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New arylpyrido-diazepine and -thiodiazepine derivatives are potent and highly selective HIV-1 inhibitors targeted at the reverse transcriptase

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Abstract

A series of pyridobenzothiodiazepindioxides such as the 11-ethyl-6,8,9-trimethyl-6,11-dihydro-pyrido[2,3-f][2,1,5]benzothiodiazepine-5,5-dioxide and arylpiridodiazepines such as the 6,7-dihydro-7-methyl-12-ethyl-pyrido[2,3-b]pyrido(2′,3′-4,5]furo[2,3-f][1,4]diazepin-6(12H)-thio and the 6,7-dihydro-7-methyl-12-ethyl-pyrido[2,3-b]pyrido- [2,3-4,5]thieno[2,3-f][1,4]diazepin-6(12H)-thione were found to inhibit human immunodeficiency virus type 1 [HIV-1(III_B)] replication at a concentration of 0.003–0.04 μM without being cytotoxic at a 3000- to 15000-fold higher concentration. These compounds proved effective against a variety of HIV-1 strains, including those that are resistant to 3′-azido-3′ deoxythymidine (AZT), but not against HIV-2, simian immunodeficiency virus or herpes simplex virus. An HIV-1 strain containing the 188 Tyr → His mutation in the reverse transcriptase displayed severely reduced sensitivity to the compounds. The specificity of these compounds is due to an interaction with the reverse transcription process. The 6,7-dihydro-7-methyl-12-ethyl-pyrido[2,3-b]pyrido [2,3-4,5]thieno[2,3-f][1,4]diazepin-6(12H)-thione (MEN 10979) enhanced the anti-HIV-1 activity of AZT and dideoxyinosine (ddI) in a synergistic manner. The new arylpyrido-diazepine and -thiodiazepine derivatives appear to be drug candidates for the treatment of HIV-1 infection.

Keywords: HIV; Non-nucleoside reverse transcriptase inhibitors

1. Introduction

During the past several years, several compounds have been found to be effective, in vitro,

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as inhibitors of human immunodeficiency viruses type 1 (HIV-1) and 2 (HIV-2). Among these there are the reverse transcriptase (RT) inhibitors which can be divided into two groups, according to their structure and action on the viral polymerase (for a review see De Clercq, 1992; De Clercq, 1994).

The first group includes: 2′,3′-dideoxynucleosides (ddNs), such as 3′-azido-3′-deoxythymidine (AZT) (Mitsuya et al., 1985), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI) (Mitsuya and Broder, 1986), 2′,3′-didehydro-2′,3′-dideoxynucleosides (D4C, D4T) (Balzarini et al., 1987; Hamamoto et al., 1987; Lin et al., 1987); and acyclic nucleosides phosphonates (ANPs), such as the 9-(2-phosphonylmethoxyethyl)adenine (PMEA), 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (FPMPA) and 9-(2-phosphonylmethoxypropyl)adenine (PMPA) (Pauwels et al., 1988a; De Clercq, 1991; Balzarini et al., 1991). These compounds specifically interact with the catalytic site of the viral polymerase, acting as competitive inhibitors with respect to the natural substrates.

The ddNs and the ANPs, after their intracellular conversion to the active 5′-triphosphate and 5′-diphosphate forms, are incorporated into the growing viral DNA, causing chain termination (Furman et al., 1986; Cheng et al., 1987). Furthermore, they show a significantly higher affinity for HIV-1 RT, HIV-2 RT and other retroviral RTs than for the cellular DNA polymerases (Vrang et al., 1988; Ono et al., 1989; Konig et al., 1989).

Another group of HIV RT inhibitors is represented by the non-nucleoside analogues (Baba et al., 1992). Their antiviral activity is specific for HIV-1 with no effect on HIV-2 or other retroviruses. They include, among several others, the tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2-(1H)-one and -thione (TIBO) derivatives (Pauwels et al., 1990; Debyser et al., 1991), nevirapine (BI-RG-587) (Merluzzi et al., 1990), the pyridinones L-697,639 and L-697,661 (Goldman et al., 1991), the bis[heteroaryl]piperazines (BHAPs) (Romero et al., 1991), the α-anilinophenylacetamide (α-APA) derivatives and new TIBO derivatives (Pauwels et al., 1993; Pauwels et al., 1994). The TIBO/nevirapine class of compounds interact with a specific allosteric site of HIV-1 RT, a 'pocket' that is close but distinct from the catalytic site where the primer-template, substrates (dNTPs) and ddN 5′-triphosphates (ddNTPs) bind (Wu et al., 1991; Kohlstaedt et al., 1992; Tantillo et al., 1994).

Another class of non-nucleoside RT inhibitors, the [1-(2-hydroxyethoxy)methyl]-6-(phenylthio)-thymine (HEPT) (Baba et al., 1989) and [2′,5′-bis-O-(tert-butyldimethyl-silyl)-3-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO) derivatives (Balzarini et al., 1992), are chemically unrelated to the TIBO/nevirapine class of compounds, yet bind to the same 'pocket' of the viral enzyme. They also show activity against HIV-1 only.

All these non-nucleoside inhibitors act as non-competitive inhibitors of HIV-1 RT, without need for previous intracellular conversion to an active metabolite (Debyser et al., 1992a; Debyser et al., 1992b).

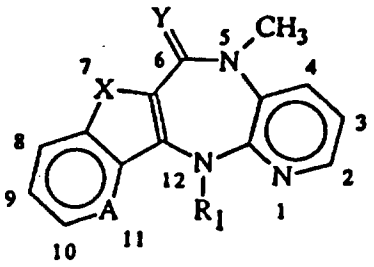
As all the non-nucleoside reverse transcriptase inhibitors (NNRTIs) have a selectivity index higher than nucleoside analogs, they appear to be interesting compounds for the therapy of HIV-1 infection. However, it has been shown that drug resistance develops rapidly (within 2–3 weeks), and this may limit their clinical usefulness as single agents (Nunberg et al., 1991; Richman et al., 1991; Mellors et al., 1992). Their role in combination therapy or in short-term application (such as post-exposure prophylaxis and prevention of fetal infection) is being considered.

Here we report on the characterisation of a new group of NNRTIs, MEN 10690, MEN 10880, and MEN 10979 (Fig. 1a and b). They specifically inhibit HIV-1 replication at nanomolar concentrations, which are 3 000- to 15 000-fold lower then their cytotoxic concentrations.

2. Materials and methods

2.1. Chemistry

The synthesis of the pyridobenzothiodiazepin-dioxides such as the 11-ethyl-6,8,9,-trimethyl-6,11-dihydro-pyrido[2,3-f][2,1,5] benzothiodiazepine-5, 5-dioxide, here called MEN 10690, and related



COMPOUND	X	Y	A	R_1	$IC_{50}(\mu M)^a$	$CC_{50}(\mu M)^b$	SI[c]
MEN 10610	O	O	=CH	C_2H_5	1.6	80	50
MEN 10992	N-C_2H_5	O	=CH	C_2H_5	0.04	25	625
MEN 10977	S	O	N	C_2H_5	0.04	73	1,825
MEN 10789	O	O	N	C_2H_5	0.02	175	8,750
MEN 10880	O	S	N	C_2H_5	0.006	>60	>10,000
MEN 10979	S	S	N	C_2H_5	0.0025	38	15,200
AZT					0.004	1,400	350,000
ddI					1	9,000	9,000
R82913					0.0015	34	22,667
Ro31-8959					0.0016	90	56,250

[a] 50 % inhibitory concentration, based on the inhibition of cytopathicity of HIV-1/IIIB in C8166 cells.
[b] 50 % cytotoxic concentration, based on the reduction of viability of mock-infected C8166 cells.
[c] selectivity index, or ratio of CC_{50} to IC_{50}.

