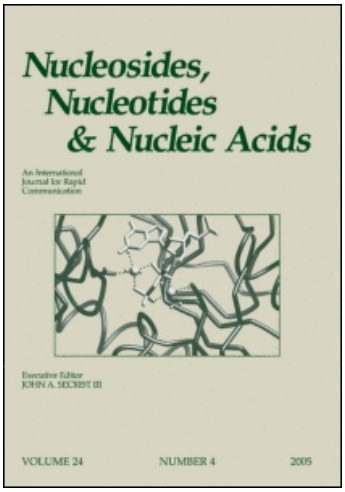

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STEREOSPECIFICITY OF 6'-*C*-NEPLANOCIN A ANALOGUES AS INHIBITORS OF S-ADENOSYLHOMOCYSTEINE HYDROLASE ACTIVITY AND HUMAN IMMUNODEFICIENCY VIRUS REPLICATION†

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ABSTRACT: The *R*- and *S*-isomers of 6'-*C*-neplanocin A analogues, which are all known as inhibitors of S-adenosylhomocysteine (AdoHcy) hydrolase, were studied for their inhibitory effects on Human Immunodeficiency Virus type 1 (HIV-1) replication and HIV-1 Tat-mediated transactivation. The *R*-isomers showed much greater activity against AdoHcy hydrolase than the *S*-isomers. The same differential activity was observed against the HIV-1 replication and the Tat transactivation.

INTRODUCTION

Several carbocyclic and acyclic adenosine analogues are potent (reversible and irreversible) inhibitors of S-adenosylhomocysteine (AdoHcy) hydrolase. In intact cells, carbocyclic adenosine analogues cause a significant increase in the intracellular AdoHcy levels which apparently results from their inhibitory effect on AdoHcy hydrolase (1-3). AdoHcy itself is a product inhibitor of methyltransferase reactions using S-adenosylmethionine (AdoMet) as methyl donor. To avoid this inhibitory effect and to

† Dedicated to the memory of Dr. T. Hata

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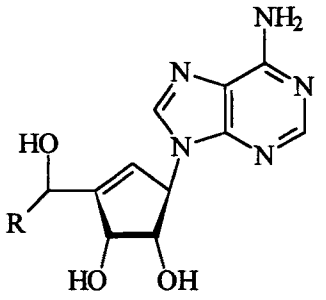
allow the methyltransferases to proceed with their action, AdoHcy has to be removed by AdoHcy hydrolase.

Acyclic and carbocyclic adenosine analogues exhibit a marked broad-spectrum antiviral activity (4, 5). A close correlation has been found between the antiviral activity of the acyclic and carbocyclic adenosine analogues and their inhibitory effect on AdoHcy hydrolase (6). More recently, the activity of 3-deazaadenosine analogues against human immunodeficiency virus type 1 (HIV-1) has been reported (7). Furthermore, adenosine analogues show a close correlation between their inhibitory effects on AdoHcy hydrolase activity and their inhibitory effects on HIV-1 replication as well as HIV-1 Tat transactivation (8). The viruses that fall within the activity spectrum of the AdoHcy hydrolase inhibitors may correspond to those that for the maturation of their viral mRNA depend on methylations requiring S-adenosylmethionine (AdoMet) as methyl donor.

One of the most potent AdoHcy hydrolase inhibitors is neplanocin A (NPA). Neplanocin A has been found to inhibit the Tat-dependent and Tat-independent transactivation of HIV-1 (7, 8). However, neplanocin A is not very specific either as AdoHcy hydrolase inhibitor or as antiviral agent. Since it can serve as a substrate for adenosine deaminase and adenosine kinase, it may be converted to various metabolites and thus act as a multifunctional drug. In attempts to increase the specificity of neplanocin A as an antiviral agent and AdoHcy hydrolase inhibitor, several neplanocin A analogues have been synthesized, such as the 6'-*C*-substituted analogues of neplanocin A (9). We investigated the inhibitory effects of both *R*- and *S*-isomers of these 6'-*C*-neplanocin A analogues on HIV-1 replication and Tat transactivation. We found that the *R*-isomers were much more active against AdoHcy hydrolase, HIV-1 Tat transactivation and HIV-1 replication than the corresponding *S*-isomers.

