Novel Zinc Chelators Which Inhibit the Binding of HIV-EP1 (HIV Enhancer Binding Protein) to NF-κB Recognition Sequence

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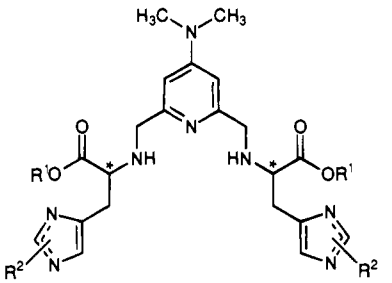
Physiologically significant proteins often contain zinc, e.g., as a cofactor of enzymes[1] and also as a key element to build up the secondary structure of proteins as seen in the zinc finger motif of transcription factors.[2] It was thought that the function of these zinc proteins could be modulated by ejecting the zinc, and this approach may provide a novel strategy for the rational design of drugs and biochemical tools. The effectiveness of this concept was exemplified by a recent report of Rice *et al.* that an aromatic *C*-nitroso compound can oxidize cysteine residues of zinc finger protein to eject zinc and inhibit HIV-1 infectivity in human lymphocyte.[3] We report herein an alternate approach using novel zinc chelators. Previously one of the authors reported human immunodeficiency virus type 1 enhancer binding protein, namely HIV-EP1[4] (also designated as PRDII-BF1[5] or MBP1[6]), which contains two C_2H_2 type zinc fingers and binds to 5′-GGGACTTTCC-3′ known as NF-κB recognition sequence.[4] The objective of this study is to construct an efficient zinc-coordinating system which can abstract zinc from HIV-EP1 to inhibit DNA binding.

Recently we reported a metal-chelating system with symmetrical structure comprising a (dimethylamino)-pyridine and histidine methyl ester, namely Me_2N-HPH (1),[7] designed by a total structural revision of our previous oxygen-activating molecules.[8] We considered that the structure of the compound 1 could be modified so as to be an efficient zinc trapper. First we tried to introduce a trityl group into the imidazole in order to alter the chelating characteristics of the imidazolyl group. We also attempted to change the methyl ester groups of compound 1 into carboxyls since carboxylate, together with imidazoles, is recognized as a key structural feature of zinc-chelating site of enzymes such as superoxide dismutase[9] and carboxypeptidase[10] in addition to the aminoacetate portion of EDTA, a strong metal chelator widely used in the demetalation procedure of biochemical protocols.[11] Thus, we prepared trityl and/or carboxyl derivatives **2**–**4** starting with Me_2N-HPH (1).[12]



Compound	R^1	R^2	Stereochemistry (*)
1	CH_3	H	*SS*
2	CH_3	Trt	*SS*
3	H	Trt	*SS*
4	H	Trt	*RS*

Treatment of metal-free 1–**4** with equimolar $ZnSO_4$ in MeOH afforded the corresponding 1:1 zinc complexes. Zinc-chelated 1–**4** thus formed were distinguished from the metal-free 1–**4** by ^1H or ^{13}C NMR spectroscopy (for NMR data, see the supplementary material).[17] ^{13}C NMR measurement also gave some information on the coordination mode of each complex in CD_3OD and/or CD_3OD–D_2O (4:1),[18] i.e., whereas the coordination of the pyridine nitrogen, the secondary amino, and the imidazole groups of 1 and **2** was assumed, two carboxyl groups of **3** and **4** appeared to bind to the zinc more significantly.[19]

The zinc affinity of the synthetic ligands **2**–**4** was compared with that of ligand 1 by competitive zinc binding experiments using ^1H NMR (eq 1). To a solution of a mixture of compound 1 (~1 equiv) and a compound to be compared (~1 equiv) in CD_3OD–D_2O (4:1),[18] $ZnSO_4 \cdot 7H_2O$ (<1 equiv) was added and the ratio of zinc-chelated compounds was estimated by ^1H NMR (Table 1). The affinity of the ligand 1 for zinc was decreased by introducing trityl group probably due to the steric hinderance of the trityl groups and reduced basicity of the imidazole. However, zinc binding power was greatly improved by changing the methyl ester to the carboxyl, and *RS*-isomer **4** showed the highest affinity for zinc. The same tendency in zinc binding was also observed in CD_3OD.

