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Synthesis, biophysical characterization, and anti-HIV-1 fusion activity of DNA helix-based inhibitors with a *p*-benzyloxyphenyl substituent at the 5′-nucleobase site

Yongjia Tang [a,c], Zeye Han [a,c], Jiamei Guo [b], Yangli Tian [a], Keliang Liu [a,*], Liang Xu [a,*]

[a] State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology and Toxicology, 27 Taiping Road, Beijing 100850, China
[b] Beijing Key Laboratory of New Drug Mechanisms and Pharmacological Evaluation Study, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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ABSTRACT

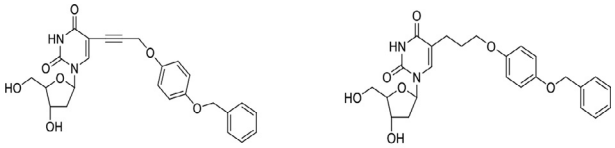
DNA helix-based HIV-1 fusion inhibitors have been discovered as potent drug candidates. Introduction of hydrophobic groups to a nucleobase provides an opportunity to design inhibitors with novel structures and mechanisms of action. In this work, two novel nucleoside analogues (**1** and **2**) were synthesized and incorporated into four DNA duplex- and quadruplex-based inhibitors. All the molecules showed anti-HIV-1 fusion activity. The effect of the *p*-benzyloxyphenyl group and the attached linker on the helix formation and thermal stability were fully compared and discussed. Surface plasmon resonance analysis further indicated that inhibitors with the same DNA helix may still have variable reaction targets, mainly attributed to the different hydrophobic modifications.

Due to the quick drug-resistant mutations of HIV-1 strains, developing inhibitors with novel structures or mechanisms of action is of great interest in the field of anti-HIV-1 drug research. Several DNA G-quadruplexes with various hydrophobic groups modified at the 5′- or 3′-hydroxyl ends have been discovered as potential HIV-1 fusion inhibitors. Both the G-quadruplex skeleton and hydrophobic groups have proven important for the inhibitory activity.[1–9] Recently, our group demonstrated that DNA duplexes and triplexes with the hydrophobic tert-butyldiphenylsilyl (TBDPS) substituent had anti-HIV-1 fusion activity.[10–12] As maintaining the balance between the hydrophobic group and the charged DNA helix is important for the activity, the TBDPS group has been introduced at the nucleobase site via *de novo* synthesis of nucleoside analogues. This modification strategy provides another alternative for introducing hydrophobic groups into the molecular structure, for duplex, triplex, and quadruplex inhibitors.

However, the structure design, especially for the duplex- and triplex-based inhibitors, is still very limited. Only the TBDPS group has been reported to be utilized by the nucleobase modification strategy for these two types of DNA helix inhibitors, and the described O-Si bond connecting the TBDPS group to the rest of the nucleoside is easily hydrolyzed in the course of deprotection of the oligonucleotide in aqueous ammonia at 55 °C.[10,13,14]

Here, two new nucleoside analogues (Fig. 1, **1** and **2**) incorporating the hydrophobic moiety *p*-benzyloxyphenyl (PBP) at the nucleobase site were synthesized, aiming to facilitate the design of novel anti-HIV-1 inhibitors with high efficiency or new mechanism of action.

The reason for choosing PBP was that it had been introduced at the 5′-hydroxyl site in a classical "TGGGAG" quadruplex inhibitor (**G3**, d(PBP-TGGGAG)$_4$, Fig. S1), and this molecule showed higher activity than any other quadruplex inhibitors ever reported.[2,15] The details for the syntheses of **1**, **2**, and their corresponding phosphoramidites are shown in Scheme 1 and the supplementary data.



1 **2**

Fig. 1. Structures of nucleoside analogues **1** and **2**.

* Corresponding authors.
E-mail addresses: keliangliu55@126.com (K. Liu), xuliang24998@yahoo.com (L. Xu).
[c] The two authors contribute equally to this work.

