Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 627–632

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Nucleomimetic Strategy for the Inhibition of HIV-1 Nucleocapsid Protein NCp7 Activities

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Received 8 October 1998; accepted 21 January 1999

Abstract : We report the synthesis and biological properties of three modified dinucleotides **T*G**, **G*T** and **T*T** in which the natural phosphodiester linkage has been replaced by a methylene carboxamide unit. They have been designed to act as nucleomimetics of a sequence recognized by the HIV-1 nucleocapsid protein NCp7 and to inhibit this interaction.

Keywords : Nucleomimetics / NCp7 / HIV-1 / Inhibitors

The HIV-1 nucleocapsid protein NCp7, a highly basic protein characterized by the presence of two zinc fingers of $CX_2CX_4HX_4C$ type, appears as an interesting target for the rational design of new antiviral agents since this protein is involved in several key steps of the retroviral life cycle, particularly by interacting with nucleic acids [1]. In addition to its chaperone activities, NCp7 seems to recognize more selectively some essential stem loops substructures of the Ψ packaging domain of HIV-1 genome (SL1-2-3-4) [2]. ^{1}H NMR studies have demonstrated that the folded CCHC boxes of NCp7 are in spatial proximity whereas the N- and C-terminal fragments remained flexible [3,4]. These structural characteristics seem to be crucial for NCp7 activities since all mutations disrupting the conformation of the finger domain led to a complete loss of infectivity due to defects in proviral DNA synthesis and provirus formation [5]. In agreement with these findings, Rice *et al.* have shown that disulfide-substituted benzamides (DIBAs) able to eject the zinc from nucleocapsid retroviral zinc fingers resulted in a loss of virus infectivity [6]. The three-dimensional structure of NCp7 complexed with d(ACGCC), which belongs to the Ψ packaging domain, was established by NMR [7]. In this complex, the tryptophan 37 was found inserted between the C2-G3 base pair and stacked on this latter, a feature also observed in the (1-55)NCp7/SL3 complex [8]. This structure was therefore used as a simplified scaffold to develop nucleomimetics of Trp^{37}-(C2-G3) insertion site. We have checked by molecular modeling that the replacement of the natural phosphodiester linkage by a methylene carboxamide unit (*) in a dinucleotide mimicking the Trp^{37} intercalation site could fulfill the previously described structural requirements for an optimal interaction with NCp7. Furthermore, the amide group is more resistant to cellular nucleases than the phosphate bond [9]. Thus, three pseudodinucleotides **T*G**, **G*T** and **T*T** have been synthesized as building blocks for the development of NCp7 inhibitors. One of the interest of

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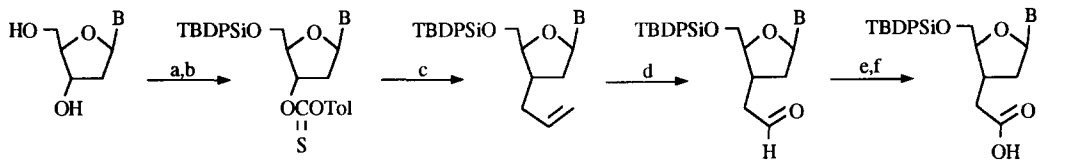
0960-894X/99/$ - see front matter
PII: S0960-894X(99)00052-9

antiviral agents directed towards NCp7 could be to overcome the problem of resistance encountered with RT and protease inhibitors. Indeed in the case of NCp7, each mutation generated by the virus in the zinc finger domain to avoid the drug recognition would unavoidably led to a loss of biological activity of the mutated NCp7.

A limited number of papers has been devoted to the synthesis of this kind of molecules [10,11] and modifications have been introduced to synthesize the three dinucleomimetics, especially **T*G**, whose preparation under an unprotected form has not previously been reported. Of the three dimers **T*G**, **G*T** and **T*T** synthesized, **T*G** is the closest mimetic of the C^2-G^3 intercalation. Moreover, it has been demonstrated that NCp7 recognize with a high affinity TG sequences [12]. **G*T** was developed to study the importance of the pyrimidine-purine versus purine-pyrimidine sequence and **T*T** was prepared since photoaddition studies have suggested that NCp7 has a predilection for pyrimidine-rich sequences [1]. Adenine was not used in the design of nucleomimetics, since it has been demonstrated that NCp7 interacts preferentially with G-containing single-stranded nucleic acids [13].