The synthetic chelators 1–**4** were found to exhibit remarkable inhibitory effect on the DNA binding of HIV-EP1, even more potent than that of EDTA, as demonstrated by electrophoretic mobility shift assay (Figure

Table 1. Competitive Zinc Binding Experiments of Compounds 1–4[a]

$$\mathbf{1} + \text{ligand} \xrightarrow[\substack{CD_3OD\text{-}D_2O \\ (4:1)}]{ZnSO_4} \mathbf{1}\text{–Zn(II)} + \text{ligand–Zn(II)} \quad (1)$$

ligand	[ligand/Zn]/[ligand] / [1–Zn]/[1]
1	1
2	0.3
3	10
4	16

[a] The ratio of zinc complexes were estimated by ^1H NMR spectroscopy.

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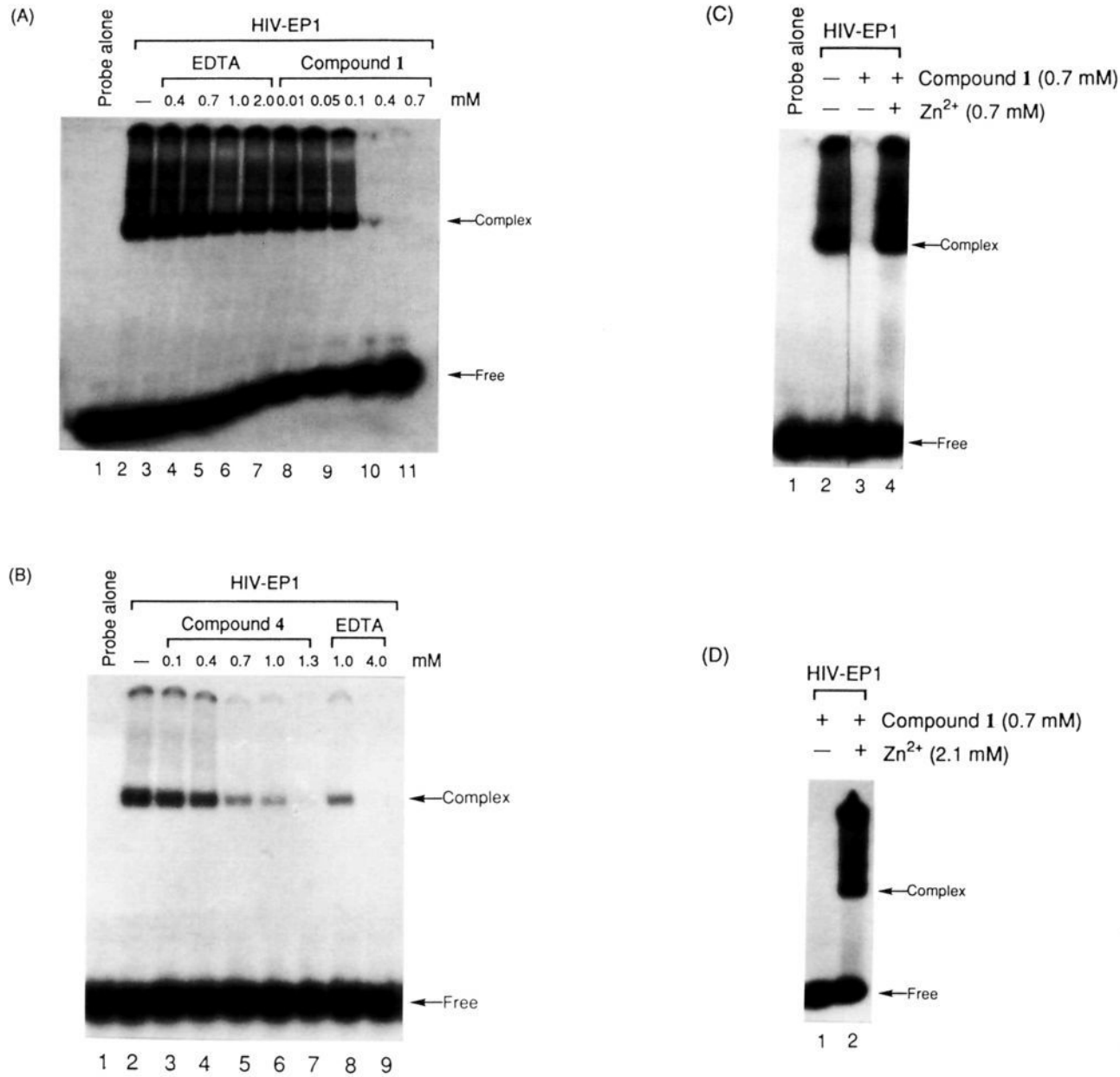

