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Characterization of human immunodeficiency virus type 1 resistant to modified cyclodextrin sulphate (mCDS71) *in vitro*

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Drug resistance of human immunodeficiency virus type 1 (HIV) to modified cyclodextrin sulphate (mCDS71) has been analysed with respect to both the *in vitro* appearance of resistance to the compound and the mechanism of the acquisition of resistance. Resistant strains could be obtained in all three strains (NL432, KK-1 and A018) tested after serial passages in MT-4 cells with a gradual increase of the concentration of mCDS71. Cross-resistance both to mCDS71 and dextran sulphate 8000 was observed. As a result of sequencing analysis of the gp120 V3-C5 region of resistant strains, the mechanism of resistance can be explained in several ways: (i) substitution of sugar chain-binding amino acids, N and S; (ii) three to five amino acid deletion in V4 loop; and (iii) several mutations in V3 and V4 regions. The real cause of the resistance may be a combination of these three mechanisms. The results suggest that the target of mCDS71 is relatively widely distributed on the viral surface glycoprotein.

Keywords: HIV-1; modified cyclodextrin sulphate; mCDS71; drug resistance; *env* region; amino acid sequence

Introduction

Treatment of human immunodeficiency virus (HIV)/AIDS has been highly improved following the licensing of protease inhibitors in 1996. Combination therapy for HIV/AIDS employing reverse transcriptase (RT) inhibitors and protease inhibitors enables the maintainance of low level viral loads in the peripheral blood (Hammer *et al.*, 1997; Gulick *et al.*, 1997) and in the lymphatic organs (Wong *et al.*, 1997; Notermans *et al.*, 1998), which are the major HIV reservoir sites. The appearance of resistance to these drugs (Larder & Kemp, 1989; Roberts, 1995; Schmit *et al.*, 1996) and complexities of the regimen for clinical use are problems that need to be solved. In particular, cross-resistance of HIV to multiple drugs (Gao *et al.*, 1993; Condra *et al.*, 1995, 1996; Chen *et al.*, 1995; Tisdale *et al.*, 1995) makes it difficult to choose effective antivirals and the development of anti-HIV drugs with mechanisms of action different from existing drugs is needed.

Sulphated polysaccharides represented by dextran sulphate (Ito *et al.*, 1987; Witvrouw *et al.*, 1994) have been known to inhibit the adsorption of HIV to the host cell surface (Baba *et al.*, 1988; Mitsuya *et al.*, 1988), but there was little *in vivo* antiviral effect of dextran sulphate during clinical trials (Abrams *et al.*, 1989). The reasons for this inefficacy were low absorption rate from the intestine and rapid degradation of the drug in the blood (Lorentsen *et al.*, 1989; Hartman *et al.*, 1990).

We have reported that a series of modified cyclodextrin sulphates, rigid circular forms made of seven glucose residues, introduced sulphate residues and modified by various functional groups, have anti-HIV activities (Moriya *et al.*, 1991, 1993; Otake *et al.*, 1994). Among them, mCDS71, in which the C-2 position was modified with a lipophilic benzyloxy group, exhibited 32- to 125-fold higher anti-HIV activity compared with the original unmodified form (Moriya *et al.*, 1993). After oral administration of mCDS71 in rats, the peripheral blood possessed anti-HIV activity (Moriya *et al.*, 1993) and anti-HIV activity of mCDS11, the prototype of mCDS71, had a half-life of 4 h in rabbits (Otake *et al.*, 1994). This drug had antiviral activity against clinical HIV-1 isolates as well as laboratory strains, and inhibited the formation of multi-nucleated cells (cytopathic effect). The target site of mCDS71 has not been identified, although it is presumed that the drug inhibits the adsorption of HIV-1 onto the cells.

In this study, the appearance of virus resistant to mCDS71 was examined *in vitro*. To elucidate the target site of this drug, the base sequence and resultant amino acid

Table 1. Sequence of primers

	Target	Code	Sequence	Location*	Reference
Outer primer	*env*	JG001	5'-CACCACTCTATTTTGTGCATCAGAT-3'	6367–6391	Genesca *et al.* (1990)
		JG002	5'-CAAGAGTAAGTCTCTCAAGCGGTGG-3'	8542–8518	Genesca *et al.* (1990)
Inner primer	V3	E80	5'-CCAATTCCCATACATTATTGTG-3'	6847–6869	Paladin *et al.* (1998)
		E95B	5'-GATGGGAGGGGCATACAT-3'	7531–7514	Paladin *et al.* (1998)
	V4 to end gp120	V3-2B	5'-CCTCAGGAGGGGACCCAGAAATTG-3'	7305–7328	
		SK69	5'-CCAGATGTGAGTTGCAACAG-3'	7927–7907	Ou *et al.* (1988)
Sequencing primer	V3	E90C	5'-CACAGTACAATGTACACATGGAAT-3'	6943–6966	Paladin *et al.* (1998)
	V4 to end gp120	SK68C	5'-CCCATAGTGCTTCCTGCTGCT-3'	7806–7786	Ou *et al.* (1988)

*Numbering of nucleotides is according to the pNL432 sequence.

sequence of the resistant strains were compared with those of wild-type susceptible strains.

Materials and Methods

Cells and viruses

MT-4 cells and CEM cells were maintained in RPMI 1640 (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Strain NL432 was prepared by single passage of the virus in MT-4 cells obtained by transfection of DNA clone pNL432 (Adachi *et al.*, 1986) into CEM cells. Strain KK-1 was isolated in our laboratory from an untreated AIDS patient and strain A018 (Larder *et al.*, 1989b) was a 3'-azido-3'-deoxythymidine (AZT)-resistant virus isolated from an AIDS patient treated with AZT. The infectivity of the virus was titrated on MT-4 cells.

Compounds

mCDS71 was synthesized as reported by Moriya *et al.* (1993). The 50% inhibitory concentration (IC_{50}) of mCDS71, determined as described below, for laboratory strain HIV-1_{LAI} on MT-4 cells was 0.77 μg/ml and the selective index (SI) was 364 when compared with the 50% cytotoxic concentration (CC_{50}). The IC_{50} for a HIV-1 clinical isolate (KK-1) on peripheral blood mononuclear cells was 0.63 μg/ml and the SI was 167. Dextran sulphate (MW 8000) and AZT were purchased from Sigma.

Selection of mCDS71-resistant virus

HIV-1 strains were infected at a m.o.i. of 0.01 to MT-4 cells and initially propagated in the presence of 0.25 μg/ml mCDS71. The appearance of CPE was monitored under a light microscope and, when it appeared, the culture fluid was transferred to fresh MT-4 cells with 1.5- to twofold elevation of the concentration of mCDS71. This procedure was repeated until the concentration of mCDS71 reached 100 μg/ml. The resultant culture fluid was passaged once in fresh MT-4 cells in the absence of mCDS71 and progeny viruses were used for the following experiments.

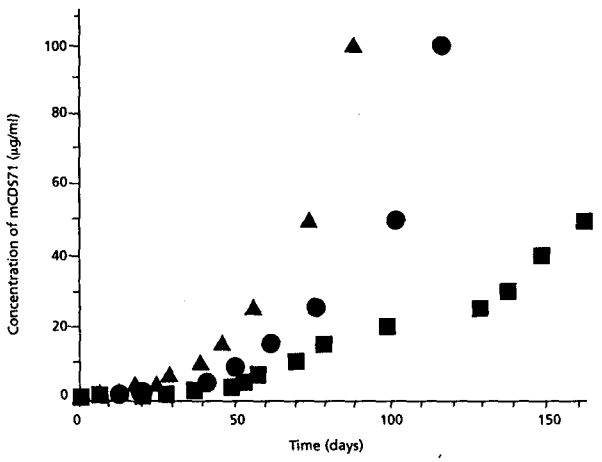
Test for susceptibility of the virus to the drug

HIV-1 strains were infected to MT-4 cells at a m.o.i. of 0.001 and incubated with serially diluted drugs at 37°C for 5 days. Viable cell count was determined by the trypan blue dye exclusion test and the IC_{50} for each virus stock was calculated.

