Pure Appl. Chem., Vol. 82, No. 1, pp. 339–347, 2010.
doi:10.1351/PAC-CON-09-01-04

Design, synthesis, and biological evaluation of novel substituted [1,2,3]triazolo[4,5-*d*]pyrimidines as HIV-1 Tat–TAR interaction inhibitors*

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Abstract: A novel series of compounds, derived from [1,2,3]triazolo[4,5-*d*]pyrimidines with a guanidyl group or amino group-terminated side chain was designed and synthesized as HIV-1 trans-activator of transcription–trans-activation responsive region (Tat–TAR) interaction inhibitors. Their ability to inhibit Tat–TAR RNA interaction was determined by a Tat-dependent HIV-1 long terminal repeat (LTR)-driven chloramphenicol acetyltransferase (CAT) assay and simian immunodeficiency virus (SIV)-induced syncytium evaluation. The binding of the compounds with TAR RNA was conducted by molecular modeling and capillary electrophoresis (CE) analysis. The results showed that all the compounds could block the Tat–TAR interaction and have antiviral activities.

Keywords: antiviral activities; heterocycle compounds; HIV; substituted purines; TAR RNA; Tat–TAR inhibitors.

INTRODUCTION

Since the 1980s, acquired immune deficiency syndrome (AIDS) has become a disastrous epidemic which greatly threatens the health of humankind and brings us crisis and challenges in the health care system, and the number of people living with HIV in 2008 was over 33 million [1]. Today, there are more than 30 drugs available in clinics for the treatment of HIV-1 infection. However, the emergence of drug-resistant viruses caused by mutations of the enzymes often results in treatment failure with existing antiretrovirals [2]. The cross-resistance to the same class of compounds is also a problem for the treatment process [3]. Thus, efforts were made to develop new antiretroviral agents with different targets for inhibition of HIV-1 replication. One such target is the interaction between HIV trans-activator of transcription (Tat) protein and trans-activation responsive region (TAR) RNA.

In the HIV-1 life cycle, transcription from the integrated proviral DNA is considered to be a crucial step for viral replication. Gene expression from proviral DNA is mainly regulated by two viral proteins, Tat and Rev, as well as several known and unknown host-cellular factors. Therefore, it appears that these molecules associated with the transcription process are potential targets for inhibition of HIV-1 replication.

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*Paper based on a presentation at the 13th International Biotechnology Symposium (IBS 2008): "Biotechnology for the Sustainability of Human Society", 12–17 October 2008, Dalian, China. Other presentations are published in this issue, pp. 1–347.

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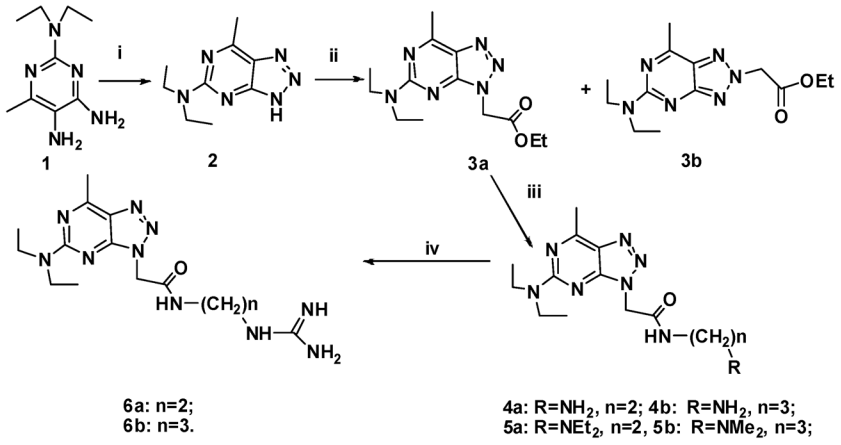
HIV-1 regulatory protein Tat stimulates transcriptional elongation through its interaction with the TAR region, a 59-nucleotide stem-loop found at the 5' untranslated end of all newly transcribed HIV mRNAs [4,5]. TAR RNA contains a six-nucleotide loop and a three-nucleotide bulge, which connects two adjacent helical stem regions. The tri-nucleotide bulge (U23, C24, U25) of HIV TAR is essential for high-affinity and specific binding of Tat protein. HIV-1 Tat is a protein with 86 amino acid residues, which includes a basic region (RKKRRQRRR, 49-57), termed arginine-rich motif (ARM), responsible for Tat–TAR specific interaction. Tat binds to this tri-nucleotide bulge of TAR and recognizes both the adjacent Watson–Crick base pairs and the positions of surrounding phosphate groups. Studies have shown that the arginine 52 residue of Tat directly binds to the tri-nucleotide bulge and its guanidyl group is largely responsible for the Tat–TAR interaction. The arginine residue can bind to TAR and induce a change in RNA conformation largely mimicking a portion of Tat–TAR complex. Furthermore, the high-affinity binding of some small molecules with amino, guanidyl group or arginine residue to TAR RNA is governed by electrostatic interaction, hydrogen bonds, and hydrophobic contacts between these groups and the region near the UCU bulge.

