Novel Zinc Chelators with Dual Activity in the Inhibition of the κB Site-Binding Proteins HIV-EP1 and NF-κB

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Both HIV-EP1 (also called PRDII-BF1 or MBP-1), a zinc finger protein, and NF-κB, a Rel family protein, bind to κB site present in the enhancer of multiple cellular and viral genes involved in immune function and inflammatory response including HIV-1 LTR and human interferon β gene. When cells are exposed to extracellular stimuli such as virus or phorbol ester, the activity of both HIV-EP1 and NF-κB is induced. Thus, κB site-directed transcription could be regulated by two distinct proteins in a cooperative way. Novel heterocyclic compounds comprising (dimethylamino)pyridine and histidine units, i.e., **1**–**4**, have been designed and synthesized, aiming at inhibition of these κB site-binding proteins to discriminate their functions. These compounds exhibited remarkable zinc-binding capability as revealed by NMR study. Compounds **1** and **2** showed a marked inhibitory effect on the DNA binding activity of HIV-EP1 by removing zinc without interfering with the DNA binding activity of NF-κB. Since it has been demonstrated that zinc somehow influences the DNA binding of NF-κB, the effect of these heterocyclic compounds and their zinc complexes on NF-κB was examined. Zinc complexes of **3** and **4** exhibited the inhibitory effect on the DNA binding of NF-κB and/or homodimeric complexes of p50 and p65 subunits of NF-κB without affecting HIV-EP1. Thus, it became possible to inhibit either one of the two κB site-binding proteins without inhibiting the other. This approach provided a basis for elucidation of the molecular mechanism by which κB site-directed transcription, such as transcription from HIV-LTR and the promoter of human interferon β, is regulated by distinct transcription factors which bind to a common recognition sequence, the κB site.

Introduction

A DNA sequence of 10 base pairs, 5′-GGGACTTTCC-3′, called the κB site, is present in various cellular genes critical for immune or inflammatory responses, e.g., genes for immunoglobulin κ light chain, interleukin-2 receptor α-chain, interleukin-2, interleukin-6, lymphotoxin, tumor necrosis factor-α, and human interferon β.[1–3] Two distinct groups of protein bind to the κB site; one group is zinc finger proteins, HIV-EP1[4] (also called PRDII-BF1[5] or MBP-1[6]), HIV-EP2,[7] and HIV-EP3,[8] and the other group is the Rel family of transcription factors such as NF-κB or Rel protein which have a Rel homology domain as a DNA binding region.[1–3] Thus, κB site-directed gene expression could be regulated by the cooperation of the two distinct transcription factors.

NF-κB is sequestered in the cytoplasm as an inactive form associated with the inhibitory protein IκB and is activated by divergent stimuli, e.g., cytokines, virus, phorbol esters, and other agents (Figure 1).[1–3] These extracellular stimuli induce phosphorylation of IκB which is thought to serve as a signal for the subsequent proteolysis of IκB.[9] These events result in translocation of the released NF-κB to the nucleus followed by the activation of transcription through binding to the κB site. NF-κB has a heterodimeric structure composed of p50 and p65 subunits. Homodimeric forms of p50 and p65 (abbreviated as $(p50)_2$ and $(p65)_2$, respectively, in this paper) can also bind to DNA.[1–3] Recent X-ray

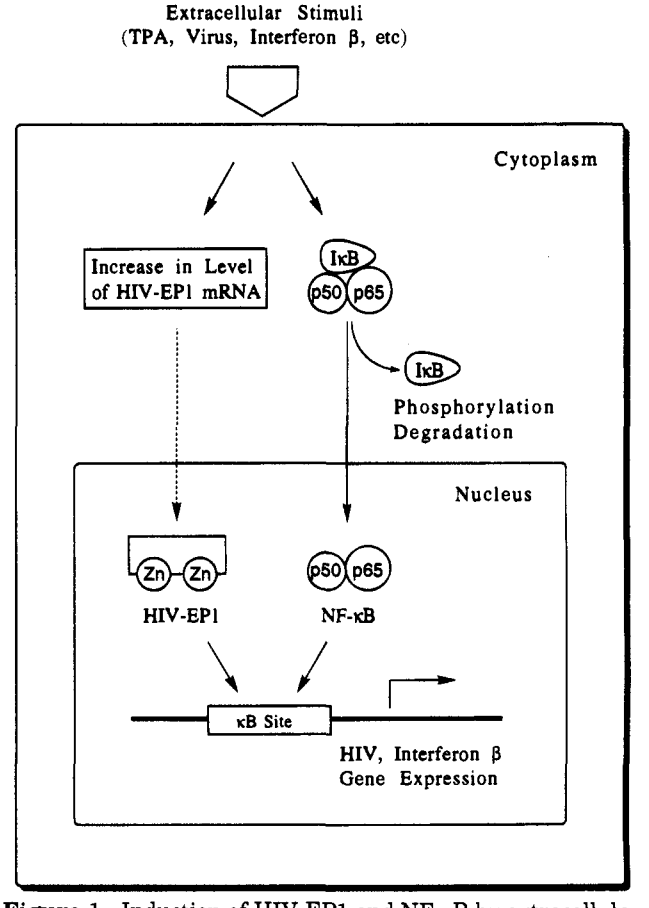

Figure 1. Induction of HIV-EP1 and NF-κB by extracellular stimuli.