Detection of mutation site(s)

DNA preparation was made from MT-4 cells infected with various HIV-1 strains. A nested PCR procedure was employed to amplify the HIV-1 genome from the *env* gp120 V3 region to the end of gp120. The primers used are summarized in Table 1. The 5' ends of inner primers, E95B and V3-2B, were labelled beforehand with biotin and ssDNA was prepared using Dynabeads M-280 streptavidin (Dynal). Using this ssDNA as the template, the sequencing reaction was performed using an AutoRead Sequencing Kit (Pharmacia Biotech) and the nucleotide sequence was determined by ALF Express (Pharmacia Biotech). Amino acid sequence was deduced after analysis by DNASIS V3.2. The conditions for the PCR were 1st

Figure 1. Emergence of mCDS71-resistant HIV-1



■,NL432 ; ●, KK-1 and ▲, A018 were cultivated in MT-4 cells in the presence of gradually increasing concentrations of mCDS71 as indicated.

Table 2. Inhibitory effect of mCDS71, DS8000 and AZT on mCDS71- or DS8000-resistant strains

Strain	IC_{50}*		
	mCDS71 (μg/ml)	DS8000 (μg/ml)	AZT (μM)
NL432/wt†	0.25 (1.0)	3.0 (1.0)	0.003
NL432/P40‡	0.3	3.0	0.004
NMR15§	5.8 (23.0)¶	17.0 (5.7)	0.003
NMR50	38.5 (154)	>500 (>167)	0.004
KK-1/wt	0.63 (1.0)	94.0 (1.0)	0.003
KK-1/P26	0.72	73.0	0.005
KMR15	4.3 (6.8)	>500 (>5.3)	0.016
KMR50	13.0 (20.6)	>500 (>5.3)	0.005
KMR100	56.0 (89.0)	>500 (>5.3)	0.009
A018/wt	1.2 (1.0)	190 (1.0)	0.14
A018/P22	1.7	225	0.41
AMR15	7.8 (6.5)	>500 (>2.6)	0.25
AMR50	19.5 (16.3)	>500 (>2.6)	0.14
AMR100	52.0 (43.0)	>500 (>2.6)	0.19
NDR500	3.9 (15.6)	105 (35.0)	0.003

*50% inhibitory concentration.
†Wild-type of NL432.
‡NL432 passaged 40 times in the absence of drug.
§NL432 capable of growing in 15 μg/ml of mCDS71.
¶Numbers in parenthesis represent the fold increase in IC_{50} as compared with wild-type IC_{50} of each strain.

PCR: 92°C for 1 min, 54°C for 1 min and 70°C for 2 min for 30 cycles and 70°C for 5 min for the extension, and for the 2nd PCR: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 30 cycles and 72°C for 10 min for the extension.

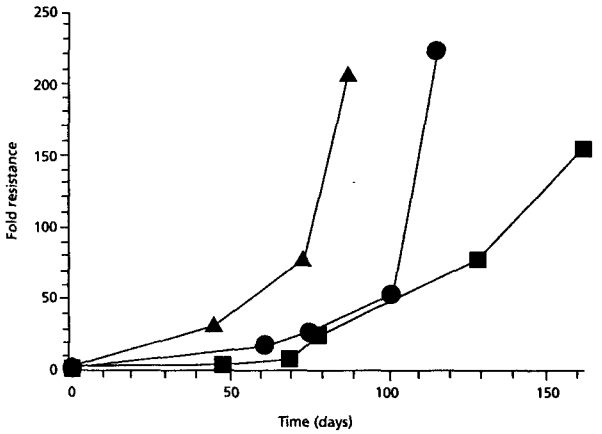
Results

Appearance of mCDS71 resistance

MT-4 cells were infected with NL432, KK-1 and A018 and the concentration of mCDS71 in culture medium was gradually elevated as shown in Figure 1. After 116 days (26 passages) for KK-1 and 88 days (22 passages) for A018, virus stocks, which could replicate in the presence of 100 μg/ml mCDS71, were obtained. They were designated as KMR100 and AMR100, respectively. In contrast, it took 161 days (40 passages) for NL432 to become resistant to 50 μg/ml of mCDS71 (NMR50) and at 100 μg/ml of the drug, no breakthrough of the virus was observed. During these procedures, drug concentration was increased 1.5- to twofold gradually. When the concentration was increased threefold or more, no breakthrough of virus growth was observed at any stage. Dextran sulphate 8000 (DS8000)-resistant NL432 was obtained after 91 days (23 passages) at a concentration of 500 μg/ml of the compound (NDR500).

mCDS71-resistant virus strains were tested for sensitivity to mCDS71, DS8000 and AZT (Table 2). Resistant strains exhibited 43- to 154-fold resistance to mCDS71. The wild-type viruses passaged similarly in the absence of these drugs showed the same sensitivity as the original wild-type after serial passages (NL432/P40, KK-1/P26 and A018/P22). The level of the drug sensitivity was expressed as the ratio of IC_{50} of each virus stock to that of NL432/wild-type (Figure 2). After 88 days of culture, the culture ratio for A018 was 208; after 116 days culture the ratio was 224 for KK-1. But, even after a period of 161 days, the ratio for NL432 reached only 154. NDR500 showed 35-fold resistance to DS8000 when compared with NL432/wild-type. Cross-resistance of these resistant strains was demonstrated for mCDS71 and DS8000. All the wild-type strains and strains resistant to mCDS71 or DS8000 were similarly sensitive to AZT, which was used as the control of the assay system.

Figure 2. Ratio of resistance of mCDS71-resistant strains relative to NL432/wild-type



At different culture days, the IC_{50} values of ■, NMR; ●,KMR and ▲,AMR were determined and compared with the IC_{50} of wild-type NL432.

Replacement of amino acids in *env* region

Since mCDS71 treatment of MT-4 cells prior to infection with various HIV-1 strains did not inhibit the infection of the cells with the virus (data not shown) and since mCDS71 strongly blocks adsorption of HIV-1 to MT-4 cells (Moriya *et al.*, 1993), the target of mCDS71 was considered to be Env. Therefore the region of Env that is important for the binding of the virus to the cell surface was analysed. The nucleotide sequences responsible for peptide sequences for V3 to the end of gp120 were analysed by nested PCR and direct sequencing; the mutations at the amino acid level are shown in Figure 3. Each resistant mutant obtained several mutation sites but the location of the mutation was variable. While resistant strains of NL432 and A018 series demonstrated mutations distributed all over the sequence from V3 to the C terminus of gp120 (C5), mutations in viruses of KK-1 series were predominantly within V4 and C4. There were no common mutations among these three virus series.

Figure 3. Amino acid substitutions in gp120 V3-C5 of mCDS71-resistant strains

	V3 loop										V4 loop								CD4 binding domain				V5			
	248	256	268	274	276	280	286	295	299	357	366	367	368	369	370	371	376	388	390	392	403	423	436	443	458	465
NL432/wt	T	V	R	R	S	Q	F	N	A	S	F	N	S	T	W	S	N	P	R	K	M	L	I	D	V	V
NL432/P40	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NMR15	.	.	.	.	R	H	.	.	T		.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.
NMR50	.	.	S	T	R	H	.	V	T	.	-	-	-	-	-	.	.	L	S	.	.	.	.	.	I	.
NDR500	.	.	.	.	.	H	.	D	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.
SID791^r				T	R	H		H	T		-	-	-	-	-			L							I	
KK-1/wt	T	V	R	R	R	-	L	D	A	S	V	N	G	N	R	S	N	P	R	K	M	L	T	N	V	V
KK-1/P26	A	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.
KMR15	A	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.
KMR50	A	.	.	.	.	-	.	.	.	.	.	.	.	T	.	N	X	.	.	N	K	I	.	D	.	.
KMR100	A	.	.	.	.	-	.	.	.	.	.	.	.	T	.	N	D	.	.	N	K	I	.	D	.	.
A018/wt	T	V	R	R	R	-	F	D	A	S	-	-	-	N	G	N	N	P	R	K	M	L	G	D	V	V
A018/P26	.	.	.	.	.	-	.	.	.	.	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.
AMR15	.	I	.	.	.	-	.	.	.	T	-	-	-	.	.	.	.	.	.	.	.	.	V	.	.	I
AMR50	.	I	.	.	.	-	L	.	.	T	-	-	-	.	.	.	.	.	S	.	.	.	V	.	.	I
AMR100	.	I	.	.	.	-	L	.	.	T	-	-	-	.	.	.	.	.	S	.	.	.	V	.	.	I

Amino acids were deduced from the nucleotide sequences obtained by PCR amplification and direct sequencing of *env* region of proviral DNA of reisistant strains. The single-letter amino acid code is used and numbering of amino acids is with reference to wild-type NL432. X represents a mixture of N and D. SID791^r is a bicyclam-resistant strain which amino acid substitutions are cited from the previous publication (De Vreese *et al.*, 1996b), and only substitutions in common with NMR50 are shown. An amino acid identical with wild-type is shown with a dot and deletion of an amino acid is shown with a hyphen.

