# A New Type of Potent Inhibitors of HIV-1 TAR RNA−Tat Peptide Binding by Zinc(II)−Macrocyclic Tetraamine Complexes

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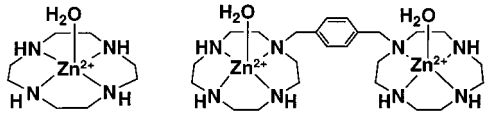
The search for small molecules that interact with RNA is currently attracting great interest for drug discovery. One typical example is AIDS therapeutics by small molecules which target viral RNA sites to prevent the formation of key RNA−protein complexes,[1−4] or to cleave specific sites of RNA.[5−7] Transcription of HIV-1 genome is facilitated by a HIV-1 regulatory protein Tat which activates the synthesis of full-length HIV-1 mRNA by its binding to a TAR (*trans*-activation responsive) element RNA.[8] A direct correlation has been found between the Tat binding to TAR RNA and up-regulation of HIV-1 mRNA transcription. The TAR element comprising the first 59 nucleotides of the HIV-1 primary transcript adopts a hairpin structure with a uracil (U)-rich bulge (UUU or UCU) located four base pairs below a six-nucleotide loop (Figure 1a). The bulge is the Tat binding site, and the loop, a homing site for cellular proteins.[8] Aminoglycoside antibiotics (e.g., neomycin) currently seem to have the highest affinity to the bulge part, showing the most potent inhibition ($IC_{50} = 1\ \mu M$) of the TAR RNA−Tat protein complex formation.[2]

We earlier discovered that zinc(II) complexes of a 12-membered macrocyclic tetraamine (1,4,7,10-tetraazacyclododecane, cyclen) (**1**) selectively interact with uridine (U) and thymidine (T) nucleotides by the specific $Zn^{2+}$-imide $N^-$ bonding with disassociation constants $K_d = 0.8$ mM and 0.3 mM (at pH 8), respectively.[9] More recently, a bis($Zn^{2+}$-cyclen) (**2**) and a tris-




**1** $Zn^{2+}$–cyclen **2** Bis ($Zn^{2+}$–cyclen)

($Zn^{2+}$-cyclen) (**3**) were found to selectively bind to a dinucleotide dTpdT and a trinucleotide dTpdTpdT, with extremely small dissociation constants $K_d = 0.6\ \mu M$ and 0.8 nM (at pH 7.4), respectively (Scheme 1).[10] The $Zn^{2+}$−cyclen complexes appended with polyaromatic rings were shown to selectively bind to T (or U)-rich sequences in double-stranded DNA (or RNA) to denature them.[11] In this study, we report that the tris($Zn^{2+}$−cyclen) complex

