (m, 2H), 5.24 (s, 2H), 4.63 (d, *J* = 5.8 Hz, 2H), 3.96−3.91 (m, 2H), 3.08−3.05 (m, 2H). ESI-MS *m*/*z*: 541.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-4-((4-phenylbutyl)amino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10k**).* Treatment of **9** with 4-phenylbutan-1-amine as outlined in general procedure A, provided **10k** as a colorless oil in 93% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.99 (t, *J* = 4.6 Hz, 1H), 10.79 (t, *J* = 5.7 Hz, 1H), 8.68−8.66 (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), 2.65 (t, *J* = 7.0 Hz, 2H), 1.81 (qd, *J* = 6.9, 3.5 Hz, 4H). ESI-MS *m*/*z*: 569.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-4-(pentylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10l**).* Treatment of **9** with pentan-1-amine as outlined in general procedure A, provided **10l** as a colorless oil in 95% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.99 (t, *J* = 4.6 Hz, 1H), 10.79 (t, *J* = 5.7 Hz, 1H), 8.68 (dd, *J* = 4.5, 1.2 Hz, 1H), 8.39 (dd, *J* = 8.2, 1.4 Hz, 1H), 7.68 (dd, *J* = 7.5, 1.5 Hz, 2H), 7.42−7.35 (m, 4H), 7.17 (dd, *J* = 8.2, 4.6 Hz, 1H), 6.86−6.78 (m, 2H), 5.25 (s, 2H), 4.63 (d, *J* = 5.7 Hz, 2H), 3.70 (td, *J* = 7.0, 5.1 Hz, 2H), 1.83−1.75 (m, 2H), 1.53−1.26 (m, 4H), 0.92 (t, *J* = 6.9 Hz, 3H). ESI-MS *m*/*z*: 507.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-4-(isopropylamino)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10m**).* Treatment of **9** with propan-2-amine as outlined in general procedure A, provided **10m** as a colorless oil in 73% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.66 (d, *J* = 8.9 Hz, 1H), 10.78 (t, *J* = 5.6 Hz, 1H), 8.71−8.67 (m, 1H), 8.28 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.69−7.67 (m, 2H), 7.40−7.34 (m, 4H), 7.19−7.16 (m, 1H), 6.86−6.78 (m, 2H), 5.26 (s, 2H), 4.63 (d, *J* = 5.7 Hz, 2H), 4.24−4.17 (m, 1H), 1.40 (d, *J* = 6.3 Hz, 6H). ESI-MS *m*/*z*: 479.2 (MH^+).

*tert-Butyl (2-((1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)ethyl)carbamate (**10n**).* Treatment of **9** with *tert*-butyl (2-aminoethyl)carbamate as outlined in general procedure A, provided **10n** as a white solid in 37% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.74 (s, 1H), 10.75 (t, *J* = 5.5 Hz, 1H), 8.69−8.56 (m, 1H), 8.34 (d, *J* = 7.9 Hz, 1H), 7.64 (dd, *J* = 7.4, 1.6 Hz, 2H), 7.36−7.31 (m, 4H), 7.17−7.13 (m, 1H), 6.82−6.74 (m, 2H), 5.20 (s, 2H), 5.09 (d, *J* = 18.3 Hz, 1H), 4.57 (d, *J* = 5.7 Hz, 2H), 3.77 (dd, *J* = 10.8, 5.4 Hz, 2H), 3.39 (dd, *J* = 11.4, 5.5 Hz, 2H), 1.36 (s, 9H). ESI-MS *m*/*z*: 580.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-4-((2-hydroxyethyl)amino)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10o**).* Treatment of **9** with 2-aminoethanol as outlined in general procedure A, provided **10o** as a white solid in 71% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.74 (t, *J* = 4.8 Hz, 1H), 10.80 (t, *J* = 5.7 Hz, 1H), 8.69−8.68 (m, 1H), 8.41 (dd, *J* = 8.2, 1.0 Hz, 1H), 7.68 (dd, *J* = 7.3, 1.5 Hz, 2H), 7.41−7.35 (m, 4H), 7.19−7.15 (m, 1H), 6.86−6.78 (m, 2H), 5.24 (s, 2H), 4.61 (d, *J* = 5.7 Hz, 2H), 3.90−3.80 (m, 2H), 3.82 (dd, *J* = 10.0, 5.1 Hz, 2H), 2.88 (bs, 1H). ESI-MS *m*/*z*: 481.1 (MH^+).

*2-((1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)ethyl Acetate (**10p**).* To a solution of **10o** (46 mg, 0.10 mmol) in CH_2CL_2 (3 mL) was added triethylamine (32 μL, 0.23 mmol) and acetic anhydride (11 μL, 0.12 mmol) and the solution was stirred at room temperature (1.5 h). Purification by CombiFlash silica gel chromatography provided **10p** as a white solid (26 mg, 52% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.93 (t, *J* = 5.2 Hz, 1H), 10.70 (t, *J* = 5.7 Hz, 1H), 8.67 (dd, *J* = 4.6, 1.6 Hz, 1H), 8.29 (dd, *J* = 8.2, 1.5 Hz, 1H), 7.64 (dt, *J* = 4.1, 2.2 Hz, 2H), 7.37−7.29 (m, 4H), 7.16 (dd, *J* = 8.2, 4.5 Hz, 1H), 6.83−6.75 (m, 2H), 5.22 (s, 2H), 4.58 (d, *J* = 5.8 Hz, 2H), 4.29 (t, *J* = 5.6 Hz, 2H), 3.89 (q, *J* = 5.5 Hz, 2H), 2.05 (s, 3H). ESI-MS *m*/*z*: 523.2 (MH^+).

*Methyl (1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)glycinate (**10q**).* Treatment of **9** with methyl 2-aminoacetate hydrochloride as outlined in general procedure A, provided **10q** as a white solid in 95% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.27 (t, *J* = 5.5 Hz, 1H), 10.70 (t, *J* = 5.6 Hz, 1H), 8.71 (dd, *J* = 4.6, 1.6 Hz, 1H), 8.22 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.67 (dd, *J* = 7.8, 1.7 Hz, 2H), 7.44−7.35 (m, 4H), 7.20 (dd, *J* = 8.2, 4.6 Hz, 1H), 6.87−6.78 (m, 2H), 5.26 (s, 2H), 4.66 (d, *J* = 5.7 Hz, 2H), 4.44 (d, *J* = 5.6 Hz, 2H), 3.81 (s, 3H). ESI-MS *m*/*z*: 509.1 (MH^+).