However, in each case, with the increase of drug resistance, the number of mutated sites were increased. The mutations in mCDS71-resistant strains were often observed at the sites of asparagine (N) or serine (S), such as R268S, S276R, S357T, N367, S368, N369T, S371N, N376D, R390S and K392N. These observations support the idea that the gain or loss of sugar chains may contribute to the change in the tertial structure of gp120, resulting in the acquisition of mCDS71 resistance. When we compared the amino acid sequence of NMR50 and NDR500, there were three amino acid exchanges observed at the same positions, Q280H and N295V or D in the V3 domain and R390S in the CD4-binding domain. The mutation Q280H could not be found in KK-1 nor A018 series because of the deletion, so it is conceivable that this position is important for low sensitivity to DS8000. Similarly, the presence of N at position 295 could be important for the anti-HIV activity of DS8000. In NMR50, five successive amino acids, FNSTW, were deleted. The mutations T248A and N443D in KMR series were also observed in passaged wild-type strain KK-1/P26, so these mutations cannot be the cause of resistance to mCDS71.

Discussion

The appearance of drug-resistant HIV-1 during the course of chemotherapy is the largest and the most troublesome problem for the treatment of HIV/AIDS. We have performed *in vitro* experiments to generate HIV-1 strains resistant to mCDS71 and to analyse the mutations responsible for the acquisition of resistance to it. It became evident that resistance to mCDS71 could be induced by successive *in vitro* cultures with a gradual increase in the concentration of the compound. The reason why resistant strains of NL432 could not easily be induced is not known, but could be because NL432 was derived from cloned DNA whereas KK-1 and A018 were composed of quasi-species.

Dextran sulphate is known as an anti-HIV compound and it inhibits adsorption of the virus to the cells with concomitant appearance of virus resistant to the compound (Esté *et al.*, 1997). HIV-1 strains resistant to DS8000 could also be induced in this laboratory. Since clinical isolates of KK-1 and A018 were already not particularly susceptible to DS8000, we tried to obtain DS8000-resistant NL432. As shown in Table 2, there was cross-resistance between mCDS71 and DS8000. Bicyclam (De Clercq *et al.*, 1992), a macrocyclic polyamine, also induces HIV-1 cross-resistance to bicyclam and DS5000 (De Vreese *et al.*, 1996a) and is known as an inhibitor of the early steps of infection, that is to say adsorption and/or membrane fusion (De Vreese *et al.*, 1996a). The presence of similar mutations in V3 regions of HIV-1 resistant to mCDS71, DS8000 or bicyclam is of interest, although we admit bicyclam inter-

Table 3. Changes of net charge in V3 and V4 loops and CD4 binding domains

Strain	Net charge		
	V3 loop	V4 loop	CD4 binding domain
NL432/wt*	+9	−3	+2
NMR50†	+9	−3	+1
KK-1/wt	+7	−2	+3
KMR100	+7	−3	+3
A018/wt	+6	−2	+4
AMR100	+6	−2	+3

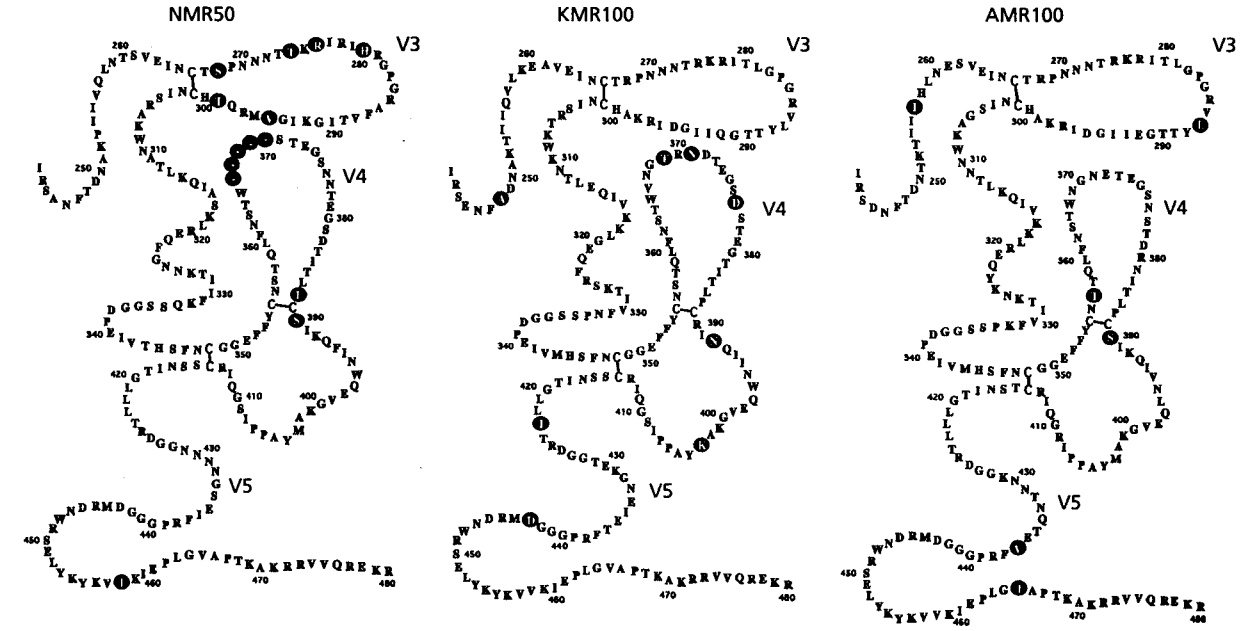
*Wild-type of NL432.
NL432 capable of growing in 50 μg/ml of mCDS71.

acts with CXCR4 (Schols *et al.*, 1997) and mCDS71 inhibits both T- and M-tropic viruses (H Mori, T Otake, I Oishi & T Kurimura, unpublished results). By *in vitro* assay (Moriya *et al.*, 1993), as well as PCR (data not shown), mCDS71 inhibits reverse transcriptase activity. mCDS71 can bind to nucleic acids, both DNA and RNA, in a non-specific manner and this binding can inhibit reverse transcription, transcription and DNA polymerase activity. Since the molecular weight of mCDS71 (approximately 3200) is not small enough to be introduced freely into the cell, inhibition of these steps by this compound is not likely to occur *in vivo*.

To analyse the target site of mCDS71, nucleotide sequences corresponding to the V3 region through to the C terminus of gp120 were analysed. Unexpectedly, we could not find any unique mutations common to all three mCDS71-resistant strains. There were five successive deletions (amino acids 366–370) in the middle of the V4 domain in NMR50. This deletion of amino acids FNSTW was also shown in HIV-1 (NL4-3) resistant to bicyclam (De Vreese *et al.*, 1996b). Wild-type A018 has a deletion of three amino acids (366–368) before cultivation in the presence of mCDS71 and this might be the cause of relative resistance of A018/wt to the compound. The presence of the N369T mutation in KMR50 and KMR100 also indicates the importance of this region for resistance, although there might be another possibility, namely that NL4-3 could have similar deletion mutations in the presence of any kind of anti-HIV substance. There were many common mutation sites between NMR50 and bicyclam-resistant NL4-3, including R274T, S276R, Q280H, N295H, A299T, P388L and V458I, but these mutations were not found in KMR100 or AMR100. Figure 4 shows all the mutation sites in the context of the V3 and V4 domains, which are composed of loop structures between disulphide-bonded cysteine residues. Most of the mutations were observed inside the V3 or V4 domains or near the disulphide bonds of the loops. The mutations causing structural changes of these loops might lead to resistance. These results indicate that the target site of mCDS71 is relatively widely distributed on the viral gp120.