Figure 1. Effect of ligands **1** and **4** on the DNA binding of HIV-EP1. HIV-EP1 was incubated with each sample in the presence of poly(dI-dC) at room temperature for 30 min. A radioactive double-stranded oligonucleotide containing a κB site from the mouse κ light-chain enhancer (5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′ and 5′-TCGACCTCTCGGAAAGTCCCCTCTGA-3′) was added. Sample was loaded onto a polyacrylamide band shift gel, and the gel electrophoresis was run. (A) Dose dependence of **1** and EDTA. (B) Dose dependence of **4** and EDTA. (C) Effect of $ZnSO_4$ (1 equiv of **1**, introduced before the addition of DNA). (D) Effect of $ZnSO_4$ (3 equiv of **1**, introduced after the addition of DNA).

1, Table 2). The most potent was compound **1** which inhibited the DNA binding almost completely at 0.4 mM concentration (Figure 1A). The most strong zinc chelator **4** showed somewhat weaker inhibition (Figure 1B). Discrepancy between the DNA-binding inhibitory effect and the zinc-binding power of ligands may be due to their relatively low solubility in aqueous media or possibly due to the difference in the dissociation of the carboxyl group of the ligand depending on the solvent constitution (CD_3OD-D_2O (4:1) for the NMR measurement and H_2O-CH_3OH (96:4) for the DNA-binding experiments). All these ligands were shown to be stronger inhibitors of DNA binding compared with EDTA, although EDTA showed stronger affinity for zinc (Figure 1, Table 2),[20] suggesting the superiority of the nitrogen-containing heterocyclic structure in terms of amino acid interaction, hydrophobic interaction, or possibly electronic effect favorable for the formation of presumed intermediary ternary complex with HIV-EP1–Zn. When zinc was introduced during (Figure 1C) or after (Figure 1D) the DNA-binding inhibition reaction with compound **1** (0.7 mM), total recovery of HIV-EP1–DNA complex was observed. Ethidium displacement and footprinting experiments indicated that **1** has virtually no interaction with DNA, consistent with the observation that metal-free **1** did not inhibit the DNA-binding of NF-κB.[21] These indicated that the inhibition was indeed caused by the removal of zinc from the zinc finger moiety of HIV-EP1 and ruled out a competition between ligand **1** and HIV-EP1 for binding to DNA.

Table 2. Inhibition of DNA Binding of HIV-EP1 by Synthetic Ligands and EDTA

no.	compound	concn (mM)	DNA-bound HIV-EP1 (%)[a]
1	**1**	0.01	100
2		0.05	81
3		0.1	68
4		0.4	4
5		0.7	0
6		1.0	0
7	**1** (0.7 mM) + $ZnSO_4$ (0.7 mM)[b]	0.7	100
8	**1** (0.7 mM) + $ZnSO_4$ (2.1 mM)[c]	0.7	100
9	**2**	1.0	7
10	**4**	0.1	76
11		0.4	58
12		0.7	28
13		1.0	21[d]
14		1.3	6
15	**4** (1.3 mM) + $ZnSO_4$ (1.3 mM)[b]	1.3	100
16	EDTA	1.0	33
17		4.0	16
18		7.0	0

[a] Quantitation of radioactivity of the electrophoresis band was conducted using a image analyzer. [b] $ZnSO_4$ was introduced before addition of DNA. [c] $ZnSO_4$ was added after addition of DNA. [d] Compound **3** showed almost same value by a visual judge.

Thus, we developed novel zinc-binding heterocycles and succeeded in the inhibition of DNA binding of a zinc finger protein HIV-EP1. Since this approach can basically be applicable to any zinc proteins and the further structural modification of the pyridine-histidine system would be easily attained, the present study may provide a basis for the control and elucidation of various biochemical processes.

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Supplementary Material Available: ^1H or ^{13}C NMR data for metal-free and Zn-chelated compounds **1**–**4**, difference in ^{13}C chemical shifts between the Zn-chelated and metal-free **1**–**4**, and HPLC for **2**–**4** (8 pages). Ordering information is given in any current masthead page.

