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Anti-HIV diarylpyrimidine–quinolone hybrids and their mode of action

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ABSTRACT

A molecular hybridization approach is a powerful tool in the design of new molecules with improved affinity and efficacy. In this context, a series of diarylpyrimidine–quinolone hybrids were synthesized and evaluated against both wt HIV-1 and mutant viral strains. The most active hybrid **5a** displayed an EC_{50} value of 0.28 ± 0.07 μM against HIV-1 III_B. A couple of enzyme-based assays clearly pinpoint a RT-targeted mechanism of action. Docking studies revealed that these hybrids could be well located in the NNIBP of HIV-1 RT despite the bulky and polar properties of a quinolone 3-carboxylic acid moiety in the molecules.

1. Introduction

HIV-1 reverse transcriptase (RT) is an essential enzyme for retroviral replication and represents an important target for the development of anti-HIV drugs. Non-nucleoside RT inhibitors (NNRTIs) bind in a noncompetitive manner to an allosteric hydrophobic site located around 10 Å away from the polymerase active site, which is unique to HIV-1 RT and absent in host cell polymerases.[1] In general, NNRTIs are minimally toxic. To date, there are five structurally diverse NNRTIs approved by FDA for clinical use: nevirapine (NVP), delavirdine (DLV), efavirenz (EFV), etravirine (ETV, TMC125) and rilpivirine (RPV, TMC278). The efficacy of the first-generation NNRTIs NVP, DLV and EFV is remarkably compromised by RT mutations.[2] The second-generation NNRTIs TMC125 and TMC278 belonging to diarylpyrimidine analogues (DAPYs, Fig. 1) overcome the deficiency of early NNRTIs, demonstrating high activity against wild-type (wt) and mutant virus.[3,4]

In this context, we explored 'molecular hybridization' strategy[5] to generate a series of diarylpyrimidine–quinolone hybrids (Fig. 2). These hybrids are characterized by a bulky and polar quinolone 3-carboxylic acid moiety as wing I, as opposed to the above features considered to be essential for the biological activity. In addition, quinolones have been reported as anti-HIV agents with diverse mechanisms of action. For instance, recently approved elvitegravir[6] (**3**, EVG, Fig. 1) is the first quinolone-based anti-HIV drug, exhibiting potent inhibitory activity against integrase-catalyzed DNA strand transfer, while anti-HIV 6-aminoquinolone **WP7-5**[7] (**4**, Fig. 1) effectively inhibits the Tat-mediated long terminal repeat driven transcription, another essential step in the HIV-1 replication cycle. As the structure of the target compounds combines the features of reverse transcriptase (RT), integrase (IN), RT-associated ribonuclease H (RNase H) and transcription inhibitors, we tested their inhibitory activity on HIV-1 RT, IN, RNase H as well as in latently infected cells to pinpoint their mode of action.

2. Chemistry

The synthesis of the diarylpyrimidine–quinolone hybrids is depicted in Scheme 1. The 2,4-difluorobenzoyl chloride **7** were coupled with ethyl 3-(dimethylamino)acrylate to afford the acrylate **8**. Substitution with appropriate aliphatic amines and followed by cyclization with potassium carbonate and subsequent hydrolysis produced quinolones **11**. The target compounds **5** were obtained from **11** by replacement of 7-F with 7-OH under basic condition and followed by condensation with 4-((4-chloropyrimidin-2-yl)amino)benzonitrile. The target compounds **6** were synthesized from **11** by treatment with 2, 4-dimethoxybenzylamine and

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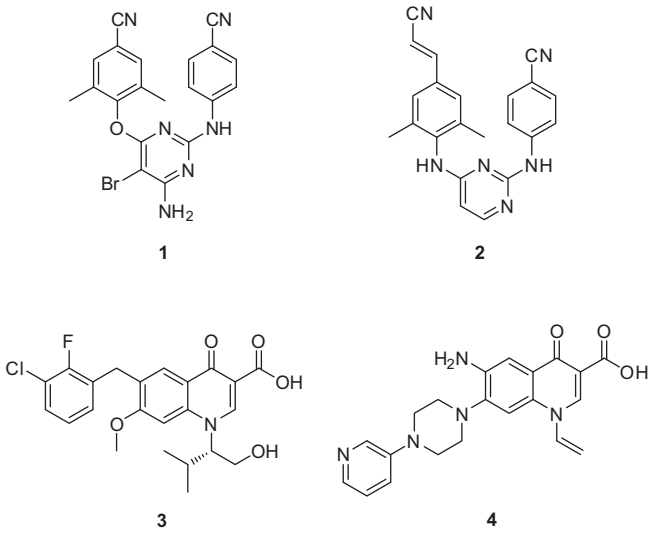


Figure 1. The structures of the inhibitors of HIV-1 RT (**1**, **2**), IN (**3**) and transcription (**4**).

followed by removal of 2, 4-dimethoxybenzyl group and subsequent condensation with 4-((4-chloropyrimidin-2-yl)amino) benzonitrile.

3. Evaluation of biological activities

3.1. Assays in MT-4 Cells

The newly synthesized diarylpyrimidine–quinolone hybrids **5a**–**i** and **6a**–**e** were evaluated for their ability to inhibit the replication of wild-type (wt) HIV-1 strain III_B in MT-4 cells. The cytotoxicity of the compounds was determined in parallel. FDA-approved drugs including the NRTI AZT, the NNRTIs NVP, EFV, ETV and the INSTI EVG were used as reference according to the same procedure.

As shown in Table 1, all the compounds with NH linker between the quinolone moiety and the central pyrimidine ring (**6a**–**e**) were devoid of antiviral activity, regardless of the substituent on *N*-1 position. Nevertheless, a few of the target molecules with *O*-linker displayed moderate inhibitory activity against wt HIV-1 replication with EC_{50} values ranging from 0.28 ± 0.07 to 1.16 ± 0.26 μM. For the compounds bearing a straight alkyl chain of variable length on the *N*-1 position, the trend of potency is ethyl (**5a**) ≈ butyl (**5e**) > propyl (**5b**). Compound **5a** showed an EC_{50} value of 0.28 ± 0.07 μM, coupled with a high SI value of 880, and the less active compounds **5b** still showed potency at the submicromolar level. The replacement of a straight chain butyl group of **5e** with branched ones (**5f**–**g**) profile was also shown by the *N*-1 propyl analogue **5b**. Although *N*-1 cyclopropyl and cyclohexyl compounds (**5d**, **5i**) proved to be inactive, the *N*-1 cyclopentyl analogue **5h** exhibited an EC_{50} value in the submicromolar range, but this activity was coupled with high cytotoxicity.

However, when assessing anti-HIV-1 activity against a typical NNRTI-resistant double mutant strain (RT K103N/Y181C), all diarylpyrimidine–quinolone hybrids totally lose their inhibitory activity, as is also the case for the first generation NNRTI NVP. EFV and ETV have EC_{50} values 26-fold and 7-fold higher, respectively, as compared to their activity against the wt virus. The NRTI AZT and the INSTI EVG are equipotent against wt and NNRTI-resistant strain.

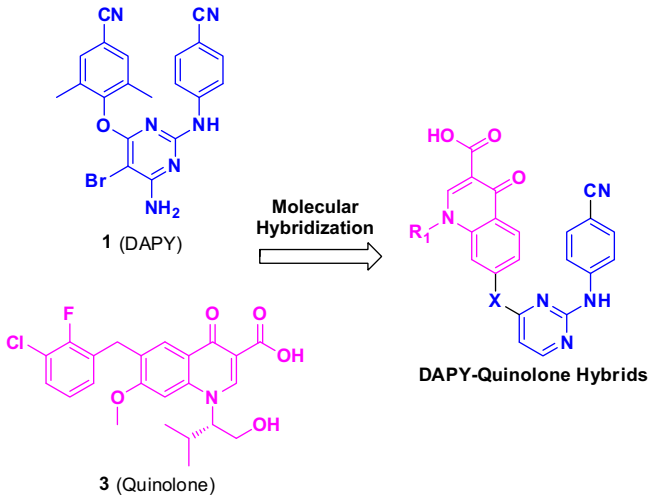


Figure 2. The design of diarylpyrimidine–quinolone hybrids through a 'merging' strategy.