According to these possible binding modes, the inhibitors have been mainly divided into two classes: (1) Compounds binding directly to TAR RNA either to the TAR RNA three-base bulge region alone or to the three-base bulge together with the lower and upper-stem/loop region. (2) Compounds binding directly to Tat protein with high affinity, thus potently inhibiting HIV-1. They both block Tat trans-activation in the formation of the Tat–TAR complex to exert antiviral activity in primary human cells [5]. Based on this idea, several series of compounds have been designed and synthesized as HIV-1 Tat–TAR inhibitors, such as acridine derivative CGP 40336A9 and diphenylfuran derivative DB60 [6].

Our previous work has demonstrated that some substituted α,α-trehalose, β-carboline, isoquinoline, and purine derivatives with a flexible side chain with a terminal amino or guanidyl group could interact with TAR and inhibit the replication of HIV-1 [7–14]. We propose a molecular model of new Tat–TAR inhibitors containing an "activator", an "anchor", and a "linker". An "activator" is defined as a group that could recognize and bind to the tri-based bulge of TAR, usually an amino or guanidyl group. An "anchor" is a functional group that could interact with TAR in different ways from the "activator", such as stacking into tri-based bulge, forming hydrogen bond with unpaired base, intercalating into the upper or lower stem, or falling into the major groove near the bulge. A "linker" is the structure linking the "activator" and the "anchor" with optimal length. [10] These studies show that the substituted purine derivatives possess more potent HIV-1 Tat–TAR inhibitory activities than β-carboline and isoquinoline derivatives due to the increased interaction between TAR RNA and the purine ring.

In this paper, we designed and synthesized a novel series of [1,2,3]triazolo[4,5-*d*]pyrimidine derivatives bearing guanidyl group or amino group-terminated side chain (Scheme 1), attempting to obtain more potential Tat–TAR inhibitors. All the compounds were evaluated for their inhibitory abilities to Tat–TAR interaction and antiviral activity by a Tat-dependent HIV-1 long terminal repeat (LTR)-driven chloramphenicol acetyltransferase (CAT) assay, and simian immunodeficiency virus (SIV)-induced syncytium evaluation. The binding of the compounds with TAR RNA was conducted by molecular modeling and capillary electrophoresis (CE) analysis.



Scheme 1 Synthesis of substituted 3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidines. Reagent and conditions: (i) $NaNO_2$/HOAc, –5 °C, 20 min; (ii) K_2CO_3/KI, ethyl chloroacetate, acetone, reflux, 8 h; (iii) ω,ω-diamino alkane (6 equiv), methanol, 8 h, reflux; (iv) $AIMSO_3H{\cdot}H_2O$ (1.2 equiv), anhydrous ethanol, 4 h, 35~45 °C.