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crystal analysis of p50 homodimer with DNA showed that the Rel homology region consists of two domains with immunoglobulin-like structure, and five loops between antiparallel β-sheets recognize the major groove of κB DNA.[10,11]

HIV-EP1 (HIV enhancer binding protein) is a zinc finger type κB site-binding protein[4] identical to PRDII-BF1[5] or MBP-1.[6] It binds to the κB site derived from HIV, human interferon β, class 1 major histocompatibility complex, and β_2-microglobulin genes. Expression of HIV-EP1 mRNA is induced upon extracellular stimulation such as virus, serum, and phorbol ester.[6,7] Thus, κB site-directed transcription could be regulated by two distinct groups of transcription factors which recognize a common sequence in a cooperative way (Figure 1). However, the presence of both transcription factors in a single cell interferes with the understanding of the function of each transcription factor and their crosstalk.

The difficulty would be circumvented if we could selectively inhibit one of the two proteins without affecting the other. To this end, we intended to develop a methodology to inhibit the function of NF-κB and HIV-EP1 discriminatively. Chemical inhibitors of NF-κB reported so far include *o*-phenanthroline which inhibits the DNA binding of NF-κB,[12] antioxidants, and antiinflammatory drugs such as sodium salicylate and aspirin which block the activation of NF-κB.[13-15] Recently, we preliminarily reported novel heterocyclic chelators which abstract zinc from HIV-EP1 and inhibit its DNA binding activity.[16] Now we further extended this approach and examined the function of our synthetic compounds to find that our compounds have discriminative activity in the inhibition of HIV-EP1 and NF-κB.

Results

Chemistry. It was thought that the function of zinc proteins could be inhibited by ejecting the zinc from the active site, 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.[17] We tried an alternative approach to develop an efficient zinc-coordinating system which abstracts zinc from the zinc finger moiety of HIV-EP1 to inhibit DNA binding. Since imidazole is an important constituent of the zinc-chelating site of proteins such as carbonic anhydrase,[18] we assumed such nitrogen heterocycles to have promising zinc-binding character. Previously we reported a metal-chelating system with symmetrical structure comprising a (dimethylamino)pyridine and histidine methyl ester **1** (Figure 2),[19] designed by total structural revision of our previous oxygen-activating molecules.[20] We considered that a compound such as **1** could be useful as a zinc trapper and tried to optimize its zinc-chelating property by further structural modifications. First, we were interested in introducing 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 carboxylates, together with imidazoles, are recognized as a key structural feature of the zinc-chelating site of enzymes such as superoxide dismutase[21] and carboxypeptidase[22] in addition to the aminoacetate portion of EDTA, a potent metal chelator widely used in the demetalation procedure of biochemical protocols.[23,24]

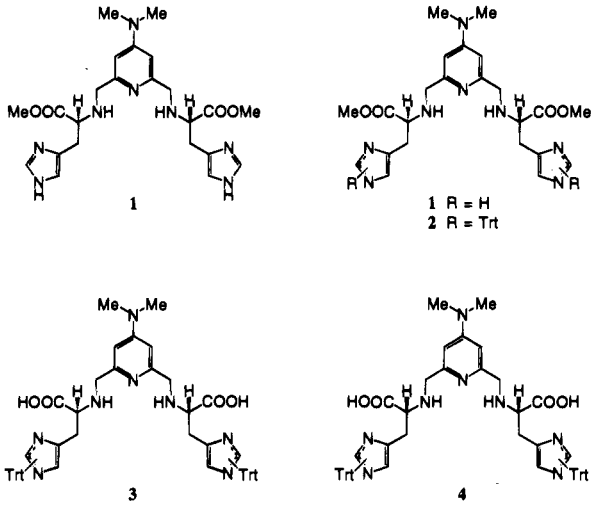


Figure 2. Structures of synthetic chelators.

Thus, we prepared trityl and/or carboxyl derivatives **2**–**4** starting with **1** (Figure 2). 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**.[25] Unexpectedly, attempts to separate diastereomers **3** and **4** by preparative TLC resulted in the capture of zinc from the TLC plate (Merck 7747; containing zinc silicate as the fluorescent indicator), affording zinc complexes of **3** (74% ee)[26] and **4**[27] in 19% and 22% yields, respectively. The identification of these zinc complexes was based on high-resolution mass spectroscopy [HRMS (FAB) $[M + H]^+$: calcd, 1003.3638; found, 1003.3685 (**3**), 1003.3661 (**4**)] and indusively coupled argon plasma atomic emission spectroscopy (48% of calculated value). This incident was the first and serendipitous execution of zinc trapping. Zinc-free diastereomers **3** and **4** were separated by employing silica gel *column* chromatography in 17% and 9%, respectively [HRMS (FAB) $[M + H]^+$: calcd, 941.4503; found, 941.4504 (**3**), 941.4505 (**4**)]. Metal-free and zinc-chelated ligands were found to be separated by use of a metal-free TLC plate (Merck 15552 Kieselgel $60F_{254S}$); for example, R_f values of metal-free and zinc-chelated **3** were 0.07 and 0.45, respectively, when developed with CH_2Cl_2–CH_3OH (1:1).[28]

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.[29] 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)[30] was added $ZnSO_4 \cdot 7H_2O$ (<1 equiv), and the ratio of zinc-chelated compounds was estimated by NMR signals for the imidazole and pyridine protons (Table 1). The affinity of the ligand **1** for zinc was decreased by introducing a 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,

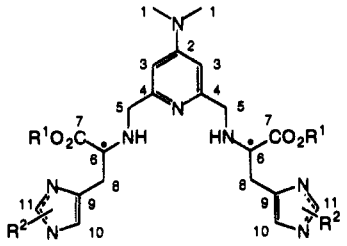
Table 1. Competitive Zinc-Binding Experiments of Compounds 1–4[a]

