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ORIGINAL ARTICLE

Synthesis, spectroscopic studies and biological activity of a novel nucleopeptide with Moloney murine leukemia virus reverse transcriptase inhibitory activity

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Abstract In this work, we report the synthesis of an alternate nucleo-alpha,epsilon-peptide based on L-lysine moieties, an in vitro study of its biological activity, and spectroscopical binding studies between the novel nucleopeptide and Moloney murine leukemia virus reverse transcriptase as well as RNA. An alternate homothymine hexamer was synthesized by a straightforward solid phase route starting from commercial materials, purified by RP-HPLC and characterized by ESI-MS. The efficiency of the novel nucleo-alpha,epsilon-peptide in interfering with the reverse transcription of eukaryotic mRNA and the noteworthy enzymatic resistance demonstrated by specific assays are in favor of the employment of this nucleopeptide in novel biomedical strategies.

Keywords PNA · RNA · Reverse transcriptase · Virus

Abbreviations

Ac_2O	Acetic anhydride
DBU	1,8-Diazabicyclo[5.4.0]undecene
DIEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
Fmoc	9-Fluorenylmethoxycarbonyl
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
PTFE	Polytetrafluoroethylene
TAE	Tris–acetate–EDTA
TFA	Trifluoroacetic acid
TMP	2,4,6-Trimethylpyridine

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Introduction

Currently, a search for synthetic DNA mimics analogs able to sequence-specifically bind to natural nucleic acids is very intensive in the scientific community due to the relevant biomedical and bioengineering potential of these compounds, leading to a great number of artificial nucleobase-containing molecules characterized by different modifications with respect to natural oligonucleotides (Briones and Martin-Gago 2006; Helene and Le Doan 1991; Kurreck 2003). Among them, peptide nucleic acids (*aeg*PNAs) (Nielsen et al. 1991) are the most powerful peptide-like oligonucleotide mimics realized till now. More particularly, these analogs contain an artificial pseudo-peptide backbone based on *N*-(2-aminoethyl)glycine in place of the sugar–phosphate chain present in DNA and RNA, and are able to sequence-specifically hybridize natural nucleic acids giving rise to complexes with high thermal stability. On the other hand, some drawbacks, such as scarce water solubility, inefficient cellular uptake and self-aggregation, which limit *aeg*PNA biomedical applications (Uhlmann et al. 1998), are expected to be overcome by chemical modification of the original *aeg*PNA structure, thus leading to the development of novel PNAs with improved characteristics.

Another important feature to be considered in the realization of novel oligonucleotide mimics with high binding specificity is the ability of the analog to distinguish between parallel and antiparallel orientations during hybridization to the complementary target. Interestingly, the introduction of chirality in PNA structure allows for improved binding mode discrimination, as reported in many cases of PNA analogs with entirely or partially chiral polyamide backbones. Indeed, a number of chiral PNAs were obtained by insertion of amino acid units (e.g., lysines and arginines) at the *aeg*PNA termini or into the PNA backbone (Sforza et al. 1999, 2002; Haimaa et al. 1996; Gangamani et al. 1999; Roviello et al. 2006, 2007, 2008, 2009a, b, c).

As a contribution to the research on chiral PNA, recently, we reported the synthesis of a new L-lysine-based nucleo-amino acid suitable for the solid phase assembly and its oligomerization to the corresponding nucleo-epsilon-peptide that we called epsilon-*lys*PNA, studied by us in binding experiments with RNA (Roviello et al. 2009b).

As an extension of our recent investigation on the epsilon-*lys*PNA, which presented interesting properties, we also wanted to study the effect to alternate in the backbone, both alpha and epsilon-*lys*PNA residues, on the binding ability of the resulting isomeric nucleopeptide. Thus, we report here, for the first time, the synthesis, the characterization and the biological studies of a novel analog, that we called alpha,epsilon-*lys*PNA, with a backbone made of L-lysine moieties linked together by means of alpha- and epsilon-peptide bonds alternated in the sequence.

Reverse transcriptases (RTs) are multi-functional enzymes derived from RNA-containing retroviruses that play a key role in the replication of retroviruses, being involved in the conversion of genomic viral RNA into proviral DNA, and they present three enzymatic activities including DNA- and RNA-dependent DNA polymerase activity, and an RNase H activity that causes the cleavage of RNA in RNA–DNA hybrids (Molling et al. 1971).

The inhibition of RT polymerase activity is a major goal for the treatment of retroviral diseases such as human immunodeficiency virus type 1 (HIV-1) infection. RT inhibitors can be subdivided into nucleoside reverse transcriptase inhibitors (NRTIs), typically modified nucleotides or nucleosides, and non-nucleoside reverse transcriptase inhibitors (NNRTIs). The inhibitory activity of NRTIs is due to their conversion in vivo into triphosphate and their incorporation into DNA, which blocks the DNA elongation. On the other hand, NNRTIs are mainly hydrophobic molecules, both natural and synthetic, known to bind all to a common allosteric site of RT, which is a hydrophobic pocket situated about 10 Å away from the polymerase site (Georgiadis et al. 1995; Motakis and Parniak 2002; Rittinger et al. 1995) that possesses a non-competitive or uncompetitive inhibitory activity with respect to the binding of DNA template–primer duplex (Huang et al. 1998; Maga et al. 2000; Yang et al. 2001).

