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A New Paradigm for Developing Antiviral Drugs Exemplified by the Development of Supremely High Anti-HIV Active EFdA

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Abstract

The fundamental concept for developing anti-viral modified nucleoside was proposed. An idea to use 4'-*C*-substituted-2'-deoxynucleoside derivatives based on the fundamental concept was also proposed to solve the problems of the existing highly active antiretroviral therapy (**HAART**).

Keywords: Anti-viral drug; Substrate selectivity of nucleic acid polymerase; Anti-HIV agent; Drug-resistant HIV; 4'-*C*-substituted-2'-deoxynucleosid

Introduction

The highly active anti-retroviral therapy (**HAART**) has dramatically improved the quality of life and the prognosis of the patients infected by HIV [1,2]. However, the existing **HAART** has critical problems to be solved. They are (i) emergence of drug-resistant HIV mutants, (ii) drug side effects, and (iii) the need to take large doses of drugs. Therefore, the development of highly potent anti-HIV drugs that prevent the emergence of drug-resistant mutants and have few side effects is required.

The fundamental concept of this study is based on the mutation of viruses. Viruses adapt themselves to the environmental change by mutation. Mutation is that viruses change their genes by taking incorrect (not-programmed) nucleosides into their genes. The fact indicates that the substrate selectivity of viral nucleic acid polymerases is not strict. On the other hand, human beings seldom change their genes. This indicates that the substrate selectivity of human nucleic acid polymerases is very strict. Therefore, by taking the advantage of the difference of the substrate selectivity between viral and human nucleic acid polymerases, it is possible to develop modified nucleosides that are more selectively active to viruses and not active to human beings.

Proposal of the Working Hypotheses to Solve the Problems

The following working hypotheses were proposed to solve the problems.

The method to prevent the emergence of resistant HIV mutants [Design of 4'-*C*-substituted-2'-deoxynucleoside (4'SdN) that could Prevent the Emergence of Drug-Resistant HIV Mutants]

All the clinical nucleoside reverse transcriptase inhibitors (**NRTIs**) belong to the family of 2',3'-dideoxynucleoside (**ddN**) (Figure 1).

The **ddN** structure has been assumed essential for the modified nucleosides to be the chain-terminator of **RT**. However, resistant **HIV** mutants against all these drugs emerged very easily and promptly.

The emergence of **HIV**-mutants resistant to **ddNRTIs** indicates that the resistant **HIV**-mutants have obtained the ability to discriminate **ddNs** from the physiologic 2'-deoxynucleoside (**dN**) and do not accept the **ddNs** into the active centre of their **RT** and/or cut off the incorporated **ddNs** from the pro-viral **DNA** terminus.

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 from **dN** by **HIV**, the modified nucleosides should have a structure resembling those of **dN** as closely as possible so that **RT** mistakes them for **dN**.

Since the striking difference of **ddN** and **dN** is whether they have 3'-OH, the modified nucleosides must have 3'-OH.

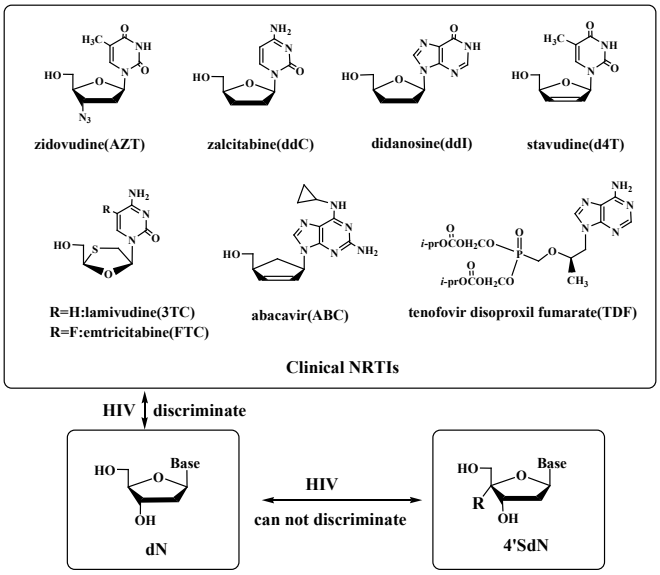

Figure 1: Structures of Nucleoside Reverse Transcriptase Inhibitor (**NRTIs**), physiologic 2'-deoxynucleoside(**dN**), and 4'-*C*-substituted-2'-deoxynucleoside (**4'SdN**).

