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Inhibition of Visna Virus Replication by 2′,3′-Dideoxynucleosides and Acyclic Nucleoside Phosphonate Analogs

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A series of acyclic nucleoside phosphonate (ANP) and 2′,3′-dideoxynucleoside (ddN) derivatives were evaluated for their inhibitory effects on visna virus replication and maedi/visna virus-induced syncytium formation in sheep choroid plexus cells. Most ANP derivatives inhibited virus replication and syncytium formation within a concentration range of 0.2 to 1.8 μM. Among the most active ANP derivatives ranked (*R*)-9-(2-phosphonomethoxypropyl)adenine, (*R*)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine, and (*S*)-9-(3-fluoro-2-phosphonomethoxypropyl)adenine. Of the ddN derivatives, 2′,3′-dideoxycytidine (ddCyd) proved to be the most inhibitory to visna virus-induced syncytium formation (50% effective concentration, 0.02 μM). The purine ddN analogs (i.e., 2′,3′-dideoxyinosine, 2′,3′-dideoxyadenosine, 2′,3′-dideoxyguanosine, and 2,6-diaminopurine-2′,3′-dideoxyribosine) were 10- to 30-fold less effective, and the thymidine derivatives 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) and 3′-azido-2′,3′-dideoxythymidine (AZT) were more than 500-fold less inhibitory to visna virus than ddCyd. The 5′-triphosphate forms of AZT and D4T were 100- to 600-fold more inhibitory to visna virus particle-derived reverse transcriptase than was the 5′-triphosphate of ddCyd. The apparent discrepancy between the inhibitory effects of these ddN derivatives on virus replication and viral reverse transcriptase activity most likely reflects differences in the metabolic conversion of ddCyd versus D4T and AZT in sheep choroid plexus cells.

Extensive investigations have been carried out with compounds that are active against the primate lentiviruses, particularly human immunodeficiency virus (HIV) and simian immunodeficiency virus (for an overview see references 1, 9, and 10) and, to a lesser extent, feline immunodeficiency virus (13, 15, 21). On the other hand, few studies have been done on compounds that are active against ungulate lentiviruses such as maedi/visna virus, which causes a slow infection in sheep (11, 12, 24). However, it is of major interest to compare the drug susceptibilities of the primate and ungulate lentiviruses in view of the characterization of their molecular and biological relationships. Also, compounds that strongly affect the replication of both subgroups of lentiviruses may be considered broad-spectrum lentivirus inhibitors. Furthermore, visna virus-infected sheep may prove to be a useful animal model for evaluating the effects of antilentivirus compounds in vivo.

In the study described here we evaluated the effect on visna virus of a number of HIV reverse transcriptase inhibitors that belong to the class of the 2′,3′-dideoxynucleoside (ddN) and acyclic nucleoside phosphonate (ANP) analogs. Whereas a good correlation was found between the anti-HIV and anti-maedi/visna virus activities of the ANPs, no such correlation could be established for the ddN analogs.

MATERIALS AND METHODS

Compounds. The syntheses of ANP derivatives have been described previously (16–19) or will be reported elsewhere. The following ANPs were tested: 9-(2-phosphonomethoxyethyl)adenine (PMEA); 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine (PMEDAP); the *S* and *R* enantiomers of the 9-(2-phosphonomethoxypropyl) derivatives of adenine (PMPA), 2,6-diaminopurine (PMPDAP), and guanine (PMPG); the *S* and *R* enantiomers of the 9-(3-fluoro-2-phosphonomethoxypropyl) derivatives of adenine (FPMPA); and the *S* enantiomer of the 9-(3-hydroxy-2-phosphonomethoxypropyl) derivatives of adenine, guanine, 2,6-diaminopurine, and cytosine (designated HPMPA, HPMPDAP, HPMPG, and HPMPC, respectively). The ddNs 2′,3′-dideoxycytidine (ddCyd), 2′,3′-dideoxyadenosine (ddAdo), and 2′,3′-dideoxyguanosine (ddGuo) were obtained from D. G. Johns (National Cancer Institute, Bethesda, Md.); 2′,3′-dideoxyinosine (ddIno) was from Bristol-Myers Squibb (Wallingford, Conn.); 2,6-diaminopurine-2′,3′-dideoxyribosine (ddDAPR), 3′-azido-2′,3′-dideoxy-2,6-diaminopurine-riboside (AzddDAPR), 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T), and 2′,3′-didehydro-2′,3′-dideoxycytidine (D4C) were synthesized by P. Herdewijn (Rega Institute, Leuven, Belgium). 3′-Azido-2′,3′-dideoxythymidine (AZT) was from Burroughs Wellcome.