All data represent mean values for at least three experiments. At an average, the upper and lower limit of SD ranged between 0.01 and 2.5 times the median 50 % values.

Fig. 1. (a) Structure-activitity relationship of MEN compounds for inhibition of HIV-1 replication and cytotoxicity in C8166 cells.

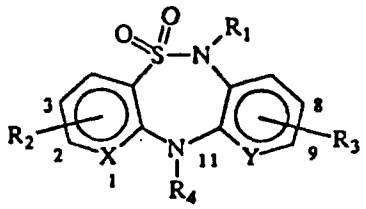
compounds are described elsewhere (Giannotti et al., 1995).

The arylpiridodiazepines such as the 6,7-dihydro-7-methyl-12-ethyl-pyrido[2,3-b]pyrido[2′,3′4,5]furo[2,3-f][14]diazepin-6(12H)-thio, MEN 10880, the 6,7-dihydro-7-methyl-12-ethyl-pyrido[2,3-b]-pyrido[2,3-4,5]thieno[2,3-f][1,4]diazepin-6(12H)-thione, MEN 10979, and related compounds, where obtained starting from the unsubstituted parent tetracyclic structure (Viti et al., 1995) by alkylation on the nitrogen in position 12 and subsequent thiation with Lawessons's reagent according to the standard procedures (Hargrave et al., 1991).

The tested compounds were dissolved in DMSO, aliquoted and stored at −20°C. AZT and dextran sulfate were purchased from Sigma Chemical Co.; Ro31-8959 and ddI were kindly provided by Roche Products Ltd. and Bristol Myers Squibb, respectively.

2.2. Viruses and cells

We used the following virus strains: HTLV-IIIB [named here HIV-1(IIIB)] (Popovic et al., 1984), obtained from the supernatants of chronically



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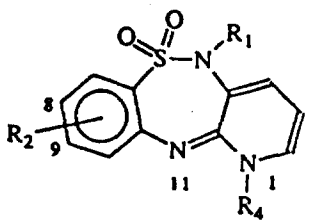

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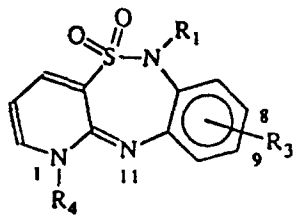

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COMPOUND	X	Y	R_1	R_2	R_3	R_4	$IC_{50}(\mu M)^a$	$CC_{50}(\mu M)^b$	SI[c]
MEN 10704 (A)	N	N	CH_3	H	H	C_2H_5	>300	300	<1
MEN 10597 (B)			CH_3	H		C_2H_5	3.7	80	22
MEN 10683 (A)	N	=CH	CH_3	H	8,9-CH_3	H	4	>125	>31
MEN 11016 (B)			CH_3	8-CH_3,9-Cl			0.28	12	43
MEN 11009 (C)			CH_3	H	8,9-CH_3	CH-$(CH_3)_2$	0.6	>30	>50
MEN 11017 (C)			CH_3	H	8,9-CH_3	C_2H_5	0.33	>30	>90
MEN 11006 (A)	N	=CH	CH_3	H	8,9-CH_3	CH-$(CH_3)_2$	0.032	>60	>1.875
MEN 10690 (A)	N	=CH	CH_3	H	8,9-CH_3	C_2H_5	0.04	150	3.750
NEVIRAPINE[d]							0.04	320	8000

[a] 50 % inhibitory concentration, based on the inhibition of cytopathicity of HIV-1/IIIB in C8166 cells.
[b] 50 % cytotoxic concentration, based on the reduction of viability of mock-infected C8166 cells.
[c] selectivity index, or ratio of CC_{50} to IC_{50}.
[d] Data taken from Baba *et al.* 1992.

All data represent mean values for at least three experiments. At an average, the upper and lower limit of SD ranged between 0.01 and 2.5 times the median 50 % values.

Fig. 1(b) Structure-activitity relationship of MEN compounds for inhibition of HIV-1 replication and cyto-toxicity in C8166 cells.

infected H9 cells (R.C. Gallo, NIH, Bethesda USA); HIV-1(PNL43) (Dr. M. Martin, AIDS Research and Reference Program, NIAID, NIH, Bethesda, MD), which displayed, in our hands, an intermediate degree of AZT-resistance; HIV-1(MN) (NIAID, AIDS Research and Reference Reagent Program); a strain with a V3-loop typical of Europe/USA, HIV-1(RF) (Popovic et al., 1984); HIV-1(NDK) (Spire et al., 1989); HIV-1(HE) (Witvrouw et al., 1991); HIV-2(ROD) (Barré-Sinoussi et al., 1983) (L. Montagnier, Pasteur Institute, Paris, France); SIV(MAC_{251}) (Franchini et al., 1987). The HIV-1 strains named R14 and $IIIB_{TIBO}$, were AZT- and TIBO-resistant strains, selected in the Institute of Virology, Rome, Italy by serial passages of HIV(IIIB) on C8166 cells with increasing drug concentrations (Antonelli et al., 1994); R14 was more than 500-fold less sensitive to AZT, while $IIIB_{TIBO}$ was 100-fold less sensitive to TIBO R82913, as compared to the wild-type. An isolate called HIV_{ID} was derived from a lymphnode biopsy of a patient treated with AZT for more than 1 year and showed a high degree of resistance to AZT ($IC_{50} > 5\mu M$). The HIV-1 mutant strains 13MB1, 3B103, 39MH1, 39MN1 and 3B188 were selected in the Rega Institute, Leuven, Belgium as described (Pauwels et al., 1993; and unpublished

data). Antiviral assays with HSV-1(HF) were performed on Hep2 cells, a line from a human squamous carcinoma. All virus strains were aliquoted and stored at −80°C.

Acute infections with HIV-1, HIV-2 and SIV were carried out in $CD4^+$ lymphoblastoid cell lines, C8166, (Clapham et al., 1987) and MT-4 (Miyoshi et al., 1982). These cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 0.1% sodium bicarbonate and 20–50 μg/ml gentamicin.

Density gradient centrifugation (Ficoll Hypaque, Pharmacia, LKB Biotechnology AB, Uppsala, Sweden) was used to separate peripheral blood mononuclear cells (PBMC) from venous blood of healthy human donors.

2.3. Virus titration

Titrations were performed in C8166 cells by the standard limiting dilution method in 96-well microtiter plates. Infectious titer was determined as described (Dianzani et al., 1988; Dianzani et al., 1989). Infectious titer, expressed as $CCID_{50}$/ml, was calculated by the method of Reed and Muench (Davis et al., 1980).

2.4. Assay of drug sensitivity

C8166 (5×10^5 cells/ml) were infected with HIV-IIIB at low multiplicity of infection (MOI 0.01–0.001); after 1 h at 37°C, cells were washed three times, resuspended in RPMI 1640 containing 10% FCS and distributed into 96-wells plates containing several dilutions of the test compounds. At 72 h later, the cells were harvested, subjected to three cycles of freeze-thawing, cell debris were removed and viral replication was assayed in the supernatants by infectivity titration and HIV-1 total antigen detection (see below). In cells infected with HIV-2 and SIV, viral replication was measured only by infectivity titration.

MT-4 cells (3×10^5 cells/ml) were infected with HIV at low multiplicity of infection (MOI 0.01–0.001). Immediately after infection, 100 ul of the cell suspension were brought into each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After 5 days incubation at 37°C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels et al., 1988b).

