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Anti-HIV and Anti-HBV Activity and Resistance Profile of 2′,3′-Dideoxy-

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A novel membrane-soluble prodrug of the 5′-monophosphate derivative of 3TC containing a phenyl group and the methyl ester of L-alanine linked to the phosphorus through a phosphoramidate bond with the primary amino moiety (designated Cf 1109) was prepared. The 3TC prodrug proved less potent an inhibitor of HIV-1 and HIV-2 replication in CEM cell cultures than 3TC, but lost only 20-fold antiviral potency in 2′-deoxycytidine kinase-deficient CEM/dCK$^-$ cells compared with a more than 2,000-fold decrease of activity of 3TC. In contrast, 3TC and Cf 1109 proved equally highly effective in inhibiting HBV release in supernatants of HBV-transfected Hep G2 2.2.15 cell cultures (50% effective concentration $\sim$ 0.02 μM). Both compounds easily selected for highly resistant HIV-1 strains at a comparable speed of breakthrough. The mutant viruses contained an 184-Ile and/or 184-Val amino acid change in their reverse transcriptase. Our data are suggestive for a relatively poor delivery of 3TC-MP in the intact CEM cells but a remarkably high delivery of 3TC and/or 3TC-MP in the intact Hep G2 2.2.15 cells.

The most recently approved 2′,3′-dideoxynucleoside (ddN) derivative for treatment of HIV infections is 2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) (Fig. 1). This drug is also subject of clinical trials in human hepatitis B virus (HBV)-infected patients. In contrast with the other approved (β-D) ddN derivatives has 3TC the β-L(−) isomeric conformation. 3TC needs to be converted to its 5′-triphosphate derivative by cellular enzymes to be inhibitory to the reverse transcriptase (RT) of HIV, and is endowed with low if any cytotoxicity (1-5). However, the first activation (phosphorylation) step is catalysed by cytosolic 2′-deoxycytidine kinase (dCK) which has a relatively poor affinity for 3TC ($K_i/K_m \sim 50$) resulting in a moderate efficiency of conversion of 3TC to its 5′-monophosphate derivative (6). In addition, a potential drawback of 3TC is the rapid emergence of drug-resistant HIV-1 strains (7-11). Indeed, mutant virus strains that emerged in the presence of 3TC were shown to contain an amino acid change from methionine (Met) to valine (Val) or isoleucine (Ile) at position 184 of the RT. Such virus is highly resistant to the inhibitory effects of 3TC in cell culture (7-11), and is reported to show low-level cross-resistance to 2′,3′-dideoxycytidine (ddC) (Fig. 1) and 2′,3′-dideoxyinosine (ddI).

In an attempt to circumvent the first intracellular phosphorylation step of 3TC, we synthesized the arylphosphoramidate derivative of 3TC containing a methylester of L-alanine linked to the 5′-phosphate of 3TC-MP through its primary amino group (Fig. 1). The corresponding 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine) analogue (So324) was previously shown to be able to deliver directly the 5′-monophosphate derivative of d4T into intact cells and to efficiently bypass the first (thymidine kinase-directed) phosphorylation of d4T (12,13). The antiviral potency and resistance profile of Cf 1109 is described compared to 3TC.

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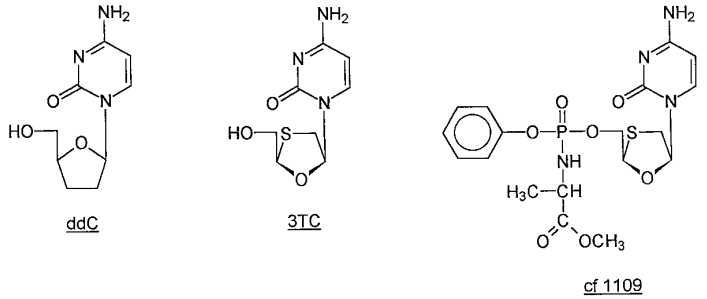

FIG. 1. Structural formulae of 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) and the arylphosphoramidate derivative of 3TC (Cf 1109).

