Anti-Hepatitis B Virus Activity of New Pyrimidine and Adenine Peptide Nucleic Acid Analogues

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A number of *N*-substituted thymine and adenine derivatives, **2a**, **b** and **3a**, **b**, were synthesized by the coupling reaction of 1-bromo-2,2-diethoxyethane with the corresponding base. The corresponding peptide nucleic acid (PNA) analogues, *N*-substituted ethylamino-3-hydroxypropanoate derivatives **5a**, **b** and ethylamino-3-hydroxybutanoate derivatives **6a**, **b**, were synthesized from the corresponding 2-[3,4-dihydro-5-methyl-2,4-dioxopyrimidin-1(2*H*)-yl]-acetaldehyde (**3a**) and 2-[6-amino-4*H*-purin-9(5*H*)-yl]-acetaldehyde (**3b**), respectively. The synthesized compounds were tested for their antiviral activity against hepatitis B virus (HBV). The plaque reduction infectivity assay was used to determine the virus count reduction as a result of the treatment with the tested compounds.

Key words: Peptide Nucleic Acid Analogues, Adenine and Thymine Nucleobases, Anti-Hepatitis B Virus

Introduction

Synthetic compounds that specifically recognize and bind to a specific DNA or RNA sequence of interest are of great significance (DeMesmaeker *et al.*, 1995; Milligan *et al.*, 1993; Uhlmann and Peymann 1990). A particular successful DNA-binding agent is a recently developed class of DNA analogues, the peptide nucleic acids (PNAs) (Fig. 1) (Nielsen *et al.*, 1991; Egholm *et al.*, 1992; Hyrup and Nielsen, 1996; Nielsen, 1998; Uhlmann *et al.*, 1998; Ganesh and Nielsen, 2000). PNAs are oligonucleotide analogues, in which the entire phosphodiester pentose backbone of DNA or RNA is replaced by a polyamide or peptide backbone (Egholm *et al.,* 1993). The complete replacement of the ribose phosphate backbone with an artificial pseudopeptide backbone results in a remarkably improved binding to complementary nucleic acid sequences occurring with both high affinity and high selectivity. These characteristics make them potentially useful as antisense and antigene drugs or molecular probes, which have numerous applications in the field of molecular and experimental medicine (Egholm *et al.*, 1995; Ray and Norden, 2000; Zhou *et al.*, 2006; Hyun *et al.*, 2006). Furthermore N^1/N^9-alkylation of nucleobases affords the important building blocks for PNAs, which have been widely described in the literature (Duehohm *et al.*, 1994; Thomson *et al.*, 1995; Will *et al.*, 1995; Alahiane *et al.*, 2003). The hybridization properties of PNAs have attracted widespread interest to this class of compounds (Zhou *et al.*, 2003; Nielsen, 1999; Seitz, 2000). Their biological stability has made PNAs attractive as therapeutic and biomolecular tools. In PNAs, the phosphoribose backbone of

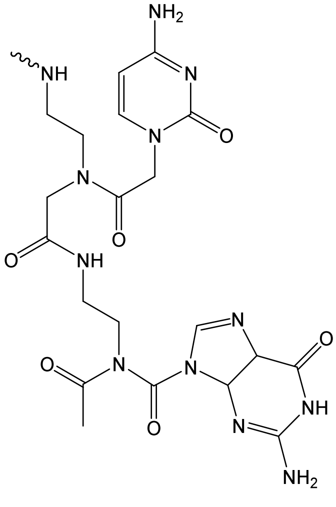

Fig. 1. Chemical structure of peptide nucleic acids.

a natural oligonucleotide has been replaced by *N*-(2-aminoethyl)glycine whereby the purine/pyrimidine base pair is attached to the glycine nitrogen atom via a methylene carbonyl linker (Debaene and Winssinger, 2003). Owing to the above significance and as a consequence of the chemical stability of PNAs and their superior hybridization properties relative to natural oligonucleotides, we here report the synthesis of new PNA analogues and acyclic analogues of nucleosides using pyrimidine and adenine as heterocyclic nucleobases.