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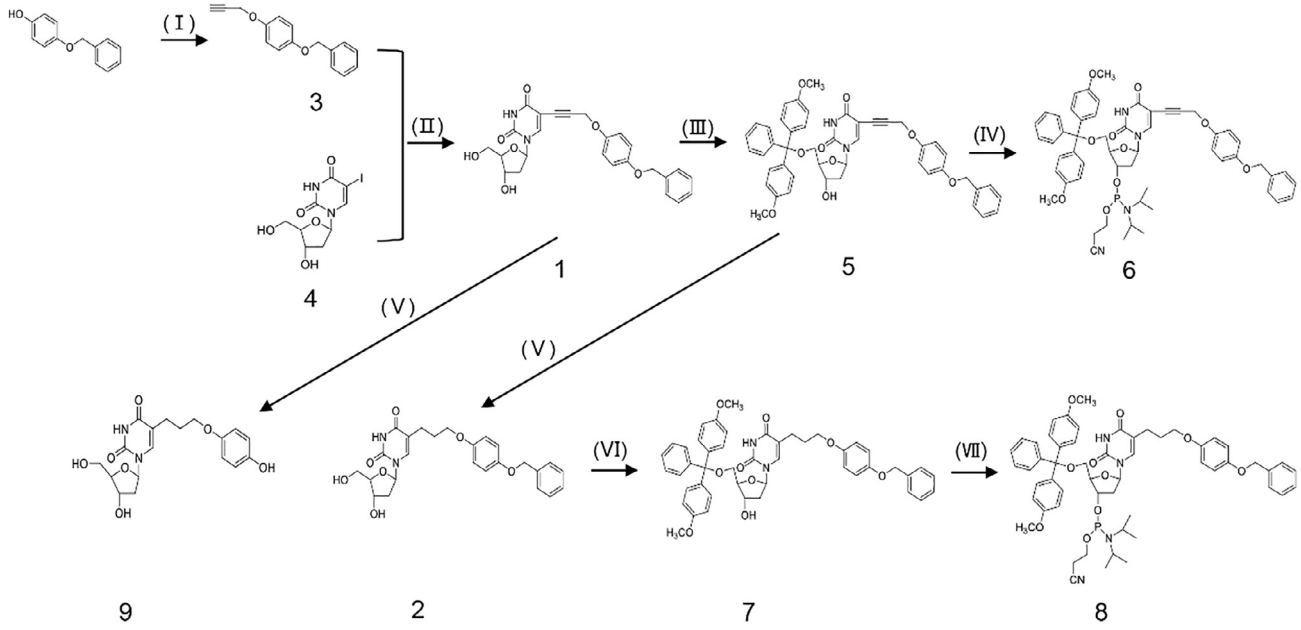

Scheme 1. The syntheses of **1**, **2**, and their corresponding phosphoramidites. (I) 3-Chloropropyne, DMF, rt; (II) CuI, $Pd(PPh_3)_4$, Et_3N, DMF, rt; (III) DMT-Cl, pyridine, rt; (IV) $[(iPr)_2N]_2P(OCH_2CH_2CN)$, $(iPr)_2EtN$, CH_2Cl_2, rt; (V) Pd/C, H_2, CH_3OH, 30 °C, 5 kgf/cm^2; (VI) DMT-Cl, pyridine, rt; (VII) $[(iPr)_2N]_2P(OCH_2CH_2CN)$, $(iPr)_2EtN$, CH_2Cl_2, rt.

It should be pointed out that, we had intended to get compound **2** from **1** directly by hydrogenation, however, compound **1** was found hard to dissolve in methyl alcohol and a primary byproduct (**9**, Scheme 1) was generated in the hydrogenation course. As an alternative method, compound **1** was reacted with DMT-Cl first and then taken hydrogenation to give compound **2**, the synthetic route was found in good purity and yield.