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

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

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

Table 2. Difference in ^{13}C Chemical Shifts between Metal-Free and Zn-Chelated 1–4



1 $R^1 = CH_3$, $R^2 = H$, *SS
2 $R^1 = CH_3$, $R^2 = Trt$, *SS
3 $R^1 = H$, $R^2 = Trt$, *SS
4 $R^1 = H$, $R^2 = Trt$, *RS

ligand	solvent[a]	carbon 1	2/4[b]	3	5	6	7	8	9/11[b]	10
1	B	0.4	≥1.4	−1.1	−1.3	1.8	−2.6	−3.4	≥2.8	−1.1
				−0.6						
2	A	0.5	≥0.6	−0.6	−0.5	2.0	−2.5	−4.9	3.3	1.2
2	B	−0.2	≥1.8	−1.0	c	0.0	−2.8	−4.9	≥2.8	1.3
3a	A	0.4	≥3.6	0.2	−3.5	1.2	−5.1	−2.1	1.4	0.9
3a	B	0.4	≥2.0	0.1	−3.2	0.8	c	−2.7	≤2.2	0.8
3b	A	0.4	≥4.8	−0.3	−1.3	1.2	c	−1.0	≤2.1	0.3
4	A	0.5	≥4.7	0.2	−3.7	0.2	c	−1.3	≤2.4	0.2
				0.6	−2.6	0.4	c	−0.8	≤1.5	0.9
4	B	0.3	≥3.1	−0.4	−2.6	1.0	c	−3.5	≤1.9	0.1
				−0.1	c	1.4	c	−2.4	≤1.4	0.4

[a] A: CD_3OD. B: CD_3OD–D_2O (4:1). [b] *x/y*: Since signals for the carbons *x* and *y* could not be distinguished from each other, differences were calculated for all possible combinations of chemical shift values for *x* and *y*. The greater one of the values for carbon *x* and *y* is shown. [c] Not clear.

and *RS*-isomer **4** showed the highest affinity for zinc. The same tendency in zinc binding was also observed in CD_3OD. 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 the zinc complex of **4** (detected by Merck TLC 15552), suggesting that all zinc was trapped by EDTA. Treatment of the zinc complex of **4** (1 equiv) with EDTA (1 equiv) in CH_3OH–H_2O (4:1) resulted in complete disappearance of **4**–Zn to afford metal-free **4** as detected by TLC (Merck 15552), indicating that EDTA abstracted the zinc from **4**–Zn. The zinc-chelating power of EDTA was stronger than that of compound **4**.

^{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) (Table 2).[30] Zinc complexation induced relatively large changes in ^{13}C-chemical shifts of carboxyl carbon of compounds **3**, while the changes of imidazole carbons of **3** and **4** were generally small compared with the case of compounds **1** and **2**. Thus, 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

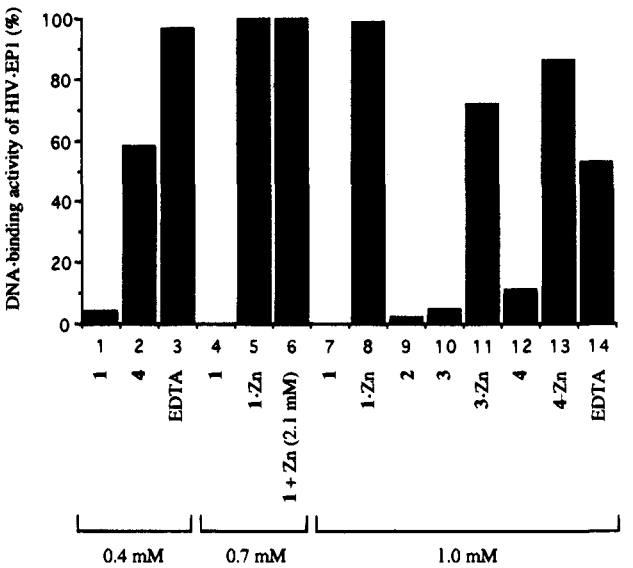


Figure 3. Effect of ligands 1–4 on the DNA binding of HIV-EP1. After HIV-EP1 was incubated with each ligand in the presence of poly(dI–dC) at room temperature for 30 min, a radioactive DNA probe (see the Experimental Section) was added. A sample was loaded onto a poly(acrylamide) band shift gel, and the gel electrophoresis was run. A histogram shows the data quantified by an image analyzer. Columns 1–4, 7, 9, 10, and 12 show inhibitory effects of **1**–**4** on the DNA binding of HIV-EP1. Columns 5, 8, and 11 show the effect of $ZnSO_4$ (1 equiv) introduced before the addition of DNA. Column 6 shows the effect of $ZnSO_4$ (3 equiv) introduced after the addition of DNA.

to contribute to the zinc binding more significantly.[28] This may be one of the reasons for the difference in the zinc affinity of these compounds as observed above.

Inhibition of DNA Binding of HIV-EP1. As the compounds **1**–**4** were found to be efficient zinc chelators, we then examined their inhibitory activity on the function of zinc finger protein. The synthetic chelators **1**–**4** exhibited remarkable inhibitory effects on the DNA binding of HIV-EP1, even more potent than that of EDTA, as demonstrated by an electrophoretic mobility shift assay (Figure 3).[16] The most potent was compound **1** which inhibited the DNA binding almost completely at 0.4 mM concentration (column 1). The most strong zinc chelator **4** showed somewhat weaker inhibition (columns 2 and 12). When zinc was introduced during (column 5) or after (column 6) the DNA-binding inhibition reaction with compound **1** (0.7 mM), total recovery of the HIV-EP1–DNA complex was observed.

