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Studies on anti-HIV quinolones: New insights on the C-6 position

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ABSTRACT

The 6-desfluoroquinolones which have been developed by our group represent a promising class of compounds for the treatment of HIV infection since they act on transcriptional regulation, a crucial step in the replication cycle that has not been clinically exploited, yet. Focussing attention on the N-1 and C-6 positions, a novel series of quinolones has been synthesized. New SAR insights have been obtained, in particular, the hydroxyl group emerged as a suitable C-6 substituent when coupled with the appropriate arylpiperazine at the neighboring C-7 position.

1. Introduction

One of the crucial steps in the HIV replication cycle that has not been clinically exploited is the transcription of viral mRNA from the HIV long terminal repeat (LTR) promoter.

The regulation of HIV-1 gene expression could be a possible intervention site for antiretroviral chemotherapy, because it gives the possibility of controlling HIV-1 replication not only in acutely but also in chronically infected cells. Inhibitors of HIV-1 transcriptional regulation could have great potential in anti-HIV drug combination therapy because they can slow down the virus replication rate or even completely shut-off virus replication giving a lifetime control of HIV infection. In addition, such inhibitors could disfavor drug resistance development due to the complex interplay of viral and cellular components involved in the transcriptional regulation process.

It is precisely at this step of the replication cycle that the 6-desfluoroquinolones (6-DFQs), identified by our group,[1–3] act, and which are therefore a very promising class of compounds for the control of the latent HIV-1 reservoir. However, to fulfill the expectations of the transcription inhibitor quinolones in anti-HIV chemotherapy field, the limitation caused by the low selectivity index observed in some cell lines needs to be overcome.

With this main purpose, further structure modifications were made in this study bearing in mind the recently published results[3] regarding the structure–activity relationship (SAR) investigations on the prototype of the 6-DFQs, **WM5**[1] (**1**, Fig. 1). In particular, the omission of the N-1 substituent did not cause a loss of activity in contrast with the well-established SAR for both antibacterial[4] and antiviral quinolone agents,[5] it rather gave derivatives showing the same anti-HIV potency as the 1-methyl counterparts coupled with a reduced cytotoxicity (from 3- to 20-fold).[3] The real novelty that emerged from our recent work,[3] came from modifications at the C-6 position where the lack of any substituent resulted in compounds endowed with markedly reduced cytotoxicity, when the appropriate 4-arylpiperazine was present at the C-7 position. In fact, the 6-hydrogenquinolones **HM12** and **HM13** (**2** and **3**, Fig. 1)[3,6] were endowed with very pronounced activity and selectivity on HIV-1 acutely infected MT-4, CEM and peripheral blood mononuclear cells (PBMCs) as well as on chronically infected HuT78 and human primary monocyte/macrophages (M/M), and

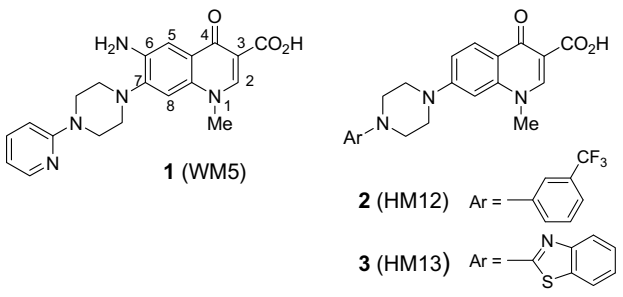

Figure 1. Potent 6-DFQs.

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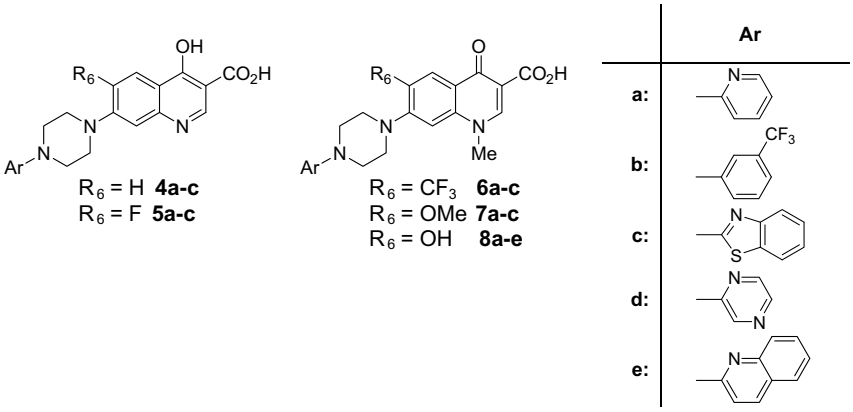


Figure 2. Structures of quinolones synthesized in this study.

latently HIV-1 infected M/M cells. This activity was also confirmed in an in vivo mice model for HIV-1 latency which is encouraging evidence for the use of quinolones in the control of HIV-1 infection in patients.[6]

Focussing our attention on the N-1 and C-6 positions, new quinolone derivatives (compounds **4–8**, Fig. 2) were designed and synthesized in this study. The 4-arylpiperazines that conferred high potency on the previous 6-DFQs were maintained at the C-7 position. In particular, in the hope of obtaining a more pronounced reduction of the cytotoxicity while conserving antiviral potency, 6-hydrogenquinolones **4a–c** were prepared, together with 6-fluoro analogs **5a–c**, which incorporate a C-6 fluorine atom, the typical substituent in antibacterial[4] and some anti-HIV quinolones[7–9] which is known to be responsible for the optimum pharmacokinetic characteristics, as well as for biological target recognition.

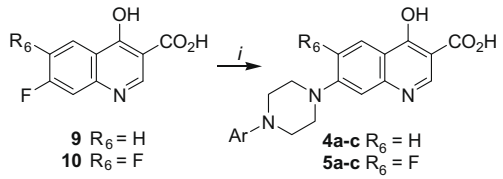
To further explore the role of the C-6 substituent in the antiviral activity/cytotoxicity, groups with different chemical physical properties have been introduced at the C-6 position to give trifluoromethyl **6a–c**, methoxy **7a–c**, and hydroxy derivatives **8a–e**. Until now, these moieties have never been explored as C-6 substituent in the anti-HIV quinolone field.