Since the V3 domain is positively charged, it is conceivable that polyanionic compounds, such as mCDS71 and DS, can bind to it. A change in the electric charge of the V3 domain was not observed, but a slight reduction of positivity was observed in V4 and the CD4 binding domain

Figure 4. Diagram of presumed secondary structures of a part of gp120 in mCDS71-resistant virus



The scheme of Leonard *et al.* (1990) was used to determine the secondary structure of the gp120 V3-C5 of mCDS71-resistant strains. Positions of amino acid mutations are represented as shaded circles. Deletions in the V4 loop in NMR50 are indicated by dashes.

(Table 3) which could, in part, be the cause of resistance. To elucidate the exact target site(s) of mCDS71, further analyses are in progress.

Although mCDS71 can be absorbed after oral administration in animal experiments and exhibits a fairly long half-life of 4 h, the absorption rate is not high enough for clinical use if administered orally. The topical use of mCDS71 as a vaginal pessary shows promise because this compound inhibits transmission of free infectious virions to the cells as well as cell-to-cell transmission. Low cytotoxicity and relatively low absorption rate may favour this method of delivery.

Stafford *et al.* (1997) reported that dextrin sulphate (D_2S) (McClure *et al.*, 1991, 1992) can be used vaginally without disturbing the function of the vaginal epithelial cells and normal bacterial flora. We can expect that the HIV-1 inhibitory activity of mCDS71 is equal to D_2S, and so the topical use of mCDS71 should be considered. Besides anti-HIV-1 activity, mCDS71 inhibits the replication of HSV-1 and HSV-2 in Vero cells. However, it requires a concentration of 20–150 μg/ml.

When mCDS71 is administered topically, contact between the drug and virus is for a short duration and this will prevent the appearance of drug-resistant HIV-1. Combined use of condoms and mCDS71 as a vaginal pessary may enable us to control heterosexual transmission of HIV, especially in developing countries.

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Potent Inhibitory Effect of a Series of Modified Cyclodextrin Sulfates (mCDS) on the Replication of HIV-1 in Vitro

According to the urgent demand for an effective and safe agent for acquired immunodeficiency syndrome (AIDS), there are numerous compounds in development with individual action mechanisms against the causative human immunodeficiency virus (HIV) of AIDS.[1] Among them, polysulfated compounds, such as dextran sulfate (DS), pentosan polysulfate (HOE/BAY-946), etc.,[2] are some of the most potent and selective inhibitors of HIV type 1 and 2 in vitro through blocking the viral adsorption to cell membrane and cell fusion (syncytium formation). However, the effectiveness in vivo has not been clarified as yet[3] because of their poor absorbability, owing to the large molecular size and unfavorable anticoagulant activity of blood.

To overcome these problems and to get a clue to rational drug design based on a structure–activity relationships, it is necessary to simplify the molecular structure to a moderate size and a more rigid skeleton, because most of the polyanionic agents with high activity that have been reported so far have a long-chained main frame, a large molecular weight (more than 5000 Da), and a high density of sulfate groups.[2f,4] Therefore to begin with, we studied and reported previously[5] that cyclodextrin sulfates (CDS)[6] which are constructed with a doughnut-like cyclodextrin frame (CD, Figure 1a), and with a pair of anionic circular moieties as shown in Figure 1b, have effective anti-HIV-1 activities. The activity of CDS increased from α- to γ-derivatives along with an increase of the constructed glucose unit. In addition to this, partially chlorinated cyclodextrin phosphate (CDP) while it was somewhat cytotoxic, showed anti-HIV-1 activity and lower anticoagulant activity than DS and CDS.

Here, we wish to report the further investigation of the separation of the anti-HIV and anticoagulant activities and the improvement of the absorbability in the gut by the introduction of hydrophobic substituents (XR) to one of the anionic moiety of CDS as illustrated in Figure 1c. More than 50 modified β-cyclodextrin sulfates (mCDS) having various sulfonate, sulfide, and amino groups on the 6-position of the cyclodextrin skeleton were synthesized according to the method as shown in Scheme I. The 6-position hydroxyl groups of β-cyclodextrin were selectively mesitylenesulfonated to 6-*O*-mesitylenesulfonylated β-cyclodextrins (I).[7] The sulfonyl groups of I were substituted for sulfide or amino groups to 6-deoxy-6-thio or 6-amino-6-deoxy β-cyclodextrins (II or III) by a reaction with thiols or amines, respectively. The hydroxy groups of these modified β-cyclodextrins (I–III) were sulfated by sulfur trioxide–pyridine complex in pyridine solution to the corresponding mCDS.[8] In the case of I, a partial replacement of the sulfonyl group to a quaternary pyridinium group occurred. The mCDSs thus formed were screened by the anti-HIV-1 activity, anticoagulant activity, and also cytotoxicity. All of the representative mCDSs (**3**, **11**, **38**) bearing different hydrophobic substituents showed superior results to the positive controls [CDS, CDP, and DS, as listed in the Table I (columns HIV-1; LAV-1/MT-4, APTT, and cytotox)]. Thus, the advantage of the introduction of hydrophobic moieties to the mCDS was clearly revealed in the potentiated anti-HIV-1 activity and the reduced unfavorable anticoagulant activity.

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(7) Heptakis(6-*O*-mesitylenesulfonyl)-β-cyclodextrin (**I**; $m = 7$, $n = 0$) was prepared according to a previous report: Tsujihara, K.; Kurita, H.; Kawazu, M. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 1567–1571. Partially 6-*O*-mesitylenesulfonylated β-cyclodextrins (**I**; $m = 1$–4, $n = 3$–6) were similarly synthesized and purified by CHP-20 (a high-polar resin supplied by Mitsubishi Kasei Co., Ltd.) column chromatography with aqueous methanol used as an eluent and obtained as mixtures of regioisomers.

(8) The number of sulfate groups was calculated from the elemental analysis.

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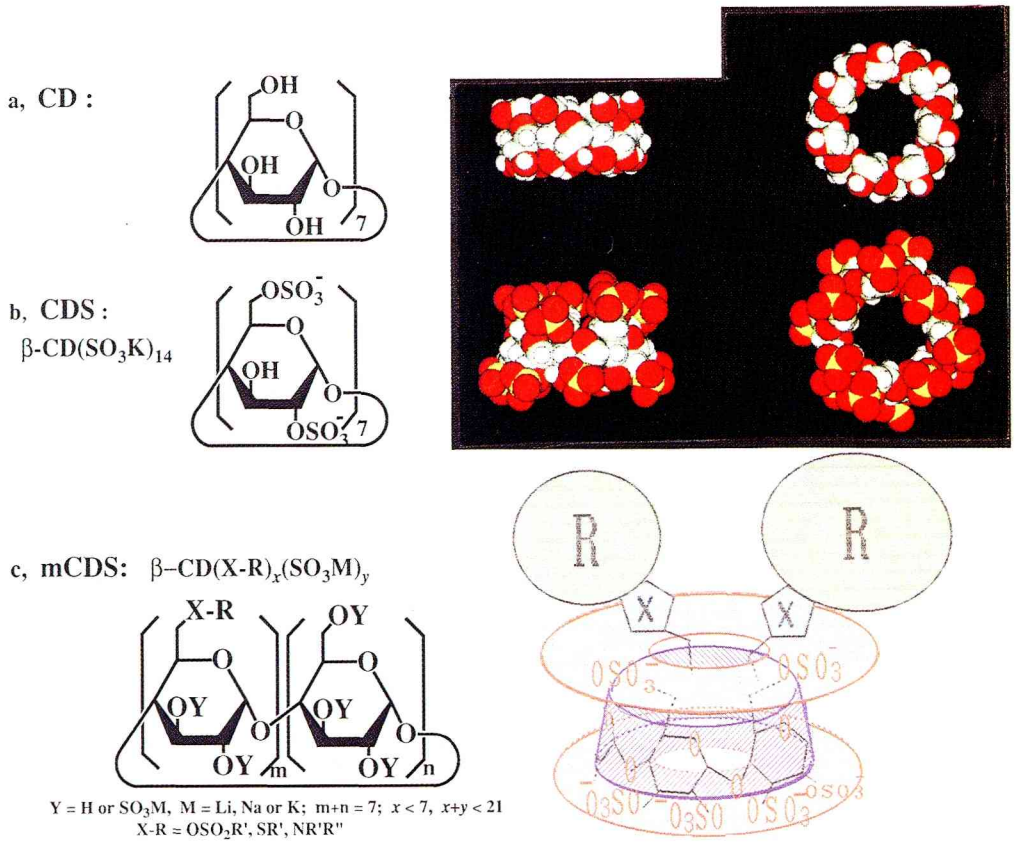

Figure 1. Side and top views of β-cyclodextrin (a) and β-cyclodextrin sulfate (b) molecules illustrated as a space field model. The color of the balls indicates the kind of atoms: white, hydrogen; gray, carbon; red, oxygen; yellow, sulfur. Part c shows illustrative structures of the β-cyclodextrin sulfate molecule which was modified with hydrophobic substituents (X–R).

