Structure Modifications of 6-Aminoquinolones with Potent Anti-HIV Activity[1]

Oriana Tabarrini,[§] Miguel Stevens,[†] Violetta Cecchetti,[§] Stefano Sabatini,[§] Micaela Dell'Uomo,[§] Giuseppe Manfroni,[§] Manlio Palumbo,[#] Christophe Pannecouque,[†] Erik De Clercq,[†] and Arnaldo Fravolini*[,§]

Dipartimento di Chimica e Tecnologia del Farmaco, Università di Perugia, Via del Liceo 1, 06123 Perugia, Italy, Dipartimento di Scienze Farmaceutiche, Università di Padova, Via Marzolo 5, 35131 Padova, Italy, and Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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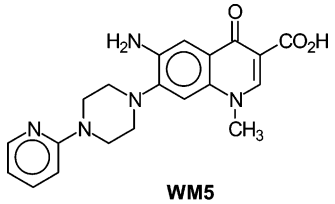
We have recently discovered that 6-aminoquinolone derivatives could be valid leads for the development of new anti-HIV agents because of their new and diversified mode of action. In fact, studies carried out on the lead WM5 showed that this derivative is able to inhibit the Tat-mediated long terminal repeat driven transcription, an essential step in the HIV-1 replication cycle. Thus, starting from lead WM5, we performed the design and synthesis of an enlarged series of 6-aminoquinolones, which permitted some very potent anti-HIV 6-amino derivatives to be obtained and the structure−activity relationship to be delineated. Some derivatives, **26c**, **26e**, **26i**, and **26j**, proved to be highly effective in inhibiting HIV replication at 50% inhibitory concentration in the range of 0.0087−0.7 μg/mL in MT-4, PBMCs and CEM cell lines coupled with positive selectivity indexes that reach values higher than 1000 on CEM cell lines for compounds **26e** and **26i**. Time-of-addition experiments clearly confirm that the new, potent 6-aminoquinolones interact at a postintegration step in the replication cycle of HIV.

Introduction

The rate of disease progression in AIDS patients has been significantly reduced through early and aggressive intervention with highly active antiretroviral therapy (HAART), which involves a combination of reverse transcriptase inhibitors and protease inhibitors. However, several serious problems still remain including multidrug resistance,[2] toxicity,[3] and high cost.[4] Moreover, the current antiretroviral regimens are unable to completely suppress viral replication, thereby allowing a latent reservoir of HIV-1 to persist which is the major documented hurdle to virus eradication[5] although other viral sanctuaries may exist.[6]

In the search for new anti-HIV agents, efforts to discover compounds with new and diversified modes of action are still a challenging task. Transcription of the viral genome (integrated proviral DNA) into its mRNA is an essential step in the HIV-1 replication cycle and is considered to be a good potential target for chemotherapeutic intervention[7] because it could allow the control of HIV-1 replication not only in acutely infected cells but also in chronically infected cells. Moreover, compounds that could interfere with this replication step would result in a lower incidence of drug resistance because HIV gene regulation requires the interplay of both viral and cellular components.[8]

In this context, quinolones have been pursued as new potential candidates for the treatment of AIDS.[9−14] Our studies in this area began with the random screening of our quinolone chemical library, including 6-fluoroquinolononones (6-FQs) and 6-desfluoroquinolononones (6-DFQs), in which we found that the 6-amino-1-*tert*-butyl-7-[4-(2-pyridinyl)-1-piperazinyl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid exhibited good anti-HIV-1 activity.[15] To pinpoint the structural features responsible for its antiviral activity and to explore the structural variations of this lead compound, we first prepared a series of 6-aminoquinolones variously substituted in the different positions of the quinolone nucleus.[14] The antiviral activity of this first series of compounds confirmed that the 6-aminoquinolones could provide valid leads for the research of new drugs for the treatment of AIDS. From this series, compound WM5 (Figure 1) was the most potent, inhibiting HIV-1 replication on de novo infected C8166 human lymphoblastoid cells, with an EC_{50} value of about 0.1 μM and a CC_{50} of 7 μM (SI = 70).[14] The same good antiviral activity was also observed in human T-lymphoid Jurkat cells and in chronically infected H9 cells, where WM5 was notably less cytotoxic with a CC_{50} value of 56 μM in Jurkat and no cytotoxicity was observed in H9 cells.[16,17] From the preliminarly structure−activity relationship (SAR) study of 6-aminoquinolones the structural features that grant the antiviral activity were determined: the presence of carboxylic acid at C-3, a small polar group at C-6, a bulky substituent at C-7, and a small substituent at N-1 of the quinolone moiety.



WM5

Figure 1. Structure of the lead compound WM5.

Regarding the mechanism of action, studies carried out on WM5 showed that this compound does not impair

* To whom correspondence should be addressed. Phone: +39 075 5855130. Fax: +39 075 585 5115. E-mail: fravo@unipg.it.
[§] Università di Perugia.
[†] Katholieke Universiteit Leuven.
[#] Università di Padova.

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the activities of the reverse transcriptase (RT), HIV integrase (I), and HIV protease (P).[17] Initially the transcomplementation assay used to examine the activity of WM5 on the replicative potential of HIV-1 in a single round of infection showed a sustained inhibition of Tat mediated long terminal repeat (LTR) driven transcription.[17] Moreover, preliminary binding studies using fluorimetric titration experiments and conducted in the presence of different RNA and DNA sequences indicated that WM5 selectively and efficiently complexes the transactivation responsive (TAR) element with a dissociation constant in the nanomolar range (19 ± 0.6 nM), suggesting a nucleic acid targeted mechanism of action.[14,17]

The indication that WM5 acts through an innovative mechanism of action validates the 6-aminoquinolones as attractive candidates for combination with the available antiretroviral drugs.

Although the 6-aminoquinolone WM5 and 6-FQs had high anti-HIV activities on all cell lines used, both acutely and chronically HIV-infected, they always displayed modest SI values that are strongly dependent on the used cell lines. Thus, in the synthesis of new quinolones, one of the main goals is to lower their cytotoxicity.

To rationalize the SAR for this promising class of anti-HIV agents, we previously performed a 3D-QSAR study[18] on a library of 6-FQs and 6-DFQs, including those taken from the literature as well as those synthesized in our laboratory. The results confirmed that high antiviral activity could be ensured by the hydrophilic region around the 4-keto-3-carboxylic moiety and a suitable hydrophobic region around the substituent at the C-7 position. Moreover, high antiviral activity seems to be related to the presence of a hydrophobic substituent at the N-1 position, which could also be a bulky group. Taking into account the indications from both our SAR and QSAR studies, further modifications at the N-1, C-6, C-7, and C-8 positions were planned in an effort to identify the structural groups capable of optimizing the antiviral activity and to reduce the cytotoxicity.

An informative survey of 6-aminoquinolone SAR showed that potency was fundamentally sensitive to substitution at the C-7 position where the 4-(2-pyridinyl)-1-piperazinyl substituent was shown to be the best. Therefore, in this study we turned our attention mainly toward the C-7 substituent where the pyridinylpiperazine was modified by replacing the pyridine ring at the N-4 piperazine core with aromatic heterocyclic or benzoheterocyclic groups such as thiazole (compound **26e**), pyrazine (compound **26i**), thiadiazole (compound **26l**), benzothiazoles (compounds **26d** and **26g)**, benzoxazole (compound **26j**), benzimidazole (compound **26h**), and benzothiazine (compound **26n**) or substituted phenyl nucleus (compounds **26b**, **26c**, **26f**, and **26k**) (Table 1). On the other hand, with the C-7 4-(2-pyridinyl)-1-piperazinyl maintained as the side chain, the NH_2 group at the C-6 position was replaced with an H atom, NO_2 group, and endocyclic N, as in compounds **45a**, **34a** and **49a**, respectively (Table 2). Three derivatives were supplemented at the C-8 position with an OMe as in compound **41a** and a fluorine atom as in compound **39a** and its nitro analogue **38a** (Table 2). The methyl substituent was always maintained at the N-1 position except for compound **27a** which had a ethyl group, compounds **28b**, **28c**, and **28d** which had a cyclopropyl group, and **29a**, **29d**, **30a**, **31a, 32a**, and **33d** characterized by a bulky substituent (Table 1).

All the synthesized quinolones were tested for their ability to inhibit both HIV-1 and HIV-2 replication in MT-4 cells and were also evaluated for cytotoxicity (Table 3). The anti-HIV activity of the most active compounds was also evaluated in the human lymphocytic CEM cell line (Table 4) and pheripheral blood mononuclear cells (PBMCs) (Table 5). Time-of-addition (TOA) experiments were also carried out to further investigate the step of the replicative cycle that was inhibited by the new 6-aminoquinolones (Figure 2).

Chemistry

The 6-aminoquinolone derivatives **26**−**33** were prepared according to the synthetic sequence outlined in Scheme 1. The key step of the synthesis involved the usual intramolecular cycloaracylation to the quinolone moiety. Thus, the reaction of acrylate **1**[19] with the appropiate amine (R_1NH_2), followed by basic cyclization, gave the crucial key intermediates **2**,[14] **3**,[20] **4**,[21] **5**,[14] **6**,[21] and **7**−**9** variously substituted at the N-1 position with a small or bulky group.

Subsequent sequential steps were nucleophilic substitution at C-7 with selected piperazines to give 6-nitro ester intermediates **10**−**17**, reduction to corresponding 6-amino ester derivatives **18**−**25**, and finally hydrolysis to the target acids **26**−**33**. The aryl or heteroarylpiperazines used were commercial products or were prepared according to literature procedures as for 1-(1,3-benzothiazol-2-yl)piperazine (**d**),[22] 1-(2-thiazolyl)piperazine (**e**),[23] 1-(2-benzimidazolyl)piperazine (**h**),[24] 1-(2-pyrazinyl)piperazine (**i**),[25] and 1-(1,3-benzoxazol-2-yl)piperazine (**j**).[23] On the other hand, 1-(5-chloro-1,3-benzothiazol-2-yl)piperazine (**g**) and 1-(5-methyl-1,3,4-thiadiazol-2-yl)piperazine (**l**) are new heteroarylpiperazines that were synthesized, as depicted in Scheme 2, starting from the corresponding 2-mercapto-5-chloro-1,3-benzothiazole and 2-mercapto-5-methyl-1,3,4-thiadiazole derivatives, which were converted into the thiomethyl derivatives and then reacted with piperazine.