MATERIALS AND METHODS

Viruses. HIV-1(III_B) and HIV-2(ROD) were kindly provided by Prof. R.C. Gallo (National Cancer Institute, National Institutes of Health, Bethesda, MD) and Dr. L. Montagnier (Pasteur Institute, Paris, France).

Cells. Wild-type CEM/0 cells were obtained from the American Tissue Cell Culture Collection (Rockville, MD), the thymidine kinase-deficient (CEM/TK^-) and 2′-deoxycytidine kinase-deficient (CEM/dCK^-) cells were kindly provided by Prof. S. Erikson (Uppsala, Sweden) and Prof. A. Karlsson (Stockholm, Sweden). Hep G2 2.2.15 hepatoblastoma cells were provided by Dr. G. Acs (Mount Sinai Medical Center, N.Y.).

Synthesis of 3TC 5′-[phenyl methoxyalaninyl]phosphate (Cf1109). Tertbutylmagnesium chloride (1.0 M in THF) (1.0 ml, 1.1 eq) was added to a slurry of 3TC (0.21 g, 0.93 mmol) in pyridine (7 ml) and THF (50 ml) with stirring, and the mixture was allowed to equilibrate for 0.5 h under a nitrogen atmosphere. Phenyl methoxyalaninyl phosphorochloridate (1.0 M in THF) (2 ml, 2 mmol, 2 eq) was added with stirring, and the mixture was allowed to stir for 2 h. The reaction mixture was diluted with dichloromethane (50 ml), quenched with saturated NH_4Cl (100 ml) and extracted with dichloromethane (3×50 ml). The combined organic layers were washed with brine (30 ml), dried over $MgSO_4$ and the solvent removed *in vacuo.* The resulting residue was purified by flash column chromatography (7% MeOH, 1% Et_3N in $CHCl_3$) to yield the product as a white solid (0.21 g, 48%) (M.Pt. 52-54°C); δ_H (DMSO-d_6) 7.71 (1H, 2d, J=7.5 Hz, H6), 7.30 (7H, m, OPhe, NH_2), 6.25 (1H, t, J=5.7 Hz, H1′), 6.14 (1H, m, Ala NH), 5.74 (1H, 2d, J=7.5Hz, H5), 5.38 (1H, 2t, H4′), 4.29 (2H, m, H5′), 3.89 (1H, m, H4′), 3.60 (3H, s, OMe), 3.42 Ala CH), 3.06 (2H, m, H2′), 1.23 (3H, 2d, J=6Hz, Ala Me); δ_p (DMSO-d_6) 4.89, 4.52; δ_C (DMSO-d_6) 176.563-176.343 (Ala C=O), 168.411 (C-4), 157.402 (C-2), 153.458-153.315 (Phe), 143.451 (C-6), 132.424 (Phe), 127.403 (Phe), 122.985-122.856 (Phe), 97.078 (C-5), 89.706 (C-1′), 84.468-84.351 (C-4′), 69.692-69.444 (C-5′), 54.709 (OMe), 52.583-52.400 (Ala CH), 38.404-38.325 (C-2′), 22.493-22.331 (Ala Me); ν_{max} (cm^{-1}): 3341.2, 3197.3, 2988.0, 2932.3, 1740.8, 1651.8.

Antiviral activity of test compounds. CEM cells were suspended at 250,000 cells per ml of culture medium and infected with approximately 100 $CCID_{50}$ (1 $CCID_{50}$ being the 50% cell culture infective dose) of HIV-1(III_B) or mutant HIV-1 strains selected for resistance against 3TC (11). Then, 100 μl of the infected cell suspensions was added to 200-μl microtiter plate wells containing 100 μl of an appropriate dilution of the test compounds (i.e. 250, 100, 20, 4, 0.8, 0.16, 0.032, 0.006, 0.001 μg/ml). The inhibitory effect of the test compounds on HIV-1-induced syncytium formation in CEM cells was examined on day 4 post infection as described previously (11). The 50% effective concentration (EC_{50}) was determined as the compound concentration required to inhibit syncytium formation by 50%.

