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Design, synthesis and bioactivities of TAR RNA targeting β-carboline derivatives based on Tat–TAR interaction

Xiaolin Yu, Wei Lin, Ruifang Pang, Ming Yang *

National Research Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100083, China

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Abstract

A series of new β-carboline derivatives **3**–**14** bearing guanidinium group or amino group-terminated side chain targeting the TAR RNA were designed and synthesized. Molecular modeling studies indicated that the minimal interaction energy was obtained for compound **11**, which contained the optimal linker of three methylene groups and the terminal guanidinium group interacted with the three-base bulge of TAR element by hydrogen bonds, which were the main contributor to the stability of drug–TAR RNA complex. To evaluate the ability of compounds **3**–**14** to block Tat–TAR interaction, we established a rapid, sensitive quantitative bioassay based on transient cotransfection of a Tat expression vector and a long terminal repeat region–chloramphenicol acetyltransferase (LTR–CAT) reporter construct in eukaryotic cells, monitoring the influence of the compounds on CAT expression levels with ELISA. Compounds **11** and **12** were the most active compounds of all in inhibiting Tat–TAR interaction bearing the terminal guanidinium group, and the optimal linker of the three methylene groups. Both compounds also exhibited anti-HIV-1 activity in MT4 cells, and their LD_{50} values of intraperitoneal acute toxicity for mice were 320.0 and 104.3 mg/kg, respectively. Furthermore, the results of capillary electrophoresis (CE) suggest that it is through targeting TAR RNA that this series of compounds block the Tat–TAR interaction.

Keywords: HIV; Tat–TAR interaction; β-Carboline; Anti-HIV activity

1. Introduction

In the past decade, remarkable clinical success in the field of target-directed AIDS treatment has been achieved with drugs against the reverse transcriptase and the protease [1]. However, the rapid development of viral resistance against existing drugs has accelerated the search for alternative targets. Recently, increasing evidence has shown that the viral trans-activator of transcription Tat protein binding to the trans-activation response region (TAR) RNA is an essential step in the human immunodeficiency virus type 1 (HIV-1) replication cycle [1,2]. Therefore, the blockage of Tat–TAR interaction is a potential route for AIDS chemotherapy. TAR RNA is a 59-base stem-loop structure located in the long terminal repeat region (LTR) at the 5′ end of all viral transcripts [3,4]. It contains a six-base loop and a three-base bulge (U23–C24–U25), which separate two helical stem regions [5,6]. Sequence and secondary structure of the apical portion of the HIV-1 TAR RNA containing the Tat binding site and apical loop is shown in Fig. 1. Deletion analyses of TAR RNA and other studies have established that Tat binds to TAR RNA at the three-base bulge and interacts with two base pairs above and

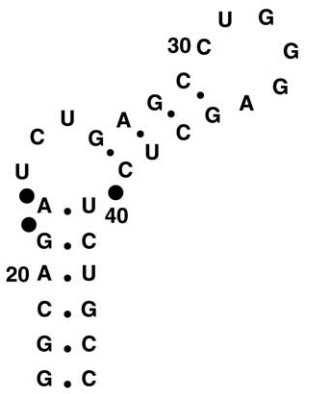

Fig. 1. Sequence and secondary structure of the HIV-1 TAR RNA containing the Tat binding site and apical loop.

* Corresponding author. Tel.: +86 10 8280 1569; fax: +86 10 8280 2062.
E-mail address: mingy@bjmu.edu.cn (M. Yang).

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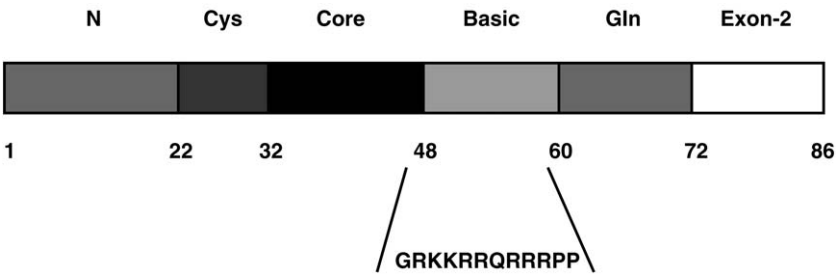


Fig. 2. Functional regions of the Tat protein and sequence of the basic region (48–59), which is one of the crucial sites of TAR recognition.

