Structure−Activity Relationships and Binding Mode of Styrylquinolines as Potent Inhibitors of HIV-1 Integrase and Replication of HIV-1 in Cell Culture

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Our prior studies showed that polyhydroxylated styrylquinolines are potent HIV-1 integrase (IN) inhibitors that block the replication of HIV-1 in cell culture at nontoxic concentrations. To explore the mechanism of action of these inhibitors, various novel styrylquinoline derivatives were synthesized and tested against HIV-1 IN and in cell-based assays. Regarding the in vitro experiments, the structural requirements for biological activity are a carboxyl group at C-7, a hydroxyl group at C-8 in the quinoline subunit, and an ancillary phenyl ring. However the in vitro inhibitory profile tolerates deep alterations of this ring, e.g. by the introduction of various substituents or its replacement by heteroatomic nuclei. Regarding the ex vivo assays, the structural requirements for activity are more stringent than for in vitro inhibition. Thus, in addition to an *o*-hydroxy acid group in the quinoline, the presence of one *ortho* pair of substituents at C-3′ and C-4′, particularly two hydroxyl groups, in the ancillary phenyl ring is imperatively required for inhibitory potency. Starting from literature data and the SARs developed in this work, a putative binding mode of styrylquinoline inhibitors to HIV-1 IN was derived.

Introduction

AIDS is essentially a viral disease and should be treated by antiretroviral agents.[1] Although a combination of antiretroviral therapy has made it possible to suppress the replication of HIV-1 in infected individuals to such an extent that it becomes undetectable in the plasma, the virus persists in reservoirs such as peripheral blood mononuclear cells or resting T-lymphocytes which went back to a latent state after an early replicative stage.[2−4] Thus, after more than 3 years of multidrug therapy, it turned out that the HIV-1 infection can be controlled but not eradicated with current treatments. Besides, the emergence of resistance against both reverse transcriptase and protease inhibitors, which was a frequent issue of the single-agent regimen, was not completely suppressed by the use of combination therapy. It is therefore important to identify new agents that could block the virus at a step of its replicative cycle which is not yet affected by current treatments. For these reasons, the integrase (IN), the third viral enzyme which is strictly required for the establishment of a stable infection,[5] is an attractive target for new antiviral agents and potential synergy with currently available HIV reverse transcriptase and protease inhibitors.[6] IN catalyzes two reactions consisting both in the nucleophilic attack of a phosphoester bond by a hydroxyl group.[7] In the first step, called 3′-processing, IN removes two nucleotides from both 3′-viral DNA ends and forms free 3′-OH groups at a conserved CA sequence. In the second step, or strand transfer, free 3′-OH's are used as nucleophilic agents and attack phosphoester bonds on opposite strands of target DNA.[8] Systematic screening of potential IN inhibitors yielded several families of active compounds, including nucleotides and oligonucleotides, peptides, and small organic molecules.[9−18] The most populated family consists of polyhydroxylated aromatic compounds. Members of this group display recurrently the *o*-diphenol (catechol) function. Among the numerous classes of HIV IN inhibitors which have emerged, only a very few of them exhibit a substantial level of antiviral activity at nontoxic concentrations. We have recently reported that polyhydroxylated styrylquinolines are potent HIV IN inhibitors, block the replication of HIV in cell culture, and are devoid of cytotoxicity.[19] However, despite these remarkable properties, the binding mode of these inhibitors remains to be elucidated. In the present paper, with the aim to explore their mechanism of action, various novel styrylquinoline derivatives were synthesized and the effects of these structural modifications were assayed against HIV-1 IN in both 3′-processing and strand-transfer reactions, as well as against HIV-1 replication in CEM cell culture.

Results and Discussion

Chemistry. Reference compounds **9**, **19**, **20**, and **23** (Table 1) have been previously described.[19] Apart from compounds **15**, **28**, and **30**, all new styrylquinolines were basically elaborated through condensation between a

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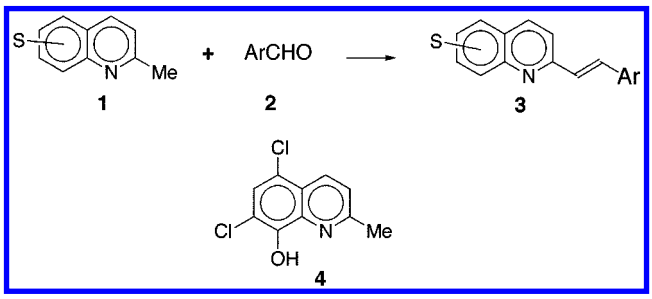
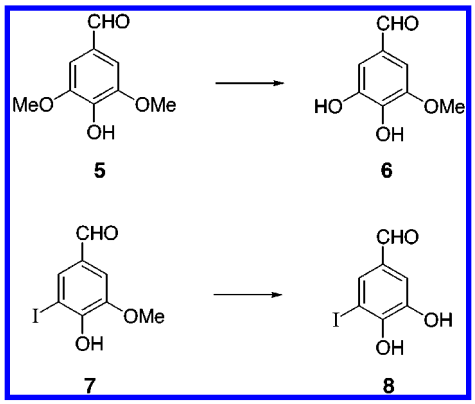
Scheme 1



Scheme 2



2-methylquinoline moiety **1** and an aromatic or heteroaromatic aldehyde **2**, followed by neutral or acidic hydrolysis. 8-Hydroxy-7-quinaldic acid (**9**) was used as starting subunit in the syntheses of derivatives **10**−**27** and **30**. Commercially available 5,7-dichloro-2-methyl-8-quinolinol (**4**) was utilized in the preparation of **29** (Scheme 1).

All aromatic or heteromatic aldehydes used are commercially available, with the exception of **6** and **8** (required for the synthesis of **24** and **27**), which were prepared through $AlCl_3$-induced demethylation[20] of syringaldehyde (**5**) and 5-iodovanillin (**7**), respectively (Scheme 2).

Styrylquinoline **15** resulted from the acidic hydrolysis of **16**, and **28** resulted from the condensation between quinaldine **9** and 3,4-dihydroxybenzaldehyde, followed by methanolysis of the crude product, using a MeOH−pyridine mixture. Compound **30** was prepared by catalytic hydrogenation of **20**.