Besides these two classes of RT inhibitors, several polynucleotides are also known to inhibit RT polymerase activity (Chan et al. 1981). Interestingly, these compounds did not show a preference regarding to the template–primer pair, inhibiting the RT reactions directed by different template–primer systems all to a similar extent (Fukui and De Clercq 1982). Presumably, the RT inhibition depends on the formation of stable inactive complexes between such polynucleotides and the enzyme (Chan et al. 1981).

Moloney murine leukemia virus (MMLV) RT is a protein having a molecular weight of approximately 75 kDa which shares structural homology with HIV-1 RT (Williams et al. 1990; Wu et al. 2005) and it is often used as a model to study the effects of novel potential RT inhibitors. The development of MMLV RT mutants that exhibit diminished RNase H activity (MMLV RT RNaseH-), but retain full RNA-directed DNA polymerase activity, allows for an efficient synthesis of long cDNA in vitro (Kotewicz et al. 1988). Because of the high degree of structural homology between HIV-1 RT and MMLV RT, the effects of newly synthesized inhibitors on MMLV RT RNaseH- can provide important informations to develop novel agents against HIV-1 RT. For this reason, in the present study, MMLV RT RNaseH- was selected as a model enzyme to investigate, by an in vitro biological assay, the inhibition of RT activity due to the novel nucleopeptide herein described. Moreover, the interaction of the new synthetic oligonucleotide with poly(A) and MMLV RT RNaseH- was also studied by circular dichroism (CD) and ultraviolet (UV) spectroscopies in order to elucidate the possible mechanism of inhibition of this novel potential antiviral agent.

Preliminary biological activity assays and CD and UV studies, performed on the new homothymine nucleopeptide, revealed interesting characteristics, such as its RT inhibitory activity and enzymatic resistance, all discussed below in this work.

Materials and methods

Chemicals

Fmoc-L-Lys(Dde)-OH, Dde-L-Lys(Fmoc)-OH and HATU were purchased from Novabiochem. Anhydroscan DMF was from LabScan. DBU and hydrazine hydrate were from Sigma-Aldrich. Solvents for HPLC chromatography and acetic anhydride were from Reidel-de Haën. TFA, TMP, Rink-amide resin were from Fluka. TFA (for HPLC) was from Romil. Diethyl ether was from Carlo Erba.

Apparatus

Centrifugations were performed on a Z 200 A Hermle centrifuge.

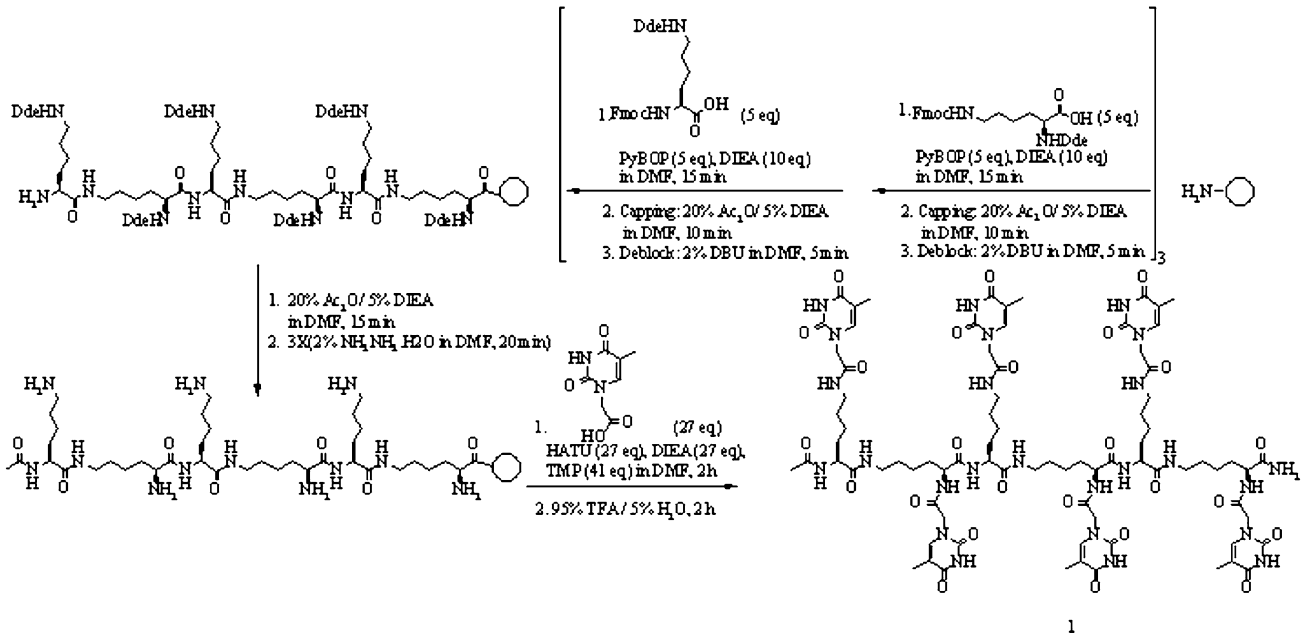
The product was analyzed and characterized by LC–MS on an MSQ mass spectrometer (ThermoElectron, Milan, Italy) equipped with an ESI source operating at 3 kV needle voltage and 320°C, and with a complete Surveyor HPLC system, comprising an MS pump, an autosampler, and a PDA detector, using a Phenomenex Jupiter C18 300 Å (5 μm, 4.6 × 150 mm) column. Gradient elution was performed using increasing amounts of acetonitrile (0.05% TFA, B) in water (0.05% TFA, A), with a linear gradient of 5% (for 5 min) to 40% B in A over 10 min (t_R = 11.7 min), monitoring at 260 nm, with a flow rate of 0.8 ml/min.