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2. In spite of having 3'-OH, the nucleoside must be the chain terminator of **RT**-catalyzed biosynthesis of pro-viral **DNA**.

Based on the following hypotheses, **4'SdN** (Figure 1) was designed as a nucleoside that could satisfy the above mentioned two conditions.

- It would be difficult for **HIV** to discriminate **4'SdN** from **dN** because **4'SdN** has all the functional groups of **dN**.
- 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** mistakes **4'SdN** for **dN**, but is too unreactive to be used for the elongation of pro-viral **DNA** by **RT**. Therefore, **4'SdN** could be the chain terminator of pro-viral **DNA** biosynthesis.
- The 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 *N*-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).
- Further, the electron-withdrawing 3'-OH makes **4'SdN** more acid stable than does **ddN** even purines. Thus, various purine derivatives can be made in this way.
- The lipophilic substituent at 4'-position imparts more lipophylicity to **4'SdN**s, thus enabling them to penetrate the cell membrane efficiently. This possibly enhances their bioavailability.

The method to decrease the toxicity of nucleoside

If human **DNA** polymerase also mistakes **4'SdN** for **dN**, **4'SdN** would be highly toxic. However, **ddN**s, which are the chain terminators of **DNA** polymerase according to Sanger Method for **DNA** sequencing [3] and therefore toxic nucleosides, have been used as anti-**HIV** drugs. These facts mean that **RT** accepts them as their substrates but **DNA** polymerase hardly does. Thus, the ability of **DNA** polymerase to discriminate substrate is superior to that of **RT**. Therefore, the substrate selectivity between **DNA** polymerase and **RT** is different. Thus, by taking the advantage of the difference of the substrate selectivity, it will be possible to develop modified nucleosides which are more selectively active to viruses and less active to human beings than the clinical **NRTI**s.

The structure of the representative nucleoside antibiotics are shown in Figure 2 [4]. Most of them are nucleoside derivatives modified at one site of the physiologic nucleosides. Though they are highly active against microorganisms, they are highly toxic, too. Therefore, they cannot be clinically used. In the 1960s and 1970s, many organic chemists modified these nucleosides expecting to get nucleoside derivatives having new and/or better biological activity. However, the additional modification of them resulted in the loss or decrease of their activity. The same results were obtained with synthetic modified nucleosides. Namely, highly active one position modified nucleosides are highly toxic, too. The modification of them also resulted in the loss or decrease of their activity. Since the loss and decrease of antibiotic activity means the loss and decrease of toxicity, there is a chance of decreasing the toxicity **4'SdN**s by additional modification.

Results and Discussion

Examination of the validity of the working hypotheses with 4'-*C*-methyl nucleosides

On the basis of the working hypotheses, the synthesis and biological evaluation of **4'SdN** were carried out. At first, to examine the validity of the working hypothesis, 4'-*C*-methyl D-*ribo*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 biological activity (both anti-**HIV** activity and toxicity), but **4'MddN** and **4'Md4N** did not show notable biological activity (Table 1).

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'MdC-TP**) and 4'-*C*-methyl-D-*arabino* furanosyl cytidine (**4'MAraC-TP**) are the chain terminator of calf thymus **DNA** polymerase α and recombinant rat **DNA** polymerase β [7]. These results indicate that **4'SdN** is **NRTI**, although further study of **4'MdC-TP** with **RT** was not performed. 4'-*C*-Methyl-D-*ribo*furanosyl nucleosides (**4'MN**s) did not show any anti-**HIV** activity

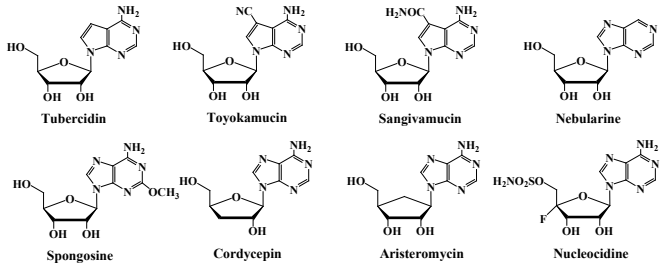


Figure 2: Structures of nucleoside antibiotics.