Cell cultures and media. Low-passage sheep choroid plexus (SCP) cells were grown in Eagle's minimum essential medium (GIBCO, Paisley, Scotland) containing 2 mM L-glutamine, 20 μg of gentamicin per ml, and 20% heat-inactivated lamb serum (Colorado Serum Comp., Denver, Colo.). Cell monolayers were maintained in the same medium with 2% lamb serum. Maintenance medium was used to dilute the test compounds and the virus stock suspensions.

Virus. Visna virus strain K796 and maedi virus strains M88 and M34 were isolated from visna virus- and maedi virus-infected Ice-landic sheep, respectively (25). Strain GPPV was isolated from a sheep with progressive pneumo-

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nia in Montana. It was kindly provided by G. J. Gibbs, National Institutes of Health, Bethesda, Md. All the virus strains were passaged 10 to 15 times in SCP cell cultures before use in the present study. The visna virus used in the reverse transcriptase (RT) assays was purified and concentrated as described previously (20).

Titration of virus. Viral infectivity was titrated by inoculation of 10-fold dilutions of the virus suspension into SCP cell monolayers in 96-well microtiter tissue culture plates (Nunc, Roskilde, Denmark). One-tenth milliliter of virus dilution was brought into each well in quadruplicate. The plates were incubated in a CO_2-controlled incubator at 37°C and were examined for cytopathic effect (CPE) at 7 and 14 days postinfection. Virus titers were calculated by the method of Reed and Muench (23).

CPE inhibition assay. Ninety-six-well microtiter plates were seeded with 6×10^3 SCP cells per well in 100 μl of growth medium, and the cell monolayers were used for the experiments after 4 to 5 days, when they were confluent. One hundred microliters of serial fivefold dilutions of the test compounds were added to the monolayer cells in duplicate. They were then infected with 10 μl of virus at a multiplicity of 0.05 50% cell culture infective doses ($CCID_{50}$s) per cell. The cultures were incubated at 37°C for 6 days. At that time, control wells without test compounds showed a widespread CPE. The monolayers were fixed in ethanol, stained with a 2% Giemsa solution, and examined for multinucleated syncytia, which represent the hallmark of visna virus CPE. The antiviral activities of the compounds were expressed as the compound concentration which caused a 50% reduction in syncytium formation (50% effective concentration [EC_{50}]).

Evaluation of cytotoxicity. To determine the effect of the test compounds against SCP cell proliferation, 96-well microtiter plates were seeded with 3×10^3 cells per well. On the following day the culture medium was replaced by growth medium containing the test compounds at concentrations varying from 4 to 100 μM, in quadruplicate. The plates were incubated at 37°C for 5 days or until the untreated control cultures reached confluency. The monolayers were then trypsinized and the cells were counted with a Coulter counter. The inhibitory effect of the test compounds on cell growth was expressed as the compound concentration that caused a 50% reduction in cell number (50% inhibitory concentration [IC_{50}]).

Virus yield reduction assay. Cell monolayers were incubated with serial dilutions of test compounds for 6 days following virus inoculation as described above for the CPE inhibition assay. The cell culture medium was then harvested from duplicate wells and the virus yields were determined. Virus titers ($\log_{10}$ $CCID_{50}$ per milliliter) were plotted against the compound concentrations, and the inhibitory effects of the test compounds on virus yield were expressed as the compound concentration which caused a 90% reduction in virus titer (IC_{90}).