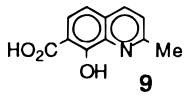
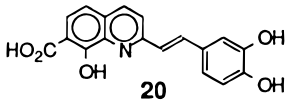
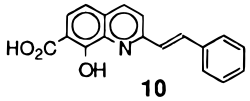
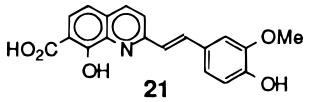
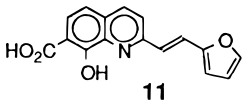
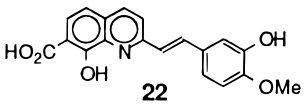
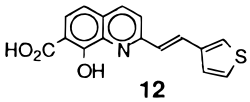
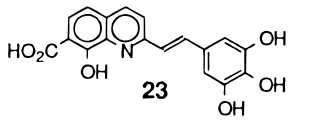
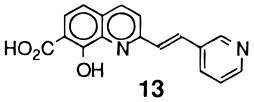
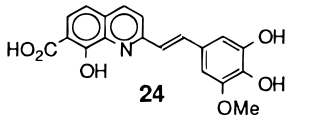
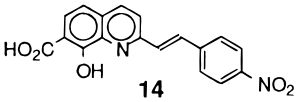
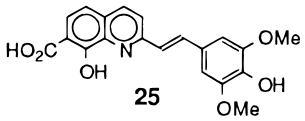
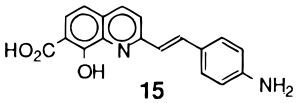
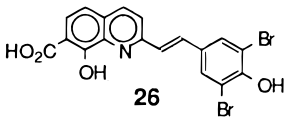
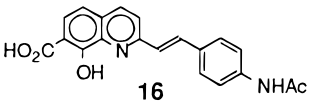
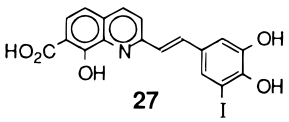
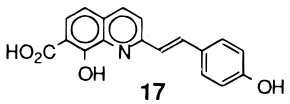
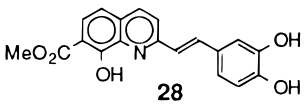
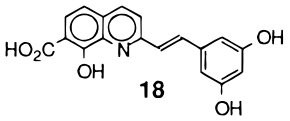
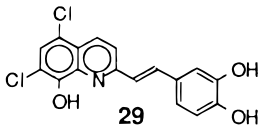
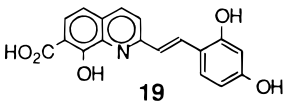
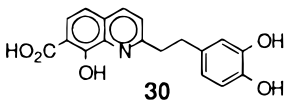
Activities against HIV-1 IN. Each new compound was screened for inhibitory activity against HIV-1 IN in both 3′-end-processing and strand-transfer reactions. Results are illustrated in Table 1. These compounds can be roughly classified as active (**10**−**27** and **30**) and inactive (**9**, **28,** and **29**). Though a complete lack of inhibitory potency was observed with the parent 8-hydroxy-7-quinaldic acid (**9**), micromolar or submicromolar activities were obtained with styrylquinolines **10**−**27**, in which this quinoline half is linked to an aromatic (or heteromatic) moiety by means of an ethylenic spacer. One striking feature of the above findings is that the replacement of the ancillary phenyl ring of prototype compound **10** by a variety of heteroatomic nuclei (2′-furyl, 3′-thienyl, and 3′-pyridyl analogues **11**−**13**, respectively) has only a limited effect on anti-HIV-1 IN activity, with IC_{50} values ranging from 1.9 to 11 μM. Also remarkable is the observation that the in vitro inhibitory potency is relatively independent of the π-electron density of the phenyl subunit, since compounds **14**−**16**, in which this ring bears a *p*-nitro, *p*-amino, and *p*-acetamido group, respectively, exhibited similar IC_{50} values, of approximately 1−4 μM. Likewise, hydroxylated analogues (4′-hydroxy **17**, 3′,5′-dihydroxy **18**, 2′,4′-dihydroxy **19**, 3′,4′-dihydroxy **20**, 3′-methoxy-4′-hydroxy **21**, 3′-hydroxy-4′-methoxy **22**, 3′,4′,5′-trihydroxy **23**, 3′,4′-dihydroxy-5′-methoxy **24**, 3′,5′-dimethoxy-4′-hydroxy **25**, 3′,5′-dibromo-4′-hydroxy **26**, and 3′,4′-dihydroxy-5′-iodo **27**) were all active in the range between 0.3 and 5 μM. In sharp contrast, compounds **28** and **29**, in which the 7-carbomethoxy-8-hydroxyquinoline and 5,7-dichloro-8-hydroxyquinoline moieties are respectively linked to a 3′,4′-catechol half by means of an ethylenic bridge, were completely devoid of biological activity. Finally, a full IN inhibitory potency (IC_{50} of approximate 2.0 μM) was restored in styrylquinoline **30**, analogue to parent compound **20** where the central ethylenic linker is replaced by an ethano bridge.

All these results clearly indicate that the presence of a 7-carboxyl-8-hydroxyl pattern in the quinoline half and an aromatic or heteroaromatic ancillary subunit are required for the biological activity of the drug. Replacements of the 7-carboxyl by a carbomethoxy group (compound **28**) or a chlorine atom (compound **29**) are indeed accompanied by a complete loss of inhibitory potency. However somewhat surprisingly, the in vitro inhibitory profile not only tolerates deep alterations of the ancillary phenyl subunit by the introduction of various substituents or its replacement by heteroaromatic nuclei but also is unaffected by the replacement of the ethylenic linker by a saturated counterpart (compound **30**). That the present inhibitors more likely act specifically against the central IN core domain rather than the end parts of IN was established by measuring the effect of "nonhydroxylated" derivatives **10**−**16** upon disintegration reaction catalyzed by the (50−212) deletion mutant. All these molecules indeed displayed good inhibitory potencies in this assay (IC_{50} range 0.8−12 μM) (Table 2). Incidentally, this experiment also confirms that the presence of hydroxyl group(s) in the aromatic (heteroaromatic) half is not an absolute requirement for in vitro activity.

Anti-HIV Activities and Cytotoxicity. Compounds **9**−**30** were evaluated for their antiviral activities against HIV-1 replication in CEM cells. They were tested for their ability to lower the viral charge in culture supernatants. CEM cells were infected with HIV-1 and subsequently treated with increasing drug concentrations. Viral load was estimated 72 h after infection. The amount of virus was tested by β-galactosidase assay, with HeLa CD4-β gal cells as reporting cells. Toxicity was estimated by MTT transformation assay. Results are listed in Table 3.

