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Synthetic Inhibitors of Regulatory Proteins Involved in the Signaling Pathway of the Replication of Human Immunodeficiency Virus 1

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Abstract—NF-κB, HIV-EP1, Sp1, and E1A are transcriptional proteins involved in the long terminal repeat-directed expression of HIV-1. The inhibitory effect of 18 dimethylaminopyridine-based compounds against these regulatory proteins was studied. Experiments using NF-κB-beads showed that histidine–pyridine–histidine compounds and their zinc complexes are inhibitory not only for the NF-κB-DNA binding, but also for the binding of NF-κB with the inhibitory protein IκB. Discriminative inhibition of the DNA binding of two distinct C_2H_2 type zinc finger proteins HIV-EP1 and Sp1 was also attempted using the synthetic compounds. Whereas some compounds inhibited the DNA binding of both HIV-EP1 and Sp1 at 300 μM, others preferentially and completely inhibited HIV-EP1 without much suppression of Sp1. Mercapto compounds were more potent and uniformly inhibitory against both HIV-EP1 and Sp1 at 30 μM. Disulfide compounds were also remarkably inhibitory against HIV-EP1 and Sp1 also at 30 μM whereas the shorter-chain disulfides **7** and **9** were effective only for HIV-EP1. *S*-Alkyl derivatives preferentially inhibited HIV-EP1 at 300 μM. The dimethylamino compound was the sole compound inhibitory only against Sp1, being non-inhibitory against HIV-EP1. Relevant combinations of these inhibitors would allow us to inhibit NF-κB, HIV-EP1, and Sp1 in any combinations. Inhibition of the TBP binding of C_4 type zinc finger protein adenovirus E1A was also examined. It was found that two compounds induced, at 50 mM concentration, effective inhibition of the TBP binding of E1A, demonstrating that it is possible in principle to inhibit the protein–protein interaction of zinc finger proteins. Copyright © 1997 Elsevier Science Ltd

Introduction

Human immunodeficiency virus 1 (HIV-1) is a retrovirus that causes acquired immune deficiency syndrome (AIDS).[1,2] Upon infection, the viral RNA is reverse-transcribed into viral DNA which is subsequently integrated into the genome of the host cell.[3–5] HIV-1 provirus thus formed contains two long terminal repeats (LTRs), three conserved structural genes *gag*, *pol*, and *env*, regulatory genes *tat*, *rev*, and *nef*, and accessory genes *vif*, *vpr*, *vpu*, *vpt*, and *tev/tnr*.[3–8] The provirus remains latent until certain stimuli or infection of other viruses activate the transcription of these genes. Expression of HIV-1 provirus is governed primarily by cellular transcription factors such as NF-κB, [9,10] HIV-EP1 (also called MBP-1 or PRDII-BF1),[11] Sp1.[12] TATA binding protein (TBP), a component of general transcription factor TFIID, also plays an important role in the basal HIV-1 gene expression.[13] The HIV-1 promoter, located in the LTR, contains binding sites for these proteins, i.e. tandem binding sites for NF-κB (κB sites), three consecutive binding sites for Sp1 (GC boxes), and a TBP binding site (TATA box) (Fig. 1).[3–5] A model for the transcriptional activation of HIV-1 promoter involving these proteins and RNA polymerase II has been well accepted.[3–5]

On the other hand, superinfection with other viruses also activates the latent HIV-1, that is, the LTR-directed expression of HIV-1 genes is activated by regulatory gene products of several DNA viruses.[3–5] In particular, we were interested in trans-activation of the LTR by E1A of adenovirus.[14] It was shown that the zinc-containing CR3 region of E1A binds to TBP and E1A-TBP complex thus formed seemed to activate HIV-1 gene expression by binding to the TATA box (Fig. 1).[15–17]

The LTR-directed activation of HIV-1 is thus regulated by signal-inducible cellular transcription factors NF-κB and HIV-EP1, constitutive transcription factor Sp1, and a protein E1A from superinfected adenovirus. Inhibition of the function of these transcriptional proteins would lead to the interference of the replication of AIDS virus. Previously we have reported novel compounds which can inhibit the κB site binding of NF-κB and HIV-EP1.[18–20] Herein we further extended our approach and examined the inhibition of the functions of Sp1, E1A, and TBP in addition to NF-κB and HIV-EP1.

Key words: NF-κB, HIV-EP1, Sp1, Adenovirus E1A, Human Immunodeficiency Virus 1.

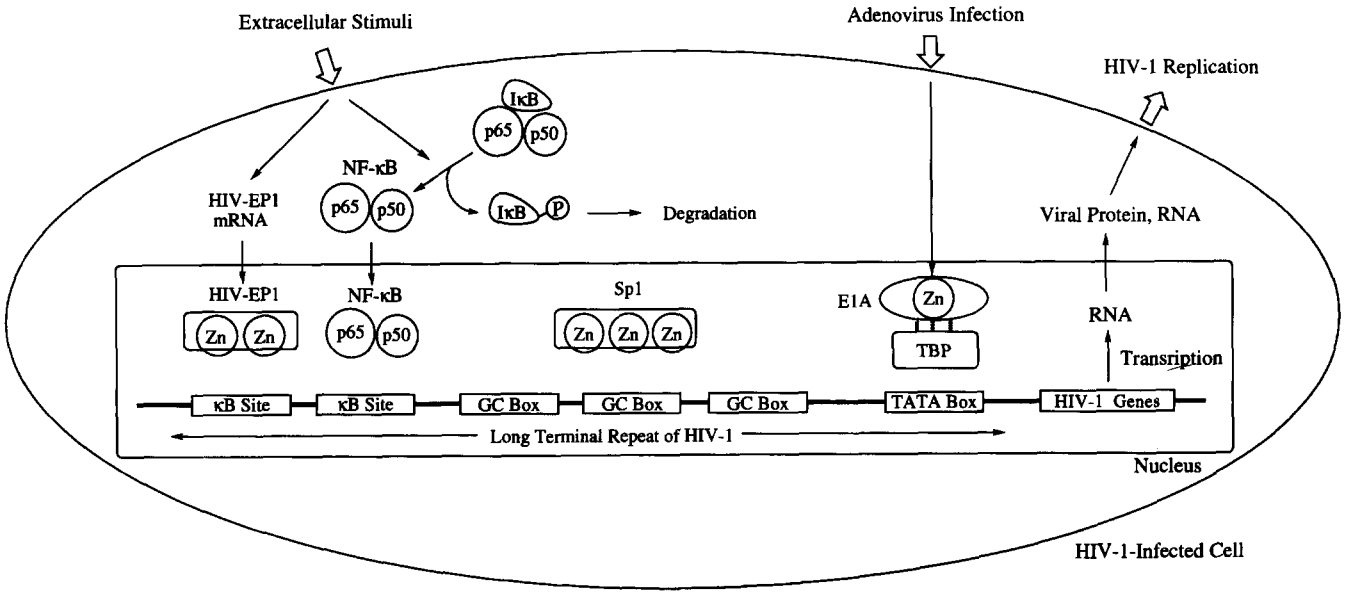


Figure 1. The LTR-directed expression of HIV-1 genes mediated by inducible transcription factors NF-κB and HIV-EP1, basal transcriptional proteins Sp1 and TBP, and a regulatory protein E1A of superinfected adenovirus.