RT inhibition assay. The endogenous RT reaction mixture (50 μl) consisted of 50 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 100 mM KCl, 4 mM dithiothreitol, 30 μg of bovine serum albumin per ml, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 10 μg of mellitin per ml. Of the four 2′-deoxynucleotide 5′-triphosphates (dNTPs), three were used at a saturating concentration of 100 μM, while the ^{3}H-labeled dNTP ([*methyl*-^{3}H]dTTP) was used at a concentration of 2.5 μM. Following the addition of various concentrations of the inhibitors (i.e., AZT-5′-triphosphate [AZT-TP] or phosphonoformic acid [PFA]) to the reaction mixture (at 0°C), 10 μl of purified, concentrated visna virions was added and the reaction mixture was incubated for 8 h at 37°C. The amount of incorporated [*methyl*-^{3}H]dTTP was determined by a standard trichloroacetic acid precipitation procedure with Whatman GF/C glass fiber filters and liquid scintillation counting (8). The RT reaction mixture (50 μl) used to evaluate AZT-TP, D4T-TP, and ddCyd-TP in the presence of artificial template and primers contained 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 300 mM glutathione, 500 μM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, 2 μCi (~2.5 μM) of [*methyl*-^{3}H]dTTP or [5-^{3}H]dCTP, a fixed concentration of the template and primer poly(A) · oligo(dT) (0.015 mM; Pharmacia, Uppsala, Sweden) or poly(I) · oligo(dC) (0.015 mM; Pharmacia), 0.06% Triton X-100, 10 μl of the inhibitor solution, and 5 μl of the RT preparation. Reaction mixtures were incubated at 37°C for 15 min, at which time 100 μl of calf thymus DNA (150 μg/ml; Sigma, St. Louis, Mo.), 2 ml of $Na_4P_2O_7$ (0.1 M in 1 M HCl), and 2 ml of trichloroacetic acid (10% [vol/vol]) were added. The solutions were kept on ice for 30 min, after which time the acid-insoluble material was washed and analyzed for radioactivity.

RESULTS

Direct inhibitory effect of the test compounds on visna virus infectivity. To determine whether any of the compounds inactivated viral infectivity, visna virus (5×10^3 $CCID_{50}$s) was incubated at 37°C for 2 h with 50 μM (each) compound in 0.1 ml of maintenance medium. Virus samples from each mixture were then titrated. None of the compounds caused a detectable reduction of virus titer compared with the virus titers in untreated control samples (data not shown).

Inhibitory effects of ddN analogs on visna virus-induced cytopathogenicity. Of the nine ddNs tested, ddCyd consistently showed the highest level of antiviral activity, with an IC_{50} of 0.02 μM (Table 1). D4C, the 2′,3′-didehydro derivative of ddCyd, proved to be about 30-fold less efficient than ddCyd in inhibiting visna virus-induced cytopathogenicity. Four other nucleoside analogs, i.e., ddIno, ddGuo, ddAdo, and ddDAPR, were 10- to 30-fold less active than ddCyd, and the 3′-azido-substituted ddDAPR was 3- to 4-fold less active than the parent compound ddDAPR. AZT showed little inhibitory effect on visna virus CPE (EC_{50}, 19 μM). Moreover, it did not completely inhibit syncytium formation at a concentration of as great as 700 μM, although there was a 50% or greater reduction in the number of syncytia at lower concentrations. Also, another thymidine analog, D4T, showed a low inhibitory effect on visna virus CPE. None of the ddNs showed detectable cytotoxicity at concentrations of up to 100 μM after exposure to the SCP cell monolayers for up to 6 days (data not shown). The IC_{50} for SCP cell proliferation was also equal to or greater than 100 μM for all compounds tested except for AzddDAPR, which proved to be cytostatic at an IC_{50} of 5 μM (Table 1).