3.2. Assays in latently infected cells

Due to the structural similarity of our newly synthesized hybrids and the reported quinolone derivatives, as for example **WP7-5**, as HIV transcription inhibitors, we also tested the target molecules **5a**, **5e** and EVG for their activity in latently HIV-1 infected promyelocytic (OM-10.1) cells, in which the virus remains in a latent state.[7] Current antiviral regimens are unable to completely eradicate the virus in HIV-1 infected individuals, which is mainly caused by the occurrence of stable latent reservoirs. In contrast to MT-4 cells, OM-10.1 cell lines should be stimulated to produce virus. After stimulation with phorbol myristate acetate (PMA), a dramatic increase in HIV-1 expression could be detected in these cells. However, none of the tested quinolone derivatives could inhibit virus production.

3.3. RT polymerase, RNase H and IN inhibition assay

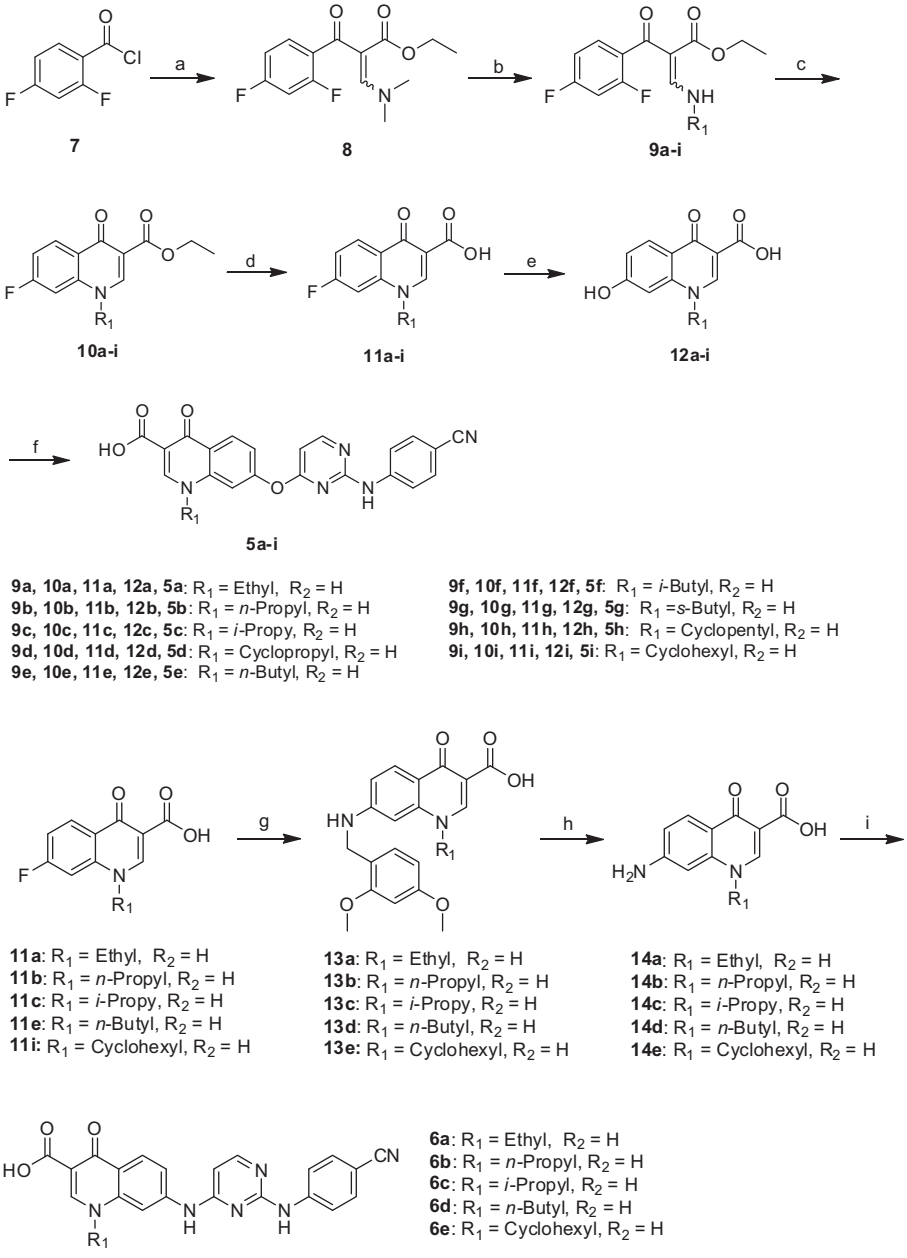
Based on the metal chelating properties of the quinolone carboxylic acid moiety of our newly synthesized compounds and the known similarities between the arrangement of the residues in the active site of HIV IN and RNase H, we investigated whether our diarylpyrimidine–quinolone hybrids could inhibit these enzyme activities in an RNase H inhibitory activity assay. All compounds in the series were totally devoid of inhibitory activity at the highest tested concentrations (⩾300 μM). Therefore the RNaseH inhibitory activity can be excluded as the mode of action of the compounds.

None of the compounds markedly inhibited the IN activity in vitro at the highest evaluated concentrations (⩾800 μM), except for compound **5e** that inhibits the IN activity with an IC_{50} value of 32.49 ± 0.09 μM, which is far above the values assessed for EVG in the same experiments (0.0018 ± 0.0013 μM).

Based on the activity spectrum of our newly synthesized compounds in cell culture, active against HIV-1, inactive against HIV-2 and total loss of activity against an NNRTI-resistant HIV-1 strain, we decided to evaluate the in vitro activity against the recombinant HIV-1 RT. As shown in Table 1, all the compounds active in cell-based assay exhibited moderate inhibitory activity against wt HIV-1 RT at micromolar level, which clearly pinpoint a RT-targeted mechanism of action.

3.4. Time-of-addition assay

In order to confirm RT is the biological target we also performed time-of-addition experiments in MT-4 cells in which the test



Scheme 1. Synthesis of diarylpyrimidine–quinolone hybrids **5a–i** and **6a–e**. Reagents and conditions: (a) ethyl 3-(dimethylamino)acrylate, Et_3N, toluene, 90 °C, 4 h; (b) aliphatic amines, THF, 50 °C, 3 h; (c) K_2CO_3, DMF, 60–90 °C, 1–6 h; (d) 5 M NaOH, THF, 50 °C, 3.5 h; (e) 12.5 M KOH, DMSO, 80 °C, 3–8 h; (f) 4-((4-chloropyrimidin-2-yl)amino)benzonitrile, K_2CO_3, DMF, 60–90 °C, 8–24 h; (g) 2,4-dimethoxybenzylamine; DMSO, 85 °C, 6 h; (h) CF_3COOH, DCM, rt, 5 h; (i) 4-((4-chloropyrimidin-2-yl)amino)benzonitrile, iPrOH, conc. HCl, reflux, 5–12 h.

compounds were added at different time points after infection of the cell cultures with HIV-1(III_B). Reference compounds with known mode of action were included such as ETV that inhibits HIV RT (3–5 h post infection), the diketo acid EVG that inhibits the strand transfer step of the integration (7–8 h post-infection) and **WP7-5** that is an inhibitor of transcription (10–16 h post-infection). Addition of the diarylpyrimidine–quinolone hybrids, **5a**, **5e** and **5f**, all can be postponed for 4–6 h post-infection, coinciding with the process of reverse transcription, which is also confirmed by a similar behavior of the NNRTI ETV in the experiments. The addition of the well-characterized INSTI EVG clearly can postpone for additional 2 h as compared to the addition of NNRTIs. As time of addition experiments only reveal the last step targeted by inhibitors, the NNRTI mode of action of the newly synthesized compounds is confirmed.

