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Design and synthesis of a novel peptidomimetic inhibitor of HIV-1 Tat–TAR interactions: Squaryldiamide as a new potential bioisostere of unsubstituted guanidine

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Abstract—By performing RNA-targeted structure–activity relationship studies, we discovered a novel peptidomimetic containing squaryldiamide as a potential bioisostere replacement for guanidine that binds transactivation responsive RNA with high affinity.

In spite of remarkable medical advances, HIV-1 infections continue to increase. Although the use of multi-drug cocktails has significantly reduced age-adjusted death rates from AIDS in developed countries, AIDS therapies still face many constraints, including inadequate therapeutic responses and intolerable drug toxicity. Another serious drawback of these therapies is that all of the current drugs can lead to the evolution of single- or multi-drug-resistant mutant viruses. In view of these realities, there is a compelling need to find new drugs and/or treatment strategies that are not limited to targeting HIV reverse transcriptase and protease. The mechanism of Tat transactivation during HIV-1 gene expression requires the interaction of transactivator of transcription (Tat) protein with the transactivation responsive (TAR) RNA, a 59-base stem-loop structure located at the 5′-end of all nascent HIV-1 transcripts.[1] Tat–TAR interactions are required to convert arrested RNA polymerase II (pol II) complexes into a processive form for an efficient production of full-length viral transcripts.[2] In the absence of Tat, pol II terminates transcription prematurely. Because binding of HIV-1 Tat to TAR RNA is essential for viral replication, developing inhibitors of this interaction is a promising starting point for the design of anti-HIV drugs. Small molecules that inhibit HIV replication have been reported, including a series of aminoglycosides and small compounds that bind TAR RNA with high affinities.[3–5] As a part of our drug discovery program for anti-HIV agents,[6–9] we have recently reported on the synthesis of a combinatorial library that was effectively screened for inhibitors of the Tat–TAR interaction. Several of these compounds showed significant TAR-RNA-binding affinities.[10] In single-round replication assay, inhibitor TR87 (**1**) showed potent, sustained anti-HIV-1 activity that did not significantly affect cell viability. To build on these results, we performed structure–activity relationship (SAR) studies with TR87 to further enhance its anti-HIV-1 activity for in vivo applications. Due to the highly basic nature of the guanidine group, guanidine-containing compounds usually display poor pharmacokinetic properties. Here, we report design and synthesis of a novel peptidomimetic containing squaryldiamide moiety as a new potential bioisostere group for guanidine replacement that binds TAR RNA with high affinity. For synthesis of peptidomimetics, carbonate monomers **10a–e** were prepared from commercially available, fully protected amino acids as shown in Scheme 1. *N*-Fmoc amino acids **8a–e** were reacted with isobutyl chloroformate in the presence of *N*-methyl morpholine to produce a series of mixed anhydrides, which were efficiently reduced with 3 equiv of aqueous $NaBH_4$, leading to high yields (>90% over two steps) of the corresponding alcohols **9a–e**.[11] By treating the resulting amino alcohols **9a–d** with *p*-nitrophenyl chloroformate, the desired carbonates **10a–d** were efficiently prepared.[12] For **10e**, Boc group on **9e** was removed and the corresponding amino alcohol was reacted with protected squarylmonoamide **12**

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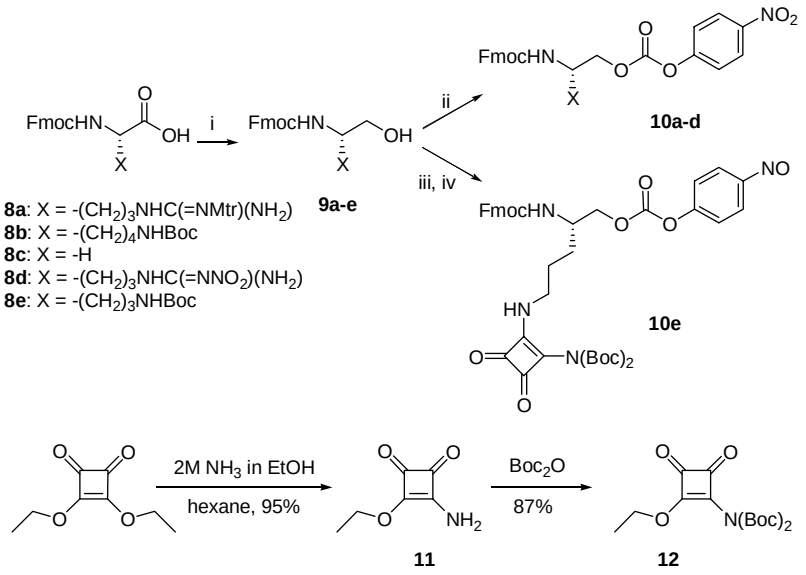