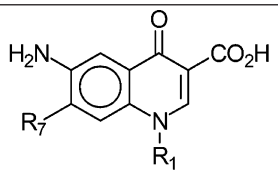
For the synthesis of derivative **10n** (Scheme 1), it was impossible to directly introduce the base 1-(1,4-benzothiazin-3-yl)piperazine by nucleophilic displacement of the C-7 chlorine atom, under various conditions. Thus, it was prepared in two steps by reacting synthone **2** with piperazine to give derivative **10m**, followed by a reaction with 3-methylthiobenzothiazine.[26]

The 6-nitrocarboxylic acid **34a** (Table 2) was obtained by direct acid hydrolysis of the corresponding ethyl 6-nitrocarboxylate **10a**.[14]

The 6-amino-8-fluoro acid **39a** was obtained by starting with acrylate **35**[27] (Scheme 3) and following the general synthetic route reported above through the intermediates **36**, **37a**, and **38a**. Intermediate **38a** was also converted to 6-amino-8-methoxycarboxylic acid **41a** by reaction with MeONa and successive catalytic reduction.

The synthesis of the two examples of 6-hydrogenquinolone derivatives, **45a** and **45b**, was accomplished starting from acrylate **42**[19] and converted in the usual

Table 1. Physical Properties for the 6-Aminoquinolones Tested in This Study



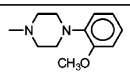
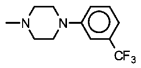
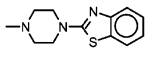
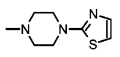
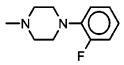
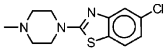
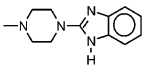
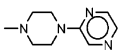
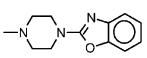
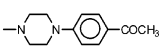
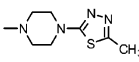
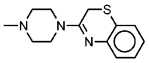
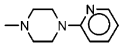
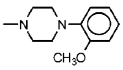
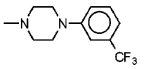
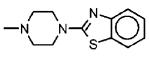
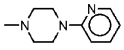
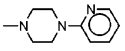
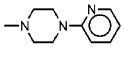
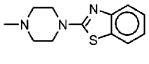
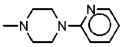
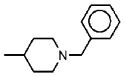
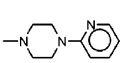
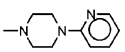
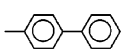
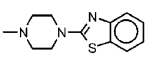
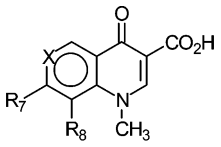
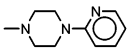
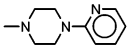
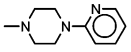
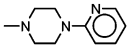
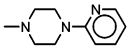
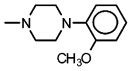
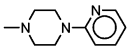
compd	R_1	R_7	Coupling reaction [a] temp./ react. time	purif. method [d]	Reduction [b] solv./ react. time	purif. method [e]	Hydrolysis [c] react. time	purif. method [f]	% yield [g]	mp, °C	Formula [h]
26b	CH_3		80-90°C 4h	**A**	DMF/EtOH 5h	**G**	7h	[i]	40	299-300	$C_{22}H_{24}N_4O_4$
26c	CH_3		80-90°C 7h	**A**	DMF 12h	**G**	7h	**J**	45	245-247	$C_{22}H_{21}F_3N_4O_3$
26d	CH_3		80-90°C 15h	**A**	DMF 48h	**G**	3 days	**J**	66	247-248	$C_{22}H_{22}ClN_5O_3S$
26e	CH_3		80°C 6h	**C**	[j]	[j]	10h	**J**	42	283-285	$C_{18}H_{19}N_5O_3S$
26f	CH_3		85°C 2h	**A**	DMF/EtOH 24h	**I**	8h	**J**	53	236-237	$C_{21}H_{22}Cl\,FN_4O_3$
26g	CH_3		80-90°C 3h	**E**	DMF 3h	**G**	3 days	**J**	15	333-334	$C_{22}H_{21}Cl_2N_5O_3S$
26h	CH_3		120°C 7h	**C**	DMF 3h	**H**	7h	**J**	40	307-308	$C_{22}H_{23}ClN_6O_3$
26i	CH_3		80-90°C 7h	**D**	DMF 3h	**H**	8h	**J**	30	327-328	$C_{19}H_{21}ClN_6O_3$
26j	CH_3		80-90°C 6h	**C**	DMF 48h	**G**	7h	**J**	36	341-343	$C_{22}H_{22}ClN_5O_4$
26k	CH_3		80-90°C 10h	**B**	DMF 5h	**G**	8h	**J**	20	229-231	$C_{23}H_{24}N_4O_4$
26l	CH_3		80-90°C 12h	**B**	DMF 1.5h	**H**	24h	**J**	19	232-235	$C_{18}H_{21}ClN_6O_3S$
26n	CH_3		[k]	[k]	DMF 2h	**G**	6h	**J**	70[l]	320-324	$C_{22}H_{23}N_5O_3S$
27a	C_2H_5		100°C 4h	**A**	DMF/EtOH 3h	**H**	30h	**K**	30	263 (dec)	$C_{21}H_{23}N_5O_3$
28b	*c*-C_3H_5		90°C 7h	**A**	DMF 9h	**G**	3h	[i]	65	200 (dec)	$C_{24}H_{26}N_4O_4$
28c	*c*-C_3H_5		80-90°C 6h	**B**	DMF/EtOH 48h	**H**	7h	**J**	44	285-286	$C_{24}H_{24}ClF_3N_4O_3$
28d	*c*-C_3H_5		80-90°C 7h	**D**	DMF 48h	**H**	6.5h	**J**	38	278.5-279	$C_{24}H_{25}Cl_2N_5O_3S$
29a			100 °C 1.5 h	**A**	DMF/EtOH 4h	**H**	7h	**K**	36	201 (dec)	$C_{28}H_{30}N_8O_3$
29d			80 °C 7 h	**A**	DMF 15h	**H**	10h	**K**	35	289-291	$C_{30}H_{30}N_8O_3S$
30a			90 °C 4 h	F	DMF 8h	**H**	5h	**J**	43	287-288	$C_{25}H_{23}ClFN_5O_3$
31a			80 °C 5 h	**A**	DMF 48h	**H**	28h	**K**	40	204-205	$C_{31}H_{34}N_6O_3$
32a			70°C 4 h	**D**	DMF 12h	**H**	6h	**K**	35	254-255	$C_{26}H_{22}N_6O_3S$
33d			80-90 °C 13 h	**E**	DMF 10h	**G**	20h	**J**	32	283-285	$C_{33}H_{27}N_5O_3S$

Table 1 (Continued)

[a] See General Procedure for Coupling Reaction in the Experimental Section. [b] See General Procedure for Reduction of 6-Nitro Group in the Experimental Section. [c] See General Procedure for Acidic Hydrolysis Reaction in the Experimental Section. [d] (A) The mixture was poured into ice/water, and the precipitate was filtered, washed with water and EtOH, and then dried. (B) After the mixture was cooled, the precipitate solid was filterd off, washed with EtOH, and dried. (C) After it was cooled, the excess side chain was filtered off and the filtrate, after concentration, gave a solid that was filtered, washed with EtOH, and dried. (D) The mixture was evaporated to dryness to give a residue that was purified by flash chromatography, eluting with a gradient of CH_2Cl_2/CH_3OH (100:0 to 90:10). (E) The mixture was concentrated to give a precipitate solid that was filtered, washed with EtOH, and dried. (F) The mixture was poured into ice/water and extracted with CH_2Cl_2. The organic solvent was evaporated to dryness and the residue was treated with EtOH to give a solid that was filtered and dried. [e] (G) The filtrate was concentrated, and after the mixture was cooled, the solid obtained was filtered, washed with EtOH, and dried. (H) The filtrate was concentrated to dryness to give a residue that was triturated with a mixture of EtOH/EtOAc to give a solid compound that was filtered and dried. (I) The filtrate was concentrated to give a residue that was purified by flash chromatography, eluting with a gradient of CH_2Cl_2/CH_3OH (100:0 to 97:3). [f] (J) After the mixture was cooled, the crystalline precipitated solid was filterd, washed with small amount of EtOH and 6 N HCl, dried, and then crystallized by MeOH/DMF. (K) After cooling at room temperature, the solution was filtered and neutralized by adding a solution of 10% NaOH. The resulting precipitate was filtered and washed with water and EtOH and then crystallized by EtOH/DMF. [g] Referenced to three steps (coupling, reduction, and hydrolysis reactions). [h] All compounds had elemental analyses within ±0.4% of the theoretical values. [i] See General Procedure for Basic Hydrolysis Reaction. [j] See in Experimental Section the reduction of **10e** to **18e**. [k] See the preparation of **10n** in the Experimental Section. [l] Referenced to the last two steps.

Table 2. Structures of Various Quinolones Tested in This Study[a]



compd	X	R_7	R_8
34a	C-NO_2		H
38a	C-NO_2		F
39a	C-NH_2		F
41a	C-NH_2		OCH_3
45a	C-H		H
45b	C-H		H
49a	N		H

[a] For the physical properties, see the Experimental Section.

way to **43** (Scheme 4). The key intermediate **43**, converted into the borine complex **44**, was then reacted with the selected arylpiperazines in DMSO and Et_3N as scavenger. The treatment with water during the workup of the reaction directly cleaved the boron ester chelate to produce the target free acids **45a** and **45b**.

Scheme 5 reports the synthesis of the 1,6-naphthyridinecarboxylic acid **49a**. Thus, the reaction of the acid chloride of 2,4-dichloropyridine-5-carboxylic acid **46**[28] with ethyl (dimethylamino)acrylate gave the adduct **47**, which was reacted at room temperature with $MeNH_2$ and successively cyclized in the presence of NaH at 0 °C to afford quinolone **48**. Nucleophilic substitution of the C-7 chlorine atom with 1-(2-pyridinyl)piperazine and successive basic hydrolysis gave the target acid **49a**.

Results and Discussion

The synthesized compounds were initially evaluated for anti-HIV activity by determining their ability to inhibit the replication of HIV-1 (III_B) and HIV-2 (ROD) in MT-4 cells. The cytotoxicity of the compounds was determined in parallel. The results are shown in Table 3. For comparative purposes, compound WM5 was assayed in the same cells.