RESULTS AND DISCUSSION

Chemistry

All of the synthetic work was carried out starting from 4-chloro-*N*,*N*-diethyl-6-methyl-5-nitropyrimidin-2-amine derived from 2-(diethylamino)-6-methyl-5-nitropyrimidin-4-ol due to its high reactivity with nucleophilic reagents, such as thiols and amines. Substituted purine analogs such as purine-8-thiol and 3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine could be easily prepared from this intermediate, and the two N-containing heterocycles are thought to be served as suitable anchors for the contained N-atoms which might reinforce the interactions with Tar RNA, especially when side chains containing amino group were introduced at N-9 of the purines [10]. Side chains were constructed by the alkylation of ethyl chloroacetate and prolonged to suitable length by further reactions with ω,ω-diamino alkanes such as ethylenediamine and propane-1,3-diamine. The reaction with ω,ω-diamino alkanes was easily accomplished in methanol with high yields by a simple final treatment. As all of the target compounds we designed were amphi- or hydrophilic, we selected anhydrous ethanol as solvent and aminoiminomethane sulfonic acid ($AIMSO_3H{\cdot}H_2O$) as guanidylation reagent. In this work, we find that using $AIMSO_3H{\cdot}H_2O$ to construct the guanidyl group at the end of the side chain is a more efficient way compared with other reagents for its high reactivity, mild condition, simple procedure, and easy final treatment, though it is not so commonly used before [15]. As all of the title compounds, such as **6a** and **6b**, were successfully obtained by this method we developed, we think the method could be efficiently used in preparing other amphi- or hydrophilic compounds containing guanidyl groups.

The route used for the preparation of the title compounds was carried out as outlined in Scheme 1. The synthetic work was started from N^2,N^2-diethyl-6-methyl-pyrimidine-2,4,5-triamine. After diazotization with $NaNO_2$ in acetic acid, N^2,N^2-diethyl-6-methyl-pyrimidine-2,4,5-triamine (**1**) was converted to 5-diethylamino-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine (**2**) via intramolecular cyclization in nearly a stoichiometric yield by a "one-pot" reaction. After the alkylation of **2** with ethyl chloroacetate in acetone under the catalysis of K_2CO_3/KI in acetone, ethyl 2-{5-(diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}acetate (**3a**) was obtained as the main product in a moderate yield. After the amination of **3a** with ethylenediamine, propane-1,3-diamine, N^1,N^1-diethylethane-1,2-diamine and N^1,N^1-dimethylpropane-1,3-diamine in methanol, respectively, compounds **4a**, **4b**, **5a**, and **5b** were obtained in good yield. Compounds **6a** and **6b** were also easily prepared from **4a** and **4b** with

$AIMSO_3H{\cdot}H_2O$ as the guanidylation regent just as the above. All the title compounds (**4a**, **4b**, **5a**, **5b**, **6a**, and **6b**) were synthesized for the first time.

Biological evaluation

Inhibition of Tat–TAR interaction in vitro

In order to reveal whether the compounds we designed could inhibit HIV-1 Tat–TAR interaction, we evaluated all six title compounds by Tat-dependent HIV-1 LTR-driven CAT gene expression colorimetric enzyme assay in human 293T cells at a concentration of 30 μM. None of the tested compounds at concentrations up to 30 μM showed any significant cytotoxicity (data not shown). Inhibitory effect of the tested compounds was measured by quantitatively determination of CAT expression. The depressed CAT expression indicated the high inhibitory activity of the compound. As shown in Fig. 1, the range of inhibited CAT expression induced by the six compounds was from 28.1 to 50.2 %, which suggested that all of these compounds could effectively block the interaction of Tat–TAR in cell-based assay. As we reported before, at the same concentration, for substituted purine derivatives, the range of inhibited CAT expression was from 34.9 to 65.7 %. These results suggested that substituted [1,2,3]triazolo[4,5-*d*]pyrimidine derivatives had better activity in the CAT assay than those substituted purine derivatives. Among the six compounds, those with a side chain terminated with a tri-substituted amino group (**5a** and **5b**) exhibited the weakest effect on CAT expression (31.6 and 50.2 %). However, unlike our previous work, the compounds bearing guanidyl group-terminated side chain (**6a** and **6b**) had the same inhibitory activities (28.4 and 28.4 %) as those compounds bearing amino group-terminated side chain (**4a** and **4b**, 29.5 and 28.1 %, respectively). It was indicated that our [1,2,3]triazolo[4,5-*d*]pyrimidine derivatives might interact with TAR in a different way from purine derivatives. Furthermore, the length of the side chain had little effect on the activities of the title compounds, for there was no significant difference in activity between the compounds, respectively.

