Review

Development of modified nucleosides that have supremely high anti-HIV activity and low toxicity and prevent the emergence of resistant HIV mutants

By Hiroshi OHRUI[*1,†]

(Communicated by Takao SEKIYA, M.J.A.)

Abstract: An idea to use 4′-*C*-substituted-2′-deoxynucleoside derivatives was proposed based on a working hypothesis to solve the problems of existing acquired immune deficiency syndrome chemotherapy (highly active antiretroviral therapy). Subsequent studies have successfully proved the validity of the idea and resulted in the development of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine and 2′-deoxy-4′-*C*-ethynyl-2-chloroadenosine, nucleoside reverse transcriptase inhibitors, which have supremely high activity against all human immunodeficiency viruses including multidrug-resistant HIV and low toxicity.

Keywords: AIDS, anti-HIV agent, nucleoside reverse transcriptase inhibitor, drug-resistant HIV, 4′-*C*-substituted-2′-deoxynucleoside

Preface

I would like to describe the development of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine and its 2-chloro congener that have high anti-HIV activity and low toxicity and prevent the emergence of resistant HIV mutants, because the development is one of my contributions to the collaboration studies, with Dr. Takeshi Kitahara, a Professor Emeritus of The University of Tokyo, on Invention and Application of Molecules with Novel Biological Functions for receiving The Japan Academy Prize in 2010.

Introduction

Since the discovery of 3′-*C*-azido-3′-deoxythymidine (**AZT**) as the anti-human immunodeficiency virus (**HIV**) agent by Dr. Hiroaki Mitsuya in 1985,[1)] many 2′,3′-dideoxy nucleoside (**ddN**) analogs have been developed as nucleoside reverse transcriptase inhibitors (**NRTI**s). However, resistant **HIV** mutants against all these **NRTI**s have emerged rapidly and easily.

A highly active antiretroviral therapy (**HAART**) using two or more nucleoside reverse transcriptase inhibitors (**NRTI**s) and protease inhibitors has dramatically improved the quality of life and prognosis of patients infected by **HIV**.[2),3)] However, the existing **HAART** has several critical problems that remain to be solved. These problems include: (i) the emergence of new drug-resistant **HIV** mutants, (ii) the need to take large dosages of drugs, and (iii) drug side effects. Therefore, the development of new, highly potent anti-**HIV** drugs that prevent the emergence of drug-resistant mutants and have few side effects is urgently needed.

These problems prompted me to speculate on the reason for the emergence of **HIV** mutant resistant to clinical **NRTI**s, which belong to the family of **ddN**. I proposed a working hypothesis for the chemical structure of a new highly anti-**HIV** active **NRTI**s that might prevent the emergence of drug-resistant mutants. The design of 4′-*C*-substituted-2′-deoxynucleoside (**4′SdN**) was based on the working hypothesis. The working hypothesis for the decrease of toxicity of nucleoside derivatives was also proposed based on my past findings of the relationship between the biological activity and structure of nucleoside derivatives. Study on the synthesis and biological evaluation of **4′SdN**s have proved the validity of the working hypotheses and resulted in the development of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine (**4′Ed2FA**) and 2′-deoxy-4′-*C*-ethynyl-2-chloroadenosine (**4′Ed2ClA**) (Fig. 1). These **NRTI**s are

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doi: 10.2183/pjab.87.53
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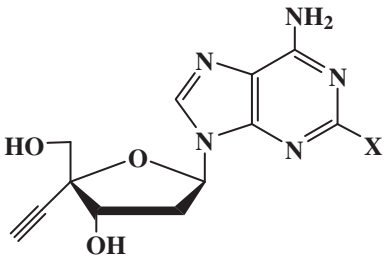


Fig. 1. Structures of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Ed2FA) and its 2-chloro congener (4′Ed2ClA).

supremely active against all **HIV**s and have prevented the emergence of drug-resistant mutants for many years since their development and expected to prevent the emergence of drug-resistant mutants, have a low toxicity, and stable to enzymatic catabolism. Hence, **4′Ed2FA** and **4′Ed2ClA** are important keys for their further development of potential therapeutics to solve the problems being encountered by the present **HAART**.

Working hypotheses of the study

1. Design of the nucleoside derivatives that might be effective for drug-resistant HIV-mutants and possibly prevent the emergence of new drug-resistant HIV mutants. The structures of the clinical **NRTI**s are shown in Fig. 2. All belong to the **ddN** family. The **ddN** structure has been considered essential for the nucleoside derivatives to be the chain terminator of the reverse transcriptase (**RT**)-catalyzed biosynthesis of proviral **DNA**. However, **HIV** mutants resistant to all **ddN-NRTI**s have emerged rapidly and easily.

The emergence of **HIV**-mutants resistant to **ddN**s indicates that **HIV**-mutants acquired the ability to distinguish **ddN**s from physiological 2′-deoxy nucleosides (**dN**s) and do not accept **ddN**s into the active center of **RT** and/or cut off the incorporated **ddN**s from the proviral **DNA** terminus.