PBMC were activated with PHA (Murex Diagnostics Limited, Dartford, England) for 72 h, and then infected with a concentrated stock of HIV (IIIB) (MOI: 0.1) for 90 min at 37°C, resuspended at 10^6 cells/ml in RPMI 1640 containing 10% FCS, gentamicin 50μg/ml and IL-2 (100U/ml) and distributed into 96-well plates containing the drugs to be tested. After 8 days of incubation at 37°C, the culture was terminated and the supernatants were tested for HIV-1 total antigen production.

2.5. Antigen detection

Assessment of HIV-1 total antigen levels in samples was carried out by ELISA (HIVAG-1, Abbott Laboratories, Chicago, IL), according to the Manufacturer's instructions.

2.6. Drug cytotoxicity

Cytotoxicity induced by drugs added to C8166, MT-4 and PBMC cultures was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-based method (Mosmann, 1983; Pauwels et al., 1988b).

2.7. Time of addition experiments

To determine where the drug interferes with the replicative cycle of HIV, C8166 cells were infected at a high multiplicity of infection (MOI: 0.1–1) with HIV(IIIB). After 1 h incubation at 37°C, unabsorbed virus was removed by three washing steps. Compounds were then added to parallel cultures in microtiter plates at different time points post infection (p.i.) at concentrations about 100-fold higher than their IC_{50} values. Viral infectivity was determined at 24 h p.i. by titration on C8166 cells.

2.8. RT assays

In the exogenous RT assay, a homopolymeric template was used. The reaction mixture (50 μl) contained 50 mM Tris. HCl pH 7.8, 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM dithiothreitol, 1mM EDTA and 0.25% NP40. The assay was performed as described by Debyser et al. (1991). The recombinant HIV-1 RT p66/p51 was purchased from Boehringer Mannheim Italia and used at a concentration of 24 ng/ml with poly(rA)-oligo(dT) and of 70 ng/ml with poly(rC)-oligo(dG). HIV-2(ROD) RT was isolated from infected C8166 cells supernatants by precipitation with PEG and subsequent lysis of virus particles. Incorporation of labelled substrates was determined by transferring RT reaction mixture (10 ul) onto DE81 paper and counting tritium on a scintillation counter. In the endogenous RT assay, a 16 S/23 S E. Coli ribosomal RNA (Boehringer Mannheim Biochemical) functioning as template was used. The reaction mixture (50 μl) consisted of 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 4mM beta-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.2% DMSO, 3% glycerol, 1 mM EDTA, 6.66 μg/ml of ribosomal RNA-primer (equivalent to 15 nM -3′-hydroxyl primers annealed to RNA), 50 μM dATP, 50 μM dGTP, 50 μM dCTP and 2 μM of tritium-labelled dTTP (Amersham International plc, specific activity 45 Ci/mmol). The recombinant HIV-1 RT p66/p51 was used at a concentration of 200 ng/ml. The assay was performed as described by Buckeit et al. (1993). The samples were precipitated with 10% TCA and processed as described by Ho et al. (1984).

2.9. Detection of HIV-1 DNA by PCR

The C8166 cells were infected at high MOI with HIV(IIIB) (MOI > 1) and then resuspended in fresh RPMI at 8×10^5 cells per ml and plated in a 96-well plate. The antiviral compounds (at different concentrations in RPMI medium) were added to the cells. After 20 hours of incubation, 2×10^5 cells for each condition were pelleted, washed twice in RPMI medium and finally resuspended in a lysis buffer containing proteinase K (0.6 mg/ml). The samples were heated at 56°C for 60 min and then at 94°C for 15 min to inactivate the proteinase K. In order to perform a semiquantitative PCR experiment, each cellular lysate was diluted from 10^{-1} to 10^{-5} and then 25 μl of each dilution were examined for HIV-1 DNA by PCR, as described in Dianzani et al. (1992).

3. Results

3.1. Screening and structure-activity relationship

Molecules belonging to the benzodiazepine family, that had been previously synthesized by Menarini Farmaceutici as potential CNS agents, were assayed for cytotoxicity and, when applicable, for anti-HIV-1 activity as described in the Materials and methods section.

As shown in Fig. 1a and b, two 'lead' compounds with different molecular structures were active against HIV-1: MEN 10610, a pyridobenzofurodiazepinone, and MEN 10597, a pyridobenzothiodiazepinedioxide.

However, these compounds display only moderate antiviral activity. Thus, new compounds were synthesized to improve the anti-HIV activity and to obtain insight in the structure-activity relationship. As to the MEN 10610 derivatives (Fig. 1a), we found that a significant (175-fold) increase in antiviral activity was obtained if a pyridine, instead of benzene, ring was condensed with the furane (MEN 10789). We also observed that the substitution of sulfur for the oxygen at position 7 gave only a 40-fold increase of antiviral activity (MEN 10977), while thiation of the central diazepine ring resulted in a greater increase of the selectivity index (SI) (MEN 10880).

Moreover, it was established that the presence of a short alkyl chain in positions 5 and 12 is essential for the activity. MEN derivatives, here not shown, that did not contain these substituents, were inactive.

Finally the presence of sulfur atoms at positions 6 and 7 of the molecular structure resulted in a compound (MEN 10979), which showed both increased anti-HIV efficacy and cytotoxicity.

Fig. 1b shows the anti-HIV-1 activity of MEN 10597-derivatives. A higher inhibitory effect was shown by compounds that were alkylated on the nitrogen at position 11 (formula A), with only 1 pyridine ring and two methyl groups at positions 8,9 (MEN 11006 and MEN 10690). MEN 10690 was the most effective compound of this series (SI: 3,700), with an antiviral activity comparable to nevirapine (IC_{50}: 0.04 μM).

Isomeric derivatives, alkylated on the nitrogen at position 1 (formula B and C), were less active (MEN 10597, MEN 11016, MEN 11017, MEN 11009). In Fig. 1b we also list an inactive compound, MEN 10704: it is interesting to note that even if its molecular structure was the most similar to that of nevirapine, MEN 10704 was completely inactive. Thus, the presence of two pyridine rings cannot be considered sufficient to generate an active compound.

3.2. Spectrum of antiviral activity

Further experiments were performed to evaluate and characterize the antiviral activity of the more interesting compounds: namely MEN 10690, MEN 10880 and MEN 10979. The inhibitory potency of these compounds against HIV-1(IIIB) varied slightly depending on the cell type (Table 1).

MEN 10979 showed a potent antiviral activity against HIV(IIIB) in C8166 and MT-4 cells, with a SI of 15 000 and 11 400, respectively. These values were comparable to those of TIBO R82913 in these cell systems. In PBMC, MEN 10979 showed a SI value of 3,100. MEN 10880 was more active against HIV-1 in C8166 cells than in MT-4 or PBMC. MEN 10690 showed lower anti-HIV-1 activity than MEN 10880 and MEN 10979, in both lymphoblastoid $CD4^+$ cell lines, but its potency remained unchanged in PBMC (IC_{50}: 0.03μM).

The anti-HIV activity of these compounds was also dependent on the virus type (Table 1). The activity of all compounds against the HIV-1 strains MN, RF, NDK and HE were comparable to those observed with the HIV-1 strain IIIB in MT-4 cells. As for TIBO and the other non-nucleoside inhibitors, all MEN compounds are inactive against HIV-2(ROD), SIV(MAC_{251}) (Table 1) and HSV-1(HF) replication (data not shown).