Among all the synthesized compounds, derivatives **26c**, **26e**, **26j**, and **28c** were the most potent inhibitors of HIV replication of the series against both HIV-1 and HIV-2, coupled with the highest selectivity index. The C-7 thiazolpiperazinyl derivative **26e** and C-7 benzoxazolpiperazinyl derivative **26j** proved to be more potent than reference compound WM5. In fact, compound **26e** showed EC_{50} values of 0.13 and 0.080 μg/mL on HIV-1 and HIV-2, respectively, and compound **26j** showed values of 0.015 and 0.0087 μg/mL, respectively. Pyrazinylpiperazine derivative **26i** also showed good antiviral activity, particularly against HIV-1 ($EC_{50} = 0.70$ μg/mL).

The biological data of the synthesized compounds led to a series of considerations that permitted a more defined SAR to be delineated for the antiviral 6-aminoquinolone class. None of the modifications at the N-1 position of the lead WM5 proved successful in improving the biological properties of the compound. The introduction of either the ethyl group **27a** or a bulky substituent, such as *p*-fluorophenyl **30a**, 1-benzyl-4-piperidinyl **31a**, and 2-benzothiazolyl gave compounds that were devoid of anti-HIV activity at an EC_{50} lower than the CC_{50}. Only compound **29a**, bearing a 4-(2-pyridinyl)-1-piperazinyl substituent at the N-1 position, was characterized by modest antiviral activity against both HIV-1 and HIV-2 coupled with low cytoxicity. Thus, within the 6-aminoquinolone series, the best activity was provided by the *N*-methyl substituent.

Variations at the C-7 position play a crucial role, as previously observed, and allow compounds with very high antiviral activity to be obtained. The introduction of a 4-(1,3-benzothiazol-2-yl)-1-piperazinyl moiety at the C-7 position gave compound **26d**, which displayed the highest anti-HIV-2 activity but which had a high level of cytotoxicity. In an attempt to reduce its cytotoxicity, the benzothiazole nucleus at the N-4 piperazine ring was replaced by thiazole **26e**, benzimidazole **26h**, pyrazine **26i**, benzoxazole **26j**, thiadiazole **26l**, and benzothiazine **26n**. Moreover, the benzothiazole was functionalized with a chlorine atom at the C-6 position, as in compound **26g**. Among these serial modifications, the replacement of the sulfur atom with an oxygen atom as in the benzoxazole derivative **26j**, as well as the elimination of the benzene ring in the thiazole derivative **26e**, gave very active compounds characterized by SI

Table 3. Anti-HIV-1 and HIV-2 Activity (EC_{50} in μg/mL) and Cytotoxicity of Quinolone Derivatives in MT-4 Cells

	HIV-1 (III_B)		HIV-2 (ROD)				
compd	EC_{50} (μg/mL)[a,c]	max % prot	EC_{50} (μg/mL)[a,c]	max % prot	CC_{50} (μg/mL)[b,c]	SI (III_B)	SI (ROD)
WM5	≥0.29	(35−59)	0.19 ± 0.099	(60−64)	0.71 ± 0.20	≤2.4	3.7
26b	>7.45	(16−21)	>7.45	(17−27)	7.45 ± 2.77	<1	<1
26c	0.31 ± 0.14	(57−110)	≥0.31	(43−78)	2.18 ± 0.28	7.0	≤7.0
26d	>0.0054	(25−30)	0.0011	(51−81)	0.0054 ± 0.0018	<1	5.1
26e	0.13 ± 0.04	(55−118)	0.080 ± 0.067	(58−83)	1.17 ± 0.44	9.0	14.6
26f	>125	(7−30)	>125	(17−31)	>125		
26g	>0.0021	(4−8)	>0.0021	(3)	0.0021 ± 0.0016	<1	<1
26h	>125	(0−1)	>125	(0)	>125		
26i	0.70 ± 0.042	(60−91)	1.54 ± 0.44	(51−55)	3.98 ± 0.98	5.7	2.6
26j	0.015 ± 0.0013	(65−86)	0.0087 ± 0.0034	(15−21)	0.098 ± 0.023	6.5	11.3
26k	>0.157	(5−9)	>0.157	(13)	0.157 ± 0.030	<1	<1
26l	>125	(29−40)	≥10.30	(29−63)	>125	<1	≤12.1
26n	>2.14	(10−27)	>2.14	(15)	2.14 ± 0.098	<1	<1
27a	>2.20	(11−24)	>2.20	(26−27)	2.20 ± 0.29	<1	<1
28b	>10.33	(9−24)	>10.33	(10)	10.33 ± 5.42	<1	<1
28c	0.27 ± 0.14	(88−115)	0.38 ± 0.18	(71−71)	3.83 ± 0.98	14.2	10.1
28d	>0.076	(16−36)	>0.076	(42)	0.076 ± 0.052	<1	<1
29a	≥17.4	(32−62)	19.20 ± 0.57	(55−58)	58.46 ± 19.90	≤3.4	3.0
29d	>0.24	(2−18)	>0.24	(0)	0.24 ± 0.20	<1	<1
30a	>22.03	(34−37)	>22.03	(22−24)	22.03 ± 4.64	<1	<1
31a	>2.36	(2−14)	>2.36	(5)	2.36 ± 0.27	<1	<1
32a	>9.13	(14−20)	>9.13	(21)	9.13 ± 5.10	<1	<1
33d	>0.67	(5−6)	>0.67	(1)	0.67 ± 0.19	<1	<1
34a	>10.07	(4−13)	>10.07	(5)	10.07 ± 2.48	<1	<1
38a	>29.57	(3−8)	>29.57	(4)	29.57 ± 9.64	<1	<1
39a	>6.08	(29−37)	>6.08	(40−42)	6.08 ± 1.01	<1	<1
41a	>4.28	(16−49)	>4.28	(21−26)	4.28 ± 1.95)	<1	<1
45a	19.20 ± 3.53	(53−59)	>65.53	(40−43)	65.53 ± 26.39	3.4	<1
45b	>125	(8−9)	>125	(6)	>125		
49a	>67.37	(32−39)	>67.37	(27−33)	67.37 ± 30.79	<1	<1

[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.

Table 4. Anti-HIV-1 and HIV-2 Activity and Cytotoxicity of Selected Quinolones in CEM 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)	SI (ROD)
WM5	0.03 ± 0.01	0.06 ± 0.01	>100	>3333	>1667
26c	0.09 ± 0.01	0.20 ± 0.16	8.00 ± 3.51	89	40
26e	0.06 ± 0.00	0.08 ± 0.00	≥100	≥1667	≥1250
26i	0.08 ± 0.02	0.20 ± 0.11	>100	>1250	>500
26j	0.01 ± 0.00	0.02 ± 0.01	2.20 ± 0.25	220	110
28c	0.80 ± 0.17	0.80 ± 0.58	9.00 ± 0.31	11	11
29a	9.40 ± 1.22	12.00 ± 0.92	>100	>11	>8
45a	1.20 ± 0.42	1.80 ± 0.17	>100	>83	>56

[a] EC_{50}: concentration of compound required to achieve 50% protection of CEM cells from HIV induced cytopathogenicity, as determined microscopically. [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 three separate experiments.

Table 5. Anti-HIV-1 Activity and Cytotoxicity of Selected Quinolones in PBMCs

compd	HIV-1 (III_B) EC_{50} (μg/mL)[a,c]	CC_{50} (μg/mL)[b,c]	SI
WM5	0.051 ± 0.05	1.45 ± 0.07	28
26c	0.056 ± 0.040	1.55 ± 0.88	28
26e	0.032 ± 0.010	1.30 ± 1.27	41
26i	0.045 ± 0.00	3.65 ± 2.33	81
26j	0.043 ± 0.020	1.12 ± 0.21	26
28c	1.09 ± 0.80	4.63 ± 0.47	4
29a	2.24 ± 1.59	≥43.30	≥19
45a	1.37 ± 1.11	>100	>73

[a] EC_{50}: concentration of compound required to achieve 50% reduction of p24 production in HIV-1 (III_B) infected PBMCs. [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.

values ranging from 6.5 to 14.6. The replacement of the benzothiazole with a pyrazine ring gave compound **26i**, which was characterized by a lower activity but coupled this time with a marked decrease in cytotoxicity. For the C-7 benzimidazole derivative **26h** and the thiadiazole derivative **26l**, the toxicity was eliminated but so was the antiviral activity. The broadening of the thiazole ring to thiazine produced benzothiazine derivative **26n** characterized by both low cytotoxicity and antiviral activity. The insertion of a chlorine atom in the benzothiazole ring as in **26g** did not affect activity or cytotoxicity. Finally, benzothiazole derivative **26d** was modified by introducing a cyclopropyl (compound **28d**) and bulky groups (compounds **29d** and **33d**) at the N-1 position; these modifications did not lead to pronounced anti-HIV activity.