At first glance, the present molecules can be categorized as inactive, cytotoxic, and active. Inactive compounds comprise quinaldine **9**, styrylquinolines **11** and **13** containing a 2′-furyl and 3′-pyridyl ring, respectively, and **17** and **18**, where the phenyl subunit is substituted by hydroxyl groups at C-4′ and at C-3′ and C-5′, respectively. Among the cytotoxic compounds, 3′-thienyl derivative **12** and **30,** in which the two subunits are linked by means of an ethano bridge, exhibited a relatively weak toxicity (TC_{50} > 60 μM). Styrylquino-

Table 1. HIV-1 IN Inhibitory Potencies Determined as Described in the Experimental Section

Compound	IC_{50} Values (μM)		Compound	IC_{50} Values (μM)	
	3'-Processing	Integration		3'-Processing	Integration
 **9**	>100	>100	 **20**	2.4	1.0
 **10**	5.3	2.1	 **21**	2.8	3.7
 **11**	1.9	5.1	 **22**	0.9	3.3
 **12**	3.4	3.0	 **23**	0.3	0.4
 **13**	4.1	11	 **24**	0.7	1.7
 **14**	1.2	1.7	 **25**	4.9	4.5
 **15**	3.5	2.2	 **26**	1.3	1.2
 **16**	1.4	1.2	 **27**	4.0	4.9
 **17**	1.6	1.6	 **28**	>100	>100
 **18**	3.2	3.2	 **29**	>100	>100
 **19**	3.7	2.8	 **30**	2.3	1.5

lines in which the ancillary phenyl nucleus is either unsubstituted (**10**) or substituted at C-4′ by a nitro group (**14**) or an acetamido function (**16**) proved to be moderately cytotoxic (TC_{50} ranging from 30 to 50 μM). Finally analogue **23,** which possesses three vicinal hydroxyl functions, and **28** and **29**, in which the carboxyl group at C-7 in the quinoline half is respectively replaced by a carbomethoxy group or a chlorine atom, showed a relatively high cytotoxicity (TC_{50} between 10 and 17 μM). Within the active compounds series, styrylquinolines where the phenyl half is substituted at C-4′ by an amino group (**15**), at C-2′ and C-4′ by two hydroxyls (**19**), and at C-3′ by a hydroxyl and at C-4′ by a methoxy group (**22**) displayed low anti-HIV-1 activities (IC_{50} ranging from 50 to 80 μM). A moderate antiviral activity (IC_{50} between 7 and 12 μM) was gained with styrylquinolines in which the phenyl moiety exhibits the 3′,4′-dihydroxy-5′-methoxy, 3′,5′-dimethoxy-4′-hydroxy, and 3′,4′-dihydroxy-5′-iodo trisubstitution patterns (**24**, **25**, and **27,** respectively). Finally, 3′,4′-dihydroxy and 3′-methoxy-4′-hydroxy derivatives (**20** and **21,** respec-

Table 2. Effect of "Nonhydroxylated" Styrylquinolines upon Disintegration Catalyzed by the (50−212) Deletion Mutant

Compound	IC_{50} (μM)
10	3.4
11	1.4
12	0.8
13	5.1
14	2.2
15	12.0
16	1.7

Table 3. Antiviral Activity and Cytotoxicity[a]

Compound	IC_{50} (μM)	TC_{50} (μM)
9	>100	>100
10	NR	31
11	>100	>100
12	NR	85
13	>100	>100
14	NR	43
15	50	100
16	NR	47
17	95	>100
18	>100	>100
19	80	>100
20	1.3	>100
21	3.0	95
22	54	95
23	1.2	10
24	12	>100
25	9.0	>100
27	6.8	>100
28	NR	9.2
29	NR	17
30	NR	61

[a] NR, not reached.

tively) showed good anti-HIV potencies (IC_{50} between 1 and 3 μM).

All these results demonstrate that the structural requirements for ex vivo activity of the drug are more stringent than for in vitro IN inhibition. Regarding the quinoline subunit, the effect of its alteration more or less parallels the in vitro observations. Thus, the presence of a free carboxyl group at C-7 is critical: its replacement by a carbomethoxy group or a chlorine atom resulted in a moderate to high cytotoxicity. In fact, the other crucial structural requirement concerns the ancillary aromatic moiety linked to the quinoline moiety. While the IN inhibitory profile tolerates a great variety of substitution of this ring or its replacement by heteroaromatic nuclei, the anti-HIV activity imperatively requires the presence of one *ortho* pair of substituents: a free hydroxyl group at C-4′ and a hydroxyl *or* a methoxy group at C-3′. Evidently, though the 3′,4′-catechol group plays a favorable role in the anti-HIV profile, its presence is not absolutely required, since the replacement of the hydroxyl at C-3′ by a methoxy group does not notably reduce the biological activity. However it is worthy of note that the presence of three vicinal hydroxyl groups (compound **23**) resulted in high cytotoxicity. Finally, replacement of the central ethylenic linker by an ethano bridge was the cause of significant collateral cytotoxicity (Table 3).

Binding Mode of Styrylquinolines. Although the precise target of styrylquinolines in the ex vivo experiments is not yet firmly identified, current data support the interpretation that these drugs block HIV-1 replication directly through inhibition of HIV-1 IN. Thus, the good correlation between activities in isolated enzyme models and the ex vivo cellular data reported here is consistent with this hypothesis. Furthermore, we believe that the inhibition of virus penetration may be ruled out for the following reasons: (i) in our conditions of infection, we observed that the entry of viruses into cells peaked after 2 h following addition of viruses to the culture medium; thus, the de novo infection assay was designed in such a manner that drugs were added to the cell culture medium only 2 h following infection and after an extensive wash of cells designed to eliminate the remaining free viruses; (ii) upon de novo infection of CEM cells, progeny viruses became detectable in culture medium after 40 h; thus the viremia estimated at 72 h was mostly due to viruses produced after only one round of infection; and (iii) we previously reported that in a "time of addition" experiment, styrylquinoline derivatives displayed a maximum activity when added between 4 and 16 h following infection, a time course compatible with the inhibition of a post-penetration step of replication. Finally, since styrylquinolines inhibited HIV IN but not reverse transcriptase in vitro, we consider that IN inhibition in infected cells is a plausible explanation for the ex vivo antiviral activity of these inhibitors.[19]