Next, four oligonucleotides (**d1**, **d2**, **g1**, and **g2**, Table 1) containing **1** or **2**, separately, and two natural oligonucleotides (**d0** and **g0**) were synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA), according to the standard protocol. Deprotection of the sequences was conducted with aqueous ammonia at 55 °C for 15 h. The oligonucleotides were purified by reverse-phase HPLC (A: 0.1 M acetic acid/triethylamine containing 5% acetonitrile; B: acetonitrile; B in A from 10% to 80% in 20 min, at 1 mL/min), and were characterized by MALDI-TOF MS (Table 1).

Three duplex structures (**D0**, **D1** and **D2**) and three tetramolecular G-quadruplex structures (**G0**, **G1** and **G2**) were obtained by annealing the corresponding oligonucleotide sequences (**d0**, **d1**, **d2**, **g0**, **g1**, or **g2**, separately) in phosphate-buffered saline buffer containing 20 mM $MgCl_2$ (for **D0**, **D1** and **D2**) or 100 mM KCl (for **G0**, **G1** and **G2**). The buffers were heated to 90 °C, held for 5 min, and cooled to 10 °C at a rate of 1 °C/min. The samples were maintained at 10 °C overnight before tests.

The melting temperatures (Tm) of **D0**, **D1**, **D2**, **G0**, **G1**, and **G2** were measured on CD spectropolarimeter (MOS-450; Bio-Logic Inc., France) with temperature rising from 15 °C to 90 °C, at step of 1 °C/min, using 4.0-nm bandwidth, 0.1-nm resolution, 0.1-cm path length, 4.0-s response time, and 50-nm/min scanning speed. Oligonucleotides were at a concentration of 8 μM (**g0**, **g1**, and **g2**) or 4 μM (**d0**, **d1** and **d2**). The wavelength was at 280 nm for **D0**, **D1** and **D2**, and 260 nm for **G0**, **G1**, and **G2**. It was found that the melting curves of G-quadruplex were not superimposable with the annealing curve, which might mainly attribute to the slow kinetics of G-quadruplex folding.[15,18] As a result, the Tm of quadruplex and duplex were both evaluated by the melting curves, and the tests were carried out after annealing and maintaining at 10 °C overnight. As shown in Table 2, all helixes showed Tm values greater than 37 °C, indicating stable helix structures at room temperature. **D1** and **D2** showed comparable Tm values to natural **D0** without significant difference ($p > 0.05$), however, increased thermal stability was observed for **G1** and **G2** when compared to unmodified **G0** ($p < 0.01$). This result indicated the positive effect of hydrophobic modification on thermal stability of quadruplex, while not on that of duplex, and the difference might mainly attribute to the discrepant helix assembly pattern. Former researches reported that hydrophobic modification at the 5′-hydroxyl end of TGGGAG quadruplex would greatly increase the thermal stability;[1,2,4,7,8] relatively, our results indicated that modification at the 5′-end of TGGGAG from nucleobase site also showed considerable effect. Moreover, **G2** was with a much higher promotion extent than that of **G1** ($p < 0.01$). The more hydrophobicity and flexibility of the propyl linker (in **2**) than the propenyl linker (in **1**), as well as

Table 1
MALDI-TOF characterization of the oligonucleotides.

No.	Sequence (5′-3′)	MS (calc)	MS (found)
d0	**T**GGTCGACC**T**	3019.0	3019.2
d1	**1**GGTCGACC**1**	3463.6	3463.0
d2	**2**GGTCGACC**2**	3471.6	3472.5
g0	**T**GGGAG	1872.3	1872.0
g1	**1**GGGAG	2095.0	2094.2
g2	**2**GGGAG	2099.0	2098.6

Table 2
The melting temperatures and *in vitro* anti-cell fusion activities of **D0** to **G2**.