below the bulge in the major groove of TAR RNA [7], and appears to involve a two-step mechanism [3]. First, in intermolecular recognition involves the binding of arginine residues within the basic region of Tat to the TAR RNA bulge, which induces a conformational change in TAR RNA. Second, recognition by the protein of functional groups presented in the major groove of the TAR bulge. This mechanism has significant implication for the discovery of novel anti-HIV compounds directed at inhibiting the essential Tat–TAR interaction. The unique structure of TAR RNA in this three-base bulge region seems to be an attractive target for designing specific TAR-binding compounds as HIV-1 Tat–TAR inhibitors [8]. Numerous studies have shown that a short region of basic amino acids in Tat (amino acid residues 48–59), termed arginine-rich motif (ARM), is responsible for Tat–TAR specific interaction (Fig. 2) [9,10], and the arginine residue in the basic region is critically important in the binding of Tat and TAR in such a way that guanidinium group interacts with the three-base bulge region of TAR [11,12]. The existing structural information should allow the possibility of rational drug discovery. And also many experiments have provided evidences that the high-affinity binding of small molecules to their RNA targets are governed by the electrostatic interaction due to amino group, guanidinium group or arginine residue [13,14]. It has been proved that acridine derivatives bearing both amino side chain and planar aromatic ring are the most potent Tat–TAR low molecular weight inhibitors known to date by inhibiting the trans-activation of Tat protein and therefore represent a significant lead for the development of new active heterocycle drugs [15]. Our previous studies have indicated that β-carboline derivatives could interact with nucleic acids for its fused planar heterocycle ring structure, the optimized linker length and also some electronic substituents were important in the binding of nucleic acids [16,17]. Therefore, with molecular modeling insights into the interaction between HIV-1 TAR RNA and small molecules, we designed 12 β-carboline derivatives (compounds **3**–**14**) bearing guanidinium group or amino group-terminated side chain targeting the TAR element. To evaluate the ability of compounds **3**–**14** to block Tat–TAR interaction, we established a rapid, sensitive quantitative bioassay based on transient cotransfection of a Tat expression vector and a LTR–CAT reporter construct in eukaryotic cells, monitoring the influence of the compounds on chloramphenicol acetyltransferase (CAT) expression levels with the colorimetric enzyme immunoassay (ELISA). Based on the bioactivity determination in transient cotransfection assay for the new compounds, their anti-HIV-1 activity in vitro and intraperitoneal acute toxicity on mice were also evaluated. In addition, we used capillary electrophoresis (CE) to study the binding specificity of this series of compounds with TAR RNA in inhibiting the Tat–TAR interaction, as CE has become a powerful analytical means in biochemical studies, because of its high efficiency, easy of automation, short analysis time, and low sample consumption in the study of protein–RNA or drug–RNA interaction [18,19].

2. Chemistry

The synthesis of the β-carboline derivatives was outlined in Scheme 1, which was carried out in three steps. First, modified β-carboline compounds **1** and **2** were synthesized using previously described procedures [20,21]. We introduced a carboxyl ester function at position 3 of β-carboline ring for use in anchoring an amino-terminated or guanidine-terminated chain via an amide linkage. Second, we synthesized six molecules (**3**–**8**) containing an amino-terminated side chain by **1** or **2** reacted with 1,2-diaminoethane, 1,3-propanediamine and 1,6-diaminohexane respectively. Third, these compounds reacted with S-methylisothiourea to yield the compounds **9**–**14** bearing guanidine-terminated side chain. New compounds were characterized by MS, ^{1}H and ^{13}C NMR spectroscopy.

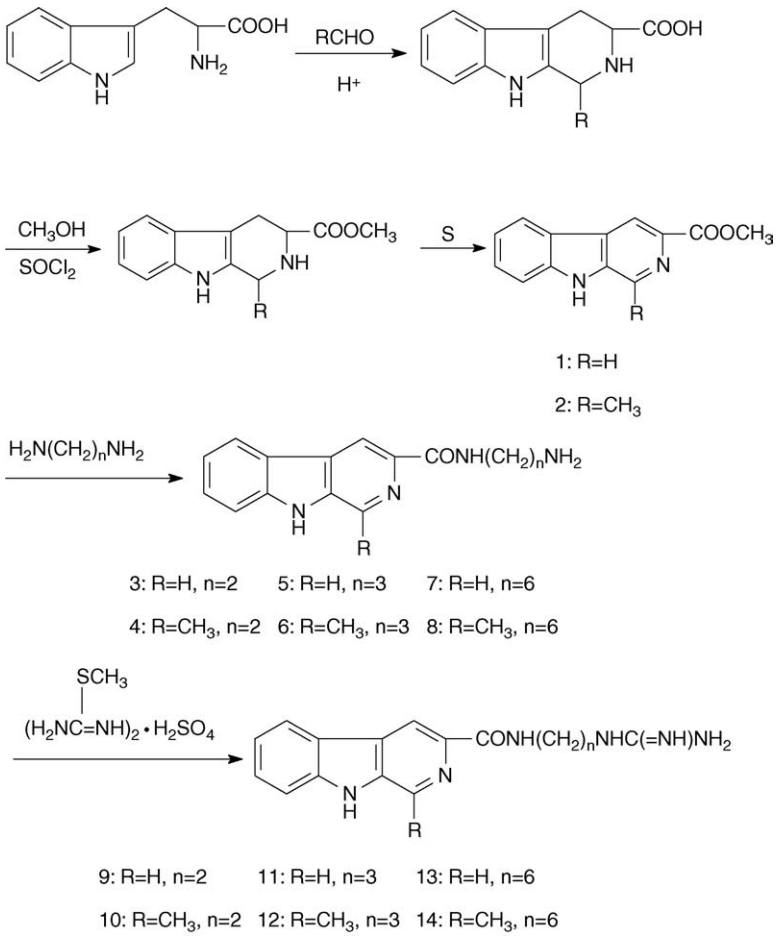
3. Molecular modeling

The calculating interaction energies of the 12 compounds with TAR RNA were presented in Table 1. Result of TAR RNA docking of compound **11** as an example was shown in Fig. 3.

4. Biological studies

4.1. Transient cotransfection assay

The results of the transient cotransfection assay for evaluating activities of compounds on Tat–TAR interaction were shown in Fig. 4.



Scheme 1. Synthesis of compounds **3**–**14**.

4.2. Anti-HIV activity and cytotoxicity assessment assays

The anti-HIV activities for compounds **5**, **6**, **11** and **12** with linker, $-(CH_2)_3-$, were determined in MT4 cells utilizing the HIV-1_{IIIB} strain of the virus, and the cytotoxic data of these compounds were assessed in the MT4 cells without any virus, which was reported in our previous paper [17].

4.3. Intraperitoneal acute toxicity studies

The LD_{50} values of intraperitoneal acute toxicity for mice of compounds **11** and **12**, which showed greater anti-HIV activities than other compounds were shown in Table 2.