I. CHEMISTRY

The formation of the amide moiety follows a common strategy for the three dimers, namely the coupling of two 2'-deoxynucleotides modified either at the 3' site by a carboxymethyl group (Scheme 1) or at the 5' site by an amino function (Scheme 2). The nucleosides **1a** and **1b** were firstly protected in the 5' position by a *tert*-butyldiphenylsilyl group (TBDPSi), then acylated in the 3' position by p-tolyl chlorothiono formate leading to **2a**, **2b**. The thiocarbonates were transformed in the allylic derivatives [14] **3a**, **3b** with retention of configuration. An oxidative cleavage of the allyl group by $OsO_4/NaIO_4$ gave the corresponding aldehydes **4a**, **4b** [11]. The oxidation of the aldehyde was performed with pyridinium dichromate and subsequent hydrolysis with sodium hydroxide furnishing the desired carboxylic acids **5a**-**5b** [10].



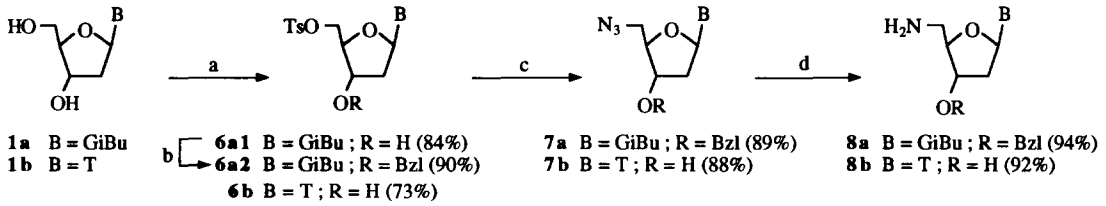
1a B = GiBu **1b** B = T **2a** B = GiBu (58%) **2b** B = T (72%) **3a** B = GiBu (60%) **3b** B = T (93%) **4a** B = GiBu (35%) **4b** B = T (55%) **5a** B = G (57%) **5b** B = T (78%)

Scheme 1. a) 2 eq. TBDPSiCl, 2 eq. imidazole, DMF, rt, 24-48h • b) 1.2 eq. DMAP, 1.2 eq. Et_3N, 2 eq. TolOCSCl, CH_2Cl_2, 24h • c) 0.7 eq. AIBN, 5 eq. $Bu_3SnCH_2CH{=}CH_2$, toluene, 80°C, 24h • d) 0.1 eq. OsO_4, 2.2 eq. $NaIO_4$, dioxane/water, rt, 6h • e) 6 eq. PDC, 6 eq. MeOH, DMF, rt, overnight • f) 2 eq. NaOH, MeOH, rt, overnight. T = thymine ; GiBu = 2-N-isobutylryl-guanine ; TBDPSi = tert-butyldiphenylsilyl.

The synthetic routes adopted to prepare the amino moieties from the protected 2'-deoxyguanosine **1a** or from the thymidine **1b** are illustrated in Scheme 2. We used a strategy different from that proposed in the literature [10,11] in order to improve global yields and to facilitate the isolation of the final products. From **1b**, the first step consisted of an activation of the 5'-alcohol by p-toluenesulfonyl followed by a substitution of the tosylate by an excess of NaN_3 [15]. The reduction of the 5'-azido **7b** was carried out using the Staudinger

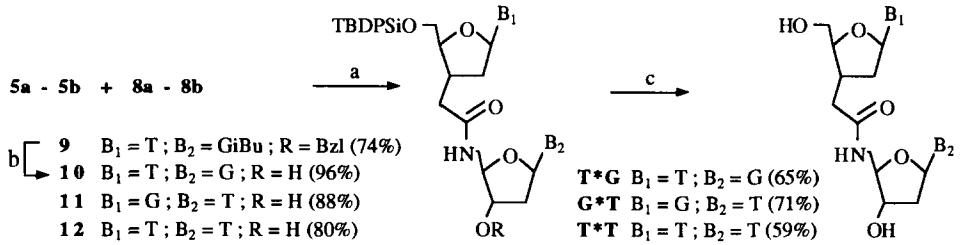
phosphine/phosphite procedure [16] to give the corresponding iminophosphorane which was subsequently hydrolyzed to afford **8b** in nearly quantitative yield.