4. Molecular modeling studies

To investigate the potential binding mode of the newly synthesized hybrids, molecular simulation was conducted by using Sybyl Surflex-Dock program and the crystallographic structures of wt RT/RPV (PDB ID: 3MEC) complex.[8] The most active compound in RT assay **5e** was chosen as a representative to be docked into the NNIBP of wt HIV-1 RT. The co-crystallized ligand was extracted and the protein was prepared by removing water molecules and adding hydrogens. Gasteiger–Hückel charges were assigned to the ligand atoms. The structure of **5e** was energy minimized using the conjugate gradient method. As shown in Figure 3, **5e** was superposed into NNIBP of wt HIV-1 RT in a horseshoe conformational shape and established several ligand-receptor interactions similar to those of ETV: (i) Two typical hydrogen bonds

Table 1
Anti-HIV-1 activity and cytotoxicity of diarylpyrimidine–quinolone hybrids **5a–i** and **6a–e** in MT-4 cells

Compd	EC_{50}[a] (μM)		IC_{50}[b] (μM)	CC_{50}[c] (μM)	SI[d]
	HIV-1(III_B)	K103N/Y181C	RT	HIV-1(III_B)	HIV-1(III_B)
5a	0.28 ± 0.07	>246.37	7.15 ± 0.51	246.37 ± 29.08	880
5b	⩾0.43	>244.84	9.12 ± 0.49	244.84 ± 25.92	⩽569
5c	>283.16	>283.16	Nd[e]	>283.16	1
5d	>221.43	>221.43	Nd	⩾221.43	⩽1
5e	0.33 ± 0.06	>199.35	3.49 ± 0.51	⩾199.35	⩾604
5f	1.16 ± 0.26	>274.44	13.98 ± 4.16	>274.44	>237
5g	>274.44	>274.44	Nd	>274.44	1
5h	0.98 ± 0.19	>4.96	6.08 ± 0.01	4.96 ± 0.40	5
5i	>3.22	>3.22	Nd	3.22 ± 0.46	<1
6a	>123.44	>123.44	7.07 ± 0.00	123.44 ± 79.38	<1
6b	>188.67	>188.67	Nd	188.67 ± 54.85	<1
6c	>10.94	>10.94	Nd	10.94 ± 4.09	<1
6d	>13.62	>13.62	Nd	13.62 ± 6.09	<1
6e	>2.18	>2.18	Nd	2.18 ± 1.24	<1
AZT	0.0056 ± 0.0011	0.0052 ± 0.0004	Nd	>94	16,785
NVP	0.244 ± 0.041	⩾11	0.62 ± 0.16	>15	>61
EFV	0.0054 ± 0.0006	0.14 ± 0.00	0.022 ± 0.0030	>6.5	>1204
ETV	0.0019 ± 0.0005	0.014 ± 0.001	0.011 ± 0.00	14.24 ± 7.49	7495
EVG	0.0031 ± 0.0020	0.0022 ± 0.0013	Nd	4.60 ± 0.69	1484

[a] Compound concentration required to protect MT-4 cells from HIV-1-induced cytopathogenic effect by 50% and the data represent the mean of at least three separate experiments.
[b] Compound concentration required to inhibit in vitro HIV-1 RT wt activity by 50%.
[c] Compound concentration that decreases the uninfected MT-4 cell viability by 50%.
[d] Selectivity index: CC_{50}/EC_{50} ratio.
[e] Not detected.

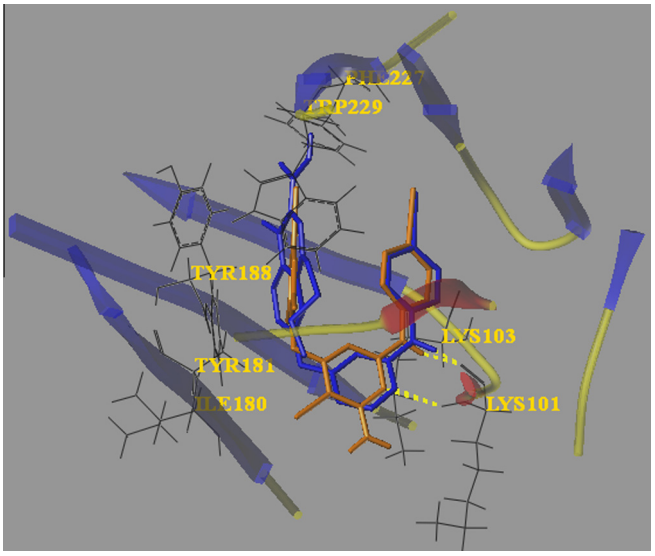


Figure 3. Predicted binding mode of **5e** (blue) within NNIBP of wt HIV-1 RT. ETV (orange) was superposed into the same pocket for comparison. Hydrogen bonds are indicated with dashed lines in yellow.

formed between the nitrogen atom of pyrimidine ring, the NH linker and Lys101, and (ii) Favorable π–π interaction of the quinolone ring with a hydrophobic pocket, as defined by the side chains of Tyr181, Tyr188, Phe227 and Tyr229.

5. Conclusion

On the basis of 'hybridization' strategy, diarylpyrimidine–quinolone hybrids were identified as a new class of potent NNRTIs. As opposed to the structural features considered to be essential for the biological activity of typical NNRTIs, these hybrids are characterized by a bulky and polar quinolone 3-carboxylic acid moiety as wing I in the molecules. Most of the hybrids with *O*-linker showed potent activity against wt HIV-1. In particular, compounds **5a**, **5e** and **5h** showed low EC_{50} value of 0.28, 0.33 and 0.98 μM, respectively.

6. Material and methods

6.1. Chemistry

Melting points were measured with a SGW X-1 microscopic melting-point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 400 MHz spectrometer. Chemical shifts are reported in δ (ppm) units relative to the internal standard tetramethylsilane (TMS). HRMS were obtained on a Bruker micrOTOF II instrument using electrospray ionization (ESI) techniques. All chemicals and solvents used were of reagent grade and were purified and dried by standard methods before use. All air-sensitive reactions were run under a nitrogen atmosphere. All the reactions were monitored by thin layer chromatography (TLC) on pre-coated silica gel G plates at 254 nm under a UV lamp using ethyl acetate/hexane as eluent. Column chromatography separations were obtained on silica gel (300–400 mesh) using dichloromethane and methanol as eluents. Analysis of sample purity was performed on an Agilent 1200 series HPLC system with a Phenomenex Synergi 4 μ Hydro-RP 80A (4.6 mm × 250 mm). HPLC conditions were the following: solvent A = water, solvent B = MeCN; flow rate = 1.0 mL/min. Condition 1: compounds were eluted with 55% MeCN/water in 40–60 min. Condition 2: compounds were eluted with 50% MeCN/water containing 0.05% TFA in 40–60 min. Purity was determined by the absorbance at 254 nm. All tested compounds have a purity of >95%.

6.1.1. Ethyl 2-(2,4-difluorobenzoyl)-3-(dimethylamino)acrylate (8)

A mixture of substituted 2, 4-difluorobenzoyl chloride (3.19 g, 18.1 mmol), ethyl 3-(dimethylamino)acrylate (2.59 g, 18.1 mmol, 1.0 equiv), and triethylamine (2.74 g, 27.1 mmol, 1.5 equiv) in toluene (30 mL) was stirred at 90 °C for 4 h. After cooling, the reaction mixture was filtered. The filtrate was concentrated under

reduced pressure. The residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate 2:1 to 1:2, v/v) to give the desired product **8** as yellow oil. Yield 65%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (s, 1H), 7.62–7.68 (m, 1H), 6.89–6.93 (m, 1H), 6.74–6.80 (m, 1H), 4.03–3.98 (q, J = 7.2 Hz, 2H), 3.31 (s, 3H), 2.89 (s, 3H), 0.95–0.99 (t, J = 7.2 Hz, 3H).

6.1.2. General procedure for the preparation of 7-fluoro-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acids (11a–i)

To a stirred solution of the acrylate **8** (0.85 g, 3.0 mmol) in THF (30 mL) was added a selected aliphatic amine (3.6 mmol, 1.2 equiv). After stirring at 50 °C for 3 h, the solution was concentrated under reduced pressure and then diluted with DMF (10 mL). To this solution was added K_2CO_3 (1.04 g, 7.5 mmol, 2.5 equiv). The resulting mixture was stirred at 60–90 °C for 1–6 h. After cooling, ice-water (40 mL) was added and the precipitate was filtered. The collected precipitate was dissolved in THF (5 mL) and 5 M NaOH (5 mL) was added. The mixture was stirred at 50 °C for 3.5 h and then concentrated under reduced pressure. The residue was poured into ice-water (5 mL) and acidified with 4 M HCl to pH ~2. The resulting precipitate was filtered, washed by water, and dried to afford **11a–i**, which was used directly for the next step without further purification.

6.1.3. General procedure for the preparation of 7-hydroxy-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acids (12a–i)

To a stirred solution of 7-fluoro-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid **11a–i** (1.0 mmol) in DMSO (5 mL) was added 12.5 M KOH (5 mL). After stirring at 80 °C for 3 to 8 h, the mixture was poured into ice-water (8 mL) and acidified with 6 M HCl to pH ~2. The resulting precipitate was filtered, washed by water, and dried to afford **12a–i** as a solid, which was used directly for the next step without further purification.

6.1.3.1. 1-Ethyl-7-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12a). Yield 68% (for 4 steps from **8**); ^{1}H NMR (400 MHz, MeOD) δ 8.86 (s, 1H), 8.33–8.31 (d, J = 8.8 Hz, 1H), 7.13–7.14 (d, J = 1.6 Hz, 1H), 7.12–7.09 (dd, J = 8.8, 1.6 Hz, 1H), 4.48–4.43 (q, J = 7.2 Hz, 2H), 1.54–1.51 (t, J = 7.2 Hz, 3H).