Experimental

General

Melting points were determined using a Büchi apparatus. IR spectra (KBr) were recorded with a Bruker-Vector22 instrument (Bruker, Bremen, Germany). ^{1}H NMR spectra were recorded with a Varian Gemini spectrometer at 300 MHz and 200 MHz. Chemical shifts were reported in δ scale (ppm) relative to TMS as internal standard and the coupling constants (*J* values) are given in Hz. The progress of the reactions was monitored by TLC using aluminum silica gel plates 60 F_{245}. EIMS spectra were recorded with a HP D5988 A 1000 MHz instrument (Hewlett Packard, Palo Alto, CA, USA). Antiviral activity against hepatitis B virus was tested at the Liver Institute, Menofia University, Egypt.

Antiviral activity

The synthesized compounds were tested for their antiviral activity against hepatitis B virus (HBV) using the HepG2.2.2.15 cell line (Sells *et al.*, 1987), a human hepatoplastoma cell line producing HBV viral particles (Korba and Gerin, 1992).

The cell line was maintained in RPMI-1640 (Glutamax) (Gibco BRL Life Technologies, Paisly, Scotland) culture medium containing 100 IU/ml nystatin (Gibco BRL Life Technologies), 380 μg/ml G418 (genetecin) (Gibco BRL Life Technologies) and 10 % fetal calf serum (FCS) (Gibco BRL Life Technologies). The transferred HEPG2.2.2.15 cells were kept in a tissue culture flask at 37 °C and 5 % CO_2. Subcultures were set up after a week by trypsination (10 % versin/trypsin; Biochrome KG, Berlin, Germany) and transferred to a 96-well tissue culture plate. Serial dilutions of the test compounds were added to the cell suspension and incubated for 6 d at 37 °C and 5 % CO_2. The antiviral activity and cytotoxic effect of the test compounds was estimated by comparing the DNA content in the culture supernatant and the viability of the cells with the test compounds to those of HepG2.2.2.15 cells without any compound added (blank cells). The drug lamivudine which is a potent selective inhibitor of HBV replication has been used as a standard positive control (Doong *et al.*, 1991). Each compound was tested in triplicate.

DNA extraction

DNA extraction was done by incubating 10 μl of diluted supernatant with 10 μl of 0.2 M NaOH at 37 °C for 1 h; then 9.6 μl of 0.2 M HCl were carefully added followed by addition of 90 μl of Tris-EDTA (TE) buffer (Gibco BRL Life Technologies).

PCR-ELISA detection of HBV DNA

The DNA content in the cell culture supernatant was determined by PCR amplification of the HBV DNA using 1 μmol/1 of each of the following primers: HCID-1 (5′-GGAAAGAAGTCAGAAGG-CA-3′) and HCID-2 (5′-TTGGGGGAGGAGAT-TAGGTT-3′), in a reaction mixture containing 14 μl extracted supernatant, 4 mmol/l $MgCl_2$, 10 μmol/l DIG-11-dUTP (Roche, Munich, Germany), 190 μmol/l dTTP, 200 μmol/l dATP, dGTP, dCTP (Roche), 1.5 U Taq polymerase (Roche) in a total volume of 50 μl. PCR reaction conditions were: 32 cycles of 10 min at 94 °C, 30 s at 58 °C and 30 s at 72 °C with a 3-s increment for each cycle in a Perkin Elmer 480 thermal cycler (Perkin Elmer, Waltham, MA, USA). The PCR product was detected by the DIG-ELISA assay (Roche). The optical density (OD) of the DNA of the test compound was compared to that of the blank culture.

Cytotoxicity assay

3-(3,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) is a colourless substrate that is transferred to a coloured product by any living cell but not by dead cells. The assay utilizes this compound to test for the viability of cells in the presence of test compound compared to the viability of the blank cells (Fouad *et al.*, 1998).