Effect of ANP analogs on visna virus CPE and SCP cell growth. A series of 14 ANPs, i.e., compounds belonging to the four different subclasses (PME, PMP, FPMP, and HPMP), were evaluated for their inhibitory effects on visna virus-induced cytopathogenicity and SCP cell proliferation.

Most of the ANP derivatives had a marked inhibitory effect on visna virus CPE at an EC_{50} of 1 μM or less (Table 1). PMEA and PMEDAP were strongly inhibitory to visna virus-induced cytopathogenicity. Their EC_{50}s were as low as 0.6 μM for PMEDAP and 1.1 μM for PMEA. PMEA was superior to PMEDAP with regard to its therapeutic index

TABLE 1. Inhibitory effects of ddN and ANP analogs on visna virus syncytium formation in SCP cell monolayers and on the proliferation of exponentially growing SCP cells

Compound	EC_{50} (μM)[a]	IC_{50} (μM)[b]	Selectivity index (ratio IC_{50}/EC_{50})
ddCyd	0.02 ± 0.007	≥100	≥5,000
ddIno	0.15 ± 0.0	ND[c]	ND
ddAdo	0.6 ± 0.2	≥100	≥167
ddGuo	0.35 ± 0.0	>100	>285
ddDAPR	0.26 ± 0.1	>100	>385
AzddDAPR	1.0 ± 0.3	5	5
D4C	0.6 ± 0.2	>100	≥167
D4T	9 ± 0.0	>100	>11
AZT	19 ± 8	≥100	≥5
PMEA	1.1 ± 0.5	60	55
PMEDAP	0.4 ± 0.3	10	17
(*S*)-PMPA	10 ± 0.3	>100	>10
(*R*)-PMPA	0.9 ± 0.2	>100	>110
(*S*)-PMPDAP	1.3 ± 0.5	>100	>77
(*R*)-PMPDAP	0.9 ± 0.3	>100	>110
(*S*)-PMPG	0.2 ± 0.02	1	5
(*R*)-PMPG	0.5 ± 0.02	20	40
(*S*)-FPMPA	0.7 ± 0.2	≥100	≥143
(*R*)-FPMPA	84 ± 40	>100	>1.2
(*S*)-HPMPA	0.7 ± 0.2	30	43
(*S*)-HPMPG	0.2 ± 0.01	10	50
(*S*)-HPMPDAP	1.8 ± 0.7	≥100	≥55
(*S*)-HPMPC	2.7 ± 1.3	>100	>37

[a] Data are the means ± standard deviation of three independent experiments.
[b] Data are the means for two independent experiments.
[c] ND, not determined.

(selectivity index [ratio IC_{50}/EC_{50}], 55 versus 17), and thus may have a higher safety range than PMEDAP at antivirally effective concentrations.

Several *R* and *S* enantiomers of the PMP and FPMP derivatives showed significant differences in antiviral activity. (*S*)-FPMPA was 120-fold more inhibitory against visna virus CPE than its *R* enantiomer. Similarly, (*R*)-PMPA proved to be 11-fold more effective than its *S* enantiomer. It should be pointed out that the *R* and *S* designations within the FPMP and PMP series are such that in terms of conformation, the *S* enantiomer of FPMPA corresponds to the *R* enantiomer of PMPA, and vice versa, the *R* enantiomer of FPMPA corresponds to the *S* enantiomer of PMPA. For the PMPDAP and PMPG derivatives, the differences in antiviral activity between the *R* and *S* enantiomers were less than 2.5-fold (Table 1). (*R*)-PMPA, (*S*)-FPMPA, and (*R*)- and (*S*)-PMPDAP had the highest selectivity indices (>77 to ≥143).

Within the HPMP series (*S*)-HPMPG showed the highest activity against visna virus CPE (EC_{50}, 0.2 μM). (*S*)-HPMPC was the least active, with an EC_{50} of 2.7 μM, whereas (*S*)-HPMPA and (*S*)-HPMPDAP showed EC_{50}s of 0.7 to 1.8 μM. Because of the virtual lack of cytotoxicity of (*S*)-HPMPDAP, it had the highest therapeutic index among the HPMP derivatives (selectivity index, ≥55). Its selectivity index was similar to or even surpassed that of the ANP prototype compound PMEA (selectivity index, 55) (Table 1).