Inhibition of the DNA Binding and the IκB Binding of NF-κB

Discriminative inhibition of NF-κB and HIV-EP1

Rel family protein NF-κB is a transcription factor found by Baltimore and coworkers that binds to the DNA sequence of 10 base pairs called the κB site (typically 5′-GGGACTTTCC-3′) 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.[21-24] Another distinct κB site-binding protein is a zinc finger protein HIV-EP1 as will be detailed in the next section.[25] Thus, κB site-directed gene expression of HIV-1 could be regulated by the co-operation of the two distinct transcription factors NF-κB[9,10] and HIV-EP1.[11]

It has been well established that 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 (Fig. 1).[21-24] These extracellular stimuli induce phosphorylation of IκB which is thought to serve as a signal for the subsequent proteolysis of IκB.[26] 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. Whereas NF-κB has a heterodimeric structure composed of p50 and p65 subunits, homodimeric form of p50 and p65 can also bind to DNA. Recent X-ray crystal analysis of DNA complex of p50 homodimer showed that the Rel homology region of p50 consists of two domains with immunoglobulin-like structure and loops between antiparallel β-sheets recognize the major groove of κB DNA.[27,28]

Chemical inhibitors of NF-κB reported so far include *o*-phenanthroline which inhibits the DNA binding of NF-κB,[29] antioxidants and anti-inflammatory drugs such as *N*-acetyl-L-cysteine,[30] pyrrolidine derivative of dithiocarbamate,[31] vitamin E derivatives,[32] cupric ion,[33] sodium salicylate,[34] and aspirin[34] which block the activation of NF-κB. We reported novel heterocyclic chelators which show discriminative activity in the inhibition of the κB site binding of HIV-EP1 and NF-κB.[18-20] We found that compounds comprising dimethylaminopyridine and histidine units **1**–**4** (Fig. 2) were efficient zinc chelators exhibiting remarkable inhibitory effect on the DNA binding of HIV-EP1 as indicated by electrophoretic mobility shift assay (EMSA).[18] We further disclosed the inhibitory effect of the compounds **3**, **4**, and their zinc complexes on the DNA binding of NF-κB.[19] The DNA binding of p50 homodimer, p65 homodimer, or heterodimeric NF-κB was inhibited by **3**, **4**, and their zinc complexes at 1 mM concentration, whereas **1**, **2**, **1**–Zn, **2**–Zn did not show significant inhibitory effect on NF-κB.[19]

Inhibition of DNA binding and IκBα binding of NF-κB-beads

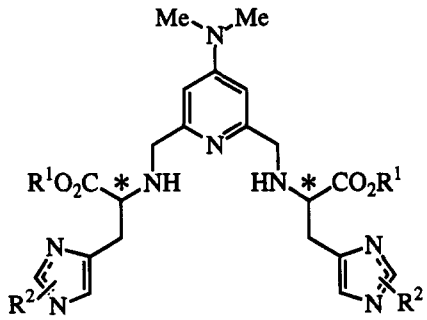
While ligands **1**–**4** seemed to abstract zinc from the zinc finger moieties of HIV-EP1 to disturb the three-dimensional structure required for the DNA binding, mechanism of inhibition of the DNA binding of NF-κB by **3**, **3**–Zn, **4**, **4**–Zn was not as clear-cut as compared with the case of HIV-EP1.[19] To this end we carried out the following experiments using p50- and p65-beads as briefly commented in the footnote of our previous report.[19]

p50- and p65-beads were prepared by binding glutathione *S*-transferase fusion protein of p50 and p65 respectively to glutathione sepharose.[35] Each

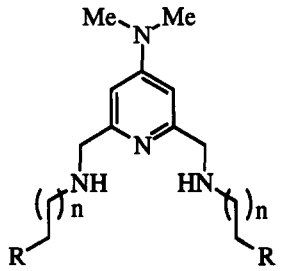
compound was incubated with p50-beads and radiolabeled DNA probe was then added. The influence of the NF-κB inhibitor **4** on the DNA binding of p50-beads was compared with that of HIV-EP1 inhibitor **1** (Fig. 3). The **4**–Zn complex inhibited the DNA binding of p50-beads at 3 mM concentration, whereas **1** or **1**–Zn did not show significant inhibitory effect. However, the inhibitory effect of zinc-free **4** was found to be weak. When **4**–Zn was added after the binding of DNA and p50-beads, the inhibitory effect of **4**–Zn was lowered. The inhibitory effect was mostly lost when the buffer solution was replaced after incubation of p50-beads and **4**–Zn, suggesting that most of the **4**–Zn was dissociated and washed away from p50 by the replacement of the buffer.

We also found that **4**–Zn inhibited IκB binding of NF-κB. p50- or p65-beads were incubated with **4**–Zn and ^{35}S-methionine-labeled IκBα was added and further incubated. The **4**–Zn complex (0.5 mM) inhibited 67% of the IκBα binding of p50-beads (Fig. 4A, C). Comparison of this with the results shown in Figure 3 indicated that the inhibitory effect of **4**–Zn against p50-beads seemed more potent towards IκBα binding than towards DNA binding. The **4**–Zn complex also inhibited p65 binding of IκBα [IκBα-bound p65 beads, 31% (0.5 mM **4**–Zn), 16% (1.0 mM **4**–Zn)] (Fig. 4B, D).