Table 1
Computer simulation of the interaction between compounds **3**–**14** and TAR RNA

Compound	E_1[a] (kcal/mol)			E_2[b] (kcal/mol)			H-bond Number	Distance (Å)
	Vdw	Elect	Total	Vdw	Elect	Total		
3	63.6102	25.7389	89.3569	–15.3925	7.61603	–7.77649	2	1.72, 2.98
4	64.1276	25.9452	90.0701	–17.6499	2.45481	–15.1951	1	1.78
5	10.6405	6.77957	17.4201	–25.4538	–1.02175	–26.4756	N	–
6	64.5641	11.2564	75.8205	–16.893	–3.53241	–20.4255	N	–
7	65.8624	16.8865	82.7489	–10.7895	–4.64092	–15.4304	N	–
8	66.3352	17.2025	83.5377	–9.57243	–2.91258	–12.485	1	1.97
9	14.3528	–9.21747	5.13538	–7.42793	–18.4944	–25.9224	1	1.63
10	65.5429	–54.21	11.3229	–14.8353	–1.94353	–16.7788	1	2.30
11	10.6448	–5.64414	5.00065	–18.7723	–20.687	–39.4592	5	2.39, 1.84, 2.20, 1.91, 1.78
12	27.5801	–15.723	11.8571	–8.1804	–20.3209	–28.5013	3	1.98, 1.62, 1.83
13	14.8106	–8.36307	6.44754	–17.6919	–14.7772	–32.4691	2	1.76, 1.94
14	64.4232	–56.7249	7.69831	–12.4073	–2.49782	–14.9051	N	–

[a] Optical conformation energy.
[b] Minimization binding energy.

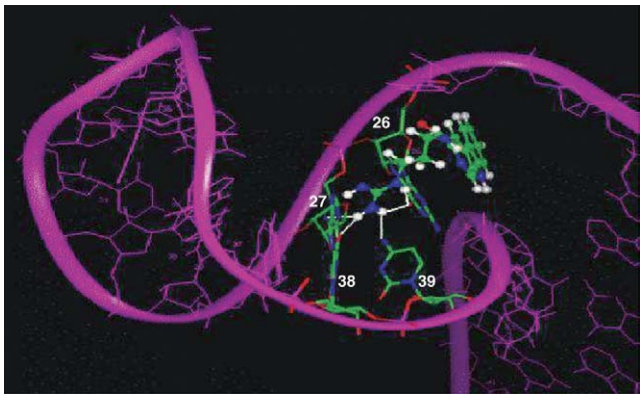


Fig. 3. Model illustrating the binding of compound **11** with HIV-1 TAR RNA.

5. Capillary electrophoresis assay

The CE electropherograms of the studies on the binding of compound **11** with TAR as well as inhibition of compound **11** on the Tat–TAR interaction as an example were shown in Fig. 5.

6. Results and discussions

A rational approach to discovering novel inhibitors of the active site of HIV-1 TAR RNA was to synthesize compounds whose structure units resembled the main features of the known Tat protein. A molecular modeling study was conducted to support the design of this inhibitory family [22,23]. Studies suggested that U23, C24 and U25 were stacked on A22 in the absence of Tat and that the arrangement of the three-nucleotide bulge was disrupted in the presence of Tat or a single arginine as well as guanidine: U23 was moved in direct proximity to G26, and the two other nucleotides became unstacked [24,25]. Tat bound to TAR RNA precisely in the region of the bulge UCU via an arginine residue and recognized both the identity of adjacent Watson-Crick base pairs and the position surrounding phosphate group. The observations that a single arginine, a single arginine amide and a single guanidine could induce similar conformational changes in TAR RNA suggested that one key arginine residue or guanidine (of the basic region of Tat protein) might be sufficient to trigger the conformational change in TAR RNA. Result of TAR RNA docking of compound **11** as an example was shown in Fig. 3. The modeling research implied that the planar β-carboline aromatic ring system had been inserted into the three-nucleotide bulge of TAR RNA sequence. In our simulation studies, we found that three nitrogen atoms in the guanidinium group of β-carboline side chain participated in hydrogen bonds, which had been shown to be important in drug–RNA interaction. It had been demonstrated that the tail chain containing polycations would contribute to the binding of small molecules with HIV-1 TAR RNA. It was also reported that specific bulge in the TAR RNA structures could be selectively recognized by intercalator-base conjugates. Newly designed molecules according to these ideas were made of β-carboline aromatic ring linked to a side chain with the terminal guanidinium group or amino group. The calculating interaction energies of the 12 compounds were presented in Table 1. Compound **11** exhibited the lowest optical conformation energy (5.0006 kcal/mol) and the lowest docking-binding energy (–39.4592 kcal/mol) among the 12 compounds, which indicated that compound **11** was not only more stable but also could form stable complex with TAR RNA more easily than the other analogues. The modeling result of compound **11** with TAR RNA implied that the planar β-carboline ring system had been inserted into the active pocket created by three-nucleotide bulge as expected and five hydrogen bonds formed between the guanidinium group of the carboxamide side chain and A27:O1, G26:O6, U38:O4, C39:N4 as well as between the NH of carboxamide and

