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A 1,8-Naphthyridone Derivative Targets the HIV-1 Tat-Mediated Transcription and Potently Inhibits the HIV-1 Replication

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The emergence of multidrug resistant HIV-1 strains and the inability of the HAART to eradicate HIV-1 virus from infected patients demand new drugs able to interfere with an alternative step of the replicative cycle. The naphthyridone **3** (**HM13N**), described in the present study, is a promising anti-HIV agent due to its ability to inhibit the HIV-1 Tat-mediated transcription and the potent antiviral activity observed in acutely, chronically, and latently infected cells. The absence of any tendency to select for resistance mutations in vitro adds to the potential clinical value of this type of compounds, especially as these compounds are drug-like and obey the Lipinski rules.

Introduction

The recent approval of the entry inhibitor maraviroc[1] and the first integrase inhibitor raltegravir,[2] which differ from existing agents with regard to their mechanism of action and resistance profiles, could revitalize the treatment regimens of multidrug-resistant HIV-1 patients. However, previous experience with the currently used anti-HIV-1 agents would presage an inevitable emergence of HIV-1 resistant strains against these new drugs once they are widely used. Resistance selection is expected to occur with any newly developed agent, particularly when it is targeting a viral protein. While the addition of these new drugs to the highly active antiretroviral therapy (HAART[a]) will allow a better management of the HIV-1 infection, it is unlikely that the virus in HIV-1-infected individuals will be completely eradicated using this new drug regimen. Some major obstacles to the eradication of HIV-1 are the occurrence of latent reservoirs, mainly in the resting $CD4^+$ lymphocytes and monocyte/macrophages (M/M), along with the residual low-level virus production that continues even in patients on optimal therapy. These cells carry an integrated provirus that is transcriptionally silent but will produce infectious particles after cellular activation that will lead to a rebound of the viral load after HAART is interrupted.[3]

Quinolone derivatives, whose potent antiviral activity is related to the inhibition of the Tat-mediated HIV-1 transcription,[4−8] are a class of molecules which may contribute to the control of the latent reservoirs. Other different classes of compounds were found to inhibit the HIV-1 transcription,[9−14] but unfortunately, none of them have still led to a successful clinical development. Inhibitors of the HIV-1 transcriptional regulation may have great potential in anti-HIV-1 drug combination therapy because they can slow down the viral replication rate or even shut it off.[9] The potent in vitro antiviral activity of these quinolones in many acutely, latently, and chronically HIV-1 infected cells, was recently confirmed in an in vivo model for HIV-1 latency where 6-hydrogenquinolone derivatives prevented virus reactivation from a human viral reservoir in mice.[6,15]

In an ongoing effort to develop analogues combining optimal antiviral and limited cytotoxic properties for clinical application, we have found that the bioisosteric replacement of the quinolone nucleus by an 1,8-naphthyridone is beneficial when a suitable arrangement of the functional groups is present at the C-6, N-1, and C-7 positions. Here we report the synthesis, the anti-HIV activity, and the mechanism of action study of the first series of 1,8-naphthyridone derivatives **1**−**4** characterized by a C-6 hydrogen atom and a N-1 methyl group coupled with a few selected 4-arylpiperazines at the C-7 position (Figure 1).

Chemistry

The 1,8-naphthyridones **1**−**4** were prepared through a cycloaracylation procedure, as shown in Scheme 1. Acrylate **5**[16] was reacted with methylamine in Et_2O/EtOH mixture at room temperature to give intermediate **6**, which was then cyclized to synthone **7** by using K_2CO_3 in DMF at 60 °C. This key intermediate was then reacted with selected 4-arylpiperazines to generate compounds **8**−**11**, which were hydrolyzed in basic condition furnishing target acids **1**−**4**.

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[a]Abbreviations: HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; IFD, induced fit docking; INSTI; strand transfer integrase inhibitor; LTR, long terminal repeat; CDK, cyclin-dependent kinase; CTD, carboxy-terminal domain; M/M, human primary monocyte/macrophages; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBMCs, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; P-TEFb, positive transcription elongation factor b; TAR, transactivating region; TNF-α, tumor necrosis factor alpha; TOA, time of addition.

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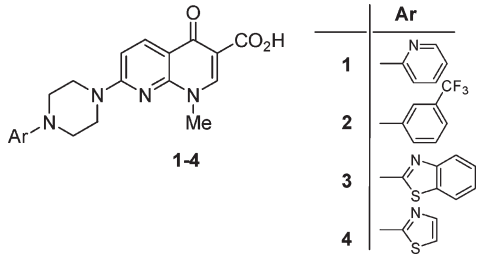
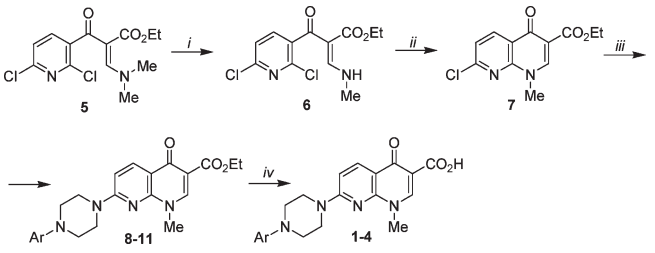


Figure 1. Structure of 1,8-naphthyridones synthesized in this study.