RESULTS AND DISCUSSION

We have investigated a series of *R*- and *S*-isomers of 6'-*C*-neplanocin A analogues (FIG. 1) with anti-AdoHcy hydrolase activity for their inhibitory effects on HIV-1 replication and Tat transactivation.



(6'*R*)-6'-*C*-methylneplanocin A R : CH_3
(6'*R*)-6'-*C*-ethylneplanocin A R : CH_2–CH_3
(6'*R*)-6'-*C*-ethenylneplanocin A R : $CH{=}CH_2$
(6'*R*)-6'-*C*-ethynylneplanocin A R : C≡CH
(6'*S*)-6'-*C*-methylneplanocin A R : CH_3
(6'*S*)-6'-*C*-ethylneplanocin A R : CH_2–CH_3
(6'*S*)-6'-*C*-ethenylneplanocin A R : $CH{=}CH_2$
(6'*S*)-6'-*C*-ethynylneplanocin A R : C≡CH

FIG. 1: Structural formulae of the 6'-*C*-neplanocin A analogues

A β-galactosidase transactivation assay, based on the expression of the β-galactosidase gene under the control of the HIV-1 promoter, allowed us to quantify the Tat-dependent transactivation of HIV-1. The HIV-1 Tat protein stimulates transcriptional initiation and elongation through interaction with a *cis*-acting element located within the HIV-1 LTR, termed the transactivation response element (TAR) (10-13, and for review, see references 14 and 15).

When tested against HIV-1 transactivation, the most potent inhibitors were (6'*R*)-6'-*C*-methylneplanocin A (IC_{50}: 0.32 μM) and (6'*R*)-6'-*C*-ethynylneplanocin A (IC_{50}: 0.90 μM) (TABLE 1). The *R*-isomer of these compounds showed a much higher activity against AdoHcy hydrolase and against the HIV-1 LTR transactivation than the *S*-isomer. For example, (6'*R*)-6'-*C*-methylneplanocin A (IC_{50}: 0.32 μM) was more than 110-fold more potent against HIV-1 Tat transactivation than the *S*-isomer (IC_{50}: > 36 μM). The same differential inhibitory activity was observed for the *R*- and *S*-isomers of 6'-*C*-ethynylneplanocin A (*R* being >232-fold more active than *S*) and 6'-*C*-ethenylneplanocin A (*R* being >215-fold more active than *S*). The (6'*R*)-6'-*C*-ethylneplanocin A had no inhibitory effect on HIV-1 Tat transactivation, probably because it is too weak an inhibitor of AdoHcy hydrolase activity (IC_{50}: 40 μM). All compounds that proved active in the Tat transactivation assay could be considered as specific inhibitors of the

TABLE 1

Inhibitory effects of *R*- and *S*-isomers of 6'-*C*-neplanocin A analogues on AdoHcy hydrolase, HIV-1 transactivation and HIV-1 replication.

	Inhibition of AdoHcy hydrolase activity	Inhibition of Tat-dependent transactivation	Inhibition of HIV-1 replication	Inhibition of Tat-independent promoter	Toxicity
	IC_{50}[a] (μM)	IC_{50}[b] (μM)	IC_{50}[c] (μM)	IC_{50}[d] (μM)	CC_{50}[e] (μM)
(6'*R*)-6'-*C*-methylNPA	0.1	0.32 ± 0.14	3.5 ± 0.5	>36	>36
(6'*S*)-6'-*C*-methylNPA	256	>36	>36	>36	>36
(6'*R*)-6'-*C*-ethynylNPA	0.25	0.90 ± 0.62	38	>209	>209
(6'*S*)-6'-*C*-ethynylNPA	16	>209	>104	>209	>209
(6'*R*)-6'-*C*-ethenylNPA	2.15	0.96 ± 0.72	6.16 ± 3	>208	>208
(6'*S*)-6'-*C*-ethenylNPA	287	>207	57 ± 52	>208	>208
(6'*R*)-6'-*C*-ethylNPA	40	>206	4.6 ± 0.34	>206	>206
(6'*S*)-6'-*C*-ethylNPA	>500	>206	67 ± 3.7	>206	>206