Most of the ANPs caused little, if any, microscopically visible alteration in the confluent SCP cell monolayers at the highest concentrations tested. Whereas (*S*)-PMPA, (*S*)-FPMPA, (*S*)-HPMPA, and (*S*)-HPMPC caused a slight clumping of the cells, (*S*)-PMPG and (*S*)-HPMPG effected a marked loss of the cells (data not shown). The inhibitory effect of the compounds on SCP cell proliferation varied markedly from one compound to another, with an IC_{50} of about 1 μM; (*S*)-PMPG was the most cytostatic; this was followed by (*S*)-HPMPG (IC_{50}, 10 μM), PMEDAP (IC_{50}, 10 μM), (*R*)-PMPG (IC_{50}, 20 μM), and (*S*)-HPMPA (IC_{50}, 30 μM). Most other compounds, i.e., (*R*)- and (*S*)-PMPA, (*R*)- and (*S*)-PMPDAP, and (*R*)- and (*S*)-FPMPA had no inhibitory effect at 100 μM (Table 1).

Inhibition of visna virus yield. Several test compounds were examined for their effects on visna virus replication as measured by titration of visna virus yield after 6 days of incubation of virus-infected SCP cell cultures with various concentrations of the test compounds. The effects on virus yield correlated well with the anti-CPE activities of the compounds. ddCyd was the most active, whereas AZT, (*S*)-PMPA, and (*S*)-HPMPC were the least active inhibitors of visna virus replication, on the basis of virus yields. However, it is noteworthy that the reduction in virus yield as a function of compound concentration varied considerably among the test compounds evaluated (data not shown). The IC_{90}s of the compounds were as follows: PMEA, 0.33 μM; PMEDAP, 0.26 μM; (*S*)-PMPA, 8.4 μM; (*R*)-PMPA, 0.35 μM; (*S*)-PMPDAP, 1.6 μM; (*R*)-PMPDAP, 0.5 μM; (*S*)-FPMPA, 0.14 μM; (*S*)-HPMPA, 0.2 μM; (*S*)-HPMPC, 2.0 μM; ddCyd, 0.03 μM; AZT, 20 μM.

Inhibitory effects of AZT-TP, D4T-TP, and ddCyd-TP on visna virus RT. AZT and D4T were only slightly active against visna virus replication and viral CPE in SCP cell cultures, whereas ddCyd showed a pronounced inhibitory activity. However, when the inhibitory effects of AZT-TP, D4T-TP, and ddCyd-TP-TP on visna virus particle-derived RT were examined, AZT-TP and D4T-TP displayed a marked inhibitory activity against RT, with IC_{50}s of 0.015 to 0.081 μM, respectively. There were no marked differences in the inhibitory effect of AZT-TP against visna virus particle-derived RT when the endogenous visna RNA template or the artificial template and primer poly(A) · oligo(dT) were used in the RT reaction mixture. In contrast, with the same primers, ddCyd-TP inhibited the enzyme by 50% at a concentration of 9 μM in the presence of poly(I) · oligo(dC) as the template and primer, a concentration that was very similar to the inhibitory activity of PFA that was evaluated against the enzyme in the presence of endogenous template (data not shown).

Inhibition of maedi virus CPE. A few ANPs [i.e., PMEA, (*R*)-PMPA, (*S*)-FPMPA, and (*S*)-HPMPC] and the ddNs ddCyd and ddDAPR were evaluated in the CPE inhibition assay against strains M88, M34, and GPPV of maedi virus. The EC_{50}s for the maedi virus strains were within the range values observed for visna virus strain K796.