Structure	Base	EC_{50}(μM)	CC_{50}(μM)	SI(CC_{50}/EC_{50})
4'MdN	Ad	2.6	2.6	1.0
	Th	7.2	104	-
	Cy	0.072	0.13	1.8
	Purine	1.9	>200	>100
4'Md4N	Ad	>500	>500	~1
	Th	21	330	16
	Cy	350	350	-
4'MddN	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-Selectivitiy index (CC_{50}/EC_{50}); 4'MdN=4'=*C*-methyl-2'-deoxynucleoside; 4'Md4N=4'-*C*-methyl-2'-3'-didehydrodi deoxynucleoside; 4'MddN=4'-*C*-methyl-2'-3'-dideoxy nucleoside; AZT=3'-azido-3'deoxythymidine; ddA=2',3'-dideoxyadenosine; d4T=2',3'-didehydro-3'-deoxythymidine

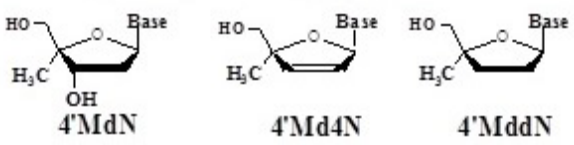


Table 1: Anti-HIV activety of 4'-*C*-methyl-2'-deoxynucleosides.

and toxicity at all, because their 5'-OH cannot be phosphorylated by kinase.

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 [8-15]. While we were working on our project, the anti-HIV activity of several **4'SdN**s was reported by the Syntex group [16-22] and others [23,24]. 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*-substitued nucleosides against **HIV** are summarized as follows:

The estimated relative order of anti-**HIV** activity is as follows:

1. CN ≥ C≡CH > N_3 > $CH=CH_2$ > Me= Et > $C\equiv C\text{-}CH_3$. Interestingly, the order is the reverse of the $-\Delta G°$ values between equatorial and axial substituents on a cyclohexane ring: CN < F < C≡CH < $CH=CH_2$ < Me ≤ Et < ^{t}Bu. 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'-deoycytidine, 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 high anti-**HIV** activity.

5. The L-isomers of **4'SdN** have no anti-**HIV** activity,[13] 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.

The biological activity of purine derivatives of 4'-*C*-Cyano-2'-deoxy- nucleoside (4'CNdNs) and 4'-*C*-ethynyl-2'-deoxynucleoside (4'EdNs)

The mentioned results led us to study the biological activity of purine derivatives of **4'CNdN** and **4'EdN** [25].

The biological activities of them are summarized in Table 3.

They are summarized as follows.

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 acceptable **SI**s.

Anti-HIV activity of 4'SdNs against drug-resistant HIV mutants [12,13,24]

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 derivatives 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 41/69/125/SG. The three purine derivatives, 2'-deoxy-4'-*C*-ethynyladenosine (**4'EdA**), 2'-deoxy-4'-*C*-ethynyl-2- amino adenosine (**4'Ed2AA**), and 2'-deoxy-4'-*C*-ethynylguanosine (**4'EdG**) except for 2'-deoxy-4'-*C*-

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-ethynylarabinofuranosylthymidine	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-ethyny-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-ethyny-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

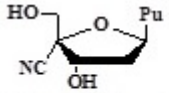
a) Anti-HIV activity was determined by MTT assay. MT-4 cells and HIV-1_{LAI} were employed. ND: not determined

Table 2: Anti-HIV activity of 4'-C-substituted-2'-deoxynucleosides.

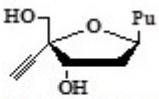
Compound	Base	EC_{50}(μM)[a)]	CC_{50}(μM)	S.I.
4'-*C*-Cyano-2'-deoxypurine	A	0.051	12	235
	I	0.051	23	451
	2AA[b)]	0.00079	0.034	43
	G	0.000188	0.034	181
	A	0.098	16	1630
	I	0.15	216	1440
	2AA	0.0003	0.82	2733
	G	0.0014	1.5	975
AZT		0.0032	29.4	9190

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

b) 2-aminoadenine



4'-*C*-Cyano-2'-deoxypurine



4'-*C*-Ethynyl-2'-deoxypuri

Table 3: Anti-HIV activity of 4'-*C*-cyano-2'-deoxypurines and 4'-*C*-ethynyl-2'-deoxypurines.