Another striking argument was provided by the elegant work of Robinson et al. who demonstrated that a single amino acid change in HIV-1 IN was sufficient to confer resistance to L-chicoric acid, an inhibitor which offers some structural resemblance to styrylquinolines.[21] Crystal structures of the core domain of HIV-1 IN mutants complexed with Mg^{2+} have been reported.[22] The magnesium is coordinated in the active site by Asp-64 and Asp-116, whereas the third residue of the catalytic amino acid triad, Glu-152, does not directly participate in metal binding. Such a binding site was unequivocally identified by X-ray structure determination of a complex between a naphthalenedisulfonic inhibitor (Y-3) and ASV IN, a protein which exhibits great similarity with HIV-1 IN.[23] The Y-3 molecule in the crystal is located in close proximity to the enzyme active site. Recently, the X-ray structure of the HIV-1 IN catalytic domain complexed with an inhibitor (5CITEP) has been determined. The inhibitor binds centrally in the active site of the IN and makes a number of close contacts with the protein. Only minor changes in the protein accompany inhibitor binding.[24]

Since it was recognized that HIV-1 IN requires a divalent cation as cofactor for its enzymatic activity (presumably Mg^{2+}), and assuming that an *o*-hydroxy

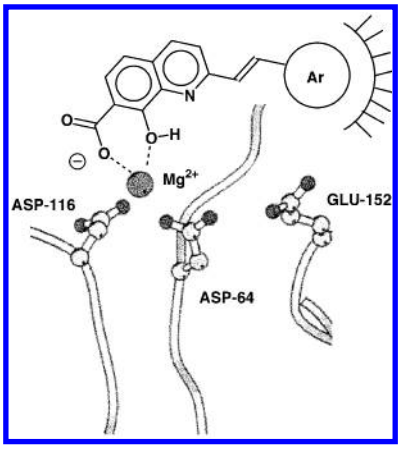


Figure 1. Presumed in vitro binding interactions between styrylquinolines and the HIV-1 IN catalytic site.

acid group in the quinoline subunit is crucially involved in both in vitro and ex vivo inhibitory activities, we hypothesized that this group lies in a possible coordination to the metal cation. Such an assumption has been recently reinforced by computational studies. Indeed, minimization of the energy of interaction between styrylquinoline drugs on the whole surface of the catalytic core domain of RSV IN revealed that the best fits were found for binding of the drugs on Mg^{2+} in the vicinity of the active site.[25]

The other prerequisite for ex vivo potency was the presence at the ancillary phenyl ring of a hydroxyl group at C-4′ and a hydroxyl or a methoxy group at C-3′. These data suggest that, in addition to an *o*-hydroxy acid, free hydroxyl group(s) may be necessary for uptake of the drugs by the cell or into the virion. Finally, besides the presence of a carboxylic acid and hydroxyl group(s), the requirement of a correct size and a geometrical fit seems also essential for anti-HIV inhibitory potency, since the replacement of the central ethylenic linker by a saturated counterpart resulted in significant cytotoxicity.

Starting from the above literature data and the SARs developed in this work, a putative binding mode of styrylquinolines to HIV-1 IN was derived (Figure 1). The platform used in designing this model was the binding site of HIV-1 IN mutants, in which a Mg^{2+} ion is coordinated by two catalytic aspartates (Asp-64 and Asp-116).[22] Binding interactions between the drug and the enzyme catalytic site were next tentatively identified. As mentioned above, since the presence at C-7 and C-8 of an *o*-hydroxy acid is required for in vitro activities, one of the sites of recognition might involve the coordination by this group of the Mg^{2+} cation. The second complementary feature for recognition is the presence of an ancillary phenyl ring. However, since the in vitro inhibitory profile tolerates deep alterations of this subunit, e.g. by the introduction of various substituents or it replacement by heteroatomic nuclei, this second site of recognition clearly involves a nonspecific binding mode, possibly through a π-stacking interaction.

Regarding the ex vivo activity, the structural requirements are more stringent than for in vitro IN inhibition. As a matter of fact, in that case two additional binding forces play a significant role in the recognition of the drug by the protein. These apparently involve hydrogen bonds at C-3′ and/or C-4′, a situation that is a specific consequence of the convergence of functional groups, particularly two hydroxyls, at this end of the molecule. A final interesting aspect of the present model is that the geometrical pattern defined by the functional group triad at C-7/C-8, C-3′, and C-4′ satisfactorily matches one of the putative three-point pharmacophores previously identified by Pommier and co-workers through a 3D database searching.[26,27]

Conclusions

Styrylquinolines have been recognized as potent HIV-1 IN inhibitors that block HIV-1 replication in cell-based assays.[19] In the present paper, with the aim to explore their mechanism of action, various novel styrylquinoline derivatives were synthesized and their biological properties were evaluated. Since styrylquinolines fall within the general category of inhibitors characterized by two aryl groups linked through a central spacer, we have examined the molecular determinants of each of these subunits, through a progressive series of analogues. Regarding the in vitro inhibition, micromolar or submicromolar activities were obtained with compounds **10**−**27** and **30**, though **9**, **28**, and **29** proved to be inactive. These results clearly demonstrate that the presence of a free carboxyl group at C-7 and a hydroxyl at C-8 in the quinoline half is required for the biological activity of the drug. The other prerequisite for in vitro potency was the presence of an ancillary aromatic ring. However the in vitro inhibitory profile tolerates the introduction of various substituents in this ring or its replacement by heteroatomic nuclei. The structural requirements for ex vivo activity are more stringent than for in vitro IN inhibition. In addition to the presence of an *o*-hydroxy acid group in the quinoline, the anti-HIV activity indeed requires the presence of one *ortho* pair of substituents in the ancillary phenyl ring: a free hydroxyl group at C-4′ and a hydroxyl or methoxy group at C-3′. Examination of SARs developed for our compounds, interpreted in the light of literature data, led to a putative binding mode of styrylquinolines to HIV-1 IN. Since the Mg^{2+} ion is a cofactor required for the enzymatic activity of HIV-1 IN, we hypothesized that the *o*-hydroxy acid group lies in a possible coordination to this metal cation. The additional binding force which plays a significant role in the recognition of the drug by the protein apparently involves the ancillary aromatic ring. However, since the exact mechanism by which styrylquinolines inhibited productive infection is unknown, the definitive mapping of the binding sites of these inhibitors awaits further investigation. In summarizing the above findings, it can be concluded that the insights so gained are likely to aid the design and development of new useful HIV-1 IN inhibitors. HIV-1 IN has indeed become an attractive target for intervention by chemotherapeutics, because of the rapid emergence of resistance against both reverse transcriptase and protease inhibitors, currently used in combination regimens for the treatment of AIDS.[28]