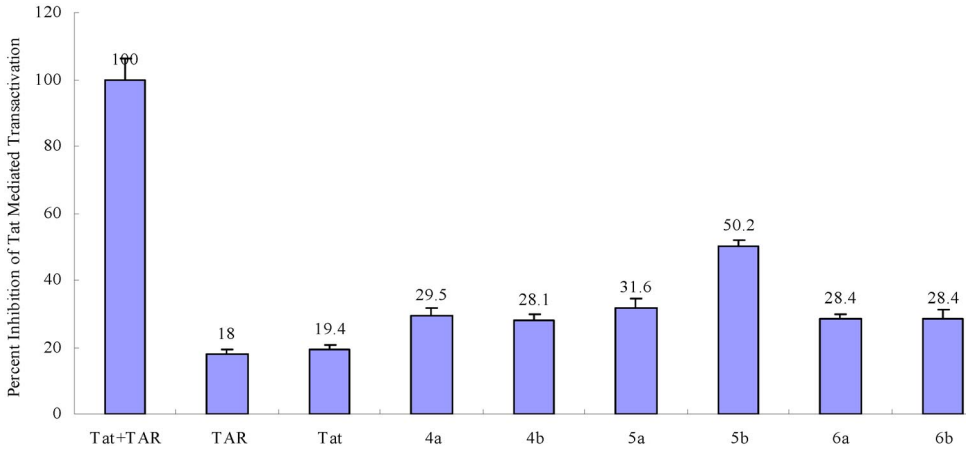


Fig. 1 Effects of the six title compounds on Tat-mediated transactivation in 293T cells.

Inhibition of viral infectivity

The biological activities of all the title compounds were evaluated by SIV-induced syncytium in CEM cells. Their EC_{50}, TC_{50}, and SI values are listed in Table 1. As shown in Table 1, each of them possessed an EC_{50} value within the range from 1.8 to 5.4 μM, and a TC_{50} value more than 100 μM. This demonstrated that all compounds possessed effective anti-SIV activity with low cytotoxicity. In addi-

tion, these results were also in agreement with those of the CAT assay study. In all the experiments reported here, compounds **4b** and **6b** exhibited the best activities.

Table 1 Inhibition effect and cytotoxicity of the title compounds on SIV-induced syncytium.

Compounds[a]	EC_{50}[b] (μM)	TC_{50}[c] (μM)	SI[d] (TC_{50}/EC_{50})
4a	2.8	>100	>35.7
4b	1.8	>100	>55.6
5a	4.3	>100	>23.3
5b	5.4	>100	>18.5
6a	2.8	>100	>35.7
6b	2.0	>100	>50

[a]AZT was used as the positive control at a concentration of 10 μM here. Its EC_{50} was 0.0122 μM and TC_{50} was above 100 μM in this system.
[b]EC_{50}: concentration required to protect cells against the cytopathogenicity of SIV by 50 %.
[c]TC_{50}: concentration required to inhibit uninfected cells proliferation by 50 %.
[d]SI: selective index.