The C-7 position was further modified by introducing an *m*-trifluoromethylphenyl ring at the N-4 piperazine moiety as in compound **26c**, which displayed good activity with EC_{50} values of 0.31 μg/mL against both HIV-1 and HIV-2 coupled with positive SI values. The

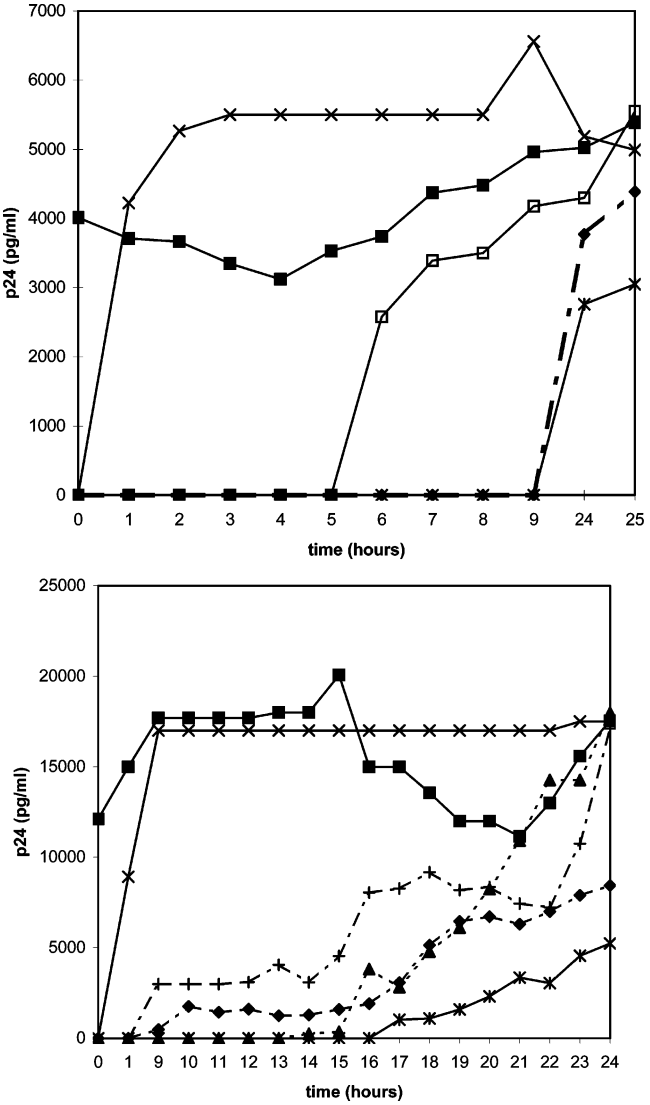


Figure 2. Time-of-addition experiment. MT-4 cells were infected with HIV-1(III_B) at an moi of 0.5, and the test compounds were added at different times postinfection. Viral p24 production was determined at 31 h postinfection and is expressed in pg/mL. Symbols represent the following: (■) control; (×) dextran sulfate; (□) AZT; (▲) **28c**; (◆) **26e**; (+) **26j**; (∗) ritonavir.

same profile was also shown by the N-1 cyclopropyl analogue **28c**.

The absence of a substituent at the C-6 position (compound **45a**), as well as the insertion of an NO_2 group (compound **34a**) or endocyclic N (compound **49a**), gave inactive or slighly active compounds confirming that the 6-NH_2 substituent was more suitable. This behavior was also confirmed by the inactivity of 6-nitro derivative **38a** and 6-hydrogen derivative **45b**. However, it is noted that the 6-hydrogen atom lowered the cytotoxicity (**45a** and **45b**).

The insertion of a fluorine atom and a methoxy group at the C-8 position gave compounds **39a** and **41a**, which did not inhibit HIV-1 and HIV-2 replication at EC_{50} values lower than the CC_{50}. This confirms what was already reported for the C-8 methyl derivative[14] and seems to indicate that the antiviral 6-aminoquinolones do not tolerate any substituent at this position in contrast to what was observed for the fluoroquinolones reported to date.[9−13]

Molecules displaying good activity on MT-4, **26c**, **26e, 26i**, **26j**, **28c**, and two molecules that are less active but with positive SI, **29a** and **45a**, were also evaluated for their anti-HIV-1 and anti-HIV-2 activity on CEM cells (Table 4), as well as against HIV-1 in PBMCs (Table 5). These two cell lines appear to be more sensitive to all the tested compounds. In fact, with the exception of **28c**, the compounds proved to be approximately 2- to 16-fold more active in PBMCs and CEM cells than in MT-4 cells; a 36-fold activity increase against HIV-2 on CEM cells was obtained with compound **45a**.

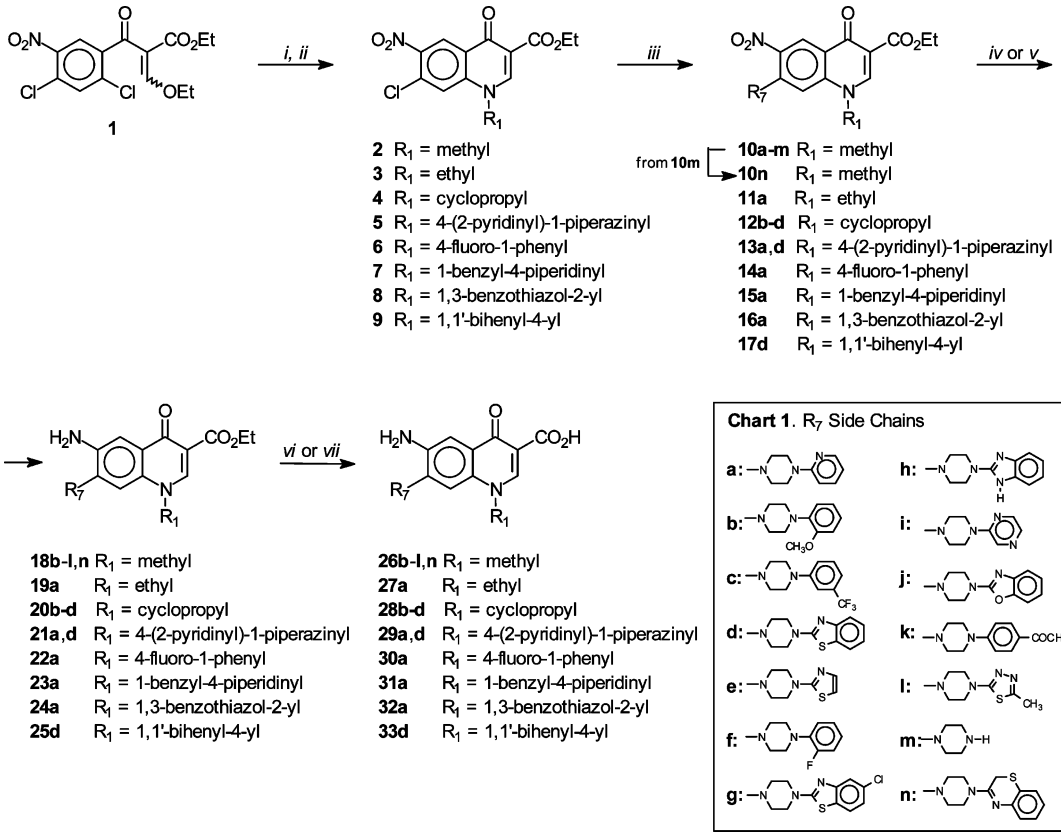
The increased antiviral activity was also coupled with a markedly lower cytotoxicity in CEM cells. In particular, derivatives **26e**, **26i**, **29a**, and **45a**, as well as the lead WM5, were devoid of any cytotoxicity (CC_{50} >100 μg/mL); thus, very high SI values were obtained particularly on HIV-1. The highest SI value was observed for reference compound WM5 (>3333), but it was also particularly good for derivatives **26e** (≥1667), **26i** (>1250), and **26j** (220). The comparison between N-1 methyl derivative **26c** and its N-1 cyclopropyl analogue **28c** showed that, contrary to their behavior on MT-4 cells, derivative **26c** was 10 and 4 times more active against HIV-1 and HIV-2, respectively, in CEM cells.

On PBMCs the compounds showed a cytotoxicity that was on the same order as that observed on MT-4 cells (Table 5). The SI values ranged from 4 for compound **28c** to 81 for compound **26i**. The 6-hydrogenquinolone **45a** was the only derivative that was not toxic (CC_{50} ≥ 100 μg/mL) in this cell line according to its behavior in the other tested cell lines. This observation appears to be very interesting and should be considered for further development of anti-HIV 6-hydrogenquinolones.

To investigate which step of the replicative cycle was inhibited by the new 6-aminoquinolones, time-of-addition experiments were carried out for derivatives **26e**, **26j**, and **28c** (Figure 2). Briefly, this experiment determines how long the addition of an anti-HIV compound can be postponed before losing its antiviral activity in the viral replication cycle. The virus was added at high multiplicity of infection (moi = 0.5) to synchronize all the steps of the viral replication. Reference compounds with a known mode of action were included: dextran sulfate, which interferes with the binding of the virus to the cells; AZT, which inhibits the reverse transcription process; and ritonavir, which is an inhibitor of the proteolytic cleavage. Addition of these compounds can be delayed for 0, 5, and 16 h postinfection, respectively (Figure 2). In the same experiment, 6-aminoquinolones lost their antiviral activities when added at 9−16 h postinfection, which clearly indicates an inhibition at a postintegrational level.

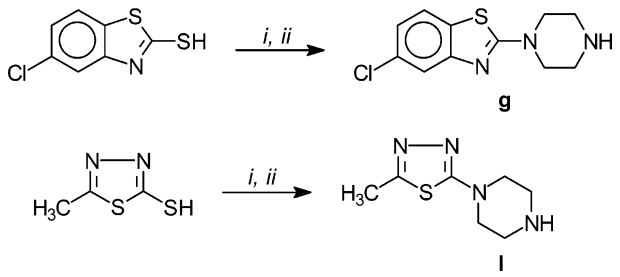
In conclusion, the structural modification around the lead WM5 permitted new, potent anti-HIV-1 and HIV-2 6-aminoquinolones **26c**, **26e**, **26i**, **26j**, and **28c** to be obtained. In general, they show the same high antiviral potency on MT-4, CEM, and PBMCs coupled with cytotoxicity that is strongly determined by the used cell. In the CEM cell line, some derivatives such as **26e** do not appear to be toxic at all. The SAR study based on MT-4 antiviral data shows that modifications of the 6-NH_2 group are detrimental for the antiviral activity; however, a hydrogen atom seems to be suitable for lowering the cytotoxicity. The suitability of a small

Scheme 1[a]



[a] Reagents: (i) R_1NH_2, EtOH/Et_2O; (ii) K_2CO_3, DMF, 100 °C; (iii) R_7H (for R_7, see Chart 1), DMF; (iv) H_2, Raney Ni, DMF or DMF/EtOH; (v) $HCOONH_4$, 10% Pd/C, MeOH/DMF; (vi) 6 N HCl, EtOH, reflux; (vii) 1 N NaOH, reflux.

Scheme 2[a]



[a] Reagents: (i) MeI, KOH, TBAB, THF; (ii) piperazine, 110 °C, closed vessel.

group such as CH_3 at the N-1 position was confirmed, which can be replaced only by a cyclopropyl; generally, bulky groups led to a decrease in activity. The C-8 position does not appear to tolerate substituents, in contrast to what was reported for known antiviral 6-fluoroquinolones. Moreover, it was confirmed that substitution at C-7 is still the best way to influence the anti-HIV activity; besides 2-pyridinylpiperazine, other aryl/heteroaryl piperazines produced potent quinolones.