Due to the low solubility of guanine derivatives in organic solvents, the preparation of the 5'-amino-2'-deoxyguanosine **8a** from the activated compound **6a1** was carried out by introducing two modifications. The hydroxyl group of **6a1** was protected by a lipophilic benzoyl moiety allowing the 5'-tosyl group of **6a2** to be easily substituted by an azido group to give **7a**. Then, the mild reduction of **7a** by tin dichloride [17] quantitatively produced the primary amine **8a**, isolated under a stable acetate salt.



Scheme 2. a) 3 eq. TsCl, pyridine, rt, 2h • b) 5 eq. BzCl, pyridine, rt, 3h • c) 2 eq. NaN_3, DMF, 80°C, 6h • d) **8a** : 1.5 eq. $SnCl_2$, CH_2Cl_2/MeOH, rt, overnight. **8b** : 2 eq. PPh_3, rt, 6h, H_2O, overnight.

The preparation of the three final dinucleomimetics **T*G**, **G*T** and **T*T** is depicted in Scheme 3. The dimers **9**, **11** and **12** were obtained with an excellent yield after activation of the carboxylic acids **5a-5b** by the O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) [18] in the presence of 1-hydroxy-benzotriazole (HOBT) followed by the addition of a stoichiometric amount of amine **8a-8b**.



Scheme 3. a) **5** 1 eq., 1.1 eq. N-methylmorpholine, 1 eq. TBTU, 0.5 eq. HOBT, DMF, rt, 2h, then **8** 1 eq., 1.5 eq. N-methylmorpholine, 24-48h • b) NH_4OH (29%)/EtOH, 55°C, 3h • c) HCl (3%)/EtOH, 2h, rt (**T*G**) ; 3 eq. Bu_4NF, THF/EtOH, 6h, rt (**G*T**, **T*T**).

In the case of **T*G**, the removal of protective groups was performed by treatment with aqueous ammoniac followed by deprotection of TBDPSi group by an ethanolic solution of gaseous HCl whereas **G*T** and **T*T** were obtained after treatment in tetra-n-butyl ammonium fluoride.

II. RESULTS AND DISCUSSION

The affinity of the three dimers **T*G**, **G*T** and **T*T** for the (12-53)NCp7 peptide was evaluated by fluorimetric experiments (Figure 1A). The fluorescence of this peptide is entirely due to Trp^{37} whose fluorescence emission is strongly quenched by complexation of the molecule with nucleic acids. Upon addition of increasing

concentrations of mimetics from $5x10^{-7}$ M up to 10^{-4} M to a $5x10^{-6}$ M solution of (12-53)NCp7, a dose-dependent quenching of Trp^{37} emission was observed. The affinity of **T*G** ($1.6x10^5$ M^{-1}) for (12-53)NCp7 was 20 times better than that of **G*T** ($9x10^3$ M^{-1}) while the affinity constant of **T*T** was too low to be accurately estimated. These results confirm the preference of NCp7 for a pyrimidine-purine sequence with G as the purine moiety, for optimal interaction with oligonucleotides [12]. Moreover, it is interesting to observe that d(ACGCC) is only 20 times more potent than **T*G** for (12-53)NCp7 binding [7]. This confirms that the stability of complexes formed between single-stranded oligonucleotides and NCp7 is essentially ensured by hydrophobic interactions, since the electrostatic contributions achieved in the case of natural nucleic acids by the negatively charged phosphate group, are absent in the dinucleomimetics. The rather good affinity of **T*G** is also probably due to a more favorable thermodynamic contribution during the NCp7 recognition process in forming the **T*G** intercalation site with subsequent insertion of the Trp^{37} aromatic ring. To the best of our knowledge, this is the first demonstration of an intercalation in these modified oligonucleotides. The easy formation of this kind of interaction could have interesting applications in homogeneous or heterogeneous duplexes formed with oligonucleomimetics.