No.	Sequence	Melting Temperature (°C)[c]	IC_{50} (μM)
D0[a]	d(**T**GGTCGACC**T**)$_2$	54.4 ± 1.6	>20
D1	d(**1**GGTCGACC**1**)$_2$	51.8 ± 1.7	6.68 ± 0.69
D2	d(**2**GGTCGACC**2**)$_2$	55.6 ± 0.8	2.19 ± 0.56
G0[b]	d(**T**GGGAG)$_4$	54.7 ± 0.5	>20
G1	d(**1**GGGAG)$_4$	59.5 ± 1.2	1.97 ± 0.23
G2	d(**2**GGGAG)$_4$	70.6 ± 2.6	0.78 ± 0.04

[a] D represents duplex.
[b] G represents G-quadruplex.
[c] Three parallel experiments were performed to derive the means ± standard deviations (SD).

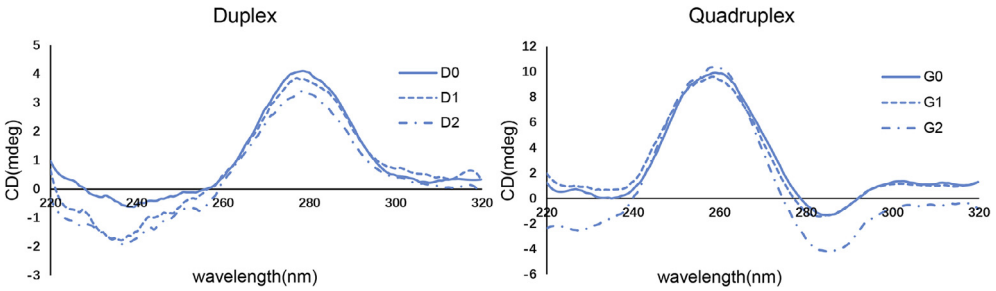

Fig. 2. CD spectrum results of duplex (**D0**, **D1**, and **D2**, left) and quadruplex (**G0**, **G1**, and **G2**, right).

the resulting more spatial freedom of the connecting hydrophobic group brought by propyl linker (correspondingly, the geometry of the propenyl linker extruded the PBP outward the helix more linearly), were supposed to be responsible for the better promotion effect.

The CD spectrum of each helix (2 μM) was obtained at 25 °C on same spectropolarimeter (MOS-450). The results are shown in Fig. 2. **D1** and **D2** showed positive peaks at approximately 280 nm and negative absorption peaks at approximately 240 nm, which were in accordance with typical CD spectra of unmodified duplex **D0**. **G1** and **G2** showed positive peaks at approximately 260 nm and negative peaks at approximately 235 nm, which were also similar to unmodified parallel quadruplex **G0**. This result indicated that the introduction of hydrophobic PBP either by propyl or propenyl linker at nucleobase site was with little influence to duplex and quadruplex structural motif, in other words, the regular helix structures were not greatly disturbed.

In addition, the slow mobility of helixes than single strand control T6 and T8 in native polyacrylamide gel electrophoresis (N-PAGE) further confirmed the formation of the duplex or quadruplex structure (Fig. S2).

As reported previously, an HIV-1 envelope protein mediated cell-cell (HL2/3 and TZM-bl cells) fusion assay was used to determine the anti-fusion activities. HL2/3 were effector cells expressed HIV-1 Gag, Env, Tat, Rev, and Nef proteins at the cell surface; HL2/3 were obtained through the AIDS Reference and Reagent Program, NIH, from Barbara K. Felber and George N. Pavlakis. TZM-bl were target cells expressing CD4 and coreceptors; TZM-bl cells were obtained through the AIDS Reference and Reagent Program, NIH, from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc. The cell-cell fusion was quantitated by a luciferase assay 8 h after cells mixing and inhibitor adding.[16,17] The results are shown in Table 2. **D1**, **D2**, **G1**, and **G2** all showed anti-fusion activity. **D2** demonstrated slightly greater activity than **D1**, and **G2** also had greater activity than **G1** ($p < 0.05$), indicating that nucleoside analogue **2** might contribute more to the activity than **1**. Considering the thermal stability of **D2** being higher than **D1** ($p < 0.05$), and **G2** being higher than **G1** ($p < 0.01$), although it was not clear whether the thermal stability was related to the anti-fusion activity, the discrepancy both in activity and thermal stability might be explained by the different spatial freedoms of the hydrophobic group in **1** and **2**.