Table 2
Intraperitoneal acute toxicity for mice of compounds **11** and **12**

Compounds	LD_{50} values (mg/kg)
11	320.0
12	104.3

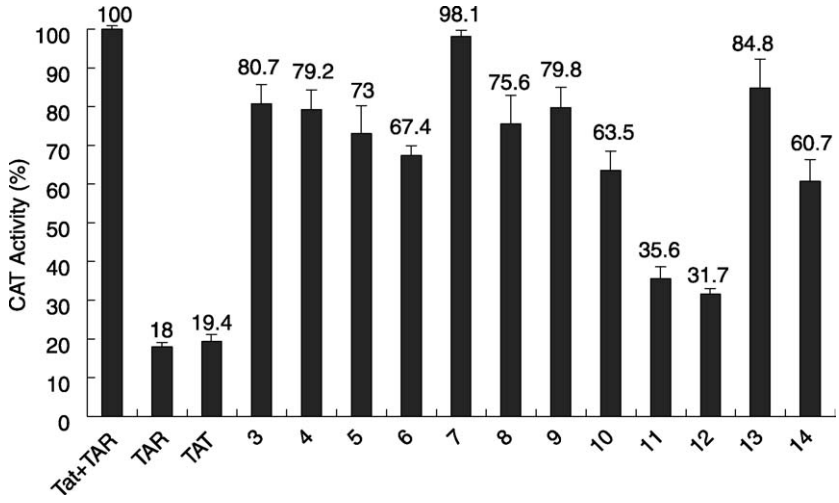


Fig. 4. Effects of compounds **3**–**14** on the expression of CAT in transient cotransfection assay.

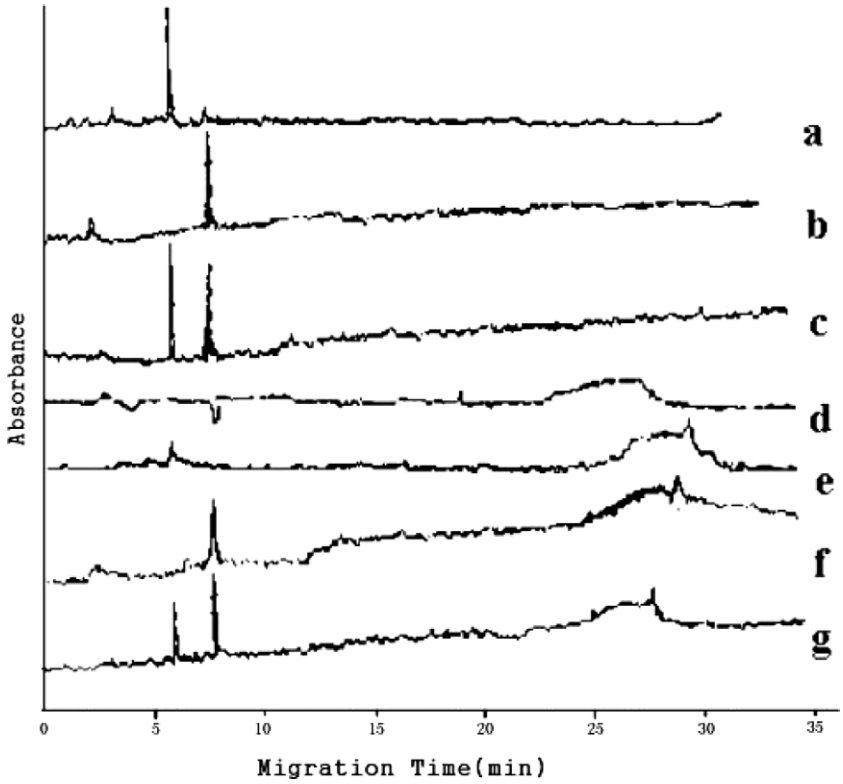


Fig. 5. CE electropherograms of the studies on the binding of compound **11** with TAR as well as inhibition of compound **11** on the Tat–TAR interaction. (a) Compound **11** alone (100 μM); (b) Tat alone (100 μM); (c) compound **11** + Tat (100 μM, each); (d) TAR alone (100 μM); (e) compound **11** + TAR (100 μM, each); (f) Tat + TAR (100 μM, each); (g) compound **11** + Tat + TAR (100 μM, each).

G26:O1 at the active sites of TAR RNA. Among the nucleotides of TAR RNA, G26 was one of essential nucleotides for Tat recognition in the Tat–TAR complex. In compound **11**, the guanidinium group was found mainly involved in hydrogen bonds to the TAR RNA. Analysis of interaction energy terms showed that the major interaction contributions were supported by the electrostatic term-hydrogen bonds.

Molecule modeling studies indicated that β-carboline derivatives might interact with TAR RNA and inhibit the Tat–TAR complex formation. The results implied that β-carboline derivatives bearing guanidinium group at the side chain (compounds **9**–**14**) had generally lower energy than the corresponding derivatives bearing amino group (compounds **3**–**8**). Compounds **4**, **6**, **8**, **10**, **12** and **14** have been designed on the basis of the same hypothesis for compounds **3**, **5**, **7**, **9**, **11** and **13**, but there was a substituted methyl group at C-1 positions on their β-carboline rings. From the data in Table 1 we can see, the substituted methyl group seemed to induce the steric hindrance, thus resulting in the relative higher docking energy. Table 1 also shows that the optimized linker chain $–(CH_2)_n–$ was an important factor in the binding of β-carboline derivatives with TAR RNA. The variation ($n = 3$) was favorable for the terminal groups to form hydrogen bonds with the three-nucleotide bulge region in TAR RNA.

