Potent Nonclassical Nucleoside Antiviral Drugs Based on the *N,N*-Diarylformamidine Concept

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Received July 23, 2003

New formamidine-3TC (3TC = 2′,3′-dideoxy-3′-thiacytidine) analogues have been synthesized through various methods, and their antiviral activities (HIV, HBV) have been evaluated in vitro. Anti-HIV-1 in acutely infected MT-4 cells and peripheral blood monocellular cells (PBMCs) showed that compounds substituted by *N,N*-diarylformamidine side chains at the 4-*N* nucleic base position (compounds **3** and **8**−**11**) had at least equivalent anti-HIV activity as 3TC (EC_{50} = 0.5 and 11.6 μM, respectively). Moreover, the newly synthesized compounds demonstrated higher anti-HBV activity (EC_{50} ranging from 0.01 to 0.05 μM) compared to the parent nucleoside 3TC (EC_{50} = 0.2 μM). It should be underlined that these new promising derivatives inhibited HIV in cells of a macrophage lineage, which are known to be cellular reservoir for HIV. These results were particularly of interest, since the antiviral activities appeared not to be mediated through the formamidine bond hydrolysis and consequently the release of free 3TC. These new analogue series were found to be highly stable to hydrolysis even after prolonged incubation in different biological media ($t_{1/2}$ ranged from 48 to 120 h). This enzymatic stability, coupled to the fact that no delay in the antiviral response was observed compared to the free 3TC antiviral response, suggest that this new *N,N*-diarylformamidine nucleoside series should not be considered as classical prodrugs.

Introduction

A great number of nucleosides drugs have received clinical approval and are widely used for their chemotherapeutic potential around the world by millions of patients suffering from viral infections and cancer. Despite the long history of nucleoside-based drugs, there is still a great need for new derivatives, notably in the field of antiviral agents. This need is further intensified by the slow development of antiviral vaccines.[1−3] Independently of the potential of vaccines, the need for chemotherapeutic drugs has remained a worldwide priority. Nucleosides represent a domain in which considerable scope for improvement exists for antiviral drug design, particularly against human immunodeficiency virus (HIV) and human hepatitis B (HBV).

Two principal approaches have guided the design of novel drugs: first, the bioisosterism approach, which consists of suitable structural modifications of the natural nucleoside building block; second, the prodrug approach, in which subtle structural modifications are made on known bioactive nucleoside drugs that allow the release of the original active drug after specific enzymatic transformations. Structural modifications have been mainly made at the 5′-*O* sugar position of the nucleosides,[4−6] which allows quite a wide diversity of metabolically cleavable linkages. In contrast, only few antiviral drugs modified at the 4-*N* position of nucleic bases have been described.[7]

It is well-established that nucleoside reverse transcriptase inhibitors (NRTIs) require triphosphorylation by cellular kinases to act as competitors of the natural 2′-deoxynucleoside triphosphate (dNTPs). Consequently, the antiviral activity of NRTIs depends primarily on the intracellular concentrations of their triphosphorylated derivative in cells. To be triphosphorylated by cellular kinases, NRTIs have to cross biological barriers such as the blood−brain barrier (BBB) or simple cellular membranes. Reduced lipophilicity may cause a decrease in cellular permeation and thus result in decreased antiviral activity. Although, the central nervous system (CNS) is a key target in HIV drug treatment, many NRTIs have difficulties in reaching this compartment. It has been also demonstrated that cytidine analogues such as 2′,3′-dideoxy-3′-thiacytidine (3TC) (Figure 1) or

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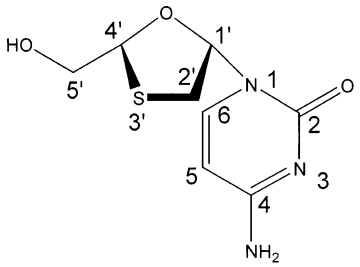
10.1021/jm0309708 CCC: $27.50
Published on Web 01/24/2004



3TC, Epivir (HIV) or Zeffix (HBV)

Figure 1. Structure of 3TC.

ddC (2′,3′-dideoxycytidine) enter the cerebrospinal fluid[8] to a reduced extent as compared to the thymidine analogues, i.e., AZT, possibly because of their lower lipophilicity and/or inferior substrate activity in the carrier-mediated uptake system.

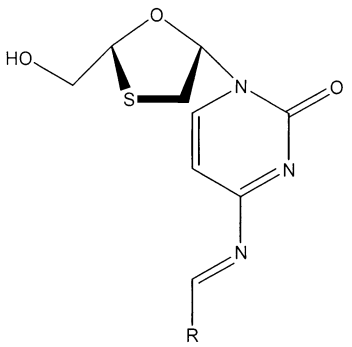
Taking all this into account, we have investigated 3TC base modifications to enhance lipophilicity and bioavailability of this antiviral nucleoside drug.

Here we report the synthesis and the antiviral activity of new 4-*N*-disubstituted-arylformamidine nucleoside antiviral drugs and discuss the possible antiviral mechanisms by which such compounds (drug or prodrug) exert their antiviral activity.

Since 1986, amidines have been investigated for capacity to protect deoxyadenosine, deoxycytidine, 5-methyldeoxycytidine, cytidine, and deoxyguanosine.[9] These formamidine nucleosides were used in oligonucleotide synthesis thanks to their enhanced stability in acidic conditions and their ease of preparation. More recently, amidines have also been used in the synthesis of oligonucleotides containing 2′-deoxyguanosine residues.[10]

These amidine protecting groups have also been used in antiviral drugs, such as the anti-HIV nucleoside analogues ddC[11] and 1-[2′,3′-dideoxy-3′-*C*-(hydroxymethyl)-β-D-erythropentofuranosyl]cytosine.[12] In the latter case, various amidine prodrugs were found to be active in different antiviral assays including DHBV (duck hepatitis B virus), HIV, and cytomegalovirus. Some of them demonstrated marked anti-HIV activities, while no activity was reported for the parent drug.[12]

Placing a variable lipophilic substituent on the exocyclic amino group of the nucleoside was expected to incrementally increase the lipophilicity and simultaneously to enhance the water solubility of the drug, due to the loss of the NH_2 group as donor of two intermolecular hydrogen bonds. The major concern in the use of amidine, as protector of the nucleic base amino group, was its stability to hydrolysis and its easy conversion to the corresponding free amino group. The hydrolysis rate of the formamidine protecting group, leading to the regeneration of the free 4-*N*-amino nucleoside, depends mainly on the structure of the substituents linked to the nitrogen atom. It has been reported that in the case of ddC series, the corresponding *N*,*N*-diisopropylformamidinyl derivative was the most stable compound to hydrolysis, in both serum or 1 N HCl, in contrast to the corresponding dimethyl-, pyrrolidino-, or morpholinoformamidinyl derivatives, which appeared to be more sensitive to hydrolysis.[11] The observed differences in hydrolysis stability were attributed to the steric and electronic effects of the formamidine side chain. Thus



where R represents various moieties such as:

-N-$(CH_3)_2$
-N-(C_4H_8O)
-N-$(CH_2\text{-Ph})_2$
-N-$(CH_2\text{-}p\text{-Me-}C_6H_5)_2$
-N-$(CH_2\text{-}p\text{-OMe-}C_6H_5)_2$
-N-$(CH_2\text{-}p\text{-F-}C_6H_5)_2$
-N-$(CH_2\text{-}p\text{-COOEt-}C_6H_5)_2$

Figure 2. Structure of the *N*,*N*-substituted formamidine 3TC derivatives.

electron-donating groups may enhance resonance stabilization of the conjugated side chain, and as far as it has been shown that the 4-*N* side chain is coplanar with the pyrimidine,[13] it has been suggested that a reinforced resonance-stabilized conjugated system may allow enhancement of their stability to hydrolysis. This concept of substituted formamidine nucleoside drugs should allow a compromise to be reached between stability to hydrolysis and sacrificing water solubility.