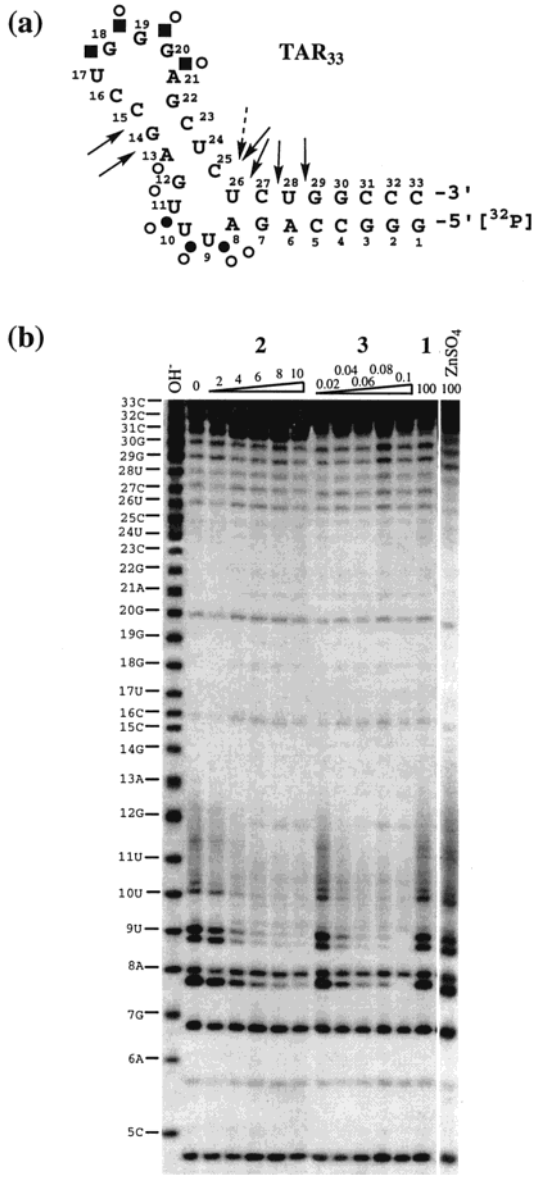



**Figure 1.** (a) A TAR model ($TAR_{33}$), containing residues 17−43 of HIV-1 mRNA and three additional GC pairs. The sequence is shown with a schematic summary of the protection sites by **3** from micrococcal nuclease (●), RNase A (■), and phosphodiesterase I (○). Enhanced cleavage sites by RNase A (dashed arrow) and phosphodiesterase I (arrow) are shown. (b) Micrococcal nuclease footprinting of $TAR_{33}$ in the presence of **1**, **2**, **3**, or $ZnSO_4$. The concentrations of compounds ($\mu$M) were shown above each lane. The lane "$OH^-$" represents the alkaline hydrolysis marker.

**3** having the unique base sequence recognition ability is the most potent inhibitor ever reported of the TAR−Tat binding due to its extremely strong binding to the UUU bulge.

(1) Mayhood, T.; Kaushik, N.; Pandey, P. K.; Kashanchi, F.; Deng, L.; Pandey, V. N. *Biochemistry* **2000**, *39*, 11532−11539.
(2) Mei, H. Y.; Cui, M.; Heldsinger, A.; Lemrow, S. M.; Loo, J. A.; Sannes-Lowery, K. A.; Sharmeen, L.; Czarnik. A. W. *Biochemistry* **1998**, *37*, 14204−12.
(3) Hamy, F.; Brondani, V.; Florsheimer, A.; Stark, W.; Blommers, M. J.; Klimkait, T. *Biochemistry* **1998**, *37*, 5086−95.
(4) Peytou, V.; Condom, R.; Patino, N.; Guedj, R.; Aubertin, A. M.; Gelus, N.; Bailly, C.; Terreux, R.; Cabrol-Bass, D. *J. Med. Chem.* **1999**, *42*, 4042−53.
(5) Mazumder, A.; Chen, C. H. B.; Gaynor, R.; Sigman, D. S. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 1503−1509.
(6) Hickerson, R. P.; Watkins, S. C. D.; Burrows, C. J.; Atkins, J. F.; Gesteland, R. F.; Felden, B. *J. Mol. Biol.* **1998**, *279*, 577−578.
(7) Cheng, C. H.; Kuo, Y. N.; Chuang, K. S.; Luo, C. F.; Wang, W. J. *Angew. Chem., Int. Ed.* **1999**, *38*, 1255−1257.
(8) Jones, K. A.; Peterlin, B. M. *Annu. Rev. Biochem.* **1994**, *63*, 717−743, and references therein.
(9) Shionoya, M.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **1993**, *115*, 6730−6737.
(10) Kimura, E.; Kikuchi, M.; Kitamura, H.; Koike, T. *Chem. Eur. J.* **1999**, *5*, 3113−3123.
(11) (a) Shionoya, M.; Ikeda, T.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **1994**, *116*, 3848−3859. (b) Kimura, E.; Ikeda, T.; Aoki, S.; Shionoya, M. *J. Biol. Inorg. Chem.* **1998**, *3*, 259−267. (c) Kikuta, E.; Murata, M.; Katsube, N.; Koike, T.; Kimura, E. *J. Am. Chem. Soc.* **1999**, *121*, 5426−5436. (d) Kikuta, E.; Katsube, N.; Kimura, E. *J. Biol. Inorg. Chem.* **1999**, *4*, 431−440. (e) Kimura, E.; Koike, T.; Kimura, E. *J. Inorg. Biochem.* **2000**, *79*, 253−259. (f) Kikuta, E.; Matsubara, R.; Katsube, N.; Koike, T.; Kimura, E. *J. Inorg. Biochem.* **2000**, *82*, 239−249. (g) For review, Kimura, E.; Kikuta, E. *J. Biol. Inorg. Chem.* **2000**, *5*, 139−155. (h) Kimura, E.; Kitamura, H.; Ohtani, K.; Koike, T. *J. Am. Chem. Soc.* **2000**, *122*, 4668−4677.

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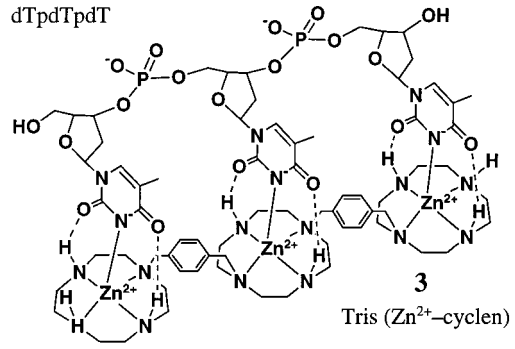
**Scheme 1**