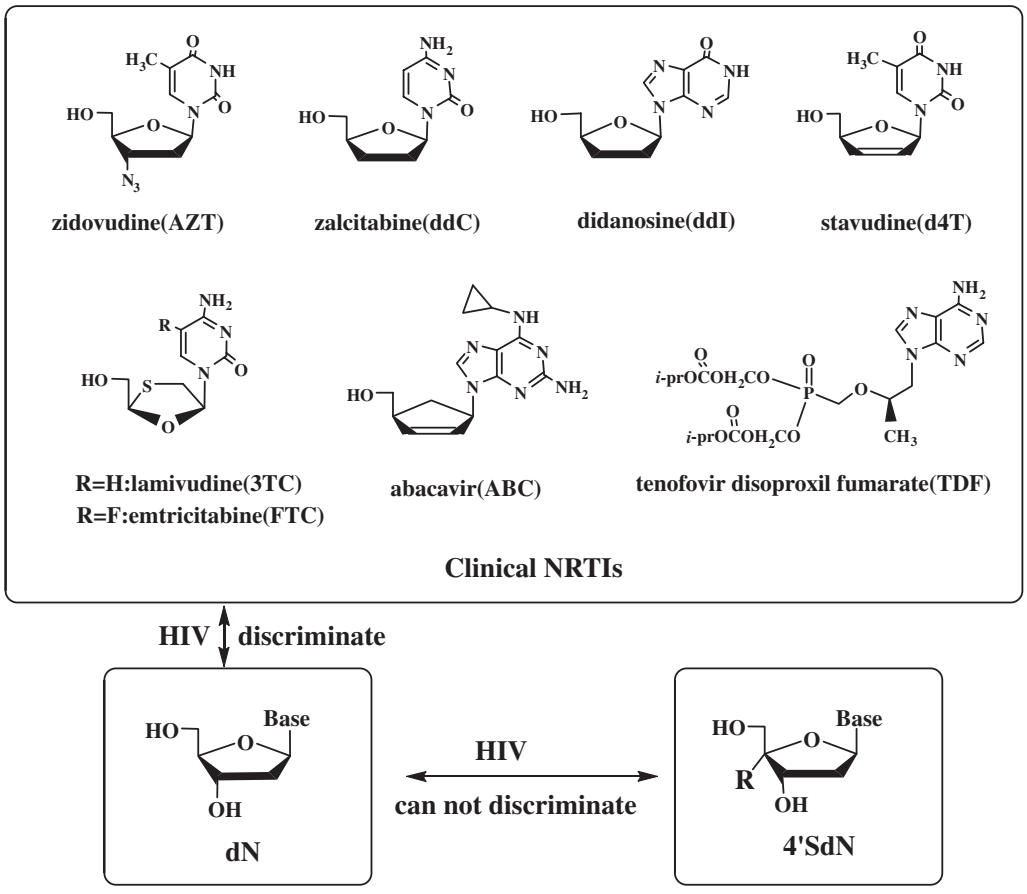


Fig. 2. Structures of clinical nucleosides reverse transcriptase inhibitors (NRTIs), physiologic 2′-deoxynucleoside (4′SdN). HIV = human immunodeficiency virus; dN = 2′-deoxynucleoside, 4′SdN = 4′-*C*-substituted-2′-deoxynucleoside.

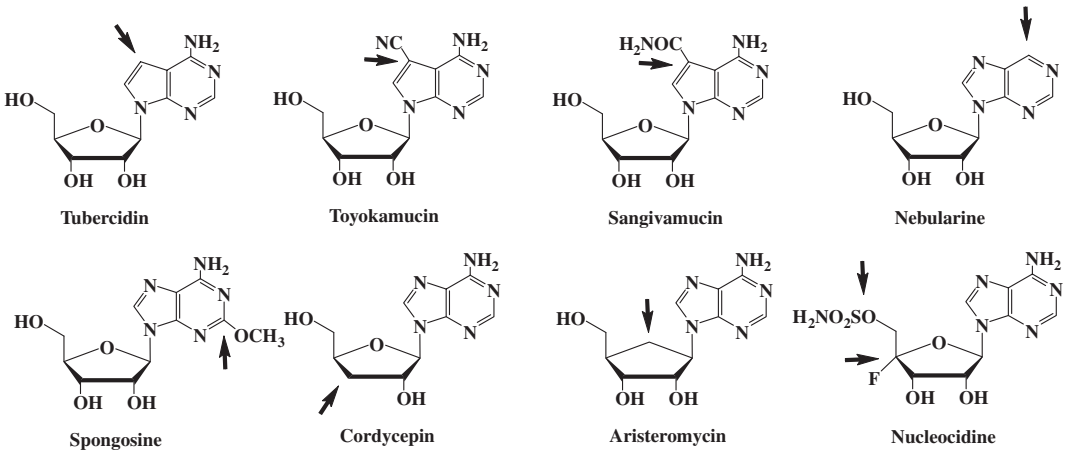


Fig. 3. Structures of the representative nucleoside antibiotics. The arrows point at the modified.

Therefore, the anti-**HIV** nucleosides that might prevent the emergence of drug-resistant **HIV** mutants must satisfy the following two conditions.

1. To prevent the discrimination by **HIV**, the modified nucleosides should have a structure resembling those of physiologic nucleosides as closely as possible so that **RT** mistakes them for physiologic nucleosides. Since the striking difference of the **ddN** and **dN** is whether they have 3′-OH, the modified nucleosides must have 3′-OH.
2. In spite of having 3′-OH, the nucleoside must be the chain terminator of **RT**-catalyzed biosynthesis of proviral **DNA**.

Based on the following hypothesis, **4′SdN** (Fig. 2) was designed as a nucleoside that could satisfy the above mentioned two conditions:

① It would be difficult for **HIV** to discriminate **4′SdN** from **dN** because **4′SdN** has all the functional groups of **dN**.

② The introduction of a substituent at 4′-position makes the 3′-OH into a very unreactive neopentyl-type secondary alcohol. Thus, the 3′-OH of **4′SdN** will be used for **HIV** to mistake **4′SdN** for **dN**, but is too unreactive to be used for the elongation of proviral **DNA** by **RT**. Therefore, **4′SdN**s could be the chain terminators of proviral **DNA** biosynthesis.

③ Steric hindrance between 3′-OH and 4′-substituent changes the conformation of the furanose ring of **4′SdN** preferably to the 3′-endo conformation (**N-type**). This results in **4′SdN** being less susceptible to both acidic and enzymatic glycolysis than **dN** and **ddN**. (In glycolysis, the oxygen atom of the furanose ring participates to form a coplanar oxocarbonium ion, but the conformational change makes it difficult for the oxygen atom to form a coplanar oxocarbonium ion.)