Herein we report the synthesis of these novel quinolone series and their anti-HIV activity evaluation in MT-4 and PBMC cell cultures which led to the identification of new antiviral compounds and broadening of the SAR at the C-6 position.

2. Chemistry

The 6-hydrogenquinolones **4a–c** and 6-fluoro analogs **5a–c** were synthesized according to Scheme 1 reacting 7-fluoro- and 6,7-difluoro-4-hydroxyquinoline-3-carboxilic acid (**9**)[10] and (**10**),[11] respectively, with 1-(2-pyridinyl)piperazine (**a**), 1-[3-(trifluoromethyl)phenyl]piperazine (**b**) and 2-(1-piperazinyl)-1,3-benzothiazole (**c**)[12] in DMSO, using Et_3N or DIPEA as HF scavenger. The nucleophilic reaction carried out on the corresponding ethyl esters, did not proceed.

The usual Gould–Jacobs procedure was applied for the synthesis of 6-trifluoromethylquinolones **6a–c** (Scheme 2). The reaction of 3-fluoro-4-(trifluoromethyl)aniline with diethylethoxymethylenemalonate (EMME) gave compound **11** which was then cyclized with polyphosphoric acid (PPA), to give the key intermediate **12**. Subsequent sequential steps were: N-1 methylation to give compound **13**, C-7 nucleophilic substitution with arylpiperazines **a–c** to intermediates **14a–c**, and basic hydrolysis to the target acids **6a–c**.



Scheme 1. Reagents and conditions: (i) Ar-piperazine (for Ar, see Fig. 2), DIPEA or Et_3N, DMSO, 120 °C.

Following an analogous procedure, 6-methoxyquinolone target acids **7a–c** were prepared starting from ethyl 7-fluoro-4-hydroxy-6-methoxyquinoline-3-carboxylate **15**[13] (Scheme 2) through intermediates **16** and **17a–c**. The nucleophilic reaction was carried out using *N*-methyl-2-pyrrolidone to make the 7-fluorine displacement possible.

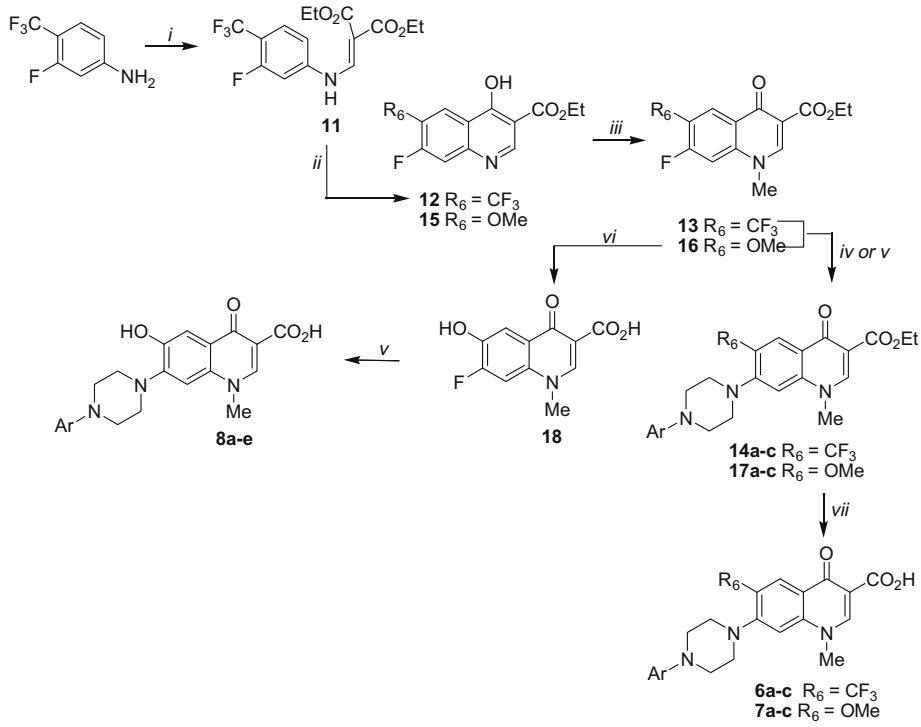
A first attempt to directly prepare the corresponding 6-hydroxy acids **8a–c** by treating the 6-methoxy esters **17a–c** with 48% HBr was disappointed since very low yield were obtained. On the other hand, the de-O-methylation did not proceed when mild conditions such as $AlCl_3$ or PBr_3, were used. In contrast, intermediate **16** was easily de-O-methylated using 48% HBr which gave the acid **18** which was then C-7 functionalized with arylpiperazines **a–c** as well as with 2-(1-piperazinyl)pyrazine (**d**),[14] and 2-(1-piperazinyl)quinoline (**e**),[15] to afford the target acids **8a–e** in good yield.

3. Results and discussion

All of the new synthesized quinolones were initially evaluated for anti-HIV-1 (III_B) and anti-HIV-2 (ROD) activity in MT-4 cells, using the MTT assay and expressed as the concentration of compound required to protect 50% (EC_{50}) of the MT-4 cells from HIV-induced cytopathogenicity. The cytotoxicity of the compounds was determined in parallel and expressed as the concentration of compound that reduces the viability of mock-infected cells by 50% (CC_{50}). For comparative purposes, compound **WM5**, nucleoside reverse transcriptase inhibitor, AZT, and non-nucleoside reverse transcriptase inhibitor, nevirapine, were assayed in the same cells.

The synthesis of the two series of N-1 unsubstituted quinolones **4a–c** and **5a–c** gave disappointing results. In fact, non-reproducible biological data (not shown) were obtained assaying the compounds in triplicate. This behavior is not attributable to their instability since this issue was tested. The low solubility in the DMSO/H_2O mixture used for the biological test, could instead account for this behavior. Consequently, these compounds did not contribute to the SAR widening but only discouraged the synthesis of further analogs.

Thus, attention was then focused exclusively on the C-6 position, keeping the methyl group at N-1 position because it gives higher solubility. Regarding the C-6 modified quinolones (Table



Scheme 2. Reagents and conditions: (i) EMME, 120 °C; (ii) PPA, 100 °C; (iii) MeI, K_2CO_3, DMF, 100 °C; (iv) Ar-piperazine, (for Ar, see Fig. 2), Et_3N, DMSO, 120 °C; (v) *N*-methyl-2-pyrrolidone, 105 °C; (vi) 48% HBr, reflux; (vii) 4% NaOH, reflux.