Starting from this premise, we have designed new 3TC derivatives substituted at the 4-*N* position by various formamidine side chains (Figure 2). The new analogues were tested for their anti-HIV and anti-HBV activities in different cell lines, and their stabilities were determined in various biological conditions (cell culture media and cellular extracts).

Chemistry

We describe herein the synthesis of some 3TC analogues via a formamidine linkage (Scheme 1).

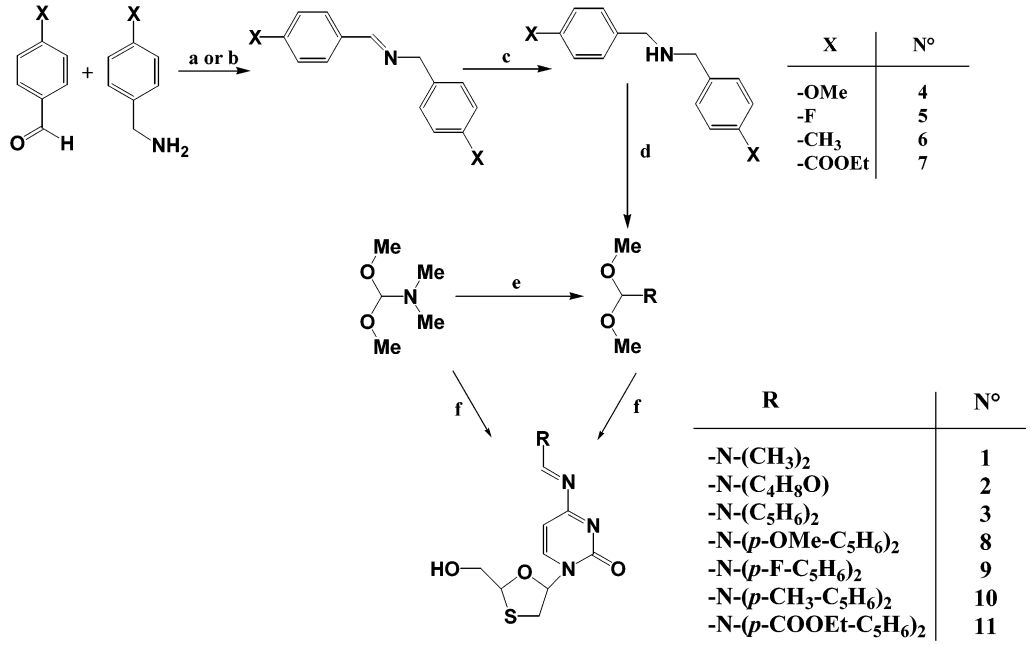
The synthesis of the new prodrug series proceeded smoothly by condensing the parent drug 3TC, obtained from Microbiologica, with an excess of the appropriate *N*,*N*-dimethylformamide diaryl acetal in anhydrous DMF, overnight at 70 °C,[11,14] as outlined in Scheme 1.

Some of these *N*,*N*-dimethylformamide diaryl acetals, which are not commercially available, have been prepared previously as described in Scheme 1. Briefly, the corresponding symmetric amines (compounds **4**−**7**) were synthesized through a coupling reaction between the suitable aldehyde and amine, followed by reduction of the resulting intermediate by sodium borohydride.[15,16] Then, the corresponding *N*,*N*-dimethylformamide diaryl acetal was synthesized through the reaction of these symmetric amines with the *N*,*N*-dimethylformamide dimethyl acetal, in anhydrous acetonitrile under reflux (Scheme 1).

In this way, 4-*N*-substituted-2′,3′-dideoxy-3′-thiacytidine (compounds **1**−**3** and **8**−**11**) were prepared and fully characterized.

It is noteworthy that we have also prepared a *N*,*N*-dibenzylformamidinyl-ddC analogue (compound **12**, not

Scheme 1. Synthesis of *N*,*N*-Dimethylformamide Dialkyl Acetal 3TC Derivatives[a]



[a] Reagents: (a) ethanol reflux, 2 h; (b) toluene reflux, Dean−Stark trap, overnight; (c) $NaBH_4$, ethanol reflux, 7 h; (d) *N*,*N*-dimethylformamide dimethyl acetal, acetonitrile reflux, overnight; (e) morpholine, acetonitrile reflux, overnight; (f) 3TC, anhydrous DMF, 70 °C, overnight.

represented in Scheme 1), to compare its antiviral activity with the corresponding analogue of the 3TC series (compound **3**). The synthesis of compound **12** was similar to that of compound **3** (see the Experimental Section).

Results and Discussion

We first studied the hydrolysis and lipophilicity of the new nucleoside drugs in various biological conditions. Then, 4-*N* formamidine-3TC derivatives were assayed for their inhibitory effects on both HIV replication and HBV DNA production in different cell systems.

Lipophilicity Studies. Partition coefficients calculated, using ACD (Advanced Chemistry Development, Inc.)/log *P* 1.0 base calculations, ranged from 0.62 for the least lipophilic derivative (compound **2**) to 5.45 for the most lipophilic (compound **7**) (Table 1). Through the substitution by the *N*,*N*-diarylformamidinyl groups, an increase in lipophilicity of 75−90 times compared to that of the parent nucleoside 3TC (log *P* = 0.06) was achieved. Moreover, this type of substitution (compounds **3** to **11**) lead to an increase of lipophilicity between 4 and 5 times that of the dialkyl series (compounds **1** and **2**).

Within the diaryl series, the contribution of different groups on the two aromatic rings to the lipophilicity of the resulting molecule could be quantified through substituent constants, σ and π, values widely used in structure−activity relationships.[17−19] These σ and π values are summarized in Table 2.

The differential effects (σ and π values) of the methoxy group compared to the other groups support the observed stability enhancement ($t_{1/2} \sim 5$ days) for the corresponding analogue **8**, compared to the other aromatic substituents. These results indicate that substituting the exocyclic amino group of the poorly water-soluble nucleoside 3TC with an appropriate side chain allows modulation of the lipophilicity through predominantly molecular conformational effects. It can be predicted that the introduction of substituents with lower π and/or σ values on the aromatic rings should allow higher hydrolysis stability for the corresponding analogues (Figure 3).

Table 1. Stability of the 3TC Analogues in Different Biological Media

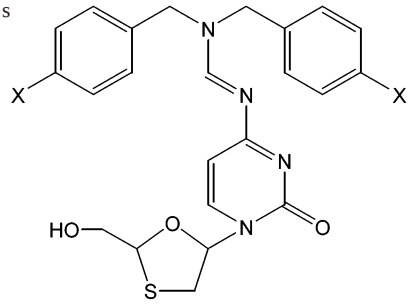
	$t_{1/2}$[a](h)					
no.	PBS[b]	HBV medium[c]	HIV medium[d]	HepG 2.2.15 lysate[e]	MT-4 lysate[f]	log *P*[g]
1	3.50	2	2.5	3	4.5	1.02
2	2.5	1	1.5	2.5	3.5	0.62
3	48	36	36	36	36	4.48
8	120	120	120	120	120	4.23
9	48	48	48	48	48	4.80
10	72	72	72	72	72	5.45
11	72	72	72	72	72	4.80
3TC						0.06
12	24	24	24	24	24	3.84
ddC						−0.58

[a] $t_{1/2}$ (half-life) is the time required for 50% of hydrolysis of compounds to 3TC at 37 °C upon incubation in different media. [b] PBS: phosphate buffer saline (PBS, 0.01 M, pH 7.4). [c] HBV medium: culture medium of HepG2.2.15 cells. [d] HIV medium: culture of MT-4 cells (RPMI 1640 + 10% of fetal calf serum). [e] HepG2.2.15 lysate: cellular lysate of HepG2.2.15. [f] MT-4 lysate: cellular lysate of MT-4 cells. [g] log P determination were performed using ACD (Advanced Chemistry Development, Inc.)/log *P* 1.0 base calculations.