Scheme 1[a]



[a] Reagents and conditions: (i) $MeNH_2$, Et_2O/EtOH; (ii) K_2CO_3, DMF, 60 °C; (iii) Ar-piperazine (for Ar, see Figure 1), DMF, 70 °C; (iii) 4% NaOH, reflux.

Table 1. Anti-HIV-1 and HIV-2 Activity and Cytotoxicity of 1,8-Naphthyridone Derivatives on MT-4 Cells

compd	HIV-1(III_B) EC_{50}(μg/mL)[a,c]	HIV-2(ROD) EC_{50} (μg/mL)[a,c]	CC_{50} (μg/mL)[b,c]	SI (III_B)[d]	SI (ROD)[d]
1	0.62 ± 0.19	0.55 ± 0.42	9.93 ± 0.94	16	18
2	> 0.41	> 0.41	0.41 ± 0.04	< 1	< 1
3	0.03 ± 0.00	0.02 ± 0.00	12.06 ± 3.11	457	522
4	3.15 ± 0.54	0.922 ± 0.322	> 125	> 40	> 136

[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cells from HIV induced cytopathogenicity, as determined by the MTT method. [b] CC_{50}: concentration of compound that reduces the viability of mock-infected cells by 50%, as determined by the MTT method. [c] All data represent mean values ± standard deviations for at least two separate experiments. [d] SI: ratio of CC_{50}/EC_{50}.

Results and Discussion

The poor solubility in aqueous medium is a typical drawback that sometimes hampers the biological evaluation of quinolones as anti-HIV agents. We therefore aimed at replacing the benzo moiety in our compounds with a pyrido moiety. As expected, the bioisosteric substitution of the phenyl ring with a pyridine increased the solubility of the new derivatives.

The newly synthesized compounds were initially evaluated for their anti-HIV-1 (III_B) and anti-HIV-2 (ROD) activity in MT-4 cells and cytotoxicity of the compounds was determined in parallel. The results reported in Table 1 show that the naphthyridone nucleus allowed a very potent and selective derivative to be obtained when a 1-(1,3-benzothiazol-2-yl)piperazine is present at the C-7 position. In fact, derivative **3** (**HM13N**) inhibited both HIV-1 and HIV-2 replication with EC_{50} values of 0.03 and 0.02 μg/mL, yielding selectivity indexes of 457 and 522, respectively. In MT-4 cells, these values are the best obtained to date among the HIV-inhibitory quinolone class of transcription inhibitors. The 1,3-benzothiazole is confirmed as an optimal aryl substituent at position N-4 of the piperazine ring in giving high anti-HIV potency. Moreover, in contrast to what was observed in the corresponding previously reported 6-desfluoroquinolone series,[5,8] when placed in the naphthyridone nucleus, the cytotoxicity also markedly decreased resulting in a double benefit.

Table 2. Anti-HIV-1 Activity and Cytotoxicity of **3** on Jurkat Cells and PBMCs

Jurkat			PBMCs		
EC_{50} (μg/mL)[a,e]	CC_{50} (μg/mL)[b,e]	SI[f]	EC_{50} (μg/mL)[c,e]	CC_{50} (μg/mL)[d,e]	SI[f]
0.34	15.13	44	0.60 ± 0.49	10.31 ± 3.09	17

[a] EC_{50}: concentration required to inhibit 50% of the level of GFP expression obtained in absence of compound. [b] CC_{50}: concentration of compound that reduces the viability of mock-infected cells by 50%, as determined by the MTT method. [c] EC_{50}: concentration of compound required to achieve 50% inhibition of p24 production in PBMC. [d] CC_{50}: concentration of compound that reduces the viability of mock-infected cells by 50%, as determined by the trypan blue exclusion method. [e] All data represent mean values ± standard deviations for at least two separate experiments. [f] SI: ratio of CC_{50}/EC_{50}.

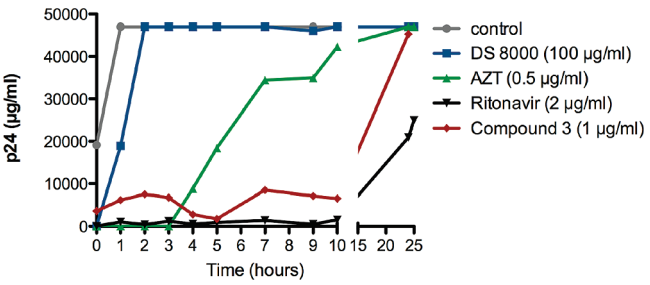


Figure 2. Time of addition experiment. MT-4 cells were infected with HIV-1 (III_B) at an moi of 0.5, and the test compounds were added at different times postinfection. Viral p24 production was determined at 31 h p.i. and is expressed in pg/mL.