To further investigate the spectrum of activity of MEN 10690 and MEN 10979 and their possible cross-reactivity with other non-nucleoside inhibitors, HIV-1 strains resistant to AZT (R14 and PNL43) or TIBO R82913 ($IIIB_{TIBO}$) were assayed for their susceptibility to all MEN compounds. In addition, the compounds were tested against a virus isolate, called HIV_{ID}, derived from a lymphnode biopsy of a patient treated with AZT for more than 1 year and displaying a high degree of resistance to AZT. The results of these experiments are shown in Table 2. MEN 10690 was fully active against PNL43 (IC_{50}: 0.15μM), while MEN 10979 and TIBO showed a 4.5-fold increase in their IC_{50} value. The AZT-resistant strain R14 was more than 500-fold resistant to AZT (IC_{50} > 1 μM); when assayed against this virus, MEN 10979 showed an IC_{50} of 0.16 μM, thus demonstrating activity also against this AZT-resistant strain. MEN 10690 and MEN 10979 showed a 2.5- and 32-fold increase in their IC_{50} against TIBO-resistant strain, thus demonstrating only limited cross-resistance with benzodiazepin-thione derivatives (particularly MEN 10979). It is worth noting that MEN 10690 and MEN 10979 also efficiently inhibited the replication of an isolate from lymphnodal PBMC of an AIDS patient, displaying high AZT-resistance.

We were able to select rapidly (upon 6/7 passages of HIV(IIIB) in the presence of increasing drug concentrations) in C8166 cells MEN 10690- and MEN 10979-resistant strains (called IIIB/MEN 10690 and IIIB/MEN 10979): IIIB/MEN 10690 showed an IC_{50} of 2 μM for MEN 10690, and an IC_{50} of 0.2 μM for MEN 10979 and TIBO R82913 in C8166 cells, thus revealing a notable degree of cross-sensitivity with these compounds (data not shown).

3.3. Sensitivity of different mutant virus strains towards MEN 10690 and MEN 10880

In another set of experiments, we evaluated the sensitivity of different mutant HIV-1 strains towards the inhibitory activity of MEN 10690 and MEN 10880 in MT-4 cells (Table 3). The muta-

Table 1
Antiretroviral activity spectrum of MEN compounds

Drug	Virus	Strain	Cell line	$IC_{50}(\mu M)$[a]	$CC_{50}(\mu M)$[b]	SI[c]
MEN 10690	HIV-1	IIIB	C8166	0.04	150	3750
		IIIB	PBMC	0.03	>50	>1666
		IIIB	MT-4	0.2	>250	>1250
		MN	C8166	0.06	150	2500
		RF	MT-4	0.3	>250	>833
		NDK	MT-4	0.3	>250	>833
		HE	MT-4	0.3	>250	>833
	HIV-2	ROD	C8166	>150	150	<1
MEN 10880	SIV	MAC251	C8166	>150	150	<1
	HIV-1	IIIB	C8166	0.006	>60	>10 000
		IIIB	PBMC	0.03	>60	>1870
		IIIB	MT-4	0.1	>250	>2500
		MN	C8166	0.006	>60	>10 000
		RF	MT-4	0.2	>250	>1250
		NDK	MT-4	0.2	>250	>1250
		HE	MT-4	0.5	>250	>500
	HIV-2	ROD	C8166	>60	>60	<1
	SIV	MAC251	C8166	>60	>60	<1
MEN 10979	HIV-1	IIIB	C8166	0.0025	38	15 200
		IIIB	PBMC	0.01	31	3100
		IIIB	MT-4	0.0035	40	11 428
		MN	C8166	0.0025	38	15 200
	HIV-2	ROD	C8166	>38	38	<1
	SIV	MAC251	C8166	>38	38	<1
TIBO R82913	HIV-1	IIIB	C8166	0.0015	20	13 333
		IIIB	PBMC	0.005	20	4000
		IIIB	MT-4	0.003	30	10 000
		MN	C8166	0.0018	30	16 666
	HIV-2	ROD	C8166	>30	30	<1
	SIV	MAC251	C8166	>30	30	<1

[a]50% inhibitory concentration, based on the inhibition of cytopathicity of HIV-1, HIV-2 or SIV strains in the indicated cell lines.
[b]50% cytotoxic concentration, based on the reduction of viability of mock-infected C8166, PBMC or MT-4 cells.
[c]Selectivity index, or the ratio of CC_{50} to IC_{50}.
All data represent median values of at least three separate experiments. At an average, the upper and lower limit of S.D. ranged between 0.01 and 2.5 times the median 50% values.

tion Y188H gave the highest degree of resistance, whereas the mutation L100I did not affect virus sensitivity, to both compounds. Also, mutations K103N and Y181C led to significant resistance to both compounds. The V106A mutation made the virus much more resistant to MEN 10880 (> 3,000-fold) than MEN 10690 (80-fold).

3.4. Mechanism of action

To elucidate the stage of the HIV-1 replicative cycle that was inhibited by the MEN molecules, we performed time of addition (TOA) experiments (Fig. 2). The results indicate that the compounds, when added at different times after infection of C8166 cells at high multiplicity of infection (MOI > 1), behaved similarly to other RT inhibitors, such as TIBO R82913, AZT and ddI. In fact, when added at 2–4 h post-infection (p.i.), these compounds showed a significant decrease of antiviral activity and, when added at 8–10 h p.i., the activity was completely lost. Conversely, the HIV protease inhibitor Ro31-8959, known to block a late stage of the HIV replicative

cycle, lost its activity when added only at 18–20 h p.i.

To test the hypothesis that these molecules acted, like the other NNRTIs, at the HIV-1 RT level, RT enzyme inhibition assays were performed (Table 4). The results show that MEN 10880 and MEN 10979 were effective against recombinant HIV-1 RT, particularly when ribosomal RNA was used as template (with an IC_{50} that is about 70-fold higher than in cell culture). Moreover, they also showed that RT inhibition was higher (about 2-fold) with primer-template poly(rC).oligo(dG) than with poly(rA).oligo(dT) (with TIBO R82913 there is a 10-fold difference). On the other hand, MEN 10690 inhibited HIV-1 RT with an IC_{50} that is independent from the primer-template used (20–30 μM). We observed no inhibition for MEN 10690 and MEN 10979 at concentrations up to 350 μM with the RT from HIV-2(ROD) or to DNA polymerase α (data not shown).

Table 2
Antiviral activity of MEN compounds against the replication of HIV-1 drug-resistant strains in C8166 cells

Drug	HIV-1 drug-resistant strain[b]	$IC_{50}(\mu M)$[a]
MEN 10690	IIIB	0.04
	$IIIB_{TIBO}$	0.1
	PNL43	0.06
	HIV/ID	0.5
	R14	N.D.
MEN 10979	IIIB	0.0025
	$IIIB_{TIBO}$	0.08
	PNL43	0.012
	HIV/ID	0.2
	R14	0.16
TIBO R82913	IIIB	0.0015
	$IIIB_{TIBO}$	0.15
	PNL43	0.005
	R14	0.06
AZT	IIIB	0.003
	$IIIB_{TIBO}$	0.003
	PNL43	0.15
	HIV/ID	>5
	R14	1

[a]50% inhibitory concentration, based on the inhibition of cytopathicity of HIV-1 drug-resistant strains in C8166 cells.
[b]IIIB: wild type HIV; $IIIB_{TIBO}$: HIV resistant to TIBO; PNL43: moderate degree of resistance to AZT; HIV/ID: fresh clinical isolate of HIV from AZT-treated patient; R14: HIV/IIIB higly resistant to AZT.
All data represent mean values for at least three separate experiments.