Calculation of IC_{50}, CC_{50}, and SI values

The 50 % inhibitory concentration of antiviral drugs (IC_{50}) was determined by plotting the DNA content of the serial dilutions of the tested compound versus the concentration of this compound. The 50 % cytotoxic effect (CC_{50}) was calculated from the average viability of the cells with 0.1 μM concentration of drugs. The selectivity index (SI) was calculated as CC_{50}/IC_{50} (Fouad *et al.*, 1998).

Results and Discussion

Chemistry

The coupling reaction at one of the nitrogen atoms in the heterocyclic base is the most effective method for introducing certain substituents with desired functionalities into the heterocyclic base. Thus, the reaction of thymine and adenine with 1-bromo-2,2-diethoxyethane was carried out in the presence of anhydrous potassium carbonate to give 1-(2,2-diethoxyethyl)-5-methylpyrimidine-2,4(1*H*, 3*H*)-dione (**2a**) and 9-(2,2-diethoxyethyl)-9*H*-purin-6-amine (**2b**), respectively. The structures of these compounds were proved by IR, ^1H NMR and mass spectra which were in agreement with the assigned structure. The ^1H NMR spectra of compounds **2a** and **2b** showed the signals of ethyl protons as triplets and quartets at δ 1.19, 3.55 ppm and δ 1.17, 3.51 ppm, respectively. Hydrolysis of **2a** and **2b** was carried out by means of 4 M hydrochloric acid to give the corresponding 2-[3,4-dihydro-5-methyl-2,4-dioxopyrimidin-1(2*H*)-yl]-acetaldehyde (**3a**) and 2-[6-amino-4*H*-purin-9(5*H*)-yl]-acetaldehyde (**3b**) in good yields. The ^1H NMR spectrum of **3a** showed the protons of $N-CH_2$ as a doublet at δ 3.62 ppm, the proton in the pyrimidine ring as a singlet at δ 7.25 ppm in addition to the NH as a singlet at δ 8.85 ppm. When **3a** and **3b** were allowed to react with L-serine and L-threonine methylester in the presence of potassium hydroxide at room temperature, they afforded the corresponding ethylamino-3-hydroxypropanoate derivatives **5a** and **5b** and ethylamino-3-hydroxybutanoate derivatives **6a** and **6b**, respectively, in moderate yields. The IR spectra of these compounds showed the characteristic absorption band at 1740–1755 cm^{-1} The ^1H NMR spectra of these compounds are in agreement with the assigned structures. For compound **5b** the spectrum showed a peak at δ 3.62 ppm corresponding to $O-CH_3$ in addition to the peak of the hydroxy group at δ 3.95 ppm and the triplet at δ 4.05 ppm corresponding to the $CH-CH_2$ group which is in agreement with the assigned structure. The ^1H NMR spectrum of compound **6a** showed the two methyl groups as a doublet at δ 1.22 ppm and a singlet at δ 1.68 ppm in addition to the peak corresponding to the $O-CH_3$ group at δ 2.43 ppm and the hydroxy group as a doublet at δ 3.75 ppm (Fig. 2).