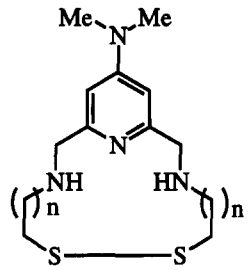
Based on these results, together with reports from other groups,[22–24] we propose the following working hypothesis. Recent X-ray analyses of the DNA-homodimeric p50 complex revealed that the DNA binding region of p50 contains many basic amino acid residues contributing base recognition.[29,30] On the other hand, the IκBα molecule consists of three distinct segments; a N-terminal region of unknown function, a middle ankyrin repeat region, and a C-terminal acidic region. It was suggested that while the ankyrin repeats interacts with the nuclear localization signal region of NF-κB, the C-terminus acidic region of IκBα recognizes the conserved arginine residues of the DNA binding motif of NF-κB and hence IκB inhibits both the nuclear localization and DNA binding of NF-κB.[36,37] More recently, it was reported that carboxy-containing Glu-284, Asp-285, and Glu-286 residues in



1 $R^1 = Me, R^2 = H, *SS$
ent-1 $R^1 = Me, R^2 = H, *RR$
2 $R^1 = Me, R^2 = Trt, *SS$
ent-2 $R^1 = Me, R^2 = Trt, *RR$
3 $R^1 = H, R^2 = Trt, *SS$
4 $R^1 = H, R^2 = Trt, *RS$



5 R = SH, n = 1
6 R = SH, n = 2
9 R = SNps, n = 1
10 R = SNps, n = 2
11 R = SBu-*t*, n = 1
12 R = SBu-*t*, n = 2
13 R = STrt, n = 1
14 $R = NMe_2$, n = 2
15 $R = CO_2Me$, n = 1
16 R = OH, n = 2



7 n = 1
8 n = 2

Figure 2. Structure of synthetic compounds.

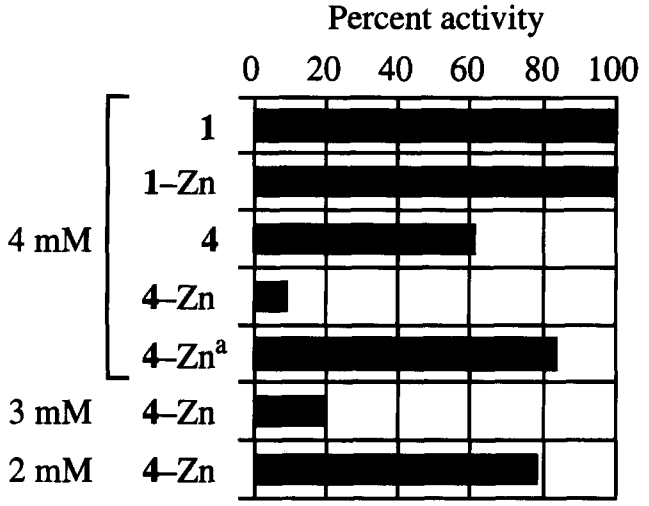


Figure 3. Effect of compounds **1**, **4**, and their zinc complexes on the DNA binding of p50-beads. After incubation of p50-beads with each compound in the presence of poly(dI-dC), radiolabeled DNA probe which contains κB site was added and the resulting mixture was further incubated. The beads were washed with binding buffer and the radioactivity of the DNA-bound beads was conducted using a scintillation counter as percent activity. [a]Buffer solution was once replaced after incubation of p50-beads with each compound followed by addition of DNA.

the C-terminus of IκBα appear to contribute to the NF-κB binding.[38] These suggest interaction between the carboxylate residues and the basic arginine residues of the DNA binding region of NF-κB. Carboxylic acids **3** and **4** were inhibitory against p50-beads in terms of binding with both DNA and IκBα but the corresponding methyl esters **1** and **2** were inactive. This could be reasonably explained if we assume that the carboxylic acid masked the conserved arginine region of p50, a dual binding site for DNA and IκBα. This could serve as a working hypothesis for the further molecular design of NF-κB inhibitors.

Discriminative Inhibition of HIV-EP1 and Sp1

Zinc-directed inhibitors

HIV-EP1 (also called PRDII-BF1[39] or MBP-1[40]) is a protein containing two tandem C_2H_2 type zinc finger motifs whose cDNA was isolated from a human B-cell λgt11 library after a search for proteins that specifically bind to the tandem κB sites in the HIV-1 enhancer.[25] Expression of HIV-EP1 mRNA is induced upon extracellular stimulation such as virus, serum, and phorbol ester.[40,41] HIV-EP1 was shown to activate the HIV-1 gene expression by transfection experiments using both eucaryotic expression vectors and antisense constructs.[11] The recent finding that the Drosophila *schnurri* gene encodes a transcription factor homologous to HIV-EP1 suggested that HIV-EP1 plays a downstream role in TGF-β/BMP signaling.[42,43] On the other hand, Sp1 found by Tijan and coworkers is also an important basal transcription factor containing three consecutive C_2H_2 type zinc fingers that binds to the DNA sequence called GC box.[44,45] Sp1 is constitutively expressed and modulates the transcriptional regulation