[a] Concentration of the inhibitor required for 50% inhibition of AdoHcy hydrolase activity.
[b] Concentration of the inhibitor required for 50% inhibition of β-Gal expression driven by the HIV-1 LTR in transfected HeLa-*tat*-III cells.
[c] Concentration of the inhibitor required for 50% inhibition of β-Gal expression induced by viral Tat in infected $CD4^+$ HeLa cells.
[d] Concentration of the inhibitor required for 50% inhibition of β-Gal expression driven by the CMV promoter in transfected HeLa-*tat*-III cells.
[e] Concentration of the inhibitor required for 50% inhibition of total protein expression in cell culture.
Data represent mean values of two to four separate experiments, each of which was performed in triplicate.

HIV-1 Tat transactivation because none of the compounds was able to inhibit the β-Gal expression from a Tat-independent cytomegalovirus (CMV) promoter (TABLE 1).

We have also investigated the inhibition of HIV-1 replication by the 6'-*C*-neplanocin A analogues in HeLa-CD4 cells with stably integrated LTR-*LacZ*. Inhibition in P4 cells (HeLa-CD4-*LacZ* cells) was measured by quantifying the transactivation of the HIV-1 LTR by viral Tat using the *LacZ* reporter gene and by quantifying p24 antigen production in the culture medium. P4 cells were infected with HIV-1_{IIIB} and treated with the compounds. The IC_{50} values for HIV-1 replication obtained for the 6'-*C*-neplanocin A derivatives are given in TABLE 1.

The most potent inhibitors were (6'*R*)-6'-*C*-methylneplanocin A (IC_{50}: 3.5 μM), (6'*R*)-6'-*C*-ethylneplanocin A (IC_{50}: 4.6 μM) and (6'*R*)-6'-*C*-ethenylneplanocin A (IC_{50}: 6.16 μM). The same differential activity of the *R*- and *S*-isomers as seen in the HIV-1 Tat transactivation assay was again observed in the HIV-1 replication assay. The *R*-isomer of 6'-*C*-methylneplanocin A (IC_{50}: 3.5 μM) was more than 10-fold more active against HIV-1 replication than the *S*-isomer (IC_{50}: > 36 μM); (6'*R*)-6'-*C*-ethynylneplanocin A was more than 3-fold more active than (6'*S*)-6'-*C*-ethynylneplanocin A, (6'*R*)-6'-*C*-ethenylneplanocin A 9-fold more than (6'*S*)-6'-*C*-ethenylneplanocin A and (6'*R*)-6'-*C*-ethylneplanocin A 14-fold more than (6'*S*)-6'-*C*-ethylneplanocin A. The compounds were not toxic to the HeLa-CD4 cells with stably integrated LTR-*LacZ* at concentrations tested as measured by total protein content by the Bradford method (TABLE 1).

When evaluated against HIV-1 replication in MT-4 cells or phytohemagglutinin-stimulated peripheral blood lymphocytes infected acutely or chronically with HIV-1, a wide range of adenosine analogues did not inhibit HIV-1 replication for 50% at subtoxic concentrations (8).

As a rule, the differential inhibitory effects of the compounds on HIV-1 replication followed the same pattern as their inhibitory effects on AdoHcy hydrolase activity and Tat-dependent transactivation (TABLE 1), except that (6'*R*)-6'-*C*-ethynylneplanocin A

was less active, and (6'*R*)-6'-*C*-ethylneplanocin A more active, against HIV-1 replication than could be anticipated from their inhibitory effects on AdoHcy hydrolase and Tat transactivation. Probably, additional factors such as cellular uptake and intracellular metabolism may contribute to the anti-HIV activity of (6'*R*)-6'-*C*-ethynylneplanocin A and (6'*R*)-6'-*C*-ethylneplanocin A.

