Biochemical Pharmacology, Vol. 38, No. 6, pp. 869–874, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

RAPID COMMUNICATIONS

5-CHLORO-SUBSTITUTED DERIVATIVES OF 2',3'-DIDEHYDRO-2',3'-DIDEOXYURIDINE, 3'-FLUORO-2',3'-DIDEOXYURIDINE AND 3'-AZIDO-2',3'-DIDEOXYURIDINE AS ANTI-HIV AGENTS

Jan BALZARINI, Arthur VAN AERSCHOT, Piet HERDEWIJN and Erik DE CLERCQ

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

(Accepted 19 December 1988)

INTRODUCTION

Various pyrimidine 2',3'-dideoxynucleoside analogues have been reported to inhibit the replication of human immunodeficiency virus (HIV) in vitro. Among the most potent inhibitors of HIV replication in vitro are 3'-azido-2',3'-dideoxythymidine (AzddThd, AZT), 3'-fluoro-2',3'-dideoxythymidine (FddThd) and 2',3'-didehydro-2',3'-dideoxythymidine (ddeThd, D4T). (For an overview, see ref. 1). Recently, we synthesized the corresponding 2',3'-dideoxyuridine derivatives [i.e. 3'-fluoro-2',3'-dideoxyuridine (FddUrd) and 3'-azido-2',3'-dideoxyuridine (AzddUrd)] and found these derivatives markedly active against HIV-1 (2). In contrast, 2',3'-didehydro-2',3'-dideoxyuridine (D4U) was inactive at subtoxic concentrations (3).

Since the introduction of an halogen atom (i.e. chlorine, bromine, iodine) in the uracil moiety of 2'-deoxyuridine (dUrd) results in molecules (i.e. 5-chloro-dUrd, 5-bromo-dUrd, 5-iodo-dUrd) which resemble thymidine (dThd), rather than dUrd, in metabolic and kinetic properties (i.e. substrate affinity for dThd kinase (4), incorporation into cell DNA (5)), we synthesized the 5-chloro-substituted derivatives of AzddUrd, FddUrd and D4U.

In the present report we describe the anti-HIV activity and several physical, kinetic and metabolic properties of D4U, FddUrd, AzddUrd and their 5-chloro-substituted derivatives i.e. FddClUrd and AzddClUrd. The latter represent two novel potent and selective anti-HIV compounds that should be further evaluated for their efficacy in the treatment of retrovirus infections. The cellular dThd kinase has a critical role in the anti-HIV action of FddClUrd, AzddClUrd and other pyrimidine 2',3'-dideoxynucleoside derivatives (i.e. FddUrd, AzddUrd and AZT).

MATERIALS AND METHODS

Compounds and radiochemicals

The synthesis of D4U, FddUrd, AzddUrd, D4T and AzddThd has been described earlier (3,6). The synthesis of FddClUrd, AzddClUrd and 2',3'-didehydro-2',3'-dideoxy-5-chlorouridine (D4CU) will be described elsewhere. Their structural formulas are depicted in Fig. 1. The other chemicals were of the highest quality obtainable. [Methyl-^{3}H]dThd (specific radioactivity : 40 Ci/mmol) was obtained from the Radiochemical Centre Amersham (Amersham, U.K.).

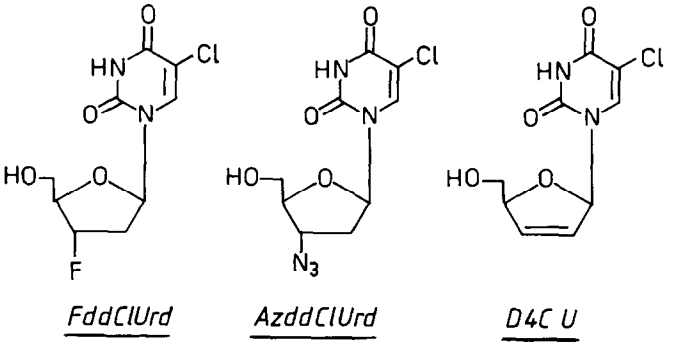

Fig. 1. Structural formulas of the 5-chloro-substituted derivatives of FddUrd, AzddUrd and D4U.