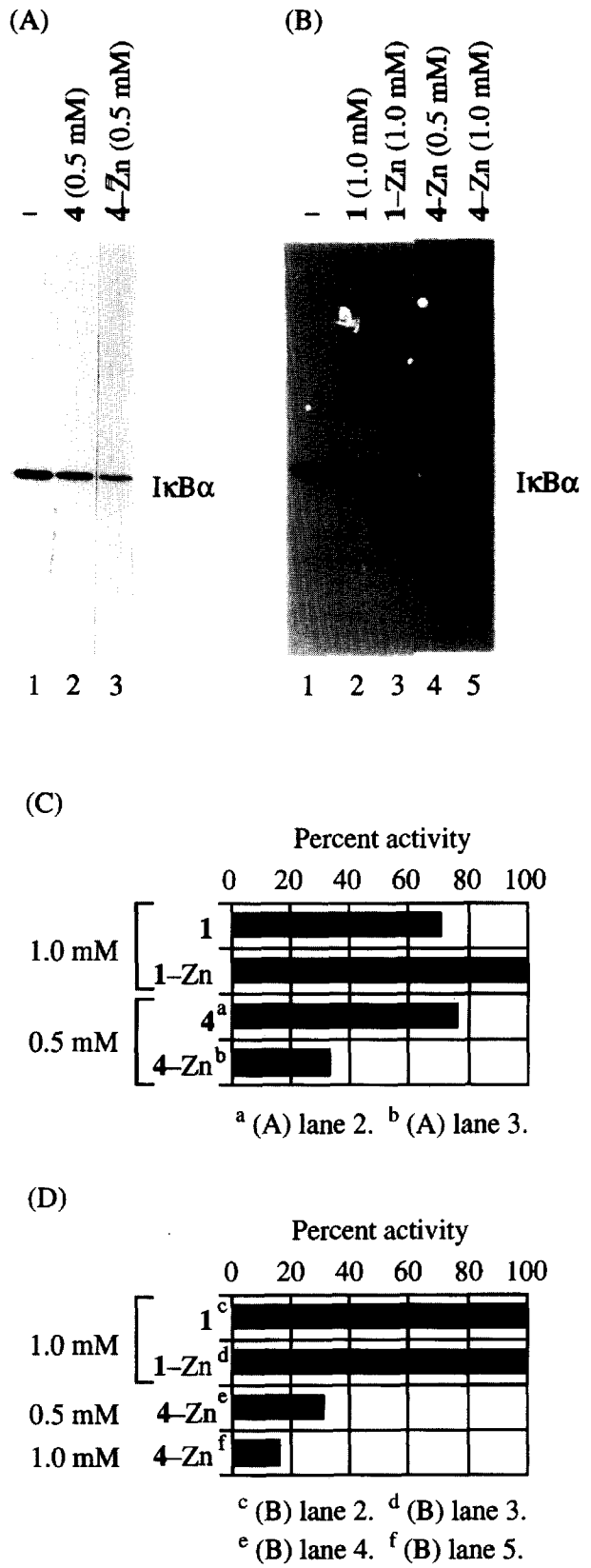


Figure 4. Effect of compounds **1**, **4**, and their zinc complexes on the IκBα binding of p50-beads (A,C) or p65-beads (B, D). After incubation of p50-beads or p65-beads with each compound, radiolabeled IκBα was added and the resulting mixture was further incubated. The beads were washed with binding buffer and SDS–PAGE was run (A, B). Quantitation of radioactivity of the SDS-PAGE band was conducted using an image analyzer (C, D).

of mammalian cells associated with other basal factors such as TFIID or TBP. It was shown that Sp1 binds to the promoter sequences within the LTR of HIV-1 to activate RNA synthesis five- to eightfold.[12] Thus, transcription of HIV-1 provirus could be regulated by two distinct group of zinc finger transcription factors HIV-EP1 and Sp1.

Previously we reported zinc-directed inhibitors of HIV-EP1, i.e. compounds **1**–**6**.[18–20] These exhibited remarkable zinc-binding capability and marked inhibitory effect on the DNA binding activity of HIV-EP1. Imdazole-pyridine-imidazole systems **1**–**4** showed inhibitory activity of the DNA binding of HIV-EP1 at 300 μM concentration.[18,19] Tenfold potentiation of the inhibitory activity was achieved by replacing the imidazoles of these inhibitors by mercapto groups, that is, compounds **5** and **6** exhibited remarkable inhibitory effect at 30 μM concentration.[20] We were interested in the effect of these HIV-EP1 inhibitors on the DNA binding of another zinc finger protein Sp1 in relation to the transcriptional regulation of HIV-1 provirus. Therefore, we prepared several novel compounds in addition to the previous inhibitors and compared the inhibitory activity on the DNA binding of HIV-EP1 and Sp1.

Imidazole-pyridine-imidazole ligands

Our previous inhibitors of HIV-EP1 **1**–**4** possess the structural characteristic of zinc enzymes that often contain imidazole residues in the zinc binding sites.[46] In order to examine the influence of the stereocheimistry of the metal site of the inhibitors we prepared enantiomers of compounds **1** and **2**, i.e. *ent*-**1** and *ent*-**2** by the procedure exactly the same as that for **1** or **2** except for the use of D-histidine. Inhibitory activity of compounds **1**–**4**, *ent*-**1**, and *ent*-**2** on the DNA binding of HIV-EP1 and Sp1 was examined by EMSA and the intensity of the electrophoresis band was quantified using an image analyzer (Fig. 5). Compounds **1**, *ent*-**1**, and **2** uniformly showed good inhibition both on HIV-EP1 and Sp1 at 300 μM concentration. We found that compound *ent*-**2** could distinguish HIV-EP1 from Sp1, that is, 300 μM concentration of *ent*-**2** completely inhibited the DNA binding of HIV-EP1 but did not suppress Sp1 very much. Inhibitory effect of *ent*-**2** against HIV-EP1 over Sp1 was confirmed by competitive inhibition experiments in the presence of both HIV-EP1 and Sp1 (data not shown). The Sp1-inhibitory effects of *ent*-**2** were evidently lower than that of **2**, suggesting that enantiomeric **2** and *ent*-**2** were discriminative in the Sp1 interaction. Imidazole-acid derivatives **3** and **4** showed stronger inhibition on Sp1 compared with HIV-EP1 at 300 μM concentration.

Sulfur-containing ligands

Our design of sulfur-containing ligands was based on the analogy to the structure of zinc finger proteins which contain a cysteine residue without exception.[47]

We compared the inhibitory effect of the ligands **5**–**13** on the DNA binding of HIV-EP1 and Sp1. Compounds **5** and **6**, stable in the presence of reducing agent such as dithiothreitol (DTT), were uniformly effective at 30 μM concentration against both HIV-EP1 and Sp1 regardless of the synthetic precursors (**7**, **8** or **9**, **10**). The inhibitory effect of