The presence of the other C-7 side chains yielded divergent results. Good activity and encouraging SI values were obtained by introducing the 1-(2-pyridinyl)piperazine moiety (compound **1**), whereas the 1-[3-(trifluoromethyl)phenyl]-piperazine moiety (compound **2**) resulted in a compound that was cytotoxic and devoid of any antiviral activity at lower concentrations. Finally, the absence of cytotoxicity, coupled to a good anti-HIV-2 activity, was achieved with the introduction of a 1-(1,3-thiazolyl)piperazine, as in compound **4**.

To better define the antiviral profile of naphthyridone derivative **3**, its anti-HIV-1 activity was also assayed on acutely infected Jurkat cells and in human PBMC (isolated from healthy HIV-1 seronegative donors). Compound **3** was about 10 times less active in these cells as compared to MT-4 cells but maintained the same low cytotoxicity (Table 2).

It has previously been reported that quinolones inhibit the HIV-1 replication by interfering with a postintegrational target of the HIV-1 replicative cycle.[17] Therefore, we aimed at pinpointing the target of action of our new naphthyridone **3**. To do so, time-of-addition (TOA) experiments were performed and the results were compared to reference compounds with a known mode of action. In contrast to the reference inhibitors, dextran sulfate 8000, AZT, and ritonavir for which addition can be delayed until about 0, 5, and approximately 18 h postinfection, addition of compound **3** could be postponed up to 10 h post infection before it lost its antiviral activity (Figure 2). In this experiment, the last time-point at which diketo acid strand transfer integrase inhibitors (INSTIs), that inhibit the very last step of the integration process, can be added for optimal antiviral activity, is 7−8 h postinfection.[18] Because addition of **3** can be postponed even

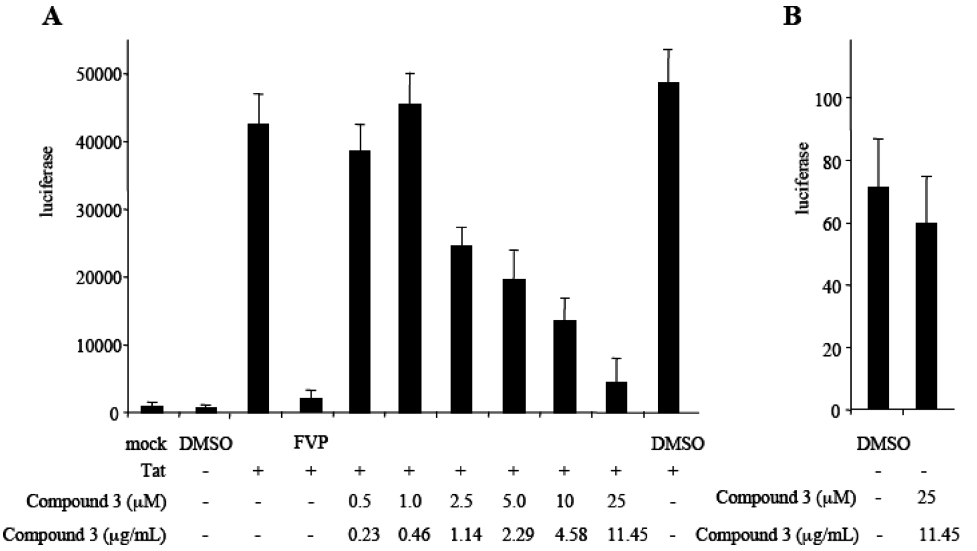


Figure 3. HIV-1 LTR transactivation assay. (A) HeLa LTR-luc were activated with Tat at suboptimal doses and treated with increasing concentration of compound **3**. Luciferase assay was performed on cell lysates 24 h after treatment. DMSO, control with compound solubilizing agent. Mock, cells not treated. FVP, flavopiridol at 10 μM. (B) HeLa LTR-luc were treated without Tat treatment to measure the basal activity of the promoter. Assay performed like in Figure 3A.

beyond 8 h before loss of activity, it is obvious that **3** acts at a postintegrational level, pointing toward a mechanism of action coinciding with the viral transcription process. Indeed, in a Tat-mediated transcription assay, performed in HeLa cell clone carrying an LTR-driven luciferase reporter, compound **3** inhibited the transcription in a dose-dependent manner in the μM range (Figure 3A); cytotoxicity was not observed in the same range (not shown). The inhibition is restricted to the Tat-mediated transcription as shown by the lack of activity in the basal expression (Figure 3B).

In highlighting the structural features of naphthyridone **3**, one notices that this compound displays striking similar features to those of diketo acid-based integrase inhibitors[19] and in particular to that of quinolone derivative GS-9137 (elvitegravir).[20] Thus, to undeniably ascertain that the anti-HIV activity of compound **3** is mainly based on the Tat-mediated transcription inhibition and to rule out any anti-integrase activity, the effect of our compound on the proliferation of wild-type versus integrase deficient HIV-1[21] was studied (Figure 4). Compound **3** efficiently inhibited the replication of the wild-type NL4.3 as well as the integrase deficient N/NTag.oriT virus, while the well-characterized integrase inhibitor L-870,810[22] was inactive against the integrase deficient virus. These data clearly demonstrate that compound **3** does not inhibit the HIV-1 integration process.