Experimental Section

Melting points were recorded on a capillary tube melting point apparatus and are uncorrected or by microthermal analysis on a Mettler FP5 apparatus. Infrared (IR) spectra were obtained as neat films between NaCl plates or KBr pellets. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$, unless otherwise stated; when diffuse, easily exchangeable

protons were not listed. Recognition of methyl, methylene, methine, and quaternary carbon nuclei in ^{13}C NMR spectra rests on the *J*-modulated spin−echo sequence. Analytical thin-layer chromatography was performed on Merck silica gel $60F_{254}$ glass precoated plates (0.25 mm layer). All liquid chromatography separations were performed using Merck silica gel 60 (230−400 mesh ASTM). Ether and tetrahydrofuran (THF) were distilled from Na-benzophenone ketyl. Methanol was dried over magnesium and distilled. Benzene and CH_2Cl_2 were distilled from calcium hydride, under a nitrogen atmosphere. All reactions involving air- or water-sensitive compounds were routinely conducted in glassware which was flame-dried under a positive pressure of nitrogen. Organic layers were dried over anhydrous $MgSO_4$. Chemicals obtained from commercial suppliers were used without further purification. Elemental analyses were obtained from the Service de microanalyse, Centre d'Etudes Pharmaceutiques, Châtenay-Malabry, France.

Condensation of 8-Hydroxy-7-quinaldic Acid (9) with Aromatic and Heteroaromatic Aldehydes. General Method A: To a solution of 1.0 equiv of acid **9** in acetic anhydride (3 mL/mmol) was added 4 equiv of aldehyde. The mixture was heated under reflux for 16 h and concentrated in vacuo. The residue was dissolved in 6 N sulfuric acid (4 mL/mmol) and the resulting solution was heated under reflux for 3 h. After beeing cooled 0 °C, 6 N potassium hydroxide was added until neutrality. The mixture was filtered and washed with water, 2-propanol and finally ether. The solid was collected and taken up into water, and the suspension was refluxed for 1 h. The mixture was then filtered; the solid was recovered and dried under vacuum. The process was repeated with CH_2Cl_2 and ether. The crude product was finally recrystallized from 2-propanol and dried in vacuo.

General Method B: Method B was identical to method A, except that recrystallization from 2-propanol was replaced by washing with hot 2-propanol.

General Method C: To a solution of 1.0 equiv of acid **9** in acetic anhydride (3 mL/mmol) was added 4 equiv of aldehyde. The mixture was heated under reflux for 16 h and concentrated in vacuo. The residue was dissolved in pyridine (4 mL/mmol), water (1 mL/mmol) was then added, and the reaction mixture was refluxed for 3 h. After cooling, the mixture was concentrated under reduced pressure. The solid residue was taken up into CH_2Cl_2 and filtered. The solid was washed with 2-propanol and ether and dried in vacuo.