Scheme 1. Synthesis of carbonate monomers **10a**–**e**. Reagents and conditions: (i) (a) *N*-methyl morpholine, *i*-BuOCOCl, CH_2Cl_2, −15 °C, 1 min; (b) $NaBH_4$ (3 equiv), water, −15 °C, 1 min, >90% over two steps; (ii) pyridine, CH_2Cl_2, *p*-NO_2—PhOCOCl, −15 °C, >85%; (iii) (a) 4 M HCl solution in dioxane, rt; (b) **12**, EtOH, Et_3N, rt; (iv) pyridine, CH_2Cl_2, *p*-NO_2—PhOCOCl, −15 °C, 71% over three steps. Fmoc = 9-fluorenylmethoxy carbonyl; Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Boc = *tert*-butoxycarbonyl.

prepared from 3,4-diethoxy-3-cyclobutene-1,2-dione and ammonia in hexane–ethanol (Scheme 1). In the literature,[13] gaseous ammonia was bubbled to the reaction mixture to give compound **11** in 75% yield. In our experiments, gaseous ammonia was replaced with 2 M ammonia solution in ethanol to produce pure squaryl-

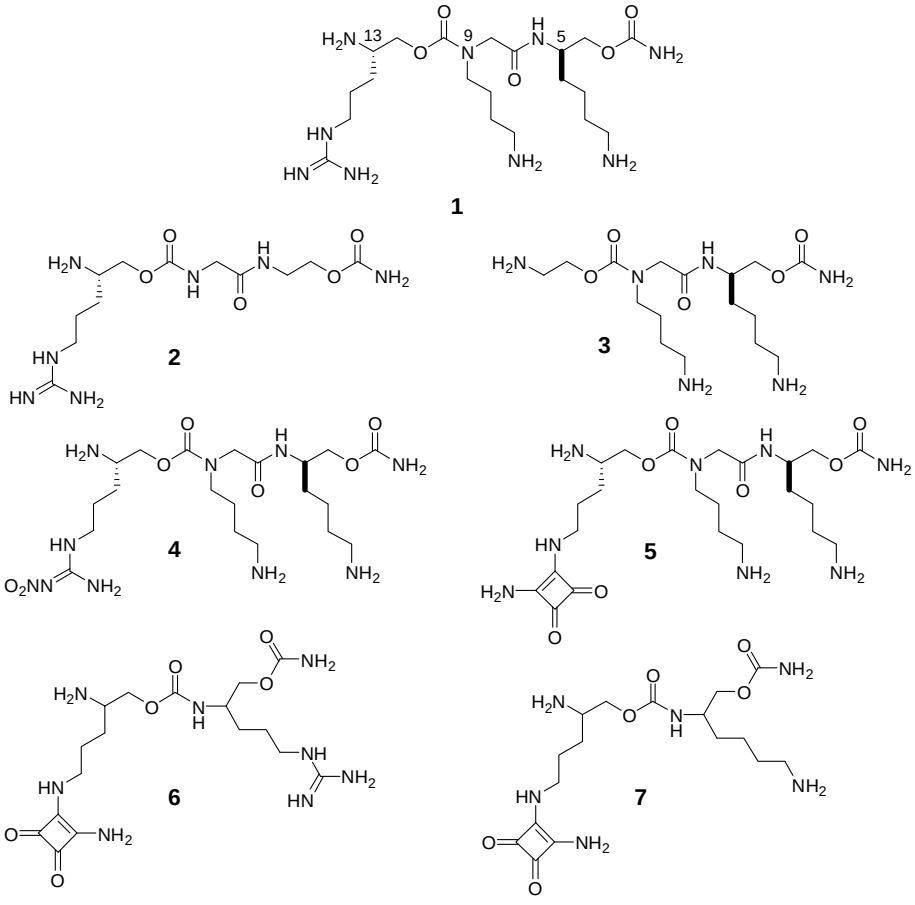

Figure 1. Structures of peptidomimetics **1**–**7**.

monoamide **11** in 95% yield through simple filtration and without further purification.

Subsequent activation of the alcohol with *p*-nitrophenyl chloroformate resulted in carbonate monomer **10e**. Small peptidomimetics **2**–**7** were manually synthesized (Fig. 1) on Rink Amide MBHA resin using the carbonate monomers **10a**–**e** according to standard solid-phase synthesis methods described previously.[6–9]

After synthesis and purification, TAR-binding affinities of these compounds were determined by competition experiments.[10] Interestingly, retaining guanidino propyl at C-13 and deletion of two alkyl amino groups at N-9 and C-5 positions led to a significant change in RNA binding, and a multiple ligand-binding pattern was observed (Table 1), compound **2**.