*Methyl (1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)-L-alaninate [(S)-**10r**].* Treatment of **9** with methyl (*S*)-2-aminopropanoate hydrochloride as outlined in general procedure A, provided (*S*)-**10r** as a white solid in 93% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.85 (d, *J* = 8.8 Hz, 1H), 10.67 (t, *J* = 5.6 Hz, 1H), 8.67−8.66 (m, 1H), 8.15 (dd, *J* = 8.2, 1.5 Hz, 1H), 7.63

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(dd, J = 7.4, 1.6 Hz, 2H), 7.37−7.31 (m, 4H), 7.17−7.14 (m, 1H), 6.83−6.75 (m, 2H), 5.23 (s, 2H), 4.64−4.58 (m, 1H), 4.61 (d, J = 6.3 Hz, 2H), 3.70 (s, 3H), 1.61 (d, J = 6.9 Hz, 3H). ESI-MS m/z: 523.2 (MH^+).

*Methyl (1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)-D-alaninate [(R)-**10r**].* Treatment of **9** with methyl (*R*)-2-zminopropanoate hydrochloride as outlined in general procedure A, provided (*R*)-**10r** as a white solid in 95% yield. ^{1}H NMR (400 MHz, cdcl$_3$) δ 11.85 (d, J = 8.8 Hz, 1H), 10.67 (t, J = 5.6 Hz, 1H), 8.67−8.66 (m, 1H), 8.15 (dd, J = 8.2, 1.5 Hz, 1H), 7.63 (dd, J = 7.4, 1.6 Hz, 2H), 7.37−7.317 (m, 4H), 7.17−7.14 (m, 1H), 6.83−6.75 (m, 2H), 5.23 (s, 2H), 4.64−4.58 (m, 1H), 4.61 (d, J = 6.3 Hz, 3H), 3.70 (s, 3H), 1.61 (d, J = 6.9 Hz, 3H). ESI-MS m/z: 523.2 (MH^+).

*Methyl (S)-2-((1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-2-phenylacetate [(S)-**10s**].* Treatment of **9** with methyl (*S*)-2-amino-2-phenylacetate hydrochloride as outlined in general procedure A, provided (*S*)-**10s** as a white solid in 91% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 12.76 (d, J = 8.3 Hz, 1H), 10.65 (t, J = 5.7 Hz, 1H), 8.61 (dd, J = 4.6, 1.6 Hz, 1H), 8.01 (dd, J = 8.2, 1.6 Hz, 1H), 7.62 (dd, J = 7.6, 1.8 Hz, 2H), 7.51 (dd, J = 7.9, 1.2 Hz, 2H), 7.39−7.31 (m, 7H), 7.01 (dd, J = 8.2, 4.6 Hz, 1H), 6.83−6.75 (m, 2H), 5.53 (d, J = 8.3 Hz, 1H), 5.20 (s, 2H), 4.67 (qd, J = 15.3, 5.8 Hz, 2H), 3.69 (s, 3H). ESI-MS m/z: 585.2 (MH^+).

*Methyl (R)-2-((1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-2-phenylacetate [(R)-**10s**].* Treatment of **9** with methyl (*R*)-2-amino-2-phenylacetate hydrochloride as outlined in general procedure A, provided (*R*)-**10s** as a white solid in 91% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 12.76 (d, J = 8.3 Hz, 1H), 10.64 (t, J = 5.6 Hz, 1H), 8.62−8.60 (m, 1H), 8.02−7.96 (m, 2H), 7.61 (dd, J = 7.3, 1.8 Hz, 2H), 7.50 (d, J = 7.4 Hz, 2H), 7.43−7.24 (m, 7H), 7.01 (dd, J = 8.2, 4.5 Hz, 1H), 6.83−6.75 (m, 2H), 5.53 (d, J = 8.3 Hz, 1H), 5.20 (s, 2H), 4.66 (qd, J = 15.3, 5.7 Hz, 2H), 3.68 (s, 3H), 3.65 (d, J = 0.8 Hz, 4H). ESI-MS m/z: 585.2 (MH^+).

*Methyl (S)-2-((1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-3-hydroxypropanoate [(S)-**10t**].* Treatment of **9** with methyl (*S*)-2-amino-3-hydroxypropanoate hydrochloride as outlined in general procedure A, provided (*S*)-**10ts** as a white solid in 83% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 11.21 (d, J = 9.7 Hz, 1H), 10.66 (t, J = 5.6 Hz, 1H), 8.67 (dd, J = 4.5, 1.6 Hz, 1H), 8.30 (dd, J = 8.2, 1.5 Hz, 1H), 7.63 (dd, J = 7.6, 1.8 Hz, 2H), 7.36−7.31 (m, 4H), 7.17 (dd, J = 8.2, 4.6 Hz, 1H), 6.83−6.75 (m, 2H), 5.22 (s, 2H), 4.60−4.57 (m, 3H), 3.99 (t, J = 4.8 Hz, 2H), 3.75 (s, 3H). ESI-MS m/z: 539.2 (MH^+).

*Methyl (R)-2-((1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-3-hydroxypropanoate [(R)-**10t**].* Treatment of **9** with methyl (*R*)-2-amino-3-hydroxypropanoate hydrochloride as outlined in general procedure A, provided (*R*)-**10t** as a white solid in 97% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 11.19 (d, J = 9.4 Hz, 1H), 10.67 (s, 1H), 8.68 (d, J = 4.2 Hz, 1H), 8.29 (d, J = 8.1 Hz, 1H), 7.63 (d, J = 6.0 Hz, 2H), 7.35−7.32 (m, 4H), 7.17 (dd, J = 8.1, 4.5 Hz, 1H), 6.82−6.76 (m, 2H), 5.22 (s, 2H), 4.60−4.58 (m, 3H), 3.99 (t, J = 5.4 Hz, 2H), 3.75 (s, 3H). ESI-MS m/z: 539.2 (MH^+).

*Ethyl (1-(Benzyloxy)-3-((2,4-difluorobenzyl)carbamoyl)-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)-L-prolinate (**10u**).* Treatment of **9** with ethyl (*S*)-pyrrolidine-2-carboxylate hydrochloride as outlined in general procedure A, provided **10u** as a white solid in 73% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.64 (dd, J = 4.6, 1.5 Hz, 1H), 8.34 (dd, J = 8.0, 1.6 Hz, 1H), 8.16 (t, J = 5.8 Hz, 1H), 7.66 (dd, J = 7.5, 1.6 Hz, 2H), 7.49 (dd, J = 15.0, 8.5 Hz, 1H), 7.37−7.32 (m, 3H), 7.23−7.19 (m, 1H), 6.86−6.75 (m, 2H), 5.24 (q, J = 8.7 Hz, 2H), 4.62 (d, J = 3.4 Hz, 2H), 4.37 (dd, J = 8.5, 3.8 Hz, 1H), 4.01−3.94 (m, 2H), 3.76−3.73 (m, 1H), 3.50−3.45 (m, 1H), 2.40−2.34 (m, 1H), 2.02−1.98 (m, 3H), 1.07 (t, J = 7.1 Hz, 3H). ESI-MS m/z: 563.2 (MH^+).