To evaluate the ability of these compounds to block HIV-1 Tat–TAR interaction, we resorted to a reporter gene-based assay. The activities of the tested compounds were measured by transient cotransfection of a Tat expression vector [pSVC-MVTAT, containing the immediate early promoter of cytomegalovirus (CMV)] and a LTR–CAT reporter construct (pLTR-CAT, containing HIV-1 LTR fused to CAT gene) to 293T cells, As such, the produced Tat would transactivate the LTR-linked gene CAT to a level that would reflect the extent of Tat–TAR interaction. Inhibitory effect of the tested compounds to trans-activation was measured by quantitatively determination of CAT reporter gene activity at 48 h post-transfection by ELISA [17,26–28]. None of the tested compounds with concentrations up to 30 μM showed any significant cytotoxicity as measured by the MTT assay (Results are not shown). Fig. 4 shows the representative results with all the tested compounds. These results indicated that CAT expression driven by the HIV-1 LTR was inhibited to different degree in the presence of the tested compounds at 30 μM. Compounds bearing guanidinium group-terminated side chain (compounds **9**–**14**) showed more inhibitory activity than those with terminal amino group (compounds **3**–**8**). It appears that the length of the flexible linker chain which connected the "activator" (guanidinium or amino group) with the "anchor" (the fused planar heterocycle ring structure in β-carboline) contributed to the inhibitory activity of the compounds to transactivation. Results demonstrated that compounds with linker, $–(CH_2)_3–$ (compounds **5**, **6**, **11** and **12**) showed greater inhibitory activity than either of the shorter ones, $–(CH_2)_2–$ (compounds **3**, **4**, **9** and **10**), or the longer ones, $–(CH_2)_6–$ (compounds **7**, **8**, **13** and **14**), which suggested that the linker chain length of the three carbon atoms seemed to be the opti-

mized linker in our experiments. These results agreed well with the molecular modeling studies. In addition, The substituted methyl group at C-1 position on the β-carboline ring of the compounds (**4**, **6**, **8**, **10**, **12** and **14**) exhibited some more inhibitory activities. These results were slightly different from the molecular modeling studies. Although results in the molecular modeling studies showed the substituent of methyl group was accompanied with the relative higher docking energy, besides the effect of the steric hindrance, the increased molecular polarity induced by the methyl group might give these compounds more binding affinities to TAR RNA, especially in the complicated eukaryotic system. The mechanism of methyl group substituent is currently a subject of investigation in our laboratories.

To increase the probability of success, easy screening methods are required for searching drugs that interfere with the HIV transactivation. We established a transient cotransfection assay that provided a virus-free system for screening compounds for their ability to interfere with Tat-mediated transactivation of HIV-1 LTR-driven gene expression. In our experiments, the CAT ELISA was used to quantitatively measure CAT expression in eukaryotic cells transfected with a plasmid bearing a CAT encoding reporter gene. The determination of CAT levels using the CAT ELISA avoided the use of radioisotopes and produced sensitivity comparable to that of the isotopic protocol. This assay provides a versatile, efficient and reproducible means for investigating the molecules involved in HIV infectivity.

The anti-HIV activities together with the cytotoxic data of compounds **5**, **6**, **11** and **12** with linker, $-(CH_2)_3-$, which showed greater inhibitory activities than other compounds in transient cotransfection assay were studied. Both compounds **11** and **12** showed inhibitory activity to HIV-1 ($EC_{50} \geq 50$ μM) and without apparent cellular toxicity ($IC_{50} \geq 100$–1000 μM), however, compounds **5** and **6** were neither inhibitory nor cytotoxic to HIV-1. These results were basically consistent with the results we have got from the transient cotransfection bioassay mentioned above.

The LD_{50} values of intraperitoneal acute toxicity for mice of compounds **11** and **12** which showed greater anti-HIV activities than other compounds were 320.0 and 104.3 mg/kg, respectively (95% confidence limits: 305.9–334.8 and 92.7–117.4 mg/kg for compounds **11** and **12**, respectively). Although the introduction of a methyl group on the heterocycle ring of the β-carboline derivatives resulted in the appearance of some more inhibitory activity to Tat–TAR interaction, it also seemed to bring more toxicity.

CE has already been proved to be an attractive method to study the interactions between biomacromolecules and small organic molecules, for they can be well separated due to their different electrophoretic mobilities. CE separation is based on the electroosmotic flow of the buffer solution, charge-to-mass ratio, size and constructer of analyte. Here it has been used to analyze the binding of compound **11** with HIV-1 TAR RNA and its inhibition of the Tat–TAR interaction, which showed inhibitory activities on Tat–TAR interaction as well as to HIV-1 in MT4 cells. As shown in Fig. 5a, the well-shaped peak of compound **11** with $t_m = 6.0$ min was clearly visible under the experimental condition used, so was Tat protein with $t_m = 7.9$ min (Fig. 5b). The migration time of free TAR RNA and its complex form with compound **11** or Tat protein was in the range of 26–30 min. Free TAR RNA migrated faster than its complex forms because of the smaller charge to mass ratio of the complexes. The binding of compound **11** with TAR RNA was observed as electrophoresis mobility shift of TAR's peak and the decrease of the height of free compound's peak in the presence of TAR. Then the inhibition of compound **11** on the binding of Tat–TAR complex was examined. Fig. 5 g showed the height of Tat's peak was higher than that of Fig. 5f, which implied that it is through targeting TAR RNA that compounds **11** blocks the Tat–TAR interaction.