Hydrolytic Stability. This series of 4-*N* substituted nucleoside are decomposed into the parent drug 3TC by hydrolysis, which involves a common 4-*N*-formyl intermediate, as shown in Scheme 2.[20]

We first investigated the stabilities of these formamidine nucleosides in DPBS, HIV and HBV cell culture media, and also in HepG2.2.15 and MT-4 cellular extracts. Enzymatic hydrolysis half-lives ($t_{1/2}$) of these new 3TC analogues and, more precisely, their conver-

Table 2. Physical Properties of Para-Functionalized 3TC Analogues



		physical properties		
no.	X	log P^a	$\sigma_p{}^b$	$\pi_p{}^c$
3	H	4.48	0	0
8	OMe	4.23	−0.268	−0.04
9	F	4.80	0.062	0.15
10	CH_3	5.45	−0.170	0.52
11	C(O)OEt	4.80	0.450	0.51

[a] log *P* calculations were performed using ACD (Advanced Chemistry Development, Inc.)/log *P* 1.0 base calculations. [b] σ_p is the Hammet coefficient (aryl substituent electronic effect) from literature data.[18,19] [c] π_p is the parameter derived from the partition coefficient, from literature data.[19]

sion into the parent nucleoside drug 3TC were determined through an HPLC method.[21−23] Table 1 lists the half-life ($t_{1/2}$) values of compounds **1**−**11**. As expected, these values ranged within a large interval of time: from 1 h (compounds **1** and **2**) to 5 days (compound **8**), depending on the substituents of the formamidine. In fact, dimethyl **1** and morpholino **2** analogues were by far the least stable ones, i.e., only a few hours (1−4 h) in almost all of the tested conditions. In contrast, the corresponding diaryl derivatives were the most stable compounds with half-life varying between 2 and 5 days in all tested biological media. These important variations in stability were due to the structural differences of the formamidine-substituted alkyl or aryl groups, leading to steric and electronic effects. The aryl substituents may protect the formamidine bond against hydrolysis through their high steric effect and their resonance stabilization compared to the compounds **1** and **2**.

Moreover, it appears that the electron-donor or -acceptor character of the substituents on the aromatic ring influenced the stability of the resulting drugs. A methoxy group significantly increased $t_{1/2}$ values (compound **8**) as compared to a fluoro substituent (compound **9**) or no substitution (compound **3**). These results are in complete agreement with our initial hypothesis based on the enhanced resonance stabilization of the 4-*N* side chain conjugation with the pyrimidine ring of the nucleoside. This stabilization can be modulated by the electronic, inductive, and mesomeric effects of the substituents on the aromatic rings.

Biological Data. The anti-HIV and anti-HBV activities of the whole series of compounds in in vitro assays are summarized in Table 3.

Anti-HIV Activities. (1) MT-4 and PBMCs in Vitro Assays. Anti-HIV activity was assayed in two systems as previously described: MT-4 cells infected with HIV-1/HTLV−III_B[24] and PBMCs infected with HIV-1/LAI.[23] In both systems, the whole series of 3TC derivatives were active, and the majority of them was found more active than the parent nucleoside.

Compound **9**, bearing the fluorobenzyl group, was about 1 order of magnitude more active than 3TC in infected PBMC cell lines and equipotent to 3TC in infected MT-4 cell lines. Compound **11**, bearing the *p*-ethyloxycarbonylbenzyl group (COOEt), was 2-fold more potent than 3TC in MT-4 cell cultures, while the dimethylamino derivative **1** was found equipotent to 3TC in MT-4 cell cultures and 2-fold less active in PBMC cultures.

These observations are of interest, since the shorter lived compounds **1** and **2** were equipotent to 3TC, while the longer lived analogues appeared to be more active than 3TC. Previously published data[11] on longer lived 4-*N* substituted ddC, bearing *N*,*N*-dialkylformamidine side chains, showed that their antiviral activities were between 4- and10-fold less active than the parent drug ddC. On the contrary, the shorter lived prodrugs were equipotent or slightly more active than the ddC. The observed anti-HIV activities for new 3TC derivatives substituted at the 4-*N* position with *N*,*N*-dialkylformamidine side chains compounds **1** and **2** (shorter lived compounds) are in agreement with the antiviral activities found for the corresponding ddC analogues. In contrast, longer lived 3TC analogues substituted at the 4-*N* position with *N*,*N*-diarylformamidine side chains were found to be more active than 3TC. These results indicate that *N*,*N*-diarylformamidine side chains increased the hydrolytic stability, enhanced lipophilicity, and seemed to increase the anti-HIV activity of the resulting drugs.

To reinforce these results, we have also synthesized and evaluated the anti-HIV property of a new ddC analogue substituted at the 4-*N* position by the *N*,*N*-dibenzylformamidine side chain (compound **12**). The results indicated that the ddC derivative **12** was about 1 log more active than ddC (Table 3). In addition, its SI was around 900, while under the same tested biological conditions the SI of ddC was 136. Consequently, this type of cytosine formamidine substitution seemed to increase the biological activities and, moreover, allowed a better SI, which represents a major pharmaceutical parameter for possible future clinical development. Furthermore, the half-life value of this ddC analogue was found to be around 24 h in all biological media (Table 1). In contrast to the previous published data,[11] *N*,*N*-diarylformamidinyl-ddC analogues were more stable than *N*,*N*-dialkylformamidinyl-ddC homologues and, in addition, showed an improved anti-HIV activity compared to the parent drug ddC. All these observations make this new *N*,*N*-diarylformamidine concept very attractive.

In addition, π values,[19] derived from partition coefficient effect,[17] seemed to be correlated with the anti-HIV activity. SAR, plotting π values versus EC_{50} values (Figure 4), showed that an increase in π value enhanced the observed anti-HIV activity. However, a similar correlation was not found with the σ Hammet constant values, which only characterized aryl substituent electronic effects.

At last, it is important to note that the new derivatives exerted no cytotoxic effect (CC_{50} > 10 μM) in activated PBMCs, as well as in MT-4 cells (Table 3). Their SI were found to be similar or higher than that of the parent nucleoside.