*1-(Benzyloxy)-N-(2,4-difluorobenzyl)-4-((2,4-dimethoxybenzyl)amino)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**10v**).* Treatment of **9** with (2,4-dimethoxyphenyl)methanamine as outlined in general procedure A, provided **10u** as a colorless oil in 89% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 12.00 (t, J = 6.0 Hz, 1H), 10.76 (t, J = 5.7 Hz, 1H), 8.65 (dd, J = 4.6, 1.5 Hz, 1H), 8.28 (dd, J = 8.2, 1.5 Hz, 1H), 7.70−7.68 (m, 2H), 7.40−7.33 (m, 4H), 7.26−7.24 (m, 1H), 7.08 (dd, J = 8.2, 4.6 Hz, 1H), 6.84−6.76 (m, 2H), 6.47−6.45 (m, 2H), 5.26 (s, 2H), 4.73 (d, J = 6.1 Hz, 2H), 4.59 (d, J = 5.7 Hz, 2H), 3.79 (s, 3H), 3.76 (s, 3H). ESI-MS m/z: 587.2 (MH^+), 609.2 (MNa^+).

*4-Amino-1-(benzyloxy)-N-(2,4-difluorobenzyl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**11**).* To a solution of **10v** (93 mg, 0.16 mmol) in CH_2Cl_2 (2.0 mL) was added TFA (1.0 mL), and the mixture was stirred at room temperature (5 min) then concentrated by rotary evaporation under reduced pressure. Purification of the resulting residue by CombiFlash silica gel chromatography provided **11** as a white solid (69 mg, 100% yield). ^{1}H NMR (400 MHz, CDCl$_3$) δ 10.62 (t, J = 5.7 Hz, 1H), 8.75 (dd, J = 4.6, 1.6 Hz, 1H), 8.04 (dd, J = 8.1, 1.6 Hz, 1H), 7.67 (dd, J = 7.7, 1.7 Hz, 2H), 7.38−7.35 (m, 4H), 7.24 (dd, J = 8.1, 4.6 Hz, 1H), 6.87−6.78 (m, 2H), 5.26 (s, 2H), 4.63 (d, J = 5.8 Hz, 2H). ESI-MS: m/z 437.1 (M+H$^+$).

*4-([1,1′-Biphenyl]-4-ylamino)-N-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5a**).* Treatment of **10a** as described under general procedure B and purification by preparative HPLC (linear gradient of 50% B to 90% B over 30 min; retention time = 22.8 min) provided **5a** as a white powder in 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.31 (s, 1H), 10.60 (t, J = 5.8 Hz, 1H), 8.61 (dd, J = 4.5, 1.7 Hz, 1H), 7.86 (dd, J = 8.2, 1.7 Hz, 1H), 7.61−7.55 (m, 4H), 7.44−7.36 (m, 3H), 7.27 (t, J = 7.3 Hz, 1H), 7.20−7.15 (m, 1H), 7.13 (d, J = 8.5 Hz, 2H), 7.07 (dd, J = 8.2, 4.6 Hz, 1H), 7.02−6.97 (m, 1H), 4.46 (d, J = 5.7 Hz, 2H). ESI-MS m/z: 499.1 (MH^+). HRMS calcd $C_{28}H_{21}F_2N_4O_3$ [MH^+], 499.1576; found, 499.1583.

*4-((9H-Fluoren-2-yl)amino)-N-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5b**).* Treatment of **10b** as described under general procedure B and purification by preparative HPLC (linear gradient of 60% B to 80% B over 30 min; retention time = 21.3 min) provided **5b** as a white powder in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.54 (s, 1H), 10.68 (t, J = 5.7 Hz, 1H), 8.59 (dd, J = 4.5, 1.6 Hz, 1H), 7.82 (dd, J = 8.2, 1.5 Hz, 1H), 7.79 (d, J = 7.5 Hz, 1H), 7.76 (d, J = 8.1 Hz, 1H), 7.49 (d, J = 7.5 Hz, 1H), 7.41 (dd, J = 15.3, 8.7 Hz, 1H), 7.31−7.29 (m, 2H), 7.24−7.20 (m, 1H), 7.20−7.17 (m, 1H), 7.07 (dd, J = 8.1, 1.8 Hz, 1H), 7.02−6.97 (m, 2H), 4.47 (d, J = 5.8 Hz, 2H), 3.79 (s, 2H). ESI-MS m/z: 511.1 (MH^+). HRMS calcd $C_{29}H_{21}F_2N_4O_3$ [MH^+], 511.1576; found, 511.1582.

*4-((4′-Cyano-[1,1′-biphenyl]-4-yl)amino)-N-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5c**).* Treatment of **10c** as described under general procedure B and purification by preparative HPLC (linear gradient of 30% B to 80% B over 30 min; retention time = 22.3 min) provided **5c** as a white powder in 39% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.13 (s, 1H), 10.53 (t, J = 6.2 Hz, 1H), 8.64 (d, J = 4.4 Hz, 1H), 7.88 (d, J = 8.2 Hz, 1H), 7.83 (bs, 4H), 7.67 (d, J = 8.4 Hz, 2H), 7.43 (dd, J = 15.6, 8.3 Hz, 1H), 7.21−7.16 (m, 3H), 7.11 (dd, J = 9.0, 3.8 Hz, 1H), 7.00 (t, J = 8.9 Hz, 1H), 4.46 (d, J = 6.1 Hz, 2H). ESI-MS m/z: 524.1 (MH^+). HRMS calcd $C_{29}H_{20}F_2N_5O_3$ [MH^+], 524.1529; found, 524.1521.

*4-((4′-Amino-[1,1′-biphenyl]-4-yl)amino)-N-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5d**).* Treatment of **10d** as described under general procedure B and purification by preparative HPLC (linear gradient of 30% B to 70% B over 30 min; retention time = 15.6 min) provided **5d** as a white powder in 51% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.42 (s, 1H), 10.89 (bs, 1H), 10.67 (t, J = 5.8 Hz, 1H), 8.62 (dd, J = 4.5, 1.6 Hz, 1H), 7.85 (dd, J = 8.2, 1.6 Hz, 1H), 7.47 (t, J = 7.9 Hz, 2H), 7.43−7.40 (m, 3H), 7.23−7.18 (m, 1H), 7.09 (d, J = 8.6 Hz, 2H), 7.02−7.00 (m, 2H), 6.76 (d, J = 8.4 Hz, 2H), 4.48 (d, J = 5.9 Hz, 2H). ESI-MS m/z: 514.1 (MH^+). HRMS calcd $C_{28}H_{22}F_2N_5O_3$ [MH^+], 514.1685; found, 514.1690.