Semi-preparative purification was performed on a Hewlett Packard/Agilent 1100 series HPLC, equipped with a diode array detector, using a Phenomenex Jupiter C18 300 Å (10 μm, 10 × 250 mm) column. Gradient elution was performed by building up a gradient starting with buffer A (0.1% TFA in water) and applying buffer B (0.1% TFA in acetonitrile) with a flow rate of 4 ml/min (monitoring at 260 nm).

Samples were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h. UV spectra were recorded on a UV-Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC-505T temperature controller using Hellma quartz Suprasil cells, with a light path of 1 cm.

Solid phase synthesis of Ac-($t_{alpha\text{-}lys}$-$t_{epsilon\text{-}lys}$)$_6$-NH_2 (**1**, Scheme 1)

Solid phase synthesis was carried out in a short PP column (4 ml) equipped with a PTFE filter, a stopcock and a cap on a Rink-amide resin using the peptide-like Fmoc chemistry. Oligomer **1** was assembled on Rink Amide-Novagel HL resin (0.63 mmol/g, 63.5 mg, 40 μmol) using the synthetic strategy described in Scheme 1. More particularly, Fmoc-L-Lys(Dde)-OH or Dde-L-Lys(Fmoc)-OH (0.13 M in DMF, 1.5 ml, 200 μmol, 5 eq), PyBOP (0.25 M in DMF, 785 μl, 200 μmol, 5 eq) and DIEA (69 μl, 400 μmol, 10 eq) were added to the NH_2 resin and the mixture was reacted for 15 min under stirring. Each condensation step was followed by capping performed with 20% Ac_2O/5% DIEA in DMF for 15 min, while Fmoc deprotection was achieved with 2% DBU in DMF (5 min). The same capping solution was used also for the acetylation of the terminal amino group. Dde protecting groups were removed from the side amino moieties of the peptide by treating the resin three times with 2% (v/v) hydrazine hydrate in DMF for 20 min (total time 1 h). After Dde removal, the free amino groups were simultaneously functionalized with thymine nucleobase by reaction with thymine-1-yl acetic acid (200 mg, 1.08 mmol, 27 eq), which was previously pre-activated with HATU (411 mg, 1.08 mmol, 27 eq), DIEA (188 μl, 1.08 mmol, 27 eq), TMP (217 μl, 1.64 mmol, 41 eq) in DMF at RT for 2 h.



Scheme 1 Synthesis of the alternate alpha,epsilon-*lys*PNA oligomer **1**

The nucleopeptide was then cleaved from the solid support by treatment with 95% TFA and 5% H_2O over 2 h and recovered by precipitation with cold diethyl ether, centrifugation and lyophilization. The hexamer **1** was purified by semi-preparative HPLC using a linear gradient of 5% (for 5 min) to 20% B in A over 20 min: $t_R = 24.1$ min; UV quantification of the purified product gave 910 nmol of **1**; ESI-MS (Fig. 1) *m/z*: 1825.26 (found), 1825.96 (expected for $[C_{80}H_{113}N_{25}O_{25} + H]^+$); 1846.87 (found), 1847.93 (expected for $[C_{80}H_{113}N_{25}O_{25} + Na]^+$); 914.30 (found), 913.48 (expected for $[C_{80}H_{113}N_{25}O_{25} + 2H]^{2+}$).

UV and CD studies

Circular dichroism spectra were obtained on a Jasco J-810 spectropolarimeter, while UV spectra were recorded on a UV-Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC-505T temperature controller, using a Hellma quartz cell with a light path of 1 cm and a Hellma Tandem quartz cell 2 × 0.4375 cm (Rocchi et al. 1972; Krzyzanowska et al. 1998).

The quantification of nucleopeptide was performed by UV absorbance measurements on solutions of the purified oligomer dissolved in a known amount of milliQ water ($T = 85$°C, absorbance value at $\lambda = 260$ nm). The epsilon value used for the quantification of the oligomer **1** (51.6 mM^{-1}) was calculated using the molar extinction coefficient of thymine *aeg*PNA monomer (8.6 mM^{-1}).

Serum stability assays

The serum stability assays were performed on oligomer **1** following a procedure already reported by us in literature (Roviello et al. 2009b). After incubation, the withdrawn samples were analyzed by HPLC on a Phenomenex Jupiter C18 300 Å (5 µm, 4.6 × 250 mm) column using a linear gradient of 5% (for 5 min) to 20% B in A over 20 min ($t_R = 24.1$ min).

Cell culture

HeLa cells (ATCC, USA) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in humidified air with 5% CO_2.