Binding of compounds and TAR RNA in vitro

CE was used in the analysis of RNA–protein interactions, which provided a quick, sensitive, and precise method to study the binding of Tat–TAR RNA and drug–TAR RNA. In our previous work, we used CE to study the TAR RNA binding property with isoquinoline derivatives [9]. Herein, we also used CE to explore whether the six title compounds bound to TAR RNA and to determine their binding constants. The calculation process was provided in Experimental. The results are shown in Table 2.

Table 2 Binding constants of the title compounds with TAR RNA.

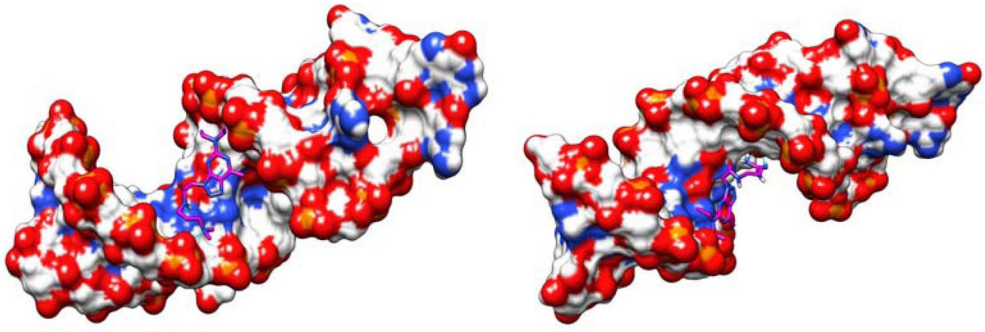
Compounds	Peak area		Linear equation	R^2	$K(\times 10^3)$
	in the absence of TAR RNA	in the presence of TAR RNA			
4a	22483.12	15849.67	C = 0.0001A + 0.1583	0.9689	2.18
4b	16454.55	11683.62	C = 0.0001A + 0.1206	0.9895	2.87
5a	19868.00	14424.26	C = 0.00009A + 0.0285	0.9956	2.78
5b	19450.47	15994.10	C = 0.0001A + 0.2034	0.9958	1.06
6a	12664.20	8151.60	C = 0.0002A – 0.1173	0.9762	3.94
6b	11567.05	7439.75	C = 0.0002A – 0.2288	0.9940	5.21

As shown in Table 2, the binding of compound with TAR RNA was observed with the decrease of the area of free compound's peak in the presence of TAR. The compounds bearing guanidyl group-terminated side chain had stronger affinities than those with terminal amino group, and the compounds with tri-substituted amino group had the weakest affinities. These results complied with those of the CAT assay. CE results also proved that our compounds inhibited Tat–TAR interaction by binding TAR RNA.

Molecular modeling

In order to verify the mechanism of the six title compounds' TAR RNA-binding properties, molecular modeling experiments were also performed by using Autodock 4.0. The free energy of **6b** was –7.95 kcal/mol, while the energy of **5b** was only –6.54 kcal/mol, which meant that **6b** had higher binding affinity than **5b** did. Hydrogen bond interactions were also observed. There were four hydrogen bonds between **6b** and TAR RNA, while only two hydrogen bonds between **5b** and the macromolecule were observed, indicating that hydrogen bond interaction may play an important role in enhancing the binding preferences of the compounds. It also confirmed our assumption that it was the hydrogen bond interaction being hindered by methyl that made **5b** have a higher EC_{50} value than **6b** did.

These two compounds might have different binding site with the macromolecule. As shown in Fig. 2, **6b** bound to the minor groove of TAR RNA with its aromatic ring and to the tri-base bulge with its terminal guanidyl group, while **5b** bound to the major groove of TAR RNA from a distance of the tri-base bulge. This might demonstrate the fact that the binding constant of **6b** was about five times higher than that of **5b**.



(a) **(b)**

Fig. 2 (a) Interaction of compound **5b** to TAR RNA; (b) Interaction of compound **6b** to TAR RNA.