*N-(2,4-Difluorobenzyl)-1-hydroxy-2-oxo-4-(phenylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5e**).* Treatment of **10e** as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 90% B over 30 min; retention time = 20.2 min) provided **5e** as a white powder in 86% yield. ^{1}H NMR

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(400 MHz, DMSO-d_6) δ 12.39 (s, 1H), 10.86 (s, 1H), 10.65 (t, J = 5.7 Hz, 1H), 8.61 (dd, J = 4.5, 1.6 Hz, 1H), 7.75 (dd, J = 8.2, 1.7 Hz, 1H), 7.42 (dd, J = 15.4, 8.7 Hz, 1H), 7.27 (t, J = 7.8 Hz, 2H), 7.22−7.17 (m, 1H), 7.13−7.00 (m, 5H), 4.47 (d, J = 5.8 Hz, 2H). ESI-MS m/z: 423.1 (MH^+). HRMS calcd $C_{22}H_{17}F_2N_4O_3$ [MH^+], 423.1263; found, 423.1269.

*N-(2,4-Difluorobenzyl)-1-hydroxy-4-(methylamino)-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 20% B to 50% B over 30 min; retention time = 26.4 min) provided **5f** as a white powder in 63% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.19 (bs, 1H), 10.08 (bs, 1H), 8.59 (d, J = 3.8 Hz, 1H), 8.55 (d, J = 8.1 Hz, 1H), 7.48 (dd, J = 15.4, 8.5 Hz, 1H), 7.22 (dd, J = 8.0, 4.5 Hz, 1H), 7.18−7.13 (m, 1H), 7.00 (dd, J = 9.7, 7.6 Hz, 1H), 4.42 (d, J = 5.6 Hz, 2H), 3.12 (s, 3H). ESI-MS m/z: 361.1 (MH^+). HRMS calcd $C_{17}H_{15}F_2N_4O_3$ [MH^+], 361.1107; found, 361.1105.

*N-(2,4-Difluorobenzyl)-4-(dimethylamino)-1-hydroxy-2-oxo-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 20% B to 45% B over 30 min; retention time = 21.6 min) provided **5g** as a yellow solid in 89% yield. ^{1}H NMR (400 MHz, DMSO-d_6) 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: 375.1 (MH^+). HRMS calcd $C_{18}H_{17}F_2N_4O_3$ [MH^+], 375.1263; found, 375.1260.

*N-(2,4-Difluorobenzyl)-1-hydroxy-4-morpholino-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. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.91 (t, J = 5.8 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 (td, 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 calcd $C_{20}H_{19}F_2N_4O_4$ [MH^+], 417.1369; found, 417.1364.

*4-(Cycloheptylamino)-N-(2,4-difluorobenzyl)-1-hydroxy-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. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.68 (bs, 1H), 10.45 (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 calcd $C_{23}H_{25}F_2N_4O_3$ [MH^+], 443.1889; 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. ^{1}H NMR (400 MHz, DMSO-d_6) 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 calcd $C_{24}H_{21}F_2N_4O_3$ [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. ^{1}H NMR (400 MHz, DMSO-d_6) 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 calcd $C_{26}H_{25}F_2N_4O_3$ [MH^+], 479.1889; found, 479.1894.

*N-(2,4-Difluorobenzyl)-1-hydroxy-2-oxo-4-(pentylamino)-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5l**).* Treatment of **10l** 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 **5l** as a yellow solid in 72% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.95 (bs, 1H), 10.47 (t, J = 5.7 Hz, 1H), 8.63 (dd, J = 4.5, 1.5 Hz, 1H), 8.54 (dd, J = 8.3, 1.5 Hz, 1H), 7.46−7.40 (m, 1H), 7.24 (dd, J = 8.2, 4.6 Hz, 1H), 7.19 (ddd, J = 10.5, 9.4, 2.6 Hz, 1H), 7.04−6.99 (m, 1H), 4.46 (d, J = 5.8 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: 417.1 (MH^+). HRMS calcd $C_{17}H_{13}F_2N_2O_4$ [MH^+], 417.1733; found, 417.1734.

*N-(2,4-Difluorobenzyl)-1-hydroxy-4-(isopropylamino)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5m**).* Treatment of **10m** as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 80% B over 30 min; retention time = 17.0 min) provided **5m** as a white solid in 80% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.47 (bs, 1H), 10.42 (t, J = 5.9 Hz, 1H), 8.64 (dd, J = 4.5, 1.6 Hz, 1H), 8.41 (dd, J = 8.2, 1.5 Hz, 1H), 7.44 (dd, J = 15.4, 8.6 Hz, 1H), 7.27 (dd, J = 8.2, 4.6 Hz, 1H), 7.22−7.17 (m, 1H), 7.02 (td, J = 8.6, 2.6 Hz, 1H), 4.46 (d, J = 5.8 Hz, 2H), 4.17−4.09 (m, 1H), 1.20 (s, 3H), 1.18 (s, 3H). ESI-MS m/z: 389.1 (MH^+). HRMS calcd $C_{19}H_{19}F_2N_4O_3$ [MH^+], 389.1420; found, 389.1422.

*4-((2-Aminoethyl)amino)-N-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5n**).* Treatment of **10n** as described under general procedure B and removal of *tert*-butyl protection [TFA−CH_2Cl_2 (1:1, 2 mL), room temperature] followed by purification using preparative HPLC (linear gradient of 0% B to 40% B over 30 min; retention time = 25.9 min) provided **5n** as a white solid in 61% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.65 (s, 1H), 9.88 (t, J = 5.9 Hz, 1H), 9.09 (s, 1H), 8.69 (dd, J = 4.5, 1.3 Hz, 1H), 8.48 (dd, J = 8.1, 1.4 Hz, 1H), 7.83 (bs, 2H), 7.65 (dd, J = 15.6, 8.8 Hz, 1H), 7.34 (dd, J = 8.1, 4.6 Hz, 1H), 7.27−7.21 (m, 1H), 7.08 (dd, J = 9.9, 7.3 Hz, 1H), 4.50 (d, J = 5.5 Hz, 2H), 3.68 (q, J = 5.9 Hz, 2H), 3.10 (bs, 2H). ESI-MS m/z: 390.1 (MH^+). HRMS calcd $C_{18}H_{18}F_2N_5O_3$ [MH^+], 390.1372; found, 390.1363.

*N-(2,4-Difluorobenzyl)-1-hydroxy-4-((2-hydroxyethyl)amino)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**5o**).* Treatment of **10o** as described under general procedure B and purification by preparative HPLC (linear gradient of 20% B to 50% B over 30 min; retention time = 21.6 min) provided **5o** as a yellow solid in 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.13 (bs, 1H), 10.49 (t, J = 5.5 Hz, 1H), 8.62 (dd, J = 4.5, 1.5 Hz, 1H), 8.55 (dd, J = 8.2, 1.6 Hz, 1H), 7.42 (dd, J = 15.3, 8.6 Hz, 1H), 7.23 (dd, J = 8.2, 4.5 Hz, 1H), 7.22−7.16 (m, 1H), 7.02 (dd, J = 9.3, 7.7 Hz, 1H), 4.46 (d, J = 5.6 Hz, 2H), 3.68−3.65 (m, 2H), 3.56 (t, J = 5.2 Hz, 2H). ESI-MS m/z: 391.1 (MH^+). HRMS calcd $C_{18}H_{17}F_2N_4O_4$ [MH^+], 391.1212; found, 391.1215.