RT-PCR

Total RNA was extracted from the cellular lysate using Tri-reagent™ (Sigma-Aldrich, St. Luis, MO) following the manufacturer's instructions. RT was performed using 0.5 µg of total RNA, 200 U of MMLV RT RNase H- (Finnzymes, Espoo, Finland), 250 ng of dT_{15} primer (Roche, Switzerland) in the presence of 0.25, 0.5, 1.25 and 7.6 µg of oligomer **1** or 6.7 µg of *aeg*PNA t_{12} and 7.0 µg of *aeg*PNA a_6 (as positive and negative controls, respectively). A preincubation mixture containing RNA and alpha,epsilon-*lys*PNA or *aeg*PNA was performed for 10 min.

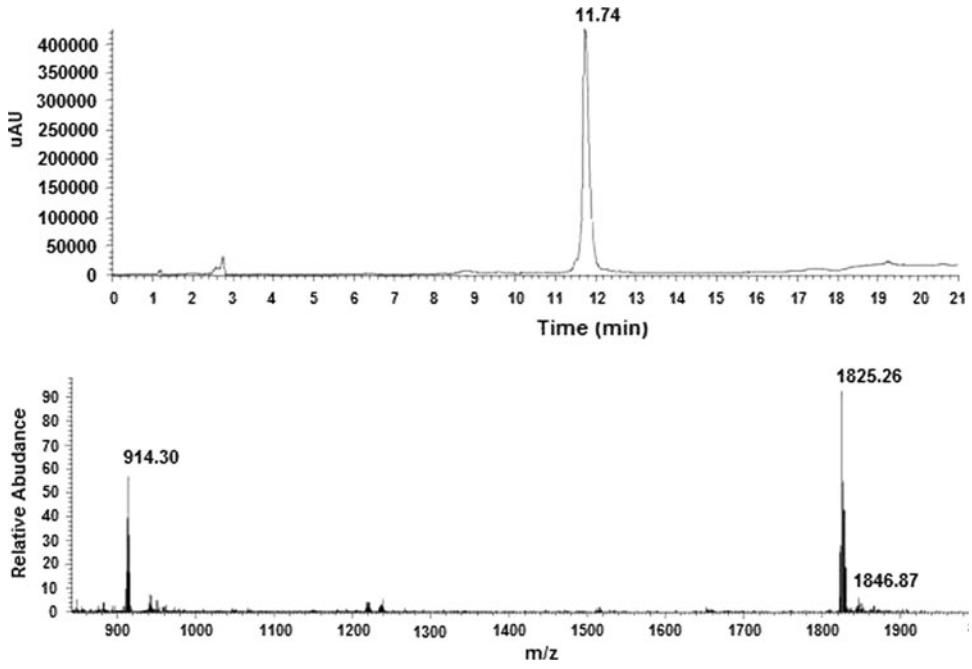


Fig. 1 LC–ESI-MS of the hexathymine oligomer **1**

Reaction temperature was set at 22°C for 1 h. RT buffer was 50 mM Tris–HCl, pH 8.3; 3 mM $MgCl_2$; 75 mM KCl; 10 mM DTT. After RT, PCR assay of GAPDH and beta-actin transcripts was carried out using the following primers: forward primer, 5′-ATGGGGAAGGTGAAGGTC-3′; reverse primer, 5′-GTCATGGATGACCTTGGC-3′ and forward primer, 5′-TGAGACCTTCAACACCCC-3′; reverse primer, 5′-CAGGAAGGAAGGCTGGAA-3′ (purchased by Sigma-Genosys Ltd), respectively. The PCR program was as follows: [(95°C, 5 min) × 1 cycle, (95°C, 1 min; 58°C, 1 min; 72°C, 1 min) × 25 cycles]. PCR products were then analyzed on 1% agarose gel with 1× TAE buffer and visualized by ethidium bromide staining. Gel images were captured by a ChemiDoc™ XRS and analyzed by Quantity-One software (Biorad, Hercules, CA).

Results and discussion

Solid phase synthesis of the alpha,epsilon-*lys*PNA

The synthesis of the homothymine alpha,epsilon-*lys*PNA hexamer **1** was performed in solid phase using starting materials such as (1) the commercial Fmoc-L-Lys(Dde)-OH and Dde-L-Lys(Fmoc)-OH amino acids for the realization of the polylysine backbone and (2) thymine-1-yl acetic acid, also commercially available, for the nucleobase derivatization of the oligomer.

In our strategy, we firstly realized an alpha,epsilon-hexalysine backbone by alternately coupling on the solid support (Rink Amide-Novagel HL) the commercial Fmoc-L-Lys(Dde)-OH and Dde-L-Lys(Fmoc)-OH derivatives following a Fmoc peptide strategy (Scheme 1). Subsequently, Dde protecting groups, stable under the Fmoc deprotection conditions used (2% DBU in DMF), were removed from the side amino moieties of the peptide under nucleophilic conditions using 2% (v/v) hydrazine hydrate in DMF. After Dde deprotection, the free amino groups were simultaneously reacted with the commercial thymine-1-yl acetic acid under ammidation conditions involving HATU as activator agent and DIEA and TMP as bases.

After cleavage from the solid support, achieved under acid treatment (95% TFA/5% H_2O), precipitation from cold diethyl ether and purification by RP-HPLC, the homothymine oligomer **1** was obtained in 2.3% yield.