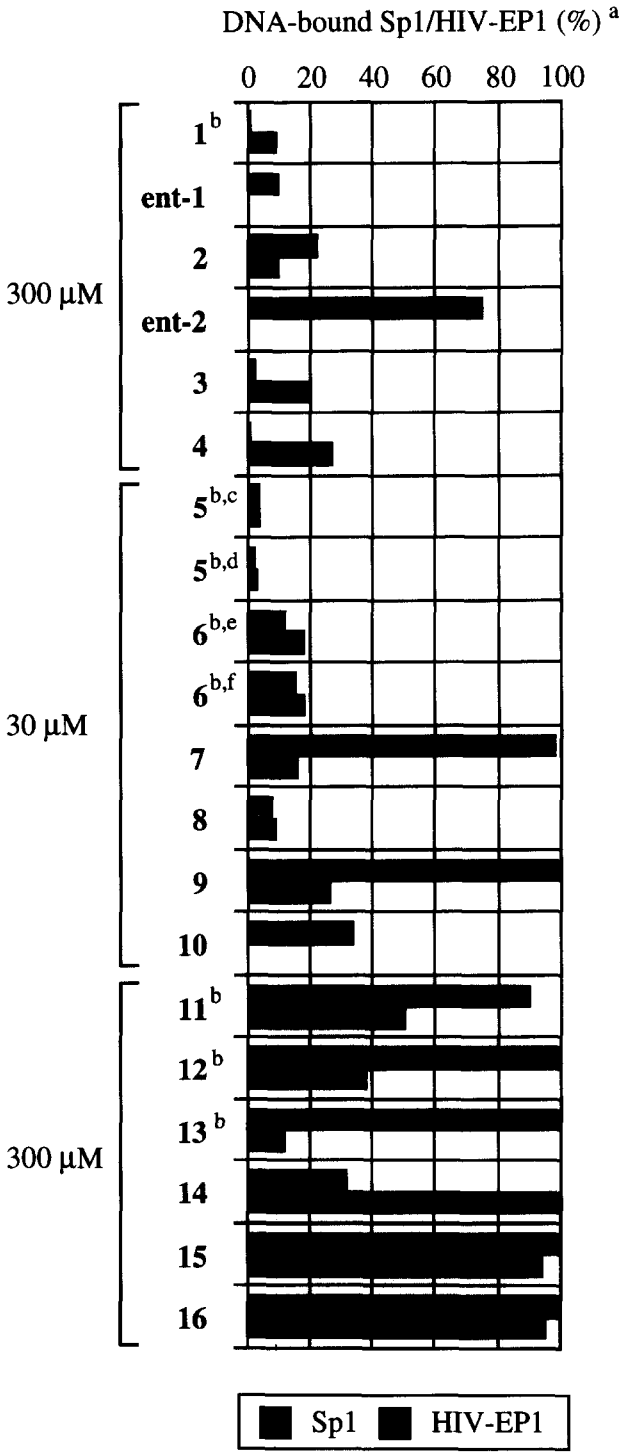


Figure 5. Effect of synthetic chelators on the DNA binding of Sp1 and HIV-EP1. [a]Quantitation of radioactivity of the electrophoresis band was conducted using an image analyzer. [b]Data for HIV-EP1 was reported in refs 18–20. [c]Generated from **7**. [d]Generated from **9**. [e]Generated from **8**. [f]Generated from **10**.

compound **5** with a shorter chain length was slightly stronger compared with **6** for both HIV-EP1 and Sp1. Disulfides **7–10** were also found to be effective inhibitors for HIV-EP1. However, the chain length of the disulfide ligand was crucial for the activity against Sp1, that is, disulfide compounds of shorter chain length (**7** and **9**) did not show an inhibitory effect at 30 μM concentration, whereas the long chain compounds **8** and **10** were remarkably inhibitory. Considering that disulfide **7** and thiol **5** were interchangeable by the reduction/oxidation process and that many drugs are known to be activated by cellular reducing agents,[48] non-inhibitory **7** and **9** could be regarded as a prodrug form that generates inhibitory **5** triggered by the reductive activation. The *S*-alkyl derivatives **11–13** were weaker inhibitors of HIV-EP1 showing moderate inhibitory activity at 300 μM concentration and found to be virtually ineffective for Sp1. The inhibitory effect of **13** against HIV-EP1 over Sp1 was also confirmed by competitive inhibition experiments (data not shown).

Ligands with the other substituents

As we experienced changes with the alteration of the substituents of the side chains on the pyridine ring of our chelating systems, we introduced a range of functional groups and prepared additional compounds with dimethylamino, methoxycarbonyl, and hydroxyl groups (**14–16**). These were prepared by the $NaBH_3CN$ reduction of Schiff base formed from the dialdehyde **16** and the respective amine (**18–20**) (Fig. 6). The dimethylamino compound, **14**, was found to be selective between Sp1 and HIV-EP1 and inhibitory against Sp1, not showing much effect on HIV-EP1.

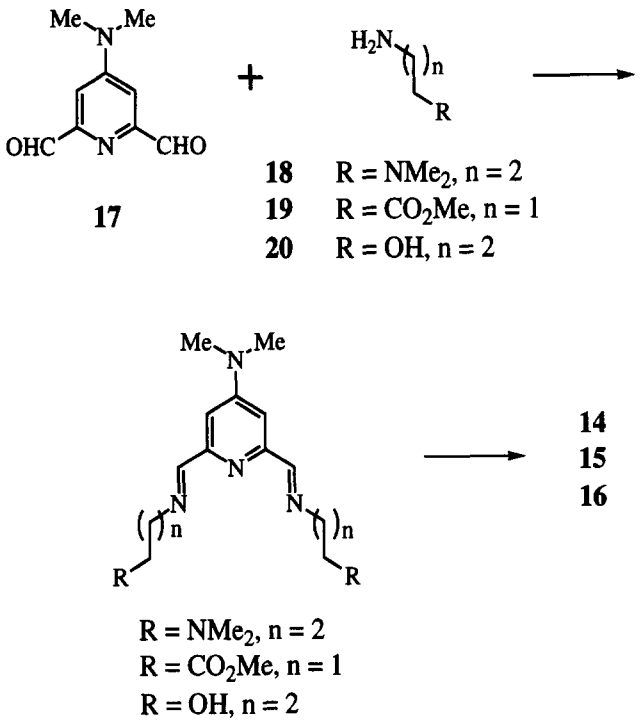


Figure 6. Synthesis of compounds **14**, **15**, and **16**.

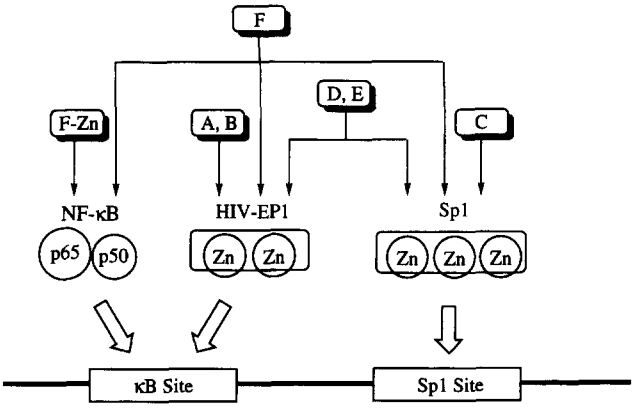


Figure 7. The inhibition profile of each class of compounds. Classification of compounds is shown in Table 1.

Compounds **15** and **16** were not effective for HIV-EP1 and Sp1 at 300 μM concentration.

Inhibitory profile

As described above we examined the activity of variously functionalized pyridine-based ligands in the inhibition of transcription factors. There is a general tendency for hydrophobic compounds preferentially to inhibit HIV-EP1. The compounds could be classified into six types based on the following inhibitory profile (Table 1, Fig. 7).

1. Compounds of class A and B are inhibitory against HIV-EP1.
2. Class B compounds are more potent.
3. Class C compound is a Sp1 inhibitor.
4. Compounds of class D and E are inhibitors of both HIV-EP1 and Sp1.
5. Class E compounds are more potent.
6. NF-κB, HIV-EP1, and Sp1 are all inhibited by class F compounds.
7. Zinc complexes of class F compounds inhibit NF-κB without affecting zinc finger proteins.