DISCUSSION

The inhibitory activities of ddCyd, ddAdo, and ddGuo have previously been determined against visna virus (14) and are in full agreement with the data obtained under our experimental conditions. However, in contrast to the present study, which consistently revealed that ddCyd was the most active compound, ddGuo was found to be the most active against visna virus replication in the previous study (14).

AZT-TP and D4T-TP proved to be as inhibitory against the visna virus-associated RT activity as against HIV RT activity (2). While ddCyd was the most active compound against visna virus replication, ddCyd-TP was much less inhibitory to visna virus RT than were AZT-TP and D4T-TP.

These data are different from those of Frank et al. (14), who reported that ddCyd-TP is almost equally inhibitory to visna virus RT as AZT-TP is. In the studies of Frank et al. (14), an mRNA primer was used, while in our studies, an artificial template and primer [poly(I) · oligo(dC)] were used, and this may at least partially explain the different sensitivities of RT to the inhibitory effects of ddCyd-TP. However, we assume that the low levels of activity of AZT and D4T against visna virus in SCP cells were due to the inability of the cells to efficiently phosphorylate these compounds. Also, it should be pointed out that the cells' conditions (essentially stationary-phase cells) used in our experiments for measuring anti-maedi/visna virus activity were quite different from those used for monitoring anti-HIV activity, in which actively dividing cells were infected with the virus. Thus, the SCP cell monolayers may have little ability to phosphorylate thymidine derivatives.

No direct metabolic studies were carried out with AZT, D4T, or ddCyd in sheep cell monolayers to prove this hypothesis. However, Dahlberg and coworkers (7) showed that in caprine Himalayan TAHR ovary cells, AZT and 2',3'-dideoxythymidine lack activity against caprine arthritis and encephalitis virus (CAEV) replication, whereas other ddNs (i.e., ddCyd and ddAdo) were exquisitely inhibitory to CAEV. These observations may be interpreted to mean that either (i) different retroviruses (i.e., CAEV and HIV) differ in their susceptibilities to the ddNs or (ii) variations in drug susceptibility among different retroviruses may result from differences in the cells' conditions. The fact that AZT was also barely active against amphoteric murine leukemia virus infection in TAHR ovary cells but was markedly effective against amphoteric murine leukemia virus and equine infectious anemia virus (EIAV) in equine dermis cells points to cellular determinants as the crucial factor in the antiviral activity of the ddN derivatives.

Differences in antiviral activity of the ddNs in different cell systems may depend on the affinities of the ddNs for the kinases that are involved in the metabolic conversion of the compounds to their 5'-triphosphate forms. Also, nucleoside and nucleotide pool sizes may be important since they are expected to influence the eventual inhibition of the retroviral RT by the ddN 5'-triphosphates. We demonstrated that AZT is very poorly phosphorylated to the 5'-triphosphate derivative in TAHR ovary cells, while ddCyd is extensively converted to ddCyd-TP (data not shown). Because of the close species relationship between caprine and sheep cell lines, the data obtained in the caprine cells can most likely be extrapolated to SCP cells.

For the PMP and FPMP derivatives, there was a better correlation between anti-HIV activity (1, 3, 4–6, 22) and anti-visna virus activity than for the ddN derivatives. These ANPs are assumed to interact with their target enzyme (i.e., RT) after phosphorylation to their diphosphate forms. The wide spectrum of retrovirus-cell systems in which the ANPs (in particular, PMEA) have been found to be active also seems to extend to the maedi/visna virus-SCP system. However, in striking contrast to HIV, the replication of visna virus could be efficiently inhibited by several HPMP derivatives [i.e., (*S*)-HPMPA, (*S*)-HPMPDAP, and (*S*)-HPMPG]. In fact, a slight but significant antiviral activity has been reported earlier for several HPMP derivatives against Moloney murine sarcoma virus-induced transformation of C3H/3T3 cells (4). Even (*S*)-HPMPC, which was never found to be inhibitory in any in vitro retrovirus model evaluated so far, showed anti-visna virus activity (EC_{50}, 2.7 μM), which is at a concentration that is two orders of magnitude lower than its toxicity threshold. It is not fully understood why (*S*)-HPMPA, whose diphosphate derivative (*S*)-HPMPApp has an inhibitory effect on HIV type 1 RT, does not show pronounced anti-HIV type 1 activity in vitro, whereas it is endowed with pronounced antiviral activity against visna virus. Therefore, the possibility of a second target for the antiviral activity of these compounds cannot be excluded.