Inhibition of DNA Binding of NF-κB. Previously it was reported that DNA binding of purified NF-κB was inhibited either by a chelator *o*-phenanthroline (2 mM) or by high concentration (1.5 and 2 mM) of Zn(II).[12] Thus, we examined the inhibitory effect of the chelators and their zinc complexes on the DNA binding of $(p50)_2$ and $(p65)_2$ of NF-κB by an electrophoretic mobility shift assay (Figure 4). The DNA binding of $(p50)_2$ was inhibited by **3**, **4**, and their zinc complexes at 1 mM concentration, whereas **1**, **2**, **1**–Zn, **2**–Zn, *o*-phenanthroline, and $ZnSO_4$ did not show significant inhibitory effects at 1 mM (Figure 4A,C). When **4**–Zn was added after $(p50)_2$ was bound to DNA, almost the same magnitude of inhibition was observed (Figure 4 C, column 9). The same tendency was observed in the case of $(p65)_2$, that is, the binding of DNA and $(p65)_2$ was inhibited by **3**, **4**, and their zinc complexes, while **1**, **2**,

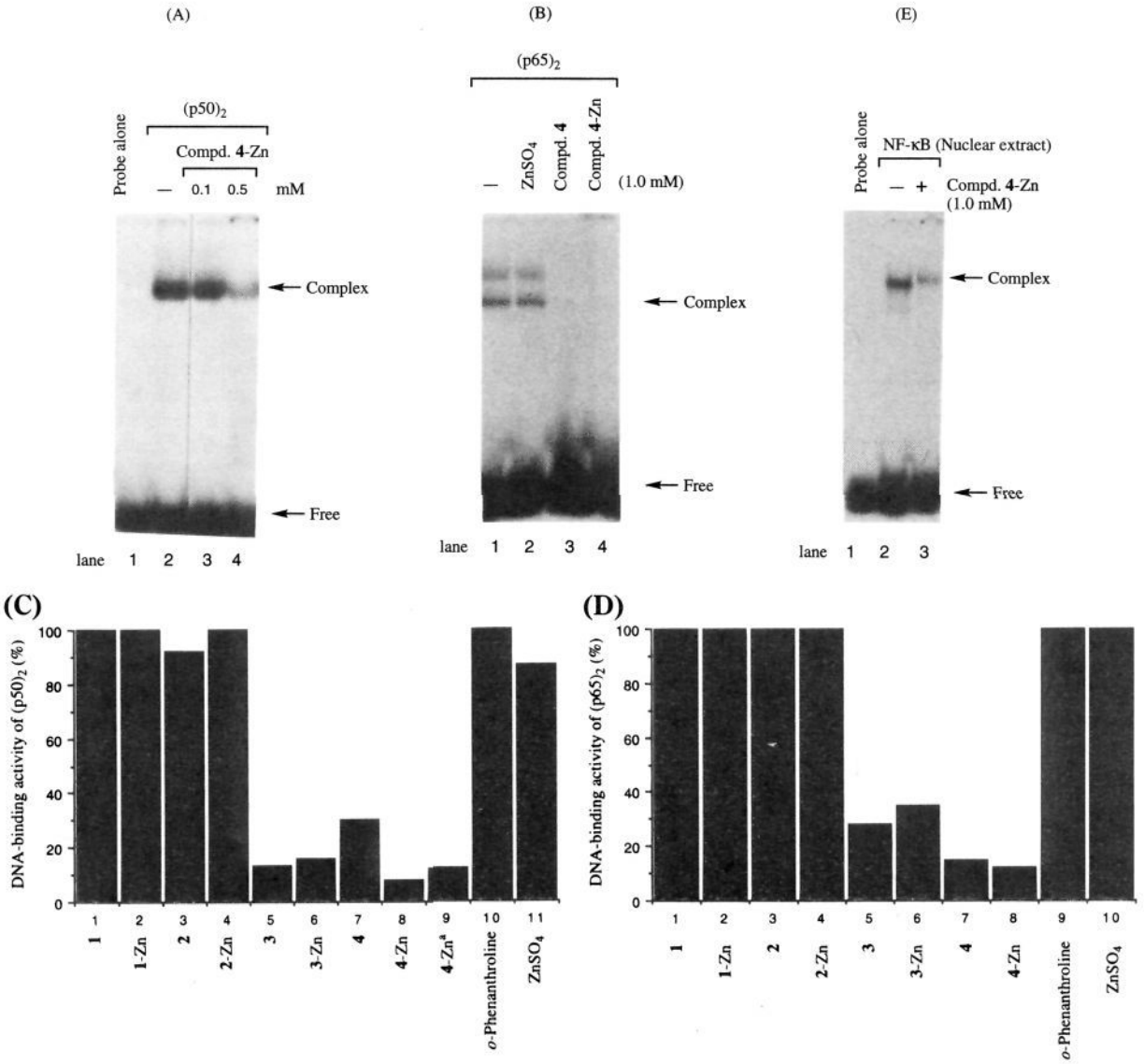


Figure 4. Effect of ligands **1**−**4** and their zinc complexes, *o*-phenanthroline, and $ZnSO_4$ on the DNA binding of $(p50)_2$, $(p65)_2$, or NF-κB. After homodimer $(p50)_2$ or $(p65)_2$ was incubated with each ligand or complex (1.0 mM) in the presence of poly(dI−dC), a radioactive DNA probe (see the Experimental Section) was added. The sample was loaded onto a poly(acrylamide) band shift gel, and the gel electrophoresis was run. (A) Inhibition of DNA binding of $(p50)_2$ by **4**−Zn. (B) Inhibition of DNA binding of $(p65)_2$ by **4** and **4**−Zn. (C) Histogram of the inhibition of DNA binding of $(p50)_2$. [a]Column 9 shows the results of a reaction in which **4**−Zn was added after $(p50)_2$ was bound to the DNA probe. (D) Histogram of the inhibition of DNA binding of $(p65)_2$. (E) Inhibition of DNA binding of NF-κB by **4**−Zn.