Table 1
Anti-HIV-1 and -HIV-2 activity and cytotoxicity of quinolones in MT-4 cells

Compound	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]
6a	>13.93	>13.93	13.93 ± 0.35	<1	<1
6b	>1.65	>1.65	1.65 ± 1.27	<1	<1
6c	>0.54	>0.54	0.54 ± 0.08	<1	<1
7a	0.47 ± 0.02	0.40 ± 0.09	2.55 ± 0.13	5	6.5
7c	>0.05	>0.05	0.05 ± 0.03	<1	<1
8a	1.85 ± 0.76	1.31 ± 0.89	>125	>68	>96
8b	⩾1.13	⩾0.133	1.13 ± 0.90	⩽1	⩽8
8c	>0.02	>0.02	0.02 ± 0.00	<1	<1
8d	>125	>125	>125	<1	<1
8e	0.080 ± 0.021	0.033 ± 0.010	0.47 ± 0.02	6	14
1[e]	0.058 ± 0.021	0.097 ± 0.053	0.84 ± 0.40	15	10
Nevirapine	0.023 ± 0.013	>4	>4	>176	
AZT	0.0015 ± 0.0005	0.0008 ± 0.0003	2.01 ± 0.35	1340	2475

[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 three separate experiments.
[d] SI: ratio of CC_{50}/EC_{50}.
[e] Data from Ref. 3.

1), the 6-trifluoromethyl derivatives **6a–c** showed variable cytotoxic effects, with **6a** being the less cytotoxic. However, all of these derivatives were completely devoid of any antiviral properties at concentrations below the cytotoxic levels. On the contrary, in the 6-methoxyquinolone series **7a–c** the presence of the 1-(2-pyridinyl)piperazine at the C-7 position resulted in derivative **7a** which was endowed with good antiviral activity against both HIV-1 and HIV-2 and showed reduced cytotoxicity compared to the lead compound **1**.[1,3] The presence of a 7-benzothiazolpiperazine gave the toxic compound **7c**, while, the antiviral results were not reproducible for the 7-(trifluoromethyl)phenylpiperazinyl derivative **7b** (not shown).

Regarding the 6-hydroxy quinolone derivatives **8a–c**, contrasting biological results were obtained, but a new interesting compound was obtained. In fact, the 7-pyridinylpiperazinyl derivative **8a** was devoid of any cytoxicity (CC_{50} >125 μg/mL) which when coupled with the good anti-HIV activity led to SI values >68 and >96 in MT-4 cells for HIV-1 and HIV-2, respectively.

Thus, in an attempt to increase the anti-HIV potency, while maintaining the absence of toxicity, the 6-hydroxy series was enlarged by synthesizing derivatives **8d** and **8e**, bearing the 2-(1-piperazinyl)pyrazine and 2-(1-piperazinyl)quinoline at the C-7 position, respectively, which are strict analogs of the 1-(2-pyridinyl)piperazine. Contrasting results were once again obtained. Derivative **8d** was neither toxic nor active while **8e** recovered a very potent anti-HIV activity against both HIV-1 and HIV-2 but also showed a more pronounced level of toxicity.

When compound **8e** was compared to clinically used drugs such as non-nucleoside and nucleoside reverse transcriptase inhibitors, nevirapine and AZT (Table 1), it was less active (particularly with

respect to AZT) and slightly more toxic. However, since 6-DFQs act on an innovative step of the replicative cycle, even though they are less potent, they could be promising candidates for use in association with the currently used cocktail of drugs to fight resistance and control HIV latency.

Molecules displaying a good profile in MT-4 cell lines, were also evaluated in acutely infected PBMCs from healthy donors (Table 2). Interestingly, all derivatives maintained good anti-HIV-1 activity confirming 6-hydroxy derivatives **8a** and **8e** as the most selective and potent quinolones obtained in this study.

4. Conclusion

In summary, starting from the novelties highlighted in the recent SAR for the anti-HIV 6-DFQs, innovative quinolone structures were designed and synthesized. While the N-1 unsubstituted quinolone series **4** and **5** was unproductive, new insights about the role of the substituents at C-6 position, emerged. The trifluoromethyl group is absolutely not suitable, while the methoxy group can occupy this position when coupled with an appropriate 4-arylpiperazine at the neighboring C-7 position. Similar observations as for the 6-methoxy derivatives but more marked were apparent in the 6-hydroxy quinolone series **8** where arylpiperazines **a** and **d** gave compounds with no observed cytotoxicity, while arylpiperazine **e** produced a very potent derivative. These data showed a strict interdependency between the C-6 and C-7 substituents in modulating the biological property, in agreement with what has been previously observed for other series of 6-DFQs.[1–3] In an efforts to understand the C-6/C-7 relationship, a molecular modeling study will be performed to determine the extent to which the arylpiperazine conformation is influenced by the nature of the C-6 substituent.

A clear indication of structure/cytotoxicity relationship emerged for the C-7 substituent itself, where, independently from the C-6 substituent, the 1-(2-pyridinyl)piperazine (**a**) produced the less toxic compounds, followed by the 1-[3-(trifluoromethyl)phenyl]piperazine (**b**) and finally by 2-(1-piperazinyl)-1,3-benzothiazole (**c**), which characterizes the most toxic derivatives.

Thus, in this study new interesting anti-HIV quinolones have been disclosed (**7a**, **8a** and **8e**), and the hydroxyl group has been identified as a new suitable C-6 substituent, together with the known NH_2 group and the H atom. However, it was not possible to reduce the cytotoxicity while maintaining the high anti-HIV potency. Perhaps this goal will only be reached when the mechanism of action of the 6-DFQs is definitively clarified. Many papers have reported that these compounds are Tat-mediated transcription inhibitors,[1,3,16–18] but their molecular target has not been fully identified. Fishing for the target is, at the moment, one of our principal interests, but it is not an easy task since there are so many cellular and viral components involved in the regulation of transcription.