7. Conclusions

Twelve β-carboline derivatives bearing guanidinium group or amino group at the terminal of the side chains were synthesized. Molecular modeling studies indicated that the minimal interaction energy was obtained for compound **11**, which contained the optimal linker of three methylene groups and the terminal guanidinium group. The simulation of compound **11** with TAR RNA showed that the β-carboline aromatic ring of the molecule was inserted into the major groove of the three nucleotides bulge, which optimally positioned the side chain guanidinium group to form five hydrogen bonds interaction with TAR RNA, this favorable electrostatic interaction in turn stabilized the drug–TAR RNA complex. In biological studies, we examined the abilities of the compounds to prevent Tat-mediated trans-activation as well as their efficacy in inhibiting viral replication in cell assay. To evaluate the ability of compounds **3**–**14** to block Tat–TAR interaction, we resorted to a rapid, sensitive quantitative bioassay based on transient cotransfection of a Tat expression vector and a LTR–CAT reporter construct in 293T cells, monitoring the influence of the compounds on CAT expression levels with ELISA. The extent of blocking Tat–TAR interaction varied with the terminal group (guanidinium or amino group), the linker length, as well as the methyl group substituent. In our study, compounds **11** and **12** remained to be the most active compounds bearing the terminal guanidinium group, and the optimal linker of the three methylene groups, which also exhibited the lowest interaction energy among the 12 compounds in molecular modeling studies. Our data demonstrated that two compounds (**11** and **12**) were active against HIV-1 in cell culture. It is not surprised that the compounds are expected to inhibit virus replication since their ability of disrupting the Tat–TAR interaction. Intraperitoneal acute toxicity studies on mice showed the LD_{50} values of compounds **11** and **12** were 320.0 and 104.3 mg/kg, respectively. Nowadays, CE has become a powerful analytical technique in biochemical studies for its high efficiency and resolving power.

Our results suggest that it is through targeting TAR RNA that this series of compounds block the Tat–TAR interaction.

The reported β-carboline derivatives could serve as a good starting point for further lead optimization by medicinal chemistry. Additional studies will be necessary to further precise the interaction of the compounds class with the three-base bulge of TAR RNA.

8. Experimental protocols

8.1. Chemistry

Melting points were determined on a XA-4 instrument and uncorrected. All NMR spectra were taken on a Varian Unity 300 NMR spectrometer with ^{1}H and ^{13}C being observed at 300 and 75 MHz, respectively and recorded as solutions in $(CD_3)_2SO$. Chemical shifts (δ) for ^{1}H and ^{13}C spectra were expressed in ppm relative to tetramethylsilane (TMS) as an internal standard. Mass spectra were measured on an ABI QSTAR spectrometer. Elemental analyses were determined on a Elementar Vario EL apparatus. Chromatography was performed on columns packed silica gel (200–400 mesh) or Sephedex LH-20. TLC was carried out on precoated silica gel F254 plates.

Unless otherwise specified, all materials, solvents and reagents were obtained from commercial suppliers.

*8.1.1. 3-Ethylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**3**)*

To a stirred 1,2-diaminoethane (1.2 ml, 17.8 mmol), a solution containing β-carboline-3-carboxylate (**1**, 0.1 g, 0.44 mmol), $CHCl_3$ (3 ml) and MeOH (1 ml) was added slowly at 80–85 °C for 2.5 h, and the resulting mixture was refluxed for 8 h. The solvent was removed under reduced pressure and the residues were dissolved in the mixture of $CHCl_3$ (5 ml) and H_2O (2 ml). The mixture was cooled overnight at 4 °C and the white solid was filtered by suction, washed with $CHCl_3$ (3 × 5 ml) and then with H_2O (3 × 5 ml). Yield 22.2%. M.p. 234–237 °C; MS (TOF): 255 (M + 1); ^{1}H NMR: δ = 12.26 (s, 1H, NH), 9.08 (s, 1H, 1-H), 8.92 (s1H, 4-H), 8.87 (d, J = 8.4 Hz, 1H, 8-H), 8.45 (d, J = 8.0 Hz, 1H, 5-H), 7.69 (d, J = 8.4 Hz, 1H, 6-H), 7.61 (t, J = 7.2 Hz, 1H, 7-H), 3.63 (m, 2H, CH_2), 3.03 (m, 2H, CH_2).

*8.1.2. 1-Methyl-3-ethylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**4**)*

To a stirred 1,2-diaminoethane (4 ml, 60.0 mmol), a solution containing methyl β-carboline-3-carboxylate (**2**, 0.4 g, 1.77 mmol), $CHCl_3$ (7 ml) and MeOH (2 ml) was added slowly at 80–85 °C for 2.5 h, and the resulting mixture was refluxed for 8 h. The solvent was removed under reduced pressure and the residues were separated by column chromatography [$CHCl_3$–MeOH (7: 2, v/v)]. The yellow oil was obtained and dried before weighted. Yield 36.2%. MS (TOF): 269 (M + 1); ^{1}H NMR: δ = 11.15 (s, 1H, NH), 8.72 (s, 1H, 4-H), 8.34 (d, J = 7.8 Hz, 1H, 8-H), 7.64 (d, J = 7.5 Hz, 1H, 5-H), 7.58 (t, J = 7.6 Hz, 1H, 6-H), 6.8 (t, 1H, J = 7.5 Hz, 7-H), 3.1–3.67 (m, 4H, 2 × CH_2), 2.83 (s, 3H, CH_3).

*8.1.3. 3-Propylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**5**) and 1-methyl-3-propylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**6**)*

3-Propylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**5**) and 1-methyl-3-propylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**6**) were prepared as described in our previous work [17].

*8.1.4. 3-Hexylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**7**)*

To a stirred 1,6-diaminohexane (0.05 g, 0.43 mmol), a solution containing **1** (0.15 g, 0.66 mmol), $CHCl_3$ (5 ml) and MeOH (0.5 ml) was added slowly at 80–85 °C for 2.5 h, and the resulting mixture was refluxed for 8 h. The solvent was removed under reduced pressure and the residues were dissolved in the mixture of $CHCl_3$ (3 ml) and H_2O (2 ml). The mixture was cooled overnight at 4 °C and the pale yellow solid was filtered by suction, washed with $CHCl_3$ (3 × 5 ml) and then with H_2O (3 × 5 ml). Yield 58.3%. M.p. 189–193 °C; MS (TOF): 311 (M + 1); ^{1}H NMR: δ = 8.93 (s, 1H, 1-H), 8.84 (s, 1H, 4-H), 8.37 (d, J = 7.8 Hz, 1H, 8-H), 7.67 (d, J = 8.1 Hz, 1H, 5-H), 7.58 (t, J = 7.5 Hz, 1H, 6-H), 7.25 (t, J = 7.2 Hz, 1H, 7-H), 3.42 (m, 2H, CH_2), 3.28 (m, 2H, CH_2), 2.78 (m, 2H, CH_2), 1.82 (m, 6H, 3 × CH_2).