Characterization of oligomer **1** was performed by LC–ESI-MS which confirmed the identity of the product (Fig. 1).

The novel nucleopeptide showed a good water solubility and did not present any tendency to self-aggregate.

In this study, we chose to realize a thymine-based alpha,epsilon-*lys*PNA oligomer since this nucleobase presents the fewest synthetic challenges, anyway the facile synthetic strategy herein reported can also be extended to the other DNA bases, allowing for the obtainment of homonucleobase nucleopeptides by the use of commercial starting materials such as Fmoc-L-Lys(Dde)-OH, Dde-L-Lys(Fmoc)-OH and nucleobase-1-yl acetic acid without requiring the synthesis of nucleo-amino acid monomers. Nevertheless, future efforts will also be devoted to the realization of lysine-based oligomers with mixed nucleobase sequences that we plan to realize by means of suitably protected nucleo-amino acid monomers.

Biological studies

The biological activity of the novel alternate nucleopeptide was tested by an in vitro assay to investigate its ability to interfere with MMLV reverse-transcription reaction using total RNA from HeLa cells. In particular, in a semi-quantitative RT-PCR experiment after RT reaction step, performed using dT_{15} primer and oligomer **1**, PCR was carried out using specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin genes. More in detail, we performed a dose-response RT inhibition assay by using different nucleopeptide amounts and the results of this experiment revealed a gene amplification reduced in the presence of different base/base ratios (from 1:1 to 1:30) of primer/alpha,epsilon-*lys*PNA (Fig. 2) in a dose-dependent manner. In particular, the reduction was quantified for the 1:1 ratio (i.e., for a 10 μg/ml concentration of **1**) as 60% for GAPDH gene (lane 3, Fig. 2a) and 58% for beta-actin gene (lane 3, Fig. 2b) when compared with PCR amplification in the absence of oligomer **1** (lane 2, Fig. 2). The gene amplification was totally inhibited in the presence of 1:30 ratio of primer/alpha,epsilon-*lys*PNA (lane 6). In the same experiment, the amplification of both genes was completely suppressed when it was performed in the presence of *aeg*PNA t_{12} as a positive control, while no reduction of gene amplification was found when PNA a_6 was used as a negative control in the same base/base ratio (data not shown). Furthermore, when oligomer **1** was used as a primer in RT reaction in the absence of dT_{15} primer, no PCR amplification products were detected (data not shown).

Moreover, the enzymatic resistance of the novel alternate nucleopeptide was investigated by incubating

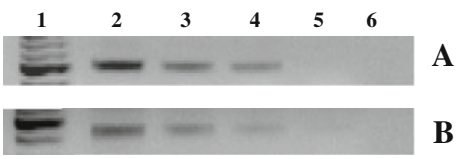


Fig. 2 Interference of addition of alpha,epsilon-*lys*PNA on semi-quantitative RT-PCR reaction. After RT, cDNA sequences were PCR amplified using specific primers for GAPDH (**a**) and beta-actin (**b**) in the presence of 0.25, 0.5, 1.25 and 7.6 μg (lanes *3–6*) of **1**. Molecular markers are in lane *1*

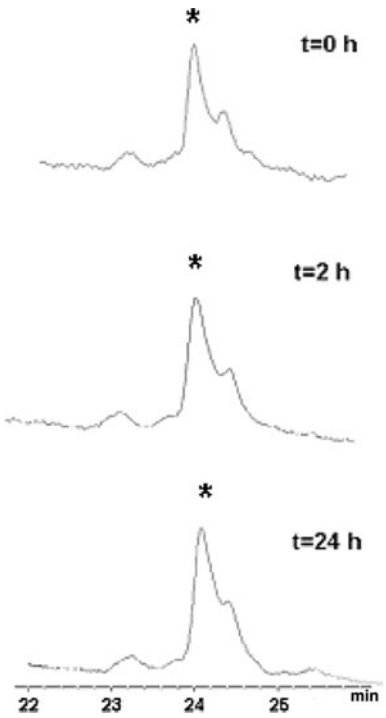


Fig. 3 Serum stability assay on alpha,epsilon-*lys*PNA. Nucleopeptide **1** (*asterisk*, $t_R = 24.1$ min) was incubated with human serum. After the indicated incubation times, samples were analyzed by C18 RP-HPLC

oligomer **1** in fresh human serum at 37°C and analyzing by HPLC samples withdrawn from the reaction mixture at various times (Fig. 3).

Even after 24 h, the alternate alpha,epsilon-*lys*PNA was still present, in contrast with natural dT_6 which completely disappeared already after 1 h (data not shown).

CD and UV studies

Since our biological assays suggested that alpha,epsilon-*lys*PNA can inhibit viral reverse transcription, a desirable property for a potential antiviral agent, we were also interested in investigating the mode of action of the novel inhibitor. In general, synthetic oligonucleotides can prevent reverse transcription in at least three different ways (Boiziau et al. 1992): (1) by direct competition with the primer (the competitor, i.e., the oligonucleotide analog, and the primer have the same sequence but the former cannot prime reverse transcription); (2) by binding to the enzyme; (3) by binding to the template RNA downstream of the primer.