Peripheral blood lymphocytes (PBL) from a normal HIV-negative human volunteer were obtained by the Ficoll/Hypaque technique, washed twice with phosphate-buffered saline (PBS) and cultured with phytohemagglutin (PHA, 2 μg/ml) for 3 days at 37°C in a CO_2-controlled incubator. PHA-stimulated PBL cells were washed twice with PBS and then infected with a concentrated HIV-1 ($HTLV\text{-}III_B$) stock in RPMI-1640 medium supplemented with 15% heat-inactivated foetal calf serum, L-glutamine (2 mM), gentamicin (50 μg/ml), recombinant interleukin-2 (10 Units/ml) and polybrene (2 μg/ml). After 60 min incubation at 37°C, non-adsorbed virus was removed by successive washing steps with fresh culture medium, and the HIV-1-infected PBL cells were then suspended at 5×10^5 cells/ml in RPMI-1640 medium and cultured in the presence of varying concentrations (20, 4, 0.8, 0.16, 0.032 and 0.006 μM) of the test compounds in 96-well culture plates (200 μl per well). HIV-1 p24 core antigen was quantified in the cell culture supernatants at 7 days after infection by an antigen-capture assay using an ELISA technique.

For analysis of extracellular HBV DNA, Hep G2 2.2.15 hepatoblastoma cells were seeded in cell culture recipients as described before (12). After seeding, culture medium, containing the drugs, was changed at days 3, 6 and 9. At day 12 culture medium was harvested and clarified by centrifugation for determination of HBV DNA. The culture

TABLE 1
Inhibitory Effect of 3TC, Cf 1109, and ddC on HIV Replication in Different CEM Cell Lines

	EC_{50} (μM)[a]				
	CEM/0			CEM/TK$^-$	CEM/dCK$^-$
Compound	HIV-1(III_B)	HIV-2(ROD)	HIV-1/184 Val	HIV-2(ROD)	HIV-1(III_B)
Cf 1109	2.5	3.0	>250	5.5	65
3TC	0.01	0.02	>250	0.07	30
ddC	0.04	0.01	0.04	0.03	20
So324	0.13	0.10	0.09	0.08[b]	0.12
d4T	0.35	0.30	0.22	33[b]	0.17

[a] 50% Effective concentration, or compound concentration required to inhibit HIV-induced cytopathicity by 50%.
[b] Data taken from ref. 13.

supernatants were prepared for dot blot analysis according to Korba and Milman (13) and applied to Hybond N+ membrane in the Convertible Filtration on Manifold.

Selection of HIV-1(III_B) mutant strains resistant to 3TC and Cf 1109 administered as single drugs at fixed concentrations. HIV-1(III_B) was subjected to seven to eight passages in 5-ml CEM cell cultures (4×10^5 cells per ml) in the presence of 2 μM 3TC or 10 μM Cf 1109 in 25-cm^2 culture flasks (Falcon, Becton Dickinson) to produce mutant HIV-1 strains. The multiplicity of the initial infection was at least 1000 times the 50% cell culture infective dose (IC_{50}). Passages were performed every 3-4 days by adding 0.5-1.0 ml of the infected culture supernatants to 4 to 4.5 ml of a suspension containing 4×10^5 uninfected CEM cells per ml. Syncytium formation was used as a parameter of virus breakthrough in the cell cultures. After the 7th or 8th passage, the test compound concentrations were gradually increased to 100 μM for Cf 1109 and 50 μM for 3TC as indicated in the footnote to Table 2.

Determination of the amino acid sequence of the RT of drug-resistant virus strains. The procedure of CEM cell infection with mutant HIV-1 strains, preparation of the samples for PCR assays, amplification of the proviral DNA, and sequencing of the 727 bp fragment covering amino acid residues 50-270 have been reported earlier (11,14). The first set of primers (5′-GTAGAATTCTGTTGACTCAGATTGG-3′ and 5′-TTCTGCCAGTTCTAGCTCTGCTTCT-3′) gave a 900-bp product of the proviral RT gene. The second set of primers (5′-CCTGAAAATCCATACAATACT-CCAGTATTTG-3′ and 5′-AGTGCTTTGGTTCCTCTAAGGAGTTTAC-3′) gave the 727-bp RT fragment covering amino acids 50-270. The PCR products were purified from 0.1% low-melting agarose gel by Magic PCR Preps (Promega) or on a microspin column, and directly sequenced with an AutoRead T-7 Sequencing kit (Pharmacia). Sequence analysis was then performed on an ALF DNA sequencer (Pharmacia).