(*E*)-8-Hydroxy-2-(2-phenylethenyl)-7-quinolinecarboxylic Acid (10). Method A using benzaldehyde afforded acid **10** in 46% overall yield as brick red crystals: mp 178−180 °C; IR (KBr, cm^{-1}) 3600−2400, 1680, 1614, 1590; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.41 (d, J = 8.7 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 7.92 (d, J = 16.4 Hz, 1H), 7.81 (d, J = 8.6 Hz, 1H), 7.71 (d, J = 6.6 Hz, 2H), 7.60 (d, J = 16.4 Hz, 1H), 7.50−7.35 (m, 3H), 7.29 (d, J = 8.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.5, 160.1, 153.7, 138.2, 137.4, 136.2 (2C), 131.0, 129.1 (3C), 127.5 (2C), 126.6 (2C), 121.7, 115.5, 111.5. Anal. ($C_{18}H_{13}NO_3 \cdot H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(2-furyl)ethenyl]-7-quinolinecarboxylic Acid (11). Method C using furfural gave acid **11** in 56% overall yield as brown crystals: mp 215−218 °C; IR (KBr, cm^{-1}) 3700−2400, 1630, 1606, 1511; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.36 (d, J = 8.7 Hz, 1H), 7.98 (d, J = 8.7 Hz, 1H), 7.82 (s, 1H), 7.80 (d, J = 8.7 Hz, 1H), 7.78 (d, J = 16.1 Hz, 1H), 7.33 (d, J = 16.1 Hz, 1H), 7.24 (d, J = 8.7 Hz, 1H), 6.81 (d, J = 3.4 Hz, 1H), 6.63 (dd, J = 3.4, 1.9 Hz, 1H); ^{13}C NMR (acetone-d_6, 50 MHz) δ 171.8, 160.7, 153.5, 152.4, 145.0, 138.2, 131.0, 127.0, 124.0, 123.5 (2C), 122.0, 115.3, 113.2, 113.0, 112.4. Anal. ($C_{16}H_{11}NO_4 \cdot H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(3-thienyl)ethenyl]-7-quinolinecarboxylic Acid (12). Method C using 3-thiophenecarboxaldehyde gave acid **12** in 33% overall yield as orange crystals: mp 175−178 °C; IR (KBr, cm^{-1}) 3400−2300, 1690, 1608, 1514; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.39 (d, J = 8.7 Hz, 1H), 8.01(d, J = 8.7 Hz, 1H), 7.93 (d, J = 16.4 Hz, 1H), 7.81 (d, J = 8.6 Hz, 1H), 7.83 (d, J = 0.5 Hz, 1H), 7.65 (dd, J = 5.0, 2.8 Hz, 1H), 7.57 (dd, J = 5.0, 0.8 Hz, 1H), 7.45 (d, J = 16.2 Hz, 1H), 7.26 (d, J = 8.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.4, 160.1, 153.8, 139.1, 138.0, 137.4, 130.7, 130.3, 127.5, 126.6, 126.4, 126.2, 125.2, 121.3, 115.2, 111.5. Anal. ($C_{16}H_{11}NO_3S \cdot {}^1/_2H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(3-pyridinyl)ethenyl]-7-quinolinecarboxylic Acid (13). Method C using 3-pyridinecarboxaldehyde gave acid **13** in 57% overall yield as yellow crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3600−3000, 2800−1800, 1633, 1592; ^1H NMR (CF_3CO_2D, 400 MHz) δ 9.15 (s, 1H), 8.93 (d, J = 8.9 Hz, 1H), 8.89 (d, J = 8.4 Hz, 1H), 7.79 (d, J = 5.8 Hz, 1H), 8.50 (d, J = 8.9 Hz, 1H), 8.31 (d, J = 8.7 Hz, 1H), 8.19 (d, J = 16.4 Hz, 1H), 8.13 (dd, J = 8.4, 5.8 Hz, 1H), 8.07 d, J = 16.4 Hz, 1H), 7.68 (d, J = 8.7 Hz, 1H); ^{13}C NMR (CF_3CO_2D, 100 MHz) δ 175.6, 155.2, 153.2, 149.5, 147.4, 144.5, 143.6, 139.8, 137.3, 134.8, 133.0, 130.7, 130.4, 127.3, 123.8, 121.2, 118.5. Anal. ($C_{17}H_{12}N_2O_3 \cdot 2H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(4-nitrophenyl)ethenyl]-7-quinolinecarboxylic Acid (14). Method B using 4-nitrobenzaldehyde afforded acid **14** in 57% overall yield as brick red crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3700−2400, 1683, 1594, 1510; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.41 (d, J = 8.6 Hz, 1H), 8.26 (d, J = 8.6 Hz, 2H), 8.09−7.96 (m, 4H), 7.83 (d, J = 8.8 Hz, 1H), 7.76 (d, J = 16.5 Hz, 1H), 7.36 (d, J = 8.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.7, 159.0, 153.4, 147.0, 142.6, 138.0, 137.3, 133.5, 131.1 (2C), 128.3 (2C), 126.7, 124.0 (2C), 122.3, 116.8, 111.0. Anal. ($C_{18}H_{12}N_2O_5 \cdot 2\ H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(4-aminophenyl)ethenyl]-7-quinolinecarboxylic Acid (15). A suspension of styrylquinoline **16** (350 mg, 1.0 mmol) in 6 N sulfuric acid (10 mL) was heated under reflux for 2 h. After being cooled at 0 °C, 6 N potassium hydroxide was added until neutrality. The mixture was filtered and washed with water, 2-propanol and finally ether. The solid was collected and taken up into water, and the suspension was refluxed for 1 h. The mixture was then filtered; the solid was recrystallized from 2-propanol and dried in vacuo to provide acid **15** (160 mg, 52% yield) as dark brown crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3700−2800, 1615, 1586, 1514; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.47 (d, J = 8.6 Hz, 1H), 8.16 (d, J = 8.6 Hz, 1H), 7.87 (d, J = 15.7 Hz, 1H), 7.83 (d, J = 8.2 Hz, 1H), 7.56−7.39 (m, 3H), 7.08 (d, J = 8.6 Hz, 1H), 6.63 (d, J = 7.6 Hz, 2H). Anal. ($C_{18}H_{14}N_2O_3 \cdot {}^1/_4H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(4-acetamidophenyl)ethenyl]-7-quinolinecarboxylic Acid (16). Method C using 4-acetamidobenzaldehyde gave acid **16** in 30% overall yield as red crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3700−2000, 1679, 1593, 1515; ^1H NMR (DMSO-d_6, 200 MHz) δ 10.10 (broad s, 1H), 8.43 (d, J = 8.7 Hz, 1H), 8.09 (d, J = 8.7 Hz, 1H), 7.86 (d, J = 16.0 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.66 (s, 4 H), 7.55 (d, J = 16.0 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 2.05 (s, 3H). Anal. ($C_{20}H_{16}N_2O_4 \cdot {}^1/_2H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(4-hydroxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (17). Method A using 4-hydroxybenzaldehyde afforded acid **17** in 54% overall yield as red crystals: mp 256−259 °C; IR (KBr, cm^{-1}) 3700−2300, 1669, 1578; ^1H NMR (DMSO-d_6, 200 MHz) δ 9.96 (broad s, 1H), 8.47 (d, J = 8.6 Hz, 1H), 8.13 (d, J = 8.6 Hz, 1H), 7.90 (d, J = 16.4 Hz, 1H), 7.81 (d, J = 8.6 Hz, 1H), 7.55 (d, J = 8.6 Hz, 2H), 7.51 (d, J = 16.4 Hz, 1H), 7.20 (d, J = 8.6 Hz, 1H), 6.84 (d, J = 8.6 Hz, 2 H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 170.8, 160.5, 159.2, 153.2, 139.4 (2C), 138.0, 130.5, 129.5 (2C), 127.0, 126.8, 121.2, 120.9, 115.9 (2C), 113.8, 112.3. Anal. ($C_{18}H_{13}NO_4 \cdot H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(3,5-dihydroxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (18). Method C using 3,5-dihydroxybenzaldehyde afforded acid **18** in 15% overall yield as red crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3700−2200, 1598, 1570; ^1H NMR (DMSO-d_6, 200 MHz) δ 9.40 (broad s, 1H), 8.42 (d, J = 8.3 Hz, 1H), 8.11 (d, J = 8.6 Hz, 1H), 7.82 (d, J = 8.3 Hz, 1H), 7.76 (d, J = 16.4 Hz, 1H), 7.45 (d, J = 16.4 Hz, 1H), 7.24 (d, J = 8.3 Hz, 1H), 6.57 (s, 2H), 6.28 (s, 1H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.3, 160.6 159.0 (2C), 153.3, 138.9, 137.7, 137.6, 137.0, 131.0, 127.0, 125.5, 121.8, 115.0, 112.1, 105.7 (2C), 104.4; MS *m/e* (rel intensity) 323 ($M^{\cdot+}$, 0.3), 278 ($(M - 45)^{\cdot+}$, 19), 159 (60), 44 (100).