Combinatorial use of these classes of inhibitors would enable us to switch off any of NF-κB, HIV-EP1, and

Table 1. Classification of compounds based on the characteristics of the inhibitory effect

		Inhibitory activity[a]	
Class	Compound	HIV-EP1	Sp1
A	*ent*-**2**, **11**, **12**, **13**	+	—
B	**7**, **9**	++	—
C	**14**	—	+
D	**1**, *ent*-**1**, **2**	+	+
E	**5**, **6**, **8**, **10**	++	++
F	**3**, **4**	+	+

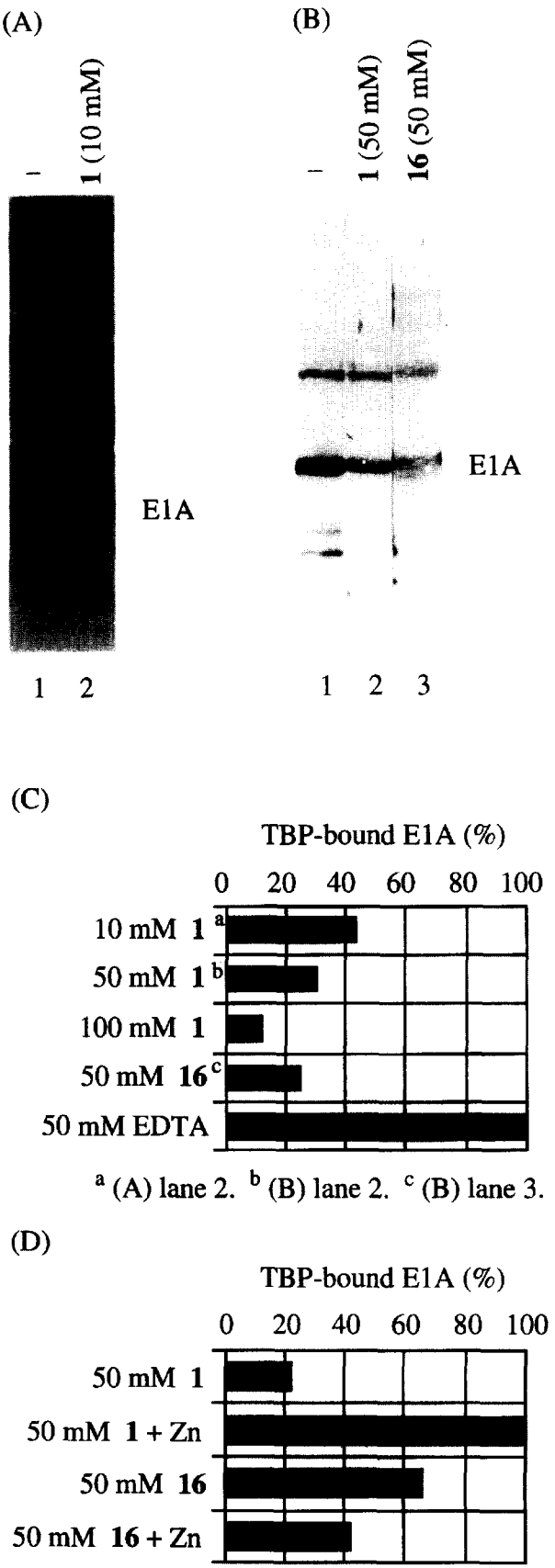
[a] + Effective at 300 μM concentration.
++ Effective at 30μM concentration.
— Not effective.

Sp1 in all possible combinations to manipulate the transcription of HIV-1 provirus.

Influence on the E1A-TBP Binding

E1A is the first viral protein induced after the infection of an adenovirus.[49,50] Two subtypes of E1A, namely 12S and 13S, are produced by the RNA splicing.[51] E1A expressed in the host cell activates the transcription of integrated genes of other superinfected viruses.[49] Particularly important is the superinfection of adenovirus and HIV-1 which results in the activation of HIV-1 genes. 13S E1A is responsible for the HIV-1 activation, while 12S does not induce HIV-1 transcription.[52] The difference between 12S and 13S E1A is the CR3 region of 46 amino acid residues present in 13S E1A that contributes to the TBP binding.[15,16] A C_4 type zinc finger motif present in the 13S E1A was shown to be responsible for the TBP binding.[17] The TATA box in the LTR of HIV-1 is required for the E1A-mediated expression of HIV-1 genes.[4,14] Thus, E1A activates the transcription of the HIV-1 genes by the formation of a E1A-TBP complex which binds to the TATA box.

We considered that HIV-1 activation by an adenovirus would be prevented by forcing dissociation of the E1A–TBP complex. We are also interested in this because E1A is different from HIV-EP1 and Sp1 in that the zinc finger of 13S E1A is C_4 type and involved in the protein–protein interaction with TBP. We now examine the effect of our compounds on E1A–TBP binding using TBP-beads. Thus, E1A was treated with each compound and further incubated with TBP-beads. Unreacted E1A was removed and E1A bound to the TBP-beads was eluted, separated by SDS–PAGE, and analysed by Western blotting. It was found that very high concentration (50–100 mM) of compounds were required for the effective inhibition of the E1A–TBP binding. We could check the activity of compounds **1** and **16** which were soluble in water and showed inhibitory effect at 50 to 100 mM concentration (Fig. 8A, B) while the others were mostly insoluble at this level of concentration. EDTA did not show inhibitory activity at 50 mM concentrations (Fig. 8C). The influence of zinc in this inhibition was examined as follows. E1A was incubated with each compound and subsequently with $ZnSO_4$ (10 mM) and the whole mixture subjected to a Sephadex G-50 column (NICK column®) to remove excess zinc and low molecular inhibitor. The eluted E1A was then incubated with the TBP-beads and the TBP-bound E1A was analysed by SDS–PAGE and Western blotting. The E1A–TBP binding inhibited by compound **1** was restored by the addition of zinc while, in the case of compound **16**, zinc did not recover the lost TBP-binding capability of E1A (Fig. 8D). The imidazole derivative **1** seemed to abstract zinc from E1A, whereas alcohol **16** appeared to bind to the zinc site of E1A. Thus, we found that high concentration of chelators **1** and **16** was required for the inhibition of C_4 type zinc finger, whereas C_2H_2 type zinc fingers were inhibited at lower concentrations.



[a] (A) lane 2. [b] (B) lane 2. [c] (B) lane 3.

Figure 8. Effect of compounds **1** and **16** on the TBP binding of E1A. E1A was incubated with each compound then TBP-beads were added and the whole mixture was further incubated. The beads were washed with buffer and SDS–PAGE was run (A, B). Quantitation of the SDS–PAGE band was conducted using a densitometer (C). The influence of zinc in the inhibition of TBP binding of E1A by compounds **1** and **1b** (D).