A close correlation was found between the inhibitory effect of a wide range of adenosine analogues on AdoHcy hydrolase activity, their inhibition of HIV-1 replication in HeLa $CD4^+$ LTR-*LacZ* cells and their inhibition of the HIV-1 Tat-dependent and Tat-independent transactivation of the LTR (8).

We conclude that the *R*-isomers of the 6'-*C*-neplanocin A analogues exert much greater activity against AdoHcy hydrolase, HIV-1 transactivation and HIV-1 replication than their *S*-isomers.

EXPERIMENTAL

Compounds. The source of the test compounds was as follows: (6'*R*)-6'-*C*-methylneplanocin A, (6'*S*)-6'-*C*-methylneplanocin A were obtained from Dr. S. Shuto (when at Toyo Jozo Co. Tagata-Gun, Shizuoka-Ken, Japan); and (6'*R*)- and (6'*S*)-6'-*C*-ethynylneplanocin A, (6'*R*)- and (6'*S*)-6'-*C*-ethenylneplanocin A and (6'*R*)- and (6'*S*)-6'-*C*-ethylneplanocin A were obtained from Dr. A. Matsuda (Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan). The structural formulae of the compounds are presented in Figure 1.

Inhibition of AdoHcy Hydrolase. AdoHcy hydrolase was purified from murine L929 cells using affinity chromatography and AdoHcy hydrolase activity was measured in the direction of AdoHcy synthesis, using 8-[^{14}C]Ado (Amersham, Buckinghamshire, England) and 2 mM D,L-homocysteine as substrates, as described earlier (16).

Inhibition of HIV-1 Transactivation. The Tat-dependent transactivation was mainly as described before (17, 18) with the following modifications. HeLa-*tat*-III cells (HeLa cells expressing HIV-1 transactivator, kindly provided by Dr. C. A. Rosen (19), were transfected with pHIV*LacZ*, kindly provided by Dr. J. J. Maio (20) or pCMVβ

(21) plasmid DNA by electroporation with an Eurogentec Genepulser (260 V, 1050 μF and infinite R). pHIV*LacZ* contains a *LacZ* gene driven by the HIV-1 LTR promoter and pCMVβ expresses the *LacZ* gene under control of the CMV promoter.

The cells (60×10^3/well) were incubated in microtiter plates for 24 hours in the presence of varying concentrations of the test compounds. Then, medium was removed by gentle aspiration, and the monolayers were washed with PBS. Cells were lysed with 25 μl of 0.5% NP-40 and β-Gal activity in 20 ml of the cell extracts was quantitated by a colorimetric assay as described by Sambrook *et al.* (22). Five μl of cell extract was used to determine total protein content by using the Bradford method (Bio-Rad). The 50% inhibitory concentration (IC_{50}) was calcultated as being the inhibitor concentration that reduces the β-Gal expression by 50%.

Inhibition of HIV-1 Replication in P4 Cells. In P4 cells (HeLa-CD4-LTR-*LacZ*) the anti-HIV-1 activity of the test compounds was determined by measuring virus-induced β-Gal expression and p24 antigen production. At day -1, the cells were plated in microtiter plates at 2×10^4 cells per well and incubated overnight at 37 °C with 5% CO_2. At day 0, the medium was removed by gentle aspiration and the cells were infected for about 1 hour with an excess of HIV-1_{IIIB} (multiplicity of infection > 1). After infection various concentrations of test compounds were added to the infected cells. Two days after infection virus replication was monitored by measuring β-Gal expression in the cell extracts and by measuring p24 antigen production. β-Gal activity was measured as described above for the transactivation assay and p24 antigen production was measured as described previously (18). The 50% inhibitory concentration (IC_{50}) was calculated as being the inhibitor concentration that reduces β-Gal expression or p24 production by 50%.

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