6.1.3.2. 7-Hydroxy-4-oxo-1-propyl-1,4-dihydroquinoline-3-carboxylic acid (12b). Yield 64% (for 4 steps from **8**); ^{1}H NMR (400 MHz, DMSO) δ 15.56 (s, 1H), 10.97 (br s, 1H), 8.92 (s, 1H), 8.24–8.22 (d, J = 8.8 Hz, 1H), 7.16–7.15 (d, J = 1.6 Hz, 1H), 7.13–7.09 (dd, J = 8.8, 1.6 Hz, 1H), 4.41–4.38 (t, J = 7.2 Hz, 2H), 1.83–1.77 (m, 2H), 0.92–0.89 (t, J = 7.2 Hz, 3H).

6.1.3.3. 7-Hydroxy-1-isopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12c). Yield 86% (for 4 steps from **8**); ^{1}H NMR (400 MHz, DMSO) δ 15.58 (s, 1H), 11.03 (br s, 1H), 8.76 (s, 1H), 8.26–8.24 (d, J = 8.8 Hz, 1H), 7.30–7.29 (d, J = 1.6 Hz, 1H), 7.14–7.12 (dd, J = 8.8, 1.6 Hz, 1H), 5.05–5.02 (m, 1H), 1.55–1.53 (d, J = 6.8 Hz, 6H).

6.1.3.4. 1-Cyclopropyl-7-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12d). Yield 81% (for 4 steps from **8**); ^{1}H NMR (400 MHz, MeOD) δ 8.82 (s, 1H), 8.29–8.27 (d, J = 8.8 Hz, 1H), 7.54 (s, 1H), 7.12–7.09 (d, J = 8.8, 1H), 3.71–3.68 (m, 1H), 1.38–1.36 (m, 2H), 1.20–1.99 (m, 2H).

6.1.3.5. 1-Butyl-7-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12e). Yield 70% (for 4 steps from **8**); ^{1}H NMR (400 MHz, DMSO) δ 15.54 (s, 1H), 10.99 (s, 1H), 8.91 (s, 1H), 8.24–8.22 (d, J = 9.2 Hz, 1H), 7.15 (s, 1H), 7.13–7.10 (d, J = 8.8 Hz, 1H), 4.45–4.41 (t, J = 7.2 Hz, 2H), 1.78–1.74 (m, 2H), 1.37–1.32 (m, 2H), 0.93–0.89 (t, J = 7.2 Hz, 3H).

6.1.3.6. 7-Hydroxy-1-isobutyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12f). Yield 69% (for 4 steps from **8**); ^{1}H NMR (400 MHz, MeOD) δ 8.77 (s, 1H), 8.31–8.29 (d, J = 8.0 Hz, 1H), 7.10 (s, 1H), 7.10–7.08 (d, J = 8.0 Hz, 1H), 4.21–4.20 (d, J = 6.8 Hz, 2H), 2.29–2.27 (m, 1H), 1.01–0.99 (d, J = 7.2 Hz, 6H).

6.1.3.7. 1-(*sec*-Butyl)-7-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12g). Yield 69% (for 4 steps from **8**); ^{1}H NMR (400 MHz, MeOD) δ 8.81 (s, 1H), 8.34–8.32 (d, J = 9.2 Hz, 1H), 7.27–7.26 (d, J = 1.2 Hz, 1H), 7.11–7.09 (dd, J = 8.8, 1.2 Hz, 1H), 4.90–4.86 (m, 1H), 2.05–1.96 (m, 2H), 1.61–1.60 (d, J = 6.4 Hz, 3H), 0.97–0.93 (t, J = 7.2 Hz, 3H).

6.1.3.8. 1-Cyclopentyl-7-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12h). Yield 90% (for 4 steps from **8**); ^{1}H NMR (400 MHz, MeOD) δ 8.82 (s, 1H), 8.34–8.32 (d, J = 9.2 Hz, 1H), 7.31–7.30 (d, J = 1.6 Hz, 1H), 7.13–7.11 (dd, J = 8.8, 1.2 Hz, 1H), 5.17–5.11 (m, 1H), 2.41–2.36 (m, 2H), 2.07–1.91 (m, 6H).

6.1.3.9. 1-Cyclohexyl-7-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (12i). Yield 78% (for 4 steps from **8**); ^{1}H NMR (400 MHz, MeOD) δ 8.88 (s, 1H), 8.34 (d, J = 8.8 Hz, 1H), 7.26 (d, J = 1.6 Hz, 1H), 7.12 (dd, J = 8.8, 1.6 Hz, 1H), 4.68–4.56 (m, 1H), 2.28–1.34 (m, 10H).

6.1.4. General procedure for the preparation of diarylpyrimidine–quinolone hybrids (5a–i)

A mixture of 7-hydroxy-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid **12a–i** (1.0 mmol), 4-((4-chloropyrimidin-2-yl)amino)benzonitrile (242 mg, 1.05 mmol, 1.05 equiv) and K_2CO_3 (345 mg, 2.5 mmol, 2.5 equiv) in DMF (10 mL) was stirred at 60–90 °C for 8–24 h. After cooling, the mixture was poured into ice-water (10 mL), acidified with HAc to pH 5–6, and filtered. The collected precipitate was purified by column chromatography on silica gel (dichloromethane/methanol 100:1 to 50:1, v/v) to give the desired product.

6.1.4.1. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)oxy)-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5a). White solid. Yield 42%; mp 291–293 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.19 (s, 1H), 10.15 (s, 1H), 9.10 (s, 1H), 8.55–8.53 (d, J = 5.6 Hz, 1H), 8.51–8.48 (d, J = 8.8 Hz, 1 H), 8.04 (s, 1H), 7.71–7.68 (d, J = 8.8 Hz, 2H), 7.62–7.59 (dd, J = 8.8 Hz, 1.6 Hz, 1H), 7.49–4.47 (d, J = 8.8 Hz, 2H), 6.77–6.75 (d, J = 5.6 Hz, 1H), 4.59–4.54 (q, J = 6.8 Hz, 2H), 1.35–1.31 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO) δ 177.71, 169.29, 166.48, 161.03, 159.41, 157.34, 150.22, 144.81, 140.99, 133.13, 128.63, 123.62, 121.66, 119.81, 118.94, 111.36, 108.48, 103.21, 100.75, 49.66, 14.97; HRMS-ESI (−) m/z calcd for $C_{23}H_{17}N_5O_4$ $[M-H]^-$: 426.1202, Found: 426.1196.

6.1.4.2. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)oxy)-4-oxo-1-propyl-1,4-dihydroquinoline-3-carboxylic acid (5b). White solid. Yield 34%; mp 252–254 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.17 (s, 1H), 10.13 (s, 1H), 9.05 (s, 1H), 8.53–8.52 (d, J = 4.2 Hz, 1H), 8.50–8.48 (d, J = 8.8 Hz, 1H), 8.02 (s, 1H), 7.67–7.65 (d, J = 8.4 Hz, 2H), 7.60–7.58 (d, J = 8.8 Hz, 1H), 7.47–7.45 (d, J = 8.8 Hz, 2H), 6.75–6.73 (d, J = 5.6 Hz, 1H), 4.49–4.46 (t, J = 7.2 Hz, 2H), 1.75–1.69 (m, 2H), 0.84–0.80 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO) δ 177.71, 169.30, 166.48, 161.05, 159.42, 157.33, 150.55, 144.82, 141.21, 133.14, 128.59, 123.61, 121.68, 119.81, 118.91, 111.51, 108.24, 103.20, 100.77, 55.49, 22.45, 10.92; HRMS-

ESI (−) *m*/*z* calcd for $C_{24}H_{19}N_5O_4$ $[M-H]^-$: 440.1358, Found: 440.1350.