*8.1.5. 1-Methyl-3-hexylamine-9-H-pyrido[3,4-b]indole-3-carboxamide (**8**)*

To a stirred 1,6-diaminohexane (0.5 g, 4.3 mmol), a solution containing **2** (0.5 g, 2.2 mmol), $CHCl_3$ (9 ml) and MeOH (2 ml) was added slowly at 80–85 °C for 2.5 h, and the resulting mixture was refluxed for 8 h. The solvent was removed under reduced pressure and the residues were dissolved in the mixture of $CHCl_3$ (3 ml) and H_2O (2 ml). The mixture was cooled overnight at 4 °C and the white solid was filtered by suction, washed with $CHCl_3$ (3 × 5 ml) and then with H_2O (3 × 5 ml). Yield 32.6%. M.p. 243 °C; MS (TOF): 325 (M + 1); ^{1}H NMR:δ = 12.01 (s, 1H, NH), 8.65 (s, 1H, 4-H), 8.33 (d, J = 7.8 Hz, 8-H), 7.64 (d, J = 8.1 Hz, 5-H), 7.57 (t, J = 6.9 Hz, 6-H), 7.27 (t, J = 7.5 Hz, 7-H), 3.35 (m, 2H, CH_2), 2.84 (s, CH_3), 2.71 (m, CH_2), 1.2–1.5 (m, 8H, 4 × CH_2).

*8.1.6. 3-(2-Guanidyl)-ethyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**9**)*

To a solution of S-methylisothiourea sulfate (0.05 g, 0.18 mmol) in water (0.03 ml) and 2 N NaOH (0.03 ml) was added the suspension of **3** (0.1 g, 0.39 mmol) in water (2 ml) slowly under 4 °C. The mixture was stirred at room temperature for 1 h, and then kept it cooled at 4 °C overnight and the precipitate was filtrated by suction, the crude was washed by cold water and cold MeOH, The solid was dissolved in a minimum volume of HCl and applied to a Sephedex LH-20 (10 g) column which was then washed with H_2O 500 ml. Subse-

quent elution was concentrated and dried under reduced pressure to afford **9** as a yellow solid. Yield 34.3%. M.p. 312–313 °C; MS (TOF): 297 (M + 1); ^{1}H NMR: δ = 8.91 (s, 1H, 1-H), 8.85 (s, 1H, 4-H), 8.38 (d, J = 7.2 Hz, 1H, 8-H), 7.67 (m, J = 7.8 Hz, 1H, 5-H), 7.60 (d, J = 6.9 Hz, 1H, 6-H), 7.30 (t, J = 7.8 Hz, 1H, 7-H), 3.52 (m, 2H, $CONHCH_2$), 1.23 (s, 2H, CH_2); ^{13}C NMR: δ = 165.53, 157.25, 141.07, 139.19, 137.27, 132.52, 128.51, 127.98, 122.14, 120.91, 119.85, 113.98, 112.30, 40.05, 37.96.

*8.1.7. 1-Methyl-3-(2-guanidyl)-ethyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**10**)*

To a solution of S-methylisothiourea sulfate (0.14 g, 0.5 mmol) in water (1 ml) and 2 N NaOH (0.5 ml) was added the suspension of **4** (0.1 g, 0.37 mmol) in water (3 ml) slowly under 4 °C. The mixture was stirred at room temperature for 1 h, and then kept it cooled at 4 °C overnight and the pale precipitate was filtrated by suction, the crude was washed by cold water and cold MeOH, The solid was dissolved in a minimum volume of HCl and applied to a Sephedex LH-20 (10 g) column which was then washed with H_2O (500 ml). Subsequent elution was concentrated and dried under reduced pressure to afford **10**. Yield 51.9%. MS (TOF): 311 (M + 1); ^{1}H NMR: δ = 11.96 (s, 1H, NH), 8.80 (s, 1H, 4-H), 8.34 (d, 1H, J = 7.5 Hz, 1H, 8-H), 7.62 (d, J = 7.8 Hz, 1H, 5-H), 7.57 (d, J = 8.7 Hz, 1H, 6-H), 7.27 (t, J = 6.6 Hz, 1H, 7-H), 3.50 (m, 2H, $CONHCH_2$), 2.86 (s, 3H, CH_3), 2.27 (m, 2H, CH_2); ^{13}C NMR: δ = 165.39, 157.33, 141.01, 140.82, 138.63, 135.91, 128.13, 127.29, 122.02, 121.39, 119.79, 112.25, 112.15, 40.57, 37.95, 20.35.

*8.1.8. 3-(3-Guanidyl)-propyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**11**) and 1-methyl-3-(3-guanidyl)-propyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**12**)*

3-(3-Guanidyl)-propyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**11**) and 1-methyl-3-(3-guanidyl)-propyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**12**) were prepared as described in our previous work [17].