and their zinc complexes were not effective (Figure 4B,D). Dose dependence was observed in the inhibition of DNA binding of $(p50)_2$ by **4**−Zn (Figure 5). Although IC_{50} of **4**−Zn was ≈500 μM in the incubation at room temperature for 30 min, it was lowered to <10 μM by incubating at 37 °C for 8 h. In addition, DNA binding of p50·p60 heterodimer (NF-κB) in nuclear extract was also inhibited by **4**−Zn at 1.0 mM concentration (DNA-bound NF-κB: 47%) as shown by an electrophoretic mobility shift assay (Figure 4E). These results demonstrated that carboxylic acids **3** and **4** are active, while methyl esters **1** and **2** are inactive in the inhibition of the DNA binding of NF-κB regardless of zinc.

Discussion

Mechanism of Inhibition. HIV-EP1 contains two C_2H_2 types of zinc finger motifs. Since our synthetic ligands **1**−**4** have strong affinity to zinc, it is most likely that these abstract zinc from the zinc finger moieties of HIV-EP1 disturb the three-dimensional structure required for the DNA binding. In fact, DNA-binding capability of HIV-EP1 was recovered by adding extra zinc, confirming the inhibition to be caused by the removal of zinc from HIV-EP1. 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 a stronger affinity for zinc (Figure 3), suggesting the superiority of the nitrogen-containing heterocyclic structure in terms of amino acid interaction, hydropho-

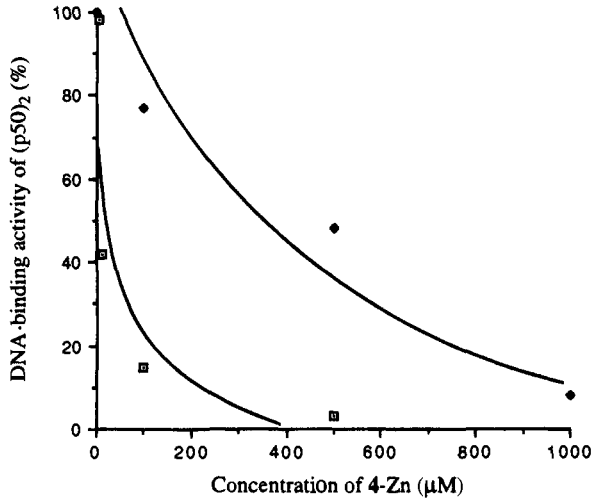


Figure 5. Dose dependence of the inhibition of DNA binding of $(p50)_2$ by 4−Zn. After incubation of $(p50)_2$ with 4−Zn in the presence of poly(dI−dC), the radioactive DNA probe AB (see the Experimental Section) was added. A sample was loaded onto a poly(acrylamide) band shift gel, and the gel electrophoresis was run. Quantitation of radioactivity of the electrophoresis band was conducted by use of an image analyzer. Condition of incubation: room temperature, 0.5 h (◆), 37 °C, 8 h (⊡).

bic interaction, or possibly electronic effect favorable for the formation of a presumed intermediary ternary complex with HIV-EP1−Zn.

Ethidium displacement and footprinting experiments indicated that **1** has virtually no interaction with DNA. Ethidium bromide displacement experiment was carried out using the same DNA as that shown in Figure 3 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 the *Bam*HI-*Sph*I fragment of pBR322 plasmid DNA. Whereas an evident footprint was observed in an experiment using elsamicin A, the footprint pattern derived from the experiment using compound **1** (1 μM) was indistinguishable from that with no drug. These are consistent with the observation that metal-free **1** did not inhibit $(p65)_2$ whose DNA-binding activity is relatively weak[3] among the three Rel family proteins examined (Figure 4). 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.

The mechanism of inhibition of the DNA binding of NF-κB and related proteins is not as clear-cut as that of HIV-EP1. Recent X-ray analyses of the DNA−$(p50)_2$ complex revealed that zinc is not involved in the DNA interaction of $(p50)_2$.[10,11] Analogously, it is probable that zinc is not involved in the DNA binding of heterodimeric NF-κB (i.e., p50·p60). However, there still remains a possibility that zinc contributes to the heterodimer formation between p50 and p65 as discussed by Baeuerle *et al.*[12] There seems to be, at least, two possible mechanisms for the inhibition of DNA binding of NF-κB, i.e., zinc-dependent and zinc-independent mechanisms. Baeuerle's finding that inhibition of DNA binding by *o*-phenanthroline (2 mM) was reversed by addition of 1 or 1.5 mM zinc[12] implies a zinc-dependent mechanism. In contrast, a zinc-independent mechanism is suggested by our observation that chelators **3** and **4** exhibited an inhibitory effect on the DNA binding of NF-κB-related proteins almost equally either in the presence or in the absence of zinc (Figure 4). On the other hand, a high concentration of zinc (1.5 or 2 mM) is inhibitory against the DNA binding of NF-κB.[12] However, it is not conceivable that a 1 mM concentration of 4−Zn could release sufficient (1.5−2 mM) free zinc required for the inhibition. Thus, the inhibition mode of **3**−Zn or 4−Zn is not a supplier of extra zinc. It was thought that 4−Zn acts not on DNA but on NF-κB because 4−Zn was inhibitory against p50 beads in terms of binding with both DNA and IκB.[31] This protein−inhibitor interaction would be noncovalent and proceed more completely by prolonged incubation under elevated temperature, resulting in the apparently lowered IC_{50} value of the inhibitor, e.g., as seen in the case of 4−Zn (Figure 5).