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** [14,15,26]. 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, and therefore, it will be toxic.

Mouse toxicity of purine derivatives of 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 $mgkg^{-1}$ of **4'EdA** and **4'EdI** by both intravenous and oral administrations, but all mice died after a single dosage of 3 $mgkg^{-1}$ of **4'Ed2AA** and **4'EdG** irrespective of the administration method (Table 5). Thus, it seemed that **4'EdA** and **4'EdI** were not toxic, but **4'E2AA** and **4'EdG** were highly toxic. Thus, **4'EdA** seemed very promising.

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, 4'-*C*-ethynyl-2'-deoxy-2-fluoroadenosine [**4'Ed2FA** which was later abbreviated as **EFdA** [29], the structure of **EFdA** is shown in Table 6, therefore, **EFdA** is used in this paper], was synthesized and evaluated for the stability to both

Compound	EC_{50}(μM)[a]									
	HXB2[b]	KH65R	L74V	4I/2I5	MI84V	MI84I	4I/69/125/SG	MDR[d]	YI8IC	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

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

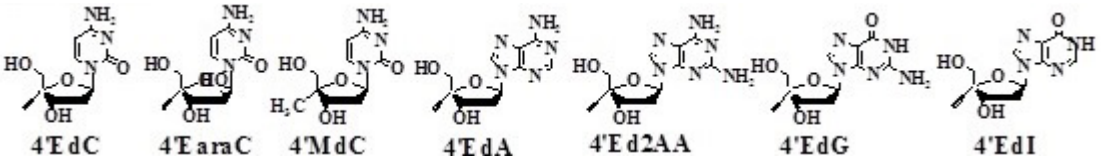


Table 4: Anti-HIV activity of selected 4'SdNs against wild type HIV and drug-resistant HIVs.

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

a) Six-week-old ICR male mice were employed.

b) Numbers in parentheses represent survival days of mice after administration

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

adenosine deaminase and acidic conditions, and for anti-**HIV** activity [30,31].

Expectedly, **EFdA** was very stable to adenosine deaminase under the conditions where **4'EdA** was completely deaminated in 60 min (Figure 3) and, further, fairly stable under acidic conditions. Thus, in 120 min only a small part (3%) of **EFdA** 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 (Figure 4).

Because **EFdA** is a nucleoside derivative modified at two positions (4'-position and 2-position) of physiologic 2'-deoxyadenosine (**dA**), the toxicity of **EFdA** is expected to be lower than that of **4'EdA**.

While we were working on this project, Haraguchi et.al reported that 4'-*C*-ethynyl d4T (**Ed4T**) is more active and less toxic than the clinical **d4T** and therefore **Ed4T** is a very promising anti-**HIV** nucleoside [32]. (The less toxicity is due to additional modification). Therefore, we synthesized dd- and d4-analogs of **EFdA** and evaluate their anti-**HIV** activity [33].

The anti-**HIV** activities of **EFdA**, 2',3'-dideoxy-4'-*C*-ethynyl-2-fluoroadenosine (**EddFA**) and 2',3'-didehydrodideoxy-4'-*C*-ethynyl-2-fluoroadenosine (**Ed4FA**) together with that of 2'-deoxy-4'-*C*-ethynyl-2-chloroadenosine (**ECldA**) are listed in Table 6 [29].

Although **Ed4FA**, **EddFA**, and **Ed4T**, which do not have 3'-OH, showed some activity against wild-type **HIV**, they significantly lost any activity against drug-resistant **HIV**s. **EFdA** and **EClda** showed very high activity against all **HIV**s and acceptable **SI**s, however, the activity of **EClda** is lower than that of **EFdA**. 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 [34].

The most resistant HIV mutant against **EFdA** emerged for the last 15 years is M184V/T165R/I142, which is 22 times more resistant than wild type **HIV** [35,36]. Thus, **EFdA** is sufficiently active against this mutant and has prevented the emergence of resistant mutant for the last 15 years.

Toxicity of EFdA to mice and inhibition of DNA polymerases

Because **EFdA** is stable to adenosine deaminase and highly active against all **HIV**s, its mouse toxicity was examined [29,30,33].