RESULTS

Antiviral activity of Cf 1109, 3TC, and ddI against HIV-1 in different CEM cell lines. The test compounds were evaluated on their antiviral efficiency against HIV in wild-type CEM/0 and the mutant thymidine kinase (TK)-deficient CEM/TK$^-$ and 2′-deoxycytidine kinase (dCK)-deficient CEM/dCK$^-$ cell cultures (Table 1). 3TC and ddCyd proved 60- to 300-fold more inhibitory against HIV-1 and HIV-2 replication in wild-type CEM/0 cell cultures than the lipophylic 3TC-MP prodrug Cf 1109. All three cytosine derivatives kept full inhibitory potential against HISV-2-infected TK-deficient CEM cells, but not against dCK-deficient CEM cells. Interestingly, both ddC and 3TC showed a decreased antiviral potency by more than 3 orders of magnitude whereas Cf 1109 lost its inhibitory potential only by 20-fold (Table 1). When evaluated against 184-Val RT-mutated HIV-1, Cf 1109 had a > 100-fold, and 3TC a 5,000-fold decreased inhibitory effect.

Antiviral activity of 3TC and CF 1109 against extracellular release of HBV from HBV-transfected Hep G2 2.2.15 cell cultures. The inhibitory effect of 3TC and Cf 1109 on the extracellular release of HBV in supernatants of Hep G2 2.2.15 cell cultures has also been evaluated (Fig. 2). Comparable results were obtained for the two drugs with an estimated 50% effective concentration of 0.02 μM, as previously described for 3TC (14).

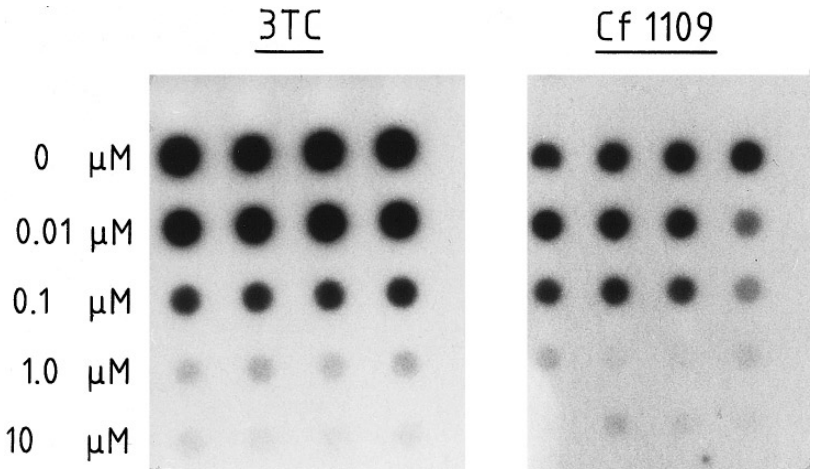

FIG. 2. Evaluation of HBV DNA by dot blot hybridization, from cell culture supernatants of Hep G2 2.2.15 after 12 days of incubation in the presence of various drug concentrations. The cell cultures were exposed to 3TC or Cf 1109 at 0 (upper horizontal row), 0.01, 0.1, 1.0 and 10 μM (lowest horizontal row). The experiment was performed in quadruplicate (vertical lanes).