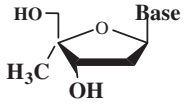
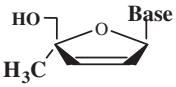
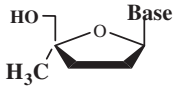
④ Further, the electron-withdrawing 3′-OH makes **4′SdN** more acid stable than does **ddN** even with purines. Thus, various purine derivatives can be made in this way.

⑤ The lipophilic substituent at the 4′-position imparts more lipophilicity to **4′SdN**s, thus enabling them to penetrate the cell membrane efficiently. This possibly enhances their bioavailability.

2. Method of decreasing the toxicity of nucleoside drugs based on my past findings of the relationship between the biological activity and structure of nucleoside derivatives. If **DNA** polymerases also mistake **4′SdN** for **dN**, **4′SdN** should be highly toxic. However, **ddN**s have been used as anti-**HIV** drugs, meaning that **DNA** polymerases hardly accept **ddN**s as their substrates. Thus, the ability of **DNA** polymerases to distinguish substrate is superior to that of **RT**. (The substrate selectivity between **DNA** polymerases and **RT** must be different.)

The structures of the representative nucleoside antibiotic are shown in Fig. 3.[4] Most of them are nucleoside derivatives modified at one site of the physiological nucleosides. Though they are highly active against microorganisms, they are highly toxic, too. Therefore, they can not be clinically used. In the 1960s and 1970s, many organic chemists synthesized nucleoside derivatives modified at two or more positions of physiologic nucleosides expecting to get nucleoside derivatives having new and/or higher

Table 1. Anti-HIV activity of 4′-*C*-methyl-2′-deoxynucleosides

Structure	Base	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
 **4'MedN**	Ad	2.6	2.6	1.0
	Th	7.2	104	
	Cy	0.072	0.13	1.8
	Purine	1.9	>200	>100
 **4'Med4N**	Ad	>500	>500	~1
	Th	21	330	16
	Cy	350	350	
 **4'MeddN**	Ad	30	400	13
	Th	>500	>500	~1
	Cy	27	27	1
AZT		0.001	>20	>2020
ddA		47	>500	>11
d4T		4.1	>500	>120

EC_{50} = 50% effective concentration; CC_{50} = cytotoxic concentration; SI = selectivity index (CC_{50}/EC_{50}); 4′MdN = 4′-*C*-methyl-2′-deoxynucleoside; 4′Md4N = 4′-*C*-methyl-2′,3′-didehydrodideoxynucleoside; 4′MddN = 4′-*C*-methyl-2′,3′-dideoxynucleoside; AZT = 3′-azido-3′-deoxythymidine; ddA = 2′,3′-dideoxyadenosine; d4T = 2′,3′-didehydro-3′-deoxythymidine.

biological activity. However, none of these modified nucleosides exhibited antimicrobial activity. These results indicated that intracellular enzymes do not recognize the nucleoside derivatives modified at two or more positions of physiologic nucleosides as substrates. Thus, there is a chance of decreasing the toxicity of **4′SdN**s further by additional modification.

Results and discussion

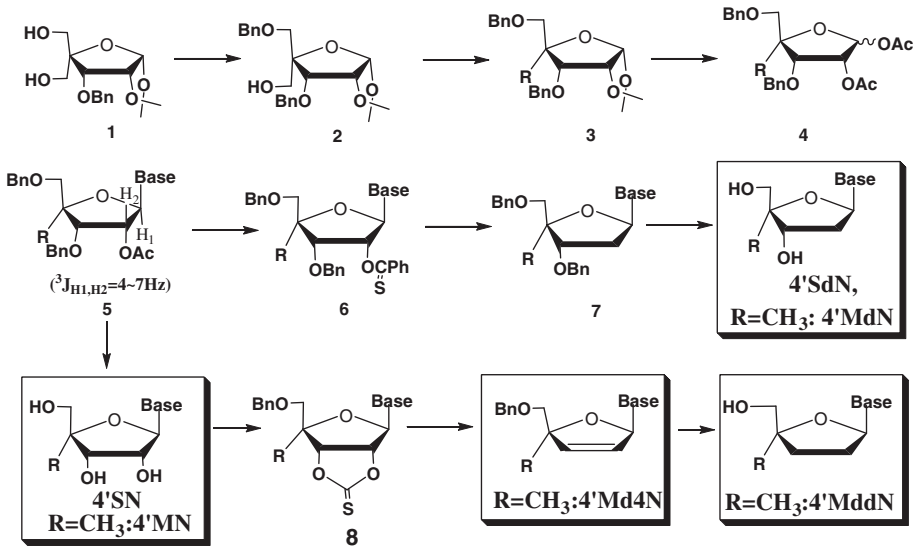
Examination of the validity of the working hypotheses with 4′-*C*-methyl nucleosides. At first, to examine the validity of the working hypothesis, 4′-*C*-methyl-nucleosides (**4′MN**s), 4′-*C*-methyl-2′-deoxynucleosides (**4′MdN**s), 4′-*C*-methyl-2′,3′-dideoxynucleosides (**4′MddN**s), and 4′-*C*-methyl-2′,3′-didehydrodideoxynucleosides (**4′Md4N**s) (Table 1) were synthesized and evaluated for their biological activity.[5),6)] **4′MdN** showed remarkable biologoical activity (both anti-**HIV** activity and toxicity), but **4′MddN** and **4′Md4N** did not show notable biological activity (Table 1). **4′MN**s did not show any anti-HIV activity at all.

These results indicate the importance of the 3′-OH for biological activity. Further, we demonstrated that 5′-*O*-triphosphate of both 4′-*C*-methyl-2′-deoxycytidine (**4′MdCTP**) and 4′-*C*-methyl-D-*arabino*-furanosyl cytidine (**4′MAraCTP**) are the chain terminator of calf thymus **DNA** polymerase α and recombinant rat **DNA** polymerase β.[7)] These results indicate that **4′SdN** was an **NRTI**, although further study of **4′MdCTP** with **RT** was not performed.