Further confirmation that the main target of action for the MEN compounds would be the viral RT, stemmed from experiments with chronically HIV-1(H9/IIIB)-infected cells (data not shown); in this system, RT inhibitors and MEN derivatives, tested at doses 100-fold higher than their IC_{50}, had no effect on cells while virus yield was significantly reduced when cells were treated with a protease inhibitor (Ro31-8959).

If the inhibition of HIV-1 RT is the most likely mechanism of action of MEN compounds, they should block the synthesis of viral DNA in acutely infected cells. Thus, the production of newly formed viral DNA in C8166 cell infected with HIV(IIIB) at high MOI was determined by semiquantitative PCR. MEN compounds were used at a concentration near to their IC_{90}. TIBO R82913 was added as reference drug. The results of these experiments are shown in Fig. 3. Synthesis of viral DNA in infected cells was inhibited by treatment with all MEN compounds. Specifically, the number of HIV(IIIB) DNA copies was reduced by about 99%, 99% or by 90% following treatment with MEN 10979, MEN 10880, or MEN 10690, respectively.

4. Discussion

The arylpyrido-diazepine and -thiodiazepine derivatives should be considered as a class of highly potent and specific anti-HIV-1 compounds. These compounds belong to distinct, although similar, chemical subtypes: MEN 10880 and MEN 10979 are tetracyclic molecules, while MEN 10690 is a tricyclic compound related to nevirapine (Merluzzi et al., 1990).

The main characteristic of MEN 10610 derivatives is the presence of a pyridofurane or a pyridothiophene system condensed with the central diazepine ring, while MEN 10597 derivatives are characterized by the presence of a sulfonamide, instead of amide, group in the diazepine ring (Giannotti et al., 1995; Viti et al., 1995).

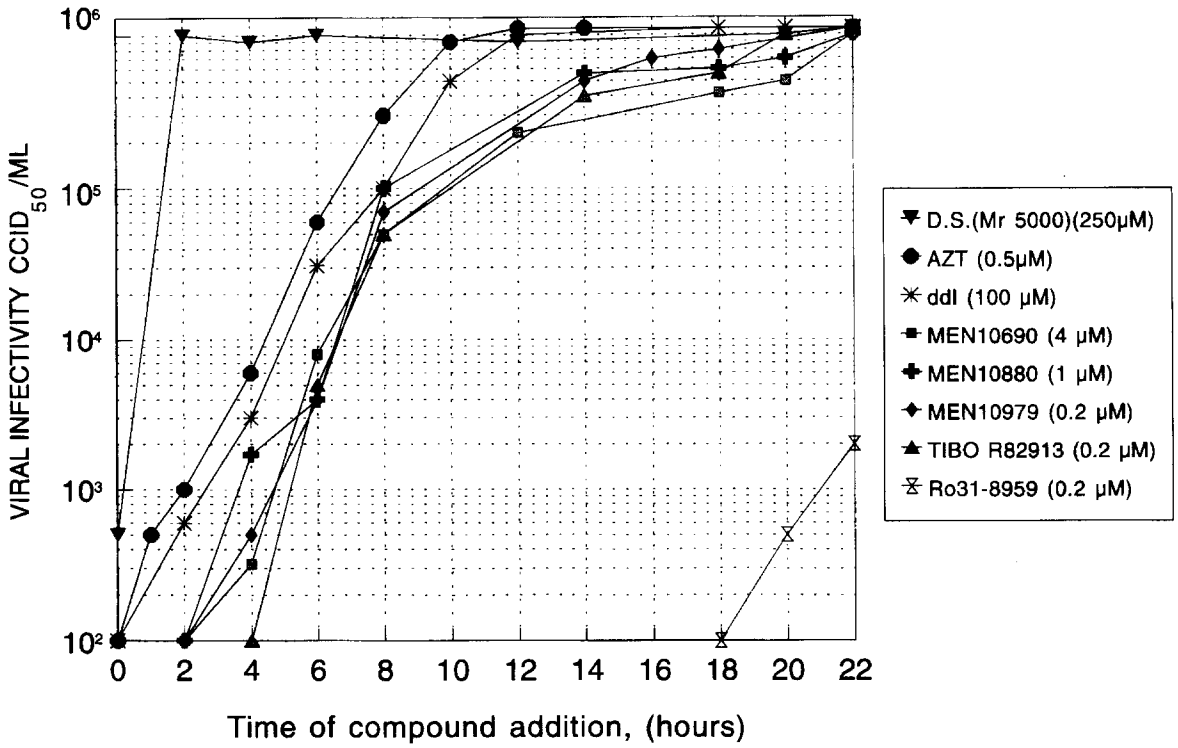

Fig. 2. Time of addition experiment.

Structure-activity studies enabled us to define the effects of some chemical groups on the anti-HIV-1 activity. It is known that the addition of a pyridine ring to Nevirapine or of two sulphur atoms to eterocyclic rings in TIBO compounds increase the antiviral activity of both substances. Consistent with these findings also in the MEN 10610 series, the addition of a pyridine ring and the presence of one or two sulphur atoms at positions 6 and 7 of the molecule, gave the most active derivatives, MEN 10880 and MEN 10979.

The anti-HIV-1 activity of this kind of molecules depends on: (1) the presence of two pyridine rings in the heterocyclic ring system, in particular the pyridine not directly condensed with the diazepine ring; (2) the presence of an alkyl moiety on nitrogen 12; (3) thiation of the amide in the diazepine ring; (4) the substitution of thiophene for the furane ring.

In the other series, MEN 10690 was the most active compound; in this case the antiviral activity seems to be influenced by the presence of only one pyridine group and two methyl residues at positions 8 and 9. Furthermore, the alkylation of nitrogen at position 11 increased the anti-HIV-1 potency. It is interesting to note that MEN 10704, where the main differences with nevirapine is the presence of a sulfonamido group on the diazepine ring, a shift in the methyl group and the presence of an ethyl moiety rather than a cyclopropyl on nitrogen 11, has no anti-HIV-1 activity at all. Our observations on the antiviral properties of MEN 10690, MEN 10880 and MEN 10979 indicate a close relationship to the TIBO/nevirapine compounds. It is known that (1) TIBO compounds and the other non-nucleoside analogues are specific inhibitors of HIV-1 RT but they are generally inactive against HIV-2 and other retroviruses; (2) they do not need to be converted intracellularly to an active metabolite; (3) in the HIV-1 RT assays they show a preference for the heteropolymer template (ribosomal RNA) and the homopolymeric primer template poly(rC).oligo(dG); (4) resistance develops rapidly

Table 3
Inhibitory effects of MEN compounds on the replication of mutant HIV-1 strains in MT-4 cells

Drug tested for antiviral activity	HIV-1 mutant strain	HIV-1 wt[b] used to develop the mutant	Drug used to develop the mutation	Cell line used to develop the mutant	Mutation	$IC_{50}(\mu M)$[a] mutant strain	$IC_{50}(\mu M)$ wt strain
MEN 10690	3B103	IIIB	R82913	MT-4	L100I	0.3	0.2
	39MH1	IIIB	R82913	CEM	K103N	12.8	0.03
	39MN1	HE	R89439	MT-4	V106A	2.4	0.8
	3B188	NDK	R89439	MT-4	Y181C	11.6	0.3
MEN 10880	13MB1	IIIB	HEPT	CEM	Y188H	14.8	0.03
	13MB1	IIIB	R82913	MT-4	L100I	0.3	0.1
	3B103	IIIB	R82913	CEM	K103N	37.2	0.03
	39MH1	HE	R89439	MT-4	V106A	>400	0.5
	39MN1	NDK	R89439	MT-4	Y181C	3.1	0.2
	3B188	IIIB	HEPT	CEM	Y188H	>800	0.03

[a]50% inhibitory concentration, based on the inhibition of cytopathicity of mutant HIV-1 (IIIB) strains in MT-4 cells.
[b]Wild type.
All data represent mean values for at least three separate experiments.