CONCLUSION

In summary, we have designed and synthesized a novel series of [1,2,3]triazolo[4,5-*d*]pyrimidine derivatives bearing guanidyl group or amino group-terminated side chain. All six title compounds could block the Tat–TAR interaction and have the antiviral activities by binding with TAR RNA, which were further confirmed by molecular modeling and CE analysis. The results also showed that the compounds containing an amino or guanidyl group at the end of the side chain displayed higher binding affinity to TAR RNA. This study provides not only some proofs and ideas for the design of new Tat–TAR inhibitors but also the applicability of CE technology in TAR RNA targeting compounds.

EXPERIMENTAL

^{1}H NMR spectra were recorded at 300 MHz, and are referenced to the residual protonated solvent peaks; δ_H 7.24 ppm for solution in $CDCl_3$. Mass analysis was performed by positive-mode electrospray ionization on a hybrid sector-time-of-flight (TOF) mass spectrometer. Analytical thin-layer chromatography (TLC) was performed on silica gel 60-F254 (Merck). All commercial reagents were used as supplied. $AIMSO_3H{\cdot}H_2O$ was prepared as reported, colorless crystal, m.p. 132~134 °C [16].

***N,N*-Diethyl-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-amine (2)**

The solution of 0.45 g $NaNO_2$ (0.0065 mol) in 2 ml water was added dropwise into the solution of 2-*N*,*N*-dimethylamino-6-methyl-pyrimidine-4, 5-diamine **1** 1.1 g (0.0056 mol) in 3 ml 36 % acetic acid and at –5 °C. **2** Precipitated out from the solution as a pale-yellow solid 20 min later. The reaction continued for more 4 h at 35 °C in water bath. When the mixture was poured into 100 ml cold water, much more **2** precipitated out. The solid was filtered out, and the filtrate was extracted with 3 × 20 ml $CHCl_3$ and the organic layer was dried with anhydrous Na_2SO_4 over night. After the solvent was removed under reduced pressure, the residue and the filter cake was combined and purified by column chromatography (silica gel, petroleum ether:ethyl acetate = 4:1, V/V = 4/1), 1.15 g, m.p. 169~171 °C, yield = 99.0 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 (t, 6 H), 1.63 (s, 1 H), 2.84 (s, 3 H), 3.81 (q, 4 H). MS (EI^+) *m*/*z* calcd: 206.13, found: 206 (M^+).

Ethyl 2-{5-(diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}acetate (**3a**) and ethyl 2-{5-(diethylamino)-7-methyl-2*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-2-yl}acetate (**3b**)

N-(2-aminoethyl)-2-{5-(diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}acetamide (**4a**)

N-(3-aminopropyl)-2-{5-(diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}actamide (**4b**)

2-{5-(Diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}-*N*-{2-(diethylamino) ethyl}acetamide (**5a**)

2-{5-(Diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}-*N*-{3-(dimethylamino) propyl}acetamide (**5b**)

2-{5-(Diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}-*N*-(2-guanidinoethyl) acetamide (**6a**)

2-{5-(Diethylamino)-7-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-3-yl}-*N*-(3-guanidinopropyl) acetamide (**6b**)

Biological evaluation

Transient transfection and CAT assays: 293T cells were grown as monolayer in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10 % (v/v) fetal calf serum, penicillin (100 U ml^{-1}), and streptomycin (100 U ml^{-1}) at 37 °C in 5 % CO_2 containing humidified air. The cells were seeded at a six-well plate 24 h prior to transfection which was performed by standard calcium phosphate coprecipitation techniques with optimum amounts of the plasmids pLTRCAT and pSVCMVTAT. After 24 h, the culture medium was removed and the cells were washed twice with phosphate-buffered saline (PBS). Then the transfected cells were added to fresh medium together with diluted compounds at final concentration of 30 μM, respectively, and incubated for another 24 h. After 48-h post-transfection, the cells were harvested and analyzed for CAT activity using a commercial CAT enzyme-linked immunoabsorbent assay (ELISA) kit (Roche Molecular Biochemicals) in accordance with the manufacturer's protocol. All data were reported as a percentage of CAT activity. Results shown were representative of three independent experiments.