An in-depth theoretical investigation of the interaction between INSTIs and an HIV-1 IN-Mg-DNA model has recently been carried out.[23] This study provided new insights into the possible mechanism of action and binding conformations of INSTIs. In particular, induced fit docking (IFD) solutions revealed that all the inhibitors (a) have a pharmacophore that can establish interactions with one/two Mg^{2+} ions, (b) interact with the donor DNA, and (c) can occupy a deep region defined by the catalytic loop and the viral DNA through their substituted aromatic tail. The same IFD protocol has now been applied to naphthyridone **3**. Only one pose came out from the IFD study, showing one-metal binding model. (Figure S1 and relative comments in Supporting Information). A comparison between the IFD conformations obtained for known INSTIs and **3** highlights that our compound appears to be too rigid to simultaneously allow the metal chelation and the occupation of the pocket defined by the catalytic loop and the viral DNA (Figure 5). The lack of such a strong and critical interaction may explain why the compound **3** cannot inhibit HIV-1 integrase.

Unlike the compounds that target an early event of the HIV-1 replication cycle (viral entry, reverse transcription, or integration), transcription inhibitors are expected to inhibit the viral production of chronically and latently infected cells in which HIV-1 proviral DNA has been integrated into the host-cell genomes.

Therefore, compound **3** was first evaluated on persistently infected HuT-78(III_B) cells. These cells continuously produced HIV-1(III_B) from the integrated proviral DNA. After having removed the already-produced viruses, the cells were exposed to a serial dilution of the compound and the quantity of newly produced virus in the presence of the inhibitor is compared with a control condition. As shown in Table 3, compound **3** had a pronounced inhibitory effect on the virus production from persistently infected HuT-78(III_B) cells, far below the cytotoxicity level, and comparable to that observed in MT-4 cells (SI values >313). Latently HIV-1 infected promyelocytic cells (OM-10.1) and promonocytic cells (U1), in which the virus remains in a dormant state, were then used. In contrast to the HuT-78(III_B) cells, these cell lines have to be stimulated in order to produce virus. Upon exposure to TNF-α or PMA, a dramatic ($\sim$1000-fold) increase in HIV-1 expression could be detected. As the concentrations of compound **3** were increased, a dose-dependent inhibition of p24 production in both HIV-1 infected cell lines was observed (Table 3). This inhibition took place at compound concentration levels that were far below the cytotoxicity level and was similar under both (TNF-α and PMA) experimental stimulation conditions. In this experimental setup, compound **3** showed very strong antiviral activities with IC_{50} values in the 0.002$-$0.004 μg/mL range; these values were 10 times lower than those observed on MT-4 and the SI value of 1333 in U1 cells stimulated with PMA.

Encouraged by the promising inhibitory activity on HIV-production, we engaged in developing resistance to this new

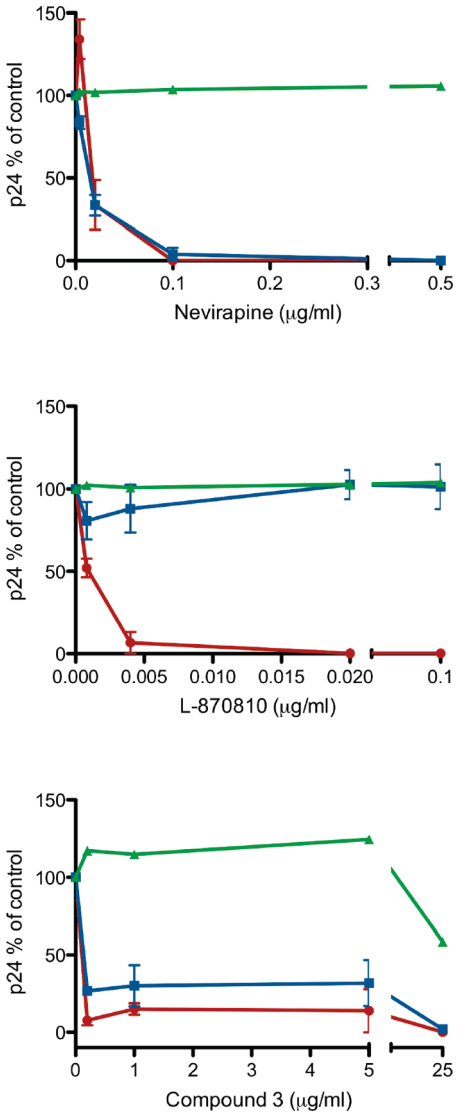


Figure 4. Concentration-dependent anti-HIV-1 effects of nevirapine, L-870,810 and compound **3**. C8166 T-cells infected with HIV-1 NL4.3 (red circles) or N/N.Tag.oriT (blue squares) were treated with the indicated compound concentrations. The definition of 100% is the level of p24 in the supernatants in control, nondrug-treated samples. Toxicity of the compounds was measured using the MTT-method (green triangles).

compound. MT-4 cells infected with HIV-1 (III_B) were subjected to serial passages (i.e., every third or fourth day of cultivation) in the presence of different concentrations of naphthyridone **3**. After 60 passages (30 weeks of selective pressure), we were unsuccessful in selecting resistance against the naphthyridone derivative **3** (Table 4).