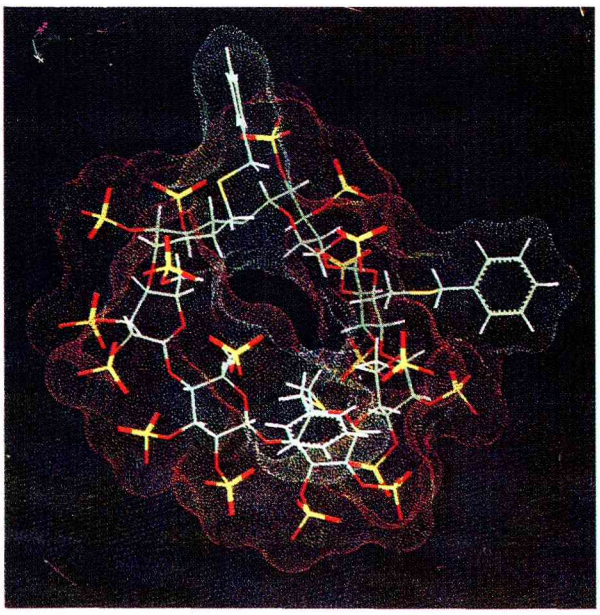

Figure 2. Computer graphics image of the one of the isomers of the mCDS **11** molecule constructed by the X-ray crystallographic data of β-cyclodextrin and the SYBIL fragment library. The red dotted regions represent the surface of the anionic part of the molecule and the others are the hydrophobic parts.

The most potent compound, mCDS **11**, having three benzylthio substituents exhibited the anti-HIV-1 activity on the HIV-1_{LAV-1}-induced cytopathic effect (CPE) in MT-4 cells at 0.98 μg/mL (Table I, column LAV-1/MT-4), inhibition of syncytia formation (G-cell) in a coculture system of MOLT-4 with persistently HIV-1_{LAV-1}-infected MOLT-4 cells at 1.4 μg/mL or with persistently HIV-2_{GH-1}-infected MOLT-4 cells at 1.7 μg/mL, and anticoagulant activity expressed by duplication of the activated partial antithrombin time (APTT) at 7.0 μg/mL. It is notable that the inhibitory activity of mCDS **11** is very strong in the syncytium formation in both the HIV-1 and HIV-2 infected cells. The cytotoxicity of mCDS **11** was above 1000 μg/mL, while the inhibition of reverse transcriptase activity (RT) was rather weak, 630 μg/mL.

It is surmised that the conventional assay system for anti-HIV-1 activity using a combination of the strain LAV-1 which has been cultured for a long time in laboratories and the targeted MT-4 cells carrying HTLV-I does not reflect the natural infection in human body. Therefore, we designed an assay system using freshly isolated HIV-1 strains (KK-1_{AIDS} isolated from an AIDS patient and KK-5_{AC} from an asymptomatic virus carrier) and peripheral blood mononuclear cells (PBMC) from a healthy donor. After preincubation of the PHA-stimulated PBMC with the either strain of HIV-1 for 3 h, mCDS effectively inhibited the replications of the HIV-1, while DS was ineffective (Table I, column KK-1_{AIDS}/PBMC and KK-5_{AC}/PBMC). This remarkable difference between mCDS and DS was also observed in an assay system which used a combination of HIV-1 (strain KK-1_{AIDS}) and MT-4 cells. Thus, the main factor which caused the differences was not due to the cell lines but due to the virus strains. Contrary to this, when the test compounds were presented at the initial infection period, both mCDS **11** and DS inhibited the replication completely (Table I, column KK-1_{AIDS}/PBMC, data in parentheses).

From the foregoing facts, the main action mechanisms of mCDS suggest that mCDS does not only inhibit the

Scheme I. Synthesis of Modified Cyclodextrin Sulfates (mCDS)

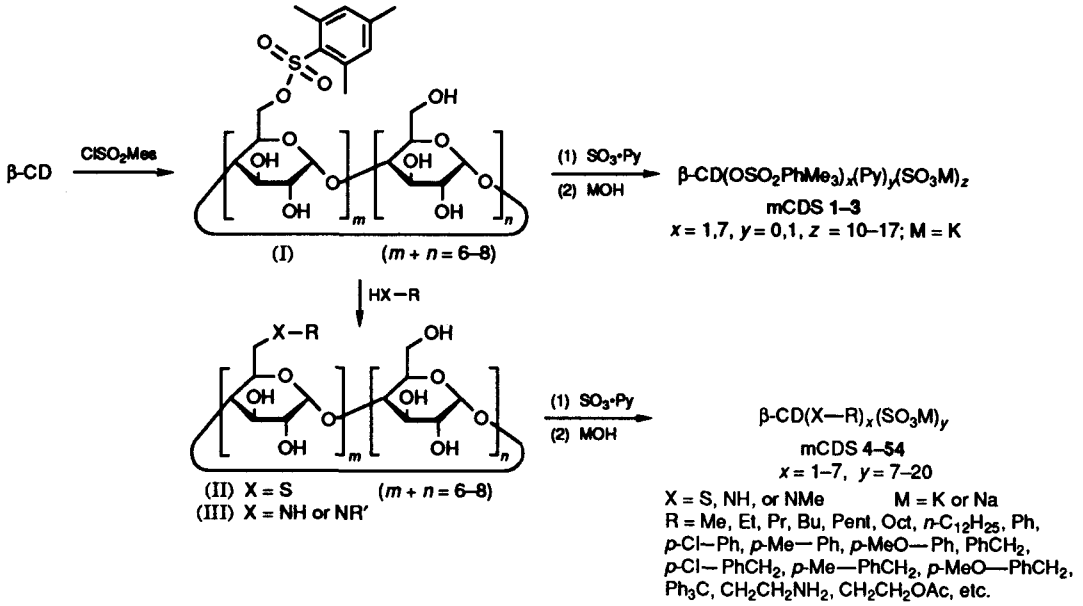

Table I. Anticoagulant Activity and Inhibitory Effect of mCDS on HIV-1 Replication, Reverse Transcriptase, Giant Cell Formation, and Cell Viability

		HIV-1					G-cell[e]			
compd	abbreviated formula[a]	LAV_{-1}[b] MT-4	$KK\text{-}1_{AIDS}$[b] MT-4	$KK\text{-}1_{AIDS}$[c] PBMC	$KK\text{-}5_{AC}$[c] PBMC	RT[d]	$HIV\text{-}1_{LAV\text{-}1}$ MOLT-4	$HIV\text{-}2_{GH\text{-}1}$ MOLT-4	APTT[f]	cytotox[g]
mCDS 3	β-CD(OSO_2PhMe_3)(SO_3K)$_{16}$-(SO_3^-)(Py^+)	1.95	62.5	382	180	>1000	42.0	64.4	4.15	>1000
mCDS 11	β-CD(SCH_2Ph)$_3$(SO_3K)$_{16}$	0.98	1.95	6.5 (0.60)	19.6	630	1.4	1.7	7.00	>1000
mCDS 38	β-CD(NHPh-4-OMe)$_7$(SO_3Na)$_{12}$	0.98	31.2	86	72.5	706	5.5	40.2	4.30	>1000
CDS	β-CD(SO_3K)$_{14}$	31.20	125	330		>1000	60.0	519	2.83	>1000
CDP	β-CD(PO_3HK)$_8Cl_5$	31.20	40			200			47.20	>1000
DS	(8000, SIGMA)	3.90	500	>500 (1.52)	500	>1000	9.9	172	3.30	>1000