6.1.4.3. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)oxy)-1-isopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5c). White solid. Yield 41%; mp 273–274 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.18 (s, 1H), 10.12 (s, 1H), 8.89 (s, 1H), 8.53–8.52 (d, J = 5.6 Hz, 1H), 8.52–8.51 (d, J = 8.8 Hz, 1H), 7.68–7.66 (d, J = 8.8 Hz, 2H), 7.62–7.60 (dd, J = 8.8, 2.0 Hz, 1H), 7.47–7.45 (d, J = 8.4 Hz, 2H), 6.75–6.73 (d, J = 5.6 Hz, 1H), 5.21–5.15 (m, 1H), 1.51–1.50 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, DMSO) δ 177.45, 169.30, 166.50, 161.03, 159.43, 157.50, 145.37, 144.83, 141.50, 133.14, 128.69, 123.66, 121.64, 119.81, 118.94, 111.06, 108.54, 103.20, 100.76, 53.36, 21.84; HRMS-ESI (−) *m*/*z* calcd for $C_{24}H_{19}N_5O_4$ $[M-H]^-$: 440.1358, Found: 440.1348.

6.1.4.4. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)oxy)-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5d). White solid. Yield 42%; mp 272–273 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.02 (s, 1H), 10.14 (s, 1H), 8.78 (s, 1H), 8.55–8.53 (d, J = 5.6 Hz, 1H), 8.47–8.45 (d, J = 8.8 Hz, 1H), 8.13–8.12 (d, J = 2.0 Hz, 1H), 7.71–7.69 (d, J = 8.4 Hz, 2H), 7.64–7.61 (dd, J = 8.8, 2.0 Hz, 1H), 7.51–7.49 (d, J = 8.8 Hz, 2H), 6.76–6.75 (d, J = 5.6 Hz, 1H), 3.79–3.76 (m, 1H), 1.27–1.25 (m, 2H), 1.16–1.15 (m, 2H); ^{13}C NMR (100 MHz, DMSO) δ 177.93, 169.14, 166.28, 161.15, 159.45, 157.08, 149.76, 144.81, 143.01, 133.21, 128.34, 123.04, 121.71, 119.84, 118.96, 111.27, 108.13, 103.25, 100.84, 36.51, 8.08; HRMS-ESI (−) *m*/*z* calcd for $C_{24}H_{17}N_5O_4$ $[M-H]^-$: 438.1202, Found: 438.1197.

6.1.4.5. 1-Butyl-7-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5e). White solid. Yield 54%; mp 267–268 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.16 (s, 1H), 10.13 (s, 1H), 9.04 (s, 1H), 8.53–8.52 (d, J = 5.6 Hz, 1H), 8.50–8.48 (d, J = 8.8 Hz, 1H), 7.98–7.97 (d, J = 1.2 Hz, 1H), 7.66–7.64 (d, J = 8.4 Hz, 2H), 7.60–7.57 (dd, J = 8.8, 1.6 Hz, 1H), 7.45–7.43 (d, J = 8.4 Hz, 2H), 6.75–6.74 (d, J = 5.6 Hz, 1H), 4.52–4.48 (d, J = 7.2 Hz, 2H), 1.68–1.64 (m, 2H), 1.27–1.21 (m, 2H), 0.77–0.73 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO) δ 177.70, 169.30, 166.47, 161.06, 159.41, 157.32, 150.46, 144.82, 141.16, 133.11, 128.63, 123.62, 121.61, 119.79, 118.87, 111.55, 108.27, 103.20, 100.80, 54.03, 31.18, 19.48, 13.83; HRMS-ESI (−) *m*/*z* calcd for $C_{24}H_{19}N_5O_4$ $[M-H]^-$: 454.1515, Found: 454.1503.

6.1.4.6. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)oxy)-1-isobutyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5f). White solid. Yield 36%; mp 251–253 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.15 (s, 1H), 10.12 (s, 1H), 9.00 (s, 1H), 8.53–8.52 (d, J = 5.6 Hz, 1H), 8.50–8.48 (d, J = 8.8 Hz, 1H), 8.00–7.99 (d, J = 1.6 Hz, 1H), 7.66–7.64 (d, J = 8.4 Hz, 2H), 7.60–7.58 (dd, J = 8.8, 1.6 Hz, 1H), 7.46–7.44 (d, J = 8.8 Hz, 2H), 4.36–4.34 (d, J = 7.2 Hz, 2H), 2.08–2.03 (m, 1H), 0.81–0.79 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, DMSO) δ 177.72, 169.27, 166.49, 161.07, 159.40, 157.30, 150.67, 144.81, 141.39, 133.13, 128.55, 123.55, 121.67, 119.79, 118.87, 111.59, 108.04, 103.19, 100.81, 60.29, 27.88, 19.54; HRMS-ESI (−) *m*/*z* calcd for $C_{24}H_{19}N_5O_4$ $[M-H]^-$: 454.1515, Found: 454.1502.

6.1.4.7. 1-(*sec*-Butyl)-7-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5g). White solid. Yield 49%; mp 273–275 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.18 (s, 1H), 10.15 (s, 1H), 8.81 (s, 1H), 8.54–8.53 (d, J = 5.6 Hz, 1H), 8.52–8.51 (d, J = 8.8 Hz, 1H), 8.19 (s, 1H), 7.68–7.66 (d, J = 8.8 Hz, 2H), 7.61–7.59 (dd, J = 8.8, 1.2 Hz, 1H), 7.48–7.46 (d, J = 8.8 Hz, 1H), 6.76–6.74 (d, J = 5.6 Hz, 1H), 5.07–4.99 (m, 1H), 1.96–1.83 (m, 2H), 1.48–1.46 (d, J = 7.2 Hz, 3H), 0.78–0.75 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO) δ 176.69, 168.85, 165.99, 160.59, 158.97, 157.11, 144.99, 144.37, 141.54, 132.68, 128.27, 123.16, 121.17, 119.33, 118.46, 110.56, 108.25, 102.73, 100.30, 57.51, 28.23, 19.43, 9.97; HRMS-ESI (−) *m*/*z* calcd for $C_{24}H_{19}N_5O_4$ $[M-H]^-$: 454.1515, Found: 454.1508.

6.1.4.8. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)oxy)-1-cyclopentyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5h). White solid. Yield 51%; mp 278–279 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.12 (s, 1H), 10.12 (s, 1H), 8.79 (s, 1H), 8.53–8.52 (d, J = 5.6 Hz, 1H), 8.51–8.49 (d, J = 8.8 Hz, 1H), 8.16 (s, 1H), 7.68–7.66 (d, J = 8.8 Hz, 2H), 7.62–7.60 (dd, J = 8.8, 1.2 Hz, 1H), 7.47–7.45 (d, J = 8.8 Hz, 2H), 6.74–6.73 (d, J = 5.6 Hz, 1H), 5.25–5.18 (m, 1H), 2.21–1.73 (m, 8 H); ^{13}C NMR (100 MHz, DMSO) δ 177.44, 169.30, 166.54, 161.04, 159.43, 157.33, 145.40, 144.82, 142.06, 133.14, 128.54, 123.69, 121.70, 119.81, 118.96, 111.60, 108.27, 103.22, 100.73, 62.92, 32.00, 23.75; HRMS-ESI (−) *m*/*z* calcd for $C_{26}H_{21}N_5O_4$ $[M-H]^-$: 466.1515, Found: 466.1500.

6.1.4.9. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)oxy)-1-cyclohexyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (5i). White solid. Yield 37%; mp 288–290 °C; ^{1}H NMR (400 MHz, DMSO) δ 15.16 (s, 1H), 10.11 (s, 1H), 8.88 (s, 1H), 8.53–8.52 (d, J = 4.8 Hz, 1H), 8.52–8.50 (d, J = 8.4 Hz, 1H), 8.18 (s, 1H), 7.69–7.67 (d, J = 8.4 Hz, 2H), 7.62–7.60 (d, J = 8.8 Hz, 1H), 7.48–7.46 (d, J = 8.4 Hz, 2H), 6.74–6.73 (d, J = 5.6 Hz, 1H), 4.83–4.77 (m, 1H), 2.03–2.01 (m, 2H), 1.85–1.77 (m, 4H), 1.64–1.49 (m, 3H), 1.33–1.22 (m, 1H); ^{13}C NMR (100 MHz, DMSO) δ 177.36, 169.25, 166.51, 161.03, 159.41, 157.45, 145.60, 144.83, 141.49, 133.14, 128.62, 123.70, 121.73, 119.80, 118.94, 110.91, 108.48, 103.22, 100.78, 60.17, 32.27, 25.28, 24.99; HRMS-ESI (−) *m*/*z* calcd for $C_{27}H_{23}N_5O_4$ $[M-H]^-$: 480.1671, Found: 480.1667.

6.1.5. General procedure for the preparation of 7-((2,4-dimethoxybenzyl)amino)-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acids (13a–e)

To a solution of 7-fluoro-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid **11a–c**, **e**, **i** (1.0 mmol) in DMSO (2 mL) was added 2,4-dimethoxybenzylamine (3.0 mmol, 3.0 equiv). After stirring at 85 °C for 6 h, the solution was poured into ice-water (5 mL), acidified with 6 M HCl to pH ~5, filtered, washed by ethanol and dried to give the desired compound as a solid.