Stimulated by the interesting profile of **3**, an in-depth investigation of its mechanism of action at the molecular level was carried out. The fishing of the target of anti-HIV quinolones is of great interest because once found it would provide the structural basis for the discovery of improved transcription inhibitors.

As previously reported, **WM5**,[24] the lead of the 6-desfluoroquinolones, interferes with the formation of the Tat/TAR complex by selectively binding to the bulge of TAR. The same effect was not seen with other potent 6-desfluoroquinolone analogues, some of which bind the TAR RNA in a nonselective way,[25] while others do not recognize any nucleic acids

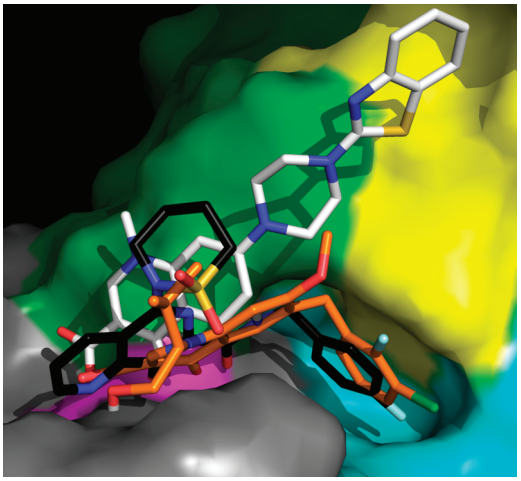


Figure 5. Comparison among IFD conformations of L-870,810 (orange), elvitegravir (black), and **3** (white). Molecular surfaces are shown for IN (gray), catalytic loop (residues 140−149; cyan), metal ions (magenta), 3′ DNA strand (green), and 5′ DNA strand (yellow). This figure was prepared using PyMol.[41]

Table 3. Antiviral Activity and Cytotoxicity of **3** in Chronically (HuT-78) and Latently (U1 and OM10.1) HIV-1 Infected Cells

cell line	stimulator	EC_{50} (μg/mL)[a,c]	CC_{50} (μg/mL)[b,c]	SI[d]
HuT-78(III_B)	none	0.032	> 10	> 313
U1	TNF-α	0.003	0.99	330
	PMA	0.002	2.67	1333
OM10.1	TNF-α	0.003	1.27	422
	PMA	0.004	0.75	189

[a] EC_{50}: concentration of compound required to achieve 50% reduction of p24 production in HIV-1 infected cells. [b] CC_{50}: concentration of compound that reduces the viability of cells by 50%, as determined by the MTT method. [c] All data represent mean values ± standard deviations for at least three separate experiments. [d] SI: ratio of CC_{50}/EC_{50}.

Table 4. Sensitivity of Mutant HIV-1 (III_B) Strains towards **3**

III_B			selected strain[e]		
EC_{50} (μg/mL)[a,c]	CC_{50} (μg/mL)[b,c]	SI[d]	EC_{50} (μg/mL)[a,c]	SI[d]	fold resistance
0.019	24.7	1280	0.017	1419	0.9

[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cells from HIV induced cytopathogenicity, as determined by the MTT method. [b] CC_{50}: concentration of compound that reduces the viability of mock-infected cells by 50%, as determined by the MTT method. [c] All data represent mean values ± standard deviations for at least two separate experiments. [d] SI: ratio of CC_{50}/EC_{50}. [e] HIV strain that emerged upon prolonged exposure to compound **3**.

(unpublished data). Consequently, the inhibitory activity of this class of compounds is not restricted to the inhibition of Tat/TAR complex formation but can also involve other cellular factors that are implicated in the Tat-mediated transcription.

In the present study, the effect of **3** on two key steps of HIV-1 transcriptional transactivation[3] that involve either the recruitment of the positive transcription elongation factor b (P-TEFb) or the recruitment of the coactivator p300 were investigated. P-TEFb, composed of Cyclin T1 and CDK9 can phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II promoting transcriptional elongation. The p300 and highly homologous cyclic adenosine monophosphate (cAMP)-responsive binding protein (CREB)-binding

protein (CBP) are acetyltransferases that act on histones to relieve the chromatin-imposed inhibition on the viral promoter, and on other proteins, including Tat itself.[26] Compound **3** did not show any effect on the kinase activity of CDK9 (measured in vitro on a recombinant CTD substrate) or on the acetyltransferase activity of p300/CBP on histones (not shown). It is concluded that **3** may act on a different step in the transactivation reaction and further experiments will be required to address this question.

Conclusions

Remarkable progress has been made in the treatment of HIV-1 infection/AIDS. However, critical problems such as the emergence of resistant strains and the inability of the HAART to eradicate the virus necessitate the identification of new compounds that can interfere with an alternative step of the replicative cycle. In this context, quinolone derivatives are very promising due to their ability to inhibit the Tat-mediated transcription. The results of the present work have shown that the bioisosteric substitution of the quinolone ring with the 1,8-naphthyridone was advantageous when a 1-(1,3-benzothiazol-2-yl)piperazine is present at the C-7 position. In addition to the improved solubility, compound **3** displayed very potent and selective anti-HIV activity in acutely, chronically, and latently infected cells. It did not show any tendency to select for resistance mutations after 60 passages. Mechanism of action studies, demonstrate that the anti-HIV activity of naphthyridone **3** is mainly due to the inhibition of the Tat-mediated transcription. In silico studies give some indications why naphthyridone **3** cannot efficiently interact with integrase enzyme.