[a] mCDS 3: Potassium 6-deoxy-6'-*O*-(mesitylenesulfonyl)-6-pyridinio-β-cyclodextrin heptadecasulfate. mCDS 11: Potassium tris(6-benzylthio-6-deoxy)-β-cyclodextrin hexadecasulfate. mCDS 38: Sodium heptakis[6-deoxy-6-(4-methoxyanilino)-β-cyclodextrin. [b] The minimum concentration for complete inhibition of HIV-1 induced CPE in MT-4 cells (IC_{100}): MT-4 cells were infected with 0.001 $TCID_{50}$ (determined by MT-4 cells on day 5 after infection) of HIV-1 (strain LAV-1 or $KK\text{-}1_{AIDS}$ from patient) per cell for 1 h and nonadsorbed virus was removed by washing. After 5 days of incubation with various concentrations (12 doses, 0.49–1000 μg/mL) of the test compound, the number of viable cells in both the HIV-1 and mock-infected cell cultures was determined by trypan blue staining. [c] Inhibition of HIV-1 replication in peripheral blood mononuclear cells (PBMC) is expressed as the inhibitory concentration, which reduces by 50% the RT activity of the culture supernatant (IC_{50}): PBMC obtained by the Ficoll–Hypaque technique from healthy donor were stimulated with 0.1% phytohemagglutinin (PHA, Difco) for 3 days. The PBMC and freshly isolated HIV-1 (strain $KK\text{-}1_{AIDS}$ or $KK\text{-}5_{AC}$ from an asymptomatic virus carrier) were incubated for 3 h with or without the test compounds. After removal of nonadsorbed virus by washing, HIV-1 infected or mock-infected PBMC was cultured in the presence of 200 unit/mL recombinant interleukin-2 (Shionogi Laboratories) and the test compounds of various concentrations (6 doses, 0.49–500 μg/mL) for 6 days. Half of the cells and culture medium were then removed and the remaining half was further incubated with the same concentrations of the compounds and the PHA-stimulated fresh PBMC in fresh medium for 4 days. HIV-1 reverse transcriptase (RT) activity of each culture supernatant was evaluated by the method of Lee et al.[14] with poly(rA)oligo(dT) used as the template primer. Mean RT activity (cpm) of the positive control (not treated with compound) was 1.2×10^6 cpm, and the negative control (not exposed to HIV-1 and not treated with compound) was 1.1×10^4 cpm. The values in parentheses were obtained when the test compounds were presented at the initial infection period. [d] The IC_{50} for inhibitory effect on reverse transcriptase of HIV-1: The direct effect of the compounds on cell-free RT activity of HIV-1(LAV-1) was determined with poly(rA)oligo(dT) as the template primer, as described by Lee et al.[14] [e] Suppressive effect on giant-cell formation: By following a modified method described by Nakashima et al.,[15] MOLT-4 and MOLT-4/$HIV\text{-}1_{LAV\text{-}1}$ or MOLT-4/$HIV\text{-}2_{GH\text{-}1}$ cells were mixed at a ratio of 1:1 (total cell number of 5×10^5 cells/mL) and the mixture was cultured for 24 h with the medium containing the test compounds. The number of viable cells was counted by the trypan blue exclusion method, and the fusion index (FI) was calculated as follows: FI = 1 − [no. of cells in test well (MOLT-4 + MOLT-4/HIV-1 or 2)]/[no. of cells in control (MOLT-4 cells)]. [f] Anticoagulation effect: Zuchker's activated partial thromboplastin time (APTT) method[16] was used. The value is indicated by the concentration (μg/mL) required to obtain 2-fold APTT. [g] Minimum concentration (μg/mL) for appearance of MT-4 cell toxicity after 5 days of incubation with the test compound. All data represent median values of 2 or 3 experiments.

initial adsorption of HIV to the target cells and cell to cell infections, like DS, but it also has an additional effect. This additional effect might play a significant role in inhibiting the replication of the freshly isolated HIV-1 strains. The structure of mCDS 11 is unique in having a hydrophobic cavity surrounded by a cloudy ring of swarmed anionic sulfate groups and lipophilic substituents which are like tentacles, as illustrated in the image of the molecule in Figure 2. It is conceivable that the cavity and the tentacles include either the nonpolar binding site on gp-120 or CD-4 such as Phe^{324} on the epitope β of gp-120,[9] Trp^{432} on gp-120 for CD4 binding,[10] and Phe^{43} on the domain D1 of CD4 molecule.[11]

From the viewpoint of therapy, conservation of the potent anti-HIV activity in vivo and oral absorbability were the most important problems that preceded developing

polysulfated compounds such as the DS and HOE/BAY946.[3,4a,12] Effectiveness of oral administration of mCDS 11 was suggested from the following ex vivo test. The HIV-1_{LAV-1}-induced CPE in MT-4 cells was completely inhibited by 50- and 160-fold diluted plasma which were prepared 2 h after giving 1 and 2 g/kg per os of mCDS 11 to male rats, respectively. On the basis of this result, the hydrophobic benzylthio groups, rigid cyclic skeleton, and the relatively small molecular size[13] of mCDS 11 are being considered to facilitate the penetration to the intestinal membrane and prevent the hydrolytic destruction of the molecule in body.

The acute toxicity of mCDS 11 was not observed at 3 g/kg per os in mice.

The elucidation and characterization of the action mechanisms of mCDS and selection of the most suitable candidate for the treatment of AIDS patients and asymptomatic virus carriers are still in progress.

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A New Candidate for an Anti-HIV-1 Agent: Modified Cyclodextrin Sulfate (mCDS71)

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The pandemic of acquired immunodeficiency syndrome (AIDS) is continuing to expand worldwide at an exponential rate, as a sexually transmitted disease. Numerous compounds with various mechanisms of action against the causative human immunodeficiency virus (HIV) of AIDS[1] are under development, but at present the only drugs approved by the US FDA for the treatment of AIDS are nucleoside derivatives: 3′-azido-3′-deoxythimidine (AZT), 2′,3′-dideoxyinosine (DDI), and 2′,3′-dideoxycytidine (DDC). Although these drugs have a potent inhibitory activity on the reverse transcriptase of HIV, serious side effects of the drugs (e.g. myelosuppression, neuropathy, and pancreatitis) and the emergence of drug-resistant strains of HIV have been reported.[1,2]

Considerable attention is currently being focused on polyanionic compounds[3] that show highly potent inhibitory activity on the replication of HIV in vitro because of their synergistic activity[4] with the nucleoside drugs and their anti-HIV action mechanism that is entirely different from that of the nucleoside drugs, i.e., they inhibit virus binding to the cell membrane resulting in the marked inhibition of cell fusion (syncytium formation). Among the polyanionic compounds, sulfated polysaccharides such as dextran sulfate (DS), pentosan sulfate (HOE/BAY-946), curdran sulfate, and others, have been investigated most actively as potentially useful agents for the treatment of AIDS. However, their effectiveness in vivo has not been established as yet[5] because of their poor absorbability owing to their large molecular size, short half-life time in the body by metabolic hydrolysis, and unfavorable anticoagulant activity in blood. Additionally, a variety of modes of action of the sulfated polysaccharides have recently been proposed.[6] This suggests manifold and complicated interaction of virus and targeted cells, and such variety could also be associated with the indefinable molecular structures showing broad ranges of molecular weight and undefined numbers of the sulfate groups and the sulfated positions.

In a previous paper,[7] we proposed a sort of guiding-star of research to overcome the above problems for development of polyanionic compounds as oral anti-HIV agents. Thus, it is necessary to simplify the molecular structure for a rational drug design based on structure–activity relationships. Additionally, down-sizing and introduction of lipophilic groups to the molecule should improve both absorbability in the gut and the separation of anticoagulant activity. In accordance with the guide, we synthesized a series of modified cyclodextrin sulfates (C-6 mCDSs) with various functional groups on the C-6 positions of the cyclodextrin (CD) frame. We found a candidate compound mCDS11 which was modified with three benzylthio groups as the lipophilic moiety, along with 16 anionic sulfate groups, as shown Figure 1. Enhancement of anti-HIV activity and separation of the defective anticoagulant activity were well established in mCDS11. The absorption of mCDS11 from the gut was confirmed by the anti-HIV activity of the plasma obtained from rats given the compound per os.