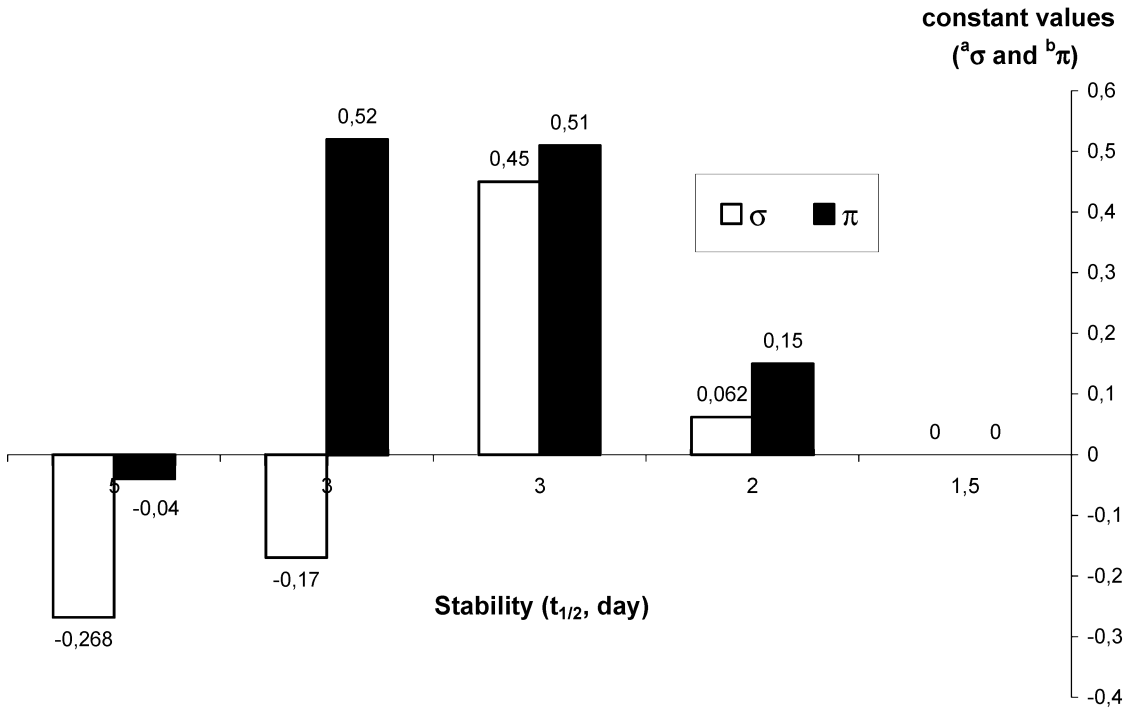
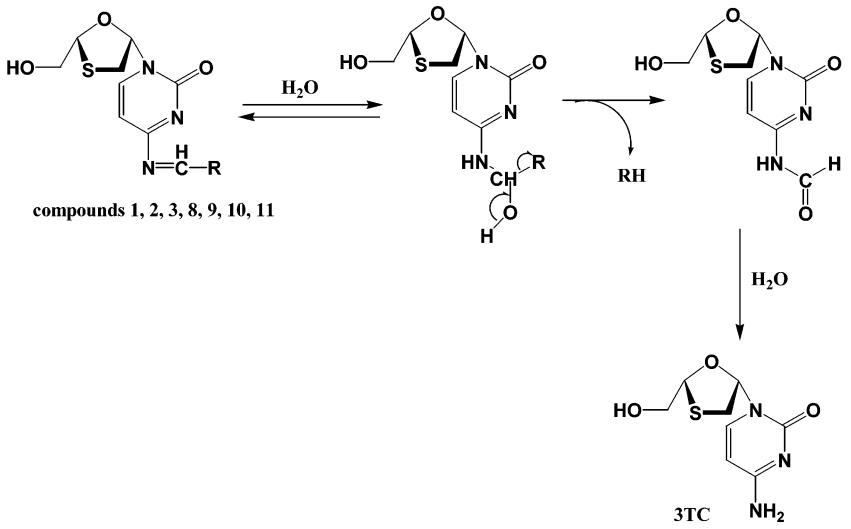


Figure 3. Stability depending on the Hammet parameters (σ and π). $^a\sigma$ is the Hammet coefficient (aryl substituent electronic effect) from literature data.[18,19] $^b\pi$ is the parameter derived from partition coefficient, from literature data.[19]

Scheme 2. Hydrolytic Pathway



(2) Macrophage in Vitro Assay. In addition to $CD4^+$ T lymphocytes, the macrophage cell lineage represents a key target for HIV infection. These cells are considered to be a major cellular reservoir of HIV-1.[25,26] The peculiar dynamics of HIV replication in macrophages, their long-term survival after HIV infection, and their ability to spread virus particles to bystander $CD4^+$ T lymphocytes point to their substantial contribution to the pathogenesis of HIV infection. Moreover, activation of the oxidative pathway in HIV-infected macrophages may lead to apoptotic death of bystander and not infected cells.[27,28] Finally, their crucial role in the pathogenesis of HIV-related encephalopathy supports the clinical relevance of therapeutic strategies able to interfere with HIV replication in macrophages. In this perspective, two prototypes of the prepared series (analogues **1** and **3**) were assayed in human monocyte-derived macrophages in vitro infected with HIV-1/Ba-L.[29] As shown in Figure 5, compound **3** blocked the viral replication with an EC_{50} value of approximately 0.1 nM, while under the same experimental conditions the EC_{50} value for the parent drug 3TC and compound **1** was about 1 nM. This result could be of interest, since it is known[30] that the penetration of nucleoside drugs in sequestered compartments (central nervous system) is rather limited and that the ability of macrophages to triphosphorylate the 5′-*O* position of nucleoside is low.

Anti-HBV Activity. The anti-HBV activity of all analogues was assayed in HepG2.2.15 cell culture, using conditions previously described.[31] All compounds exhibited significant in vitro anti-HBV activities with EC_{50} values ranging from 0.01 to 0.05 μM, without significant toxicity below 100 μM. These new 3TC derivatives

Table 3. Anti-HIV and Anti-HBV Evaluation of 3TC Derivatives on MT-4 Cells (HIV-1 III_B), PBMCs (HIV-1 LAI), and HepG2.2.15 (HBV)

	MT-4 (HIV-1 III_B)			PBMCs (HIV-1 LAI)			inhibition of HBV in HepG2.2.15:
no.	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]	EC_{50}[a] (nM)	CC_{50}[b] (μM)	SI[c]	EC_{50}[d,e] (μM)
1	0.570	>440	>772	23.7	>10	>422	0.01
2	0.40	>265	>692	6.2	>10	>1.613	0.05
3	0.69	164	235	9.9	>10	>1.010	0.05
8	1.50	144	96	8.9	>10	>1.124	0.03
9	0.50	150	298	0.78	>10	>12.82	0.01
10	0.24	32	133	nd[f]	nd[f]	nd[f]	nd[f]
11	0.28	113	404	nd[f]	nd[f]	nd[f]	nd[f]
3TC	0.50	>44	>88	11.6	>10	>0.862	0.2
12	0.14	129	921	nd[f]	nd[f]	nd[f]	/
ddC	1.04	141	135	nd[f]	nd[f]	nd[f]	/

[a] EC_{50}: concentration in μM required to inhibit by 50% the cytopathicity of HIV-1 in MT-4 cells or to inhibit by 50% HIV-1 replication in PBMCs. [b] CC_{50}: concentration in μM required to cause 50% death of uninfected MT-4 cells or PBMCs. [c] SI: selectivity index = CC_{50}/EC_{50}. [d] EC_{50}: concentration in μM required to inhibit 50% of HBV DNA formation by HepG2.2.15 stably transfected by HBV. [e] EC_{50}: cytotoxicity were evaluated as already described;[36] unless indicated, no apparent cytotoxicity was observed below 100 μM for each compound; no SI was given since the CC_{50} values could not be accurately determined in the conditions used for the antiviral assays. [f] nd: not determined.