Cells and viruses

Human T4-lymphocyte MT4, human B-lymphoblast-like Raji/O and Raji/TK$^-$ cells (the latter being a dThd kinase-deficient mutant cell line derived from the wild-type Raji/O cells) and murine embryo fibroblast C3H cells were grown and characterized as described earlier (2,7).

Stock suspensions of HTLV-III$_B$ (designated HIV-1) and LAV-2 (designated HIV-2) were prepared from supernatants of infected HUT-78 and CEM cells, respectively. Simian AIDS-related virus (SRV) was prepared from the supernatants of infected Raji/O cells. Moloney murine sarcoma virus (MSV) was prepared from tumors induced in 3-day old NMRI mice according to a previously reported procedure (8).

Antiretroviral assays

The methodology of the antiretroviral assays has been described previously (2). Briefly, MT-4 cells (5×10^5 cells/ml) were suspended in fresh culture medium and infected with HIV-1 or HIV-2 at 200 $CCID_{50}$ per ml cell suspension (1 $CCID_{50}$ being the dose infective for 50 % of the cell cultures). Then, 100 µl of the infected cell suspension was mixed with 100 µl of the appropriate dilution of test compound [in the presence or absence of 1000 µM 2'-deoxycytidine (dCyd) or 1000 µM dCyd + 250 µM dThd], transferred to microplate wells and further incubated at 37°C for 5 days. The numbers of viable cells were determined for both virus-infected and mock-infected cell cultures. The 50 % effective dose (ED_{50}) and 50 % cytotoxic dose (CD_{50}) were defined as the compound concentration required to reduce by 50 % the number of viable cells in the virus-infected and mock-infected cell cultures, respectively.

Raji/O and Raji/TK$^-$ cells were suspended at 250,000 and 350,000 cells per ml culture medium, respectively and infected with SRV at 40 $CCID_{50}$ per ml (Raji/O) or 100 $CCID_{50}$ per ml (Raji/TK$^-$). Then, 100 µl of the infected cell suspension were added to 200-µl microtiter plate wells, containing 100 µl of an appropriate dilution of test compound. After 4 days incubation at 37°C, 100 µl culture medium was removed and 50 µl of the concentrated cell suspension was diluted 4-fold with fresh culture medium and transferred to new 200-µl wells. Four days later (= day 8 of the experiment), 100 µl culture medium was removed and 35 µl of the remaining cell suspension was diluted 5 times with fresh culture medium and transferred to new wells. Finally, at day 12 of the experiment, the total number of giant cells present in the cell cultures was estimated and the ED_{50} of the test compounds was defined as the compound concentration that caused a reduction of the number of giant cells by 50 %.

C3H cells were seeded at 20,000 cells per ml into wells of Tissue Culture Cluster Plates (48 wells/plate). Following a 24-hour incubation period, cell cultures were infected with 80 foci-forming units of MSV during 120 min, whereafter the culture medium was replaced by 1 ml fresh medium containing appropriate concentrations of the test compounds. After 6 days, transformation of the cell cultures was examined microscopically.

Enzyme assay

dThd kinase was prepared from exponentially growing MT-4 cells. The enzyme fraction precipitated between 30 % and 70 % $(NH_4)_2SO_4$ was used in our experiments and [methyl-^{3}H]dThd served as the radiolabeled substrate for dThd kinase. The method for preparing the enzyme extract and the assay procedures have been described previously (4).

Determination of the lipophilicity of the compounds

To estimate the lipid solubility of the test compounds, the partition of the test compounds between 1-octanol and 10 mM potassium phosphate buffer pH 7.5 was measured spectrophotometrically as described earlier (9).

RESULTS AND DISCUSSION

When the 5-chloro-substituted derivatives of D4U, FddUrd and AzddUrd were examined for their inhibitory effect on the cytopathogenicity of HIV-1 and HIV-2 in MT-4 cells, FddClUrd and AzddClUrd proved highly potent in protecting the cells against destruction by the virus (Table 1). Their selectivity index (S.I.) *in vitro* [ratio CD_{50} / ED_{50}] amounted to 1446 and 296, respectively, which was higher than the S.I. of the parent compounds FddUrd and AzddUrd. The S.I. of FddClUrd and AzddClUrd was comparable to that of AZT and D4T, respectively (Table 1). In contrast, introduction of a chlorine atom at C-5 of D4U did not result in an increase of activity or selectivity. The ED_{50} values of the compounds for HIV-2 replication were quite comparable to those found for HIV-1 (Table 1).