As mentioned above, a former most potent quadruplex inhibitor, d(PBP-TGGGAG)$_4$ (named as **G3**, synthesized according to Fig. S1 and Ref. 2), was tested for anti-fusion activity as a control. In this work, **D2** and **G2** showed equivalent or slightly greater activity than **G3** (1.84 ± 0.12 μM) under the same test condition, indicating their potential use as novel lead compounds.

In addition, the natural control, **D0** and **G0**, showed no obvious activities in our test condition, indicating the importance of hydrophobic groups. However, very recently, Romanucci reported the surprising anti-HIV activity of the natural G-quadruplex d(TGGGAG)$_4$.[19] As the kinetic for natural G-quadruplex folding was very slow, the distinct results might mainly contribute to their 14 days of incubation before anti-HIV assays. Besides, the paper also supposed different target and action mechanism of d(TGGGAG)$_4$ from traditional 5′-end conjugated ODNs.

Unlike the quadruplex inhibitors first discovered about 30 years ago; limited information is known about the mechanisms of action of the newly established DNA duplex inhibitors. Therefore, surface plasmon resonance (SPR) analysis was carried out in this work, for the first time, to explore the interaction between the duplex inhibitors and the HIV-1 surface glycoproteins gp120 and gp41 directly. The test was performed on a Biacore T100 instrument (GE Healthcare, Uppsala, Sweden) at 25 °C in HBS-P buffer (10 mM HEPES, 150 mM NaCl, 10 mM $CaCl_2$, and 0.05% surfactant P20; pH 7.4). Recombinant HIV-1(IIIB) gp120 (Immuno Diagnostics Inc., Woburn, MA) or HIV-1(IIIB) gp41 (ProSpec-Tany TechnoGene Ltd., Israel) was covalently immobilised on a CM5 sensor chip using standard amine coupling chemistry, separately. Samples were injected for 1 min at a flow rate of 30 μL/min and followed by a dissociation phase of 2 min. The CM5 sensor chip surface was regenerated with injection of 2.5 M NaCl at a flow rate of 30 μL/min for 1 min.

The results of the duplex-based inhibitors (**D1** and **D2**) interacting with gp120 and gp41 are shown in Fig. S3 and Table 3. **D2** and **D1** both showed an interaction with gp41; however, **D2** showed a much higher absorption and K_D value than **D1**. Besides, **D2** showed a K_D value when interacting with gp120, while **D1** did not. As **D1** and **D2** have the same sequence composition and the same hydrophobic group, PBP, the conformational difference between **D1** and **D2** at the PBP connection site might be the key factor to explain these results. To the best of our knowledge, these findings are the first to indicate that inhibitors possessing the same duplex structural motif may bind to their reaction targets differently, based on the structure and spatial conformation of the hydrophobic substituent. In addition, **D0**, which has the same sequence composition as **D1** and **D2** but does not contain a hydrophobic group, showed no interaction with gp120 or gp41. This result further indicated the importance of the hydrophobic group for the interaction between the inhibitor and receptor.

Similar results were observed for **G1** and **G2**. For gp41, **G2** demonstrated a higher absorption and K_D value than those of **G1**. For gp120, **G2** also showed an interaction, while **G1** did not. It should be mentioned that the **G2** and **D2** containing the nucleoside

Table 3
The K_D[a] (mol/L) values of different molecules.