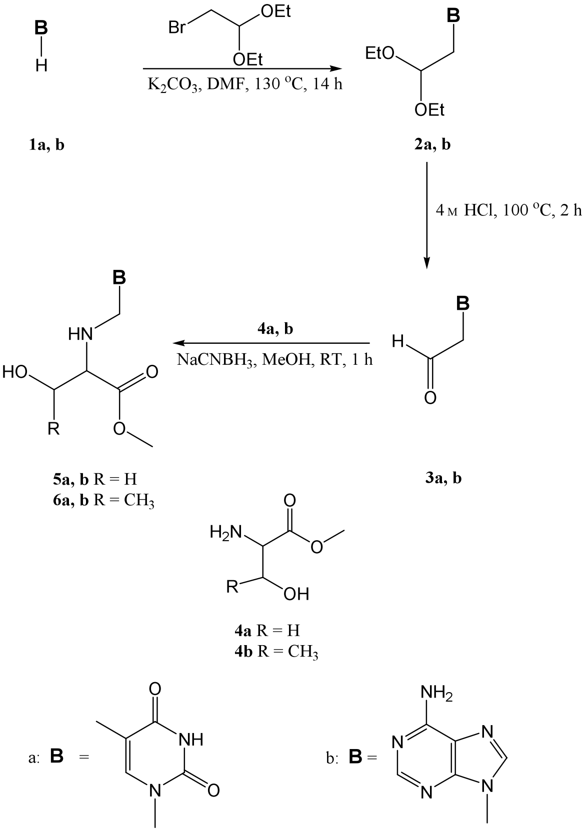

Fig. 2. Pyrimidine and adenine peptide nucleic acid analogues.

Antiviral testing

The results of the antiviral activity measurements against HBV are shown in Tables I and II. Preliminary screening indicated that **5b** and **6b** showed

Table I. Cytotoxic effect (CC_{50})*, inhibitory concentration (IC_{50}), and selectivity index (SI) of the newly synthesized compounds.

Compound	HBV DNA IC_{50} [μM]	SI
Lamivudine	0.1	1000
2a	0.6	166.6
2b	0.5	200.0
3a	0.7	142.8
3b	0.6	166.6
5a	0.5	200.0
5b	0.2	500.0
6a	0.3	333.3
6b	0.2	500.0

* HepG2.2.2.15 CC_{50} for all tested compounds was 100 μM.

Table II. Inhibition of HBV replication by the tested compounds.

Compound	Concentration [μM]	HBV DNA in supernatant	HepG2 viable cells
Lamivudine	0.1	0.25	1.00
2a	0.1	0.17	0.56
2b	0.1	0.13	0.19
3a	0.1	0.28	0.35
3b	0.1	0.19	0.33
5a	0.1	0.13	0.19
5b	0.1	0.83	0.90
6a	0.1	0.21	0.63
6b	0.1	0.29	0.44

the highest activity against HBV with an effective concentration of 0.2 μM and the selectivity index 500, followed by **6a** whereas **2a**, **3a** and **3b** showed the lowest activity (Table I).

Alahiane A., Taourirte M., Rochdi A., Redwane N., Sebti S., Engels J. W., and Lazrek H. B. (2003), Building blocks for polyamide nucleic acids: facile synthesis using potassium fluoride doped natural phosphate as pasic catalyst. Nucleosides, Nucleotides, Nucleic Acids **22**, 109–114.

Debaene F. and Winssinger N. (2003), Azidopeptide nucleic acid, an alternative strategy for solid-phase peptide nucleic acid (PNA) synthesis. Org. Lett. **5**, 4445–4447.

DeMesmaeker A., Haner R., Martin P., and Moser H. E. (1995), Antisense oligonucleotides. Acc. Chem. Res. **28**, 366–374.

Doong S. L., Tsai C. H., Chinazi R. F., Liotta D. C., and Cheng Y. C. (1991), Inhibition of the replication of hepatitis B virus *in vitro* by 2′,3′-dideoxy-3′-thiacytidine and related analogues. Proc. Natl. Acad. Sci. USA **88**, 4895–8499.

Duehohm K. L., Egholm M., Behrens C., Christensen L., Hansen H. F., Vulpius T., and Peterson K. H. (1994), Synthesis of peptide nucleic acid monomers containing the four natural nucleobases: thymine, cytosine, adenine, and guanine and their oligomerization. J. Org. Chem. **59**, 5767–5773.

Egholm M., Buchardt O., Nielsen P. E., and Berg R. H. (1992), Peptide nucleic acids (PNA). Oligonucleotide analogs with an achiral peptide backbone. J. Am. Chem. Soc. **114**, 1895–1897.