dT⁻pdT⁻pdT⁻ - **3** complex

Footprinting analysis using micrococcal nuclease (ribo- and deoxyribonuclease), has revealed the UUU bulge to be strongly protected by **2** and **3** in the TAR model sequence ($TAR_{33}$) (Figure 1). The micrococcal nuclease intrinsically hydrolyzes 5′-phosphates of A, T, and U sites of denatured or single-stranded DNA and RNA.[11c,12] The sequencing ladder in the absence of **2** or **3** actually shows that the 5′-phosphates of A6, A8, U9, and U10 (see Figure 1a) were cleaved to produce the 3′-phosphate and/or 2′,3′-cyclic phosphate termini of 5C, 7G, 8A, and 9U in Figure 1b, respectively. At the U9, U10, and U11 (see Figure 1a) hydrolysis, we saw double products, which we provisionally assigned to the 2′, 3′-cyclic phosphate termini and 3′-phosphate termini fragments of 8A, 9U, and 10U in Figure 1b.[13] One of these doublets matched to the RNA fragments 8A, 9U, and 10U by alkaline hydrolysis (at pH 9.2 and 90 °C for 3 min), see the ladder under $OH^-$ in Figure 1b. Whatever the hydrolysis products may be, the micrococcal nuclease hydrolysis of the bulge region was strongly inhibited by **2** and **3** (see the disappearing products at 8A, 9U, and 10U). In Figure 1a, those protected sites are indicated by filled circle marks. The 50% inhibition concentrations ($IC_{50}$) of the 5′-phosphate hydrolysis of U10 (the dose-dependently disappearing doublet products are shown at 9U in Figure 1b) were determined as 3 $\mu$M for **2** and 25 nM for **3**, while it was >100 $\mu$M for **1**.[14] $Zn^{2+}$ ion alone (100 $\mu$M) did not influence the nuclease digestion. For further footprinting analysis, we have used RNase A, which indicated that **2** and **3** protected the loop region (U17-G20, see filled square in Figure 1a) with the $IC_{50}$ values of 60 $\mu$M and 4 $\mu$M, respectively.[15] The enhanced cleavage by RNase A at the 3′-phosphate of C25 (dashed arrow in Figure 1a) in the presence of **2** or **3** implies that the conformational change led to its exposure to the nuclease.[16] Phosphodiesterase I footprinting showed the protection of the bulge and loop by **3**, and enhanced cleavage at the vicinities of G14 and C25 (see open circles and arrows in Figure 1a). The RNase A and phosphodiesterase I footprinting procedures and results are all shown in Supporting Information (Figure S1).

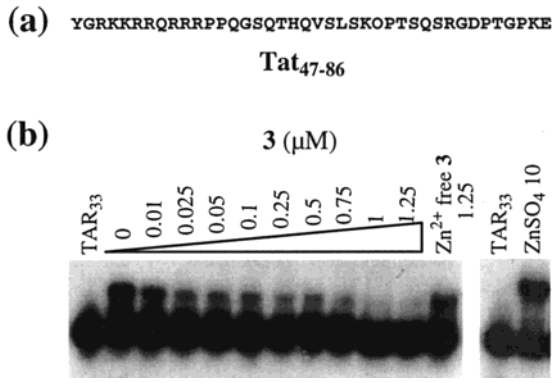



**Figure 2.** (a) Chemically synthesized $Tat_{47-86}$ (Tat-derived peptide contains an arginine-rich basic domain of Tat protein). (b) Gel mobility shift of the complex of $TAR_{33}$ and $Tat_{46-86}$ (9.1 nM) in the presence of increasing concentration of **3** (0−1.25 $\mu$M) or $ZnSO_4$ (10 $\mu$M). Lane "$TAR_{33}$" represents uncomplexed $TAR_{33}$.[20]

The inhibition of the complexation of $TAR_{33}$ with a Tat-consensus peptide ($Tat_{47-86}$, Figure 2a) was studied by gel mobility shift assay, as described by Long et al.[17] First, the $K_d$ value for the $TAR_{33}$−$Tat_{47-86}$ complexation was determined as 15 nM (Figure S3). Among the $Zn^{2+}$−cyclen complexes tested, **3** was the most potent inhibitor of the $TAR_{33}$−$Tat_{47-86}$ complexation with an extremely low $IC_{50}$ value of 20 nM (Figure 2b).[18] Neither the $Zn^{2+}$-free ligand of **3** nor $Zn^{2+}$ alone had inhibitory effect. The well-established arginine-diphosphate binding for the TAR−Tat interaction[19] would be strongly hindered by the UUU−**3** complexation.