(*E*)-8-Hydroxy-2-[2-(4-hydroxy-3-methoxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (21). Method A using vanillin gave acid **21** in 67% overall yield as orange crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3500−2400, 1621, 1581; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 9.60 (broad s, 1H), 8.48 (d, J = 8.8 Hz, 1H), 8.13 (d, J = 8.8 Hz, 1H), 7.90 (d, J = 16.2 Hz, 1H), 7.82 (d, J = 8.6 Hz, 1H), 7.56 (d, J = 16.2 Hz, 1H), 7.30 (s, 1H), 7.21 (d, J = 8.6 Hz, 1H), 7.13 (d, J = 8.1 Hz, 1H), 6.85 (d, J = 8.1 Hz, 1H), 3.87 (s, 3H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 170.9, 160.6, 153.2, 148.8, 148.1, 139.5, 138.3, 135.8, 130.5, 127.4, 127.2, 122.4, 121.4, 120.8, 115.8, 113.8, 112.4, 110.3, 55.6. Anal. ($C_{19}H_{15}NO_5 \cdot H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(3-hydroxy-4-methoxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (22). Method A using isovanillin gave acid **22** in 54% overall yield as orange crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3700−2400, 1603; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.52 (d, J = 8.6 Hz, 1H), 8.20 (d, J = 8.6 Hz, 1H), 7.90 (d, J = 16.8 Hz, 1H), 7.84 (d, J = 8.1 Hz, 1H), 7.51 (d, J = 16.8 Hz, 1H), 7.26 (d, J = 8.1 Hz, 1H), 7.15 (m, 2H), 7.01 (d, J = 8.6 Hz, 1H), 3.87 (s, 3H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.0, 159.3, 153.0, 149.9, 146.9, 140.4, 139.5, 134.3, 130.5, 128.5, 127.5, 120.9 (3C), 114.4, 113.8, 112.8, 112.2, 55.7. Anal. ($C_{19}H_{15}NO_5 \cdot {}^1/_2H_2O$) C, H, N.

3,4-Dihydroxy-5-methoxybenzaldehyde (6). To a solution of 3,5-dimethoxy-4-hydroxybenzaldehyde (**5**) (510 mg, 2.8 mmol) in CH_2Cl_2 (5 mL) was added portionwise aluminum chloride (450 mg, 3.3 mmol). Anhydrous pyridine (1.0 mL, 12.4 mmol) was then added dropwise to the resulting suspension. After being stirred 72 h at room temperature, the mixture was poured into 1 N HCl and was extracted with CH_2Cl_2. The organic extracts were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was recrystallized from $CHCl_3$ to give aldehyde **6** (300 mg, 63%) as colorless crystals: mp 180−181 °C (lit.[20] mp 181−182 °C); ^{1}H NMR (DMSO-d_6, 200 MHz) δ 9.68 (s, 1H), 6.98 (s, 2H), 3.80 (s, 3H).

(*E*)-8-Hydroxy-2-[2-(4,5-dihydroxy-3-methoxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (24). Method A using 4,5-dihydroxy-3-methoxybenzaldehyde **(6)** gave acid **24** in 53% yield as brick red crystals: mp 215−217 °C; IR (KBr, cm^{-1}) 3700−2200, 1588, 1512; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.42 (d, J = 8.6 Hz, 1H), 8.14 (d, J = 8.6 Hz, 1H), 7.84 (m, 2H), 7.51 (d, J = 16.2 Hz, 1H), 7.18 (d, J = 8.6 Hz, 1H), 6.88 (s, 1H), 6.81 (s, 1H), 3.85 (s, 3H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 170.9, 160.8, 153.0, 148.7, 146.0, 139.6, 138.9, 136.8, 135.8, 130.6, 127.4, 126.4, 121.5, 120.8, 113.5, 112.8, 109.4, 103.2, 55.9. Anal. ($C_{19}H_{15}NO_6 \cdot {}^3/_2H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(4-hydroxy-3,5-dimethoxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (25). Method A using 3,5-dimethoxy-4-hydroxybenzaldehyde (**5**) gave acid **25** in 38% overall yield as brick red crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3700−2500, 1612, 1515; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.90 (broad s, 1H), 8.45 (d, J = 8.6 Hz, 1H), 8.10 (d, J = 8.6 Hz, 1H), 7.89 (d, J = 16.3 Hz, 1H), 7.81 (d, J = 8.6 Hz, 1H), 7.59 (d, J = 16.3 Hz, 1H), 7.21 (d, J = 8.6 Hz, 1H), 7.02 (s, 2 H), 3.84 (s, 6 H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.8, 161.4, 154.0, 149.0 (2C), 140.1, 139.1, 138.5, 137.0, 131.3, 127.7, 127.0, 123.0, 121.6, 115.1, 113.0, 105.9 (2C), 56.8 (2C). Anal. ($C_{20}H_{17}NO_6 \cdot {}^1/_2H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(3,5-dibromo-4-hydroxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (26). Method B using 3,5-dibromo-4-hydroxybenzaldehyde gave acid **26** in 55% yield, as brick red crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3650−2400, 1675, 1611, 1481; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.35 (d, J = 8.2 Hz, 1H), 8.05−7.70 (m, 3H), 7.90 (s, 2H), 7.50 (d, J = 16.0 Hz, 1H), 7.25 (d, J = 8.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.4, 160.0, 153.3, 151.3, 137.8, 137.6, 133.0, 131.0 (2C), 130.7, 128.2, 126.6 (2C), 121.5, 115.3, 112.3 (2C), 111.8. Anal. ($C_{18}H_{11}NBr_2O_4 \cdot H_2O$) C, H, N.

(*E*)-8-Hydroxy-2-[2-(3-iodo-4,5-dihydroxyphenyl)ethenyl]-7-quinolinecarboxylic Acid (27). Method B using 3-iodo-4,5-dihydroxybenzaldehyde (**8**) gave acid **27** in 49% yield, as brick red crystals: mp > 260 °C; IR (KBr, cm^{-1}) 3700−2500, 1619, 1587, 1513; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 10.20 (broad s, 1H), 9.70 (broad s, 1H), 8.46 (d, J = 8.4 Hz, 1H), 8.11 (d, J = 8.4 Hz, 1H), 7.90−7.70 (m, 2 H), 7.53 (s, 1H), 7.42 (d, J = 16.4 Hz, 1H), 7.20 (d, J = 8.6 Hz, 1H), 7.14 (s, 1H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 170.9, 160.4, 153.0, 147.7, 145.2, 137.0, 136.9, 135.9, 130.7, 129.3 (2C), 127.4, 122.4, 121.1, 113.9, 113.8, 112.5, 85.4. Anal. ($C_{18}H_{12}INO_6 \cdot {}^3/_2H_2O$) C, H, N.