In order to elucidate the possible mechanism of RT inhibition by our nucleopeptide, we performed several spectroscopic studies with poly(A) and MMLV RT. Regarding the first hypothesis, we explored the ability of our alpha,epsilon-*lys*PNA to bind complementary RNA, a characteristic which is required for a potential competitor of the oligothymine DNA primer in MMLV RT inhibition, and it is also important for many biomedical applications of RNA-binding molecules.

More in detail, the structural characteristics of the L-lysine-based nucleopeptide as well as its RNA-binding ability were investigated by CD spectroscopy. Firstly, the CD profile of the nucleopeptide single strand in 10 mM phosphate buffer, pH 7.5, was analyzed in order to evaluate an helical pre-organization of the alpha,epsilon-*lys*PNA. By examining the CD behavior of the nucleopeptide in the range 190–240 nm at different temperatures (5, 10 and 25°C), no significant helical contribution was detected for the single strand (data not shown).

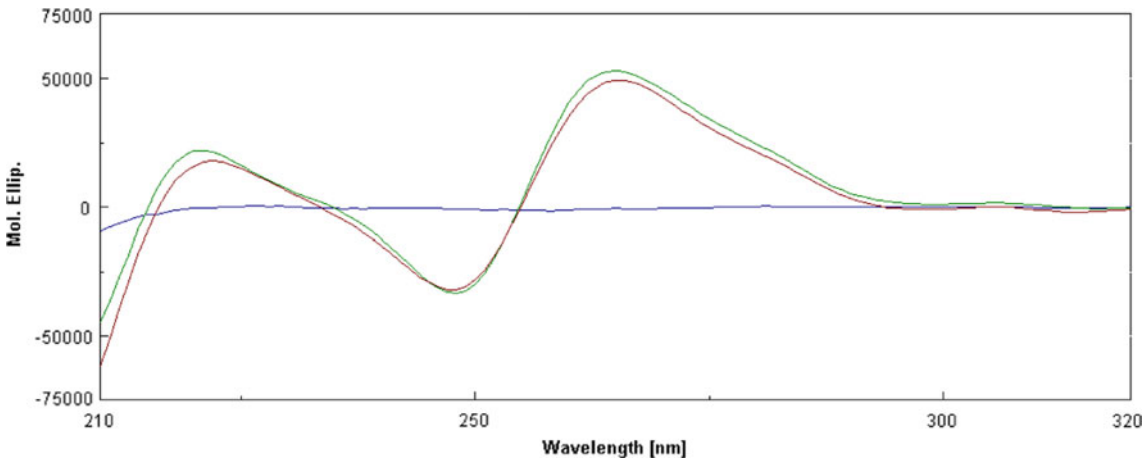


Fig. 4 CD spectra, recorded at 5°C by a 1 cm Hellma quartz cell, relative to (i) a solution of *lys*PNA (80 μM in thymine, 10 mM phosphate buffer, pH 7.5, *blue line*); (ii) a solution of polyadenylic RNA (8 μM in adenine, 10 mM phosphate buffer, pH 7.5, *green line*); (iii) a solution of *lys*PNA (80 μM in thymine, 10 mM phosphate buffer, pH 7.5) plus polyadenylic RNA (8 μM in adenine, 10 mM phosphate buffer, pH 7.5), *red line*

CD binding experiments on the homothymine alpha,epsilon-*lys*PNA with the complementary poly(A) were then performed in order to evaluate not only its potential as antisense tool, but also to investigate a possible mechanism of RT inhibition. The CD profile relative to the solution of the nucleopeptide single strand (80 μM in thymine, 10 mM phosphate buffer, pH 7.5, blue line, Fig. 4) resulted very weak as compared to the strong bands of the RNA (8 μM in adenine, 10 mM phosphate buffer, pH 7.5, green line, Fig. 4).

When a tenfold amount in nucleobase of thymine alpha,epsilon-*lys*PNA was added to a solution of polyadenylic RNA in 10 mM phosphate, pH 7.5, a certain change was detected in the shape and intensity of the CD profile of the polyA (red line, Fig. 4). This finding suggests the formation of a weak complex between the *lys*PNA and RNA at 5°C. However, no significant difference in the CD profile was detected when the same experiment was performed at 22°C, in the same RT-PCR conditions, in the presence of different amounts of oligomer **1** (data not shown).

Interestingly, the novel analog was characterized by an average number of bonds (5) between the atoms bearing the nucleobases identical to that already explored by us in *dab*PNA (Roviello et al. 2006, 2008). In both cases, no significant nucleic binding ability was found with complementary strands of DNA (data not shown) and RNA.

Subsequently, we investigated also the ability of the L-lysine-containing nucleopeptide to bind to the MMLV RT. Thus, we performed UV and CD binding experiments in a tandem cell recording the "sum" spectra of the separated components and the "mix" spectra, recorded after mixing, relative to the same excess of alpha,epsilon-*lys*PNA with respect to MMLV RT (67:1 lysPNA:MMLV nanomolar ratio) used in PCR experiments to totally inhibit RT activity. More in detail, 0.02 nmol of MMLV RT and 1.34 nmol of alpha,epsilon-*lys*PNA were dissolved each in 0.8 ml of 10 mM phosphate buffer, pH 8.0.