In conclusion, the quinolone structure is confirmed as "privileged" to obtain potent anti-HIV agents, capable of inhibiting different steps of the viral replicative cycle such as the integration (i.e., GS-9137) or transactivation (i.e., compound **3**) based on the different quinolone nucleus substitution pattern.

Taking into account the promising anti-HIV results and drug-like nature of the compounds, it is certainly worthwhile to spend efforts on establishing the activity of compound **3** in the SCID-mice model.

Experimental Section

All reactions were routinely checked by thin-layer chromatography (TLC) on silica gel $60F_{254}$ (Merck) and visualized by using UV. Flash column chromatography separations were carried out on Merck silica gel 60 (mesh 230−400). Melting points were determined in capillary tubes (Büchi Electrothermal model 9100) and are uncorrected. Elemental analyses were performed on a Fisons elemental analyzer, model EA1108CHN, and the data for C, H, and N are within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 200 MHz (Bruker DPX 200) using residual solvents such as chloroform (δ = 7.26) or dimethylsulfoxide (δ = 2.48) as an internal standard. Chemical shifts are given in ppm (δ), and the spectral data are consistent with the assigned structures. Reagents and solvents were purchased from common commercial suppliers and were used as such. Organic solutions were dried over anhydrous Na_2SO_4, filtered, and concentrated with a Büchi rotary evaporator at reduced pressure. Yields are of purified product and were not optimized. All starting materials were commercially available unless otherwise indicated.

Ethyl 2-[(2,6-Dichloro-3-pyridinyl)carbonyl]-3-(methylamino)-2-propenoate (6). A mixture of **5**[16] (1.0 g, 3.15 mmol) in EtOH/Et_2O (1:4, 20 mL) was treated with a 33% solution of methylamine in EtOH (0.44 mL, 3.78 mmol). After 30 min, the mixture reaction was concentrated in vacuo, obtaining a residue which was treated with cyclohexane, to give a solid which was filtered and dried to give **6** (0.9 g, 94%): mp 156−157 °C. ^{1}H NMR (DMSO-d_6) δ 0.75 and 0.95 (each t, J = 7.1 Hz, 3H, CH_2CH_3), 3.15−3.20 (m, 3H, CH_3), 3.85−3.95 (m, 2H, CH_2CH_3), 7.55 (d, J = 7.9 Hz, 1H, aromatic CH), 7.75 and 7.78 (each d, J = 7.9 Hz, 1H, aromatic CH), 8.20 and 8.25 (each d, J = 15.0 Hz, 1H, *CH*NH), 9.50−9.55 and 10.70−10.75 (each m, 1H, NH).

Ethyl 7-Chloro-1-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (7). A mixture of acrylate **6** (0.9 g, 2.96 mmol) and K_2CO_3 (1.22 g, 8.90 mmol) in DMF (7 mL) was heated at 60 °C for 2 h. After cooling the reaction mixture was poured into ice/water, obtaining a precipitate which was filtered, washed with water, and Et_2O and dried to give **7** (0.67 g, 85%): mp 199−200 °C. ^{1}H NMR ($CDCl_3$) δ 1.40 (t, J = 7.1 Hz, 3H, CH_2CH_3), 3.95 (s, 3H, CH_3) 4.40 (q, J = 7.1 Hz, 2H, CH_2CH_3), 7.40 (d, J = 8.2 Hz, 1H, H-6), 8.60 (s, 1H, H-2), 8.70 (d, J = 8.2 Hz, 1H, H-5).

General Procedure for Coupling Reaction. Ethyl 1-Methyl-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (8). A mixture of **7** (0.1 g, 0.37 mmol) and 1-(2-pyridinyl)piperazine (0.18 g, 1.12 mmol) in DMF (3 mL), was heated at 70 °C for 4 h. The reaction mixture was then poured into ice/water giving a precipitate which was filtered, and washed with water and EtOH, to give **8** (0.14 g, 98%): mp 169−170 °C. ^{1}H NMR (DMSO-d_6) δ 1.25 (t, J = 7.1 Hz, 3H, CH_2CH_3), 3.70−3.75 (m, 4H, piperazine CH_2), 3.80−3.90 (m, 7H, piperazine CH_2 and CH_3), 4.25 (q, J = 7.1 Hz, 2H, CH_2-CH_3), 6.75 (t, J = 5.9 Hz, 1H, pyridine CH), 6.90 (d, J = 8.4 Hz, 1H, pyridine CH), 7.10 (d, J = 9.0 Hz, 1H, H-6), 7.60 (t, J = 7.4 Hz, 1H, pyridine CH), 8.20−8.30 (m, 2H, H-5 and pyridine CH), 8.65 (s, 1H, H-2).