*8.1.9. 3-(6-Guanidyl)-hexyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**13**)*

To a solution of S-methylisothiourea sulfate (0.2 g, 0.72 mmol) in water (4 ml) and 2 N NaOH (0.5 ml) was added the suspension of **7** (0.2 g, 0.57 mmol) in water (2 ml) slowly under 4 °C. The mixture was stirred at room temperature for 1 h, and then kept it cooled at 4 °C overnight and the pale precipitate was filtrated by suction, the crude was washed by cold water and cold MeOH, The solid was dissolved in a minimum volume of HCl and applied to a Sephedex LH-20 (10 g) column which was then washed with H_2O (500 ml). Subsequent elution was concentrated and dried under reduced pressure to afford **13** as a pale yellow solid. Yield 64.2%. M.p. 181–183 °C; MS (TOF): 353 (M + 1); ^{1}H NMR: δ = 12.08 (s, 1H, NH), 8.91 (s, 1H, 1-H), 8.81 (s, 1H, 4-H), 8.37 (d, J = 7.5 Hz, 1H, 8-H), 7.63 (d, J = 7.8 Hz, 1H, 5-H), 7.56 (d, J = 8.1 Hz, 1H, 6-H), 7.28 (t, J = 7.2 Hz, 1H, 7-H), 3.33 (m, 2H, $CONHCH_2$), 3.03 (m, 2H, NH_2CH_2), 1.23–1.55 (m, 8H, $CH_2 \times 4$).

*8.1.10. 1-Methyl-3-(6-guanidyl)-hexyl-9-H-pyrido[3,4-b]indole-3-carboxamide (**14**)*

To a solution of S-methylisothiourea sulfate (0.1 g, 0.5 mmol) in water (1 ml) and 2 N NaOH (1 ml) was added the suspension of **8** (0.04 g, 0.12 mmol) in water (1 ml) and ethanol (95%, 1.2 ml) slowly under 4 °C. The mixture was stirred at room temperature for 1 h, and then kept it cooled at 4 °C overnight and the pale precipitate was filtrated by suction, the crude was washed by cold water and cold MeOH, the solid was dissolved in a minimum volume of HCl and applied to a Sephedex LH-20 (10 g) column which was then washed with H_2O (500 ml). Subsequent elution was concentrated and dried under reduced pressure to afford **14**. Yield 34.5%. M.p. 196–198 °C; MS (TOF): 367 (M + 1); ^{1}H NMR: δ = 12.03 (1H, NH), 8.65 (s, 1H, 4-H), 8.32 (d, J = 7.2 Hz, 1H, 8-H), 7.62 (d, 2H, 5-H, 6-H), 7.51 (t, 1H, 7-H), 3.05 (m, 2H, CH_2), 2.84 (s, 3H, CH_3), 1.53 (m, 2H, CH_2), 1.34–0.83 (m, 8H, $CH_2 \times 4$).

8.2. Molecular modeling

The docking study of the designed compounds with TAR RNA (PDB code: 1ANR) as target was carried out using Insight II from Accelrys. Energy was computed with AMBER force field and minimization was performed using the steepest gradient algorithm with 1000 iteration steps or until convergence. The geometric constraints were created with the specific command "genericdist" of the DISCOVER module. All calculations were performed on a SGI IRIX6.5 workstation in Peking University Health Science Center.

8.3. Biological studies

8.3.1. Transient cotransfection assay

The cotransfection was performed in 293T cells by standard calcium phosphate coprecipitation techniques with optimum amounts of reporter DNA (pLTRCAT) and effector (pSVCMVTAT). Twenty-four hours later, the transfected cells were washed with PBS and added to fresh medium together with diluted compounds at final concentration 30 μM respectively and incubated for another 24 h before they were harvested and lysed. The aliquots of the lysate were used to measure CAT activities that were normalized to the protein concentration as determined using a commercial CAT ELISA kit (Roche Molecular Biochemicals) in accordance with the manufacturer's protocol [17]. All data were reported as a percentage of CAT activity (±S.D.). Unless otherwise indicated, the relative CAT activities represent the average of three identical transfections performed in duplicate.

8.3.2. Intraperitoneal acute toxicity studies

Mice weighting 18–20 g were purchased from the Experimental Animal Department of Peking University Health Sci-

ence Center. They were kept for 1 week before the beginning of the experiments at controlled temperature (21 ± 1 °C) and humidity (60–70%), using dust free poplar chips for bedding. Ten mice were treated with each dose level of compound **11** (204.8, 256, 320, 400, and 500 mg/kg) or compound **12** (64, 80, 100, 125 and 156.2 mg/kg) by intraperitoneal injection in a volume of 20 ml/kg body weight. Control animals received the vehicle alone (20 ml/kg of 0.9% NaCl). After intraperitoneal injection, the mice were observed for 14 days and signs of toxicity and mortality were recorded. Surviving animals were sacrificed 14 days after the treatment. Each mouse was submitted to necroscopic examination immediately after death [29].

8.4. Capillary electrophoresis assay

To ensure proper folding of the TAR RNA structure, the RNA solutions were annealed by heating for 3 min at 95 °C and cooled slowly. The solutions of complexes were mixed well, incubated at 4 °C for 1 h before CE analysis.

CE experiments were carried out on a Beckman P/ACE 2100 CE system using a Beckman 57 cm × 75 μm I.D. bare fused-silica capillary, 50 cm to the UV detector. 50 mM phosphate buffer (pH 8.0) was used as running buffer. Reverse polarity, a constant voltage of 11 kV, a temperature of 25 ± 0.1 °C and pressure injection for 10 s at 20 psi (1 psi = 6894.76 Pa) were used. Absorption of samples was detected at 210 nm. Prior to use, the capillary was pre-treated with 0.2 M NaOH for 60 min, water for 30 min and finally with the running buffer until the baseline become smooth. To decrease the adsorption of the samples on the capillary wall through electrostatic interaction and to insure the reproducibility of migration time, the capillary was washed between runs with 0.2 M NaOH, water and running buffer each for 4 min orderly. Solutions were filtered through a 0.22 μm PTFE membrane before use [17,19].

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