Upon mixing of the two ligand solutions, a difference was observed between the "sum" (green line) and "mix" CD spectra (blue line, Fig. 5a) suggesting a complex formation between the two molecules in conditions similar to those employed in the RT-PCR assays. Also the hypochromic effect in UV spectrum observed after mixing the two solutions ("sum" spectrum: green line; "mix" spectrum: blue line, Fig. 5b) was in accordance with the formation of a complex between the novel nucleopeptide and the reverse transcriptase.

These results are interesting because they suggest that the inhibition of MMLV reverse transcription by our chiral nucleopeptide cannot be due to the competition between the analog and the DNA primer (no RNA binding in the RT-PCR conditions), but to the direct interaction with the enzyme. Interestingly, the RT inhibitory activity, previously reported (Ray and Nordén 2000) for the achiral *aeg*PNA, was not related to a PNA-RT interaction but to the PNA antisense activity (i.e., *aeg*PNA competed with the primer having the same base sequence). This was confirmed also by our experiments: in fact, t_6, and not a_6 *aeg*PNA, inhibited MMLV RT in the presence of the oligothymine primer. On the other hand, the preliminary in vitro studies presented in this work suggest a direct interaction between the chiral alpha,epsilon-*lys*PNA and RT, which could play an important role in the inhibition of the RT activity and encourage further studies on this novel class of molecules with antiviral potential.

Conclusions

The biological data herein reported demonstrated that the newly synthesized serum-resistant alpha,epsilon-*lys*PNA was able to inhibit the viral reverse transcriptase activity in a dose-dependent manner. Furthermore, our studies suggest that the MMLV RT inhibition can only be due to a direct RT binding of the new oligonucleotide analog to the enzyme, hypothesis supported by our preliminary spectroscopic results, in analogy to other reports on antiviral polynucleotides (Chan et al. 1981; Fukui and De Clercq 1982), because no ability of alpha,epsilon-*lys*PNA to bind RNA in the RT-PCR conditions was found, as required for a primer competitor.

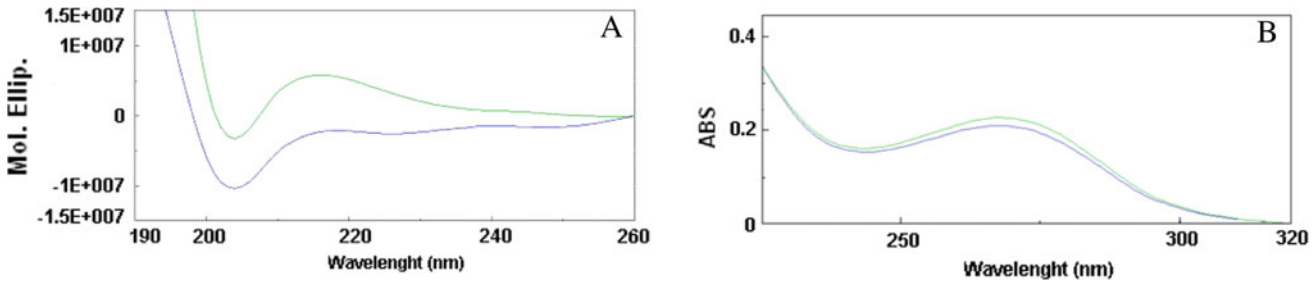


Fig. 5 "Sum" (*green line*) and "mix" (*blue line*) CD (**a**) and UV (**b**) spectra, recorded at 22°C by a tandem cell, relative to MMLV reverse transcriptase (0.02 nmol) and alpha,epsilon-*lys*PNA (1.34 nmol) dissolved each in 0.8 ml of 10 mM phosphate buffer, pH 8.0. The volume of solution after mixing was 1.6 ml (0.8 ml + 0.8 ml)

Taken together, all these considerations allow us to conclude that the alternate alpha,epsilon-*lys*PNA, synthesized by a convenient and straightforward solid phase route, is an enzymatically stable nucleoderivative, with antiviral potential of considerable interest for future biomedical applications.

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References

Boiziau C, Thuong NT, Toulmé JJ (1992) Mechanisms of the inhibition of reverse transcription by antisense oligonucleotides. Proc Natl Acad Sci USA 89:768–772

Briones C, Martin-Gago JA (2006) Nucleic acids and their analogs as nanomaterials for biosensor development. Curr Nanosci 2:257–273

Chan EW, Lee CK, Dale PJ, Nortridge KR, Hom SS, Seed TM (1981) Antiviral properties of polyinosinic acids containing thio and methyl substitutions. J Gen Virol 52:291–299

Fukui T, De Clercq E (1982) Inhibition of murine leukaemia virus reverse transcriptase by 2-halogenated polyadenylic acids. Biochem J 203:755–760

Gangamani BP, Kumar VA, Ganesh KN (1999) Chiral analogues of peptide nucleic acids: synthesis of 4-aminoprolyl nucleic acids and DNA complementation studies using UV/CD spectroscopy. Tetrahedron 55:177–192

Georgiadis MM, Jessen SM, Ogata CM, Telesnitsky A, Goff SP, Hendrickson WA (1995) Mechanistic implications from the structure of a catalytic fragment of Moloney murine leukemia virus reverse transcriptase. Structure 3:879–892