General Procedure for Hydrolysis Reaction. 1-Methyl-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic Acid (1). A suspension of 8 (0.1 g, 0.25 mmol) in 4% NaOH (2 mL) was refluxed for 4 h. After cooling, the mixture reaction was treated with 2 N HCl to obtain a precipitate (∼pH = 6). The solid was filtered and washed with water and EtOH, to give 1 (0.08 g, 90%): mp 270−271 °C. ^{1}H NMR (DMSO-d_6) δ 3.80−3.85 (m, 4H, piperazine CH_2), 3.90 (s, 3H, CH_3), 3.95−4.00 (m, 4H, piperazine CH_2), 6.80 (t, J = 5.4 Hz, 1H, pyridine CH), 7.10−7.20 (m, 2H, H-6 and pyridine CH), 7.75−7.85 (m, 1H, pyridine CH), 8.10 (d, J = 5.3 Hz, 1H, pyridine CH), 8.30 (d, J = 9.0 Hz, 1H, H-5), 8.95 (s, 1H, H-2), 15.80 (bs, 1H, COOH). Anal. ($C_{19}H_{19}N_5O_3$) C, H, N.

1-Methyl-4-oxo-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydro-1,8-naphthyridine-3-carboxylic Acid (2). The title compound was prepared through the general procedure for coupling reaction replacing 1-(2-pyridinyl)piperazine with 1,3-(trifluoromethyl)phenyl]piperazine to give intermediate **9** in 78% yield, followed by the general procedure for hydrolysis to give **2** in 70% yield: mp 279−281 °C. ^{1}H NMR (DMSO-d_6) δ 3.35−3.45 (m, 4H, piperazine CH_2), 3.85 (s, 3H, CH_3), 3.90−4.00 (m, 4H, piperazine CH_2), 6.90−7.00 and 7.05−7.10 (m, each 1H, aromatic CH), 7.20−7.30 (m, 2H, H-6 and aromatic CH), 7.40−7.50 (m, 1H, aromatic CH), 8.25 (d, J = 9.0 Hz, 1H, H-5), 8.60 (s, 1H, H-2). Anal. ($C_{21}H_{19}F_3N_4O_3$) C, H, N.

7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-1-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic Acid Hydrochloride (3). The title compound was prepared through the general procedure for coupling reaction replacing 1-(2-pyridinyl)-piperazine with 1-(1,3-benzothiazol-2-yl)piperazine[27] to give intermediate **10** in 53% yield, followed by the general procedure for hydrolysis to give **3** in 99% yield: mp 338−339 °C. ^{1}H NMR (DMSO-d_6) δ 3.65−3.75 (m, 4H, piperazine CH_2), 3.95 (s, 3H, CH_3), 3.95−4.05 (m, 4H, piperazine CH_2), 7.10 (t, J = 7.3 Hz, 1H, benzothiazole CH), 7.25 (d, J = 9.1 Hz, 1H, H-6), 7.30 (t, J = 7.3 Hz, 1H, benzothiazole CH), 7.50 and 7.80 (d, J = 8.0 Hz, each 1H, benzothiazole CH), 8.30 (d, J = 9.1 Hz, 1H, H-5) 8.90 (s, 1H, H-2). Anal. ($C_{21}H_{20}ClN_5O_3S$) C, H, N.

1-Methyl-4-oxo-7-[4-(1,3-thiazol-2-yl)piperazin-1-yl]-1,4-dihydro-1,8-naphthyridine-3-carboxylic Acid (4). The title compound was prepared through the general procedure for coupling reaction replacing 1-(2-pyridinyl)piperazine with 1-(1,3-thiazol-2-yl)piperazine[28] to give intermediate **11** in 87% yield, followed by the general procedure for hydrolysis to give **4** in 99% yield: mp 292−293 °C (d). ^{1}H NMR (DMSO-d_6) δ 3.70−3.80 (m, 4H, piperazine CH_2), 3.95−4.05 (m, 7H, piperazine CH_2 and CH_3), 7.10 (d, J = 3.9 Hz, 1H, thiazole CH), 7.25 (d, J = 9.1 Hz, 1H, H-6), 7.35 (d, J = 3.9 Hz, 1H, thiazole CH), 8.30 (d, J = 9.1 Hz, 1H, H-5) 8.90 (s, 1H, H-2). Anal. ($C_{17}H_{17}N_5O_3S$) C, H, N.

In Vitro Antiviral Assays. Evaluation of the antiviral activity of the compounds against HIV-1 strain III_B and HIV-2 strain (ROD) in MT-4 cells was performed using the MTT assay as previously described.[29,30] Stock solutions (10× final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock-and HIV-infected cells at the beginning of each experiment. Serial 5-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman Instruments, Fullerton, CA). Untreated control HIV-and mock-infected cell samples were included for each sample.

HIV-1(III_B)[31] or HIV-2 (ROD)[32] stock (50 μL) at 100−300 $CCID_{50}$ (50% cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compound on uninfected cells in order to assess the cytotoxicity of the test compound. Exponentially growing MT-4 cells[33] were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6 × 10^5 cells/mL and 50 μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT assay.