Table 2
Anti-HIV-1 activity and cytotoxicity of selected quinolones in PBMCs

Compound	HIV-1(III_B) EC_{50} (μg/mL)[a,c]	CC_{50} (μg/mL)[b,c]	SI[d]
7a	1.78 ± 0.17	8.46 ± 5.61	5
8a	0.39 ± 0.05	⩾17.16	⩾44
8b	0.40 ± 0.06	1.61 ± 0.52	4
8e	0.26 ± 0.12	1.91 ± 0.12	7

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

5. Experimental

5.1. Chemistry

All reaction 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 Mod. 9100) and are uncorrected. Elemental analyses were performed on a Carlo Erba elemental analyzer, Model 1106, 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 solvent 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 commercial suppliers and were used as such. Organic solvents were dried over anhydrous Na_2SO_4 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.

5.2. General procedure for coupling reaction (method A)

A mixture of the appropriate synthone (**9**,[15] **10**[11] and **13**) (1 equiv), selected arylpiperazine (3 equiv) and DIPEA or Et_3N (4 equiv), in DMSO was heated at 120 °C until no starting material could be detected by TLC (6–48 h). The reaction mixture was then poured into ice/water and the precipitate was filtered, washed with water and EtOH, and crystallized by EtOH/DMF.

5.2.1. 4-Hydroxy-7-(4-pyridin-2-ylpiperazin-1-yl)quinoline-3-carboxylic acid (4a)

The title compound was prepared starting from synthone **9**[15] through the general procedure for coupling reaction (method A), using 1-(2-pyridinyl)piperazine (**a**) and DIPEA (46 h) in 59% yield: mp 267–268 °C; ^{1}H NMR (DMSO-d_6) δ 3.50–3.55 and 3.65–3.75 (m, each 4H, piperazine CH_2), 6.65 (dd, J = 7.1 and 4.9 Hz, 1H, pyridine CH), 6.90 (d, J = 8.5 Hz, 1H, pyridine CH), 6.95 (br s, 1H, H-8), 7.35 (d, J = 9.2 Hz, 1H, H-6), 7.50–7.60 (m, 1H, pyridine CH), 8.10 (d, J = 9.2 Hz, 1H, H-5), 8.15–8.25 (m, 1H, pyridine CH), 8.70 (s, 1H, H-2), 12.80 (br s, 1H, OH), 15.80 (s, 1H, COOH). Anal. Calcd for $C_{19}H_{18}N_4O_3$: C, 65.13; H, 5.18; N, 15.99. Found: C, 65.23; H, 5.00; N, 16.10.

5.2.2. 4-Hydroxy-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}quinoline-3-carboxylic acid (4b)

The title compound was prepared starting from synthone **9**[15] through the general procedure for coupling reaction (method A), using 1-[3-(trifluoromethyl)phenyl]piperazine (**b**) and DIPEA (27 h) in 55% yield: mp 281–283 °C; ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 and 3.50–3.60 (m, each 4H, piperazine CH_2), 7.00 (s, 1H, H-8), 7.10 (d, J = 7.6 Hz, 1H, aromatic CH), 7.25 (s, 1H, aromatic CH), 7.30 (d, J = 8.5 Hz, 1H, aromatic CH), 7.40 (dd, J = 9.2 and 2.0 Hz, 1H, H-6), 7.40–7.50 (m, 1H, aromatic CH), 8.10 (d, J = 9.2 Hz, 1H, H-5), 8.75 (s, 1H, H-2), 12.80 (br s, 1H, OH), 15.80 (s, 1H, COOH). Anal. Calcd for $C_{21}H_{18}F_3N_3O_3$: C, 60.43; H, 4.35; N, 10.07. Found: C, 60.05; H, 4.55; N, 10.31.

5.2.3. 7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-4-hydroxyquinoline-3-carboxylic acid (4c)

The title compound was prepared starting from synthone **9**[15] through the general procedure for coupling reaction (method A), using 2-(1-piperazinyl)-1,3-benzothiazole (**c**)[12] and DIPEA (48 h) in 60% yield: mp 275–277 °C; ^{1}H NMR (DMSO-d_6) δ 3.50–3.60

and 3.70–3.80 (m, each 4H, piperazine CH_2), 7.00 (br s, 1H, H-8), 7.10 and 7.30 (t, J = 7.8 Hz, each 1H, benzothiazole CH), 7.35 (br d, J = 9.2 Hz, 1H, H-6), 7.50 and 7.80 (d, J = 7.8 Hz, each 1H, benzothiazole CH), 8.10 (d, J = 9.2 Hz, 1H, H-5), 8.70 (s, 1H, H-2), 12.50 (br s, 1H, OH), 15.80 (s, 1H, COOH). Anal. Calcd for $C_{21}H_{18}N_4O_3S$: C, 62.05; H, 4.46; N, 13.78. Found: C, 62.10; H, 4.62; N, 13.45.

5.2.4. 6-Fluoro-4-hydroxy-7-(4-pyridin-2-ylpiperazin-1-yl)quinoline-3-carboxylic acid (5a)

The title compound was prepared starting from synthone **10**[11] through the general procedure for coupling reaction (method A), using 1-(2-pyridinyl)piperazine (**a**) and Et_3N (11 h) in 58% yield: mp 279–280 °C; ^{1}H NMR (DMSO-d_6) δ 3.25–3.35 and 3.65–3.75 (m, each 4H, piperazine CH_2), 6.65–6.75 (m, 1H, pyridine CH), 6.85 (d, J = 8.9 Hz, 1H, H-8), 7.30 (d, J = 7.0 Hz, 1H, pyridine CH), 7.50–7.65 (m, 1H, pyridine CH), 7.85 (d, J = 13.4 Hz, 1H, H-5), 8.10–8.20 (m, 1H, pyridine CH), 8.80 (s, 1H, H-2), 13.25 (br s, 1H, OH), 15.40 (s, 1H, COOH). Anal. Calcd for $C_{19}H_{17}FN_4O_3$: C, 61.95; H, 4.65; N, 15.21. Found: C, 62.15; H, 4.60; N, 15.14.