*2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)ethyl Acetate (**5p**).* Treatment of **10p** as described under general procedure B and purification by preparative HPLC (linear gradient of 30% B to 50% B over 30 min; retention time = 23.6 min) provided **5p** as a white powder in 79% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.55 (bs, 1H), 10.34 (t, J = 5.5 Hz, 1H), 8.69 (d, J = 4.5 Hz, 1H), 8.55 (d, J = 8.2 Hz, 1H), 7.52 (dd, J = 15.4, 8.6 Hz, 1H), 7.32 (dd, J = 8.1, 4.3 Hz, 1H), 7.27−7.22 (m, 1H), 7.07 (t, J = 7.4 Hz, 1H), 4.51 (d, J = 5.7 Hz, 2H), 4.19 (t, J = 5.1 Hz, 2H), 3.84 (d, J = 4.9 Hz, 2H), 2.00 (s, 3H). ESI-MS m/z: 433.1 (MH^+). HRMS calcd $C_{20}H_{19}F_2N_4O_5$ [MH^+], 433.1318; found, 433.1308.

*Methyl 2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)acetate (**5q**).* Treatment of **10q** as described under general procedure B and purification by preparative HPLC (linear gradient of 20% B to 50% B over 30 min; retention time = 28.4 min) provided **5q** as a white solid in 44% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.43 (brs, 1H), 10.09 (t, J = 5.7

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Hz, 1H), 8.62 (dd, J = 4.5, 1.3 Hz, 1H), 8.43 (dd, J = 8.2, 1.4 Hz, 1H), 7.50 (dd, J = 15.5, 8.7 Hz, 1H), 7.25 (dd, J = 8.2, 4.6 Hz, 1H), 7.20−7.12 (m, 1H), 7.00 (td, 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 calcd $C_{19}H_{17}F_2N_4O_5$ [MH^+], 419.1162; found, 419.1163.

*Methyl (S)-2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)propanoate [(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 40% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (t, J = 5.8 Hz, 1H), 10.02 (d, J = 8.4 Hz, 1H), 8.70 (dd, J = 4.5, 0.9 Hz, 1H), 8.46 (d, J = 8.2 Hz, 1H), 7.57 (dd, J = 15.4, 8.5 Hz, 1H), 7.34−7.31 (m, 1H), 7.23 (dd, J = 13.8, 6.1 Hz, 1H), 7.07 (td, J = 8.6, 2.7 Hz, 1H), 4.78−4.71 (m, 1H), 4.49 (qd, J = 15.5, 6.0 Hz, 2H), 3.61 (d, J = 0.5 Hz, 3H), 1.47 (d, J = 6.9 Hz, 3H). ESI-MS m/z: 433.1 (MH^+). HRMS calcd $C_{20}H_{19}F_2N_4O_5$ [MH^+], 433.1318; found, 433.1305.

*Methyl (R)-2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)propanoate [(R)-**5r**].* Treatment of (*R*)-**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 (*R*)-**5r** as a white solid in 40% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (t, J = 5.8 Hz, 1H), 10.02 (d, J = 9.6 Hz, 1H), 8.70 (dd, J = 4.6, 1.5 Hz, 1H), 8.46 (dd, J = 8.2, 1.5 Hz, 1H), 7.57 (dd, J = 15.5, 8.8 Hz, 1H), 7.33 (dd, J = 8.2, 4.6 Hz, 1H), 7.24 (ddd, J = 10.6, 9.4, 2.6 Hz, 1H), 7.10−7.05 (m, 1H), 4.74 (dq, J = 13.9, 6.9 Hz, 1H), 4.49 (qd, J = 15.2, 5.7 Hz, 2H), 3.61 (s, 3H), 1.47 (d, J = 6.9 Hz, 3H). ESI-MS m/z: 433.1 (MH^+). HRMS calcd $C_{20}H_{19}F_2N_4O_5$ [MH^+], 433.1318; found, 433.1303.

*Methyl (S)-2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-2-phenylacetate [(S)-**5s**].* Treatment of (*S*)-**10s** as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 70% B over 30 min; retention time = 22.6 min) provided (*S*)-**5r** as a white solid in 51% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.72 (d, J = 8.0 Hz, 1H), 10.57 (t, J = 5.9 Hz, 1H), 8.67 (dd, J = 4.5, 1.2 Hz, 1H), 8.46 (dd, J = 8.2, 1.4 Hz, 1H), 7.49 (dd, J = 15.4, 8.6 Hz, 1H), 7.37 (d, J = 4.2 Hz, 3H), 7.35−7.31 (m, 1H), 7.29−7.24 (m, 2H), 7.09 (dd, J = 9.8, 7.3 Hz, 1H), 6.01 (d, J = 8.1 Hz, 1H), 4.55 (d, J = 5.7 Hz, 2H), 3.61 (s, 3H). ESI-MS m/z: 495.1 (MH^+). HRMS calcd $C_{25}H_{21}F_2N_4O_5$ [MH^+], 495.1475; found, 495.1457.

*Methyl (R)-2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-2-phenylacetate [(R)-**5s**].* Treatment of (*R*)-**10s** as described under general procedure B and purification by preparative HPLC (linear gradient of 40% B to 60% B over 30 min; retention time = 26.3 min) provided (*R*)-**5s** as a white solid in 31% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.72 (d, J = 8.3 Hz, 1H), 10.57 (t, J = 5.4 Hz, 1H), 8.67 (d, J = 4.5 Hz, 1H), 8.46 (d, J = 8.2 Hz, 1H), 7.49 (dd, J = 15.2, 8.6 Hz, 1H), 7.37−7.36 (m, 2H), 7.33 (dd, J = 8.1, 4.1 Hz, 1H), 7.29−7.24 (m, 2H), 7.09 (dd, J = 9.8, 7.1 Hz, 1H), 6.01 (d, J = 8.1 Hz, 1H), 4.55 (d, J = 5.6 Hz, 2H), 3.61 (s, 3H). ESI-MS m/z: 495.1 (MH^+). HRMS calcd $C_{25}H_{21}F_2N_4O_5$ [MH^+], 495.1475; found, 495.1460.