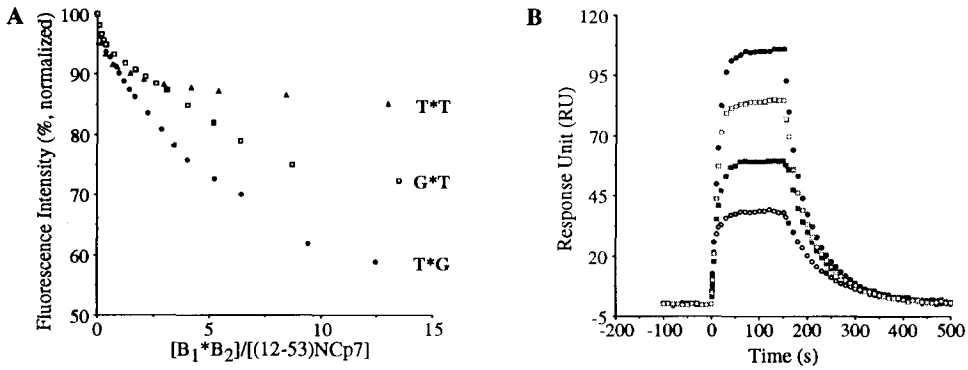


Figure 1. **A)** Titration of (12-53)NCp7 by the dimers **T*G**, **G*T** and **T*T** using tryptophan fluorescence as a binding signal (Hepes 5 mM, pH = 7.0). Apparent affinities were assumed by Scatchard treatment • **B)** Inhibition of (12-53)NCp7/SL3 complex formation upon addition of **T*G** (● : 0 ; □ : 0.1 mM ; ■ : 0.5 mM ; ○ : 1 mM) followed by SPR (Hepes 10 mM, NaCl 100 mM, p20 0.005%, pH = 7.4).

The potency of **T*G** to compete with nucleic acids upon binding to (12-53)NCp7 was further evaluated by real-time biospecific interaction analysis (BIA) based on a surface plasmon resonance (SPR) technology (Figure 1B). It has been demonstrated that NCp7 binds with a high affinity SL3 stem loop of the Ψ packaging sequence [2,8]. Therefore, 300 RU (Response Unit) of 5'-biotinylated SL3 DNA sequence were immobilized on the biosensor dextran surface. Under the described conditions, 110 RU of (12-53)NCp7 (100 nM) were reproducibly bound to the 5'-biotinSL3. Apparent kinetic rates of the (12-53)NCp7/SL3 complex were calculated using the BIAcore 2.0 analytical software (ka = $2.5x10^5$ $M^{-1}.s^{-1}$; kd = $2.2x10^{-3}$ s^{-1} ; K_{Dapp} = 8.8 nM). Figure 1B represents an overlay of the sensorgrams obtained from successive injections of the preformed (12-53)NCp7/**T*G** complex on the immobilized SL3. Upon addition of increasing concentrations of **T*G** from 100 µM up to 1 mM, a gradual decrease in SPR signals was observed, correlated with the amount of (12-53)NCp7 bound to SL3. At a concentration of $5x10^{-4}$ M, **T*G** was able to inhibit 50% of (12-53)NCp7/SL3 recognition.

In the ^{1}H NMR study of the 1/1 **T*G**/(12-53)NCp7 (1 mM) complex (Figure 2A), significant broadening and chemical shift variations were observed for Val^{13}, Phe^{16}, Thr^{24}, Ala^{25}, Arg^{26}, Trp^{37} and Gln^{45}. Similar modifications also occurred for the (12-53)NCp7/d(ACGCC) complex [7]. The large upfield shifts observed for the aromatic protons of Trp^{37} and G^2 in **T*G** confirm the critical role played by the intercalation of Trp^{37} in the stabilization of the complex. Inspection of NOESY data in the complex confirmed the presence of a number of intramolecular NOEs accounting for the internally folded structure of the protein and more precisely for the proximity of both zinc fingers as in the free (12-53)NCp7 [3]. All these results indicate that as expected, the structure of the (12-53)NCp7/**T*G** complex is similar to that found for the d(ACGCC)/(12-53)NCp7 complex at the level of the Trp^{37} intercalation site.