Thus, introduction of a chlorine at the C-5 position of the uracil base resulted in a substantial increase of the anti-HIV selectivity of the 3'-fluoro- or 3'-azido-substituted ddUrd analogues but not of D4U. The increased antiviral selectivity of FddClUrd was due to a marked decrease in the cytotoxicity, rather than an increased antiviral potency of the test compounds. The molecular basis of the decreased cytotoxicity remains subject for further investigation.

and FddThd, were 6-, 550- and 200-fold more effective in the MSV assay system than was AzddClUrd (2). D4U and D4CU were virtually ineffective as inhibitors of MSV. The fact that the 3'-fluoro analogues of ddUrd and ddClUrd were less effective anti-MSV agents than their 3'-azido-counterparts may be related to the observations of Bazin et al. (10) who found several 3'-fluoro-ddUrd 5'-triphosphate analogues to be substantially less effective as substrates for murine leukemia retrovirus-associated reverse transcriptase than HIV reverse transcriptase. This may explain why FddUrd and FddClUrd failed to inhibit murine retrovirus replication in vitro.

The test compounds were also evaluated for their cytostatic and antiviral effects in Simian AIDS related virus (SRV)-infected Raji/O and Raji/TK$^-$ cells (the latter cell line being deficient in cytosol dThd kinase). In contrast with the 2',3'-unsaturated 2',3'-dideoxynucleosides D4U, D4CU and D4T, the antiviral and cytostatic effects of the other test compounds were exquisitely dependent on the presence of cellular dThd kinase. Indeed, FddUrd, FddClUrd, AzddUrd, AzddClUrd and AZT were substantially less active as antiviral or cytostatic agents in Raji/TK$^-$ than Raji/O cells (Table 2). These observations clearly point to a pivotal role of dThd kinase in the metabolic activation of 3'-fluoro- and 3'-azido-ddUrd and -ddClUrd analogues, but not 2',3'-unsaturated ddUrd, ddClUrd and ddThd derivatives. This is in keeping with our other findings that the anti-HIV-1 activities of FddUrd, FddClUrd, AzddUrd and AzddClUrd, but not D4U and D4CU, were markedly decreased (as much as 250- to > 1000-fold) when combined with 250 μM dThd (in the presence of 1000 μM dCyd to avoid cytotoxicity of dThd) (data not shown). The data obtained in Raji/O and Raji/TK$^-$ cells provide direct evidence for the important role of the cytosol dThd kinase in the antiretroviral activity of several but not all pyrimidine 2',3'-dideoxynucleosides.

The test compounds were also evaluated for their potential to inhibit [methyl-^{3}H]dThd phosphorylation by partially purified dThd kinase extracted from MT-4 cells. (Table 3).

Table 3. Effect of introduction of a chlorine at C-5 of the uracil part on the affinity of D4U, FddUrd and AzddUrd for MT-4 cell dThd kinase

Compound	K_i (μM)	K_i/K_m [a]	Type of inhibition
D4U	>> 2000 [b]	>> 1000	-
D4CU	1975	1241	slope-linear mixed
FddUrd	392	302	competitive
FddClUrd	6.1	4.7	competitive
AzddUrd	49	48	competitive
AzddClUrd	1.7	1.7	competitive

[a] K_m values obtained in the individual experiments ranged from 0.84 to 2.5 μM and were graphically obtained from the Lineweaver-Burk plots. The K_i values for the respective compounds were calculated from following formula : $K_i = [I]/[(K_{m_{app}} \times K_m^{-1}) - 1]$.

[b] at 2000 μM, no significant inhibition of [methyl-^{3}H]dThd phosphorylation by dThd kinase was observed.

FddClUrd and AzddClUrd showed a 30- to 60-fold higher affinity for dThd kinase than FddUrd and AzddUrd. Both FddClUrd and AzddClUrd competitively inhibited [methyl-^{3}H]dThd phosphorylation by dThd kinase at relatively low concentrations (Fig. 2).