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(12) Trityl derivative **2** was obtained in 88% yield by treatment of compound **1** with triphenylmethyl bromide [HRMS(FAB) [M + H]$^+$ calcd 969.4816, found 969.4824]. Alkaline hydrolysis of methyl ester **2** afforded carboxylic acid **3** along with its diastereomer **4**.[13] Unexpectedly, attempt to separate diastereomers **3** and **4** by preparative TLC resulted in the capture of zinc from the TLC plate,[14] affording zinc complexes of **3** (74% ee)[15] and **4**[16] in 14% and 21% yields, respectively. The idenfication of these zinc complexes was based on high resolution mass spectroscopy [HRMS(FAB) [M + H]$^+$ calcd 1003.3642, found 1003.3685 (**3**); 1003.3661 (**4**)] and indusively coupled argon plasma atomic emission spectroscopy (48% of calculated value). Zinc-free diastereomers **3** and **4** were separated by employing silica gel *column* chromatography in 16% and 9%, respectively [HRMS-(FAB) [M + H]$^+$ calcd 941.4503, found 941.4504 (**3**); 941.4505 (**4**)]. Homogeneity of new compounds **2**–**4** was confirmed by HPLC (see the supplementary material). We thank Prof. M. Matsui and Dr. Y. Sohrin, Institute for Chemical Research, Kyoto University, for the measurement of indusively coupled argon plasma atomic emission spectra, and K. Tanaka, JEOL LTD., for the measurement of fast atom bombardment high-resolution mass spectra.
(13) Isomers **3** and **4** were formed in a ratio **3**:**4** = 60:40. The ratio did not change throughout the reaction or by conducting the hydrolysis of ester **2** at 5 °C.
(14) Merck 7747 silica gel containing zinc silicate as fluorescent indicator.
(15) Optical purity was determined by HPLC (column: CHIRALPAK AD, 0.46 × 25 cm, eluted with EtOH:*n*-hexane:Et_2NH = 60:40: 0.1, retention time: *SS*-isomer, 7.4 min; *RR*-isomer, 10.2 min).
(16) Stereochemistry of the isomers **3** and **4** was determined by the specific rotation and considering the symmetry of each zinc complex. The compound showing unsymmetrical NMR signals was assigned to be a zinc complex of *RS*-isomer **4**.
(17) Zinc complex of compound **3** exists in two different coordination geometries in a ratio 70:30 in CH_3OH-H_2O (4:1) and 83:17 in CH_3OH.
(18) Since compounds **2**–**4** were insoluble in water, it was necessary to use CD_3OD in order to gain concentrations sufficient for the NMR measurement.
(19) Change of the chemical shifts in ^{13}C NMR spectrum upon complexation was examined. Zinc complexation induced relatively large changes in ^{13}C-chemical shifts of the carboxyl carbon of compounds **3** while the changes of the imidazole carbons of **3** and **4** were small compared with the case of compounds **1** and **2** (see the supplementary material).
(20) Zinc-chelating power of EDTA was stronger than that of compound **4**. Addition of $ZnSO_4$ (0.9 equiv) to a mixture of **4** (1 equiv) and EDTA (1 equiv) in CH_3OH-H_2O (4:1) resulted in no formation of zinc complex **4**, suggesting that all zinc was trapped by EDTA. Treatment of zinc complex of **4** (1 equiv) with EDTA (1 equiv) in CH_3OH-H_2O (4:1) afforded metal-free **4** as detected by TLC, indicating that EDTA abstracted the zinc from **4**–Zn.
(21) Ethidium bromide displacement experiment was carried out using the same DNA as that shown in Figure 1 except the ^{32}P label. Whereas distamycin A (105 μM) caused an evident displacement of ethidium bromide (2.1 μM) bound to the DNA, compound **1** (105 μM) induced no observable change in the fluorescence spectrum of the complex system of the DNA and ethidium bromide (2.1 μM). DNase I footprinting experiment was carried out using *Bam*HI-*Sph*I fragment of pBR322 plasmid DNA. Whereas an evident footprint was observed in an experiment using elsamicin A, gel electrophoresis of the experiment using compound **1** (1 mM) was indistinguishable from that with no drug. Compounds **1** and **2** did not inhibit the DNA binding of either p50 homodimer or p65 homodimer, the both being the components of NF-κB. Detailed account on the DNA-binding inhibition experiments and comparison with other metal chelators including EDTA and *o*-phenanthroline will be published elsewhere. We thank Mr. Motonari Uesugi, Institute for Chemical Research, Kyoto University, for his assistance in the ethidium displacement and footprinting experiments.