Conclusion

Herein we studied the inhibitory activity of 18 dimethylaminopyridine-based compounds against transcriptional proteins NF-κB, HIV-EP1, Sp1, and E1A involved in the LTR-directed expression of HIV-1. Experiments using NF-κB-beads disclosed that histidine-pyridine-histidine compounds **3**, **4**, and their zinc complexes are inhibitory not only for the DNA binding but also for the IκB binding of NF-κB. This can be explained by assuming that the inhibitors masked the arginine-rich region of NF-κB that interacts with both DNA and IκB. We also attempted to discriminate two distinct C_2H_2 type zinc finger proteins HIV-EP1 and Sp1 in the inhibition of the DNA binding using artificial compounds. Whereas compounds **1**, *ent*-**1**, and **2** inhibited the DNA binding of both HIV-EP1 and Sp1 at 300 μM, compound *ent*-**2** completely inhibited HIV-EP1 without suppressing Sp1 much. Mercapto compounds **5** and **6** were more potent and uniformly inhibitory against both HIV-EP1 and Sp1 at 30 μM. Disulfide compounds with a long side chain, **8** and **10**, were also remarkably inhibitory against HIV-EP1 and Sp1 at 30 μM, whereas the shorter chain length counterparts, **7** and **9**, were effective only for HIV-EP1. *S*-Alkyl derivatives **11–13** preferentially inhibited HIV-EP1 at 300 μM. The dimethylamino compound **14** was the sole compound that showed inhibitory preference against Sp1 being non-inhibitory against HIV-EP1. Relevant combination of these inhibitors would allow us to inhibit NF-κB, HIV-EP1, and Sp1 in any combination. Previous X-ray analysis of a zinc finger protein Zif268 by Pabo and coworkers revealed the zinc binding at the His_2-Cys_2 residues and the α-helix–β-sheet module structure that interacts DNA.[53] This seemed to be a common structural feature of C_2H_2 type zinc finger in general and it appeared to be difficult to distinguish zinc finger proteins from each other in inhibition. However, the above results demonstrated that it is possible to differentiate inhibitors so as to recognize each C_2H_2 zinc finger protein by simply replacing the structural units of our chelator system. We also examined the inhibition of the TBP binding of C_4 type zinc finger protein adenovirus E1A and found that compounds **1** or **16** induced, albeit at high concentration, effective inhibition of the TBP binding of E1A. This demonstrated a possibility in principle to inhibit the protein–protein interaction of zinc finger proteins.

The inhibitory activity on the zinc proteins seemed not solely dependent on the zinc-binding affinity of the inhibitors. It was considered that the inhibitor forms a ternary complex protein–Zn–inhibitor that, in turn, could dissociates to zinc-bound inhibitor and zinc-free protein. The formation of the ternary complex could account for the results in the case of the SH-containing inhibitors; it was considered that SH groups of the inhibitor replace the imidazole residues of the C_2H_2-type zinc finger to form a $(SH)_4Zn$ complex, resulting in the interference of the DNA binding which was not restored by the addition of extra zinc.[20] On the other hand, in the case of imidazole-containing inhibitors, the inhibitors seemed to abstract zinc from Zn-bound protein via a intermediary protein-Zn-inhibitor ternary complex. Favorable formation of this intermediary complex would lead to efficient inhibition and this could explain the observed discrepancy between the zinc-binding affinity and the inhibitory effect of the chelators.[18,19] Control of the formation of this ternary complex could be a clue to attain the transcription factor specificity in the molecular design of new inhibitors.

Transcriptional proteins could be promising targets for drug design since several antitumor drugs have been known to interfere with the regulatory machinery of gene expression.[54] In many cases transcription factors form highly ordered protein complexes to mediate the gene expression. For instance, E1A plays an important role in activating transcription as a bridge between TBP and one CRE (cAMP responsive element)-binding protein CRE-BP1 (also called ATF-2).[55,56] In addition, unidentified factor is thought to form a complex with CRE-BP1 through the C_2H_2 type metal finger structure in the amino-terminal region of CRE-BP1.[17,55,56] Thus, the targeted dissociation of transcriptional protein complex could be a new principle for the pinpoint inhibition of a specific gene with higher accuracy and is currently under active investigation in these laboratories.

Experimental

General procedure

NMR spectra were measured on Varian VXR-200 spectrometers using TMS as the internal standard. Infrared spectra were measured on a Hitachi 260–50 spectrometer. High resolution mass spectra were measured on a JEOL JMS-DX 300 mass spectrometer. Homogeneity of the new compounds was confirmed by NMR.

4-(Dimethylamino)-2,6-bis[[[3-(dimethylamino)propyl]-amino]methyl]pyridine (14). Activated powdered molecular sieves 3 Å (0.55 g) and dialdehyde **17** (48.8 mg, 0.274 mmol) were successively added to a soln of *N*,*N*-dimethyl-1,3-propanediamine **18** (56.0 mg, 0.548 mmol) in MeOH (5.4 ml) at room temperature. After being stirred at room temperature for 16 h, $NaBH_3CN$ (95%) (59.5 mg, 0.900 mmol) was added. The mixture was stirred at room temperature for 7 h and filtered. The filtrate was acidified to pH 2 with 1N HCl, stirred at room temperature for 1 h, neutralized with aq satd $NaHCO_3$, and concd in vacuo. The residue was dried over P_2O_5 and purified by chromatography on silica gel (CH_2Cl_2:MeOH, 10:1→1:5) to give **14** (39.9 mg, 42%) as an oil. ^1H NMR ($CDCl_3$): δ 2.16 (quint, J=6.0 Hz, 4H), 2.41 (s, 12H), 2.72 (t, J=6.0 Hz, 4H), 3.00 (s, 6H), 3.12 (t, J=6.0 Hz, 4H), 4.05 (s, 4H), 4.64 (br, 2H), 6.44 (s, 2H); ^{13}C NMR (CD_3OD): δ 25.3, 40.4, 45.3, 48.3, 53.5, 58.7, 106.5, 155.0, 158.6; IR (KBr) 3380, 2920, 2680, 1600, 1540, 1460, 1390, 1210, 1150, 980, 830 cm^{-1}; HRMS, calcd for $C_{19}H_{38}N_6$ 350.3157, found 350.3146.