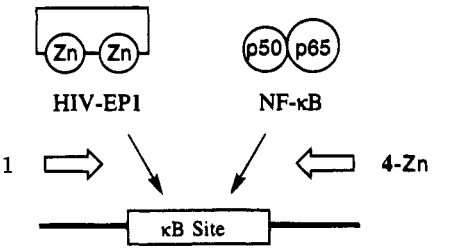


Figure 6. Discriminative inhibition of κB site binding of HIV-EP1 and NF-κB.

Conclusion. We developed novel zinc-binding heterocycles and succeeded in the separation of inhibitory activity against the two types of κB site-binding proteins, HIV-EP1 and NF-κB. Compound **1** inhibited the DNA binding of zinc finger protein HIV-EP1 most effectively (Figure 3), while it showed no inhibitory activity against NF-κB (Figure 4). On the other hand, 4−Zn showed an inhibitory effect on the DNA binding of NF-κB (Figure 4E) without affecting the DNA binding of HIV-EP1 (Figure 3, column 13). Thus, it became possible to independently inhibit the DNA binding of either HIV-EP1 or NF-κB and to switch off either one of the two signaling pathways by choosing **1** or 4−Zn (Figure 6). We believe that the present approach could provide a basis for the understanding and manipulation of signal transduction leading to acquisition of antitumor or anti-HIV activities.

Experimental Section

General Procedures. NMR spectra were measured on Varian VXR-200 and JEOL JNM-GX 400 spectrometers using TMS as the internal standard. High-resolution mass spectra were measured on a JEOL JMS-SX102A mass spectrometer by courtesy of JEOL Ltd. Homogeneity of new compounds **2**−**4** was confirmed by HPLC (see supporting information), TLC, and ^1H NMR.

4-(Dimethylamino)-2,6-bis[[[(*S*)-1-(methoxycarbonyl)-2-[(triphenylmethyl)imidazol-4-yl]ethyl]amino]methyl]-pyridine (2). To a solution of 1 (94.3 mg, 0.195 mmol) in methylene chloride (16 mL) was added triphenylmethyl bromide (0.146 g, 0.451 mmol) followed by pyridine (47 μL, 0.58 mmol) at 5 °C. The mixture was stirred at room temperature for 19 h. Water (1 mL) was added, and the mixture was neutralized with aqueous $NaHCO_3$. The organic layer was separated, dried with magnesium sulfate, and concentrated in vacuo. The residue was washed with hexane (15 mL × 5) to afford a pale yellow solid (0.166 g, 88%): mp 94−96 °C; $[\alpha]^{20}_D$ −2.6° (c = 1.0, $CHCl_3$); R_f 0.30 (Merck 15696 developed with CH_3OH); ^1H NMR (CD_3OD) δ 2.91 (d, J = 6.0 Hz, 4H), 2.98 (s,

6H), 3.0–3.5 (m, 2H), 3.4–3.6 (m, 2H), 3.57 (s, 6H), 3.70 (d, J = 14.0 Hz, 2H), 6.58 (s, 2H), 6.68 (s, 2H), 7.0–7.5 (m, 32H); ^{13}C NMR (CD_3OD) δ 33.5, 40.2, 53.1, 54.7, 63.0, 77.6, 105.5, 122.1, 130.05, 130.14, 131.7, 138.5, 140.1, 144.3, 158.2, 160.2, 176.5; IR (KBr) 3420, 1730, 1640, 1490, 1440, 1160, 1130, 1000, 750, 700 cm^{-1}. HRMS (FAB). Calcd for $C_{61}H_{61}O_4N_8$: 969.4816. Found: 969.4824.