Substitution of guanidino propyl at C-13 position with hydrogen, led to a complete loss in Tat–TAR RNA inhibitor activity (Table 1) compound **3**. Since the two alkyl amino groups at N-9 and C-5 positions could not be removed and the guanidine group was essential for disrupting Tat–TAR interactions, we planned to synthesize novel bioisosteres that could mimic guanidine functionality and analyzed their binding affinities for TAR RNA. Attaching electron-withdrawing groups such as cyano, nitro, acyl, or sulfonyl to the nitrogen atom to attenuate the basicity of guanidine is one classical technique used in medicinal chemistry for bioisosteric replacement.[14] However, as shown in Table 1, nitro-containing compound **4** exhibited significantly lower binding affinity for TAR RNA (~528-fold less) than compound **1**. We then analyzed 1,2-diaminocyclobutene-3,4-dione (squaryldiamide), which has been reported as a bioisostere of *N*-cyanoguanidine in pinacidil,[15] but has not been used for unsubstituted guanidine. The squaryldiamide derivative **5** was synthesized and tested as a Tat–TAR inhibitor. Compound **5** was able to bind TAR RNA with high affinity (K_D = 7.7 μM). Squaric acid amide has been used as a carboxylic acid and phosphate equivalent;[16] however, there are no examples of squaryldiamide in the literature that successfully demonstrate bioisosteric replacement of unsubstituted guanidine functionality. In addition, we synthesized the two dimeric carbamates **6** and **7**, containing the squaryldiamide moiety. These compounds show poor TAR-binding capabilities (Table 1; 741 μM for **6**, no binding for **7**), indicating that a wide range of backbone structures is not tolerated in targeting TAR structure. As shown in Figure 2, quenching of TAR-linked fluorescein (F-TAR) through fluorescence resonance energy transfer (FRET) occurs upon the formation of rhodamine-labeled Tat-(47–58) peptide (Rh-Tat) and F-TAR complexes.

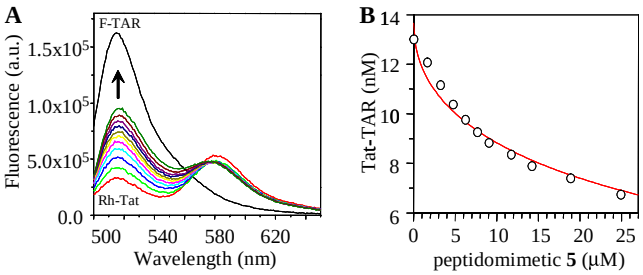

Figure 2. Competitive binding of peptidomimetic **5** with a preformed complex between F-TAR and Rh-Tat-(47–58) was monitored by FRET. (A) Fluorescence emission spectra. (B) Binding isotherm monitored by increase in emission measured at 515 nm.

Upon titration of increasing amounts of peptidomimetic **5**, this quenching is relieved, which corresponds to decreased Rh-Tat–F-TAR binding. These results demonstrate that **5** directly competes with Rh-Tat for binding to F-TAR. Notably, when 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay[17] using MT-4 cells was carried out, we found compound **5** to be a very potent inhibitor of HIV-1 replication in MT-4 cells. Its 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) were 12.4 and 1600 μM, respectively. In summary, 1,2-diaminocyclobutene-3,4-dione (squaryldiamide) has been identified as a new potential bioisostere for unsubstituted guanidine functionality in peptidomimetics. This group was easily incorporated into a carbonate monomer, which could be coupled to produce peptidomimetics for Tat–TAR antagonists.

Table 1. Tat–TAR-binding affinity (K_D) of compounds **1**–**7**[a]

Compound	K_D
1	1.8
2	mb
3	nb
4	952
5	7.7
6	741
7	nb

Binding affinities were measured at room temperature using a fluorescence spectrophotometer. To examine the interaction between TAR RNA and the peptidomimetics in the presence of Tat peptide, we used an in vitro competition assay based on fluorescence resonance energy transfer (FRET).[10] Compounds **1**–**7** were titrated incrementally to the mixture of TAR RNA labeled with donor fluorescein at its 5′-end (F-TAR) and Tat (amino acids 47–58) peptide labeled with acceptor rhodamine (Rh-Tat).

[a] The values are in micromolar. mb, multiple binding; nb, no binding.

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