6.1.5.1. 7-((2,4-Dimethoxybenzyl)amino)-1-ethyl-4-oxo-1,4-dihydroqui noline-3-carboxylic acid (13a). Yield 100%; ^{1}H NMR (400 MHz, DMSO) δ 16.03 (s, 1H), 8.75 (s, 1H), 8.01–7.99 (d, J = 8.8 Hz, 1H), 7.44–7.41 (t, J = 5.2 Hz, 1H), 7.21–7.19 (d, J = 8.4 Hz, 1H), 6.96–6.93 (dd, J = 1.6, 8.8 Hz, 1H), 6.59–6.57 (m, 2H), 6.49–6.47 (dd, J = 8.4, 2.0 Hz, 1H), 4.37–4.30 (m, 4H), 3.83 (s, 3H), 3.72 (s, 3H), 1.30–1.27 (t, J = 7.2 Hz, 3H).

6.1.5.2. 7-((2,4-Dimethoxybenzyl)amino)-4-oxo-1-propyl-1,4-dihydroqui noline-3-carboxylic acid (13b). Yield 96%; ^{1}H NMR (400 MHz, DMSO) δ 16.02 (s, 1 H), 8.74 (s, 1H), 8.02–7.99 (d, J = 8.8 Hz, 1H), 7.44–7.42 (t, J = 4.8 Hz, 1H), 7.18–7.16 (d, J = 8.0 Hz, 1H), 6.97–6.94 (d, J = 8.8 Hz, 1H), 6.60–6.59 (d, J = 1.6 Hz, 1H), 6.52 (s, 1 H), 6.49–6.46 (dd, J = 8.4, 2.0 Hz, 1H), 4.32–4.30 (d, J = 5.2 Hz, 2H), 4.28–4.24 (t, J = 7.2 Hz, 2H), 3.82 (s, 3H), 3.72 (s, 3H), 1.68–1.63 (m, 2 H), 0.84–0.81 (t, J = 7.2 Hz, 3 H).

6.1.5.3. 7-((2,4-Dimethoxybenzyl)amino)-1-isopropyl-4-oxo-1,4-dihydro quinoline-3-carboxylic acid (13c). Yield 95%; ^{1}H NMR (400 MHz, DMSO) δ 15.78 (s, 1 H), 8.74 (s, 1H), 8.30–8.28 (d, J = 8.8 Hz, 1H), 7.28 (s, 1H), 7.22–7.20 (d, J = 8.4 Hz, 1H), 6.86–6.84 (d, J = 8.8 Hz, 1H), 6.59 (s, 1 H), 6.53 (s, 1 H), 6.49–6.47 (d, J = 8. 0 Hz, 1H), 5.01–4.98 (t, J = 5.2 Hz, 1H), 4.84–4.81 (m, 1H), 4.42–4.40 (d, J = 5.6 Hz, 2 H), 1.60–1.59 (d, J = 6.4 Hz, 6 H).

6.1.5.4. 1-Butyl-7-((2,4-dimethoxybenzyl)amino)-4-oxo-1,4-dihydro quinoline-3-carboxylic acid (13d). Yield 95%; ^{1}H NMR (400 MHz, DMSO) δ 15.98 (s, 1 H), 8.74 (s, 1H), 8.02–8.00 (d, J = 8.4 Hz, 1H), 7.46 (s, 1H), 7.16–7.14 (d, J = 8.0 Hz, 1H), 6.98–6.96 (d, J = 8.4 Hz, 1H), 6.60 (s, 1H), 6.48 (s, 1H), 6.48–6.46 (d, J = 8.0 Hz, 1H), 4.32–4.29 (m, 4H), 3.83 (s, 3H), 3.72 (s, 3H), 1.59–1.56 (m, 2 H), 1.25–1.19 (m, 2 H), 0.84–0.81 (t, J = 7.2 Hz, 3 H).

6.1.5.5. 1-Cyclohexyl-7-((2,4-dimethoxybenzyl)amino)-4-oxo-1,4-dihydro quinoline-3-carboxylic acid (13e). Yield 100%; ^{1}H NMR (400 MHz, DMSO) δ 16.02 (s, 1 H), 8.59 (s, 1H), 8.05–8.03 (d, J = 9.2 Hz, 1H), 7.39 (s, 1H), 7.16–7.14 (d, J = 8.4 Hz, 1H), 6.98–6.96 (d, J = 9.2 Hz, 1H), 6.66 (s, 1H), 6.60–6.59 (d, J = 2.0 Hz, 1H), 6.47–6.44 (dd, J = 2.0, 8.0 Hz, 1H), 4.44–4.38 (m, 1H), 4.35–4.33 (d, J = 5.2 Hz, 1H), 3.82 (s, 3H), 3.72 (s, 3H), 1.95–1.67 (m, 7 H), 1.51–1.25 (m, 3 H).

6.1.6. General procedure for the preparation of 7-amino-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (14a–e)

To a solution of 7-((2,4-dimethoxybenzyl)amino)-1-alkyl-4-oxo- 1,4-dihydroquinoline-3-carboxylic acids **13a–e** (5.0 mmol) in dichloromethane (30 mL) was added trifluoroacetic acid (1.71 g, 15.0 mmol, 3.0 equiv). After stirring at rt for 5 h, the solution was poured into ice-water (250 mL) and stirred violently for 20 min. The resulting precipitate was collected, dissolved in dichloromethane (20 mL) containing 5% methanol and filtered. The filtrate was concentrated under reduced pressure to afford the desired compound (**14a–e**) as a solid.

6.1.6.1. 7-Amino-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (14a). Yield 93%; ^{1}H NMR (400 MHz, DMSO) δ 16.02 (s, 1H), 8.77 (s, 1H), 8.02–8.00 (d, J = 8.8 Hz, 1H), 6.87–6.85 (d, J = 9.2 Hz, 1H), 6.78 (s, 1H), 6.49 (s, 2H), 4.37–4.32 (q, J = 6.8 Hz, 2H), 1.40–1.37 (t, J = 6.8 Hz, 3 H).

6.1.6.2. 7-Amino-4-oxo-1-propyl-1,4-dihydroquinoline-3-carboxylic acid (14b). Yield 95%; ^{1}H NMR (400 MHz, DMSO) δ 16.02 (s, 1H), 8.76 (s, 1H), 8.02–8.00 (d, J = 8.8 Hz, 1H), 6.87–6.85 (dd, J = 1.2, 9.2 Hz, 1H), 6.77–6.76 (d, J = 0.8 Hz, 1H), 6.49 (s, 2H), 4.29–4.25 (t, J = 7.2 Hz, 2H), 1.83–1.77 (m, 2H), 0.92–0.89 (t, J = 7.2 Hz, 3H).

6.1.6.3. 7-Amino-1-isopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (14c). Yield 96%; ^{1}H NMR (400 MHz, DMSO) δ 16.05 (s, 1H), 8.63 (s, 1H), 8.05–8.03 (d, J = 8.8 Hz, 1H), 6.93 (s, 1H), 6.89–6.86 (dd, J = 1.2, 8.8 Hz, 1H), 6.49 (br s, 2H), 4.91–4.84 (m, 1H), 1.54–1.52 (d, J = 6.4 Hz, 6H).

6.1.6.4. 7-Amino-1-butyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (14d). Yield 97%; ^{1}H NMR (400 MHz, DMSO) δ 16.00 (s, 1H), 8.76 (s, 1H), 8.02–8.00 (d, J = 9.2 Hz, 1H), 6.87–6.85 (d, J = 8.8 Hz, 1H), 6.76 (s, 1H), 6.50 (s, 2H), 4.29–4.31 (t, J = 7.2 Hz, 2H), 1.79–1.72 (m, 2H), 1.37–1.31 (m, 2H), 0.94–0.90 (t, J = 8.8 Hz, 3H).

6.1.6.5. 7-Amino-1-cyclohexyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (14e). Yield 84%; ^{1}H NMR (400 MHz, DMSO) δ 16.00 (s, 1H), 8.63 (s, 1H), 8.05–8.03 (d, J = 8.8 Hz, 1H), 6.94 (s, 1H), 6.89–6.86 (dd, J = 2.0, 8.8 Hz, 1H), 6.48 (s, 2H), 5.74 (s, 1H), 4.45–4.39 (m, 1H), 2.07–1.70 (m, 7H), 1.57–1.31 (m, 3H).