These findings showed that the unique U-rich sequence recognizing $Zn^{2+}$-cyclen complexes efficiently and selectively recognize a U-rich single-strand element in a biologically important RNA and may serve as a new type of HIV-1 RNA targeting small molecules. An additional binding of **3** to the loop might serve to further hinder the TAR RNA binding to host cell proteins, which might work to enhance the anti-HIV activity. It is of particular pertinence to note that we have very recently found **3** possessing a strong anti-HIV activity,[21] as already reported for bis($Zn^{2+}$−cyclen) complexes and bis(macrocyclic polyamines) by us[22] and Bridger's group.[23]

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**Supporting Information Available:** Experimental details of the preparation procedure of $TAR_{33}$ RNA, nuclease footprinting analysis, CD spectroscopy, and gel mobility shift assay (PDF). This material is available free or charge via the Internet at http://pubs.acs.org.

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(12) Alexander, M.; Heppel, L. A.; Hurwitz, J. *J. Biol. Chem.* **1961**, *236*, 3014−3019.

(13) However, longer RNA fragments (>15 nucleotides) with 2′,3′-cyclic phosphate termini seem to migrate similarly to those with the corresponding 3′-phosphate termini. (Jorge, C. R.; Kenneth, J. P.; Laura, N. R.; Mita, M.; Barbara, S. W. *Biochemistry* **1998**, *37*, 6059−6064).

(14) Neomycin earlier was shown to bind to the UCU bulge of $TAR_{31}$ for protection from nuclease V1 hydrolysis at the concentration range 200−1000 $\mu$M and from RNase A hydrolysis at 10−50 $\mu$M (ref 2). We have found with our $TAR_{33}$ that neomycin (100 $\mu$M) also protected the UUU bulge (9U, 10U, and 11U) and its vicinity 5C from RNase A.

(15) We previously reported that $Zn^{2+}$−cyclen complexes had minor interaction with guanine base (refs 11a, b, f, and h). Bis($Zn^{2+}$−cyclen) **2** also showed a weak interaction with dinucleotide d(GpT) to form a 1:1 complex by guanine N(7)−$Zn^{2+}$ and thymine N(3)−$Zn^{2+}$ coordinations with $K_d$ value of 7.7 $\mu$M at pH 7.4 (ref 10). So, we figure that $Zn^{2+}$−cyclen complexes **2** and **3** are likely to interact with the U and G regions in the loop.

(16) The conformational change of $TAR_{33}$ upon the $Zn^{2+}$−cyclen complexes binding was monitored by circular dichroism (CD) spectroscopy. The binding of Tat peptide causes a modest ellipticity decrease of positive peak around 265 nm, which is due to perturbations in base stacking. (see Loret, E. P.; Georgel, P.; Johnson, W. C., Jr.; Ho, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, 89, 9734−9738). The binding of $Zn^{2+}$−cyclen complexes altered the CD spectrum of $TAR_{33}$ (1 $\mu$M), **3** (1−10 $\mu$M) decrease the ellipicity of the positive peak (Supporting Information Figure S2).

(17) Long, K. S.; Crothers, D. M. *Biochemistry* **1995**, *34*, 8885−8895.

(18) Under the same conditions, neomycin inhibited the $TAR_{33}$−$Tat_{47-86}$ complexation with an $IC_{50}$ value of 50 $\mu$M.

(19) Perreault, D. M.; Cabell, L. A.; Anslyn, E. V. *Bioorg. Med. Chem.* **1997**, *5*, 1209−1220.

(20) See Supporting Information for experimental details of gel mobility shift assay.

(21) Kimura, E.; Katsuura, K.; Yokota, T.; Fujiwara, M.; Okamoto, M. Unpublished results.

(22) (a) For review; Kimura, E.; Koike, T.; Inouye, Y. *Perspectives on Bioinorganic Chemistry*; JAI Press Inc: London, UK, 1999; Vol. 4, pp 145−164. (b) Inouye, Y.; Kanamori, T.; Yoshida, T.; Koike, T.; Shionoya, M.; Fujioka, H.; Kimura, E. *Biol. Pharm. Bull.* **1996**, *19*, 456−458 (c) Inouye, Y.; Kanamori, K.; Sugiyama, M.; Yoshida, T.; Shionoya, M.; Fujioka, H.; Kimura, E. *Antiviral, Chem. Chemother.* **1995**, *6*, 337 (d) Inouye, Y.; Kanamori, T.; Yoshida, T.; Bu, X.; Shionoya. M.; Koike, T.; Kimura, E. *Biol. Pharm. Bull.* **1994**, *17*, 243−250.

(23) (a) For review; Bridger, G. J.; Skerlj, R. T. *Advances in Antiviral Drug Design*; JAI Press Inc: London, UK, 1999; Vol. 3, pp 161−229. (b) Bridger, G. J.; Skerlj, R. T.; Padmanabhan; Martellucci, S. A.; Henson, G. W.; Struyf, S.; Witurouw, M.; Schols, D.; De Clarcq, E. *J. Med. Chem.* **1999**, *42*, 3971−3981.