PMEA, (*R*)-PMPA, and (*R*)-PMPDAP exerted marked inhibitory effects on visna virus replication in vitro and should be considered prime candidate compounds to be pursued further in vivo, i.e., in lambs with maedi/visna virus infection. Results of such studies should be instructive as to the potential of these compounds to affect retrovirus-related neurological disorders.

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REFERENCES

1. **Balzarini, J., and E. De Clercq.** Acyclic purine nucleoside phosphonates as retrovirus inhibitors. *In* J. Jeffries and E. De Clercq (ed.), Antiviral chemotherapy, in press. John Wiley & Sons, Chichester, Great Britain.
2. **Balzarini, J., P. Herdewi jn, and E. De Clercq.** 1989. Differential patterns of intracellular metabolism of 2',3'-didehydro 2',3'-dideoxythymidine and 3'-azido-2',3'-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. J. Biol. Chem. **264**:6127–6133.
3. **Balzarini, J., A. Holy, J. Jindrich, H. Dvorakova, Z. Hao, R. Snoeck, P. Herdewi jn, D. G. Johns, and E. De Clercq.** 1991. 9-[(2*RS*)-3-Fluoro-2-phosphonylmethoxypropyl] derivatives of purines: a class of highly selective antiretroviral agents *in vitro* and *in vivo*. Proc. Natl. Acad. Sci. USA **88**:4961–4965.
4. **Balzarini, J., A. Holy, J. Jindrich, L. Naesens, R. Snoeck, D. Schols, A. Holy, and E. De Clercq.** 1993. Differential antiherpesvirus and antiretrovirus effects of the (*S*) and (*R*) enantiomers of acyclic nucleoside phosphonates: potent and selective in vitro and in vivo antiretrovirus activities of (*R*)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine. Antimicrob. Agents Chemother. **37**:332–338.
5. **Balzarini, J., L. Naesens, P. Herdewi jn, I. Rosenberg, A. Holy, R. Pauwels, M. Baba, D. G. Johns, and E. De Clercq.** 1989. Marked *in vivo* antiretrovirus activity of 9-(2-phosphonylmethoxyethyl)adenine, a selective anti-human immunodeficiency virus agent. Proc. Natl. Acad. Sci. USA **86**:332–336.
6. **Balzarini, J., L. Naesens, J. Slachmuylders, H. Niphuis, I. Rosenberg, A. Holy, H. Schellekens, and E. De Clercq.** 1991. 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) effectively inhibits Simian immunodeficiency virus (SIV) infection in Rhesus monkeys. AIDS **5**:21–28.
7. **Dahlberg, J. E., H. Mitsuya, S. B. Blam, S. Broder, and S. A. Aaronson.** 1987. Broad spectrum antiretroviral activity of 2',3'-dideoxynucleosides. Proc. Natl. Acad. Sci. USA **84**:2469–2473.
8. **Debyser, Z., A.-M. Vandamme, R. Pauwels, M. Baba, J. Desmyter, and E. De Clercq.** 1992. Kinetics of inhibition of endogenous HIV-1 reverse transcription by ddNTP, TIBO and HEPT derivatives. J. Biol. Chem. **267**:11769–11776.
9. **De Clercq, E.** 1989. New acquisitions in the development of anti-HIV agents. Antiviral Res. **12**:1–20.
10. **De Clercq, E.** 1991. Broad-spectrum anti-DNA virus and antiretrovirus activity of phosphonylmethoxyalkylpurines and -py-