6.1.7. Procedure for the preparation of diarylpyrimidine–quinolone hybrids (6a–e)

A mixture of 7-amino-1-alkyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid **14a–e** (1.0 mmol), 4-((4-chloropyrimidin-2-yl)amino)benzonitrile (345 mg, 1.5 mmol, 1.5 equiv) in a solution of isopropanol (5 mL) containing 20% conc. HCl was stirred at reflux for 5–12 h. The mixture was cooled and filtered. The filter cake was washed by isopropanol, neutralize with $NaHCO_3$ to pH 5–6, and filtered. The crude was purified by column chromatography on silica gel (dichloromethane/methanol 20:1, v/v) to give the desired product.

6.1.7.1. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)amino)-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (6a). White solid. Yield 28%; mp >300 °C; IR ν 3427, 3322 (OH, NH), 2220 (C≡N), 1710 (C=O acid), 1629 (C=O ketone) cm^{-1}; ^{1}H NMR (400 MHz, DMSO) δ 10.90 (s, 1H), 10.41 (s, 1H), 9.02 (s, 1H), 8.35–8.32 (d, J = 8.8 Hz, 1H), 8.25–8.23 (d, J = 6.0 Hz, 1H), 8.12 (s, 1H), 8.09–8.07 (d, J = 8.8 Hz, 1H), 7.87–7.85 (d, J = 8.8 Hz, 2H), 7.78–7.76 (d, J = 8.8 Hz, 2H), 6.62–6.61 (d, J = 6.0 Hz, 1H), 4.45–4.40 (q, J = 7.2 Hz, 2H), 1.36–1.33 (t, J = 7.2 Hz, 3H); HRMS-ESI (−) m/z calcd for $C_{23}H_{18}N_6O_3$ [M−H]$^-$: 425.1362, Found: 425.1341.

6.1.7.2. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)amino)-4-oxo-1-propyl-1,4-dihydroquinoline-3-carboxylic acid (6b). Offwhite solid. Yield 32%; mp >300 °C; IR ν 3313, 3186, 3090 (OH, NH), 2220 (C≡N), 1698 (C=O acid), 1608 (C=O ketone) cm^{-1}; ^{1}H NMR (400 MHz, DMSO) δ 15.48 (s, 1H), 10.22 (s, 1H), 9.83 (s, 1H), 8.98 (s, 1H), 8.33–8.31 (d, J = 9.2 Hz, 1H), 8.26–8.25 (d, J = 5.6 Hz, 1H), 8.19 (s, 1H), 8.06–8.04 (d, J = 9.2 Hz, 1H), 7.94–7.92 (d, J = 8.8 Hz, 2H), 7.72–7.70 (d, J = 8.8 Hz, 2H), 6.50–6.49 (d, J = 5.6 Hz, 1H), 4.43–4.40 (t, J = 7.2 Hz, 2H), 1.82–1.76 (m, 2H), 0.86–0.82 (t, J = 7.2 Hz, 3H); HRMS-ESI (−) m/z calcd for $C_{24}H_{20}N_6O_3$ [M−H]$^-$: 439.1518, Found: 439.1511.

6.1.7.3. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)amino)-1-isopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (6c). Offwhite solid. Yield 30%; mp >300 °C; IR ν 3323, 3203, 3105 (OH, NH), 2221 (C≡N), 1699 (C=O acid), 1611 (C=O ketone) cm^{-1}; ^{1}H NMR (400 MHz, DMSO) δ 15.50 (s, 1H), 10.23 (s, 1H), 9.87 (s, 1H), 8.80 (s, 1H), 8.36–8.34 (d, J = 8.8 Hz, 1H), 8.26 (m, 2H), 8.11–8.09 (d, J = 8.4 Hz, 1H), 7.93–7.91 (d, J = 7.6 Hz, 2H), 7.71–7.70 (d, J = 7.6 Hz, 2H), 6.50–6.49 (d, J = 5.6 Hz, 1H), 5.01–4.95 (m, 1H), 1.52–1.51 (d, J = 5.2 Hz, 6H); HRMS-ESI (−) m/z calcd for $C_{24}H_{20}N_6O_3$ [M−H]$^-$: 439.1518, Found: 439.1519.

6.1.7.4. 1-Butyl-7-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (6d). Offwhite solid. Yield 35%; mp 293–295 °C; IR ν 3343, 3176, 3074 (OH, NH), 2221 (C≡N), 1700 (C=O acid), 1606 (C=O ketone) cm^{-1}; ^{1}H NMR (400 MHz, DMSO) δ 15.50 (s, 1H), 10.24 (s, 1H), 9.85 (s, 1H), 8.97 (s, 1H), 8.33–8.30 (d, J = 9.2 Hz, 1H), 8.26–8.25 (d, J = 5.6 Hz, 1H), 8.21 (s, 1H), 8.03–8.01 (d, J = 9.2 Hz, 1H), 7.94–7.92 (d, J = 8.8 Hz, 2H), 7.72–7.70 (d, J = 8.8 Hz, 2H), 6.51–6.49 (d, J = 5.6 Hz, 1H), 4.47–4.43 (t, J = 6.8 Hz, 2H), 1.76–1.72 (m, 2H), 1.30–1.24 (m, 2H), 0.85–0.81 (t, J = 7.2 Hz, 3H); HRMS-ESI (−) m/z calcd for $C_{25}H_{22}N_6O_3$ [M−H]$^-$: 453.1675, Found: 453.1677.

6.1.7.5. 7-((2-((4-Cyanophenyl)amino)pyrimidin-4-yl)amino)-1-cyclo hexyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (6e). White solid. Yield 38%; mp 227–230 °C; IR ν 3511, 3400, 3048 (OH, NH), 2229 (C≡N), 1715 (C=O acid), 1605 (C=O ketone) cm^{-1}; ^{1}H NMR (400 MHz, DMSO) δ 15.49 (s, 1H), 10.26 (s, 1H), 9.93 (s, 1H), 8.79 (s, 1H), 8.38 (s, 1H), 8.36–8.34 (d, J = 8.8 Hz, 1H), 8.28–8.26 (d, J = 5.6 Hz, 1H), 8.01–7.98 (d, J = 8.8 Hz, 1H), 7.96–7.94 (d, J = 8.8 Hz, 2H), 7.72–7.69 (d, J = 8.8 Hz, 2H), 6.51–6.50 (d, J = 5.6 Hz, 1H), 4.61–4.60 (m, 1H), 2.09–2.06 (m, 2H), 1.83–1.81 (m, 4H), 1.61–1.58 (m, 1H), 1.41–1.21 (m, 3H); HRMS-ESI (−) m/z calcd for $C_{27}H_{24}N_6O_3$ [M−H]$^-$: 479.1831, Found: 479.1810.

6.2. Biology and biochemistry

6.2.1. Anti-HIV activity assays

Evaluation of the antiviral activity of the compounds against HIV-1 strain III_B in MT-4 cells was performed using the MTT assay as previously described.[9] 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 mad 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 samples.

HIV-1(III_B)[10] stock (50 μL) at 100–300 $CCID_{50}$ (cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells[11] 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[12] were centrifuged for 5 min at 220 g 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 coloured 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 (Infinite M1000, Tecan, Mechelen, Belgium), 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}).

6.2.2. Reverse transcriptase assay

Recombinant wt p66/p51 HIV-1 RT was expressed and purified as described by Auwerx et al.[13] The RT assay is performed with the EnzCheck Reverse Transcriptase Assay kit (Molecular Probes, Invitrogen), as described by the manufacturer. The assay is based on the dsDNA quantitation reagent PicoGreen. This reagent shows a pronounced increase in fluorescence signal upon binding to dsDNA or RNA-DNA heteroduplexes. Single-stranded nucleic acids generate only minor fluorescence signal enhancement when a sufficiently high dye: base pair ratio is applied.[14] This condition is met in the assay.

A poly(rA) template of approximately 350 bases long, and an oligo(dT)16 primer, are annealed in a molar ratio of 1:1.2 (60 min at rt). Fifty-two ng of the RNA/DNA is brought into each well of a 96-well plate in a volume of 20 μL polymerization buffer (60 mM Tris–HCl, 60 mM KCl, 8 mM $MgCl_2$, 13 mM DTT, 100 μM dTTP, pH 8.1). Five μL of RT enzyme solution, diluted to a suitable concentration in enzyme dilution buffer (50 mM Tris–HCl, 20% glycerol, 2 mM DTT, pH 7.6), is added. The reactions are incubated at 25 °C for 40 min and then stopped by the addition of EDTA (15 mM fc). Heteroduplexes are then detected by addition of PicoGreen. Signals are read using an excitation wavelength of 490 nm and emission detection at 523 nm using a spectrofluorometer (Safire2, Tecan). To test the activity of compounds against RT, 1 μL of compound in DMSO is added to each well before the addition of RT enzyme solution. Control wells without compound contain the same amount of DMSO. Results are expressed as relative fluorescence, that is, the fluorescence signal of the reaction mix with compound divided by the signal of the same reaction mix without compound.