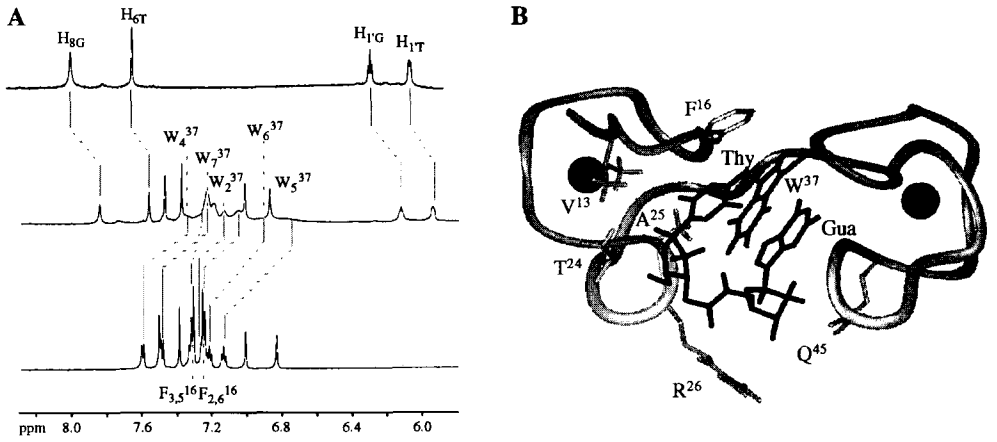


Figure 2. A) Chemical shift variations of the aromatic residues of (12-53)NCp7 upon **T*G** binding in D_2O, pH = 6.0, T = 293K (top : free **T*G** ; middle : (12-53)NCp7/**T*G** : 1/1 • bottom : free (12-53)NCp7) • **B)** Docking of **T*G** in (12-53)NCp7 obtained by template-forcing and energy-minimization.

However, the number of intermolecular NOEs observed in the two-dimensional ^{1}H NMR spectra was too limited for the three-dimensional structure of the complex to be determined. Only a model (Figure 2B) of the **T*G**/(12-53)NCp7 structure could be proposed. In this model, the sugar moieties are facing the N-terminal zinc finger, which stabilizes **T*G** through several possible hydrophobic contacts in agreement with the observed chemical shift changes and accounting for the few intermolecular NOEs observed, for instance between H_β Ala^{25} and H_1'.T. Hγ Val^{13} and Hγ Thr^{24} are well disposed to establish hydrophobic contacts with H_1'.T and εNH_2 Gln^{45} is able to create hydrogen bonds with the $H_{2'',2'}$ of G. Chemical shift variations observed for Phe^{16} and Trp^{37} aromatic protons are in agreement with the short distances measured between the aromatic rings of Phe^{16}/T and Trp^{37}/G. In addition, the side chain of Arg^{26} is well disposed to interact with the carbonyl of the **T*G** amide linker thus mimicking the salt-bridge [7] formed by the positively charged guanidinium group of this amino acid with the 5' phosphate of G^3 (3 to 3.5 Å). In consequence, the replacement of the natural phosphodiester backbone by an amide unit does not alter significantly the structure of the dinucleotide, allowing an easy formation of a Trp^{37} intercalation site.

In conclusion, the replacement of the natural phosphodiester linkage by an amide unit allowed the synthesis of a building block **T*G**, which is recognized by NCp7. This modified dinucleotide binds the protein with a

similar mechanism of protein recognition, characterized by the intercalation of Trp^{37} between the nucleotide bases. This is also confirmed by the ability of **T*G** to compete with a selective single-stranded DNA sequence during the formation of NCp7/nucleic acid complex.

When tested in a cellular assay using an HIV-1 infected cell line, **T*G** was able to induce a 20% decrease of reverse transcriptase acitivity at a concentration of 10 μM (not shown). This encouraging but low antiviral activity is probably due to a still too low bioavailability of the compound coupled to a relatively weak affinity for the NCp7/nucleic acid complex. The first problem could be overcome by introducing lipophilic moieties in a **T*G** prodrug. Thus, **T*G** can be considered as a primary unit which could help us to develop specific sequences recognized by NCp7. The design of such molecules is now in progress in the laboratory.

ACKNOWLEDGMENTS

We thank T. Huynh Dinh and H. de Rocquigny for helpful discussions, N. Gresh for modeling study and A. Bousseau (Rhone Poulenc Rorer-Vitry) for cellular tests. The authors wish to thank C. Dupuis for expert manuscript drafting.This work was supported by the French programs against AIDS (ANRS and Sidaction).

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