4-(Dimethylamino)-2,6-bis[[[2-(methoxycarbonyl)ethyl]-amino]methyl]pyridine (15). $NaBH_3CN$ (95%) (90.0 mg, 1.36 mmol) was added to a soln of dialdehyde **17** (100 mg, 0.562 mmol) and β-alanine methyl ester hydrochloride (**19**) (157 mg, 1.13 mmol) in MeOH (11 ml) at room temperature. After being stirred for 36 h at room temperature, the mixture was acidified to pH 2 with conc HCl, stirred at room temperature for 1 h, neutralized with aq satd $NaHCO_3$, and concd in vacuo. The residue was dried over P_2O_5 and purified by chromatography on silica gel (CH_2Cl_2:MeOH, 20:1→1:1) to give **15** (130 mg, 66%) as a solid. ^1H NMR (CD_3OD): δ 2.71 (t, J=6.5 Hz, 4H), 3.03 (t, J=6.5 Hz, 4H), 3.10 (s, 6H), 3.70 (s, 6H), 4.00 (s, 4H), 6.71 (s, 2H); ^{13}C NMR (CD_3OD): δ 33.9, 40.5, 45.7, 53.5, 53.6, 106.1, 155.5, 158.8, 175.5; IR (KBr) 3390, 2920, 1730, 1610, 1440, 1390, 1210, 1080, 1000, 830 cm^{-1}; HRMS, calcd for $C_{17}H_{28}N_4O_4$ 352.2110, found 352.2093.

4-(Dimethylamino)-2,6-bis[[(3-hydroxypropyl)amino]-methyl]pyridine (16). Compound **16** was prepared according to the same procedure as that for **14** using 3-amino-1-propanol (**20**) and was obtained as an oil in 17% yield. ^1H NMR (CD_3OD): δ 1.98 (quint, J=6.0 Hz, 4H), 3.05 (s, 6H), 3.18 (t, J=6.0 Hz, 4H), 3.72 (t, J=6.0 Hz, 4H), 4.20 (s, 4H), 6.69 (s, 2H); ^{13}C NMR (CD_3OD): δ 30.7, 40.3, 48.2, 53.1, 61.5, 106.7, 153.6, 158.4; IR (KBr) 3360, 2920, 1610, 1550, 1440, 1400, 1220, 1070, 840 cm^{-1}; HRMS, calcd for $C_{15}H_{28}N_4O_2$ 296.2211, found 296.2211.

Assay for inhibition of the DNA binding of p50-beads

A double-stranded oligonucleotide containing a κB site from the mouse immunoglobulin κ light chain enhancer

```
5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3'
    3'-AGTCTCCCCTGAAAGGCTCTCCAGCT-5'
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was phosphorylated with polynucleotide kinase in the presence of [γ-^{32}P]ATP (Amersham, >5000 Ci/mmol) and purified by a Sephadex G-50 spin column. A mixture containing each compound, p50-beads,[35] binding buffer (15 mM Tris·3HCl, pH 7.0, 75 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 7.5% glycerol, 0.3% NP-40, 1 μg/μl BSA), 4% MeOH, and 5.0 μg poly(dI-dC) was shaken at room temperature for 40 min, then the 5′-end labeled DNA prepared as above was added. The mixture in a volume of 300 μl was further shaken at room temperature for 20 min. The beads were washed with binding buffer (650 μl × 3), and radioactivity of the beads was conducted using a scintillation counter.

Assay for inhibition of IκBα binding of p50 beads and p65 beads

A mixture containing each compound, p50-beads or p65-beads,[35] binding buffer (20 mM Tris·3HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100), and 4% MeOH was shaken at room temperature for 40 min, then ^{35}S-methionine-labeled IκBα translated in vitro was added. The mixture in a vol of 200 μl was further shaken at room temperature for 30 min. The beads were washed with binding buffer (800 μl × 4). A mixture containing IκBα-bound beads, 0.5 M Tris·3HCl (pH 6.8), sodium dodecylsulfate, 2-mercaptoethanol was boiled and the sample was loaded onto 10% SDS–PAGE and electrophoresis was run. Quantitation of the radioactivity of the band was conducted by an image analyzer.

Electrophoresis mobility shift assay (EMSA)

5′-End labeled DNA containing a κB site was prepared as described above. Double-stranded oligonucleotide containing a GC box

```
5'-CTAGTGGGGCGGGGCCT-3'
    3'-ACCCCGCCCCGGAGATC-5'
```

was phosphorylated with polynucleotide kinase in the presence of [γ-^{32}P]ATP (Du Pont, >5000 Ci/mmol). The DNA-binding domain of HIV-EP1 was expressed as a fusion protein with β-galactosidase in bacteria. The DNA-binding domain of Sp1 was expressed in bacteria. About 500 ng of each protein was used for EMSA. A mixture containing each compound, HIV-EP1 or Sp1, binding buffer (15 mM Tris·3HCl, pH 7.0, 75 mM NaCl, 1.5 mM EDTA, (1.5 mM dithiothreitol in the case of HIV-EP1, except **7**–**10**), 7.5% glycerol, 0.3% NP-40, 1 μg/μl BSA), 4% MeOH, 2.4 μg of poly(dI-dC) was incubated at room temperature for 30 min, then the 5′-end labeled DNA prepared as above was added. The mixture was further incubated at room temperature for 15 min. The sample in a volume of 24 μl was loaded onto 4% polyacrylamide gel and electrophoresis was run. Quantitation of the bands was conducted by an image analyzer.

Assay for inhibition of E1A-TBP binding using TBP-beads

TBP-beads were obtained by binding glutathione *S*-transferase fusion protein of TBP to glutathione sepharose (Pharmacia, LKB). E1A 13S was expressed as an insoluble form in bacteria.[57] A mixture containing each compound, 1 μg E1A, binding buffer (50 mM HEPES, pH 7.2, 250 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 0.3% NP-40, 0.1% Triton X-100, 0.1 mM PMSF), and 4% MeOH was stood for 30 min at room temperature, then TBP-beads, which contains about 20 μg of GST-TBP, were added (total volume 230 μl). The resulting mixture was stood for 1 h on ice with tapping at 15 min intervals. The beads was washed with binding buffer (the same constitution as above except omitting EDTA) (500 μl × 5). Beads-bound E1A was loaded onto 12% SDS–PAGE and electrophoresis was run. The band was detected by Western blotting [primary antibody: Adenovirus-2 E1A (13S-5) rabbit polyclonal IgG; Santa Cruz Biotechnology, secondary antibody: HRP-Goat Anti-Rabbit IgG (H + L) DS Grd;

2YMED] and the inhibitory activity was assessed using a laser densitometer (LKB Model 2222 Ultro-Scan XL).

The effect of zinc in the E1A-TBP binding inhibition was examined as follows. A mixture containing each compound, E1A, binding buffer (the same constitution as above), 4% MeOH was stood for 30 min at room temperature, then $ZnSO_4$ was added. After being stood for a further 5 min, the mixture was subjected to Sephadex G-50 column (NICK column®) eluted by binding buffer to remove excess zinc and synthetic compound added. TBP-beads were added to macromolecular fraction (total amount 410 μl). The resulting mixture was stood for 1 h on ice with tapping at 15 min intervals. Washing of the beads, SDS–PAGE, and quantitation of the band were carried out as described above.

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