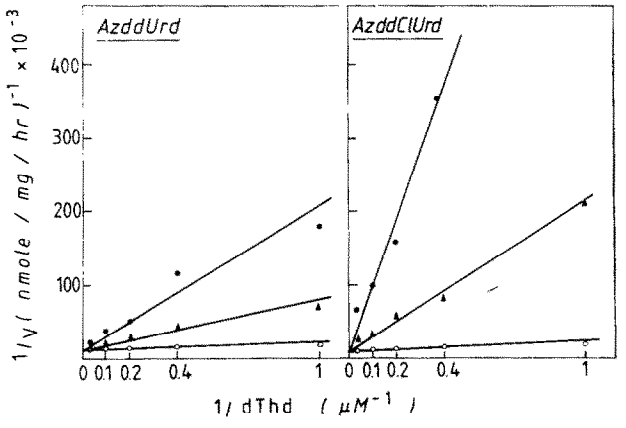

Fig. 2. Double-reciprocal plots for the inhibition of MT-4 cell dThd kinase by AzddUrd (left Panel) and AzddClUrd (right Panel). Inhibitor concentrations : 0 mM (○), 100 μM (●) and 40 μM (▲). Substrate : [methyl-^{3}H]dThd.

As we reported in a previous paper (2), FddUrd, in contrast with AzddUrd, is unable to inhibit MSV-induced transformation of murine C3H cells. We now found that FddClUrd also is inactive as an anti-MSV agent, while AzddClUrd is as effective as AzddUrd. However, D4T, AZT

Table 1. Anti-HIV-1 and -HIV-2 effects of 2',3'-dideoxyuridine analogues and their 5-chloro-substituted congeners

Compound	HIV-1-induced cytopathogenicity in MT-4 cells			HIV-2-induced cytopathogenicity in MT-4 cells
	ED_{50}[a] (μM)	CD_{50}[b] (μM)	S.I.[c]	ED_{50}[a] (μM)
D4U	> 20	39±1.3	< 2	> 20
D4CU	> 20	44±1.7	2.2	> 20
FddUrd	0.06±0.02	1.1±0.2	25	0.16±0.03
FddClUrd	0.38±0.06	535±41	1408	0.69±0.15
AzddUrd	0.43±0.21	39±3.7	90	0.10±0.01
AzddClUrd	0.72±0.22	213±29	296	0.45±0.01
D4T	0.05±0.001	19±3.6	380	0.09±0.01
AZT	0.003±0.001	4.8±2.5	1603	0.004±0.003

[a] 50 % effective dose or dose required to inhibit virus-induced cytopathogenicity by 50 %.
[b] 50 % cytostatic dose or dose required to reduce the number of living MT-4 cells by 50 % after a 5-day incubation period.
[c] Selectivity index or ratio CD_{50}/ED_{50}.

Table 2. Anti-SRV and -MSV effects of 2',3'-dideoxyuridine analogues and their 5-chloro-substituted congeners

Compound	SRV-induced syncytium formation in Raji/O and Raji/TK⁻ cells				MSV-induced transformation of C3H cells		
	Raji/O		Raji/TK⁻				
	ED_{50}[a] (μM)	CD_{50}[b] (μM)	ED_{50}[a] (μM)	CD_{50}[b] (μM)	ED_{50}[c] (μM)	MCC[d] (μM)	S.I.[e]
D4U	> 100	261±1	> 100	252±7.0	> 200	> 200	< 1
D4CU	> 20	105±32	> 20	68±4.2	≥ 100	500	≤ 5
FddUrd	40	> 500	> 500	> 500	> 500	> 500	-
FddClUrd	4	> 500	> 500	> 500	457±7	> 500	> 1.1
AzddUrd	40	> 500	> 500	> 500	15[f]	> 400[f]	> 27
AzddClUrd	2	258±39	≥ 500	> 500	12±2.0	> 500	> 41
D4T	4	280±8.0	10	254±9	2.1±0.7	> 200	> 95
AZT	0.1	57.8±14	≥ 200	> 500	0.023[f]±0.01	> 500[f]	>22,000

[a] 50 % effective dose or dose required to reduce the number of giant cells by 50 %.
[b] 50 % cytostatic dose or dose required to inhibit proliferation of Raji cells by 50 % after a 3-day incubation period.
[c] 50 % effective dose or dose required to inhibit virus-induced transformation by 50 %.
[d] Minimal cytotoxic concentration or concentration of the compound that causes a microscopically morphological alteration of the cells.
[e] Selectivity index or ratio MCC/ED_{50}.
[f] Data taken from ref. 2.