*Methyl (S)-2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-3-hydroxypropanoate [(S)-**5t**].* Treatment of (*S*)-**10t** as described under general procedure B and purification by preparative HPLC (linear gradient of 30% B to 50% B over 30 min; retention time = 15.9 min) provided (*S*)-**5t** as a white solid in 44% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (bs, 1H), 10.62 (bs, 1H), 10.37 (t, J = 5.9 Hz, 1H), 8.70 (dd, J = 4.5, 1.5 Hz, 1H), 8.38 (dd, J = 8.2, 1.5 Hz, 1H), 7.52 (dd, J = 15.4, 8.7 Hz, 1H), 7.31 (dd, J = 8.2, 4.6 Hz, 1H), 7.25 (ddd, J = 10.5, 9.4, 2.6 Hz, 1H), 7.10−7.06 (m, 1H), 4.77−4.72 (m, 1H), 4.51 (qd, J = 15.3, 5.6 Hz, 2H), 3.81 (qd, J = 11.1, 4.3 Hz, 2H), 3.63 (s, 3H). ESI-MS m/z: 449.1 (MH^+). HRMS calcd $C_{20}H_{19}F_2N_4O_6$ [MH^+], 449.1267; found, 449.1256.

*Methyl (R)-2-((3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)amino)-3-hydroxypropanoate [(R)-**5t**].* Treatment of (*R*)-**10t** as described under general procedure B and purification by preparative HPLC (linear gradient of 25% B to 50% B over 30 min; retention time = 20.5 min) provided (*R*)-**5t** as a white solid in 42% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.61 (d, J = 8.7 Hz, 1H), 10.37 (t, J = 5.6 Hz, 1H), 8.70 (dd, J = 4.5, 1.4 Hz, 1H), 8.38 (dd, J = 8.2, 1.4 Hz, 1H), 7.52 (dd, J = 15.6, 8.7 Hz, 1H), 7.32 (dd, J = 8.2, 4.6 Hz, 1H), 7.25 (ddd, J = 11.2, 10.6, 6.4 Hz, 1H), 7.07 (dd, J = 9.7, 7.3 Hz, 1H), 4.75 (dt, J = 8.8, 4.3 Hz, 1H), 4.51 (qd, J = 15.4, 5.8 Hz, 2H), 3.81 (qd, J = 11.0, 4.4 Hz, 2H), 3.63 (s, 3H). ESI-MS m/z: 449.1 (MH^+). HRMS calcd $C_{20}H_{19}F_2N_4O_6$ [MH^+], 449.1267; found, 449.1255.

*Ethyl (S)-1-(3-((2,4-Difluorobenzyl)carbamoyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridin-4-yl)pyrrolidine-2-carboxylate [(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. ^{1}H NMR (400 MHz, DMSO-d_6) 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 (dd, J = 15.1, 6.3 Hz, 1H), 4.38−4.30 (m, 2H), 3.95 (q, J = 7.1 Hz, 2H), 3.64 (dd, J = 15.2, 6.4 Hz, 1H), 3.18 (dd, J = 12.8, 8.1 Hz, 1H), 2.11−1.93 (m, 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 calcd $C_{23}H_{23}F_2N_4O_5$ [MH^+], 473.1631; found, 473.1618.

*4-Amino-N-(2,4-difluorobenzyl)-1-hydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxamide (**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. ^{1}H NMR (400 MHz, DMSO-d^6) 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, 1H), 4.46 (d, J = 5.8 Hz, 2H). ESI-MS m/z: 347.1 (MH^+). HRMS calcd $C_{16}H_{13}F_2N_4O_3$ [MH^+], 347.0950; found, 347.0953.

Integrase Biochemical Assays. Determination of IN 3′- P and ST inhibitory values using an in vitro assay IN reactions were carried out using [γ-^{32}P]-labeled DNA as previously described.[9,22]

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.[9]

Single-Round HIV-1 Infectivity Assays. As previously described,[9] the human embryonic kidney cell line 293T was transfected with the pNLNgoMIVR$^-$ΔLUC vector, which was made from pNLNgoMIVR$^-$Δ*E*nv.HSA by removing the HSA reporter gene and replacing it with a luciferase reporter gene between the NotI and XhoI restriction sites.[28] VSV-*g*-pseudotyped HIV was produced transfecting 293T cells as described previously.[29] On the day prior to transfection, 293T cells were plated on 100 mm diameter dishes at a density of 1.5 × 10^6 cells per plate. 293T cells were transfected with 16 μg of pNLNgoMIVR$^-$ΔLUC and 4 μg of pHCMV-g (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 100 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

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Software, Reading, PA) was used to perform regression analysis on the data. EC_{50} values were determined from the fit model.

Vector Constructs. pNLNgoMIVR$^-$ΔEnv.LUC has been described previously.[28] The IN coding region was removed from pNLNgoMIVR$^-$ΔEnv.LUC (between *Kpn*I and *Sal*I sites) and inserted between the *Kpn*I and *Sal*I 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, Q148H, Q148 K, N155H, G140S + Q148H, G140A + Q148 K, and E138 K + Q148 K. The following sense with cognate antisense (not shown) oligonucleotides (Integrated DNA Technologies, Coralville, IA) were used in the mutagenesis: G118R, 5′-GTACATACAGACAATCGCAGCAATTTCACCAGTAC-3′; E138 K, 5′-GGCGGGGATCAAGCAGAAATTTGGCATTCCCTA-3′; G140A, 5′-GGGGATCAAGCAGGAATTTGCCATTCCCTA-CAATC-3′; G140S, 5′-GGGGATCAAGCAGGAATTTAG-CATTCCCTACAATC-3′; Y143R, 5′-GCAGGAATTTGG-CATTCCCCGCAATCCCCAAAGTCAAGGA-3′; Q148H, 5′-CATTCCCTACAATCCCCAAAGTCATGGAGTAATAGAATCTA-3′; Q148 K, 5′-CATTCCCTACAATCCCCAAAGTAAAGGAGTAA-TAGAATCTATGAA-3′; N155H, 5′-CCAAAGTCAAGGAGTAATA-GAATCTATGCATAAAGAATTAAAGAAAATTATAGGACA-3′. The double mutation G140S + Q148H was constructed by using the previously generated Q148H mutant and the appropriate oligonucleotide for the second mutation, G140S. The double mutation G140A + Q148 K was made by using the Q148 K mutant and the appropriate oligonucleotide for the second mutation, G140A. The double mutation E138 K + Q148 K was made by using the Q148 K mutant and the appropriate oligonucleotide for the second mutation, E138 K. The DNA sequence of each construct was verified independently by DNA sequence determination. The mutant IN coding sequences from pBluescript II KS+ were then subcloned into pNLNgo-MIVR$^-$ΔEnv.LUC (between the *Kpn*I and *Sal*I sites) to produce the full-length mutant HIV-1 IN constructs. These DNA sequences were checked independently by DNA sequence determination.