They were synthesized by glycosidation of 1,2-di-*O*-acetyl-3,5-di-*O*-benzyl-4-*C*-methyl-D-*ribo*-furanose (4), prepared from D-glucose through 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-*ribo*-furanose (1),[8)] and nucleobases and then deoxygenation of the hydroxyl groups of **4′MN**s (Scheme 1).

It should be noted that the 4′-*C*-methyl *ribo*-furanose derivatives took longer reaction time to complete the glycosidation reaction with silylated nucleoside bases than that of normal *ribo*-furanose derivatives, indicating the lower reactivity of the 4′-*C*-methyl *ribo*-furanoses than normal *ribo*-furanoses. In addition, the $^3J_{1',2'}$ values of 4′-*C*-methyl *ribo*-furanosyl nucleoside were larger (4–7 Hz) than those of the normal β-*ribo*-nucleoside (0–1 Hz), indicating the change of the conformation of the furanose ring of the 4′-*C*-substituted nucleosides.

Structure–activity relationship (SAR) of 4′SdNs. Next, to study the **SAR** of **4′SdN**s and develop **4′SdN**s having more potent anti-**HIV** activity and less toxicity than **4′MdN**s, **4′SdN**s having various kinds of 4′-*C*-substituents and nucleobases were synthesized and evaluated for their biological activity.[9)–16)] While we were working on our project, the anti-HIV activity of several **4′SdN**s

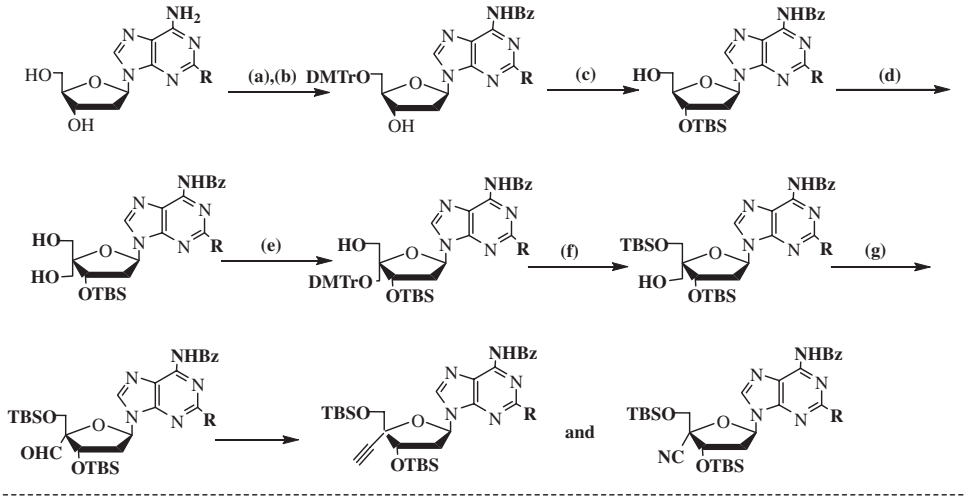


Scheme 1. General synthetic scheme of 4′-*C*-substituted-2′-deoxynucleoside (4′SdNs) from D-glucose. 4′SdN = 4′-*C*-substituted-2′-deoxynucleoside; 4′MN = 4′-*C*-methylnucleoside; 4′Md4N = 4′-*C*-methyl-2′,3′-didehydrodeoxynucleoside; 4′MddN = 4′-*C*-methyl-2′,3′-dideoxynucleoside.

Table 2. Anti-HIV activity of 4′-*C*-substituted-2′-deoxynucleosides

Compound	EC_{50} (mM)[a)]	CC_{50} (mM)	S.I.
4′-*C*-cyanothymidine	0.002	1	500
4′-*C*-azidothymidine	0.01	8	300
4′-*C*-ethynylthymidine	0.83	>400	>482
4′-*C*-ethynylarabinofuranosylthymine	119	>400	>3.4
4′-*C*-azidomethylthymidine	2.1	333	159
4′-*C*-methylthymidine	7.2	104	14
4′-*C*-ethylthymidine	>400	400	ND
4′-*C*-methoxythymidine	8.49	200	24
4′-*C*-vinylthymidine	>400	>400	ND
4′-*C*-hydroxymethylthymidine	7.0	>400	>57
4′-*C*-propylthymidine	>100	>100	ND
4′-*C*-cyano-2′-deoxycytidine	0.0012	0.17	142
4′-*C*-azido-2′-deoxycytidine	0.004	0.21	52
4′-*C*-ethynyl-2′-deoxycytidine	0.0048	2.2	458
L-4′-*C*-ethynyl-2′-deoxycytidine	>400	>400	ND
4′-*C*-ethynyl-2′-deoxy-5-fluorocytidine	0.030	>100	>3333
4′-*C*-ethynylarabinofuranosylcytidine	0.043	2.0	46.5
4′-*C*-methyl-2′-deoxycytidine	0.015	1.0	66.7
4′-*C*-fluoromethyl-2′-deoxycytidine	0.0068	0.12	18
4′-*C*-methyl-2′-deoxyadenosine	2.6	2.6	1
4′-*C*-azido-2′-deoxyadenosine	0.13	50	385
4′-*C*-ethynyl-2′-deoxyadenosine	0.098	16	1630
2′,3′-dideoxy-3′-thia-L-cyrtidine (3TC)	0.10	>100	>1000
3′-azido-3′-deoxythymidine (AZT)	0.0032	29.4	9190

a) Anti-HIV activity was determined by MTT assay. MT-4 cells and HIV-1_{LAI} were employed.
ND: not determined.