Egholm M., Buchardt O., Christensen L., Behrens C., Freier S. M., Driver D. A., Berg R. H., Kim S. K., Norden B., and Nielsen P. E. (1993), PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature **365**, 566–568.

Egholm M., Christensen L., Dueholm K. L., Buchardt O., Coull J., and Nielsen P. E. (1995), Efficient pH-independent sequence-specific DNA binding by pseudoisocytosine-containing bis-PNA. Nucleic Acids Res. **23**, 217–222.

Fouad T., Nielsen C., Brunn L., and Pederson E. B. (1998), Use of standardization cell culture assay to assess activities of some potent anti-HIV nucleoside analogues against hepatitis B virus replication. Sc. J. Az. Med. Fac. (GIRLS) **19**, 1173–1187.

Ganesh K. N. and Nielsen P. E. (2000), Peptide nucleic acids: analogs and derivatives. Curr. Org. Chem. **4**, 931–943.

Hyrup B. and Nielsen P. E. (1996), Peptide nucleic acids (PNA): synthesis, properties and potential applications. Bioorg. Med. Chem. **4**, 5–23.

Hyun S., Lee K. H., and Yu J. (2006), A strategy for the design of selective RNA binding agents. Preparation and RRE RNA binding affinities of a neomycin-peptide nucleic acid heteroconjugate library. Bioorg. Med. Chem. Lett. **16**, 4757–4759.

Korba B. E. and Gerin J. L. (1992), Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. Antiviral Res. **19**, 55–70.

Milligan J. F., Matteucci M. D., and Martin J. C. (1993), Current concepts in antisense drug design. J. Med. Chem. **36**, 1923–1937.

Nielsen P. E. (1998), Structural and biological properties of peptide nucleic acid (PNA). Pure Appl. Chem. **70**, 105–110.

Nielsen P. E. (1999), Peptide nucleic acid. A molecule with two identities. Acc. Chem. Res. **32**, 624–630.

Nielsen P. E., Egholm M., Berg R. H., and Buchardt O. (1991), Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science **254**, 1497–1500.

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

Seitz O. (2000), Solid-phase synthesis of doubly labelled peptide nucleic acids as probes for the real-time detection of hybridization. Angew. Chem. Int. Ed. **39**, 3249–3252.

Sells M. A., Chem M. L., and Acs G. (1987), Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. USA **84**, 1005–1009.

Thomson J. A., Josey S. A., Cadilla R., Gaul M. D., and Hassman C. F. (1995), Synthesis of cell-permeable peptide nucleic acids and characterization of their hybridization and uptake properties. Tetrahedron **51**, 6179–6194.

Uhlmann E. and Peymann A. (1990), Antisense oligonucleotides: a new therapeutic principle. Chem. Rev. **90**, 543–584.

Uhlmann E., Peyman A., Breipohl G., and Will D. W. (1998), PNA: Synthetic polyamide nucleic acids with unusual binding properties. Angew. Chem. **110**, 2954; Angew. Chem. Int. Ed. **37**, 2797–2823.

Will D. W., Breipohl G., Langner D., Knolle J., and Uhlmann E. M. (1995), The synthesis of polyamide nucleic acids using a novel monomethoxytrityl protecting-group strategy. Tetrahedron **51**, 12069–12082.

Zhou P., Wang M., Du L., Fisher G. W., Waggoner A., and Ly D. H. (2003), Novel binding and efficient cellular uptake of guanidine based peptide nucleic acids (GPNA). J. Am. Chem. Soc. **125**, 6878–6879.

Zhou P., Dragulescu-Andrasi A., Bhattacharya B., O'Keefe H., Vatta P., Hyldig-Nielsenb J. J., and Lya D. H. (2006), Synthesis of cell-permeable peptide nucleic acids and characterization of their hybridization and uptake properties. Bioorg. Med. Chem. Lett. **16**, 4931–4935.