CE assay

CE experiments were carried out on a Beckman P/ACE 2100 CE system using a 50 cm × 50 μM ID bare fused-silica capillary (Beckman). Phosphate buffer (50 mM, pH 8.0) was used as running buffer. Electrophoresis was started at 15 kV and 20 ± 0.1 °C. Samples were injected at 10 kV for 10 s and detected at 260 nm. Prior to use, the capillary was pretreated successively with 0.1 M NaOH for 60 min, water for 30 min, and finally with running buffer until the baseline became smooth. Between the runs, the capillary was washed sequentially with 0.1 M NaOH, water, and running buffer for 4 min each. The

solutions were filtered through a 0.22 μM PTFE membrane prior to use. 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 TAR-compound was incubated for 30 min at 4 °C (binding buffer: 10 mM Tris-HCl, 70 mM NaCl, 0.2 mM EDTA, and 5 % glycerol, pH 7.4) before CE analysis.

We assumed for simplicity that TAR RNA had a single site for the title compounds and developed a simplified reaction model for the TAR-compound system:

$$T + C = TC \quad K = [CT]/[T][C] = \gamma/[C] \tag{1}$$

where [*TC*], [*T*], and [*C*] are at concentration of TAR-compound complex, nonbonding TAR, and nonbonding compound in the mixture.

By the law of mass action, the molar concentration of all TAR-compound complexes ([*TC*]) is equal to the concentration of bound compound, and the net concentration of bound compound is equal to the total compound concentration (C_C) less the concentration of free compound ([*C*]) remaining at equilibrium. The TAR binding ratio γ is calculated as eq. 2.

$$\gamma = [TC]/[T] = (C_C - [C])/[T] \tag{2}$$

The concentration of free TAR RNA ([*T*]) in the equilibrium mixture was set equal to the total concentration of TAR (C_T) added to the mixture minus the amount of bound RNA in the complex (equal to [*TC*]). To estimate the binding constant of TAR-compound complex using our CE method, we developed eq. 3.

$$\gamma = (C_C - [C])/(C_T - C_C + [C]) \tag{3}$$

In our method, linear equations by which concentration of the compounds in the mixture could be calculated were obtained. Thus, the ratio γ and the binding constant K were calculated and summarized in Table 2 [9].

Inhibition of SIV-induced syncytium in CEM174

Cell cultures were measured in a 96-well microplate containing 2×10^5 CEM cells/ml infected with 100 $TCID_{50}$ of SIV per well and containing appropriate dilutions of the tested compounds. After 5 days of incubation at 37 °C in 5 % CO_2 containing humidified air, CEM giant (syncytium) cell formation was examined microscopically. The EC_{50} was defined as the compound concentration required to protect cells against the cytopathogenicity of SIV by 50 %. Azidothymidine (AZT) was used as the positive control at a concentration of 10 μM here.

Molecular modeling

The initial structures of our compounds were subjected to minimization using MOPAC in Chemoffice 2006 and the 3D structure of HIV-1 TAR RNA in complex with its inhibitor rbt 158 was recovered from the Protein Database (<http://www.PDB.org>) with the code as 1UUI [17]. The advanced docking program Auto-dock 4.0 was used to remove the small molecule and perform the automatic molecular docking with our compounds. The number of enerations, energy evaluation, and docking runs were set to 370 000, 1 500 000, and 30, respectively, and the kinds of atomic charges were taken as Kollman-all-atom for HIV-1 TAR RNA and Gasteiger-Hücel for the compounds.

ACKNOWLEDGMENTS

This project was supported by the National Natural Science Foundation of China (Grant No. 30670415).

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