In this respect, FddClUrd and AzddClUrd more closely resembled to the corresponding thymidine derivatives (FddThd and AZT) (2) than the uridine derivatives, a kinetic behaviour that could be expected based on our previous knowledge of the kinetic properties of 5-chloro-dUrd relative to those of dThd and dUrd (4,5). In contrast, D4CU appeared to be a weak inhibitor of the phosphorylation of [methyl-^{3}H]dThd by MT-4 cell extracts, and the kinetics followed a slope-linear mixed type inhibition (Fig. 3).

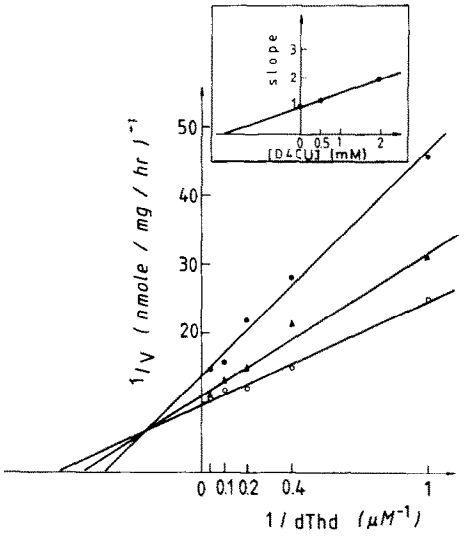

Fig. 3. Double reciprocal plots for the inhibition of MT-4 cell dThd kinase by D4CU. Inhibitor concentrations : 0 mM (o), 2 mM (●) and 0.5 mM (▲). Substrate : [methyl- H]dThd.

Thus, from our data we may infer that the phosphorylation of the 5-chloro-substituted FddUrd and AzddUrd derivatives by dThd kinase is a prerequisite for their antiretroviral action. For D4U and D4CU no such dependence as phosphorylation by dThd kinase could be demonstrated. Which cellular enzyme(s), if any, are responsible for the metabolic conversion of D4U and D4CU remains to be elucidated.

In view of the requirement of an anti-AIDS compound to cross the blood brain barrier, we also determined the effect of the introduction of the 5-chloro-substituent on the lipophilicity of D4U, FddUrd and AzddUrd. As a rule, the 5-chloro-substituted ddUrd derivatives were invariably 2- to 2.5-fold more lipophilic than their unsubstituted counterparts (Table 4).

Table 4. Effect of introduction of a chlorine at C-5 of the uracil part on the partition of D4U, FddUrd and AzddUrd between 1-octanol and 10 mM potassium phosphate buffer.

Compound	p[a]
D4U	0.086 ± 0.048
D4CU	0.218 ± 0.023
FddUrd	0.279 ± 0.035
FddClUrd	0.678 ± 0.192
AzddUrd	0.506 ± 0.003
AzddClUrd	1.188 ± 0.080
D4T	0.154 ± 0.008
AZT	0.964 ± 0.038

[a]Partition coefficient or ratio of the compound concentration present in the apolar (1-octanol) phase to the compound concentration present in the polar (water) phase.

Consequently, FddClUrd had a partition coefficient (P) that was only slightly inferior to that of AZT (P : 0.678 and 0.964, respectively) while AzddClUrd was clearly more lipophilic than AZT. From a clinical viewpoint, the relatively high lipophilicity of FddClUrd and AzddClUrd may make these compounds therapeutically advantageous over their unsubstituted congeners FddUrd and AzddUrd.

CONCLUSIONS

The 5-chloro-substituted derivatives of FddUrd and AzddUrd, but not D4U proved considerably more selective in their anti-HIV-1 and HIV-2 activity than their unsubstituted parents. Phosphorylation by dThd kinase appeared to be a prerequisite for the antiviral and cytostatic effects of 3'-fluoro- and 3'-azido-substituted analogues of both ddUrd and ddClUrd. The potent and selective activity of FddClUrd and AzddClUrd against HIV-1 and HIV-2 justifies further studies with these novel compounds to assess their therapeutic potential as candidate anti-AIDS drugs.