Further studies are needed to better delineate the structure−activity relationships in order to meet the important challenge of recognizing the structural features that are responsible for the antiviral activity and/or cytotoxicity.

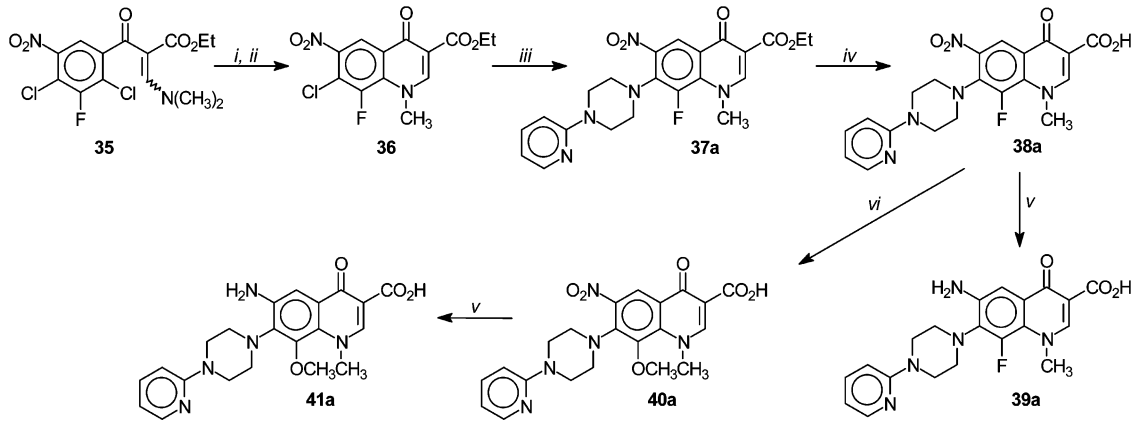
Experimental Section

Melting points were determined in capillary tubes (Büchi melting point apparatus) 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) with Me_4Si as the 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. Flash column chromatography separations were carried out on Merck silica gel 60 (mesh 230−400). Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a Büchi rotary evaporator at low pressure. Yields are of purified product and were not optimized. All starting materials were commercially available unless otherwise indicated.

General Procedure for Cycloaracylation Reaction. A stirred solution of the appropiate acrylate (**1**, **35**, **42**) (1 equiv) in EtOH and Et_2O (3:1) was treated dropwise with a selected R_1NH_2 group (1.1 equiv), and the mixture was reacted at room temperature for 8 h. The solution was then evaporated to dryness to give an oil that was washed with a mixture of cyclohexane/MeOH and then solubilized in DMF. To this solution K_2CO_3 (2 equiv) was added, and the mixture was heated at 100 °C for 1 h. After cooling, the reaction mixture was poured into ice/water, giving a precipitate that was filtered, dried, and purified by flash chromatography, eluting with a gradient of CH_2Cl_2/MeOH (100:0 to 98:2).

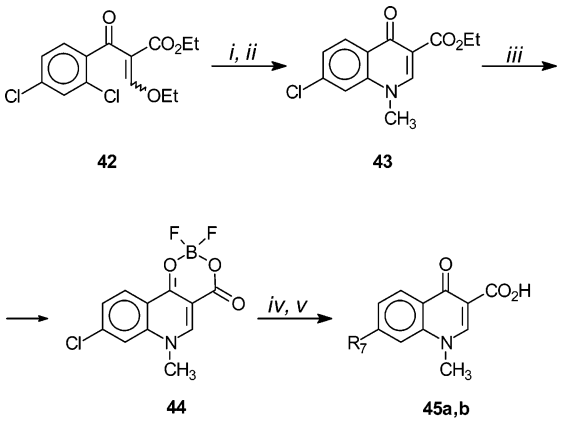
Ethyl 1-(1-Benzyl-4-piperidinyl)-7-chloro-6-nitro-4-oxo-1,4-dihydroquinolin-3-carboxylate (7). Following the general procedure for cycloaracylation reaction, starting from ethyl 2-(2,4-dichloro-5-nitrobenzoyl)-3-etoxyacrylate **1**[19] and using 4-amino-1-benzylpiperidine, the title compound was obtained in 35% yield: mp 170−172 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (3 H, t, J = 7.0 Hz, CH_3), 2.10−2.50 (6 H, m, piperidine CH_2), 3.15−3.35 (2 H, m, piperidine CH_2), 3.70 (2 H, s, benzyl CH_2), 4.25−4.50 (3 H, m, CH_2CH_3 and piperidine CH), 7.30−7.45 (5 H, m, benzyl CH), 7.75 (1 H, s, H-8), 8.75 (1 H, s, H-5), 9.05 (1 H, s, H-2).

Scheme 3[a]



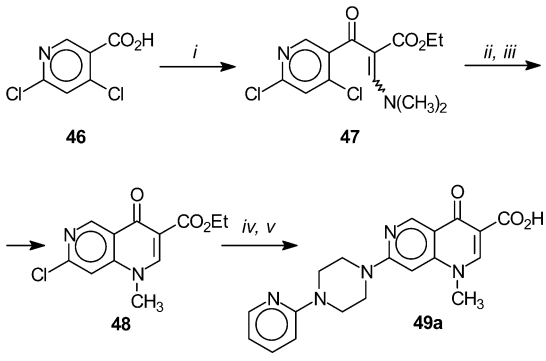
[a] Reagents: (i) $MeNH_2$, EtOH/Et_2O; (ii) K_2CO_3, DMF, 100 °C; (iii) 1-(2-pyridinyl)piperazine, DMF; (iv) 6 N HCl, EtOH, reflux; (v) H_2, Raney Ni, DMF; (vi) MeONa, DMF/MeOH.

Scheme 4[a]



[a] Reagents: (i) $MeNH_2$, EtOH/Et_2O; (ii) K_2CO_3, DMF, 100 °C; (iii) 48% HBF_4, 90−100 °C; (iv) R_7H (for R_7, see Chart 1), DMSO; (v) H_2O.

Scheme 5[a]



[a] Reagents: (i) (a) $SOCl_2$; (b) $Me_2NCH{=}CHCO_2Et$, Et_3N, toluene; (ii) $MeNH_2$, EtOH/Et_2O; (iii) 60% NaH, THF; (iv) 1-(2-pyridinyl)piperazine, DMF; (v) 4% NaOH reflux.

Ethyl 1-(1,3-Benzothiazol-2-yl)-7-chloro-6-nitro-4-oxo-1,4-dihydroquinolin-3-carboxylate (8). Following the general procedure for cycloaracylation reaction, starting from ethyl 2-(2,4-dichloro-5-nitrobenzoyl)-3-etoxyacrylate **1**[19] and using 2-aminobenzothiazole, the title compound was obtined in 32% yield. In this case, the reaction mixture with amine was heated at 60 °C for 2 h: mp 249−250 °C; ^{1}H NMR ($CDCl_3$) δ 1.45 (3 H, t, J = 7.0 Hz, CH_3), 4.50 (2 H, q, J = 7.0 Hz, CH_2), 7.55−7.80 (3 H, m, benzothiazole CH), 8.00 (1 H, s, H-8), 8.20−8.25 (1 H, m, benzothiazole CH), 8.80 (1 H, s, H-5), 9.05 (1 H, s, H-2).

Ethyl 1-(1,1′-Biphenyl-4-yl)-7-chloro-6-nitro-4-oxo-1,4-dihydroquinolin-3-carboxylate (9). Following the general procedure for cycloaracylation reaction, starting from ethyl 2-(2,4-dichloro-5-nitrobenzoyl)-3-etoxyacrylate **1**[19] and using 1,1′-biphenyl-4-amine, the title compound was obtained in 35% yield: mp 189−191 °C; ^{1}H NMR ($CDCl_3$) δ 1.40 (3 H, t, J = 7.0 Hz, CH_3), 4.40 (2 H, q, J = 7.0 Hz, CH_2), 7.00−7.10 and 7.25−7.35 (each 3 H, m, aromatic CH), 7.50−7.55 (1 H, m, aromatic CH), 7.60−7.85 (3 H, m, aromatic CH and H-8), 8.75 (1 H, s, H-5), 9.00 (1 H, s, H-2).

Ethyl 7-Chloro-8-fluoro-1-methyl-6-nitro-4-oxo-1,4-dihydroquinolin-3-carboxylate (36). Following the general procedure for cycloaracylation reaction, starting from ethyl 2-(2,4-dichloro-3-fluoro-5-nitrobenzoyl)-3-(dimethylamino)acrylate **35**[27] and using $MeNH_2$, the title compound was prepared in 52% yield: mp 174−176 °C; ^{1}H NMR (DMSO-d_6) δ 1.30 (3 H, t, J = 7 Hz, CH_2CH_3), 4.15 (3 H, d, J = 9 Hz, CH_3), 4.25 (2 H, q, J = 7 Hz, CH_2CH_3), 8.65 (1 H, d, J = 2.0 Hz, H-5), 8.75 (1 H, s, H-2).

Ethyl 7-Chloro-1-methyl-4-oxo-1,4-dihydroquinolin-3-carboxylate (43). Following the general procedure for cycloaracylation reaction, starting from ethyl 2-(2,4-dichlorobenzoyl)-3-etoxyacrylate **42**[19] and using $MeNH_2$, the title compound was prepared in 57% yield: mp 178−180 °C; ^{1}H NMR (DMSO-d_6) δ 1.45 (3 H, t, J = 7 Hz, CH_2CH_3), 3.90 (3 H, s, CH_3), 4.45 (2 H, q, J = 7 Hz, CH_2CH_3), 7.45 (1 H, dd, J = 1.8 and 7.0 Hz, H-6), 7.45 (1 H, bs, H-8), 8.45−8.55 (2 H, m, H-5 and H-2).

Ethyl 7-Chloro-1-methyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylate (48). A mixture of acid **46**[28] (1.3 g, 6.8 mmol) and thionyl chloride (6 mL) was refluxed for 2 h. The excess thionyl chloride was removed by distillation under reduced pressure to give an oil residue that was dissolved in dry toluene (15 mL) and added to ethyl 3-(dimethylamino)-acrylate[29] (1.35 g, 9.47 mol). The resulting solution was heated at 120 °C for 4 h. After cooling, the insoluble material was filtered and the solvent was evaporated to dryness. The residue was purified by flash chromatography, eluting with a mixture of petroleum ether/EtOAc (70:30) to give **47** (0.8 g, 37%).