Table 4
Inhibition of HIV-1 RT

Template/primer	IC_{50}(uM)[b]				
	MEN 10690	MEN 10880	MEN 10979	TIBO 82913	AZT-TP[a]
poly(rA).oligo(dT)$_{12-18}$	22 ± 2	15 ± 2	15 ± 5	3.6 ± 0.5	0.007 ± 0.002
poly(rC).oligo(dG)$_{12-18}$	20 ± 5	7 ± 4	6 ± 0.5	0.4 ± 0.05	>100
Ribosomal RNA[c]	30	0.3	0.18	0.05	0.2

[a]AZT 5′-triphosphate.
[b]Data were mean values for at least three experiments.
[c]These data were mean values for two experiments.

to non-nucleoside RT inhibitors (Pauwels et al., 1990; Debyser et al., 1991; Mellors et al., 1992).

We found that, as for other NNRTIs, the antiviral activity of MEN molecules is restricted to HIV-1. Moreover, in TOA experiments, they appear to behave more similarly to TIBO R82913 than to AZT and ddI, thus confirming not only their action on the viral RT but probably the absence of some chemical intermediate responsible for RT inhibition. Furthermore, when MEN 10880 and MEN 10979 were tested in the RT inhibition assay, they showed greater potency when the viral enzyme was assayed with ribosomal RNA than with poly(rA).oligo(dT) or poly(rC).oligo(dG) as the template/primer. The discrepancy between their IC_{50} values in the RT assay with ribosomal RNA as template/primer and the IC_{50} observed in cell cultures was 70-fold, just as described for TIBO R82913 (White et al., 1991), while with poly(rC).oligo(dG) as template/primer the difference was about 1000–2000-fold, probably because the homopolymeric template is a more artificial system. On the other hand, MEN 10690 behaved similarly to nevirapine (Merluzzi et al., 1990), because its RT inhibition was independent from the primer/template used: but the difference with data obtained on cell cultures always remains very high (about 500-fold).

This could raise the question whether inhibition of HIV-1 RT is the sole mechanism of action of these compounds, and in particular MEN 10690. However, when HIV-1 DNA was monitored by PCR, there was a reduction of the viral reverse transcription products, with a good correlation for all MEN compounds between the doses inhibiting viral infectivity and those inhibiting viral DNA synthesis.

The partial cross-resistance that MEN 10979 shows with a IIIB/TIBO-resistant strain, is further evidence for the functional resemblance between these two compounds; however, since the TIBO-resistant virus remained more sensitive to MEN 10979 than to TIBO R82913, it is likely that these molecules interact with distinct residues of the RT enzyme. MEN 10690 showed only a low degree of cross-resistance, thus confirming its belonging to a different kind of non-nucleoside HIV-1 inhibitors. These observations were confirmed by the evaluation of the sensitivity of different NNRTI mutant virus strains towards the inhibitory activity of MEN 10690 and MEN 10880 (Table 3) in MT-4 cells. These results suggest that MEN 10690 and MEN 10880 interact with the NNRTI ('TIBO') pocket site at HIV-1 RT in a way that is different from that of the other NNRTIs, and MEN 10690 and MEN 10880 differ from one another in the way they interact with this pocket site.

We also obtained the rapid selection of IIIB/MEN 10690 and IIIB/MEN 10979 resistant strains; studies to sequence the 'pol' region of these viruses are in progress. However the above data seem to suggest that their clinical usefulness could be relatively limited or short-lived. We also found that MEN 10979 acts synergistically with AZT or ddI in inhibiting HIV-1 replication in MT-4 cells which point to the clinical potential of such drug combinations.

Studies are in progress to establish the toxicological and pharmacokinetic properties of these compounds, as well as the synthesis of new derivatives of both chemical series (MEN 10610 and MEN 10597) to further improve their anti-HIV-1 activity and safety profile.

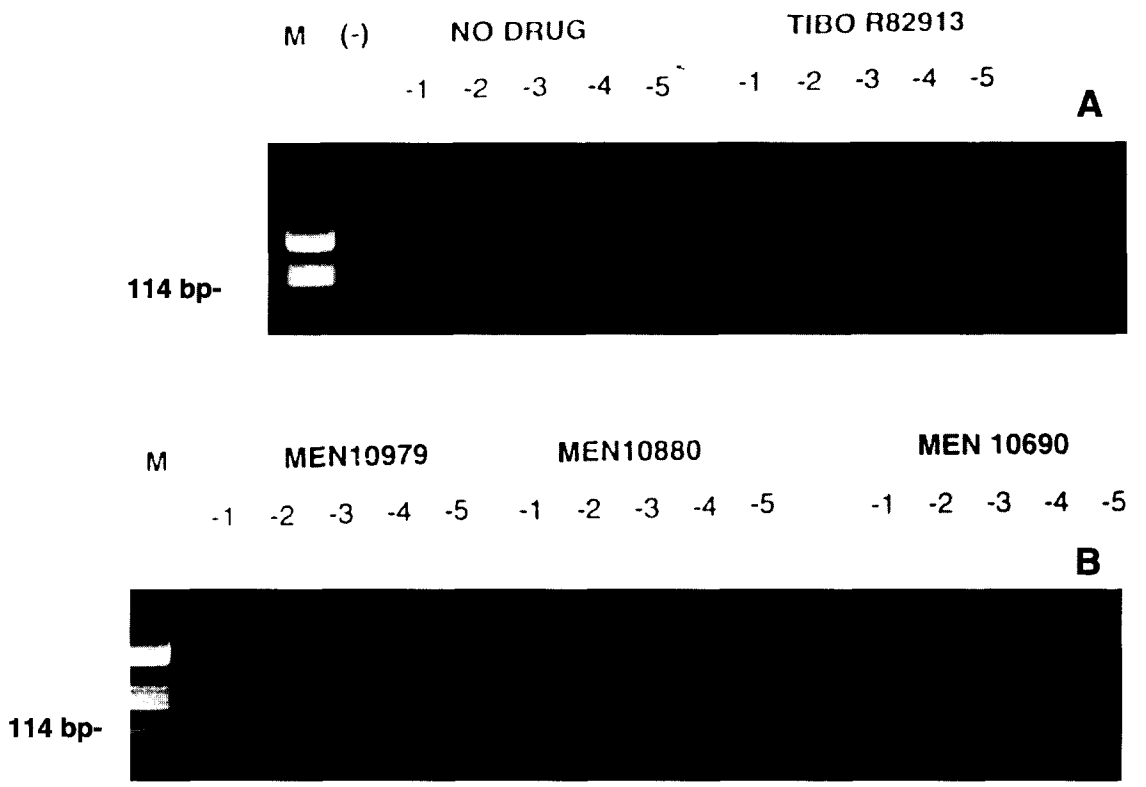

Fig. 3. Reduction of viral DNA synthesis by MEN compounds in MT-4 cells. Panel A, lanes: M = size markers; (-) = mock-infected cells; no DRUG = cells infected with HIV-1(IIIB); TIBO R82913 (1 μM) = cells infected with HIV-1(IIIB) and treated with TIBO R82913. Panel B, lanes: M = size markers; MEN 10979 (2 μM), MEN 10880 (2 μM) and MEN 10690 (4 μM) = cells infected with HIV-1(IIIB) and treated with MEN 10979, MEN 10880 and MEN 10690, respectively.