5.2.5. 6-Fluoro-4-hydroxy-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}quinoline-3-carboxylic acid (5b)

The title compound was prepared starting from synthone **10**[11] through the general procedure for coupling reaction (method A), using 1-[3-(trifluoromethyl)phenyl]piperazine (**b**) and Et_3N (6 h) in 77% yield: mp 315–316 °C; ^{1}H NMR (DMSO-d_6) δ 3.30–3.50 (m, 8H, piperazine CH_2), 7.10 (d, J = 7.5 Hz, 1H, aromatic CH), 7.20–7.35 (m, 3H, H-8 and aromatic CH), 7.40–7.50 (m, 1H, aromatic CH), 7.85 (d, J = 13.4 Hz, 1H, H-5), 8.80 (s, 1H, H-2), 15.50 (s, 1H, COOH). Anal. Calcd for $C_{21}H_{17}F_4N_3O_3$: C, 57.93; H, 3.94; N, 9.65. Found: C, 57.99; H, 4.12; N, 9.48.

5.2.6. 7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-6-fluoro-4-hydroxyquinoline-3-carboxylic acid (5c)

The title compound was prepared starting from synthone **10**[11] through the general procedure for coupling reaction (method A), using 2-(1-piperazinyl)-1,3-benzothiazole (**c**)[12] and Et_3N (24 h) in 37% yield: mp 313–315 °C; ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 and 3.70–3.85 (m, each 4H, piperazine CH_2), 7.05–7.15 (m, 1H, benzothiazole CH), 7.25–7.35 (m, 2H, benzothiazole CH and H-8), 7.50 (d, J = 7.8 Hz, 1H, benzothiazole CH), 7.65–7.75 (m, 1H, benzothiazole CH), 7.85 (d, J = 13.4 Hz, 1H, H-5), 8.85 (s, 1H, H-2), 15.50 (s, 1H, COOH). Anal. Calcd for $C_{21}H_{17}FN_4O_3S$: C, 59.42; H,4.04; N 13.20. Found: C, 59.47; H, 4.36; N, 13.00.

5.2.7. Ethyl 1-methyl-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-6-(trifluoromethyl)-1,4-dihydroquinoline-3-carboxylate (14a)

The title compound was prepared starting from synthone **13** through the general procedure for coupling reaction (method A), using 1-(2-pyridinyl)piperazine (**a**) and Et_3N (24 h) in 58% yield: mp 136–137 °C; ^{1}H NMR ($CDCl_3$) δ 1.45 (t, J = 7.1 Hz, 3H, CH_2CH_3), 3.15–3.25 and 3.75–3.85 (m, each 4H, piperazine CH_2), 3.90 (s, 3H, CH_3), 4.45 (q, J = 7.1 Hz, 2H, CH_2CH_3), 6.65–6.85 (m, 2H, pyridine CH), 7.10 (s, 1H, H-8), 7.60–7.65 (m, 1H, pyridine CH), 8.15–8.25 (m, 1H, pyridine CH), 8.48 (s, 1H, H-5), 8.75 (s, 1H, H-2).

5.2.8. Ethyl 1-methyl-4-oxo-6-(trifluoromethyl)-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydroquinoline-3-carboxylate (14b)

The title compound was prepared starting from synthone **13** through general procedure for coupling reaction (method A), using 1-[3-(trifluoromethyl)phenyl]piperazine (**b**) and Et_3N (48 h) in 71% yield: mp 177–178 °C; ^{1}H NMR (DMSO-d_6) δ 1.50 (t, J = 7.0 Hz, 3H, CH_2CH_3), 3.15–3.25 and 3.35–3.45 (each 4H, m, piperazine CH_2), 4.20 (s, 3H, CH_3), 4.40 (q, J = 7.0 Hz, 2H, CH_2CH_3), 7.25–7.35 (m, 1H, aromatic CH), 7.45–7.55 (m, 2H, H-8 and aromatic CH), 7.60–7.70 and 7.80–7.85 (m, each 1H, aromatic CH), 8.65 (s, 1H, H-5), 8.85 (s, 1H, H-2).

5.2.9. Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-methyl-4-oxo-6-(trifluoromethyl)-1,4-dihydroquinoline-3-carboxylate (14c)

The title compound was prepared starting from synthone **13** through general procedure for coupling reaction (method A), using 2-(1-piperazinyl)-1,3-benzothiazole (**c**)[12] and Et_3N (36 h). After flash column chromatography purification (1% MeOH/CH_2Cl_2) the title compound was obtained in 26% yield: mp 215–216 °C; ^{1}H NMR ($CDCl_3$) δ 1.45 (t, J = 7.2 Hz, 3H, CH_2CH_3), 3.15–3.25 (m, 4H, piperazine CH_2), 3.80 (s, 3H, CH_3), 3.85–3.95 (m, 4H, piperazine CH_2), 4.45 (q, J = 7.2 Hz, 2H, CH_2CH_3), 7.10–7.20 (m, 2H, H-8 and aromatic CH), 7.30–7.40, 7.55–7.65 and 7.70–7.75 (m, each 1H, aromatic CH), 8.45 (s, 1H, H-5), 8.80 (s, 1H, H-2).

5.3. Diethyl 2-({[3-fluoro-4-(trifluoromethyl)phenyl]amino}methylene)malonate (11)

A mixture of 3-fluoro-4-(trifluoromethyl)aniline (5.0 g, 27.9 mmol) and EMME (6.02 g, 27.9 mmol) was heated at 120 °C for 2.5 h. The mixture was then evaporated to dryness to give a residue which was triturated with cyclohexane to give a solid which was filtered and dried to afford compound **11** (5.8 g, 59%): mp 88–89 °C; ^{1}H NMR ($CDCl_3$) δ 1.30–1.45 (m, 6H, CH_2CH_3), 4.20–4.40 (m, 4H, CH_2CH_3), 6.90–7.00 (m, 2H, H-2 and H-6), 7.55–7.65 (m, 1H, H-5), 8.45 (d, J = 13.2 Hz, 1H, vinyl-H), 11.10 (d, J = 13.2 Hz, 1H, NH).