The MTT assay is based on the reduction of yellow-colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue−purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland) at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of tree wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD540) of the mock-infected control sample by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

Peripheral Blood Mononuclear Cells (PBMCs). PBMCs from healthy donors were isolated by density centrifugation (Lymphoprep; Axis-Shield, PoC AS, Oslo, Norway) and stimulated with phytohemagglutin (PHA) (Sigma Chemical Co., Bornem, Belgium) for 3 days. The activated cells (PHA-stimulated blasts) were washed with PBS, and viral infections were done as described by the AIDS clinical trial group protocols.[34] Briefly, PBMCs (2 × 10^5/200 μL) were plated in the presence of serial dilutions of the test compound and were infected with HIV stocks at 1000 $CCID_{50}$ per mL. At day 4 postinfection, 125 μL of the supernatant of the infected cultures was removed and replaced with 150 μL of fresh medium containing the test compound at the appropriate concentration. At 7 days after plating the cells, p24 antigen was detected in the culture supernatant by an enzyme-linked immunosorbent assay (Perkin-Elmer, Boston, MA).

J-Lat GFP Clone A72 Jurkat Cells.[35] J-Lat GFP clone A72 Jurkat cells (30000 cells per 96-well) were infected with respective viruses in the absence or presence of test compound. After 5 days incubation, cells were harvested, washed, and fixed in 3% paraformaldehyde solution. Evaluation of the antiviral activity of the compounds was performed using flow cytometry using a FACSCanto II flow cytometry system (Becton-Dickinson) equipped with a 488 nm argon-ion laser and a 530/30 bandpass filter. Acquisition was stopped when 10000 events were counted. Cytotoxicity was monitored using the MTT-method as described above.

Latently Infected OM-10.1 and U1 Cells. The activity values of the naphtyridones against chronic HIV-1 infection were based on the inhibition of p24 antigen production in OM-10.1 an U1 cells after stimulation with TNF-α (Roche Diagnostics, Belgium) and PMA (Sigma Chemical Co., Bornem Belgium). Briefly, OM-10.1 and U1 cells (5 × 10^5 cells/mL) were incubated in the presence or absence of the compounds for 2 h in 48 wells plates. After this short incubation period, the cells were stimulated with 1 ng/mL of TNF-α or 0.02 μM of PMA, followed by transfer of 2 × 200 μL into a 96-well plate for toxicity evaluation. After a 2-day incubation at 37 °C, the cell culture supernatants were collected from the 48-well plates and examined for their p24 antigen levels with the HIV-1 p24 ELISA kit (Perkin-Elmer, Boston, MA). Cytotoxicity of the compounds for both latently HIV-1-infected OM-10.1 and U1 cell lines in the 96-well plates were based on the MTT cell viability staining as described previously.[29]

Persistently Infected HuT-78 Cells. Chronically infected HuT-78 cells were washed with PBS and pelleted. The supernatant was discarded. This manipulation was repeated 3 times. The test compounds at required concentrations were transferred into the cups of a 48-well plate. Then 200000 cells were added to each cup (final volume per cup is 1 mL). The cultures were allowed to grow for 44 h. Supernatant was collected and p24 concentrations were determined by ELISA. Nevirapine (NNRTI) and ritonavir (PI) were included as negative and a positive control, respectively.

Time-of-Addition Experiment. MT-4 cells were infected with HIV-1 (III_B) at a multiplicity of infection (moi) of 0.5 to synchronize all the steps of the viral replication. The test compounds were added at different times after infection (from 0 to 25 h).[36] Viral p24 Ag production was determined at 31 h post infection by ELISA (Perkin-Elmer, Boston, MA). Reference compounds with a known mode of action were included: AZT, which inhibits the reverse transcription process, and ritonavir, an inhibitor of the proteolytic cleavage of precursor proteins, were added at 100 times their 50% inhibitory concentration (IC_{50}) obtained in the MT-4 cells/MTT assay. Addition of these compounds can be delayed for 5 and 16 h postinfection, respectively.

Transactivation Assay. The inhibition of basal and Tat-mediated transactivation of the HIV-1 long terminal repeat (LTR) in a HeLa cell clone carrying an LTR-driven luciferase reporter was performed as described previously.[37] Cells were either mock transfected or treated with subsaturating concentrations of Tat to activate the viral promoter and incubated with increasing amounts of the compound.

In Vitro Resistance Selection. An attempt to generate an HIV-1 strain resistant to compound **3** in MT-4 cells was undertaken by passaging the virus in the presence increasing concentrations of compound **3** during 60 passages (= 30 weeks).

Kinase Assay. The CDK9 kinase activity was measured in vitro on a recombinant GST-CTD substrate as described previously.[38]

Histone Acetyltransferase (HAT) Activity of p300/CBP. The HAT activity of p300/CBP was detected using a recombinant GST-HAT fragment on a histone substrate as described previously.[39,40]

Cellular Integrase Assay. This assay was carried out as previously reported.[21]

Induced Fit Docking Studies. The IFD protocol used in this study was carried out as previously reported.[23] The carboxylic acid of compound **3** was modeled as carboxylate.

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Supporting Information Available: IFD poses for compound **3** and elvitegravir; elemental analysis data for target compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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