EFdA did not show any acute toxicity to mice by either oral or intravenous administration up to 100 $mgkg^{-1}$ (Figure 5 and Table 7).

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 ($\mathbf{EC_{50}}$) of 2'-deoxy-4'-*C*-ethyny-2-fluoroadenosine-5-*O*-triphosphate (**EFdA-TP**) 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 (**ddA-TP**) [30,35]. The $\mathbf{EC_{50}}$ values of **EFdA-TP** against **DNA** polymerase α and β were higher than 200 μM. These results indicate that the **DNA** polymerases scarcely recognize **EFdA-TP**, a derivative modified at two positions of physiologic **dATP**, as their substrate but that **RT** does [30,35,36].

It should be noted that **EFdA** is highly active to Simian Immunodeficiency Virus (**SIV**) and did not show any detectable side effects to macaques within 6 months of continuous therapy [37].

Intracellular metabolism of EFdA [35]

The amounts of all fractions of intracellular **EFdA** metabolites, (**EFdA**-monophosphate (**EFdA-MP**), **EFdA**-diphosphate (**EFdA-DP**), and **EFdATP**) increased proportionately with an increase in the concentration of intracellular **EFdA**, while compared to **AZT**-diphosphate and **AZT**- triphosphate (**AZT-TP**), only **AZT**-monophosphate markedly increased with an increase in intracellular **AZT** concentration. The intracellular half-life ($\mathbf{T_{1/2}}$) of **EFdA-TP** 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 **AZT-TP** was 3 h). About 50% of the cells were protected against the infection of **HIV** for 24 h after removal of extracellular **EFdA** in both MT4 cells and MAGI cells cultured in the presence of 0.1 μM of **EFdA**.

These results indicate that **EFdA**, **EFdA-DP** and **EFdA-TP** are very stable against intracellular enzymatic catabolism.

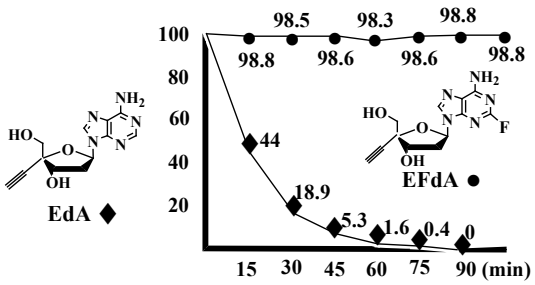


Figure 3: Stability of EFdA to Adenosine Deaminas.

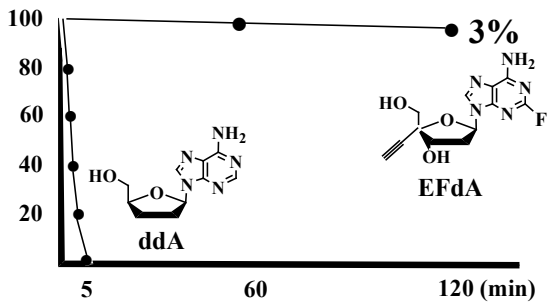


Figure 4: Stability of EFdA at pH 1.06, 36°C.

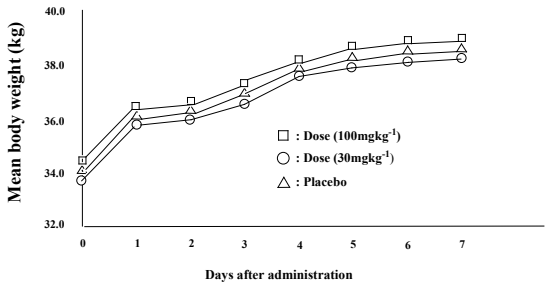


Figure 5: Body weight change of mice after a single dosage of 2'-deoxy-4'-*C*-ethynyl-2-fluoroadenosin, administrated orally or intravenously to ICR mice.

A rationalization of the inhibition of RT and DNA polymerase by 4'SdNs

The one position modified **4'SdN**s in Figure 6 are highly anti-**HIV** active and highly toxic, too. These results show that both **RT** and **DNA** polymerase accept these one position modified nucleosides. On the other hand, the two positions modified **4'SdN**s are highly anti-**HIV** active but very low toxic. These results show that **RT** accepts very easily these two positions modified **4'SdN**s but **DNA** polymerase hardly does. These results showed that the substrate selectivity is different between **RT** (**RNA**-dependent **DNA** polymerase) and **DNA** polymerase (**DNA** dependent **DNA** polymerase).