Acknowledgements- HIV-1 (HTLV-III_B) and HIV-2 (LAV-2) were a gift from Dr. R.C. Gallo and Dr. M. Popovic [National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD, USA], and Dr. L. Montagnier [Institut Pasteur, Paris, France], respectively. Simian AIDS related virus (RSV) was kindly provided by Dr. H. Schellekens and Dr. H. Niphuis from TNO, Rijswijk, The Netherlands. MT-4 cells were kindly supplied by Dr. N. Yamamoto [Yamaguchi University, Yamaguchi, Japan]. Dr. P. Herdewijn is a Research Associate of the Belgian N.F.W.O. ("Nationaal Fonds voor Wetenschappelijk Onderzoek"). Arthur Van Aerschot is a fellow of the Janssen Research Foundation.

These investigations were supported in part by the AIDS Basic Research Programme of the European Community, by grants from the Belgian F.G.W.O. ("Fonds voor Geneeskundig Wetenschappelijk Onderzoek", projects no. 3.0040.83 and 3.0097.87) and the Belgian G.O.A. ("Geconcerteerde Onderzoeksacties", project no. 85/90-79). We thank Ann Absillis, Miette Stuyck, Lizette van Berckelaer and Guy Schepers for their excellent technical help and Christiane Callebaut and Dominique Brabants for their fine editorial assistance.

REFERENCES

1. De Clercq E, Van Aerschot A, Herdewijn P, Baba M, Pauwels R, Balzarini J. Anti-HIV-1 activity of 2',3'-dideoxynucleoside analogues : structure-activity relationship. Nucleosides & Nucleotides, in press, 1988.
2. Balzarini J, Baba M, Pauwels R, Herdewijn P, De Clercq E, Anti-retrovirus activity of 3'-fluoro- and 3'-azido-substituted pyrimidine 2',3'-dideoxynucleoside analogues. Biochem Pharmacol 37:2847-2856, 1988.
3. Herdewijn P, Balzarini J, De Clercq E, Pauwels R, Baba M, Broder S, Vanderhaeghe H, 3'-Substituted 2',3'-dideoxynucleoside analogues as potential anti-HIV (HTLV-III/LAV) agents. J Med Chem 30:1270-1278, 1987.
4. Balzarini J, De Clercq E, Mertes MP, Shugar D, Torrence PF, 5-Substituted 2'-deoxyuridines: correlation between inhibition of tumor cell growth and inhibition of thymidine kinase and thymidylate synthetase. Biochem Pharmacol 31:3673-3682, 1982.
5. Balzarini J, De Clercq E, Ayusawa D, Seno T, Incorporation of 5-substituted pyrimidine nucleoside analogues into DNA of a thymidylate synthetase-deficient murine FM3A carcinoma cell line. Meth and Find Exptl Clin Pharmacol 7:19-28, 1985.
6. Herdewijn P, Van Aerschot A, Kerremans L, Synthesis of nucleosides fluorinated in the sugar moiety. The application of diethylaminosulfur trifluoride to the synthesis of fluorinated nucleosides. Nucleosides & Nucleotides, in press, 1988.
7. Balzarini J, De Clercq E, Torrence PF, Mertes MP, Park JS, Schmidt CL, Shugar D, Barr PJ, Jones AS, Verhelst G, Walker RT, Role of thymidine kinase in the inhibitory activity of 5-substituted-2'-deoxyuridines on the growth of human and murine tumor cell lines. Biochem Pharmacol 31:1089-1095, 1982.
8. De Clercq E, Merigan TC, Moloney sarcoma virus-induced tumors in mice: inhibition or stimulation by (poly rI).(poly rC). Proc Soc Exp Biol Med 137:590-594, 1971.
9. Balzarini J, Baba M, Pauwels R, Herdewijn P, Wood SG, Robins MJ, De Clercq E, Potent and selective activity of 3'-azido-2,6-diaminopurine-2',3'-dideoxyriboside, 3'-fluoro-2,6-diaminopurine-2',3'-dideoxyriboside, and 3'-fluoro-2',3'-dideoxyguanosine against human immunodeficiency virus. Mol Pharmacol 33:243-249, 1988.
10. Bazin H, Chattopadhyaya J, Datema R, Gilljam G, Johansson NG, Koshida R, Öberg B, Stening G, Vrang L, Wahren B, Wu J-C, Inhibition of replication of HIV and MULV by some 3'-blocked pyrimidine analogs. In: Abstracts of the International Symposium on "Basic and Clinical Approaches to Virus Chemotherapy", Helsinki, Finland, June 19-22, 1988. Abstracts, p. 48:T42, 1988.