Scheme 2. Synthetic scheme of purine derivatives of 4′-*C*-cyano-2′-deoxynucleoside and 4′-*C*-ethynyl-2′-deoxynucleoside from 2′-deoxynucleosides. TFA = trifluoroacetic acid; DMF = dimethylformamide; rt = room temperature.

was reported by the Syntex group[17)–21)] and others.[22),23)] Therefore, the anti-**HIV** activities of **4′SdN**s that we studied together with those reported by other groups are listed in Table 2.

The **SAR**s of 4′-*C*-substituted nucleosides against **HIV** are summarized as follows:

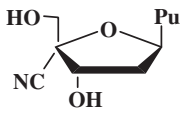
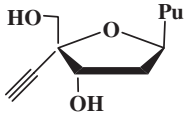
1. The estimated relative order of anti-**HIV** activity is as follows: $CN \geq C{\equiv}CH > N_3 > CH{=}CH_2 > Me = Et > C{\equiv}C{-}CH_3$. Interestingly, the order is the reverse of the $-\Delta G^\circ$ values between equatorial and axial substituents on a cyclohexane ring: $CN < F < C{\equiv}CH < CH{=}CH_2 < Me \leq Et < {}^tBu$. Thus, these results indicate that the sterically less demanding substituent at the 4′-position gives more potent anti-HIV activity.
2. Purine analogs are generally less toxic than pyrimidine. Although 2′-deoxy-4′-*C*-ethynyl-5-fluorocytidine, which is a nucleoside derivative modified at two positions of physiologic 2′-deoxycytidine, gave a very acceptable Selectivity Index (**SI** = CC_{50}/EC_{50}) with MT-4 cells, it was toxic with other cells (Kohgo, Yamasa Corporation, private communication).
3. *Arabino* analogs are less active and less toxic compared with their corresponding 2′-deoxy counterparts.
4. **4′SddN**s do not show anti-**HIV** activity.
5. The L-isomers of **4′SdN** have no anti-**HIV** activity,[14)] although it is known that the L-enantiomer of 2′,3′-dideoxy-3′-thia-L-cytidine (**3TC**) is as active as the D-enantiomer and less toxic than the D-isomer.[24)] This may be due to that the L-isomers are too much modified to be recognized by **RT** as its substrates.

Synthesis of purine derivatives of 4′-*C*-cyano-2′-deoxynucleoside (4′CNdNs) and 4′-*C*-ethynyl-2′-deoxynucleoside (4′EdNs) and their anti-HIV activity. The mentioned results led us to study the biological activity of purine derivatives of **4′CNdN** and **4′EdN**.[25)] Although **4′SdN**s in our project had been synthesized by the glycosidation of 4-*C*-substituted-D-*ribo*-furanose derivatives and nucleobases, this synthetic route incurred some problems, as follows:

1. Preparation of 4-*C*-substituted-D-*ribo*-furanoses and their conversion to the desired **4′SdN**s require multistep reactions, and their total yields are low.
2. 4-*C*-Substituited-D-*ribo*-furanose derivatives have low reactivity in glycosidation reactions, especially when the substituent is an electron-withdrawing group such as a cyano group. (In contrast, the low reactivity of the anomeric position of 4-*C*-substituted furanose derivatives indicates that **4′SdN** will be more stable to acidic hydrolysis than **dN**, **ddN**, and **d4N** will.)

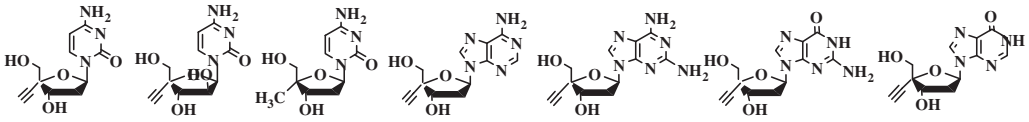
These problems prompted us to develop another method of preparing purine derivatives of **4′SdN**, which starts from **dN**s (Scheme 2).[25)]

Table 3. Anti-HIV activity of purine derivatives of 4′-*C*-cyano-2′-deoxy (4′CNd) and 4′-*C*-ethynyl-2′-deoxynucleosides

Structure	Base	EC_{50} (μM)[a)]	CC_{50} (μM)	S.I.
	A	0.051	12	235
	I	0.051	23	451
	2AA[b)]	0.00079	0.034	43
	G	0.000188	0.034	181
	A	0.098	16	1630
	I	0.15	216	1440
	2AA	0.0003	0.82	2733
	G	0.0014	1.5	975
AZT		0.0032	29.4	9190

a) Anti-HIV activity was determined by MTT assay. MT-4 cells and HIV-1_{LAI} were employed.
b) 2-aminoadenine.

Table 4. Anti-HIV activity of selected 4′-*C*-substituted-2′-deoxynucleoside against wild type HIV and drug-resistant HIVs



4'EdC **4'EaraC** **4'MdC** **4'EdA** **4'Ed2AA** **4'EdG** **4'EdI**

Compound	EC_{50} (μM)[a)]									
	HXB2[b)]	KH65R	L74V	41/215	M184V	M184I	41/69/125/SG	MDR[c)]	Y181C	CC_{50} (μM)
4′EdC	0.0012	0.0008	0.0013	0.006	0.0024	0.0026	0.015	0.0012	0.0021	>200
4′EaraC	0.0071	0.015	0.026	0.026	0.71	0.48	0.17	0.0079	0.016	>200
4′MedC	0.0058	0.0071	0.0062	ND	0.2	0.74	ND	0.0033	ND	>200
4′EdA	0.008	0.0033	0.004	0.012	0.047	0.022	0.065	0.0062	0.011	>200
4′Ed2AA	0.0014	0.00035	0.0007	0.0017	0.0059	0.0027	0.0041	0.001	0.0008	>200
4′EdG	0.007	0.001	0.0012	0.019	0.008	0.0041	0.0068	0.0048	0.01	52
4′EdI	0.81	0.25	0.61	1.3	1.6	1.5	2.2	0.51	ND	>200
AZT	0.022	0.02	0.02	0.3	0.01	0.017	1.6	15.3	0.014	>100
3TC	0.71	ND	ND	ND	>100	>100	9.9	1.1	ND	>100
ddC	0.2	3.0	1.5	ND	2.2	ND	1.3	5.5	ND	>100
ddI	3.9	12.7	19.5	3.6	10.1	ND	12.2	25	ND	>100

a) Anti-HIV activity was determined with MAGI assay, ND: not determined. b) wild type HIV. c) multidrug-resistant HIV.