5.4. Ethyl 7-fluoro-1-methyl-4-oxo-6-(trifluoromethyl)-1,4-dihydroquinoline-3-carboxylate (13)

A mixture of **11** (5.5 g, 15.7 mmol) and PPA was heated at 100 °C for 2 h, then poured into ice/water and neutralized with 10% NaOH. The obtained precipitate was filtered, washed with water and with a mixture of $CHCl_3$/EtOH and dried to give ethyl 7-fluoro-4-hydroxy-6-(trifluoromethyl)quinoline-3-carboxylate (**12**) (1.5 g, 31%), that was used in the next step without further characterization due to its low solubility in the common deuterated solvent. To a mixture of synthone **12** (1 g, 32 mmol) and K_2CO_3 (1.13 g, 82 mmol) in DMF (5 mL), a solution of MeI (0.93 g, 65 mmol) in DMF (2 mL) was added. The mixture was heated to 100 °C for 12 h and then poured into ice/water. The obtained precipitate was filtered, dried and purified by flash chromatography, eluting with MeOH/$CHCl_3$ (2%) to give **13** (0.5 g, 49%): mp 177–178 °C; ^{1}H NMR ($CDCl_3$) δ 1.48 (t, J = 7.1 Hz, 3H, CH_2CH_3), 3.80 (s, 3H, CH_3), 4.40 (q, J = 7.1 Hz, 2H, CH_2CH_3), 7.25 (d, J = 11.2 Hz, 1H, H-8), 8.50 (s, 1H, H-2), 8.80 (d, J = 7.9 Hz, 1H, H-5).

5.5. General procedure for basic hydrolysis

A suspension of selected ester (**14a–c** and **17a–c**) (0.3 m equiv) in 4% NaOH (5 mL) was refluxed until no starting material could be detected by TLC (15 min–6 h) and worked and purified as defined in the description of the compounds.

5.5.1. 1-Methyl-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-6-(trifluoromethyl)-1,4-dihydroquinoline-3-carboxylic acid hydrochloride (6a)

The title compound was prepared starting from **14a** through the general procedure for basic hydrolysis (6 h): after cooling, the precipitate was filtered, suspended in 2 N HCl (2 mL) and refluxed for 1 h. After cooling, the hydrochloride was filtered, washed with water, EtOH and finally recrystallized by EtOH/DMF to give **6a** in

40% yield: mp 287–288 °C; ^{1}H NMR (DMSO-d_6) δ 3.20–3.30 and 3.80–3.90 (m, each 4H, piperazine CH_2), 4.15 (s, 3H, CH_3), 6.85–6.95 and 7.30–7.40 (m, each 1H, pyridine CH), 7.75 (s, 1H, H-8), 7.85–8.00 and 8.10–8.20 (m, each 1H, pyridine CH), 8.60 (s, 1H, H-5), 9.10 (s, 1H, H-2). Anal. Calcd for $C_{21}H_{20}ClF_3N_4O_3$: C, 58.33; H, 4.43; N, 12.96. Found: C, 58.18; H, 4.27; N, 12.99.

5.5.2. 1-Methyl-4-oxo-6-(trifluoromethyl)-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydroquinoline-3-carboxylic acid (6b)

The title compound was prepared starting from **14b** through general procedure for basic hydrolysis, (2 h): after cooling, the mixture was completely acidified by adding 2 N HCl and the solid obtained was collected by filtration, washed with water, EtOH and finally recrystallized by EtOH/DMF to give **6b** in 4% yield: mp 233–234 °C; ^{1}H NMR (DMSO-d_6) δ 3.20–3.30 and 3.70–3.80 (m, each 4H, piperazine CH_2), 4.20 (s, 3H, CH_3), 7.10–7.20 (m, 1H, aromatic CH), 7.25–7.35 (m, 2H, aromatic CH and H-8), 7.40–7.50 and 7.70–7.80 (m, each 1H, and aromatic CH), 8.60 (s, 1H, H-5), 9.10 (s, 1H, H-2), 14.80 (s, 1H, COOH). Anal. Calcd for $C_{23}H_{19}F_6N_3O_3$: C, 55.31; H, 3.83; N, 8.41. Found: C, 55.55; H, 3.98; N, 8.21.

5.5.3. 7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-1-methyl-4-oxo-6-(trifluoromethyl)-1,4-dihydroquinoline-3-carboxylic acid (6c)

The title compound was prepared starting from **14c** through general procedure for basic hydrolysis (1 h): after cooling, the mixture was completely acidified by adding 2 N HCl and the solid obtained was collected by filtration, washed with water and EtOH and finally recrystallized by EtOH/DMF to give **6c** in 49% yield: mp 326–327 °C; ^{1}H NMR (DMSO-d_6) δ 3.20–3.30 and 3.35–3.45 (m, each 4H, piperazine CH_2), 4.15 (s, 3H, CH_3), 7.10–7.20, 7.25–7.35 and 7.40–7.50 (m, each 1H, aromatic CH), 7.75–7.85 (m, 2H, H-8 and aromatic CH), 8.60 (s, 1H, H-5), 9.10 (s, 1H, H-2), 14.75 (s, 1H, COOH). Anal. Calcd for $C_{23}H_{19}F_3N_4O_3S$: C, 56.55; H, 3.92; N, 11.47. Found: C, 83; H, 3.75; N, 11.59.

5.5.4. 6-Methoxy-1-methyl-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid hydrochloride (7a)

The title compound was prepared starting from **17a** through general procedure for basic hydrolysis (30 min): after cooling, the precipitate was filtered, suspended in 2 N HCl and refluxed for 1 h. After cooling, the precipitate was filtered, washed with water, EtOH and finally recrystallized by EtOH/DMF to give **7a** in 98% yield: mp 286–288 °C; ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 and 3.75–3.85 (m, each 4H, piperazine CH_2), 3.95 and 4.05 (s, each 3H, CH_3 and OCH_3), 6.80–6.90 (m, 1H, pyridine CH), 7.10 (s, 1H, H-8), 7.20–7.30 (m, 1H, pyridine CH), 7.65 (s, 1H, H-5), 7.80–7.90 and 8.10–8.20 (m, each 1H, pyridine CH), 8.90 (s, 1H, H-2), 15.75 (br s, 1H, COOH). Anal. Calcd for $C_{21}H_{23}ClN_4O_4$: C, 63.95; H, 5.62; N, 14.20. Found: C, 64.09; H, 5.73; N, 14.02.