4'-*C*-ethynyl group has special affinity to RT

The facts that **Ed4T** is more active than **d4T**, and that **EFdA-TP** is two times better substrate for **RT** than the physiologic substrate 2'-deoxy-**ATP** [38] had indicated that the 4'-*C*-ethynyl group will have special affinity to **RT**. The indication was confirmed first by Yang and his co-workers using **Ed4T** and X-ray crystallographic method [39]. They showed that the 4'-C-ethynyl group fits into a hydrophobic pocket defined **RT** residual Ala-114, Try-115, Phe-160, Met-184, and the aliphatic chain of Asp-185.

One year later, the same result was obtained by Michailidis and his co-workers using **EFdA** [38]. Further, they named **EFdA** Translocation-Defective Reverse Transcriptase Inhibitor (**TDRTI**) because the affinity of **EFdA** to **RT** by both 4'-*C*-ethynyl and 3'-OH groups is so strong that the 3'-**EFdA-MP**-terminated primer strand on the **RT** does not translocate from the pre-translocation site (N-site) to the post-translocation site (P-site) to accept the next deoxynucleoside triphosphate (**dNTP**). Therefore, the next **dNTP** cannot react with the 3'-**EFdA-MP**-teminus.

Therefore, **EFdA** has supremely high anti-**HIV** activity.

The validity of all the working hypotheses is proved and we have developed **EFdA**, which could prevent the emergence of resistant **HIV**-mutants, and has the anti-HIV activity of 400 times more active than **AZT** and several orders of magnitude more active than the other clinical **NRTI**s, and low toxicity.

Thus, **EFdA** could solve all the problems of the existing **HAART**.

EFdA	ECldA	Ed4FA	EddFA	Ed4T
Compound	Anti HIV activity (Magi assay, μM)			
	$HIV\text{-}1_{wild}$	$HIV\text{-}1_{MDR}$	$HIV\text{-}1M_{184V}$	SI
EFdA	0.00020	0.00014	0.0031	110,000
ECldA	0.0019	0.0084	0.01	330,000
Ed4FA	0.80	0.15	1.8	
EddFA	0.94	8.7	97	
AZT	0.17	74.3	0.13	
3TC	1.0	2.8	>100	
Ed4T	1.5	1.1	17	>50,000
d4T	7.6	64	5.6	

MAGI = multinuclear activation of galactosesidase indicator, HIV = human immune deficiency virus, AZT = 3'-azido-3'-deoxythymidine; 3TC = 2',3'-dideoxy-3'-thia-L-cytidime; d4T = 2',3'-didehydrodideoxythymidine.

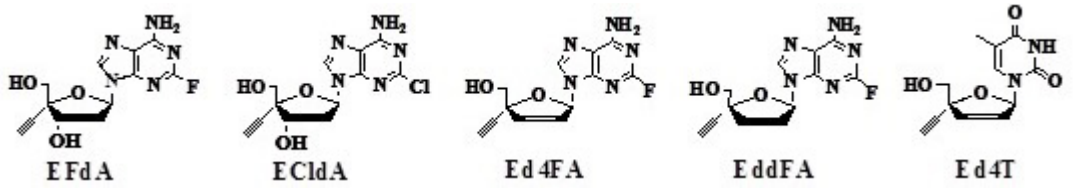
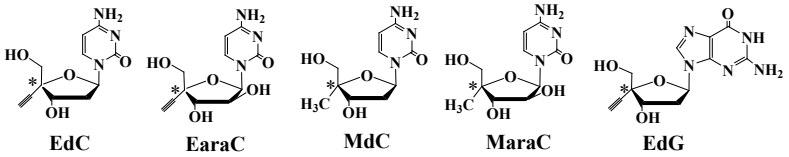
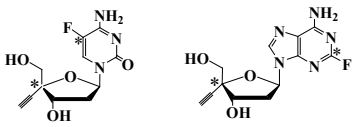


Table 6: Anti HIV activity of 4'-*C*-substituted-2'-deoxy-2-haloadenosines.