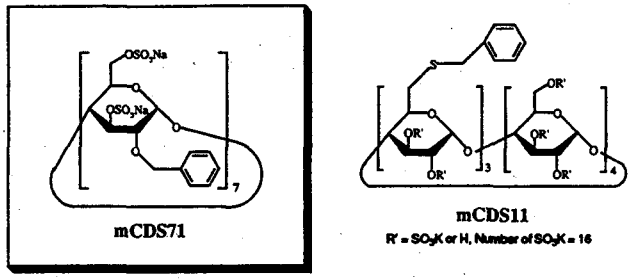

Figure 1.

In this paper, we report an advanced investigation of mCDS and a new candidate mCDS71. A series of C-2 modified cyclodextrin sulfates (C-2 mCDS) were synthesized instead of C-6 modified analogs for the development of a more satisfactory harmless oral anti-HIV agent with high activity. The introduction of lipophilic substituents to the C-2 position of CD was carried out according to the method of Takeo et al.[8] with slight modification, as shown in Scheme I. The most reactive seven C-6 hydroxyl groups of CD were protected with *tert*-butyldimethylsilyl (TBS) groups before modification of the C-2 position hydroxyl groups. The resultant C-6 silyl CD derivative (I) was reacted with various alkyl halides, using a barium oxide–barium hydroxide mixture as a base in dimethyl formamide (DMF), and the silyl groups of the formed 6-silyl-2-alkylated-cyclodextrin derivative (II) were then removed by tetrabutylammonium fluoride. The C-2 modified cyclodextrin (III) thus obtained was sulfated by sulfur trioxide–pyridine complex in pyridine to afford the objective C-2 mCDS. The compound mCDS78, in which the C-6 hydroxyl groups are not sulfated, was prepared by direct sulfation of II and by successive deprotection under acidic conditions.

The C-2 mCDS thus prepared were screened in the same way as reported previously.[7] Anti-HIV-1 activity which was estimated by determining the inhibition of the cytopathic effect (CPE) in MT-4 cells, using two strains of LAV-1 (a well-established cultured strain in laboratories) and KK-1_{AIDS} (a clinically isolated HIV-1 strain from a Japanese AIDS patient); anticoagulant activity was examined in human serum, and cytotoxicity was investigated in MT-4 cells.

Three of four mCDS compounds had roughly equal inhibitory activity against the two strains of HIV-1 tested.

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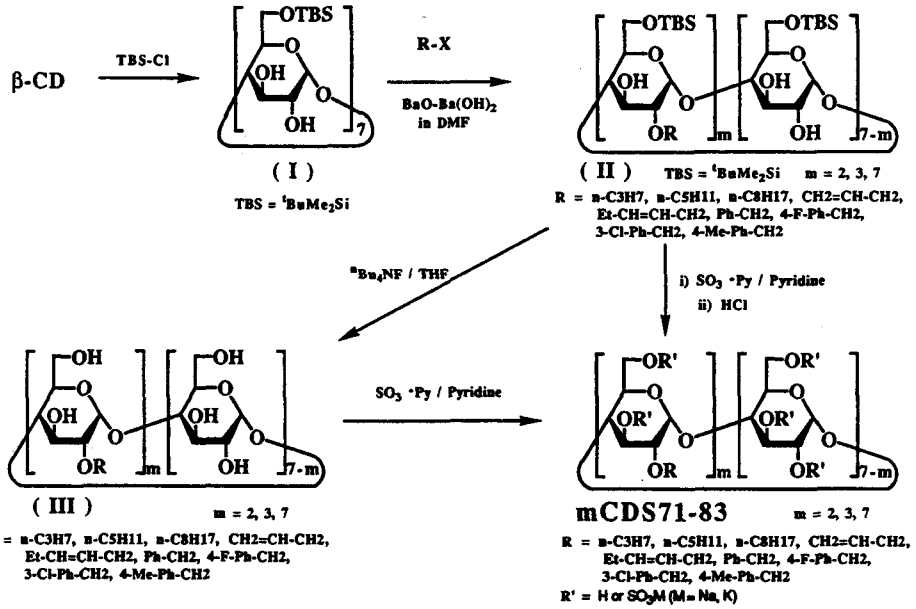
Table I. Anticoagulant Activity and Inhibitory Effect of mCDSs on HIV-1 Replication and Cell Toxicity

compound [abbreviated formula][a]	anti-HIV-1[b] (IC_{100}, μg/mL) LAV-1	anti-HIV-1[b] (IC_{100}, μg/mL) $KK\text{-}1_{AIDS}$	APTT[c] (μg/mL)	cytotox[d] (TD_{50}, μg/mL)
mCDS71 [β-CD(2-*O*-CH_2Ph)$_7$(SO_3Na)$_{14}$]	0.98	0.98	14.8	1000
mCDS77 [β-CD(2-*O*-CH_2Ph)$_3$(SO_3K)$_{16}$]	1.95	3.90	4.0	>1000
mCDS78 [β-CD(2-*O*-CH_2Ph)$_3$(SO_3K)$_9$]	7.80	15.60	13.5	>1000
mCDS11 [β-CD(6-*S*-CH_2Ph)$_3$(SO_3K)$_{16}$]	0.98	1.95	7.1	>1000
CDS [β-CD(SO_3K)$_{14}$]	31.26	125	2.8	>1000
DS [DS8000]	3.90	250	3.3	>1000

[a] mCDS71: Tetradecasodium heptakis(2-*O*-benzyl)-β-cyclodextrin tetradecasulfate. mCDS77: Hexadecasodium tris(2-*O*-benzyl)-β-cyclodextrin hexadecasulfate. mCDS78: Nonasodium tris(2-*O*-benzyl)-β-cyclodextrin nona(2- and 3-sulfate). mCDS11: Hexadecapotassium tris(6-(benzylthio)-6-deoxy)-β-cyclodextrin hexadecasulfate. CDS: Tetradecapotassium β-cyclodextrintetradecasulfate. DS: Dextran sulfate purchased from Sigma Chemical Co. prepared from average molecular weight of 8000. [b] The minimum concentration for complete inhibition of HIV-1-induced CPE in MT-4 cells (IC_{100}): MT-4 cells were infected with 0.001 $TCID_{50}$ (determined by MT-4 cells on day 5 after infection) on HIV-1 (strain LAV-1 or $KK\text{-}1_{AIDS}$, a strain clinically isolated from an AIDS patient) per cell for 1 h, and nonadsorbed virus was removed by washing. After 5 days of incubation with various concentrations (12 doses, 0.49–1000 μg/mL) of the test compound, the number of viable cells in both the HIV-1- and mock-infected cell cultures was determined by trypan blue staining. [c] Anticoagulation effect: Zuchker's activated partial thromboplastin time (APTT) method[11] was used. The value is indicated by the concentration (μg/mL) required to obtain 2-fold APTT. [d] Minimum concentration (μg/mL) for appearance of MT-4 cell toxicity after 5 days of incubation with the test compound. All data represent median values of two or three experiments.

Scheme I. Synthesis of 2-O-Modified β-Cyclodextrin Sulfates



All mCDS compounds had enhanced potency when compared to CDS. These findings strongly suggest that the introduction of lipophilic groups to the polyanionic compound CDS particularly enhanced anti-HIV-1 activity.

The anticoagulant activity of mCDS71 and 78 was very weak: this was mainly attributed to the location of the sulfate groups at one side of the CD ring (in the case of mCDS78) or to masking of the C-3 sulfate groups by C-2 lipophilic groups (in the case of mCDS71). Thus, the sulfate groups located at top and bottom of the CD ring appear to behave as the site corresponding to the two binding sites of heparinoides to antithrombin III.[9]

The most potent compound among the C-2 mCDS, mCDS71 [tetradecasodium heptakis(2-*O*-benzyl)-β-cyclodextrin tetradecasulfate, $C_{91}H_{98}O_{77}S_{14}Na_{14}$, MW 3194.71], has seven uniformly modified glucose units bearing a benzyloxy group at the C-2 position and sodium sulfate groups at the C-3 and C-6 positions as shown in Figure 1. Therefore, it has a C_7 symmetry axis at the center of the doughnut-like β-cyclodextrin molecular frame. The extremely orderly constructed structure was suggested from the sharp and well-assignable NMR spectra. The uniformity of mCDS71 should serve to elucidate the mechanisms of action of a desirable polyanionic anti-HIV agent and should also help in the development of such agents. mCDS71 exhibited anti-HIV-1 activity at 0.98 μg/mL [the minimum concentration for complete inhibition (IC_{100}) of both the $HIV_{LAV\text{-}1}$- and $HIV_{KK\text{-}1/AIDS}$-induced cytopathic effect (CPE) in MT-4 cells]. The anticoagulant activity was exerted at 14.80 μg/mL [the concentration for doubling of the activated partial thrombin time (APTT)], which corresonded to only half and one-fourth of the anticoagulant activity of mCDS11 and DS, respectively. The cytotoxicity of mCDS71 as low as 1000 μg/mL did not affect MT-4 cells.