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References

Antonelli, G., Dianzani, F., Bellarosa, D., Turriziani, O., Riva, E. and Gentile, A. (1994) Drug combination of AZT and ddI: synergism of action and prevention of appearance of AZT-resistance. Antiviral Chem. Chemother. 5, 51–55.

Baba, M., Tanaka, H., De Clercq, E., Pauwels, R., Balzarini, J., Schols, D., Nakashima, H., Perno, C.-F., Walker, R.T. and Miyasaka, T. (1989) Highly specific inhibition of human immunodeficiency virus type 1 by a novel 6-substituted acyclouridine derivative. Biochem. Biophys. Res. Commun. 165, 1375–1381.

Baba, M., Debyser, Z., Shigeta, S. and De Clercq, E. (1992) Highly potent and selective inhibition of human immunodeficiency virus type 1 (HIV-1) by the HIV-1-specific reverse transcriptase inhibitors. Drugs of the Future 17, 891–897.

Balzarini, J., Kang, G.J., Dalal, M., Herdewijn, P., De Clercq, E., Broder, S. and Johns, D.G. (1987) The anti-HTLV-III(anti-HIV) and cytotoxic activity of 2′,3′-didehydro-2′,3′-dideoxyribonucleosides: a comparison with their parental 2′,3′- dideoxyribonucleosides. Mol. Pharmacol. 32, 162–167.

Balzarini, J., Holy, A., Jindrich, J., Dvorakova, H., Hao, Z., Snoeck, R., Herdewijn, P., Johns, D.G. and De Clercq, E. (1991) 9-[(2RS)-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.

Balzarini, J., Perez-Perez, M.J., San-Felix, A., Velazquez, S., Camarasa, M.J. and De Clercq, E. (1992) [2′,5′,bis-O-(tert-butyldimethylsilyl)]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO) derivatives of purine and pyrimidine nucleosides as potent and selective inhibitors of human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 36, 1073–1080.

Barré-Sinoussi, F., Chermann, J.C., Rey, F. Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W. and Montagnier, L. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for AIDS. Science 22, 868–871.

Buckeit, R.W. Jr., Hollingshead M.G., Germany-Decker J., White E.L., McMahon J.B., Allen L.B., Ross L.J., Don Decker W., Westbrook L., Shannon W.M., Weislow O., Bader J.P. and Boyd M.R. (1993) Thiazolobenzimidazole: biological and biochemical anti-retroviral activity of a new nonnucleoside reverse transcriptase inhibitor. Antiviral Res. 21, 247–265.

Cheng, Y.-C., Dutchman, G.E., Bastow, K.F., Sarngadharan, M.O. and Ting, R.Y.C. (1987) Human immunodeficiency virus reverse transcriptase: general properties and its interaction with nucleoside triphosphate analogs. J. Biol. Chem. 262, 2187–2189.

Clapham, P.R., Weiss, R.A., Dalgleish, A.G., Exley, M., Whitby, D. and Hogg, N. (1987) Human immunodeficiency virus infection of monocytic and T-lymphocyte cells: receptor modulation and differentiation induced by phorbol ester. Virology 158, 44–51.

Davis, B.D., Dulbecco, R., Eisen, H.N. and Ginsberg, H.S. (1980) The nature of viruses: quantitative aspects of infection. In: Microbiology, including immunology and molecular genetics. 3rd ed. Hagerstown, MD: Harper and Row, 880–883.

Debyser, Z., Pauwels, R., Andries, K., Desmyter, J., Kukla, M., Janssen P.A.J. and De Clercq, E. (1991) An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo-[4,51-jk] [1,4]benzodiazepin-2(1H)-one and -thione derivatives. Proc. Natl. Acad. Sci. USA, 88, 1451–1455.

Debyser, Z., Pauwels, R., Andries, K., Desmyter, J., Engelborghs, Y., Janssen, P.A.J. and De Clercq, E. (1992a) Allosteric inhibition of human immunodeficiency virus type 1 reverse transcriptase by tetrahydroimidazo[4,5,1-jk][1,5]benzodiazepin-2(1H)-one and -thione compounds. Mol. Pharmacol. 41, 203–208.

Debyser, Z., Pauwels, R., Baba, M., Desmyter, J. and De Clercq, E. (1992b) Common features in the interaction of tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one and -thione and 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymine derivatives with the human immunodeficiency virus type 1 reverse transcriptase. Mol. Pharmacol. 41, 963–968.

De Clercq, E. (1991) Broad-spectrum anti-DNA virus and anti-retrovirus activity of phosphonylmethoxyalkylpurines and -pyrimidines. Biochem. Pharmacol. 42, 963–972.

De Clercq, E. (1992) HIV inhibitors targeted at the reverse transcriptase. AIDS Res. Hum. Retrovir. 8, 119–133.

De Clercq, E. (1994) HIV resistance to reverse transcriptase inhibitors. Biochem. Pharmacol. 47, 155–169.

Dianzani, F., Antonelli, G., Capobianchi, M.R., De Marco, F. (1988). Replication of human immunodeficiency virus: yield of infectious virus under single growth cycle conditions. Arch. Virol. 103, 127–131.

Dianzani, F., Capobianchi, M.R., Antonelli, G., Amicucci, P. and De Marco, F. (1989). Susceptibility of human immunodeficiency virus to antiviral agents measured by infectious virus yield reduction. Antiviral Res. 11, 229–306.

Dianzani, F., Antonelli G., Turriziani O., Dong G., Capobianchi M.R. and Riva E. (1992). In vitro selection of human immunodeficiency virus type 1 resistant to 3′-azido-3′-deoxythymidine. Antiviral Res. 18, 39–52.

Franchini, G., Gurgo, C., Guo, H.G., Gallo, R.C., Collalti, E., Fargnoli, K.A., Hall, L.F., Wong-Staal, F. and Reitz, M.J. Jr. (1987) Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses. Nature (London) 328, 539–543.

Furman, P.A., Fyfe, J.A., St. Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Nusinoff-Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H. and Barry, D.W. (1986) Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83, 8333–8337.

Giannotti, D., Viti, G., Nannicini, R., Pestellini, V. and Bellarosa, D. (1995) Synthesis and anti-HIV-1 activity of new thiadiazepindioxides. Biorg. Med. Chem. Lett. 5(14), 1461–1466.

Goldman, M.E., Nunberg, J.H., O'Brien, J.A. Quintero, J.C., Schleif, W.A., Freund, K.F., Gaul, S.L., Saari, W.S., Wai, J.S., Hoffman, J.M., Anderson, P.S., Hupe, D.J., Emini, E.A. and Stern, A.M. (1991) Pyridinone derivatives: specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity. Proc. Natl. Acad. Sci. USA 88, 6863–6867.

Hamamoto, Y., Nakashima, H., Matsui, T., Matsuda, A., Ueda, T. and Yamamoto, N. (1987) Inhibitory effect of 2′,3′-didehydro-2′,3′-dideoxynucleosides on infectivity, cytopathic effects, and replication of human immunodeficiency virus. Antimicrob. Agents Chemother. 31, 907–910.

Hargrave, K.D., Proudfoot, J.R., Grozinger, K.G., Cullen, E., Kapadia, S.R., Patel, U.R., Fuchs, V.U., Mauldin, S.C., Vitous, J., Behnke, M.L., Klunde, J.M., Pal, K., Skiles, J.W., McNeil, D.W., Rose, J.M., Chow, G.C., Skoog, M.T., Wu, J.C., Schmidt, G., Engel, W.W., Eberlein, W.G., Saboe, T.D., Campbell, S.J., Rosenthal, A.S. and Adams, J. (1991) Novel non-nucleoside inhibitors of HIV-1 reverse transcriptase. 1: tricyclic pyridobenzo- and -dipyridodiazepinones. J. Med. Chem. 34, 2231–2241.