6.2.3. Time-of-addition experiments

Time-of-addition experiments were adapted from Pauwels et al. and Daelemans et al.[15] Briefly, MT-4 cells were infected with HIV-1(III_B) or the RT double mutant virus (K103 N/Y181C) at an m.o.i. of 0.5. Following a 1 h adsorption period cells were distributed in a 96-well tray at 45,000 cells/well and incubated at 37 °C. Test compounds were added at different times (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 24, and 25 h) after infection. HIV-1 production was determined at 31 h postinfection via a p24 enzyme-linked immunosorbent assay (Perkin Elmer, Brussels, Belgium). Dextran sulfate was used at 12.5 μM, AZT at 1.9 μM, ETV at 0.19 μM, EVG at 0.31 μM, the 6-aminoquinolone derivative **WP7-5** at 0.25 μM and the diarylpyrimidine–quinolone hybrids were added at their 100-fold their EC_{50} concentration obtained in the MT-4/MTT assay.

6.2.4. RNaseH assay

The RNaseH assay was developed also employing the dsDNA quantitation reagent PicoGreen. A RNA/DNA heteroduplex is formed by annealing a 40 bases long RNA oligonucleotide (5′-CC AGCAGGAAACAGCUAUGACGAUC UGAGCCUGGGAGCU-3′) and 120 bases long DNA oligonucleotide (5′-AGCTC CCAGGCTCAGATCGTC ATAGCTGTTTCCTGCTGGCAGCTCCCAGGCTCAGATCGTCATAGCTGTT TCCTGCTGGCAGCTCCCAGGCTCAGATCGTCATAGCTGTTTCCTGCTGG C-3′) in a 4:1 molar ratio.

An amount of 76 ng of the annealed complex is brought into each well of a 96-well plate in a volume of 20 μL RNaseH buffer (60 mM Tris–HCl, 60 mM KCl, 8 mM $MgCl_2$, 13 mM DTT, pH 8.1) and 5 μL of suitably diluted RT solution is added. Reactions are stopped after 60 min. at 25 °C by the addition of EDTA (15 mM fc). PicoGreen is then added to measure the amount of heteroduplexes and thus the decrease in signal upon RNA hydrolysis in the presence of enzyme activity and signals are read as described for the reverse transcriptase assay. The results are expressed relative to the amount of heteroduplexes measured in a negative control sample without RT enzyme, after subtraction of the background signal obtained with only ssDNA.

To test compounds for activity against RNaseH, 1 μL of compound in DMSO is added to each well before the addition of RT enzyme solution. Control wells without compound contain the same amount of DMSO. Positive compounds were tested for autofluorescence in a separate test.

6.2.5. Integrase assay

The inhibitory activity of the reference compound EVG and the newly synthesized was assessed using the HIV-1 Integrase Kit (catalog number EZ-1700) of XpressBio (Thurmont, MD, USA) according to the manufacturer's protocol.

6.2.6. Anti-HIV-1 and cytotoxic assays in latently infected OM-10.1 cells

The activity values of the compounds against latent HIV-1 infection were based on the inhibition of p24 antigen production in OM-10.1 cells after stimulation with PMA (Sigma Chemical Co., Bornem Belgium). Briefly, OM-10.1 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 0.02 μM of PMA followed by transfer of 2 times 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 latently HIV-1-infected OM-10.1 cell line in

the 96-well plates were based on the MTT cell viability staining as described previously.[14]

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References and notes

1. (a) Tronchet, J. M.; Seman, M. *Curr. Top Med. Chem.* **2003**, *3*, 1496; (b) Ren, J.; Stammers, D. K. *Virus Res.* **2008**, *134*, 157.
2. (a) Domaoal, R. A.; Demeter, L. M. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 1735; (b) Mehellou, Y.; De Clercq, E. *J. Med. Chem.* **2010**, *53*, 521.
3. Ludovici, D. W.; De Corte, B. L.; Kukla, M. J.; Ye, H.; Ho, C. Y.; Lichtenstein, M. A.; Kavash, R. W.; Andries, K.; De B_ethune, M. P.; Azijn, H.; Pauwels, R.; Lewi, P. J.; Heeres, J.; Koymans, L. M.; De Jonge, M. R.; Van Aken, K. J.; Daeyaert, F. F.; Das, K.; Arnold, E.; Janssen, P. A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2235.
4. Janssen, P. A.; Lewi, P. J.; Arnold, E.; Daeyaert, F.; De Jonge, M.; Heeres, J.; Koymans, L.; Vinkers, M.; Guillemont, J.; Pasquier, E.; Kukla, M.; Ludovici, D.; Andries, K.; De Béthune, M. P.; Pauwels, R.; Das, K.; Clark, A. D., Jr.; Frenkel, Y. V.; Hughes, S. H.; Medaer, B.; De Knaep, F.; Bohets, H.; De Clerck, F.; Lampo, A.; Williams, P.; Stoffels, P. *J. Med. Chem.* **2005**, *48*, 1901.
5. (a) Viegas-Junior, C.; Danuello, A.; Bolzani, V.; Barreiro, E. J.; Fraga, C. A. M. *Curr. Med. Chem.* **2007**, *14*, 1829; (b) Lazar, C.; Kluczyk, A.; Kiyota, T.; Konishi, Y. *J. Med. Chem.* **2004**, *47*, 6973.
6. Sato, M.; Motomura, T.; Aramaki, H.; Matsuda, T.; Yamashita, M.; Ito, Y.; Kawakami, H.; Matsuzaki, Y.; Watanabe, W.; Yamataka, K.; Ikeda, S.; Kodama, E.; Matsuoka, M.; Shinkai, H. *J. Med. Chem.* **2006**, *49*, 1506.
7. Tabarrini, O.; Massari, S.; Daelemans, D.; Meschini, F.; Manfroni, G.; Bottega, L.; Gatto, B.; Palumbo, M.; Pannecouque, C.; Cecchetti, V. *ChemMedChem* **2010**, *5*, 1880.
8. Lansdon, E. B.; Brendza, K. M.; Hung, M.; Wang, R.; Mukund, S.; Jin, D.; Birkus, G.; Kutty, N.; Liu, X. *J. Med. Chem.* **2010**, *53*, 4295.
9. (a) Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, J.; De Clercq, E. *J. Virol. Methods* **1988**, *20*, 309; (b) Pannecouque, C.; Daelemans, D.; De Clercq, E. *Nat. Protoc.* **2008**, *3*, 427.
10. Popovic, M.; Sarngadharan, M. G.; Read, E.; Gallo, R. C. *Science* **1984**, *224*, 497.
11. Barré-Sinoussi, F.; Chermann, J. C.; Nugeyre, M. T.; Chamaret, S.; Grest, J.; Dauget, C.; Axler-Blin, C.; Vézinet-Brun, F.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. *Science* **1983**, *220*, 868.
12. Miyoshi, I.; Taguchi, H.; Kobonishi, I.; Yoshimoto, S.; Ohtsuki, Y.; Shiraishi, Y.; Akagi, T. *Gann. Monogr.* **1982**, *28*, 219.
13. Auwerx, J.; North, T. W.; Preston, B. D.; Klarmann, G. J.; De Clercq, E.; Balzarini, J. *Mol. Pharmacol.* **2002**, *61*, 400.
14. Singer, V. L.; Jones, L. J.; Yue, S. T.; Haugland, R. P. *Anal. Biochem.* **1997**, *249*, 228.
15. (a) Pauwels, R.; Andries, K.; Desmyter, J.; Schols, D.; Kukla, M. J.; Breslin, H. J.; Raeymaeckers, A.; Van Gelder, J.; Woestenborghs, R.; Heykants, J.; Schellekens, K.; Janssen, M. A. C.; De Clercq, E.; Janssen, P. A. J. *Nature* **1990**, *343*, 470; (b) Daelemans, D.; Pauwels, R.; De Clercq, E.; Pannecouque, C. *Nat. Protoc.* **2011**, *6*, 925.