Thus, a stirred solution of **47** (0.7 g, 2.2 mmol) in EtOH (20 mL) and Et_2O (8 mL) was treated dropwise with $MeNH_2$ (33% solution in EtOH) (0.476 mL, 3.53 mmol). After 1.5 h at room temperature the solvent was evaporated to dryness and washed with Et_2O to give 0.6 g of methylamine acrylate as a white solid that was solubilized in THF (30 mL). To this solution maintained at 0 °C, NaH (60% in an oil suspension) (0.09 g, 2.2 mmol) was added portionwise. After 1 h at 0 °C, the mixture was evaporated to dryness and the residue was treated with water to give a solid that was filtered and dried to give **48** (0.3 g, 51%): mp 165−168 °C; ^{1} H NMR ($CDCl_3$) δ 1.40 (3 H, t, J = 7 Hz, CH_3), 3.80 (3 H, s, NCH_3), 4.40 (2 H, q, J = 7 Hz, CH_2), 7.30 (1 H, s, H-8), 8. 45 (1 H, s, H-5), 9.40 (1 H, s, H-2).

General Procedure for Coupling Reaction. A mixture of the appropiate synthone (**2-9**, **36**, **48**) (1 equiv) and selected arylpiperazine (3 equiv) in dry DMF was heated at 90−120 °C until no starting material could be detected by TLC (usually 2−15 h). The reaction mixture was then worked up and purified as defined in footnote d in Table 1.

Ethyl 7-[4-(2*H*-1,4-Benzothiazin-3-yl)-1-piperazinyl]-1-methyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (10n). Following the general procedure for coupling reaction, synthone **2** was reacted with piperazine for 4 h at 80 °C. After the mixture was cooled, the precipitate was filtered, washed with EtOH, and dried to give **10m** in 80% yield. A mixture of compound **10m** (0.4 g, 1,1 mmol) and 3-(methylthio)-2*H*-1,4-benzothiazine[26] (1.3 g, 6.6 mmoli) in dry DMF (10 mL) was refluxed for 16 h. The reaction mixture was then evaporated to dryness, put into water, and extracted several times with EtOAc. The organic layers were concentrated to give a solid that was filtered and dried to afford compound **10n** (0.250 g, 45%): mp 297−298 °C; ^{1}H NMR (DMSO-d_6) δ 1.25 (3 H, t, J = 7.0 Hz, CH_2CH_3), 3.25−3.50 (6 H, m, piperazine CH_2 and SCH_2), 3.75−4.00 (7 H, m, piperazine CH_2 and NCH_3), 4.25 (2 H, q, J = 7.0 Hz, CH_2CH_3), 6.90−7.20 (5 H, m, aromatic H and H-8), 8.55 (1 H, s, H-5), 8.70 (1 H, s, H-2).

General Procedure for Reduction of 6-Nitro Group. A stirred solution of a selected 6-nitro derivate in DMF or in a mixture of DMF/EtOH was hydrogenated over a catalytic amount of Raney nickel at room temperature and atmospheric pressure until no starting material could be detected by TLC (usually 2−10 h). The mixture was then filtered over Celite, and the filtrate was worked up and purified as described in footnote e in Table 1.

Ethyl 6-Amino-1-methyl-7-[4-(1,3-thiazol-2-yl)-1-piperazinyl]-4-oxo-1,4-dihydroquinolin-3-carboxylate (18e). Ammonium formate (0.49 g, 7.9 mmol) and 10% Pd/C (0.7 g) were added to a solution of nitro derivate **10e** (0.7 g, 1.58 mmol) in dry MeOH (20 mL) and dry DMF (10 mL). The mixture was stirred for 12 h at room temperature and then filtered over Celite, washing several times with hot DMF. The filtrate was evaporated to dryness, and the residue was crystallized by DMF to give derivate **18e** (0.080 g, 13%): mp 297−298 °C; ^{1}H NMR (DMSO-d_6) δ 1.20 (3 H, t, J = 7.0 Hz, CH_2CH_3), 2.95−3.05 and 3.50−3.60 (each 4 H, m, piperazine CH_2), 3.75 (3 H, s, CH_3), 4.10 (2 H, q, J = 7.0 Hz, CH_2), 5.20 (2 H, bs, NH_2), 6.75 (1 H, d, J = 9.4 Hz, thiazole CH), 7.00 (1 H, s, H-8), 7.10 (1 H, d, J = 9.4 Hz, thiazole CH), 7.40 (1 H, s, H-5), 8.40 (1 H, s, H-2).

General Procedure for Acidic Hydrolysis Reaction. A mixture of selected 6-amino ester (0.3 mequiv) in EtOH (2 mL) and 6 N HCl (2 mL) was refluxed until no starting material could be detected by TLC (usually 7−72 h) and worked up and purified as defined in footnote f in Table 1.

By use of this procedure, the target acids **26c**, **26d, 26e**, **26f**, **26g**, **26h**, **26i**, **26j**, **26l**, **26n**, **27a**, **28c**, **28d**, **29a**, **29d**, **30a**, **31a**, **32a**, and **33d** were obtained. Further experimental details are given in Table 1, while their spectral data are listed below.

Compound 26c: ^{1}H NMR (DMSO-d_6) δ 3.20−3.30 and 3.50−3.60 (4 H, m, CH piperazine), 4.15 (3 H, s, CH_3), 7.15 (1 H, d, J = 8.0 Hz, aromatic CH), 7.25−7.40 (3 H, m, aromatic CH and H-8), 7.60 (1 H, s, H-5), 8.80 (1 H, s, H-2). Anal. ($C_{22}H_{21}F_3N_4O_3$) C, H, N.

Compound 26d: ^{1}H NMR (DMSO-d_6) δ 3.10−3.25 and 3.75−3.90 (each 4 H, m, piperazine CH_2), 4.00 (3 H, s, CH_3), 7.05−7.15 (1 H, m, benzothiazole CH), 7.25−7.40 (2 H, m, benzothiazole CH and H-8), 7.45−7.55 (1 H, m, benzothiazole CH), 7.60 (1 H, s, H-5), 7.75−7.85 (1 H, m, benzothiazole CH), 8.75 (1 H, s, H-2), 16.00 (1 H, bs, COOH). Anal. ($C_{22}H_{22}ClN_5O_3S$) C, H, N.

Compound 26e: ^{1}H NMR (DMSO-d_6) δ 3.10−3.25 and 3.60−3.70 (each 4 H, m, piperazine CH_2), 4.00 (3 H, s, CH_3), 5.50 (2 H, bs, NH_2), 6.80 (1 H, d, J = 3,4 Hz, thiazole CH), 7.20−7.30 (2 H, m, thiazole CH and H-8), 7.50 (1 H, s, H-5), 8.75 (1 H, s, H-2), 16.00 (1 H, bs, COOH). Anal. ($C_{18}H_{19}N_5O_3S$) C, H, N.

Compound 26f: ^{1}H NMR (DMSO-d_6) δ 3.20−3.35 (8 H, m, piperazine CH_2), 4.10 (3 H, s, CH_3), 6.90−7.25 (4 H, m, aromatic CH), 7.35 (1H, s, H-8), 7.75 (1 H, s, H-5), 8.90 (1 H, s, H-2). Anal. ($C_{21}H_{22}ClFN_4O_3$) C, H, N.

Compound 26g: ^{1}H NMR (DMSO-d_6) δ 3.00−3.10 and 3.70−3.80 (each 4 H, m, piperazine CH_2), 4.00 (3 H, s, CH_3), 7.05 (1 H, dd, J = 8.6 and 2.0 Hz, H-6′), 7.20 (1 H, s, H-8), 7.40 (1 H, d, J = 2.0 Hz, H-4′), 7.50 (1 H, s, H-5), 7.75 (1 H, d, J = 8.6 Hz, H-7′), 8.70 (1 H, s, H-2), 16.00 (1 H, bs, COOH). Anal. ($C_{22}H_{21}Cl_2N_5O_3S$) C, H, N.

Compound 26h: ^{1}H NMR (DMSO-d_6) δ 3.20−3.35 and 4.00−4.15 (each 4 H, m, piperazine CH_2), 4.00 (3 H, s, CH_3), 7.25−7.35 (3 H, m, aromatic H and H-8), 7.40−7.50 (2 H, m, aromatic H), 7.05 (1 H, s, H-5), 8.75 (1 H, s, H-2). Anal. ($C_{22}H_{23}ClN_6O_3$) C, H, N.

Compound 26i: ^{1}H NMR (DMSO-d_6) δ 3.10−3.25 and 3.80−3.95 (each 4 H, m, piperazine CH_2), 4.05 (3 H, s, CH_3), 7.30 (1 H, s, H-8), 7.75 (1 H, s, H-5), 7.90, 8.20 and 8.50 (each 1 H, s, pyrazine CH), 8.80 (1 H, s, H-2). Anal. ($C_{19}H_{21}ClN_6O_3$) C, H, N.

Compound 26j: ^{1}H NMR (DMSO-d_6) δ 3.10−3.30 (4 H, m, piperazine CH_2), 3.80−4.15 (7 H, m, piperazine CH_2 and CH_3), 7.10−7.50 (5 H, m, aromatic H and H-8), 7.65 (1 H, s, H-5), 8.75 (1 H, s, H-2). Anal. ($C_{22}H_{22}ClN_5O_4$) C, H, N.

Compound 26k: ^{1}H NMR (DMSO-d_6) δ 2.45 (3 H, s, $COCH_3$), 3.15−3.25 and 3.55−3.65 (each 4 H, m, piperazine CH_2), 4.00 (3 H, s, NCH_3), 7.00 (2 H, d, J = 7.0 Hz, aromatic CH), 7.25 (1 H, s, H-8), 7.60 (1 H, s, H-5), 7.80−7.90 (2 H, m, aromatic CH), 8.80 (1 H, s, H-2). Anal. ($C_{23}H_{24}N_4O_4$) C, H, N.