Ho, D.D., Schooley, R.T., Rota, T.R., Kaplan, J.C., Flynn, T., Salahuddin, S.Z., Gonda, M.A. and Hirsch, M.S. (1984) HTLV-III in the semen and blood of a healthy homosexual man. Science 226, 451–453.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A. and Steitz, T.A. (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256, 1783–1790.

Konig, H., Behr, E., Lower, J. and Kurth, R. (1989) Azidothymidine triphosphate is an inhibitor of both human immunodeficiency virus type 1 reverse transcriptase and DNA polymerase gamma. Antimicrob. Agents Chemother. 33, 2109–2114.

Lin, T., Schinazi R.F. and Prusoff W.H. (1987). Potent and selective in vitro activity of 3′-deoxythymidin-2′-ene (3′-deoxy-2′,3′-didehydrothymidine) against human immunodeficiency virus. Biochem. Pharmacol. 36, 2713–2718.

Mellors, J.W., Dutschman, G.E., Im, G.-J., Tramontano, E., Winkler, S.R. and Cheng, Y.-C. (1992) In vitro selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. Mol. Pharmacol. 41, 446–451.

Merluzzi, V.J., Hargravre, K.D., Labadia, M., Grozinger, K., Skoog, M. Wu, J.C., Shih, C.-K., Eckner, K., Hattox, S. Adams, J., Rosenthal, A.S., Faanes, R., Eckner, R.J., Koup, R.A. and Sullivan, J.L. (1990) Inhibition of HIV-1 replication by a non-nucleoside reverse transcriptase inhibitor. Science 250, 1411–1413.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St.Clair, M.H., Lehrman, S.N., Gallo, R.C., Bolognesi, D., Barry, D.W. and Broder, S. (1985) 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA 82, 7096–7100.

Mitsuya, H. and Broder S. (1986) Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLVIII/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA 83, 1911–1915.

Miyoshi, I., Taguchi, H., Kuboniski, I., Yoskimoto, S., Ohtsuki, Y., Shiraishi, Y. and Akagi, T. (1982) Type C virus-producing cell lines derived from adult T-cell leukemia. Gann. Monogr. 28, 219–228.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J. Immunol. Meth. 65, 55–63.

Nunberg J.H., Schleif W.A., Boots E.J., O'Brien J.A., Quintero J.C., Hoffman J.M., Emini E.A. and Goldman M.E. (1991) Viral resistance to human immunodeficiency type 1-specific pyridinone reverse transcriptase inhibitors. J. Virol. 65, 4887–4892.

Ono, K., Nakame, H., Herdewijn, P., Balzarini, J. and De Clercq, E. ((1989) Differential inhibitory effects of several pyrimidine 2′,3′-dideoxynucleoside 5′-triphosphate on the activities of reverse transcriptase and various cellular DNA polymerases. Mol. Pharmacol. 35, 578–583.

Pauwels, R., Balzarini, J., Schols, D., Baba, M., Desmyter, J., Rosenberg, I. and De Clercq, E. (1988a) Phosphonylmethoxyethyl purine derivatives, a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. 32, 1025–1030.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and De Clercq, E. (1988b) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Meth. 20, 309–321.

Pauwels, R., Andries, K., Desmyter, J., Schols, D., Kukla, M.J., Breslin, H.J., Raeymaeckers, A., Van Gelder, J., Woestenborghs, R., Heykants, J., Schellekens, K., Janssen, M.A.C., De Clercq, E. and Janssen, P.A.J. (1990) Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. Nature 343, 470–474.

Pauwels, R., Andries, K., Debyser, Z., Van Daele, P., Schols, D., Stoffels, P., De Vreese, K., Woestenborghs, R., Vandamme, A.-M., Janssen, C.G.M., Anné, J., Cauwenbergh, G., Desmyter, J., Heykants, J., Janssen, M.A.C., De Clercq, E. and Janssen, P.A.J. (1993) Potent and highly selective human immunodeficiency virus type 1 (HIV-1) inhibition by a series of 6-anilinophenylacetamide derivatives targeted at HIV-1 reverse transcriptase. Proc. Natl. Acad. Sci. USA 90, 1711–1715.

Pauwels, R., Andries, K., Debyser, Z., Kukla, M.J., Schols, D., Breslin, H.J., Woestenborghs, R., Desmyter, J., Janssen, M.A.C., De Clercq, E. and Janssen, P.A.J. (1994) New tetrahydroimidazo [4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and -thione derivatives are potent inhibitors of human immunodeficiency virus type 1 replication and are synergistic with 2′,3′-dideoxynucleoside analogs. Antimicrob. Agents Chemother. 38, 2863–2870.

Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. (1984) Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 497–500.

Richman, D., Shih, C.-K., Lowy, I., Rose, J., Prodanovich, P., Goff, S. and Griffin, J. (1991) Human immunodeficiency virus type 1 mutants resistant to non-nucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc. Natl. Acad. Sci. USA, 88, 11241–11245.

Romero, D.L., Busso, M., Tan, C.-K., Reusser, F., Palmer, J.R., Poppe, S.M., Aristoff, P.A., Downey, K.M., So, A.G., Resnick, L. and Tarpley, W.G. (1991) Non-nucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. USA 88, 8806–8810.

Spire, B., Sire, J., Zachar, V., Rey, F., Barré-Sinoussi, F., Galibert, F., Hampe, A. and Chermann, J.-C. (1989). Nucleotide sequence of HIV-1-NDK: a highly cytopathic strain of the human immunodeficiency virus. Gene 81, 275–284.

Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R.G., Boyer, P.L., Hughes, S.H., Pauwels, R., Andries, K., Janssen, P.A. and Arnold, E. (1994) Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. J. Mol. Biol. 243: 369–387.

Viti, G., Giannotti, D., Nannicini, R., Balacco, G., Pestellini, V., Paoli, P. and Dapporto, P. (1995) Synthesis of tetracyclic arylpyridodiazepines as potential anti-HIV-1 agents. Heterocycles, 41, in press.

Vrang, L., Oberg, B., Lower, J. and Kurth, R. (1988) Reverse transcriptases from human immunodeficiency virus type 1 (HIV-1), HIV-2 and simian immunodeficiency virus (SIV_{MAC}) are susceptible to inhibition by foscarnet and 3′-azido-3′-deoxythymidine triphosphate. Antimicrob. Agents Chemother. 32, 1733–1734.

White, L.E., Buckheit, R.W.Jr., Ross, L.J., Germany, J.M., Andries, K., Pauwels, R., Janssen, P.A.J., Shannon, W.M. and Chirigos, M.A. (1991) A TIBO derivative, R82913, is a potent inhibitor of HIV-1 reverse transcriptase with heteropolymer templates. Antivir. Res. 16, 257–266.

Witvrouw, M., Schols, D., Andrei, G., Snoeck, R., Hosoya, M., Pauwels, R., Balzarini, J. and De Clercq, E. (1991) Antiviral activity of low-MW dextran sulphate (derived from dextran MW1,000) compared to dextran sulphate samples of higher MW. Antiviral Chem. Chemother. 2, 171–179.

Wu, J.C., Warren, T.C., Adams, J., Proudfoot, J., Skiles, J., Raghavan, P., Perry, C., Potocki, I., Farina, P.R. and Grob, P.M. (1991) A novel dipyridodiazepinone inhibitor of HIV-1 reverse transcriptase acts through a nonsubstrate binding site. Biochemistry 30, 2022–2026.