Reverse Transcriptase (RT) Assays. The assay used to determine whether the compounds inhibit the of polymerase activity of RT has been previously described.[30] Briefly, the −47 sequencing primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′; New England Biolabs) was 5′ end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. After purification, the labeled primer was annealed to single-stranded M13mp18 DNA (1 μg of DNA for each sample to be assayed) by heating and slow cooling. For each sample, 0.1 μg of wild-type RT (approximately 17 nM final) added to the labeled primer template (approximately 9.0 nM) in 25 mM Tris−HCl (pH 8.0), 75 mM KCl, 10 mM $MgCl_2$, 100 μg of BSA per mL, and 10 mM CHAPS. The reaction mixture was supplemented with 0.5 μM (each) of dATP, dCTP, dGTP, and TTP. The compounds were added to give final concentrations of 0, 0.02, 0.1, 0.5, 1, or 10 μM. The reactions were allowed to proceed at 37 °C for 60 min and were stopped by the addition of EDTA. The samples were precipitated by the addition of two volumes of EtOH, fractionated by electrophoresis on a 6.0% polyacrylamide gel, and the gel was autoradiographed. Reactions were carried out in duplicate.

RNaseH Assays. This procedure has been previously described.[30] Briefly, RNA oligonucleotide (5′-GGGGCCACUUUUUAAAA-GAAAAGGGGGGACUGGAAGGGCUAAUUCACUCAC-3′) was obtained from Dharmacon Research, Inc. The RNA oligonucleotide was 5′-end labeled and was then annealed to a DNA oligonucleotide (5′-GAGTGAATTAGCCCTTCCAGTCCC-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 $MgCl_2$, 100 μg of bovine serum albumin/mL, 10 mM CHAPS, and 1 U of Superasin (Ambion). The compounds were added to the reactions to give the following final concentrations (0, 0.1, 0.5, 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 2× 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; IC_{50}, half-maximum inhibitory concentration; EC_{50}, half maximal effective concentration; WT, wild-type; DMF, dimethylformamide; HPLC, high-pressure liquid chromatography; HRMS, high-resolution mass spectrometry

REFERENCES

(1) Cherepanov, P.; Maertens, G. N.; Hare, S. Structural insights into the retroviral DNA integration apparatus. *Curr. Opin. Struct. Biol.* **2011**, *21*, 249−256.

(2) Croxtall, J. D.; Scott, L. J. Raltegravir: in treatment-naive patients with HIV-1 infection. *Drugs* **2010**, *70*, 631−642.

(3) Wills, T.; Vega, V. Elvitegravir: a once-daily inhibitor of HIV-1 integrase. *Expert Opin. Invest. Drugs* **2012**, *21*, 395−401.

(4) Marchand, C.; Maddali, K.; Metifiot, M.; Pommier, Y. HIV-1 IN inhibitors: 2010 update and perspectives. *Curr. Top. Med. Chem.* **2009**, *9*, 1016−1037.

(5) Mesplede, T.; Quashie, P. K.; Wainberg, M. A. Resistance to HIV integrase inhibitors. *Curr. Opin. HIV AIDS* **2012**, *7*, 401−408.

(6) Geretti, A. M.; Armenia, D.; Ceccherini-Silberstein, F. Emerging patterns and implications of HIV-1 integrase inhibitor resistance. *Curr. Opin. Infect. Dis.* **2012**, *25*, 677−686.

(7) Katlama, C.; Murphy, R. Dolutegravir for the treatment of HIV. *Expert Opin. Invest. Drugs* **2012**, *21*, 523−530.

(8) Ballantyne, A. D.; Perry, C. M. Dolutegravir: first global approval. *Drugs* **2013**, *73*, 1627−1637.

(9) Zhao, X. Z.; Smith, S. J.; Metifiot, M.; Marchand, C.; Pommier, Y.; Hughes, S. H.; Burke, T. R., Jr. 4-Amino-1-hydroxy-2-oxo-1,8-naphthyridine-containing compounds having high antiviral potency against cells harboring raltegravir-resistant integrase mutants. *J. Med. Chem.* **2014**, *57*, 1573−1582.