The biological activities of the purine derivatives of **4′CNdN** and **4′EdN** are summarized as follows (Table 3):

1. Some of the purine derivatives of **4′CNdN** have high anti-**HIV** activity, but none of them gives an acceptable **SI**.
2. All the purine derivatives of **4′EdN** have both high anti-**HIV** activity and an acceptable **SI**.

Anti-HIV activity of 4′SdNs against drug-resistant HIV mutants.[13),14),25)] Many **4′SdN**s showed very high anti-HIV activity against wild-type **HIV**. However, the most important point of our study is whether they are active against drug-resistant **HIV**-mutants. The anti-**HIV** activity of selected **4′SdN**s against **HIV** mutants resistant to various **NRTI**s is listed in Table 4.

It is noteworthy that the three cytidine derivtives maintained their activity against the drug-resistant HIV mutants, although the activity of 4′-*C*-ethynyl *D-arabino*-furanosyl cytosine (**4′EaraC**) and **4′MdC** decreased significantly against M184V, M184I, and

Table 5. Toxicity of purine derivatives of 4′-*C*-ethynyl-2′-deoxynucleosides to mice[a)]

	Intraveneous administration		Oral administration	
	Dose (mg/Kg)	Mortality (%)	Dose (mg/Kg)	Mortality (%)
4′EdA and 4′EdI	100	0	100	0
	10	0	10	0
	3	0	3	0
	1	0	1	0
4′Ed2AA	100	100 (1 day)[b)]	100	100 (1 day)
	10	100 (2 days)	10	100 (2 days)
	3	0	3	100 (2 days)
	1	0	1	0
4′EdG	100	100 (1 day)	100	100 (1 day)
	10	100 (2 days)	10	100 (4 days)
	3	100 (4 days)	3	100 (4 days)
	1	0	1	0

a) Six-week-old ICR male mice were employed.
b) Numbers in parentheses represent survival days of mice after administration.

41/69/125/SG. The three purine derivatives, 2′-deoxy-4′-*C*-ethynyladenosine (**4′EdA**), 2′-deoxy-4′-*C*-ethynyl-2-aminoadenosine (**4′Ed2AA**), and 2′-deoxy-4′-*C*-ethynylguanosine (**4′EdG**) except for 2′-deoxy-4′-*C*-ethynylinosine (**4′EdI**) were highly potent against all drug-resistant HIV-mutants (**4′EdI** was much less active than the former three derivatives, especially against M184V). Additionally, the three were also active against a non-nucleoside reverse transcriptase inhibitor-resistant Y181C. Further, the three purine derivatives were highly potent against the **HIV**s isolated from seven heavily drug-experienced patients with acquired immune deficiency syndrome (**AIDS**) as efficiently as against wild-type **HIV**.[15),16),26)] Thus, **4′EdA**, **4′Ed2AA**, and **4′EdG** were highly potent against all the existing **HIV**s.

These results let us suppose that the three purine **4′EdN**s could even prevent the emergence of drug-resistant **HIV**s. It should be noted that **4′EdG** showed toxicity to *Hela* cells at 52 μM.

Mouse toxicity of purine 4′EdNs. Because the three purine derivatives of **4′EdN**s showed high activity against all **HIV**s and acceptable **SI**s, the mouse toxicity of these **4′EdN**s was next examined (Table 5).[25),26)]

All eight mice survived after a single dosage of 3–100 mg kg^{-1} of **4′EdA** and **4′EdI** by both intravenous and oral administrations, but all mice died after a single dosage of 3 mg kg^{-1} of **4′Ed2AA** and **4′EdG** irrespective of the administration method (Table 5). Thus, it seemed that **4′EA** and **4′EI** were not toxic, but **4′E2AA** and **4′EdG** were highly toxic.

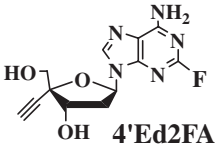
However, in mice, it was found that **4′EdA** and **4′Ed2AA** were easily converted to **4′EdI** and **4′EdG**, respectively, by adenosine deaminase.[25),26)] These results showed that the actual toxicity of **4′EdA** and **4′Ed2AA** to animals is hard to estimate.

Anti-HIV activity of 4′EdA derivatives stable to adenosine deaminase. The fact that both **4′EdA** and **4′Ed2AA** are deaminated by adenosine deaminase prompted us to prepare **4′EdA** derivatives stable to the enzyme. It has been known that the adenine derivatives having a halogen atom at the 2-position of the base are stable to adenosine deaminase.[27),28)] Therefore, at first, **4′Ed2FA** (Fig. 1) was synthesized and evaluated for anti-**HIV** activity[28)–31)] and stability to both adenosine deaminase and acidic conditions.

Because **4′Ed2FA** is a nucleoside derivative modified at two positions (4′-position and 2-position) of physiologic 2′-deoxyadenosine, the toxicity of **4′Ed2FA** is expected to be lower than that of **4′EdA**. As shown in Table 6, **4′Ed2FA** is highly potent against all HIVs including multidrug-resistant and M184V HIV mutants and has an acceptable **SI** (110,000).[28)–31)]

Expectedly, **4′Ed2FA** was completely stable to adenosine deaminase under the conditions where **4′EdA** was completely deaminated in 60 min and, further, fairly stable under acidic conditions. Thus, in 120 min only a small part (3%) of **4′Ed2FA** was decomposed under the acidic conditions of gastric juices (pH 1.06) at 24 °C, while 2′,3′-dideoxyadenosine (**ddA**) was completely decomposed in 5 min.