Compound 26l: ^{1}H NMR (DMSO-d_6) δ 2.45 (3 H, s, CH_3), 3.00−3.10 and 3.50−3.60 (each 4 H, m, piperazine CH_2), 3.95 (3 H, s, NCH_3), 7.20 (1 H, s, H-8), 7.60 (1 H, s, H-5), 8.70 (1 H, s, H-2). Anal. ($C_{18}H_{21}ClN_6O_3S$) C, H, N.

Compound 26n: ^{1}H NMR (DMSO-d_6) δ 3.10−3.40 (4 H, m, piperazine CH_2), 4.00 (3 H, s, NCH_3), 4.10−4.30 (6 H, m, piperazine CH_2 and CH_2), 7.15−7.45 (5 H, m, aromatic H and H-8), 7.50 (1 H, s, H-5), 8.70 (1 H, s, H-2), 12.10 (1 H, bs, COOH). Anal. ($C_{23}H_{23}N_5O_3S$) C, H, N.

Compound 27a: ^{1}H NMR (DMSO-d_6) δ 1.40 (3 H, t, J = 7.0 Hz, CH_3), 3.00−3.15 and 3.60−3.75 (each 4 H, m, piperazine CH_2), 4.55 (2 H, q, J = 7.0 Hz, CH_2CH_3), 5.50 (2 H, bs, NH_2), 6.50−6.65 (1 H, m, pyridine CH), 6.90 (1 H, d, J = 8.5 Hz, pyridine CH), 7.25 (1 H, s, H-8), 7.50−7.65 (2 H, m, pyridine CH and H-5), 8.20 (1 H, d, J = 4.5 Hz, pyridine CH), 8.90 (1 H, s, H-2), 16.00 (1 H, bs, COOH). Anal. ($C_{21}H_{23}N_5O_3$) C, H, N.

Compound 28c: ^{1}H NMR (DMSO-d_6) δ 1.05−1.40 (4 H, m, cyclopropyl CH_2), 3.15−3.30 and 3.45−3.65 (each 4 H, m, piperazine CH_2), 3.80−3.90 (1 H, m, cyclopropyl CH), 7.00−7.50 (4 H, m, aromatic CH), 7.70 (2 H, s, H-8 and H-5), 8.60 (1 H, s, H-2). Anal. ($C_{24}H_{24}ClF_3N_4O_3$) C, H, N.

Compound 28d: ^{1}H NMR (DMSO-d_6) δ 1.05−1.20 and 1.30−1.45 (each 2 H, m, cyclopropyl CH_2), 3.20−3.30 (4 H, m, piperazine CH_2), 3.75−4.00 (5 H, m, piperazine CH_2 and cyclopropyl CH), 7.05−7.20 and 7.30−7.40 (each 1 H, m, aromatic H), 7.45−7.55 (2 H, m, aromatic H and H-8), 7.65 (1 H, s, H-5), 7.70−7.80 (1H, m, aromatic H), 8.50 (1 H, s, H-2). Anal. ($C_{24}H_{25}Cl_2N_5O_3S$) C, H, N.

Compound 29a: ^{1}H NMR ($CDCl_3$) δ 3.10−3.50 (8 H, m, piperazine CH_2), 3.70−3.90 and 4.30−4.60 (each 4 H, piperazine CH_2), 6.60−6.90 (4 H, m, pyridine CH), 7.50−7.70 (2 H, m, pyridine CH), 7.80 (1 H, s, H-8), 7.90 (1 H, s, H-5), 8.20−8.40 (2 H, m, pyridine CH), 8.90 (1 H, s, H-2), 15.50 (1 H, bs, COOH). Anal. ($C_{22}H_{21}F_3N_4O_3$) C, H, N. Anal. ($C_{28}H_{30}N_8O_3$) C, H, N.

Compound 29d: ^{1}H NMR ($CDCl_3$) δ 3.10−3.45 (8 H, m, piperazine CH_2), 3.80−4.00 and 4.25−4.60 (each 4 H, piperazine CH_2), 6.75−6.90 (2 H, m, aromatic CH), 7.10−7.40 (4 H, m, aromatic CH), 7.50 (1 H, s, H-8), 7.60−7.70 (2 H, m, aromatic CH), 7.85 (1 H, s, H-5), 8.20−8.30 (1 H, m, aromatic

CH), 8.90 (1 H, s, H-2), 15.50 (1 H, bs, COOH). Anal. ($C_{30}H_{30}N_8O_3S$) C, H, N.

Compound 30a: ^{1}H NMR (DMSO-d_6) δ 2.90−3.00 and 3.90−4.00 (each 4 H, m, piperazine CH_2), 6.50 (1 H, s, H-8), 6.90 (1 H, t, J = 7.0 Hz, pyridine CH), 7.35−7.60 (3 H, m, aromatic H and pyridine CH), 7.70−7.80 (3 H, m, aromatic H and H-5), 7.95−8.05 (2 H, m, pyridine H), 8.50 (1 H, s, H-2). Anal. ($C_{25}H_{23}ClFN_5O_3$) C, H, N.

Compound 31a: ^{1}H NMR (DMSO-d_6) δ 1.75−2.00 (4 H, m, piperidine CH_2), 2.90−3.20 (6 H, m, piperidine CH_2 and piperazine CH_2), 3.60−3.90 (6 H, m, piperazine CH_2 and piperidine CH_2), 4.70−4.80 (1 H, m, piperidine CH), 5.50 (2 H, s, benzyl CH_2), 6.55 (1 H, t, J = 7.0 Hz, H-5′), 6.80 (1 H, d, J = 8.5 Hz, H-3′), 7.20−7.40 (6 H, m, aromatic CH and H-8), 7.45−7.55 (2 H, m, H-4′ and H-5), 8.10 (1 H, d, J = 4.0 Hz, H-6′), 8.50 (1 H, s, H-2). Anal. ($C_{31}H_{34}N_6O_3$) C, H, N.

Compound 32a: ^{1}H NMR (DMSO-d_6/$CDCl_3$) δ 2.90−3.10 and 3.35−3.55 (each 4 H, m, piperazine CH_2), 4.60 (2 H, bs, NH_2), 6.10−6.35 (2 H, m, aromatic H), 6.90 (1 H, s, H-8), 7.10−7.40 (5 H, m, aromatic H and H-5), 7.50−7.70 (4 H, m, aromatic H), 8.50 (1 H, s, H-2), 14.50 (1 H, bs, COOH). Anal. ($C_{26}H_{22}N_6O_3S$) C, H, N.

Compound 33d: ^{1}H NMR (DMSO-d_6) δ 2.75−2.95 and 3.75−3.95 (each 4 H, m, piperazine CH_2), 6.30 (1 H, s, H-8), 7.00−7.50 (10 H, m, aromatic CH and H-5), 7.60−7.90 (4 H, m, aromatic CH), 8.60 (1 H, s,H-2). Anal. ($C_{33}H_{27}N_5O_3S$) C, H, N.

General Procedure for Basic Hydrolysis Reaction. A suspension of selected 6-amino ester (0.3 mequiv) in 1 N NaOH (5 mL) was refluxed until the suspension became a solution (usually 2−3 h). After the mixture was cooled to room temperature, the precipitate obtained was filtered and solubilized in water and the solution was brought to pH 6 by adding a 2 N HCl solution. The resulting precipitate was filtered and washed with water to give the corresponding acid. By use of this general procedure, the target acids **26b** and **28b** were obtained. Further experimental details are given in Table 1, while their spectral data are listed below.

Compound 26b: ^{1}H NMR (DMSO-d_6) δ 3.10−3.50 (8 H, m, piperazine CH_2), 3.75 and 4.10 (each 3 H, s, CH_3), 5.50 (2 H, bs, NH_2), 6.80−7.00 (4 H, m, aromatic CH), 7.25 (1 H, s, H-8), 7.50 (1 H, s, H-5), 8.75 (1 H, s, H-2), 16.00 (1 H, bs, COOH). Anal. ($C_{22}H_{24}N_4O_4$) C, H, N.

Compound 28b: ^{1}H NMR ($CDCl_3$) δ 1.10−1.40 (4 H, m, cyclopropyl CH_2), 3.10−3.25 (7 H, m, piperazine CH_2 and OCH_3), 3.75−3.85 (5 H, m, piperazine CH_2 and cyclopropyl CH), 5.50 (2 H, bs, NH_2), 6.90−7.00 (4 H, m, aromatic CH), 7.50 (1 H, s, H-8), 7.70 (1 H, s, H-5), 8.50 (1 H, s, H-2). Anal. ($C_{24}H_{26}N_4O_4$) C, H, N.

1-Methyl-6-nitro-7-[4-(2-pyridinyl)-1-piperazinyl]-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (34a). Starting from nitro ester **10a**[14] and using the above general procedure for acidic hydrolysis (4 h at reflux, purification method J as reported in footnotes of Table 1), the title compound was obtained in 80% yield: mp 295−300 °C; ^{1}H NMR (DMSO-d_6) δ 3.50−3.60 and 3.80−3.90 (each 4 H, m, piperazine CH_2), 4.10 (3 H, s, CH_3), 6.90 (1 H, t, J = 7.0 Hz, H-5′), 7.20 (1 H, d, J = 7.5 Hz, H-3′), 7.30 (1 H, s, H-8), 7.85−8.15 (2 H, m, H-4′ and H-6′), 8.75 (1 H, s, H-5), 9.00 (1 H, s, H-2). Anal. ($C_{20}H_{19}N_5O_5$) C, H, N.

8-Fluoro-1-methyl-6-nitro-7-[4-(2-pyridinyl)-1-piperazinyl]-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (38a). The title compound was obtained starting from synthone **36** and using the general procedure for the coupling reaction (8 h at 70−80 °C, purification method C in Table 1) to give compound **37a** (86% yield) that was then acid-hydrolyzed to **38a** (general method for acidic hydrolysis, 2 h at reflux, purification method K, 82% yield): mp 248−250 °C; ^{1}H NMR (DMSO-d_6) δ 3.25−3.40 and 3.75−3.90 (each 4 H, m, piperazine CH_2), 4.25 (3 H, d, J = 9.0 Hz, CH_3), 6.95 (1 H, t, J = 7.0 Hz, H-3′), 7.40 (1 H, d, J = 9.0 Hz, H-5′), 8.00 (1 H, t, J = 7.0 Hz, H-4′), 8.10 (1 H, d, J = 4.0 Hz, H-6′), 8.50 (1 H, d, J = 1.5 Hz, H-5), 9.00 (1 H, s, H-2). Anal. ($C_{20}H_{18}FN_5O_5$) C, H, N.