4-(Dimethylamino)-2,6-bis[[[(*S*)-1-carboxy-2-[(triphenylmethyl)imidazol-4-yl]ethyl]amino]methyl]pyridine (3) and 4-(Dimethylamino)-2-[[[(*S*)-1-carboxy-2-[(triphenylmethyl)imidazol-4-yl]ethyl]amino]methyl]-6-[[[(*R*)-1-carboxy-2-[(triphenylmethyl)imidazol-4-yl]ethyl]amino]methyl]pyridine (4). To a solution of **2** (19.5 mg, 0.0201 mmol) in methanol (2.5 mL) was added 0.155 M aqueous LiOH (1.3 mL, 0.20 mmol). The mixture was stirred at room temperature for 16 h, neutralized with 1 N aqueous HCl, and concentrated in vacuo. The residue was dried with phosphorus pentoxide and chromatographed on silica gel (eluted with CH_3OH) to afford pale yellow solids **3** (3.2 mg, 17%) and **4** (1.7 mg, 9%). **3**: mp 213–215 °C; $[\alpha]^{20}_D$ +2.2° (c = 1.0, $CHCl_3$); R_f 0.58 [Merck 5714 developed with CH_2Cl_2–CH_3OH (5:1)], 0.47 (Merck 15696 developed with CH_3OH); ^{1}H NMR (CD_3OD) δ 2.6–3.5 (m, 6H), 2.83 (s, 6H), 3.49 (ABq, $\Delta_{\nu AB}$ = 38.0 Hz, J = 28.0 Hz, 4H), 6.15 (s, 2H), 6.65 (s, 2H), 6.8–7.5 (m, 30H), 7.31 (s, 2H); ^{13}C NMR (CD_3OD) δ 32.7, 40.1, 56.9, 66.2, 77.7, 105.0, 121.8, 130.0, 130.1, 131.8, 139.4, 141.0, 144.6, 160.2, 162.2, 182.8; IR (KBr) 3380, 1600, 1490, 1440, 1390, 1130, 750, 700 cm^{-1}. HRMS (FAB). Calcd for $C_{59}H_{57}O_4N_8$: 941.4503. Found: 941.4504. **4**: mp 192–194 °C; R_f 0.39 [Merck 5714 developed with CH_2Cl_2–CH_3OH (5:1)], 0.47 (Merck 15696 developed with CH_3OH); ^{1}H NMR (CD_3OD) δ 2.6–3.5 (m, 8H), 3.01 (s, 6H), 3.78 (d, J = 36.0 Hz, 2H), 6.39 (s, 2H), 6.77 (s, 2H), 7.0–7.5 (m, 32H); ^{13}C NMR (CD_3OD) δ 32.3, 40.1, 55.5, 66.4, 77.4, 104.9, 121.8, 129.9, 131.5, 139.9, 140.7, 144.3, 160.4, 162.1, 182.4; IR (KBr) 3390, 1600, 1490, 1440, 1390, 1130, 750, 700 cm^{-1}. HRMS (FAB). Calcd for $C_{59}H_{57}O_4N_8$: 941.4503. Found: 941.4505.

Zinc Complex of 1. To a solution of 1 (24.0 mg, 0.0495 mmol) in CD_3OD–D_2O (4:1) was added zinc sulfate heptahydrate (15.7 mg, 0.0546 mmol) to afford **1**–Zn: ^{1}H NMR (CD_3OD) δ 2.7–3.5 (m, 6H), 3.11 (s, 3H), 3.13 (s, 3H), 3.71 (s, 6H), 4.16 (ABq, $\Delta_{\nu AB}$ = 86.0 Hz, J = 18.0 Hz, 4H), 6.68 (s, 2H), 7.15 (s, 2H), 8.45 (s, 2H); ^{13}C NMR (CD_3OD–D_2O (4:1)) δ 27.9, 39.8, 52.0, 53.4, 63.6, 103.8, 104.3, 117.0, 136.2, 139.2, 155.9, 158.4, 158.6, 173.7; IR (KBr) 3120, 2910, 1730, 1620, 1540, 1440, 1390, 1110, 1030, 840, 610 cm^{-1}. HRMS (FAB). Calcd for $C_{23}H_{31}O_4N_8Zn$: 547.1760. Found: 547.1794.

Zinc Complex of 2. Complex **2**–Zn was synthesized from **2** according to the same procedure as that for 1–Zn: ^{1}H NMR (CD_3OD) δ 2.68 (d, J = 8.0 Hz, 4H), 3.14 (s, 6H), 2.9–3.4 (m, 2H), 3.33 (s, 6H), 4.21 (ABq, $\Delta_{\nu AB}$ = 212.0 Hz, J = 20.0 Hz, 4H), 6.72 (s, 2H), 6.93 (s, 2H), 6.9–7.6 (m, 30H), 8.68 (s, 2H); ^{13}C NMR (CD_3OD) δ 28.6, 40.7, 53.1, 54.2, 65.0, 79.1, 104.9, 123.3, 130.4, 130.5, 131.6, 137.6, 143.4, 157.7, 159.6, 174.0; IR (KBr) 3410, 1730, 1620, 1490, 1440, 1120, 1030, 750, 700 cm^{-1}. HRMS (FAB). Calcd for $C_{61}H_{59}O_4N_8Zn$: 1031.3951. Found: 1031.3949.

Zinc Complexes of 3 and 4. To a solution of **2** (42.6 mg, 0.0440 mmol) in methanol (5.9 mL) was added 0.155 M aqueous LiOH (2.8 mL, 0.43 mmol). The mixture was stirred at room temperature for 8 h, acidified to pH 4 with 1 N aqueous HCl, and concentrated in vacuo. The residue was dried with phosphorus pentoxide and purified by preparative TLC (Merck 7747, CH_2Cl_2:MeOH = 5:1) to afford pale yellow solids **3**–Zn (8.5 mg, 19%; complex A:complex B = 83:17) and **4**–Zn (9.7 mg, 22%). **3**–Zn: ^{1}H NMR (CD_3OD) δ 2.71 (d, J = 4.4 Hz, 4H), 3.10 (s, 6H), 3.28 (t, J = 4.4 Hz, 2H), 3.92 (ABq, $\Delta_{\nu AB}$ = 161.6 Hz, J = 16.9 Hz, 4H), 6.61 (s, 2H), 6.92 (s, 2H), 7.1–7.4 (m, 30H), 8.40 (s, 2H) (complex A); ^{13}C NMR (CD_3OD) δ 30.6, 40.5, 53.4, 67.4, 79.2, 105.2, 122.7, 130.3, 130.4, 131.6, 139.5, 142.4, 143.7, 156.6, 159.8, 177.7 (complex A); ^{13}C NMR (CD_3OD) δ 31.7, 40.5, 55.6, 67.4, 78.2, 104.7, 122.1, 130.1, 130.2, 131.6, 138.9, 141.2, 144.2, 155.4, 159.8 (complex B); IR (KBr) 3410, 2920, 1620, 1490, 1450, 1390, 1260, 1090, 1040, 800, 750, 700 cm^{-1}. HRMS (FAB). Calcd for $C_{59}H_{55}O_4N_8Zn$: 1003.3638. Found: 1003.3685. **4**–Zn: ^{1}H NMR (CD_3OD) δ 2.7–3.4 (m, 6H), 3.09 (s, 6H), 3.6–3.8 (br, 1H), 3.98 (ABq, $\Delta_{\nu AB}$ = 208.0 Hz, J = 20.0 Hz, 2H), 4.2–4.4 (br, 1H), 6.55 (s, 1H), 6.59 (s, 1H), 6.80 (s, 1H), 6.95 (s, 1H), 7.0–7.5 (m, 31H), 8.49 (s, 1H); ^{13}C NMR (CD_3OD) δ 31.0, 31.5, 40.6, 51.8, 52.9, 66.6, 66.8, 77.8, 79.1, 105.1, 105.5, 122.0, 122.7, 130.1, 130.2, 130.3, 130.4, 131.6, 131.7, 139.2, 139.9, 140.6, 142.3, 143.6, 144.5, 155.7, 156.6, 159.8; IR (KBr) 3410, 2920, 1620, 1490, 1450, 1390, 1260, 1090, 1030, 800, 750, 700 cm^{-1}. HRMS (FAB). Calcd for $C_{59}H_{55}O_4N_8Zn$: 1003.3638. Found: 1003.3661.