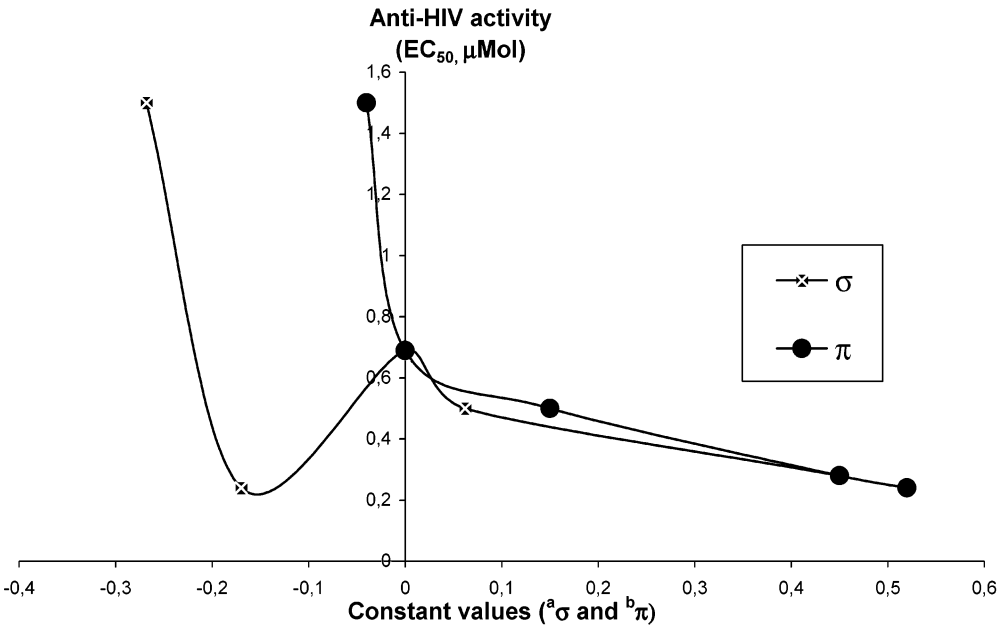


Figure 4. SAR relative to electronic effects of the Hammet parameters (σ and π). [a]σ is the Hammet coefficient (aryl substituent electronic effect) from literature data.[18,19] [b]π is the parameter derived from partition coefficient, from literature data.[19]

appeared to be 2−10-fold more active than the parent drug 3TC. Moreover, the half-life values of the different analogues (Table 1) did not seem to influence the anti-HBV activities.

It is well-known that the performance of an antiviral drug depends on its physicochemical properties, among which aqueous solubility and drug stability play a major role. Studies have shown that the acidic lability of 2′,3′-dideoxynucleosides is significantly increased upon removal of the hydroxyl groups in the 2′- and 3′-positions; therefore, the solution stability of dideoxynucleosides has become a significant concern for dosage form delineation.

The results of this investigation demonstrated that 3TC or ddC analogues *N*-substituted at the 4 position with various *N*,*N*-diarylformamidine side chains proved superior to the parent drug 3TC or ddC in terms of antiviral (HIV and HBV) efficacy. These new promising antiviral compounds, which inhibit HIV not only in $CD4^+$ T lymphocytes but also in macrophages, may provide crucial hits in therapeutic strategies. The optimal combination of desirable physicochemical properties, enhanced lipophilicity, and high stability suggest favorable pharmacokinetic characteristics.

Despite of these facts, the question of the mechanism by which these new nucleoside drugs exert their antiviral activity remains unclear. Indeed, their antiviral activities and stability studies suggest that their mechanism of action could be different from that of the parent drug 3TC (or ddC).

The important role played by the substituents on the formamidine moiety should also be taken into consideration. Their π constant values, which are correlated with their corresponding stabilities, should be taken into account in the design of new nucleoside formamidine analogues. In fact, the results reported in the case of ddC analogues *N*-substituted at the 4 position with various *N*,*N*-dialkylformamidine side chains have shown that the compounds with shorter half-life values were more active than their homologues with longer half-life values.[10] In contrast, we found that in the case of 3TC (or ddC) analogues *N*-substituted at the same position

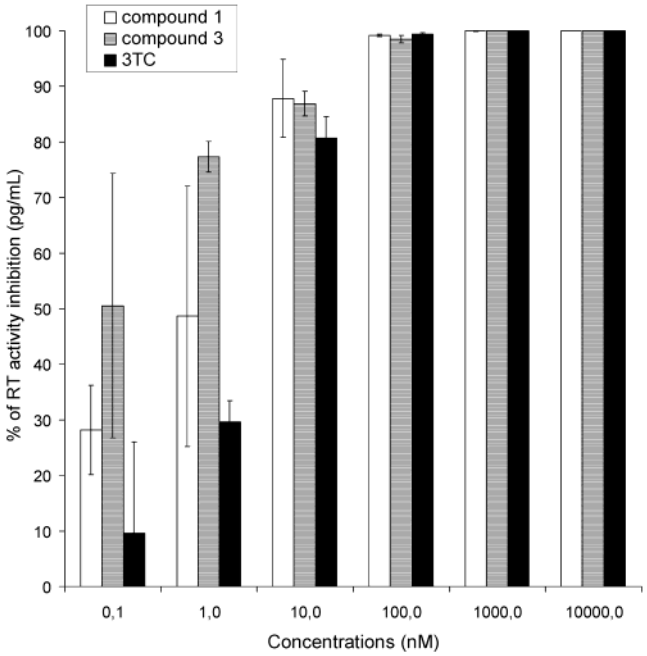


Figure 5. Antiviral activity of compounds **1** and **3** in macrophage cell lines.

with *N*,*N*-diarylformamidine side chains the most potent compounds had the longest half-life values.

One hypothesis could be that these *N*,*N*-diarylformamidinyl analogues with enhanced lipophilicity are favorably transported across the cellular membrane in their intact form and are subsequently partially phosphorylated under their 4-*N* formamidine form, because of their high stability. It would be likely that 3TC *N*,*N*-diarylformamidinyl derivatives were phosphorylated first to their monophosphate form and then metabolically transformed into the parent monophosphate drug 3TCMP. Such a pathway has been already observed for Abacavir (Ziagen), a carbocyclic nucleoside analogue of Carbovir. This compound was first anabolized intracellularly to its 5′-*O*-monophosphate analogue by the enzyme adenosine phosphotransferase, then deaminated to (−)carbovir-5′-*O*-monophosphate, and finally converted into its 5′-*O*-diphosphate and 5′-*O*-triphosphate derivatives of carbovir.[32] Moreover, it would be unprecedented, but possible, that these compounds could be triphosphorylated in their N-protected form. Then, the resulting triphosphorylated 4-*N*-formamidine 3TC analogues could directly interact with the HIV or HBV reverse transcriptase, or could be hydrolyzed to free triphosphorylated 3TC (Scheme 3).

It should emphasized that, "kinetically speaking", the antiviral effects observed with the new highly stable formamidine analogues were identical to that of the parent drug 3TC. Indeed, one could expect a delay in the antiviral response. This observation seems to be in favor of the original pathway presented in Scheme 3.

Conclusion. It is still too early to predict the potential for in vivo therapeutic use of these new nucleoside formamidine antiviral drugs. Nevertheless, this new nucleoside 4-*N*-diaryl-substituted formamidine approach could be promising, since in both in vitro models (HIV and HBV) some of the new drugs are more potent than the parent drug 3TC. However, as far as the "drug or prodrug" action mechanism has not been totally elucidated, it is difficult to evaluate their clinical potential. This approach could be generalized to other drugs, whether antiviral or not, to improve their biological and pharmacological activities.