6-Amino-8-fluoro-1-methyl-7-[4-(2-pyridinyl)-1-piperazinyl]-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (39a). By use of the general procedure for reduction of the 6-nitro group (purification method H), the title compound **39a** was obtained starting from nitroacid **38a**. After crystallization by EtOH, it was obtained in 22% yield: mp 288−289 °C; ^{1}H NMR (DMSO-d_6) δ 3.10−3.40 (8 H, m, piperazine CH_2), 4.15 (3 H, d, J = 9.0 Hz, CH_3), 5.95 (2 H, bs, NH_2), 6.70 (1 H, t, J = 7.0 Hz, pyridine CH), 6.80 (1 H, d, J = 8.5, pyridine CH), 7.35 (1 H, s, H-5), 7.60 (1 H, t, J = 7.0 Hz, pyridine CH), 8.10 (1 H, d, J = 4.0 Hz, pyridine CH), 8.70 (1 H, s, H-2). Anal. ($C_{20}H_{20}FN_5O_3$) C, H, N.

6-Amino-8-methoxy-1-methyl-7-[4-(2-pyridinyl)-1-piperazinyl]-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (41a). A solution of MeONa (0.36 g, 6.67 mmol) in dry DMF (5 mL) and dry MeOH (3 mL) was added to a suspension of 8-fluoro derivate **38a** (0.5 g, 1.17 mmol) in dry DMF (10 mL). The mixture was heated to 50 °C for 3 h and then poured into ice/water. The solution was acidified to pH 6 with 2 N HCl, and the solid so obtained was filtered and dried to give 0.44 g (92%) of 8-methoxy-6-nitro derivative **40a**: mp 210−211 °C;^{1}H NMR (DMSO-d_6) δ 3.20−3.30 and 3.60−3.75 (each 4 H, m, piperazine CH_2), 3.80 (3 H, s, CH_3), 4.25 (3 H, s, CH_3), 6.70 (1 H, t, J = 7.0 Hz, H-5′), 6.90 (1 H, d, J = 8.5 Hz, H-3′), 7.60 (1 H, t, J = 7.0 Hz, H-4′), 8.15 (1 H, d, J = 4 Hz, H-6′), 8.40 (1 H, s, H-5), 8.90 (1 H, s, H-2), 14.55 (1 H, bs, COOH).

Compound **40a** was then converted to 6-amino acid **41a** following the general procedure for reduction (purification method H) to give a 95% yield: mp 272−274 °C; ^{1}H NMR (DMSO-d_6) δ 3.10−3.40 (8 H, m, piperazine CH_2), 3.50 (3 H, s, CH_3), 4.05 (3 H, s, CH_3), 5.50 (2 H, bs, NH_2), 6.70 (1 H, t, J = 7.0 Hz, H-5′), 6.90 (1 H, d, J = 8.5 Hz, H-3′), 7.45 (1 H, s, H-5), 7.60 (1 H, t, J = 7.0 Hz, H-4′), 8.10 (1 H, d, J = 4 Hz, H-6′), 8.60 (1 H, s, H-2), 15.80 (1 H, bs, COOH) Anal. ($C_{21}H_{23}N_5O_4$) C, H, N.

7-Chloro-1-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid BF_2 Chelate (44). A mixture of **43** (0.4 g, 1.5 mmol) and 48% HBF_4 in water was heated at 90−100 °C for 30 min. After the mixture was cooled, the white solid was filtered, washed with water, and dried to give borine chelate **44** (0.4 g, 93%): ^{1}H NMR. (DMSO-d_6) δ 4.40 (3 H, s, CH_3), 8.00 (1 H, dd, J = 2.0 and 8.9 Hz, H-6), 8.50 (1 H, d, J = 2.0 Hz, H-8), 8.55 (1 H, d, J = 8.9, H-5), 9.60 (1 H, s, H-2).

7-[4-(2-Methoxyphenyl)-1-piperazinyl]-1-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (45b). A mixture of borine complex **44** (0.65 g, 2.3 mmol), 1-(2′-methoxyphenyl)-piperazine (2.18 g, 11.4 mmol), and Et_3N (0.69 g, 6.8 mmol) in dry DMSO (30 mL) was heated to 100 °C for 4 h. The mixture was then evaporated to dryness to give a residue that was treated with ice/water. The obtained solid was filtered, dried, and purified by flash chromatography, eluting with MeOH/$CHCl_3$ (2:98) to give acid **45b** (0.18 g, 20%): mp 290−310 °C; ^{1} H NMR (DMSO-d_6) δ 3.10−3.20 (4 H, m, piperazine CH_2), 3.70−3.90 (7 H, m, piperazine CH_2 and CH_3), 4.25 (3 H, s, CH_3), 6.80−7.10 (4 H, m, aromatic CH), 7.15 (1 H, d, J = 2.0 Hz, H-8), 7.70 (1 H, dd, J = 2.0 and 8.9 Hz, H-6), 8.25 (1 H, d, J = 8.9 Hz, H-5), 9.25 (1 H, s, H-2). Anal. ($C_{22}H_{23}N_3O_4$) C, H, N.

1-Methyl-7-[4-(2-pyridinyl)-1-piperazinyl]-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (45a). Following the procedure of **45b**, starting from borine complex **44** and substituting 1-(2-methoxyphenyl)piperazine with 1-(2-pyridinyl)piperazine, the title compound was prepared (26%): mp 302−303 °C; ^{1} H NMR(DMSO-d_6) δ 3.60−3.90 (8 H, m, piperazine CH_2), 4.25 (3 H, s, NCH_3), 6.70 (1 H, t, J = 7.0 Hz, pyridine CH), 6.90 (1 H, d, J = 8.5 Hz, pyridine CH), 7.10 (1 H, bs, H-8), 7.50−7.70 (2 H, m, pyridine CH), 8.15 (1 H, d, J = 4 Hz, pyridine CH), 8.25 (1 H, d, J = 8.9 Hz, H-5), 9.30 (1 H, s, H-2). Anal. ($C_{20}H_{20}N_4O_3$) C, H, N.

1-Methyl-7-[4-(2-pyridinyl)-1-piperazinyl)]-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic Acid (49a). Starting from synthone **48** and with the general procedure for coupling (100 °C for 72 h, purification method D in Table 1) followed by hydrolysis as reported in the general procedure

for basic hydrolysis, the title compound was obtained in 50% yield: 319−320 °C; ^{1}H NMR (DMSO-d_6/$CDCl_3$) δ 3.50−3.70 (4 H, m, piperazine CH_2), 3.80−4.00 (7 H, m, piperazine CH_2 and CH_3), 6.50−6.65 (2 H, m, pyridine CH and H-8), 6.80 (1 H, d, J = 8.5 Hz, pyridine CH), 7.40−7.55 (1 H, m, pyridine CH), 8.10−8.20 (1 H, m, pyridine CH), 8.85 (1 H, s, H-5), 9.10 (1 H, s, H-2), 15.00 (1 H, bs, COOH). Anal. ($C_{19}H_{19}N_5O_3$) C, H, N.

1-(5-Chloro-1,3-benzothiazol-2-yl)piperazine (g). A mixture of 2-mercapto-5-chloro-1,3-benzothiazole, (2 g, 9.93 mmol), MeI (1.41 g, 9.93 mmol), KOH (0.056 g, 9.93 mmol), and TBAB (0.30 g, 1.0 mmol) in dry THF was reacted at room temperature for 3 h. The mixture was then poured into water to give a white solid that was filtered, dried, and recrystallized from MeOH, affording 2-methylmercapto-5-chloro-1,3- benzothiazole (1.7 g, 79%).

The mixture of the above compound (0.6 g, 2.78 mmol) and piperazine (1.436 g, 16.7 mmol) was heated in a closed vessel for 5 h at 110 °C. After cooling, the mixture was treated with MeOH to give a solid that was filtered to afford base **g** (0.7 g, 94%); mp 128−132 °C; ^{1}H NMR ($CDCl_3$) δ 3.00−3.20 and 3.60−3.70 (each 4 H, m, piperazine CH_2), 7.05 (1 H, dd, J = 13.2 Hz, H-6), 7.50−7.65 (2 H, m, H 4 and H-7).

1-(5-Methyl-1,3,4-thiadiazol-2-yl)piperazine (l). By use of the above procedure, replacing 2-mercapto-5-chloro-1,3-benzothiazole with 2-mercapto-5-methyl-1,3,4-thiadiazole, the title compound was obtained in 88% yield: mp 115 °C; ^{1} H NMR ($CDCl_3$) δ 2.60 (3 H, s, CH_3), 3.00−3.10 and 3.50−3.60 (each 4 H, m, piperazine CH_2), 4.20 (1 H, bs, NH).

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.[30] Stock solutions (10× final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells 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 2000 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 (50μL) 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 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 an amount of 50 μL volumes was 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 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 (OD_{540}) 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}).

Evaluation of the antiviral activity of compounds against HIV-1 strain III_B and HIV-2 strain ROD-induced cytopathic effect in CEM cells was performed using the MTS assay as previously described.[34] This cell-based microtiter assay quantitates the ability of a compound to inhibit the HIV-induced cell killing. Briefly, exponentially growing CEM cells[35] were collected by centrifugation and suspended in fresh tissue culture medium at 250 000 cells per mL. The cells were infected with 100 50% cell culture infective doses ($CCID_{50}$) of HIV-1(III_B) or HIV-2 (ROD). Then, 100 μL of the infected cell suspensions was added to 200 μL microtiter plate wells containing 100 μL of an appropriate dilution of the test compounds. The inhibitory effects of the test compounds on HIV-induced syncytium formation in CEM cells were examined on day 4 postinfection, as previously described.[36,37]

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 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.[38] 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 (NEN, Paris, France).

Time-of-Addition Experiment. MT-4 cells were infected with HIV-1 (III_B) at a multiplicity of infection (moi) of 0.5. The test compounds were added at different times after infection (from 0 to 25 h).[39] Viral p24 Ag production was determined at 31 h postinfection by ELISA (NEN, Brussels, Belgium). The reference compounds were added at 100 times their 50% inhibitory concentration (IC_{50}) obtained in the MT-4 cells/MTT assay.

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Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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