(10) Summa, V.; Petrocchi, A.; Matassa, V. G.; Taliani, M.; Laufer, R.; De Francesco, R.; Altamura, S.; Pace, P. HCV NS5b RNA-dependent RNA polymerase inhibitors: From alpha, gamma-diketoacids to 4,5-dihydroxypyrimidine- or 3-methyl-5- hydroxypyrimidinonecarboxylic acids. Design and synthesis. *J. Med. Chem.* **2004**, *47*, 5336−5339.
(11) Summa, V.; Petrocchi, A.; Matassa, V. G.; Gardelli, C.; Muraglia, E.; Rowley, M.; Paz, O. G.; Laufer, R.; Monteagudo, E.; Pace, P. 4,5-Dihydroxypyrimidine carboxamides and *N*-alkyl-5-hydroxypyrimidinone carboxamides are potent, selective HIV integrase inhibitors with good pharmacokinetic rofiles in preclinical species. *J. Med. Chem.* **2006**, *49*, 6646−6649.
(12) Williams, P. D.; Venkatraman, S.; Langford, H. M.; Kim, B.; Booth, T. M.; Grobler, J. A.; Staas, D.; Ruzek, R. D.; Embrey, M. W.; Wiscount, C. M.; Lyle, T. A. Preparation of 1-hydroxynaphthyridin-2(1*H*)-one derivatives as anti-HIV agents. (Merck & Co., Inc.) WO2008010964A1, 2008.
(13) Su, H.-P.; Yan, Y.; Prasad, G. S.; Smith, R. F.; Daniels, C. L.; Abeywickrema, P. D.; Reid, J. C.; Loughran, H. M.; Kornienko, M.; Sharma, S.; Grobler, J. A.; Xu, B.; Sardana, V.; Allison, T. J.; Williams, P. D.; Darke, P. L.; Hazuda, D. J.; Munshi, S. Structural basis for the inhibition of RNase H activity of HIV-1 reverse transcriptase by RNase H active site-directed inhibitors. *J. Virol.* **2010**, *84*, 7625−7633.
(14) Williams, P. D.; Staas, D. D.; Venkatraman, S.; Loughran, H. M.; Ruzek, R. D.; Booth, T. M.; Lyle, T. A.; Wai, J. S.; Vacca, J. P.; Feuston, B. P.; Ecto, L. T.; Flynn, J. A.; Di, S. D. J.; Hazuda, D. J.; Bahnck, C. M.; Himmelberger, A. L.; Dornadula, G.; Hrin, R. C.; Stillmock, K. A.; Witmer, M. V.; Miller, M. D.; Grobler, J. A. Potent and selective HIV-1 ribonuclease H inhibitors based on a 1-hydroxy-1,8-naphthyridin-2(1*H*)-one scaffold. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6754−6757.
(15) Costi, R.; Metifiot, M.; Esposito, F.; Cuzzucoli Crucitti, G.; Pescatori, L.; Messore, A.; Scipione, L.; Tortorella, S.; Zinzula, L.; Marchand, C.; Pommier, Y.; Tramontano, E.; Di Santo, R. 6-(1-Benzyl-1*H*-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acids as dual inhibitors of recombinant HIV-1 integrase and ribonuclease H, synthesized by a parallel synthesis approach. *J. Med. Chem.* **2013**, *56*, 8588−8598.
(16) Esposito, F.; Tramontano, E. Past and future. Current drugs targeting HIV-1 integrase and reverse transcriptase-associated ribonuclease H activity: single and dual active site inhibitors. *Antivir. Chem. Chemother.* **2013**, *23*, 129−144.
(17) Zhao, X. Z.; Maddali, K.; Vu, B. C.; Marchand, C.; Hughes Stephen, H.; Pommier, Y.; Burke Terrence, R., Jr. Examination of halogen substituent effects on HIV-1 integrase inhibitors derived from 2,3-dihydro-6,7-dihydroxy-1*H*-isoindol-1-ones and 4,5-dihydroxy-1*H*-isoindole-1,3(2*H*)-diones. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2714−2717.
(18) Kawasuji, T.; Johns, B. A.; Yoshida, H.; Taishi, T.; Taoda, Y.; Murai, H.; Kiyama, R.; Fuji, M.; Yoshinaga, T.; Seki, T.; Kobayashi, M.; Sato, A.; Fujiwara, T. Carbamoyl pyridone HIV-1 integrase inhibitors. 1. Molecular design and establishment of an advanced two-metal binding pharmacophore. *J. Med. Chem.* **2012**, *55*, 8735−8744.
(19) Kawasuji, T.; Johns, B. A.; Yoshida, H.; Weatherhead, J. G.; Akiyama, T.; Taishi, T.; Taoda, Y.; Mikamiyama-Iwata, M.; Murai, H.; Kiyama, R.; Fuji, M.; Tanimoto, N.; Yoshinaga, T.; Seki, T.; Kobayashi, M.; Sato, A.; Garvey, E. P.; Fujiwara, T. Carbamoyl pyridone HIV-1 integrase inhibitors. 2. Bi- and tricyclic derivatives result in superior antiviral and pharmacokinetic profiles. *J. Med. Chem.* **2013**, *56*, 1124−1135.
(20) Johns, B. A.; Kawasuji, T.; Weatherhead, J. G.; Taishi, T.; Temelkoff, D. P.; Yoshida, H.; Akiyama, T.; Taoda, Y.; Murai, H.; Kiyama, R.; Fuji, M.; Tanimoto, N.; Jeffrey, J.; Foster, S. A.; Yoshinaga, T.; Seki, T.; Kobayashi, M.; Sato, A.; Johnson, M. N.; Garvey, E. P.; Fujiwara, T. Carbamoyl pyridone HIV-1 integrase inhibitors 3. A diastereomeric approach to chiral nonracemic tricyclic ring systems and the discovery of dolutegravir (S/GSK1349572) and (S/GSK1265744). *J. Med. Chem.* **2013**, *56*, 5901−5916.
(21) Pratt, J. K.; Donner, P.; McDaniel, K. F.; Maring, C. J.; Kati, W. M.; Mo, H.; Middleton, T.; Liu, Y.; Ng, T.; Xie, Q.; Zhang, R.; Montgomery, D.; Molla, A.; Kempf, D. J.; Kohlbrenner, W. Inhibitors of HCV NS5B polymerase: synthesis and structure−activity relationships of *N*-1-heteroalkyl-4-hydroxyquinolon-3-yl-benzothiadiazines. *Bioor. Med. Chem. Lett.* **2005**, *15*, 1577−1582.
(22) Zhao, X. Z.; Semenova, E. A.; Vu, B. C.; Maddali, K.; Marchand, C.; Hughes, S. H.; Pommier, Y.; Burke, T. R., Jr. 2,3-Dihydro-6,7-dihydroxy-1*H*-isoindol-1-one-based HIV-1 integrase inhibitors. *J. Med. Chem.* **2008**, *51*, 251−259.
(23) Malet, I.; Delelis, O.; Valantin, M.-A.; Montes, B.; Soulie, C.; Wirden, M.; Tchertanov, L.; Peytavin, G.; Reynes, J.; Mouscadet, J.-F.; Katlama, C.; Calvez, V.; Marcelin, A.-G. Mutations associated with failure of raltegravir treatment affect integrase sensitivity to the inhibitor in vitro. *Antimicrob. Agents Chemother.* **2008**, *52*, 1351−1358.
(24) Delelis, O.; Thierry, S.; Subra, F.; Simon, F.; Malet, I.; Alloui, C.; Sayon, S.; Calvez, V.; Deprez, E.; Marcelin, A.-G.; Tchertanov, L.; Mouscadet, J.-F. Impact of Y143 HIV-1 integrase mutations on resistance to raltegravir in vitro and in vivo. *Antimicrob. Agents Chemother.* **2009**, *54*, 491−501.
(25) Metifiot, M.; Maddali, K.; Naumova, A.; Zhang, X.; Marchand, C.; Pommier, Y. Biochemical and pharmacological analyses of HIV-1 integrase flexible loop mutants resistant to raltegravir. *Biochemistry* **2010**, *49*, 3715−3722.
(26) Metifiot, M.; Maddali, K.; Johnson, B. C.; Hare, S.; Smith, S. J.; Zhao, X. Z.; Marchand, C.; Burke, T. R., Jr.; Hughes, S. H.; Cherepanov, P.; Pommier, Y. Activities, crystal structures and molecular dynamics of dihydro-1*H*-isoindole derivatives, inhibitors of HIV-1 integrase. *ACS Chem. Biol.* **2013**, *8*, 209−217.
(27) Jacobo-Molina, A.; Ding, J.; Nanni, R. G.; Clark, A. D.; Lu, X.; Tantillo, C.; Williams, R. L.; Kamer, G.; Ferris, A. L.; Clark, P. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 6320−6324.
(28) Zhao, X. Z.; Maddali, K.; Metifiot, M.; Smith, S. J.; Vu, B. C.; Marchand, C.; Hughes, S. H.; Pommier, Y.; Burke, T. R., Jr. Bicyclic hydroxy-1*H*-pyrrolopyridine-trione containing HIV-1 integrase inhibitors. *Chem. Biol. Drug Des.* **2012**, *79*, 157−165.
(29) Brachmann, A.; Koenig, J.; Julius, C.; Feldbruegge, M. A reverse genetic approach for generating gene replacement mutants in *Ustilago maydis*. *Mol. Genet. Genomics* **2004**, *272*, 216−226.
(30) Ivetac, A.; Swift, S. E.; Boyer, P. L.; Diaz, A.; Naughton, J.; Young, J. A. T.; Hughes, S. H.; McCammon, J. A. Discovery of novel inhibitors of HIV-1 reverse transcriptase through virtual screening of experimental and theoretical ensembles. *Chem. Biol. Drug Des.* **2014**, *83*, 521−531.