No.	gp120	gp41
D1	Very low or not detected	2.80×10^{-5}
D2	5.25×10^{-6}	5.37×10^{-6}
G1	Very low or not detected	1.27×10^{-6}
G2	6.61×10^{-6}	7.67×10^{-7}

[a] K_D represents the dissociation constant.

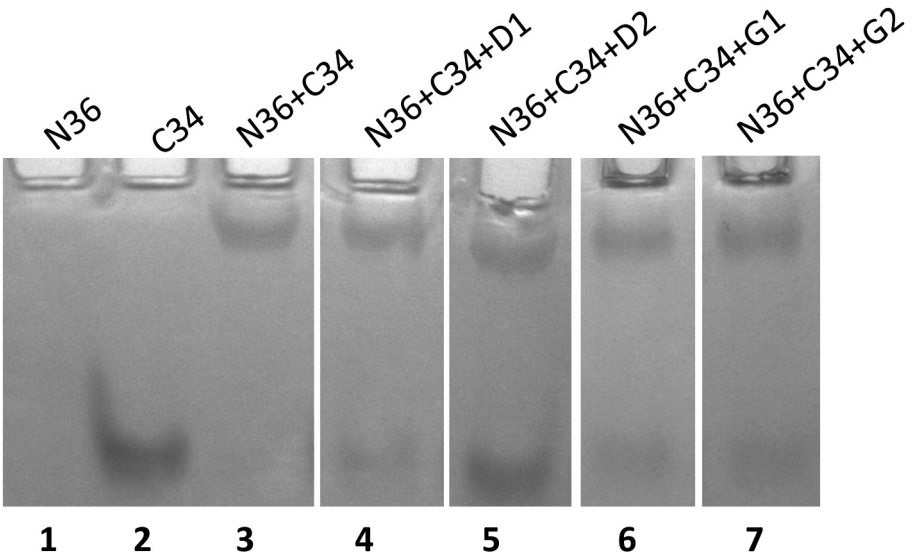

Fig. 3. Inhibition of formation of the HIV-1 gp41 6HB. For lanes 1–7, N36, C34, **D1**, **D2**, **G1**, and **G2** at a concentration of 50 mM were used.

analogues **2**, had higher activities than **G1** and **D1** containing the nucleoside analogues **1**. These results further indicated that the anti-fusion activity could be attributed to different interactions between the helix inhibitor and the receptors.

The formation of a 6-helical bundle (6HB) region between the N-terminal heptad repeat and the C-terminal heptad repeat of the HIV-1 surface glycoprotein gp41 plays a critical role in virus–cell fusion. Many HIV-1 fusion inhibitors have been reported inhibiting the 6HB formation. To evaluate the effects of **D1**, **D2**, **G1**, and **G2** on 6HB formation, N-PAGE was carried out according to a previously reported method.[10] The results are shown in Fig. 3. N36 alone (lane 1) exhibited no band. This peptide carries net positive charge and may migrate off the gel. In contrast, C34 alone (lane 2) displayed a band at a lower position on the gel. The band for the mixture of N36 and C34 (lane 3) was located in the upper portion of the gel, corresponding to that of the 6HB. Lines 4–7 show the results for **D1**, **D2**, **G1**, and **G2** incubated with N36 and C34, all at equimolar concentrations. For all mixtures, unbound C34 was observed at the lower position on the gel, indicating that **D1**, **D2**, **G1**, and **G2** all inhibited the formation of the 6HB between N36 and C34.

In conclusion, two novel nucleoside analogues containing the hydrophobic group PBP at the nucleobase site were synthesized and utilized for constructing four new DNA duplex- and quadruplex-based HIV-1 fusion inhibitors. All the inhibitors showed anti-HIV-1 fusion activity, and their target receptor varied, depending on the hydrophobic group substitution. This work not only provided more opportunity to design anti-HIV-1 DNA helixes with new structures and mechanisms, but it also added important information regarding their structure-activity relationships.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.04.012.

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