One position modified 4'SdNs



Two positions modified 4'SdNs

Figure 6: Structures of one position modified 4'SdNs and two positions modified 4'SdNs.

Substrate selectivity of viral RNA polymerase is different from that of human RNA polymerase

One of the important findings in our study is that the substrate selectivity of **RT** (**RNA**-dependent **DNA** polymerase) is different from that of human **DNA** polymerase (**DNA**-dependent **DNA** polymerase).

This finding yielded a new question; whether the substrate selectivity of viral **RNA** polymerase (**RNA**-dependent **RNA** polymerase) is different from that of human **RNA** polymerase (**DNA**-dependent **RNA** polymerase).

Eldrup and his co-workers synthesized two position modified 2'-*C*-methyl-7-deazaadenosine (**C**), the hybrid of 2'-*C*-methyl adenosine (**A**) and the antibiotic tubercidine (**B**) [40] (Figure 7). The one position modified **A** is highly active to Hepatitis C Virus (**HCV**) and highly toxic and the one position modified **B** is also highly active and highly toxic. They found that the two position modified **C** is highly anti-**HCV** active and very low toxic [40]. These results showed that the modification of the highly toxic one position modified nucleosides decreased their toxicity and more significantly that **HCV**-**RNA** polymerase accepts the two positions modified but human **RNA** polymerase hardly does, thus, the substrate selectivity of **HCV**-**RNA** polymerase is different from that of human **RNA** polymerase.

On the other hand, Smith and his co-workers reported that 4'-*C*-azidocytidine (**D**) is anti-**HCV** active [41]. This is a striking different point between 4'-*C*-azidocytidine and 4'-*C*-alkyl cytidines. (4'-*C*-alkyl cytidines do not have any biological activity because they cannot be phosphorylated by kinase). Further, they reported that 4'-*C*-azido*arabino*cytidine (**E**) and 4'-*C*-azido-2'-deoxy-2'-β-fluorocytidine (**F**) and 4'-*C*-azido-2'-deoxy-2',2'-difluorocytidine (**G**) (Figure 7) are more active than **D** and low toxic [41], and further these are chain terminators of **HCV**-**RNA** polymerase. Surprisingly, **HCV**-**RNA** polymerase accepted these 2'-deoxynucleoside derivatives.

Dose ($mgKg^{-1}$)	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

Table 7: Toxicity of 2'-deoxy-4'-*C*-ethynyl-2-fluoroadenosine (EFdA) after a single dosage to ICR mice.

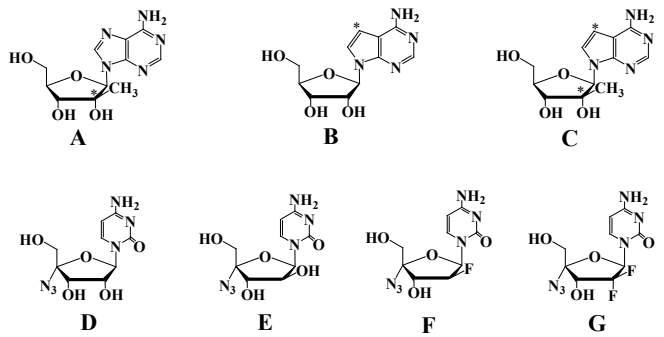


Figure 7: Structures of 4'-*C*-methyladenosine (**A**), tubercidine (**B**), the hybride of **A** and **B** [7-deza-2'-*C*-methyladenoine (**C**)], 4'-*C*-azidecytidine (**D**), 4'-*C*-azidearaC (**E**), 4'-*C*-azido-2'-deoxy-2'-β-fluorocytidine (**F**), and 4'-*C*-azide-2'-deoxy-2',2'-difluorocytidine (**G**).

Our preliminary experiments suggested that **4'EdA** and its derivative are anti-Flu Virus active (unpublished). These results indicate that the substrate selectivity of viral **RNA** polymerase is different from that of human **RNA** polymerase.

Conclusion

The substrate selectivity of viral RNA-dependent nucleic acid polymerases is different from that of human DNA-dependent nucleic acid polymerases. Therefore, by taking the advantage of the difference, it will be possible to develop modified nucleosides which are highly selectively active to viruses and not active to human beings.

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