Haimaa G, Lohse A, Buchardt O, Nielsen PE (1996) Peptide nucleic acids (PNAs) containing thymine monomers derived from chiral amino acids: hybridization and solubility properties of D-lysine PNA. Angew Chem Int Ed Engl 35:1939–1942

Helene C, Le Doan T (1991) Oligonucleotides: problems and frontiers of practical applications. Trends Biotechnol 9:339–340

Huang H, Chopra R, Verdine GL, Harrison SC (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science 282:1669–1675

Kotewicz ML, Sampson CM, D'Alessio JM, Gerard GF (1988) Isolation of cloned Moloney murine leukemia virus reverse transcriptase lacking ribonuclease H activity. Nucleic Acids Res 16:265–277

Krzyzanowska D, Lisowski M, Kochman M (1998) UV-difference and CD spectroscopy studies on juvenile hormone binding to its carrier protein. J Pept Res 51:96–102

Kurreck J (2003) Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 270:1628–1644

Maga G, Ubiali D, Salvetti R, Pregnolato M, Spadari S (2000) Selective interaction of the human immunodeficiency virus type 1 reverse transcriptase nonnucleoside inhibitor efavirenz and its thio-substituted analog with different enzyme–substrate complexes. Antimicrob Agents Chemother 44:1186–1194

Molling K, Bolognesi DP, Bauer H, Busen W, Plassmann HW, Hausen P (1971) Association of viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA–DNA hybrids. Nat New Biol 234:240–243

Motakis D, Parniak MA (2002) A tight-binding mode of inhibition is essential for anti-human immunodeficiency virus type 1 virucidal activity of nonnucleoside reverse transcriptase inhibitors. Antimicrob Agents Chemother 46:1851–1856

Nielsen PE, Egholm M, Berg RM, Buchardt O (1991) Sequence selective recognition of DNA by strand displacement with thymine-substituted polyamide. Science 254:1497–1500

Ray A, Nordén B (2000) Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. FASEB J 14:1041–1060

Rittinger K, Divita G, Goody RS (1995) Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. Proc Natl Acad Sci USA 92:8046–8049

Rocchi R, Borin G, Marchiori F, Moroder L, Peggion E, Scoffone E, Crescenzi V, Quadrifoglio F (1972) Interaction of S-protein with S-peptide and with synthetic S-peptide analogs. A spectroscopic and calorimetric investigation. Biochemistry 11:50–57

Roviello GN, Moccia M, Sapio R, Valente M, Bucci EM, Castiglione M, Pedone C, Perretta G, Benedetti E, Musumeci D (2006) Synthesis, characterization and hybridization studies of new nucleo-gamma-peptides based on diaminobutyric acid. J Pept Sci 12:829–835

Roviello GN, Musumeci D, Moccia M, Castiglione M, Sapio R, Valente M, Bucci EM, Perretta G, Pedone C (2007) *dab*PNA: design, synthesis and DNA binding studies. Nucleos Nucleot Nucleic Acids 26:1307–1310

Roviello GN, Musumeci D, Bucci EM, Castiglione M, Cesarani A, Pedone C, Piccialli G (2008) Evidences for complex formation between L-*dab*PNA and *aeg*PNA. Bioorg Med Chem Lett 18:4757–4760

Roviello GN, Musumeci D, Moccia M, Castiglione M, Cesarani A, Bucci EM, Saviano M, Pedone C, Benedetti E (2009a) Evidences of complex formation between DABA-based nucleo-gamma-peptides with alternate configuration backbone. J Pept Sci 15:147–154

Roviello GN, Musumeci D, Castiglione M, Bucci EM, Pedone C, Benedetti E (2009b) Solid phase synthesis and RNA-binding studies of a serum resistant nucleo-ε-peptide. J Pept Sci 15:155–160

Roviello GN, Musumeci D, Pedone C, Bucci EM (2009c) Synthesis, characterization and hybridization studies of an alternate nucleo-ε/γ-peptide: complexes formation with natural nucleic acids. Amino Acids. doi:10.1007/s00726-008-0214-7

Sforza S, Haaima G, Marchelli R, Nielsen PE (1999) Chiral peptide nucleic acids (PNAs): helix handedness and DNA recognition. Eur J Org Chem 1:197–204

Sforza S, Galaverna G, Dossena A, Corradini R, Marchelli R (2002) Role of chirality and optical purity in nucleic acid recognition by PNA and PNA analogs. Chirality 14:591–598

Uhlmann E, Peyman A, Breipohl G, Will DW (1998) PNA: synthetic polyamide nucleic acids with unusual binding properties. Angew Chem Int Ed Engl 37:2796–2823

Williams KJ, Loeb LA, Fry M (1990) Synthesis of DNA by human immunodeficiency virus reverse transcriptase is preferentially blocked at template oligo(deoxyadenosine) tracts. J Biol Chem 265:18682–18689

Wu L, Huang MH, Zhao JL, Yang MS (2005) Study of MMLV RT-binding with DNA using surface plasmon resonance biosensor. Acta Biochim Biophys Sin (Shanghai) 37:634–642

Yang SS, Cragg GM, Newman DJ, Bader JP (2001) Natural product-based anti-HIV drug discovery and development facilitated by the NCI developmental therapeutics program. J Nat Prod 64:265–277