Experimental Section

General Methods. Nuclear magnetic resonance spectra were recorded at 250 MHz for ^{1}H and 62.9 MHz for ^{13}C on a Brüker AC-250 spectrometer. Chemical shifts are expressed as δ units (part per million) downfield from TMS (tetramethylsilane). Electrospray mass spectral analysis and LC−MS analysis were obtained from Dr. Drouot, Miss Maux, and Miss Saint-Pé (Trophos, Faculté des Sciences de Luminy, Marseille, France) on a Waters Micromass ZMD spectrometer for the ES-MS analysis by direct injection of the sample solubilized in acetonitrile. The LC−MS analysis was carried out by using a Waters model 2690 pump and a Waters C18 Symmetry column with a two mobile phase system (0.1% formic acid in water and 0.1% formic acid in acetonitrile). IR spectra were recorded on a Perkin-Elmer FTIR 1605 spectrometer. Microanalyses were carried out by the Service Central d'Analyses du CNRS (Venaison, France) and were within 0.4% of the theoretical values. Analytical thin layer chromatographies (TLC) and preparative thin layer chromatographies (PLC) were performed using silica gel plates 0.2 mm thick and 1 mm thick, respectively ($60F_{254}$ Merck). Preparative flash column chromatographies were carried out on silica gel (230−240 mesh, G60 Merck). Analytical HPLC was performed on a Waters 600E instrument with a M991 detector using the following conditions: 4.6 × 150 mm column (Waters Spherisorb S5 ODS2, 5 μM); mobile phases, A = 0.1% TFA in H_2O, B = 0.1% TFA in acetonitrile, C = MeOH; flow rate 1 mL/min. All reagents were of commercial quality (Aldrich Co.) from freshly opened containers. Methylene dichloride (CH_2Cl_2) was distilled over P_2O_5 just prior to use. Dimethylformamide (DMF) was of anhydrous quality from commercial suppliers (Aldrich, Carlo Erba Reagents).

Biological Methods. 1. Cell lines and Viruses. (i) MT-4 cells[33] were grown and maintained in RPMI 1640 cell culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1% sodium bicarbonate, and gentamicin (20 μg/mL). The medium was replaced twice a week. The origin of the HIV-1 (III_B) stock has been described elsewhere.[34]

(ii) Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy HIV-, HCV-, HBV-seronegative blood donor by Ficol-Hypaque density gradient centrifugation. These cells were activated for 3 days with 1 μg/mL phytohemagglutinin-P (PHA-P) and 5 IU/mL recombinant human interleukin-2 (rhIL-2). Then, PBMCs were dispensed into 96-well microplates (100 000 cells per well) in 200 μL of medium A (RPMI 1640 cell culture medium, 10% heat-inactivated FCS (56 °C for 45 min) and 1% triantibiotic mixture (penicillin, streptomycin, neomycin) supplemented with 20 IU/mL rhIL).

(iii) Macrophages were separated from PBMCs thought three cycles of adherence and differentiated and cultured as previously described.[35]

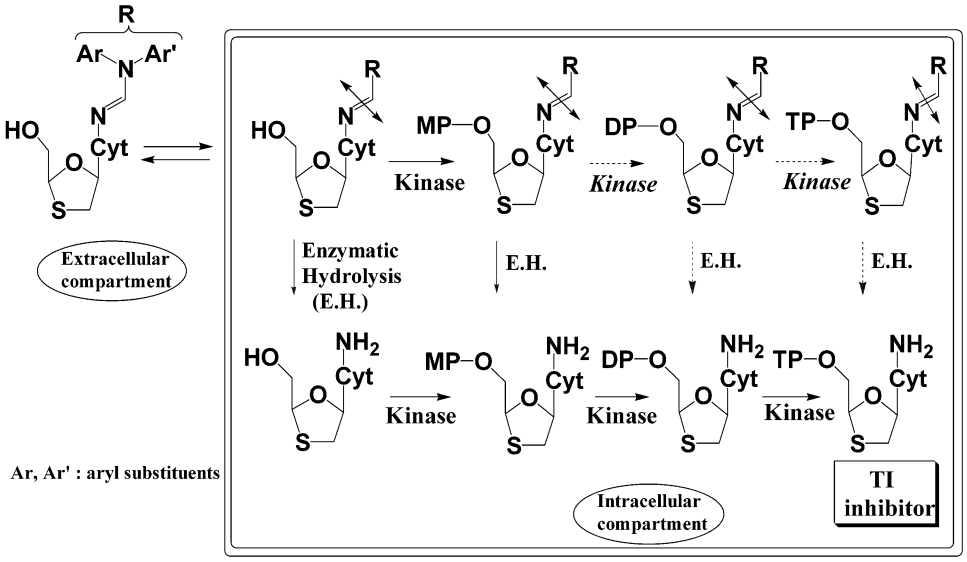
(iv) The HepG2.2.15 cells were cloned derivates of HepG2 cells that were transfected with a plasmid containing HBV DNA. The HepG2.2.15 cells were maintained in minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum. Cell viability was estimated by incorporation of neutral red according to a protocol already described.[36]

All the different types of cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2. Anti-HIV Activity Assays. (i) The inhibitory effects of 3TC and its derivatives on HIV-1 replication were monitored by the inhibition of the virus-induced cytopathicity in MT-4 cells 5 days after infection, as already described.[24] The cytotoxicity of the compounds was determined by measuring the viability of mock-infected cells at day 5.

(ii) PBMCs (100 000) were pretreated for 30 min by different concentrations of molecules and infected with 75 $TCID_{50}$ of the

Scheme 3. Different Mechanism of Action Pathways



reference lymphotropicHIV-1/LAI strain. Cell supernatants were collected at day 7 postinfection and stored at −20 °C. Cells were microscopically observed to assess possible drug-induced cytotoxicity. HIV replication was measured by the dosage of reverse transcriptase (RT) activity in cell culture supernatants using the RetroSys RT kit (Innovagen). Experiments were performed in triplicate, and results were expressed as the mean of RT activity ± standard deviation (SD). Fifty percent effective doses (EC_{50}) were calculated using percents of untreated controls and microcomputer software (Chou, J.; Chou, T. C., Biosoft, Cambridge, UK).

(iii) The evaluation of the antiviral activity of compounds in macrophage lineage was the same as in PBMCs. Only the virus dose was different—macrophages were infected with 30 000 $TCID_{50}$ of HIV-1/Ba-L—and results are expressed as cumulative RT activity, the sum of RT activity obtained for each time-point in each individual cell culture well.

3. Anti-HBV Activity Assays.[31] (i) Cells were incubated at 37 °C in a moist atmosphere containing 5% of CO_2. The HepG2.2.15 cells were inoculated at a density of 2×10^5 cells per mL in 12-well culture plates and grown at confluency. The compounds studied were added to the medium at 3 days after the inoculation. Cells were grown in the presence of drug for 10 days with changing of the medium every day. After incubation, the medium was centrifuged (10 min, 2000*g*). HBV DNA from culture supernatants was detected by a specific dot blot hybridization assay. For this purpose 600 μL of culture supernatants was spotted onto a nitrocellulose membrane, then denaturized with NaOH (0.2 M)/NaCl (1 M), neutralized with Tris-HCl (0.5 M), pH 7.4/NaCl (1 M), washed with 2× SSC, and fixed by baking for 2 h at 80 °C. HBV DNA was detected with a full-length HBV genomic DNA probe labeled with [α-^{32}P]CTP. A quantitative analysis was carried out with use of the PhosphorImager SI system.[37] The limit of detection of serum viral DNA by this assay was 100 pg/mL (equivalent to 5 pg of HBV DNA per 50 μL sample on a dot blot point). The amount of HBV-specific DNAs was similar in separate experiments performed in duplicate.

4. MT-4 and HepG2.2.15 Lysate Preparations. HepG-2.2.15 cells (Dr. O. Hantz, INSERM U271, Lyon, France) or MT-4 cells (Dr. I. Hirsch, INSERM U372, Luminy, Marseille, France) were washed two times with cold PBS buffer. The lysis solution, containing Tris HCl (50 mM), NaCl (150 mM), EDTA (0.1 mM), NP40 (0.5%), and protease inhibitor (Protease Inhibitor Cocktail 1 tablet/10 mL, Roche-Boehringer), was added at 4 °C to the cells in a range of about 10×10^6 cells per mL of lysis solution. The lysis was performed by stirring the solution for 10 min on ice. Then, the lysate was homogenized by centrifugation (10 min, 10 000 rpm). The supernatant fluid was recovered and frozen at −80 °C.