Electrophoretic Mobility Shift Assay (EMSA). Double-stranded oligonucleotide containing a κB site from the mouse immunoglobulin κ light-chain enhancer

5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′

3′-AGTCTCCCCTGAAAGGCTCTCCAGCT-5′

was phosphorylated with polynucleotide kinase in the presence of [γ-^{32}P]ATP (Amersham, >5000 Ci/mmol) and purified by a G-50 Sephadex spin column. The DNA-binding domain of HIV-EP1 was expressed as a fusion protein with β-galactosidase in bacteria. p50 and p65 subunits of NF-κB expressed as a fusion protein with glutathione *S*-transferase (GST) in bacteria were purified by glutathione-agarose (Sigma). Nuclear extract obtained from Jurkat human T cell line was used as the p50/p65 heterodimer. About 500 ng of each protein was used for EMSA. After incubation of each reaction mixture containing pH 7.0 binding buffer (15 mM Tris·HCl, pH 7.0, 75 mM NaCl, 1.5 mM EDTA,[33] 1.5 mM dithiothreitol, 7.5% glycerol, 0.3% NP-40, 1 μg/μL of BSA), 4% methanol, 2.4 μg of poly(dI–dC), DNA-binding protein, and each compound at room temperature for 30 min, labeled DNA probe (30 000 cpm) was added, and the mixture was further incubated at room temperature for 15 min. The sample in a volume of 24 μL was loaded onto 4% poly(acrylamide) gels and electrophoresed at 150 CV.

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Supporting Information Available: HPLC for **2**–**4** (3 pages). Ordering information is given on any current masthead page.

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(25) 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.
(26) The optical purity of **3**–Zn 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).
(27) The stereochemistry of 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**.
(28) A larger R_f value [Merck 5714 developed with CH_2Cl_2–CH_3OH (5:1)] of **3**–Zn (R_f, 0.58) compared with **2**–Zn (R_f, 0) suggests neutralization of the carboxylate anion by a coordinated zinc cation.
(29) The 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.
(30) 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. Assignment of ^{13}C signals was based on the off-resonance measurements.
(31) p50 beads or p65 beads were obtained by binding glutathione *S*-transferase fusion protein of p50 or p65 to glutathione-sepharose (Pharmacia, LKB).[32] Assay for the inhibition of DNA binding of p50 beads was carried out as follows. After shaking each reaction mixture containing a pH 7.0 buffer, 4% methanol, 5.0 μg of poly(dI–dC), p50 beads, and each compound at room temperature for 40 min, labeled κB oligonucleotide was added, and the mixture in a volume of 300 μL was further shaken at room temperature for 20 min. The beads were washed with 650 μL of binding buffer three times, and radioactivity associated with beads was conducted. Assay for the inhibition of IκB-α binding of p50 beads was carried out as follows. After shaking of each reaction mixture containing pH 7.5 binding buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100), 4% methanol, p50 beads, and each compound at room temperature for 40 min, ^{35}S-methionine-labeled IκB-α translated *in vitro* was added, and the mixture in a volume of 200 μL was further shaken at room temperature for 30 min. The beads were washed with 800 μL of binding buffer four times, and the amount of IκB-α associated with p50 beads was analyzed using 10% poly(acrylamide)/SDS gel. DNA binding activities of p50 beads were 20% and 9% in the presence of 3 and 4 mM of **4**–Zn, respectively. IκB-α binding activity of p50 beads was 33% in the presence of 0.5 mM of **4**–Zn.
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(33) EDTA (1.5 mM) was added to the binding buffer in order to remove potentially contaminated metals in the preparation of proteins which might interfere with the action of compounds **1**–**4**. As it has been demonstrated that HIV-EP1 binds to DNA probe in the presence of 1.5 mM EDTA (ref 16, Figure 1A, lane 2); a uniform condition using this binding buffer was employed herein for all the EMSA experiments. Since the capability of EDTA to abstract zinc from HIV-EP1 is much weaker compared with that of **1**–**4**, EDTA would not affect the activity of **1**–**4**. In fact, **1**–**4** smoothly exerted their function to remove zinc from HIV-EP1 in the presence of EDTA (Figure 3).

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