Table 6. Anti-HIV activity of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Ed2FA)

HIV-1	Compound	EC_{50} (nM)	CC_{50} (nM)	SI
Wild-type	4′Ed2AA	0.34	900	2,600
	4′Ed2FA	0.068	7,500	110,000
	AZT	3.2	29,400	9,190
M184V	4′Ed2FA	3.1	 **4'Ed2FA**	m.p. (decomp.) 224.4–224.6 °C $[\alpha]_D$ +4.64 (c = 0.5, DMSO)
	AZT	16.0		
MDR	4′Ed2FA	0.15		
	AZT	15,300.00		

EC_{50} = 50% effective concentration; CC_{50} = 50% cytotoxic concentration; SI = selectivity index = CC_{50}/EC_{50}

Appearance of two papers claiming that 3′-OH of 4′EdNs is the cause of the toxicity of 4′EdNs. While we were working on our project, two papers on the role of 3′-OH of **4′SdN**s appeared in which the authors claimed that 3′-OH was the cause of the toxicity of **4′SdN**s. Tanaka and coworkers reported[32] that 4′-*C*-ethynyl-2′,3′-didehydro-3′-deoxythymidine (**4′Ed4T**) was more potent against wild-type **HIV** and multi-drug resistant **HIV** mutant but a little less potent against M184V-mutant and much less toxic than **d4T**. Further, they claimed that 3′-OH of **4′SdN**s was the cause of the toxicity.

In contrast, Marquez and coworkers reported[33] that 3′-OH of **4′SdN**s played an important role in the phosphorylation of 5′-OH by cellular kinases, but was the cause of the toxicity of **4′SdN**s. This determination was based on their results that 4′-*C*-ethynyl-2′,3′-dideoxycytidine (**4′EddC**) was inactive against **HIV** in cellular systems, but its 5-O-triphosphate (**4′EddCTP**) was more active than 3′-azido-3′-deoxythymidine-5′-O-phosphate (**AZTTP**) against the **RT** of wild-type **HIV**. They also reported that **4′EddCTP** was much less active against **RT** of the M184V mutant than against the **RT** of wild-type **HIV**. In addition, the L-isomer of **4′EddCTP** was not active against the **RT** of the M184V mutant.

Anti-HIV activity of 2′,3′-dideoxy (dd-) and 2′,3′-didehydrodideoxy (d4) analogs of 4′Ed2FA. The two papers previously cited caused us to synthesize the dd- and d4-analogs of **4′Ed2FA** and evaluate their anti-**HIV** activity.[31] The anti-**HIV** activities of 2′,3′-dideoxy-4′-*C*-ethynyl-2-fluoroadenosine (**4′Edd2FA**) and 2′,3′-didehydrodideoxy-4′-*C*-ethynyl-2-fluoroadenosine (**4′Ed42FA**) together with that of 2′-deoxy-4′-*C*-ethynyl-2-chloroadenosine (**4′Ed2ClA**) are listed in Table 7.

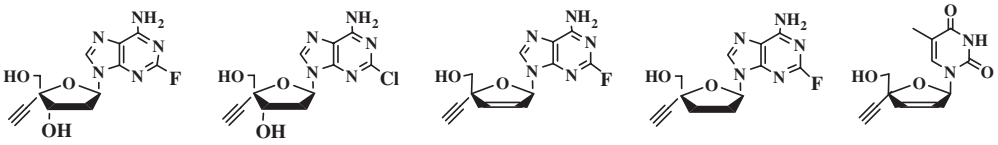
Although both **4′Ed42FA** and **4′Edd2FA** showed some activity against wild-type **HIV**, they significantly lost any activity against drug-resistant **HIV**s. **4′Ed2ClA** is highly active against all **HIV**s, however, its activity is lower than that of **4′Ed2FA**. These results indicated that the 3′-OH played important roles not only for the phosphorylation of 5′-OH, but also for the activity against drug-resistant **HIV**s. Further, these results together with those reported by Tanaka and coworkers[32] indicated that the 4′-*C*-ethynyl group played an important role for the anti-HIV activity. Recently, it was reported that the active center of the **RT** of **HIV**s has a narrow lipophylic cavity to accept preferably the ethynyl group, thus 4′-*C*-ethynyl nucleoside derivatives have potent anti-**HIV** activity.[34]

Toxicity of 4′Ed2FA to mice and inhibition of DNA polymerases. Because **4′Ed2FA** is stable to adenosine deaminase, its mouse toxicity was examined.[29)–31),33),35] **4′Ed2FA** did not show any acute toxicity to mice by either oral or intravenous administration up to 100 mg kg^{-1} (Fig. 4; Table 8).

It is known that the toxicity of **NRTI**s to animals is caused by their inhibition of mitochondrial **DNA** polymerase γ. The 50% effective concentration (**EC_{50}**) of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine-5-*O*-triphosphate (**4′Ed2FATP**) to inhibit the incorporation of 2′-deoxyadenosine-5-*O*-triphosphate (**dATP**) mediated by human mitochondrion DNA polymerase was 10 µM, which was significantly higher than the 0.2 µM of 2′,3′-dideoxyadenosine-5-*O*-triphosphate (**ddATP**).[30),31),35] The **EC_{50}** values of **4′Ed2FATP** against **DNA** polymerase α and β were higher than 200 µM. These results indicate that the **DNA** polymerases scarcely recognize **4′Ed2FATP**, a derivative modified at two positions of physiologic **dATP**, as their substrate but that **RT** does.