6. Hydrolysis of the Compounds in Biological Media.[21−23] To 990 μL of the appropriate medium was added 10 μL of a solution of the tested compound in DMSO (final concentration around 5×10^{-4}M), and the mixture was incubated at 37 °C in a water bath. At various time intervals, the samples (100 μL) were withdrawn and added immediately to ice-cold methanol (400 μL). The resulting samples were centrifuged (5 min, 3000 rpm). The supernatants were filtered through nylon filters (0.45 μm) and then analyzed by HPLC using the following methods: (i) method I, 30% of B in C to 100% of B in 10 min; (ii) method II, 30% of B in A to 100% of B in 10 min. The absorption maximum for all the 3TC analogues was at 317 nm; therefore, this wavelength was used for their HPLC detection. The absorption maximum for the ddC analogues was at 339 nm; therefore, this wavelength was used for the HPLC detection. Peak retention times (t_r) were 6.3 min for compound **1** (method I), 2.6 min for compound **2** (method I), 5.5 min for compound **3** (method I), 10.2 min for compound **8** (method II), 10.3 min for compound **9** (method II), 11.5 min for compound **10** (method II), 11.6 min for compound **11** (method II), and 9.9 min for compound **12** (method II). The $t_{1/2}$ values calculated from peak areas for all the compound studies in the various media are summarized in Table 1.

Chemical Synthesis. 4-*N*-(*N*,*N*-Dimethylformamidinyl)-2′,3′-dideoxy-3′-thiacytidine 1. To a solution of 3TC (100 mg, 0.436 mmol, 1 equiv) in dry methanol (5 mL) was added *N*,*N*-dimethylformamide dimethyl acetal (260 mg, 2.18 mmol, 5 equiv) under N_2. The reaction mixture was stirred for 6 h, then solvent was removed under reduced pressure and coevaporated with toluene to afford compound **1** (120 mg, 97%): $R_f = 0.51$ (CH_2Cl_2/MeOH 85:15); MS (ES+) 307 $(M + Na)^+$, 285 $(M + H)^+$. Anal. ($C_{11}H_{16}N_4O_3S$) C, H, N.

4-*N*-(Morpholinoformamidinyl)-2′,3′-dideoxy-3′-thiacytidine 2. To a solution of *N*,*N*-dimethylformamide dimethyl acetal (1 g, 8.4 mmol, 5 equiv) in dry acetonitrile (15 mL) was added morpholine (2.2 g, 25.2 mmol, 15 equiv) under N_2. The reaction mixture was refluxed for 20 h and then evaporated to dryness, and traces of remaining dimethylformamide dimethyl acetal were removed by two successive coevaporations with toluene. The crude residue (704 mg, 4.37 mmol, 5 equiv) was dissolved in anhydrous DMF and added to a solution of 3TC (200 mg, 0.873 mmol, 1 equiv) in anhydrous DMF. The resulting solution was stirred at 70 °C overnight, and solvents were removed under reduced pressure. The residual oil was triturated with cold Et_2O and centrifuged, and the formed precipitate was dried under vacuum to give compound **2** (140 mg, 49%): $R_f = 0.52$ (CH_2Cl_2/MeOH 90:10); MS (ES+) 327 $(M + H)^+$. Anal. ($C_{13}H_{18}N_4O_4S$) C, H, N.

4-*N*-(Dibenzylformamidinyl)-2′,3′-dideoxy-3′-thiacytidine 3. To a solution of *N*,*N*-dimethylformamide dimethyl acetal (500 mg, 4.2 mmol, 5 equiv) in dry acetonitrile (10 mL) was added dibenzylamine (2.5 g, 12.6 mmol, 15 equiv) under N_2. The reaction mixture was refluxed for 20 h and then evaporated to dryness, and traces of remaining dimethylformamide dimethyl acetal were removed by two successive coevaporations with toluene. The crude residue (596 mg, 2.2 mmol, 5 equiv) was dissolved in anhydrous DMF and added to a solution of 3TC (100 mg, 0.436 mmol, 1 equiv) in anhydrous DMF. The resulting solution was stirred at 70 °C overnight, and solvents were removed under reduced pressure. The residual oil was dissolved in CH_2Cl_2, washed with 5% aqueous citric acid, and extracted with CH_2Cl_2. The organic layers were dried over $MgSO_4$ and filtered to afford, after purification by flash chromatography on silica gel (CH_2Cl_2/MeOH, 96:4), compound **3** (150 mg, 79%): $R_f = 0.32$ (CH_2Cl_2/MeOH 95:5); MS (ES+) 437 $(M + H)^+$. Anal. ($C_{23}H_{24}N_4O_3S$) C, H, N.

General Procedure A for the Formation of Dibenzylamine Derivatives 4 and 5. A mixture of the appropriate benzylamine derivative (1 equiv) and the corresponding benzaldehyde (1 equiv) was refluxed in dry ethanol under N_2 for 2 h. Afterward, solvents were evaporated under reduced pressure, and the residual oil was triturated with MeOH to give the corresponding imine quantitatively. This new intermediate was immediately dissolved in dry MeOH, cooled to 0 °C, and reduced by $NaBH_4$ (1 equiv). The reaction mixture was stirred at 40 °C for 40 min and refluxed for 2 h, and then solvents were removed under vacuum. The residual oil was dissolved in CH_2Cl_2, washed with 5% aqueous $NaHCO_3$, dried over $MgSO_4$, and filtered. Evaporation of the solvents afforded the desired dibenzylamine derivative.

***N*,*N*-Bis(*p*-methoxybenzyl)amine 4.** According to general procedure A, the reaction between 4-methoxybenzylamine (2 g, 14.58 mmol, 1.9 mL, 1 equiv) and *p*-anisaldehyde (1.99 g, 14.58 mmol, 1.8 mL, 1 equiv) afforded the title compound **4** (3.15 g, 84%): $R_f = 0.63$ (CH_2Cl_2/MeOH 90:10); MS (ES+) 258 $(M + H)^+$. Anal. ($C_{16}H_{19}NO_2$) C, H, N.

***N*,*N*-Bis(*p*-fluorobenzyl)amine 5.** According to general procedure A, the reaction of 4-fluorobenzylamine (1.9 g, 15.2 mmol, 1.74 mL, 1 equiv) and 4-fluorobenzaldehyde (1.9 g, 15.2 mmol, 1.62 mL, 1 equiv) afforded the title compound **5** (1.4 g, 50%): $R_f = 0.76$ (CH_2Cl_2/MeOH 90:10); MS (ES+) 234 $(M + H)^+$. Anal. ($C_{14}H_{13}F_2N$) C, H, N.

***N*,*N*-Bis(*p*-methylbenzyl)amine 6.** A mixture of 4-methylbenzylamine (1.7 g, 14 mmol, 1 equiv) and 4-methylbenzaldehyde (1.68 g, 14 mmol, 1 equiv) in toluene (20 mL), in a flask equipped with a Dean−Stark trap, was refluxed overnight. Afterward, solvents were evaporated under reduced pressure, and the residual oil obtained was directly dissolved in ethanol (20 mL). Sodium borohydride ($NaBH_4$; 0.53 g, 14 mmol, 1 equiv) was added at 0 °C, and the reaction mixture was stirred at 40 °C for 20 min and then refluxed for about 7 h. The solvents were removed, the crude product was dissolved in CH_2Cl_2, and the formed precipitate was eliminated. The filtrate was concentrated and washed with a 5% aqueous solution of $NaHCO_3$. The organic layers were dried over $MgSO_4$, filtered, and concentrated. The residual compound was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH, 95:5) to give compound **6** (2.77 g, 88%): $R_f = 0.64$ (CH_2Cl_2/MeOH 95:5); MS (ES+) 226 $(M + H)^+$. Anal. ($C_{16}H_{19}N$) C, H, N.