To determine which compound, mCDS71 or -11, is a more suitable development candidate as an AIDS treatment, the biological studies of each were further conducted. Anti-HIV-1 activity was reexamined in the presence of 50% fresh human serum (HS) in the medium to reflect the *in vivo* situation by the experimental conditions and also to account for the suppressive effect of HS, which had been reported to reduce the activity of DS.[5d] As shown in Table II, excellent results for mCDS71 were documented in all the anti-HIV-1 activity assay systems tested. That is, the undesirable influence of HS on mCDS71 was negligible; high activity (IC_{50} = 0.87 μg/mL) was shown on the CPE assay system constructed with a conventional combination of MT-4 cells and the LAV-1 strain, whereas

Table II. Inhibitory Effect of mCDS71 and -11 on HIV-1 Replication and Giant Cell Formation in the Presence of Human Serum

compound [abbreviated formula]	anti-HIV-1 (IC_{50}, μg/mL) LAV-1 MT-4[a]	anti-HIV-1 (IC_{50}, μg/mL) $KK\text{-}1_{AIDS}$ MT-4[a]	anti-HIV-1 (IC_{50}, μg/mL) $KK\text{-}1_{AIDS}$ PBMC[b]	suppression of G-cell formation[c] (IC_{50}, μg/mL) MOLT-4/$MOLT\text{-}4_{LAV\text{-}1}$
mCDS71 [β-CD(2-*O*-$CH_2Ph)_7(SO_3Na)_{14}$]	0.87	5.80	11.50 (3.6)	0.81 (0.46)
mCDS11 [β-CD(6-*S*-$CH_2Ph)_3(SO_3K)_{16}$]	4.50	11.00	27.50 (6.5)	12.70 (2.00)
DS8000	6.40	350	>500 (500)	21.00 (6.60)

[a] Concentration that causes 50% inhibition of the CPE, estimated in the same way as described in Table I, in the presence of 50% human serum (HS) in the culture medium. [b] Inhibition of HIV-1 replication in peripheral blood mononuclear cells (PBMC) is expressed as the inhibitory concentration that reduces the RT activity of the culture supernatant by 50% (IC_{50}): PBMC, obtained by the Ficoll-Hypaque technique from a healthy donor, were stimulated with 0.1% phytohemagglutinin (PHA, Difco) for 3 days. The PBMC were infected with 0.001 $TCID_{50}$ (determined by PBMC on day 10 after infection) of HIV-1 (strain $KK\text{-}1_{AIDS}$ from a patient) per cell for 3 h. After removal of nonadsorbed virus by washing, HIV-1-infected or mock-infected PBMC were cultured in the presence of 200 units/mL recombinant interleuken-2 (Shionogi Laboratories) and the test compounds, in various concentrations (6 doses, 2.1–500 μg/mL), for 6 days. Half of the cells and culture medium were then removed. The remaining half was further incubated with the same concentrations of the compounds and the PHA-stimulated fresh PBMC in fresh medium for 4 days. HIV-1 reverse transcriptase (RT) activity of each culture supernatant was evaluated by the method of Lee et al.[13] with poly(rA)oligo(dT) used as the template primer. Mean RT activity (cpm) of the positive control (not treated with compound) was 1.2×10^6 cpm; the negative control (not exposed to HIV-1 and not treated with compound) was 1.1×10^4 cpm. The values in parentheses were obtained in the presence of 20% FCS instead of HS. [c] Suppressive effect on giant-cell formation (IC_{50}): Via the modified method described by Nakashima et al.,[13] MOLT-4 and $MOLT\text{-}4/HIV_{LAV\text{-}1}$ cells were mixed at a ratio of 1:1 (total cell number of 5×10^5 cells/mL). The mixture was cultured for 24 h with medium containing the test compounds and 50% HS. The number of viable cells was counted by the trypan blue exclusion method, and the fusion index (FI) was calculated as follows: FI = 1 – [no. of cells in test well (MOLT-4 + MOLT4/HIV-1)]/[no. of cells in control (MOLT-4 cells)]. The values in parentheses were obtained in the presence of 10% FCS instead of HS.

the activity of mCDS11 was reduced, being shown at 4.50 μg/mL. The excellence of mCDS71 was also shown in the natural isolated HIV-1 strain: the cytopathogenicity of the clinically prepared $HIV_{KK\text{-}1/AIDS}$ was well inhibited by mCDS71 in MT4 cells at 5.80 μg/mL and the replication of $HIV_{KK\text{-}1/AIDS}$ in PBMC was blocked at 11.50 μg/mL. These activities were approximately 2-fold the corresponding activities of mCDS11. (As a reference, the inhibition concentrations of the replication of $HIV_{KK\text{-}1/AIDS}$ in PBMC in 20% FCS are shown in parentheses.) The most remarkable superiority of mCDS71 to mCDS11 was shown in the inhibition of giant cell formation. mCDS71 inhibited syncytium formation at 0.81 μg/mL, and its activity was more than 10 times that of mCDS11, due to the smaller reduction of this activity by HS. (Compare the effective concentrations in 10% FCS given in parentheses.)

To elucidate these different undesirable influences of HS on the anti-HIV activity of polyanionic compounds, binding to serum proteins was estimated by determining the anti-HIV-1 activity of an ultrafiltered HS solution of the agents after removal of the formed agent–protein complexes. Surprisingly, the most striking binding, 98.4%, was observed in mCDS71, with the binding magnitudes of mCDS11 and DS being 93.8% and 87.4%, respectively, indicating a reciprocal relation to the influence of HS. These findings suggested that mCDS71 binds nonselectively to serum proteins in plasma and then rebinds specifically to the surface of HIV virions and/or HIV infected cells, if present, thereby exerting potent inhibition of viral replication and especially of syncytium formation.

In addition, the synergistic antiviral effect of mCDS71 with AZT was detected in terms of an inhibitory effect on the replication of clinically isolated $HIV\text{-}1_{KK\text{-}1/AIDS}$ in PBMC cultures in medium containing 50% HS. Elion's[10] fractional inhibitory concentration (FIC) values were between 0.5 and 1.0.

From the viewpoint of therapy, conservation of potent anti-HIV activity *in vivo* and the bioavailability of the agent after oral administration (oral bioavailability) are the most important aspects to be focused on in the development of polysulfated compounds such as DS and HOE/BAY-946. The oral absorbability of mCDS71 was estimated to be about 3-fold that of mCDS11. The plasma level of mCDS71, determined by the inhibition of 320-fold diluted plasma on $HIV_{LAV\text{-}1}$-induced CPE in MT-4 cells, was 320 μg/mL at 2–3 h after oral administration of 1 g/kg in male rats.

On the basis of the above result, the hydrophobicity of mCDS71 increased by the seven benzyloxy groups, the rigid cyclic skeleton, and the relatively small molecular size are considered to facilitate penetration of mCSD71 through the intestinal wall and prevent the hydrolytic destruction of the molecule in the body.

In an oral toxicity test of mCDS71 in male mice, no toxicity was exhibited at 2.0 g/kg/day for 5 days.

The elucidation and characterization of the mechanisms of action and toxicity of mCDS71 are still in progress. Of particular importance will be to determine if the biological diversity of HIV-1 affects the inhibitory potency of mCDS71. We are presently in the process of testing this compound against a panel of reference strains of HIV isolates and will report this data in a full paper.

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Supplementary Material Available: Experimental details for the synthesis of tris(2-*O*-benzyl)heptakis(6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (**IIb**), tris(2-*O*-benzyl)-β-cyclodextrin (**IIIb**), tetradecasodium heptakis(2-*O*-benzyl)-β-cyclodextrin tetradecasulfate (mCDS71), and nonasodium tris(2-*O*-benzyl)-β-cyclodextrin nonasulfate (mCDS78). (4 pages). Ordering information in given on any current masthead page.

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