5.5.5. 6-Methoxy-1-methyl-4-oxo-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydroquinoline-3-carboxylic acid (7b)

The title compound was prepared starting from **17b** through general procedure for basic hydrolysis (15 min): after cooling, the mixture was neutralized by adding 2 N HCl and the solid obtained was collected by filtration, washed with water, EtOH and finally recrystallized by EtOH/DMF to give **7b** in 53% yield: mp 258–259 °C; ^{1}H NMR (DMSO-d_6) δ 3.30–3.45 (m, 8H, piperazine CH_2), 3.95 and 4.05 (s, each 3H, CH_3 and OCH_3), 7.05–7.15 (m, 2H, H-8 and aromatic CH), 7.20–7.35 (m, 2H, H-5 and aromatic CH), 7.40–7.50 and 7.65–7.75 (m, each 1H, aromatic CH), 8.85 (s, 1H, H-2), 15.75 (s, 1H, COOH). Anal. Calcd for $C_{23}H_{22}F_3N_3O_4$: C, 59.87; H, 4.81; N, 9.11. Found: C, 60.05; H, 4.83; N, 9.07.

5.5.6. 7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-6-methoxy-1-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (7c)

The title compound was prepared starting from **17c** through general procedure for basic hydrolysis (15 min): after cooling, the mixture was neutralized by adding 2 N HCl and the solid obtained was collected by filtration, washed with water, EtOH and finally recrystallized by EtOH/DMF to give **7c** in 68% yield: mp 279–280 °C; ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 and 3.75–3.85 (m, each 4H, piperazine CH_2), 3.90–4.10 (m, each 3H, CH_3 and OCH_3), 7.10–7.20 (m, 2H, H-8 and aromatic CH), 7.30–7.40 (m, 1H, aromatic CH), 7.50–7.90 (m, 3H, H-5 and aromatic CH), 8.90 (s, 1H, H-2). Anal. Calcd for $C_{23}H_{22}N_4O_4S$: C, 61.32; H, 4.92; N, 12.44. Found: C, 61.70; H, 4.69; N, 12.54.

5.6. Ethyl 7-fluoro-6-methoxy-1-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (16)

Starting from intermediate **15**[13] and following the procedure as used for the synthesis of compound **13** (75 °C, 1 h), the title compound was obtained in 95% yield: mp 220–221 °C; ^{1}H NMR (DMSO-d_6) δ 1.25 (t, J = 6.7 Hz, 3H, CH_2CH_3), 3.85 (s, 3H, CH_3), 3.95 (s, 3H, OCH_3), 4.20 (t, J = 6.7 Hz, 2H, CH_2CH_3), 7.70–7.80 (m, 2H, H-5 and H-8), 8.60 (s, 1H, H-2).

5.7. General procedure for coupling reaction (method B)

A mixture of the appropriate synthone (**16** and **18**) (1 equiv) and selected arylpiperazine (6 equiv) in *N*-methyl-2-pyrrolidone, was heated at 105 °C until no starting material could be detected by TLC (15–24 h) and worked and purified as defined in the description of the compounds.

5.7.1. Ethyl 6-methoxy-1-methyl-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-1,4-dihydroquinoline-3-carboxylate (17a)

The title compound was prepared starting from synthone **16** through general procedure for coupling reaction (method B), using 1-(2-pyridinyl)piperazine (**a**) (24 h). The reaction mixture was poured into ice/water giving a solid which was filtered, washed with water and Et_2O to give **17a** in 60% yield: mp 206–207 °C; ^{1}H NMR (DMSO-d_6) δ 1.25 (t, J = 7.0 Hz, 3H, CH_2CH_3), 3.20–3.30 and 3.55–3.65 (m, each 4H, piperazine CH_2), 3.90 (s, 6H, CH_3 and OCH_3), 4.20 (q, J = 7.0 Hz, 2H, CH_2CH_3), 6.60–6.70 and 6.80–6.90 (m, each 1H, pyridine CH), 6.95 (s, 1H, H-8), 7.50–7.65 (m, 2H, pyridine CH and H-5), 8.10–8.20 (m, 1H, pyridine CH), 8.55 (s, 1H, H-2).

5.7.2. Ethyl 6-methoxy-1-methyl-4-oxo-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydroquinoline-3-carboxylate (17b)

The title compound was prepared starting from synthone **16** through general procedure for coupling reaction (method B), using 1-[3-(trifluoromethyl)phenyl]piperazine (**b**) (24 h). The reaction mixture was poured into ice/water and extracted with EtOAc; the organic layers were evaporated to give an oil which by treatment with H_2O/MeOH gave a solid which was filtered and dried to give **17b** in 55% yield: mp 127–128 °C; ^{1}H NMR (DMSO-d_6) δ 1.25 (t, J = 7.0 Hz, 3H, CH_2CH_3), 3.30–3.40 and 3.70–3.80 (m, each 4H, piperazine CH_2), 3.85–3.95 (br s, 6H, CH_3 and OCH_3), 4.20 (q, J = 7.0 Hz, 2H, CH_2CH_3), 6.90–7.00 and 7.05–7.15 (m, each 1H, aromatic CH), 7.20–7.30 (m, 2H, aromatic CH and H-8), 7.45–7.55 (m, 1H, aromatic CH), 7.65 (s, 1H, H-5),8.55 (s, 1H, H-2).

5.7.3. Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-6-methoxy-1-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (17c)

The title compound was prepared starting from synthone **15** through general procedure for coupling reaction (method B), using 2-(1-piperazinyl)-1,3-benzothiazole (**c**)[12] (24 h). The reaction mixture was poured into ice/water and extracted with EtOAc; the organic layers were evaporated to give an oil which by treatment with H_2O/MeOH gave a solid which was filtered and dried to give **17c** in 47% yield: mp 153–154 °C; ^{1}H NMR (DMSO-d_6) δ 1.25 (t, J = 6.6 Hz, 3H, CH_2CH_3), 3.25–3.40 and 3.70–3.80 (m, each 4H, piperazine CH_2), 3.90 (s, 6H, CH_3 and OCH_3), 4.20 (q, J = 6.6 Hz, 2H, CH_2CH_3), 6.95 (s, 1H, H-8), 7.05–7.15, 7.20–7.30 and 7.40–7.50 (m, each 1H, aromatic CH),7.65 (s, 1H, H-5) 7.75–7.85 (m, 1H, aromatic CH), 8.55 (s, 1H, H-2).