***N*,*N*-Bis(*p*-ethyloxycarbonylbenzyl)amine 7.** A mixture of ethyl 4-formylbenzoate (1.8 g, 8.3 mmol, 1 equiv) and ethyl 4-(aminomethyl)benzoate hydrochloride (1.48 g, 8.3 mmol, 1 equiv) in toluene (20 mL), in a flask equipped with a Dean−Stark trap, was refluxed overnight. Then, solvents were evaporated under reduced pressure, and the residual oil obtained was directly dissolved in ethanol (20 mL). $NaBH_4$ (0.345 g, 9.13 mmol, 1.1 equiv) was added in one portion at 0 °C, the reaction mixture was stirred at room temperature for 2 h. Afterward, another portion of $NaBH_4$ was added and the mixture was stirred overnight. Once the reaction was finished, the solution was acidified at pH 2 with a 1 N solution of hydrochloric acid. The solvents were removed, the crude product was dissolved in CH_2Cl_2, and the formed precipitate was eliminated. The filtrate was concentrated and washed with a solution of 1 N NaOH. The aqueous layer was extracted with CH_2Cl_2, the organic layers were dried over $MgSO_4$, filtered, and concentrated. The residual compound was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH, 97:3) to give compound **7** (1.6 g, 57%): $R_f = 0.63$ (CH_2Cl_2/MeOH 95:5); MS (ES+) 342 $(M + H)^+$. Anal. ($C_{20}H_{23}NO_4$) C, H, N.

General Procedure B for the Preparation of the *N*,*N*-Disubstituted Dibenzyl Formamidine 3TC Derivatives. *N*,*N*-Dimethylformamide dimethyl acetal (5 equiv) and the appropriate dibenzylamine derivatives (15 equiv) were refluxed in dry acetonitrile for 20 h. The reaction mixture was evaporated to dryness, and traces of remaining dimethylformamide dimethyl acetal were removed by two successive coevaporations with toluene. The crude residue (5 equiv) was dissolved in anhydrous DMF and added to a solution of 3TC (1 equiv) in anhydrous DMF. The resulting solution was stirred at 70 °C overnight, then solvents were removed under reduced pressure. The residual oil was dissolved in CH_2Cl_2 and washed with 5% aqueous citric acid, and the organic layer was dried over $MgSO_4$ and filtered. The crude residue was purified by flash chromatography on silica gel to afford the desired compound.

4-*N*-(*N*,*N*-Bis(*p*-methoxybenzyl)formamidinyl)-2′,3′-dideoxy-3′-thiacytidine 8. According to general procedure B the reaction of *N*,*N*-dimethylformamide dimethyl acetal (400 mg, 3.36 mmol, 5 equiv) with the title compound **4** (2.59 g, 10.1 mmol, 15 equiv) and then 3TC (150 mg, 0.655 mmol, 1 equiv) afforded, after purification by flash chromatography on silica gel (CH_2Cl_2/MeOH, 96:4), compound **8** (167 mg, 51%): $R_f = 0.47$ (CH_2Cl_2/MeOH 95:5); MS (ES+) 497 $(M + H)^+$. Anal. ($C_{25}H_{28}N_4O_5S$) C, H, N.

4-*N*-(*N*,*N*-Bis(*p*-fluorobenzyl)formamidinyl)-2′,3′-dideoxy-3′-thiacytidine 9. According to general procedure B, the reaction of *N*,*N*-dimethylformamide dimethyl acetal (300 mg, 2.52 mmol, 5 equiv) with the title compound **5** (1.4 g, 6.01 mmol, 15 equiv) and then 3TC (150 mg, 0.655 mmol, 1 equiv) afforded, after purification by flash chromatography on silica gel (CH_2Cl_2/MeOH, 97:3), compound **9** (59.8 mg, 19%): $R_f = 0.34$ (CH_2Cl_2/MeOH 95:5); MS (ES+) 473 $(M + H)^+$. Anal. ($C_{23}H_{22}F_2N_4O_3S$) C, H, N.

4-*N*-(*N*,*N*-Bis(*p*-methylbenzyl)formamidinyl)-2′,3′-dideoxy-3′-thiacytidine 10. According to the general procedure B, the reaction of *N*,*N*-dimethylformamide dimethyl acetal (477 mg, 4 mmol, 5 equiv) with the title compound **6** (2.7 g, 12 mmol, 15 equiv) and then 3TC (150 mg, 0.655 mmol, 1 equiv) afforded, after purification by flash chromatography on silica gel (CH_2Cl_2/MeOH, 96:4), compound **10** (244 mg, 80%): $R_f = 0.32$ (CH_2Cl_2/MeOH 95:5); MS (ES+) 465 $(M + H)^+$. Anal. ($C_{25}H_{28}N_4O_3S$) C, H, N.

4-*N*-(*N*,*N*-Bis(*p*-ethyloxycarbonylbenzyl)formamidinyl)-2′,3′-dideoxy-3′-thiacytidine 11. According to the general procedure B the reaction of *N*,*N*-dimethylformamide dimethyl acetal (186 mg, 1.56 mmol, 5 equiv) with the title compound **7** (1.6 g, 4.8 mmol, 15 equiv) and then 3TC (100 mg, 0.437 mmol, 1 equiv) afforded, after purification by flash chromatography on silica gel (CH_2Cl_2/MeOH, 95:5), compound **11** (17 mg, 7%): $R_f = 0.36$ (CH_2Cl_2/MeOH 95:5); MS (ES+) 581 $(M + H)^+$. Anal. ($C_{29}H_{32}F_2N_4O_7S$) C, H, N.

4-*N*-(Dibenzylformamidinyl)-2′,3′-dideoxycytidine 12. To a solution of *N*,*N*-dimethylformamide dimethyl acetal (200 mg, 1.68 mmol, 5 equiv) in dry acetonitrile (6 mL) was added dibenzylamine (993 mg, 5.04 mmol, 15 equiv) under N_2. The reaction mixture was refluxed for 20 h and then evaporated to dryness, and traces of remaining dimethylformamide dimethyl acetal were removed by two successive coevaporations with toluene. The crude residue (250.2 mg, 0.923 mmol, 5 equiv) was dissolved in anhydrous DMF and added to a solution of ddC (39 mg, 0.185 mmol, 1 equiv) in anhydrous DMF (3 mL). The resulting solution was stirred overnight at 70 °C, and solvents were removed under reduce pressure. The residual oil was dissolved in CH_2Cl_2, washed with 5% aqueous

citric acid, and extracted with CH_2Cl_2. The organic layers were dried over $MgSO_4$ and filtered to afford after purification by flash chromatography on silica gel (CH_2Cl_2/MeOH, 92:8) compound **12** (25 mg, 32%): $R_f = 0.44$ (CH_2Cl_2/MeOH 90:10); MS (ES+) 419 $(M + H)^+$. Anal. ($C_{24}H_{26}N_4O_3$) C, H, N.

Acknowledgment. We gratefully acknowledge Pr. K. Dudley (INSERM U-382, Université Méditerranée, Luminy-Marseille, France) for manuscript revisions. We are grateful to Liesbet de Dier and Cindy Heens for their excellent technical assistance. This research was supported by grants from LAPHAL Laboratories and PACA regional Council (C.A.). INSERM is acknowledged for financial support.

Supporting Information Available: NMR and IR spectroscopic data and elemental analyses of the desired compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0309708