Breakthrough of mutant HIV-1(III_B) strains in the presence of 3TC and Cf 1109. The ability of 3TC and Cf 1109 to suppress mutant virus breakthrough was then evaluated in wild-type CEM cell cultures (Table 2). HIV-1-infected CEM cell cultures were treated with Cf 1109 and 3TC at 10 μM and 2 μM, respectively, that is at a 5- and 50-fold higher concentration than their EC_{50}. Initial (microscopic) signs of virus breakthrough occurred at day 10 (for Cf 1109) and day 18 for 3TC. Full cytopathocity was reached under continued drug exposure at days 21 and 24 for Cf 1109 and 3TC, respectively (Table 2). Gradual increases of drug concentrations to 100 μM for Cf 1109 or 50 μM for 3TC did not markedly affected virus replication in the cell cultures. Stocks of the mutant virus strains were prepared at day 45 post infection.

Molecular characterisation of the mutant HIV-1 strains that emerged in the presence of Cf 1109 and 3TC. The amino acid substitutions in the RT of the virus strains that emerged in the presence of Cf 1109 and 3TC were determined. The virus strain that emerged in the continued presence of 3TC contained a 184 Met → Ile amino acid change in its RT (codon ATG → ATA). The virus that emerged in the presence of Cf 1109 consisted of a mixture of

TABLE 2
Suppression of HIV-1-Induced Cytopathicity in CEM Cell Cultures (% of Control)

Compound	Concentration (μM)	Days post infection												
		3	7	10	14	18	21	24	27	31	35	38	42	45
Cf 1109	10	0	0	3	6.5	50	100	100[a]	100	100	100	100	100	100[c]
3TC	2	0	0	0	0	25	37	100	100[b]	100[b]	87	100	100	100[c]

[a] Initial Cf 1109 concentration was increased from 10 to 20 μM on day 24, from 20 to 50 μM on day 27, and from 50 to 100 μM on day 31 post infection and further subcultured in the presence of the test compound.

[b] Initial 3TC concentration was increased from 2 to 8 μM on day 27 and from 8 to 40 μM on day 31 post infection and further subcultured in the presence of the test compound.

[c] Virus stocks were prepared on day 45 post infection.

TABLE 3
Inhibitory Effect of NRTIs and NNRTIs on Mutant HIV-1 Replication in CEM Cell Cultures

	EC_{50} (μg/ml)[a]		
	Mutant HIV-1(III_B) strains containing the following amino acid changes in their RT		
Compound	III_B (wild-type)	184-Ile (HIV-1/3TC)	184-Val/Ile (HIV-1/Cf 1109)
3TC[b]	0.05	3.6	30
Cf 1109[b]	1.1	60	>250
ddC[b]	0.02	0.04	0.05
ddI[b]	2.3	5.1	6.5
AZT[b]	0.008	0.007	0.004
PMEA[b]	7.0	10	6.5
Nevirapine	0.022	0.021	0.010
BHAP U90,152	0.007	0.006	0.004
TIBO R82,913	0.023	0.020	0.011
α-APA	0.005	0.015	0.004
HBY 097	0.0007	0.025	0.041
TSAO-m ^{3}T	0.018	0.025	0.041
UC-10	0.013	0.009	0.008
UC-781	0.001	0.002	0.001
UC-82	0.002	0.007	0.002

[a] 50% Effective concentration, or compound concentration required to inhibit HIV-1-induced giant cell formation in CEM cell cultures by 50%.
[b] Data expressed in μM.

virus strains that contained a 184 Met → Val (codon ATG → GTG) or 184 Met → Ile (codon ATG → ATA) in their RTs.

Sensitivity/resistance spectrum of the mutant virus strains that emerged in the presence of 3TC and Cf 1109. The sensitivity/resistance spectrum of the mutant viruses that broke through in the presence of 3TC and Cf 1109 in the HIV-1-infected CEM cell cultures was determined for a variety of nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) and compared with the parental wild-type virus (Table 3). Among the NRTIs, only 3TC (and Cf 1109) showed a markedly decreased antiviral activity against both HIV-1/184 Ile and HIV-1/184 Val/Ile viruses. The activity of 3TC was almost 100-fold (for HIV-1/184 Ile) to 1,000-fold (for HIV-1/184 Val) decreased. Cf 1109 completely lost its inhibitory potential against both mutant viruses at 250 μg/ml. In contrast, DDC nor ddI, AZT and PMEA lost their antiviral activity against the mutant virus strains. Also, prototype members of a variety of 7 different NNRTI classes virtually kept full inhibitory potential against mutant HIV-1/184 Ile and HIV-1/184 Val/Ile when compared with wild-type virus (Table 3).