5.7.4. 6-Hydroxy-1-methyl-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (8a)

The title compound was prepared starting from synthone **18** through the general procedure for coupling reaction (method B), using 1-(2-pyridinyl)piperazine (**a**) (15 h). After cooling, the mixture was poured into ice/water and acidified with 2 N HCl (pH 6) giving a solid which was filtered, washed with water, dried and recrystallized by DMF to give **8a** in 44% yield: mp 314–315 °C; ^{1}H NMR (DMSO-d_6) δ 3.35–3.45 and 3.65–3.75 (m, each 4H, piperazine CH_2), 4.10 (s, 3H, CH_3), 6.65–6.75 and 6.90–7.00 (m, each 1H, pyridine CH), 7.10 (s, 1H, H-8), 7.50–7.60 (m, 1H, pyridine CH), 7.75 (s, 1H, H-5), 8.10–8.20 (m, 1H, pyridine CH), 8.80 (s, 1H, H-2), 10.70 (s, 1H, OH), 16.00 (s, 1H, COOH). Anal. Calcd for $C_{20}H_{20}N_4O_4$: C, 63.15; H, 5.30; N, 14.73. Found: C, 63.28; H, 5.17; N, 14.92.

5.7.5. 6-Hydroxy-1-methyl-4-oxo-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydroquinoline-3-carboxylic acid (8b)

The title compound was prepared starting from synthone **18** following the same procedure as used for the synthesis of compound **8a**, replacing the 1-(2-pyridinyl)piperazine (**a**) with the 1-[3-(trifluoromethyl)phenyl]piperazine (**b**) in 56% yield: mp 318–319 °C; ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 (m, 8H, piperazine CH_2), 4.15 (s, 3H, CH_3), 7.05–7.15 (m, 2H, H-8 and benzothiazole CH), 7.20–7.30 (m, 2H, benzothiazole CH), 7.40–7.50 (m, 1H, benzothiazole CH), 7.65 (m, 1H, H-5), 8.80 (s, 1H, H-2), 10.50 (br s, 1H, OH), 16.00 (s, 1H, COOH). Anal. Calcd for $C_{22}H_{20}F_3N_3O_4$: C, 59.06; H, 4.51; N, 9.39. Found: C, 59.21; H, 4.38; N, 9.27.

5.7.6. 7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-6-hydroxy-1-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (8c)

The title compound was prepared starting from synthone **17** following the same procedure as used for the synthesis of compound **8a**, replacing the 1-(2-pyridinyl)piperazine (**a**) with the 2-(1-piperazinyl)-1,3-benzothiazole (**c**)[12] in 57% yield: mp >330 °C; ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 and 3.75–3.85 (m, each 4H, piperazine CH_2), 4.10 (s, 3H, CH_3), 7.05–7.15 (m, 2H, H-8 and aromatic CH), 7.25–7.35 and 7.45–7.55 (m, each 1H, aromatic CH), 7.70 (s, 1H, H-5), 7.75–7.85 (m, 1H, aromatic CH), 8.80 (s, 1H, H-2), 10.70 (s, 1H, OH), 16.00 (s, 1H, COOH).

Anal. Calcd for $C_{22}H_{20}N_4O_4S$: C, 60.54; H, 4.62; N, 12.84. Found: C, 60.57; H, 4.62; N, 12.99.

5.7.7. 6-Hydroxy-1-methyl-4-oxo-7-(4-pyrazin-2-ylpiperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (8d)

The title compound was prepared starting from synthone **17** following the same procedure as used for the synthesis of compound **8a**, replacing the 1-(2-pyridinyl)piperazine (**a**) with the 2-(1-piperazinyl)pyrazine (**d**)[14] in 57% yield: mp >330 °C; ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 and 3.80–3.90 (m, each 4H, piperazine CH_2), 4.10 (s, 3H, CH_3), 7.10 (s, 1H, H-8), 7.70 (s, 1H, H-5), 7.90, 8.20 and 8.45 (s, each 1H, pyrazine CH), 8.80 (s, 1H, H-2), 10.70 (s, 1H, OH), 16.00 (s, 1H, COOH). Anal. Calcd for $C_{19}H_{19}N_5O_4$: C, 59.84; H, 5.02; N, 18.36. Found: C, 59.92; H, 5.00; N, 18.48.

5.7.8. 6-Hydroxy-1-methyl-4-oxo-7-(4-quinolin-2-ylpiperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (8e)

The title compound was prepared starting from synthone **18** following the same procedure as used for the synthesis of compound **8a**, replacing the 1-(2-pyridinyl)piperazine (**a**) with the 2-(1-piperazinyl)quinoline (**e**)[15] in 71% yield: mp 297 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.40–3.50 and 3.90–4.00 (m, each 4H, piperazine CH_2), 4.10 (s, 3H, CH_3), 7.10 (s, 1H, H-8), 7.30–7.50 (m, 2H, quinoline CH), 7.60–7.80 (m, 4H, H-5 and quinoline CH), 8.15–8.20 (m, 1H, quinoline CH), 8.80 (s, 1H, H-2), 10.70 (s, 1H, OH), 16.00 (s, 1H, COOH). Anal. Calcd for $C_{24}H_{22}N_4O_4$: C, 66.97; H, 5.15; N, 13.02. Found: C, 66.96; H, 5.21; N, 12.98.

5.8. 7-Fluoro-6-hydroxy-1-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (18)

A mixture of compound **16** (0.1 g, 0.3 mmol) in 48% HBr (10 mL) was refluxed for 5 h. After cooling, the hydrobromide salt was filtered, solubilized in water and neutralized by adding 10% NaOH. The resulting precipitate was filtered, washed with water and EtOH, and dried to give the acid **18** in 82% yield: mp >330 °C; ^{1}H NMR ($CDCl_3$) δ 4.00 (s, 3H, CH_3), 7.90–8.00 (m, 2H, H-5 and H-8), 9.00 (s, 1H, H-2), 11.00 (s, 1H, OH), 15.40 (s, 1H, COOH).

5.9. In vitro anti-HIV 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.[19] Briefly, 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)[20] or HIV-2 (ROD)[21] 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[22] 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) from healthy donors were isolated by density centrifugation (Lymphoprep; Nycomed Pharma, AS Diagnostics, Oslo, Norway) and stimulated with phytohemagglutin (PHA) (Sigma Chemical Co., Bornem Belgium) for three 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.[23] 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 post-infection, 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 seven days after plating the cells, p24 antigen was detected in the culture supernatant by an enzyme-linked immunosorbent assay (Perkin Elmer, Brussels, Belgium).

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