rimidines. Biochem. Pharmacol. **42:**963–972.
11. **Duckworth, D. M., M. R. Harnden, R. M. Perkins, and D. N. Planterose.** 1991. Antiviral 9-[2-(phosphonomethoxy)alkoxy]purines. Antiviral Chem. Chemother. **2:**229–241.
12. **Duckworth, D. M., M. R. Harnden, and D. N. Planterose.** 1991. 9-(2-Phosphonylmethoxy)alkoxy purines, a new series of antiviral acyclonucleotides. Nucleosides Nucleotides **10:**427–430.
13. **Egberink, H., M. Borst, H. Niphuis, J. Balzarini, H. Neu, H. Schellekens, E. De Clercq, M. Horzinek, and M. Koolen.** 1990. Suppression of feline immunodeficiency virus infection *in vivo* by 9-(2-phosphonomethoxyethyl)adenine. Proc. Natl. Acad. Sci. USA **87:**3087–3091.
14. **Frank, K. B., P. A. McKernan, R. A. Smith, and D. F. Smee.** 1987. Visna virus as an in vitro model for human immunodeficiency virus and inhibition by ribavirin, phosphonoformate, and 2′,3′-dideoxynucleosides. Antimicrob. Agents Chemother. **31:** 1369–1374.
15. **Hartmann, K., A. Donath, B. Beer, H. F. Egberink, M. C. Horzinek, H. Lutz, G. Hoffmann-Fezer, I. Thum, and S. Thefeld.** 1992. Use of two virustatica (AZT, PMEA) in the treatment of FIV and FeLV seropositive cats with clinical symptoms. Vet. Immunol. Immunopathol. **35:**167–175.
16. **Holy, A.** 1993. Syntheses of enantiomeric *N*-(3-hydroxy-2-phosphonomethoxypropyl) derivatives of purine and pyrimidine bases. Collect. Czech. Chem. Commun. **58:**649–674.
17. **Holy, A., and I. Rosenberg.** 1987. Synthesis of 9-(2-phosphonylmethoxyethyl)adenine and related compounds. Collect. Czech. Chem. Commun. **52:**2801–2809.
18. **Holy, A., and I. Rosenberg.** 1987. Synthesis of isomeric and enantiomeric *O*-phosphonylmethyl derivatives of 9-(2,3-dihydroxypropyl)adenine. Collect. Czech. Chem. Commun. **52:** 2775–2791.
19. **Holy, A., I. Votruba, A. Merta, J. Cerny, J. Vesely, J. Vlach, K. Sediva, I. Rosenberg, M. Otmar, H. Hrebabecky, M. Travnicek, V. Vonka, R. Snoeck, and E. De Clercq.** 1990. Acyclic nucleotide analogues: synthesis, antiviral activity and inhibitory effects on some cellular and virus-encoded enzymes in vitro. Antiviral Res. **13:**295–312.
20. **Lin, F. H., and H. Thormar.** 1970. Ribonucleic acid-dependent deoxyribonucleic acid polymerase in visna virus. J. Virol. **6:**702–704.
21. **North, T. W., G. T. North, and N. C. Pedersen.** 1989. Feline immunodeficiency virus, a model for reverse transcriptase-targeted chemotherapy for acquired immune deficiency syndrome. Antimicrob. Agents Chemother. **33:**915–919.
22. **Pauwels, R., J. Balzarini, D. Schols, M. Baba, J. Desmyter, I. Rosenberg, A. Holy, and E. De Clercq.** 1988. Phosphonylmethoxyethyl purine derivatives: a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. **32:**1025–1030.
23. **Reed, L. H., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
24. **Sundquist, B., and E. Larner.** 1977. Phosphonoformate inhibition of visna virus replication. J. Virol. **30:**847–851.
25. **Thormar, H.** 1965. Physical, chemical and biological properties of visna virus and its relationship to other animal viruses, p. 335–340. *In* D. C. Gajdusek, C. J. Gibbs, Jr., and M. Alpers (ed.), Slow, latent and temperate virus infections. NINDB Monograph No. 2. U.S. Public Health Service Publications, Washington, D.C.