Intracellular metabolism of 4′Ed2FA.[29] The amounts of all fractions of intracellular **4′Ed2FA**

Table 7. Anti-HIV activity of 4′-*C*-substituted-2′-deoxy-2-haloadenosines



4'Ed2FA **4'Ed2ClA** **4'Ed42FA** **4'Edd2FA** **4'Ed4T**

Compound	Anti-HIV activity (MAGI assay, μg)			
	HIV-1_{wild}	HIV-1_{MDR}	HIV-$1M_{184V}$	SI
4′Ed2FA	0.00020	0.00014		110,000
4′Ed2ClA	0.0019	0.0084	0.01	330,000
4′Ed42FA	0.80	0.15	1.8	
4′Edd2FA	0.94	8.7	97	
AZT	0.17	74.3	0.13	
3TC	1.0	2.8	>100	
4′Ed4T	1.5	1.1	17	>50,000
d4T	7.6	64	5.6	

MAGI = multinuclear activation of galactosidase indicator; HIV = human immunodeficiency virus; AZT = 3′-azido-3′-deoxythymidine; 3TC = 2′,3′-dideoxy-3′-thia-L-cytidime, d4T = 2′,3′-didehydrodideoxythymidine.

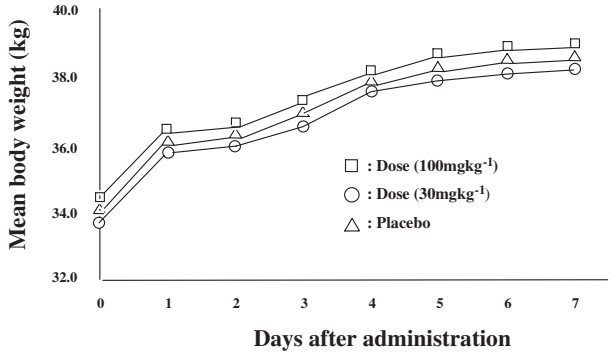


Fig. 4. Body weight change of mice after a single dosage of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosin, administrated orally or intravenously to ICR mice.

Table 8. Toxicity of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Ed2FA) after a single dosage to ICR mice

Dose ($mg\,Kg^{-1}$)	Survivors/total	
	p.o	i.v.
Placebo	8/8	8/8
1	8/8	8/8
3	8/8	8/8
10	8/8	8/8
30	8/8	8/8
100	8/8	8/8

metabolites, (**4′Ed2FA**-monophosphate, **4′Ed2FA**-diphosphate, and **4′Ed2FATP**) increased proportionately with an increase in the concentration of intracellular **4′Ed2FA**, while compared to **AZT** diphosphate and **AZTTP**, only **AZT** monophosphate markedly increased with an increase in intracellular **AZT** concentration. The intracellular half-life ($\mathbf{T_{1/2}}$) of **4′Ed2FATP** was ∼18 h in complete expansion media (CEM) cells, MT4 cells, and multinuclear activation of galactosidase indicator (MAGI)-CCR5 cells ($\mathbf{T_{1/2}}$ of **AZTTP** was 3 h). About 50% of the cells were protected against the infection of **HIV** for 24 h after removal of extracellular **4′Ed2FA** in both MT4 cells and MAGI cells cultured in the presence of 0.1 μM of **4′Ed2FA**.[30),31),36)] These results indicate that **4′Ed2FA**, 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine-5-*O*-diphosphate (**4′Ed2FADP**), and **4′Ed2FATP** are very stable against intracellular enzymatic catabolism.

Summary

A study of the synthesis and biological evaluation of **4′SdN**s was conducted according to the proposed working hypotheses based on the fundamentals of both organic chemistry and biochemistry. All scientific evidences proved the validity of the working hypotheses and resulted in the development of **4′Ed2FA**, which is highly potent against all **HIV**s, is stable to intracellular enzymatic catabolism and acidic degradation, has a very long

intracellular $\mathbf{T_{1/2}}$, does not greatly inhibit **DNA** polymerase γ, and does not have acute mouse toxicity.

These results strongly suggest that **4′Ed2FA** deserves further study for the development of a highly potent therapeutic agent for **HIV** infection (**AIDS**), and which may solve the problems of the existing **HAART**.

It should be noted that **4′Ed2ClA** is also highly potent against all **HIV**s, though it is a little less potent than **4′Ed2FA**, and has better **SI** than **4′Ed2FA**, Therefore, **4′Ed2ClA** deserves also for further study.

The above study together with the recent studies by others[37),38)] on the development of anti-**HCV** active and/or anti-tumor active modified nucleosides which are chain-terminators of **RNA** polymerase, prompted the author to propose the structure of modified nucleosides expected to have high antiviral activity and low toxicity.[39)]

Acknowlegement

The work described in this paper was achieved through the tremendous efforts of the many co-workers whose names appear in the cited references.

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(Received Nov. 24, 2010; accepted Jan. 14, 2011)

Profile

Hiroshi Ohrui was born in 1942. He received his B.S. (1965) and Ph.D. degrees (1971) from The University of Tokyo under the direction of Professor Masanao Matsui. He joined the Institute for Physical and Chemical Research (RIKEN) in 1966 as a researcher. In 1981, he moved to Tohoku University as Associate Professor in the Department of Food Chemistry, Faculty of Agriculture. In 1997, he was promoted to Full Professor, Graduate School of Life Science. In 2006, he moved to Yokohama College of Pharmacy as Professor. He worked for Dr. J. J. Fox at Sloan-Kettering Institute for Cancer Research (1972–1973) as Research Associate, and for Dr. J. G. Moffatt at Syntex Research (1973–1974) as a postdoctoral fellow. He has received several awards including The Agricultural Society of Japan Award for Young Scientists (1974), Inoue Prize for Science (2001), Japan Prize of Agricultural Sciences (2004), The Japan Society for Analytical Chemistry Award (2004), and the Japan Academy Prize (2010). His research interests cover organic synthesis, chiral discrimination, and medicinal chemistry.