(*E*)-5,7-Dichloro-8-hydroxy-2-[2-(3,4-dihydroxyphenyl)ethenyl]-7-quinoline (29). To a solution of 5,7-dichloro-8-hydroxy-2-methyl-7-quinoline (**4**) (2.28 g, 10 mmol) in acetic anhydride (30 mL) was added 3,4-dihydroxybenzaldehyde (5.52 g, 40 mmol). The mixture was heated under reflux for 16 h and concentrated in vacuo. The residue was dissolved in pyridine (40 mL), water (10 mL) was then added and the reaction mixture was refluxed for 3 h. After cooling, the mixture was concentrated under reduced pressure. The solid residue was taken up into CH_2Cl_2 and filtered. The crude product was finally recrystallized from 2-propanol and dried in vacuo to give quinoline **29** (5.2 g, 44% yield) as ocher crystals: mp 250−252 °C; IR (KBr, cm^{-1}) 3700−2500, 1614, 1599, 1554; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 9.40 (broad s, 1H), 8.36 (d, J = 8.4 Hz, 1H), 8.08 (d, J = 16.5 Hz, 1H), 7.85 (d, J = 8.8 Hz, 1H), 7.66 (s, 1H), 7.16 (d, J = 16.5 Hz, 1H), 7.11(s, 1H), 7.02 (d, J = 8.1 Hz, 1H), 6.80 (d, J = 8.1 Hz, 1H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 156.7, 149.4, 148.0, 146.5, 139.7, 138.0, 133.9, 128.7, 127.3, 124.4, 124.2, 123.0, 121.1, 120.0, 116.8, 116.2, 115.1. Anal. ($C_{17}H_{11}Cl_2NO_3$) C, H, N.

8-Hydroxy-2-[2-(3,4-dihydroxyphenyl)ethyl]-7-quinolinecarboxylic Acid (30). To a solution of styrylquinoline **20** (200 mg, 0.62 mmol) in DMF (14 mL) was added 50 mg of 10% Pd/C. The mixture was vigorously stirred for 24 h at 20 °C under hydrogen atmosphere (4 bar). The catalyst was removed by filtration through Celite and the filter pack was washed repeatedly with CH_2Cl_2. The filtrate was concentrated in vacuo. The solid residue was recrystallized in acetic acid to afford acid **30** as orange crystals (60 mg, 30% yield): mp 264−266 °C; IR (KBr, cm^{-1}) 3700−2400, 1656, 1599; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.69 (broad s, 2H), 8.43 (d, J = 8.4 Hz, 1H), 7.83 (d, J = 8.6 Hz, 1H), 7.64 (d, J = 8.4 Hz, 1H), 7.18 (d, J = 8.6 Hz, 1H), 6.61 (s, 1H), 6.59 (d, J = 8.2 Hz, 1H), 6.47 (dd, J = 7.8, 1.8 Hz, 1H), 3.26 (t, J = 6.8 Hz, 2H), 2.88 (t, J = 6.8 Hz, 2H); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 171.1, 160.8, 159.5, 145.1, 143.6, 139.9, 136.2, 131.6, 130.7, 127.2, 124.3, 119.1, 115.9, 115.6, 113.6, 112.2, 34.5, 33.2. Anal. ($C_{18}H_{15}NO_5 \cdot {}^1/_2H_2O$) C, H, N.

Preparation of DNA Substrates. Oligonucleotides U5B 5′-GTGTGGAAAATCTCTAGCA, U5AACTGCTAGAGATTTTCCACAC, and D38U3 TGCTAGTTCTAGCAGGCCCTTGGGCCGGCGCTTGCGCC[29] were purchased from Eurogentec and further purified by denaturing 18% acrylamide gel. For processing and disintegration assays, 100 pmol of U5A and D30U5 oligonucleotides were radiolabeled respectively using T4 polynucleotide kinase (Biolabs) and 50 μCi of [γ-^{32}P]ATP (s.a. 3000 Ci/mmol). Kinase was heat inactivated and unincorporated nucleotide were removed by passage through Sephadex G-10 (Pharmacia). NaCl was added to the final concentration of 0.1 M and complementary unlabeled strand U5B was added to U5A. The mixture was heated to 90 °C for 2 min and the DNA was annealed by slow cooling.

HIV-1 IN Assay. Purified recombinant full-size HIV-1 IN was a generous gift of Rhône-Poulenc-Rorer. Plasmid encoding the His-tagged soluble deletion mutant F(185)K corresponding to the (50−212) core domain of HIV-1 IN was generously provided by Dr. R. Craigie. The protein was expressed and purified as described.[30] Processing assay was performed using 0.5 pmol of U5A/U5B substrate in the presence of 1 pmol of IN in buffer containing 20 mM Tris (pH 7.2), 30 mM NaCl, 10% (w/v) glycerol, 10 mM DTT, 0.01% NP40, supplemented with 10 mM $MnCl_2$. Disintegration assays were performed in the presence of 0.5 pmol of D38U3 substrate and 2 pmol of IN core domain. Gels were analyzed using a STORM Molecular Dynamics phosphorimager. Inhibition in the presence of drugs was expressed as percent of fractional product compared with the control.

Antiviral Assays. The lymphocyte cell line CEM was maintained in a RPMI-1640 (GIBCO Laboratories) supple-

mented with 10% fetal calf serum. Hela-CD4$^+$-βGal (P4) cells, kindly provided by P. Charneau (Institut Pasteur, Paris), were grown in DMEM with 10% fetal calf serum and 0.5 mg/mL geneticin. Cell-free viral supernatants were obtained by transfection of P4 cells with HIV-1 PLN4-3 genomic clone. Cells were plated in triplicate on a 96-well plates (100 μL) and infected with cell-free virus. Viral supernatants were removed 2 h after infection and drugs dissolved in DMSO were added in fresh medium. 72 h later, supernatants were used to infect Hela-CD4$^+$-βGal cells. The P4 cultures were subsequently incubated for 24 h and subsequently lysed in a phosphate buffer containing 50 mM 2-mercaptoethanol, 10 mM $MgSO_4$, 25 mM EDTA, 0.125% NP40. 20 μL of lysate was incubated with 100 μL of CPRG-containing buffer.[31] The red staining intensity was quantified on a multiscan photometer at 570 nm. Cell viability was estimated by the MTT (Sigma) assay. 20 μL of a solution of MTT (7.5 mg/mL) in phosphate buffer was added to each well of the microtiter trays. The plates were further incubated at 37 °C in a CO_2 incubator for 4 h. Solubilization of the formazan crystals was achieved by adding 100 mL of 10% SDS, 10 mM HCl. Absorbances were read in a multiscan photometer at 570 nm. In some cases, antiviral activity was measured by p24 antigen ELISA.

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