DISCUSSION

Recently, we developed a successful approach to efficiently deliver the 5′-monophosphate of d4T into intact CEM cells (15,16). The arylphosphoramidate derivative of d4T (So324) proved at least as effective if not more efficient in inhibiting HIV replication in cell culture than the parental nucleoside d4T. We could also show that So324, in sharp contrast with d4T, kept its full antiviral efficacy in thymidine kinase-deficient CEM cell cultures. The latter observation, together with metabolism experiments in CEM/0 and CEM/TK^- cell cultures, using radiolabeled So324 and d4T, strongly pointed to the ability of So324 to deliver directly d4T-MP in the intact cells. To overcome the dependence on phosphorylation of 3TC by cytosolic dCyd kinase, an analogous approach was followed.

Surprisingly, the 3TC prodrug Cf 1109 proved ~ 250-fold less active against HIV than 3TC. This is in sharp contrast with the analogous prodrugs of AZT (designated So221) (17) and d4T (designated So324) (15,16) that showed virtually comparable, if not superior, antiviral activity than the parental AZT and d4T nucleosides. This observation can be explained by a much poorer uptake of the 3TC prodrug compared to the d4T and AZT prodrugs, and/or to a markedly lower efficiency of the intracellular release of the nucleoside or nucleotide. Although we do not have experimental proof, we do believe that the first possibility is unlikely since these highly lipophilic compounds are thought to enter the cells by passive diffusion which is supposedly rather independent on the nature of the nucleoside sugar part. Therefore, a relatively low efficiency of intracellular release of 3TC and 3TC-MP from the prodrug Cf 1109 is more likely to explain the lower antiviral activity of Cf 1109 than 3TC in CEM cells.

3TC and Cf 1109 are virtually equally effective against HBV in Hep G2 2.2.15 cells. Obviously, the enzymes that may release 3TC and/or 3TC-MP from Cf 1109 clearly differ in the human hepatoblastoma Hep G2 2.2.15 and lymphocyte CEM cells. It is also noteworthy that the d4T-MP prodrug So324, that showed a higher anti-HIV efficacy than d4T in cell culture, was not endowed with a marked anti-HBV activity (EC_{50} for d4T and So324: > 10 μM) in the Hep G2 2.2.14 hepatoblastoma cell cultures. Thus, whereas So324 proved superior to Cf 1109 against HIV, the d4T-MP prodrug was inferior to the 3TC-MP prodrug against HBV.

In contrast with the d4T-MP prodrug So324, Cf 1109 markedly lost (20-fold) antiviral potency in HIV-infected CEM cell cultures that lacked the enzyme phosphorylating 3TC (i.e. CEM/dCK$^-$). However, our observation that the parental nucleoside 3TC lost more than 1,500-fold its antiviral potential under identical experimental conditions points to some delivery of 3TC-MP within the intact cells, albeit at relatively low efficiency (estimated to be ~ 5%). This relatively low delivery of 3TC-MP from Cf 1109 in the intact CEM cells contrasts with the markedly more efficient delivery of d4T-MP from So324 (15,16) and may likely be due to different substrate specificities of esterases and/or other enzyme activities that may determine the eventual delivery of 3TC and/or 3TC-MP from Cf 1109. The pathways followed by So324 and Cf 1109 to deliver d4T-MP and 3TC-MP, respectively, are currently under investigation. However, modifications in the aryl part and/or the amino acid part of the 3TC prodrug may be required to improve the stability and/or depot-function of Cf 1109 for 3TC and 3TC-MP.

The fact that both Cf 1109 and 3TC virtually give an identical resistance spectrum (both in selection of mutant virus strains and the sensitivity/resistance profile) is strongly suggestive for a common mechanism of HIV (and HBV) inhibition by these compounds: i.e. conversion of the compounds to 3TC-TP, followed by inhibition of the HIV RT and HBV DNA polymerase by 3TC-TP.

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